Towards application of genetic engineering in citriculture:

1) assessing dispersal, long-term stability and phenotypic impact of transgenes in citrus trees and

2) improving nutri-functional quality of orange fruit through metabolic engineering

Elsa Pons Bayarri, September 2014
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Dissertation submitted in partial fulfillment of the requirements for obtaining the degree of Doctor (PhD) in Biotechnology

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El Doctor Leandro Peña García, Investigador científico del Instituto Valenciano de Investigaciones Agrarias,

CERTIFICA:

Que la presente memoria titulada “Towards application of genetic engineering in citriculture: 1) assessing dispersal, long-term stability and phenotypic impact of transgenes in citrus trees and 2) improving nutri-functional quality of orange fruit through metabolic engineering”, ha sido realizada por Elsa Pons Bayarri, Ingeniero Agrónomo por la Universitat Politècnica de València, bajo su dirección y constituye su Memoria de Tesis para optar al grado de Doctor en Biotecnología.

Fdo: Dr. Leandro Peña García

Valencia, 25 de julio de 2014

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Que Dña. Elsa Pons Bayarri, Ingeniero Agrónomo por la Universitat Politècnica de València, ha realizado bajo su tutela el trabajo que, con el título “Towards application of genetic engineering in citriculture: 1) assessing dispersal, long-term stability and phenotypic impact of transgenes in citrus trees and 2) improving nutri-functional quality of orange fruit through metabolic engineering”, presenta para optar al grado de Doctor en Biotecnología por la Universidad Politécnica de Valencia.

Y para que así conste a los efectos oportunos, firma el presente certificado

Dr. Vicente Moreno Ferrero

Valencia, 25 de julio de 2014

A Ricardo
y a Saúl

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Abstract

Despite the huge potential benefits offered by genetically modified (GM) citrus, field releases raise concerns about their potential environmental impact and the possibility to show unexpected deleterious effects from an agronomic view. The main concerns raised by the use of genetic transformation to improve this long-lived crop, of vegetative propagation and complex reproductive biology are: (1) the transfer of transgenes via pollen to compatible varieties of *Citrus* and relatives; (2) the stability of the transgenes in the long-term; (3) the occurrence of adverse pleiotropic effects derived from the integration and expression of the transgenes on the main agronomic and phenotypic crop characteristics. All these issues have been extensively studied in other annual GM crops that are already commercial or non-commercial yet. However, since the use of genetic transformation in improving fruit trees is still in its infancy, currently there is very little information on biosafety and transgene stability for these crops. Therefore, the future of transgenic trees in commercial agriculture remains uncertain, though we already have the technology to produce them. On the other hand, in the case of citrus, there are neither commercial transgenic varieties nor unequivocal evidence that this tool could be really useful to deal successfully with specific improvement goals. Achieving improvement goals so important as improving the nutri-functional quality of citrus fruits through genetic engineering could contribute to a wider acceptance of this technology by the public, since it is an improvement addressed to the consumer first.

In this work, we have faced some of the aspects which are greatly limiting the acceptance and marketing of GM citrus, by (1) conducting a field release experiment with GM citrus to assess their environmental safety and the lack of adverse agronomic effects (2) addressing an objective to improve the nutri-functional quality of orange fruit through metabolic engineering in order to strengthen their healthy properties.

The field experiment consisted of a planting of transgenic citrus trees carrying only the *uidA* and *nptII* marker genes, and its purpose was to study the feasibility of genetic transformation in improving commercially important citrus genotypes. This experimental orchard allowed us to estimate the maximum frequency of transgenic pollen dispersal under conditions of open pollination and to study genetic, phenological and environmental factors that determined it, in order to propose appropriate transgene containment measures for future GM citrus plantings. It also served as a first approach to address basic issues as the study of the stability of transgene expression in the long term (after 7 years of establishment in the field) under real agricultural conditions and its potential impact on the morphology, phenology and fruit quality of transgenic citrus. These studies, though do not solve all concerns regarding GM citrus, provide crucial information about environmental biosafety and behaviour in the field, so far non-existent, which can serve as a basis to design future field trials with GM citrus and to guide case-by-case regulatory policies for new plantings.

Moreover, in this work we have succeeded in developing a strategy to induce early fruit production and increase the content of β-carotene (pro-vitamin A, with high antioxidant capacity) in the pulp of a sweet orange variety by metabolic engineering. This strategy consisted of RNAi-mediated silencing of a β-carotene hydroxylase gene from orange (*CsβCHX*), involved in the conversion of β-carotene into xanthophylls, combined with overexpression of the *FLOWERING LOCUS T* gene from orange (*CsFT*) in juvenile transgenic plants of Pineapple sweet orange. Subsequent tests with the animal model *Caenorhabditis elegans* demonstrated that the enriched orange exerted an *in vivo* antioxidant effect 20% higher than isogenic control oranges. This is the first successful example of metabolic engineering to increase the content of β-carotene (or any phytonutrient) in orange and demonstrates the potential of genetic engineering for nutritional enrichment of woody fruit crops.

Resumen

A pesar de los enormes beneficios potenciales que ofrecen los cítricos genéticamente modificados (GM), su liberación en campo suscita preocupaciones acerca de su potencial impacto ambiental y posibilidad de que muestren efectos deletéreos inesperados desde un punto de vista agronómico. Las principales preocupaciones que plantea el uso de la transformación genética para la mejora de este cultivo de vida larga, propagación vegetativa y compleja biología reproductiva son: (1) la transferencia de los transgenes vía polen a variedades compatibles de especies de *Citrus* y afines; (2) la estabilidad de los transgenes a largo plazo; (3) la aparición de efectos pleiotrópicos adversos derivados de la integración y la expresión de los transgenes sobre las principales características agronómicas y fenotípicas del cultivo. Todas estas cuestiones han sido ampliamente estudiadas en otros cultivos anuales GM que ya son o no comerciales. Sin embargo, puesto que el empleo de la transformación genética en la mejora de árboles frutales todavía se encuentra en sus inicios, actualmente se dispone de muy poca información al respecto para estos cultivos. Por todo ello, el futuro de los arboles transgénicos en el ámbito comercial permanece aún incierto, aunque actualmente se dispone de la tecnología para producirlos. Por otro lado, en el caso concreto de los cítricos, no existen variedades transgénicas comerciales ni evidencias inequívocas de que esta herramienta sea realmente útil para afrontar con éxito objetivos de mejora concretos. Lograr cumplir objetivos de mejora tan importantes como la mejora de la calidad nutri-funcional de los frutos cítricos mediante ingeniería genética podría contribuir a una mayor aceptación de esta tecnología por parte del público, puesto que se trata de una mejora dirigida primeramente al consumidor.

En este trabajo nos hemos planteado afrontar parte de los aspectos que en gran medida limitan la aceptación y comercialización de cítricos GM, mediante (1) la realización de un experimento de campo con cítricos GM para evaluar su seguridad ambiental y la ausencia de efectos agronómicos adversos (2) el abordaje de un objetivo de mejora de la calidad nutri-funcional de la naranja concreto mediante ingeniería metabólica con la finalidad de reforzar sus propiedades saludables.

El experimento de campo consistió en una plantación de cítricos transgénicos que portaban únicamente los genes marcadores *uidA* y *nptII* cuya finalidad fue estudiar la viabilidad de la transformación genética en la mejora de genotipos cítricos comercialmente importantes. Este huerto experimental nos sirvió para estimar la frecuencia máxima de dispersión de los transgenes por polen bajo condiciones de polinización abierta y estudiar los factores ambientales, genéticos y fenológicos que la determinan, para así poder proponer medidas de contención apropiadas en futuras plantaciones de cítricos GM. También sirvió como primera aproximación para abordar cuestiones básicas como el estudio de la estabilidad de la expresión de los transgenes a largo plazo (tras 7 años de establecimiento en campo) bajo condiciones reales de cultivo y su potencial impacto sobre la morfología, fenología y calidad de

la fruta de los cítricos transgénicos. Los estudios realizados, aunque no resuelven todas las preocupaciones concernientes a los cítricos GM, aportan información crucial relativa a su seguridad y comportamiento en campo, inexistente hasta el momento, que puede servir como base para futuros ensayos de campo con cítricos GM y como guía para las políticas de regulación de su plantación (caso-a-caso).

Por otro lado, en este trabajo se ha logrado desarrollar una estrategia para inducir producción temprana de fruta e incrementar el contenido de b-caroteno (pro-vitamina A, con elevada capacidad antioxidante) en la pulpa de una variedad de naranjo dulce mediante ingeniería metabólica. Dicha estrategia consistió en el silenciamiento mediado por RNAi del gen de una β-caroteno hidroxilasa de naranjo (*CsβCHX*), implicada en la conversión de b-caroteno en xantofilas, combinado con la sobreexpresión del gen *FLOWERING LOCUS T* de naranjo (*CsFT*) en plantas transgénicas juveniles de naranjo dulce cv Pineapple. Posteriores ensayos con el animal modelo *Caenorhabditis elegans* demostraron que la naranjas enriquecidas ejercían un efecto antioxidante *in vivo* un 20% mayor que las naranjas control isogénicas. Este es el primer ejemplo exitoso de ingeniería metabólica para incrementar el contenido de β-caroteno (o cualquier fitonutriente) en naranjas y demuestra el potencial que tiene la ingeniería genética para el enriquecimiento nutricional de cultivos frutales leñosos.

Resum

A pesar dels enormes beneficis potencials que ofereixen els cítrics genèticament modificats (GM), el seu alliberament en camp suscita preocupacions sobre el seu potencial impacte ambiental i la possibilitat que mostrin efectes deleteris inesperats des d'un punt de vista agronòmic. Les principals preocupacions que planteja l'ús de la transformació genètica per a la millora d'aquest cultiu de vida llarga, propagació vegetativa i complexa biologia reproductiva són: (1) la transferència dels transgens via pol·len a varietats compatibles d'espècies de Citrus i afins; (2) l'estabilitat dels transgens a llarg termini; (3) l'aparició d'efectes pleiotròpics adversos derivats de la integració i l'expressió dels transgens sobre les principals característiques agronòmiques i fenotípiques del cultiu. Totes aquestes qüestions han sigut àmpliament estudiades en altres cultius anuals GM que ja són o no comercials. No obstant això, ja que l'ús de la transformació genètica en la millora d'arbres fruiters encara es troba en els seus inicis, actualment es disposa de molt poca informació al respecte per aquests cultius. Per tot això, el futur dels arbres transgènics en l'àmbit comercial roman encara incert, encara que actualment es disposa de la tecnologia per produir-los. D'altra banda, en el cas concret dels cítrics, no existeixen varietats transgèniques comercials ni evidències inequívoques que aquesta eina sigui realment útil per afrontar amb èxit objectius de millora concrets. Aconseguir complir objectius de millora tan importants com la millora de la qualitat nutri-funcional dels fruits cítrics mitjançant l'enginyeria genètica podria contribuir a una major acceptació d'aquesta tecnologia per part del públic, ja que es tracta d'una millora dirigida primerament al consumidor.

En aquest treball ens hem plantejat afrontar part dels aspectes que en gran mesura limiten l'acceptació i comercialització de cítrics GM, mitjançant (1) la realització d'un experiment de camp amb cítrics GM per avaluar la seva seguretat ambiental i l'absència d'efectes agronòmics adversos (2) l'abordatge d'un objectiu de millora de la qualitat nutri-funcional de la taronja concret mitjançant enginyeria metabòlica amb la finalitat de reforçar les seves propietats saludables.

L'experiment de camp va consistir en una plantació de cítrics transgènics que portaven únicament els gens marcadors *uid*A i *nptII*, quina finalitat va ser estudiar la viabilitat de la transformació genètica en la millora de genotips cítrics comercialment importants. Aquest hort experimental ens va servir per estimar la freqüència màxima de dispersió dels transgens per pol·len baix condicions de pol·linització oberta i estudiar els factors ambientals, genètics i fenològics que la determinen, per així poder proposar mesures de contenció apropiades en futures plantacions de cítrics GM. També va servir com a primera aproximació per abordar qüestions bàsiques com l'estudi de l'estabilitat de l'expressió dels transgens a llarg termini (després de 7 anys d'establiment en camp) en condicions reals de cultiu i el seu potencial impacte sobre la morfologia, fenologia i qualitat de la fruita dels cítrics transgènics. Els estudis realitzats, encara que no resolen totes les preocupacions concernents als cítrics GM, aporten informació crucial relativa a la seva seguretat i comportament en camp, inexistent fins al

moment, que pot servir com a base per a futurs assajos de camp amb cítrics GM i com a guia per les polítiques de regulació de la seva plantació (cas-a-cas).

D'altra banda, en aquest treball s'ha aconseguit desenvolupar una estratègia per induir producció precoç de fruita i incrementar el contingut de β-carotè (provitamina A, amb elevada capacitat antioxidant) a la polpa d'una varietat de taronger dolç mitjançant enginyeria metabòlica. Aquesta estratègia va consistir en el silenciament mediat per RNAi del gen d'una β-carotè hidroxilasa de taronger (*CsβCHX*), implicada en la conversió de β-carotè en xantofilas, combinat amb la sobreexpressió del gen *FLOWERING LOCUS T* de taronger (*CsFT*) en plantes transgèniques juvenils de taronger dolç cv Pineapple. Posteriors assajos amb l'animal model *Caenorhabditis elegans* van demostrar que la taronges enriquides exercien un efecte antioxidant *in vivo* un 20% major que les taronges control isogèniques. Aquest és el primer exemple exitós d'enginyeria metabòlica per incrementar el contingut de β-carotè (o qualsevol fitonutrient) en taronges i demostra el potencial que té l'enginyeria genètica per a l'enriquiment nutricional de cultius fruiters llenyosos.

1. INTRODUCTION

1. Citrus improvement by genetic transformation

1.1. Citrus

1.1.1. Taxonomy, origin, and distribution

The genus *Citrus* is one of the 33 genera in the subfamily *Aurantoideae* of the family *Rutaceae*. Within this subfamily, most taxonomists recognize that "true citrus fruit trees" belong to the tribe *Citreae*, subtribe *Citrinae*, being three genus of economic importance: *Poncirus*, *Fortunella* and *Citrus*. Among them, the genus *Citrus* is by far the most important, but *Fortunella* and *Poncirus* are also playing a relevant role in citriculture.

Fortunella is a genus with several genotypes known as kumquats, all being small trees with a later flowering time than *Citrus* species, relatively cold tolerant and resistant to *citrus canker* and *Phytophthora spp.* They bear small fruits with sweet tasting rind. Kumquats have been cultivated extensively in China for long time and are recently used as parents in citrus breeding programs. *Poncirus* includes only the *Poncirus trifoliata* (L.) Raf. species. It is used exclusively as a rootstock in many areas and as parent in rootstock breeding programs, due to its resistance to *Citrus Tristeza Virus*, the citrus nematode *Tylenchulus semipenetrans*, *Phytophthora parasitica* and *Phytophthora citrophthora* and its cold tolerance. Its derived hybrids with sweet oranges, [mainly `Carrizo´ and `Troyer´ citranges (*C. sinensis* x *P. trifoliata*)] are the main rootstocks used in Spain; a cross between *Poncirus* and grapefruit known as Swingle citrumelo (*C. paradisi* x *P. trifoliata)* is also used as a rootstock by many citrus industries.

The taxonomy of the genus *Citrus* is controversial. The system most commonly used comes from the classification of Swingle with modifications provided by the much more complex Tanaka's classification. While Swingle recognizes 10 and 6 species, respectively, in the two subgenera *Citrus* and *Papeda* (Swingle and Reece, 1967), Tanaka identifies up to 162 species in different groups and subgroups (Tanaka, 1954, 1977). From the ten *Citrus* species designated by Swingle, eight are of commercial importance: *C. sinensis* (L.) Osb. (sweet oranges), *C. reticulata* Blanco (mandarins), *C. paradisi* Macf. (grapefruits), *C. grandis* (L.) Osb. (pummelos), *C. limon* (L.) Burm. f. (lemons), *C. aurantifolia* (Christm.) Swing. (limes), *C. aurantium* L. (sour oranges), and *C. medica* L. (citrons). Tanaka´s system is better adapted to horticultural traits paying also special consideration to cultivated species. This concerns to *Citrus* genotypes that are widely cultivated and of high economic importance, such as clementine mandarins (*C. clementina* Hort. ex Tan.), satsuma mandarins (*C. unshiu* (Mak.) Marc.), or Rangpur lime (*C. limonia* (L.) Osb.) among others, for which most citrus researchers use the Tanaka's classification (Krueger and Navarro, 2007). From an agronomical point of view, Tanaka' s classification is better adapted to the characteristics of the different agronomic groups, and it is widely used to manage germplasm collections (Krueger and Navarro, 2007).

The area of origin of *Citrus* is believed to be southeastern Asia, including south China, the Indo-Chinese peninsula, northeastern India and Burma (Webber, 1967). This is a wide area,

but attempts to localize more precisely the centers of origin of the most important *Citrus* types are still now controversial. It has become clear in recent times that only citron (*Citrus medica*), mandarin (*C. reticulata*), and pummelo (*C. grandis*) are "true species" within genus *Citrus*, being other important *Citrus* types, as sweet orange, sour orange, lemon, lime, grapefruit and other mandarins originated from hybridization between these ancestral species followed by subsequent frequent somatic mutations (Davies and Albrigo, 1994; Ollitrault, et al., 2012). This view was convincingly supported by classical (Mabberley, 1997) and molecular (Nicolosi, et al., 2000; Xu, et al., 2013; Wu, et al., 2014) phylogenetic studies. Since the three ancestral species only reproduce sexually and are original from the same geographical area, several generations of hybridization among these species would generate the highest levels of genetic diversity within the genus *Citrus* and sexually-compatible relatives. Therefore, southeastern Asia would not only be the site of origin of most important *Citrus* types but also its major center of diversity. Domestication could have started in this area and expanded progressively in all directions (revised by Webber (1967)). In the case of genus *Fortunella* and *Poncirus* all authors coincide in ascribing their origin to central China, since both genera are most cold-hardy than *Citrus* and are reported as growing wild in the Yellow river area in ancient Chinese literature.

Due to their apomictic character (ability of nucellar cells from seeds to develop embryos that are genetically identical to mother plant), most *Citrus* varieties were propagated as seedlings during many centuries. In the case of monoembryonic genotypes (that is, genotypes producing seeds that only develop sexual embryos), propagation by seeds led to generation of a lot of genetic variation and horticultural diversity, as it is exemplified by the high number of different mandarin types that have been grown in China and Japan during many years. Although there are ancient Chinese references reporting the graft of mandarins onto *Poncirus trifoliata*, grafting only became a common practice in citriculture from the mid-19th century, after sweet orange seedlings grown in Europe were seriously affected by *Phytopthothora* epidemics. Nowadays, the citrus industry relies on trees composed of two different genotypes: a mature fruit-producing *Citrus* scion grafted onto a highly apomictic juvenile rootstock.

1.1.2. Citrus biology: some clues on growth and development

1.1.2.1. Vegetative development

Seed and seedling

Sowing is practiced mainly with rootstock cultivars which are grown in nurseries and prepared for grafting. The rate of seedling development varies considerably among cultivars and is greatly dependent upon genetic characteristics such as nucellar embriony rate and vigor, and environmental conditions, including temperature, soil type, irrigation, and particularly, nitrogen fertilization. Citrus seedlings are juvenile, much more so than rooted cuttings or other vegetatively propagated plants. The period from seed to first fruiting is known as the juvenile period. Its length in citrus is often four to six years, but it could be much longer, highly

depending on the genetic and the environmental context for each citrus type. Juvenility is generally associated with inability to flower, but the juvenile growth habit is revealed also in upright, unbranched growth, abundance of thorns, and in certain cultivars (e. g. Shamouti orange) by very large leaves (Spiegel-Roy and Goldschmidt, 1996). Moreover, even with the advent of fruiting, some of its characteristics, such as thorniness and undesirable fruit shape, often persist. While there is a definite genetic component on the length of the juvenile period – oranges are slow to come into bearing compared with most mandarins – environmental conditions are also highly influential, being juvenility shorter in tropical areas.

Shoot development

Shoot growth occurs in most types of citrus in several well-defined waves (flushes). Citrus trees have a sympodial growth habit, that is, a lateral growth pattern in which the apical meristem is terminated (with either the abortion of apical meristem or its conversion into a flower, inflorescence or a specialized structure), and growth is continued by expanding shoots from a lateral meristem, which repeats the process (Lord and Eckard, 1987). Under cool climatic conditions only two flushes appear annually, while three to five flushes occur in warmer, subtropical regions. Under wet, tropical conditions shoot growth occurs uninterruptedly, throughout the year. Lemons, citrons and acid limes retain their tropical nature even in cooler climates and new shoots emerge year-round. In most citrus areas, the spring flush is the most important one containing both vegetative and reproductive shoots. The midsummer and subsequent flushes are generally vegetative, with fewer but longer, vigorously growing shoots and larger leaves. As trees get older, the spring flush comprises mainly short, reproductive shoots (leafy and leafless inflorescences). For its vegetative growth the tree is dependent upon the summer flushes (Spiegel-Roy and Goldschmidt, 1996).

An axillary bud occurs in the axil of each citrus leaf. The axillary bud consists of an apical meristem, covered by several prophylls (bud scales). Accessory buds develop in the axis of the prophylls; thus, multiple buds are present in the axis of leaves. Axillary thorns may subtend the buds, occurring opposite the first prophyll. Thorns are particularly prominent in juvenile, vigorously growing shoots (Spiegel-Roy and Goldschmidt, 1996). All *Citrus* types are evergreen and do not show winter dormancy but just a bud resting period. However, the *Poncirus* relative is deciduous, showing winter leaf abscission and bud dormancy (Peña, et al., 2008).

Leaves are unifoliate and in most species the petioles are winged. *Poncirus* shows trifoliolate leaves, reminiscent of other *Aurantoideae* genera with composite leaves. Elongated leaf shape and larger petiole wings are considered juvenile characters (Spiegel-Roy and Goldschmidt, 1996).

1.1.2.2. Reproductive development/biology

The Flowering

The transition of the vegetative, leaf producing meristem into the reproductive floral meristem is the initial event in the long chain of developmental processes leading to seed and fruit production. The environmental and endogenous control of flower bud differentiation is quite complex and varies considerably from one species to another. Citrus trees, like other fruit trees, are polycarpic plants undergoing repeated cycles of flowering and fruiting. Fruit trees never commit all their buds to flowering – a certain number of buds must be retained under the vegetative, non-differentiated state to ensure the tree's future. Flower bud differentiation is induced photoperiodically in subtropical areas when the day becomes shorter during winter months. Cold temperatures are also important in floral induction. In the deciduous *Poncirus*, flower bud induction is initiated during late summer. In tropical areas, without photoperiod changes, water stress is the major flower-inducing signal.

In *Citrus*, blooming usually occurs in spring, following flower development. As evergreen, reproductive and vegetative developments are intimately related, and four main shoot types can be distinguished: vegetative shoots, leafy inflorescences, leafless inflorescences and solitary flowers. *Poncirus* and *Fortunella* also flower in spring but usually sooner and later than *Citrus*, respectively. Dates and duration of bloom are highly variable even for the same cultivar – differences of up to 40 days in the commencement of anthesis from one year to the next are not uncommon. Slight climatic differences between locations also affect the date of blooming. The rate of flower development from budbreak to anthesis is rather closely dependent upon the accumulation of heat units above a minimum threshold temperature (Lomas and Burd, 1983). The duration of the flowering period is also largely dependent upon the prevailing temperatures. Warmer than usual weather will bring about opening of flowers within a few days, resulting in a concentrated wave of bloom, petal fall and fruit set. Cool spring weather, on the other hand, may lead to an extended period of diffuse flowering. Such seasonal differences may have important consequences for the chances of pollination and fruit set, particularly in self-incompatible cultivars (e. g. mandarin hybrids) where overlapping with pollination is critical.

Hybridization and parthenocarpy

Citrus flowers are attractive to insects due to abundant pollen, nectar, typical perfume, and the conspicuous corolla. Most citrus species are valuable honey-producing plants. While thrips and mites also abound on flowers, honey bees are the main agent in natural cross pollination. Wind is a minor, irrelevant factor in citrus pollination. Self-pollination may occur in self-compatible genotypes. Self-pollination usually takes place in the unopened or opening flower, often allowing pollination before anthesis. Temperature has considerable effect on pollination efficiency, affecting the rate of pollen-tube growth as well as bee activity. Pollen viability and ovule fertility are also influenced by temperature.

Absolute or a high degree of gametic sterility is encountered in numerous citrus cultivars. The percentage of functional pollen varies among species and cultivars. Some of the most widely used commercial cultivars are deficient in this respect. Navel orange produces no

viable pollen; satsuma mandarin and Marsh grapefruit very little; lemons and most orange cultivars often have low amounts. Most cultivars of mandarin and pummelo produce largely functional pollen. Cultivars with a problem of non-functional pollen very often show comparable ovule abortion; thus the pollen-sterile Washington Navel and, more so, satsuma mandarins have (few) functional ovules. Degeneration before meiosis is also encountered. In addition to absolute gametic sterility, self and, to some extent, cross incompatibility are also present in citrus. Incompatibility is widespread in pummelos. Self-incompatibility is a genetically controlled phenomenon preventing seed set in self-pollinated plants producing functional gametes. Nagai and Tanikawa (1928) found that some self-incompatible accessions produced seedless fruits when they were self-pollinated. Almost all pummelos, some mandarins and several natural or artificial hybrids are self-incompatible (Hearn, 1969). The list of self-incompatible cultivars is extensive (including accessions such as Clemenules clementine, Imperial mandarin, Sukega grapefruit, Siames pummelo, Ellendale tangor, Orlando and Minneola tangelos, etc.) and is on the increase. Seedlessness and pollen sterility have been reviewed (Iwamasa, 1966; Nicolosi, 2007).

Fertilization leading to seed formation is generally a prerequisite for fruit set and lack of fertilization will inevitably end up in drop of the ovary. There are, nevertheless, numerous plants which produce seedless fruit. Production of fruit without seeds is parthenocarpy (Frost and Soost, 1968). The setting of fruit without any external stimulation is defined as autonomic parthenocarpy. The term stimulative parthenocarpy is used to describe the cases in which some kind of stimulus is required. In stimulative parthenocarpy, pollination, pollen germination and pollen tube growth, unaccompanied by fecundation, provide sufficient stimulation to for set of seedless fruit. Thus, self-pollination may exert a sufficient stimulus in self-incompatible genotypes for the setting of seedless fruit. In some cases of parthenocarpy, fruit with occasional fruit seeds can be found as a result of incomplete female sterility (Washington Navel orange, Marsh seedless grapefruit). Parthenocarpic tendency and ovule sterility may vary independently. Some usually seeded cultivars may be capable of a variable degree of parthenocarpy, especially self-incompatible ones. Vary, et al. (1988) state that the potential for pollen-stimulated parthenocarpic fruit is rather widespread in citrus. Ovule fertility and the presence of compatible pollen mask stimulative parthenocarpy. In natural and induced seedlessness, the seedless condition is generally accompanied by irregularities of meiosis. In a few cases in citrus, a phenomenon resembling stenospermocarpy (fecundation followed by post-zygotic abortion) has been noted. For a cultivar incapable of seed production to be horticultural acceptable, a high parthenocarpic tendency is essential.

Poliembriony/apomixis

Polyembryony, a feature widespread in citrus (Koltunow, et al., 1996), is the development of two or more embryos in one seed. Extra embryos are commonly produced apomictically from cells of the seed parent (nucellar embryony). Nucellar embryos develop asexually by ordinary mitotic division of cells of the nucellus. The apomictic process thus

generates seeds containing embryos of a purely maternal genetic constitution. In apomictic citrus genotypes, sexual and apomictic processes occur within the same ovule. Nucellar embryos are initiated from the nucellar tissue in the region around the developing sexual embryo sac (Koltunow, 1993; Chiancone and Germana, 2013). The growth of the zygotic embryo is often slower when compared with that of the nucellar embryos. The zygotic embryo may also not complete its development.

A good summary on citrus biology can be found in Spiegel-Roy and Goldschmidt (1996).

1.2. The citrus fruits

1.2.1. Commercialization and socio-economic importance

Because of their preferred flavor, delightful taste, affordable economic reach, and consumer awareness of their increasingly recognized potential health properties, the commercial production, processing, and global trade of citrus have significantly increased in the last several decades, placing citrus as the most important fruit tree in the world (Ting, 1980; UNCTAD, 2004). In 2012, the global citrus acreage was 8.7 million hectares and citrus production was about 131 million tons. Citrus is grown in more than 140 countries in tropical, subtropical and Mediterranean climates. Major producing countries include China, Brazil, USA, India, Mexico, Spain, Egypt, Nigeria, Turkey, Italy, Iran, Argentina, South Africa, Pakistan, Morocco, Indonesia, Thailand, Colombia, Argelia, Peru, and Japan, from major to minor. The first three countries account for about 50% of the citrus world production. Production trends indicate that oranges constitute about 60% of the total citrus output, followed by the group formed by mandarins, clementines, satsumas, and tangerines, which comprise about 20% of the output. The group of lemons and limes constitutes 11–12%, and grapefruit and pummelos comprise roughly 5–6% (FAO statistics, 2012). Brazil and USA (Florida and California) were leading producers of sweet oranges. USA is the primary producer of grapefruit. China, Spain and Japan produce 65% of the tangerines grown in the world. Lemons are produced primarily in Argentina, Spain and USA, while Mexico is the largest producer of small fruited limes. Lime is also a traditional crop in South Asia and the Middle East (FAO statistics, 2012). Many citrus species have industrial significance as a raw material for cosmetic and pharmaceutical products.

Although many citrus fruits, such as oranges, tangerines, and grapefruits can be eaten fresh, about a third of citrus fruit worldwide is utilized after processing, and orange juice production accounts for nearly 85% of total processed consumption (USDA, 2006). Among the 86 million metric tons (valued at $9.3 billion) of citrus products traded in 2012, sweet orange accounted for more than a half of citrus production for both fresh fruit and processed juice consumption. According to 2008-2012 data from the Food and Agriculture Organization of the United Nations (FAO), about 40% of sweet orange produced yearly in the world is processed.

Traditionally, oranges were consumed as fresh fruits but in the last 50 years consumption of processed oranges (mainly as concentrated fruit juice) has increased extraordinarily all over the world, and especially in Europe and USA. It represents the primary force supporting expanded world consumption and is the basis of Brazilian and Florida citrus industries.

Citrus cultivation not only is remunerative, but it also generates employment, and as detailed bellow (in the paragraph 3 of the introduction), fruits have nutritive and therapeutic value.

1.2.2. Morphology/anathomy, development and maturation

The fruits can have different forms (for example, round, oblong, or elongated) and various sizes from 3.8 to 14.5 cm in diameter (Ranganna, et al., 1983). The citrus fruit is a hesperidium, namely a berry arising from growth and development of the ovary, consisting of fleshy parts divided by segments, the whole being surrounded by a separable skin. It is composed of two major regions: the pericarp, commonly known as the peel, and the endocarp, often called the pulp. The pericarp is composed of external colored peel known as flavedo (with oil sacs producing aromatic oils), and the internal usually white layer known as albedo (a spongy layer below the flavedo, source of flavanones) (Spiegel-Roy and Goldschmidt, 1996). The inner flesh or pulp consists of segments surrounding the central axis of the fruit, the ovarian locules, enclosed in a locular membrane in which seeds and juice sacs (vesicles) grow (Agustí, et al., 2003). Juice vesicles are elongate multicellular structures, each attached to the endocarp through a filament and which are oriented towards the interior of the locule. Mature vesicles are formed by highly vacuolated cells containing juice (Tadeo, et al., 2003). Structural and physiological differences between peel and pulp of citrus fruit have already been pointed out in the foregoing discussion of fruit development. During maturation peel and pulp behave in most respects as separate organs, although some coordination does exist (Spiegel-Roy and Goldschmidt, 1996).

Growth and development of citrus fruit follows a typical sigmoid growth curve, divided into three clear-cut stages (Bain, 1958). The initial phase, or phase I, encompasses from anthesis until the end of the physiological fruit drop, and is characterized by rapid growth of the fruit caused by cell division, thus increasing the number of cells in all developing tissues except the central axis. During this period, the increase in fruit size is due primarily to the growth of the peel. Thereafter, in the rapid growth period (phase II), which extends from the end of the physiological drop until the start of the color break, fruit experiences a huge increase in size by cell enlargement and water accumulation. During this period, fruit growth is largely due to accumulation of juice in the vesicles and all tissues reach their maximum size. Finally, in phase III or maturation period, growth is mostly arrested and fruits undergo a non-climacteric process while maintained in the tree. This phase comprises most of the external and internal changes associated with maturation. On one hand, the dark green, photosynthetically active flavedo transforms its chloroplasts in to carotenoid-rich chromoplasts, resulting in the color break of the

fruit. On the other hand, maturation of the pulp is generally characterized by a decline in acidity and an increase in sugars. As Koch (1984) demonstrated, many organic acids are synthesized in the fruit during Phase I and, generally, they are reduced during phases II and III of fruit development -except for lemons, where level of acids remains high (Bain, 1958). Despite this reduction, mature citrus fruits have an elevated concentration of organic acids, being citric acid the most abundant by far, and among which is noteworthy the ascorbic acid (vitamin C) because of its nutritional relevance. Although citrus fruits are not the single supplier of vitamin C, they are particularly rich and a popular dietary source among vegetables and fruits, providing average vitamin C concentration ranging from 23 to 83 mg/100 g fresh weight (Koch, 1984), the variability of vitamin C content in fresh citrus fruits and their commercial products is greatly influenced by variety, maturity, climate, handling, processing, and storage conditions (Nagy, 1980; Lee and Kader, 2000).

1.2.3. Quality attributes of fruit and juice

The fruit quality attributes are classified into two groups: 1) *internal quality attributes*, including texture/mouthfeel, seed number, juice percentage, juice color, flavor (governed by the balance sugar:acid content plus the concentration of certain volatile compounds); recently, there is a tendency to provide also toxicological and nutritional attributes, giving consumers more information on the characteristics of citrus fruits and juices; and 2) *external quality attributes*, related to the appearance and especially important for fruit intended for fresh consumption, such as size, shape, rind color, presence of alterations and defects on the surface (blemishes, puffing,…), etc.; this also includes attributes related to post-harvest shelf life of the fruit (such as antifungal wax treatments, cold storage time and conditions).

The quality attributes have a strong economical relevance since they are related to consumer perception and ultimately determine marketability, price and use of fruits. They may eventually constrain the success of the citrus industry. Therefore, their evaluation is necessary and there exist many measurement methods to accomplish it. Many quality attributes can be evaluated by subjective methods. The organoleptic quality or sensory evaluation is subjective and based on the response of human senses to external and internal fruit quality. On the other hand, quality attributes can be measured by objective methods that could be grouped into three categories: physical, chemical, and physiological, on the basis of analytical process and principles involved. Moreover, microbial quality is routinely evaluated in processed citrus fruit (Ladaniya, 2008).

Citrus production faces diverse problems in different regions of the world, and fruit quality varies with agro-climatic conditions. In subtropical regions, under arid conditions with low humidity, fruit quality is excellent, with very few blemishes on the fruit's surface, and pack-out can be as high as 95 percent if fruit meets the size requirements. In tropical climates with high humidity, however, pack-out can be less (50 percent of the produce harvested) because of blemishes on the fruit's surfaces. This is evident from the differences in the produce of Florida

and California, two well-known citrus-growing regions. In tropical areas of India and many other Southeast Asian countries and Brazil, the incidence of fruit surface blemishes is high, and fruit rind color remains green even when the fruit is internally mature. In the cool climates (subtropical) and arid conditions of northwestern India, fruit quality is excellent with respect to color, size, and taste. For fresh citrus fruits, there are certain fixed standards of internal and external quality, based on which its grade, utility, and marketability is decided. But, due to the variation in fruit quality among producing regions, fruit grades and internal standards differ among the domestic markets of many countries. The draft codex standards of Food and Agricultural Organization (FAO) are being evolved through discussions and consensus for world trade. Similarly, rules and regulations under sanitary and phytosanitary (SPS) treaties have been finalized and are being discussed under the new World Trade Organization (WTO) regime (Ladaniya, 2008).

1.2.3.1. Quality standards for fresh citrus fruits

Internal Standards (Indices of Maturity)

Sweet oranges, mandarins, grapefruits, and pummelos are considered mature when their juice content and total soluble solids:acidity ratio have attained certain minimum limits for palatability. Total soluble solids (TSS) comprise 10-20% of the fresh weight of the fruit and consist mainly of sugars (75-85%), of which, fructose, glucose and sucrose are the most abundant (Agustí, et al., 2003; Tewari, et al., 2008). Thus, the content of TSS, usually measured by a refractometer and expressed as º Brix, serves as an estimate of the sugar content of the juice. The acidity of the juice of citrus fruits is largely due to high contents of citric acid, being malic acid and fumaric acid the next in abundance (Feryal, 2003). The total acidity or titratable acidity (TA) of the juice is usually determined by titration with NaOH and expressed as the percentage of anhydrous citric acid by weight. In citrus fruits, maturity is determined mainly on the basis of the ratio of TSS to TA. This ratio is called the maturity index (MI) and it is closely related with taste. However, the reliance on this ratio alone can be deceptive. A minimum sugar or TSS content is required for palatability, thus, these parameter should also be the part of maturity indices. Likewise, juice content is also an accepted criteria for judging maturity (Sites and Reitz, 1949), and it is usually determined as a percentage by weight or volume of fruit. Lastly, although in most citrus fruits color break (i., e., change of fruit color from light green to yellow-orange) is generally related to the degree of maturation, this parameter cannot be considered a maturity index in tropical areas, where the flavedo remains green after maturation.

External Standards (Fruit Grades)

Citrus fruit grades are mostly related to size, appearance, extent of defects, shape, and color of the fruit. European citrus-growing countries, South Australia, California, and other places with Mediterranean-type climates (cool winter nights, bright days, and low rainfall) can

rely almost entirely on external standards to sell their fruit. As Grierson and Ting (1978) put it, the real basis for fresh citrus fruit grades and standards is economics. What is economically justified under one situation may not be so in another. Hence almost all the countries have their own standards for domestic markets and these standards also vary as per early-, mid- and late-season crop fruit. The same variety of citrus also performs differently in different climatic conditions and this also leads to setting of different standards. The grade standards of citrus fruits are published by international bodies and national governments of different countries. The Organization for Economic Cooperation and Development (OECD) introduced standards for marketing fruit between countries. The Economic Commission for Europe (ECE) also publishes standards for grades of fruits and vegetables including citrus. For fruit to be palatable, all grades of fruit must meet minimum internal maturity standards. Besides international standards published by the FAO Codex committee, several countries have their own fruit-quality standards.

1.3. Genetic improvement of citrus

1.3.1. Needs for genetic improvement: special focus on scion breeding goals

The vast majority of citrus rootstocks and varieties grown commercially nowadays arose by budsport mutations and chance seedlings that were selected directly by growers due to their excellent fruit quality, performance and stress resistance (Peña, et al., 2008). However, as with most agricultural crops, many factors are known to limit the production and processing of citrus. Most are dependent on problems related to scion and rootstock deficiencies. Major constrains to citrus production involve management inefficiencies, susceptibility to pests and diseases, and environmental challenges. Many different citrus genotypes are commercially grown in a wide diversity of soil and climatic conditions, implicating that trees are subjected to important abiotic and biotic stresses that limit the production and, in some instances, the use of certain rootstocks and varieties. There are pests and diseases that additionally cause quarantine restrictions for the movement of fresh fruit from affected areas (Graham, et al., 2004). At the same time, there is an increasing (and changing) consumer interest for fruit (and juice) quality attributes, and competition in international markets is growing tremendously. Even in domestic markets, citrus fruit quality and price have to be competitive with other fruits. Thus, new and improved scion and rootstocks cultivars aimed at controlling these production and marketing constrains have been the primary breeding efforts. Since the last century, several citrus improvement programs have been performed using both traditional breeding techniques (e. g., hybridization, selecting clones from spontaneous or induced mutations) as new biotechnological tools (based on *in-vitro* cell, protoplast, tissue and organ culture, and genetic transformation). The specific breeding goals addressed in these programs were, in principle, different depending on whether improved rootstocks or scions would like to be generated.

Major current goals of rootstock breeding are resistance to *Citrus tristeza virus* (CTV) and *Phytophthora* sp., cold-hardiness in citrus areas as Japan, Florida or New Zealand, scion size-controlling abilities, higher tolerance to calcareous and saline soils in areas with poor-quality water, and resistance to the citrus and the burrowing nematodes, particularly in Florida.

On the other hand, scion breeding is mainly focused in resistance against major pests and diseases that limit fruit commercialization, and in fruit quality aspects. Regarding pests, some of them directly affect the tree and/or produced fruit, as the Mediterranean fruit fly (*Ceratitis capitata*), spider mites (*Tetranychus urticae*) and the California red scale (*Aonidiella aurantii*), and others are vectors of diseases such as the psyllid *Diaphorina citri*, transmitting the bacteria *Candidatus* Liberibacter spp., or the aphid *Toxoptera citricidus*, a very effective vector of CTV. At the present, measures used to control pests of citrus are fundamentally aggressive agrochemical treatments, and they do not pose a lasting solution, neither economically or ecologically sustainable at medium-term. Diseases that cause considerable damage in orchards include Huanglongbing (HLB, *ex* citrus greening) in Asia, South Africa and recently in North and South America, Citrus canker in most tropical and subtropical areas and Citrus black spot in tropical and subtropical climates (NRC 2000, 2010). Moreover, post-harvest diseases also affect fruit commercialization, being Green mold the most widespread on citrus. All these diseases cause important economic losses and the lack of efficient means of control against some of them poses a serious threat to the current citrus industry. In this context, resistance to biotic stresses becomes a major goal on genetic improvement of citrus varieties.

Improving fruit quality is also an important objective in scion breeding programs. In relation with specific market demands, the main goal of breeding may vary between the production areas. However, some general trends can be outlined. For juice processing, prime goals are high productivity, high juice content of the fruit, good juice color, lack of bitterness, and availability of juice along the whole year (Ollitrault, et al., 2007; Peña, et al., 2008). For the fresh fruit market, major goals include improvement of organoleptic qualities (attractive color and taste/aroma, compensated acid/sugar content) and pomological qualities (easy peeling, seedlessness, external appearance, adequate size, good storage and shipment) of fruits (Roose, et al., 2002; Navarro, et al., 2005; Aleza, et al., 2010). Besides this interest on new fruit varieties with improved functional attributes in the form of organoleptic, chemical, and physical properties, recently, more attention has been paid to the improvement of the nutri-functional quality of citrus fruits. As a result of World Health Organization recommendations, nowadays, consumers demand high sensory, nutritional and health-related qualities of fruit and their derivative products. The health benefits of fresh citrus fruit have been the subject of extensive research and it is well established that some of their phytonutrients promote health and protection against chronic diseases. The protective effects of citrus fruit have been mainly attributed to the high concentrations of bioactive compounds which have antioxidant properties, such as vitamin C, phenolic compounds and carotenoids (Knekt, et al., 2002; Franke, et al., 2005; Dauchet, et al., 2006; Tripoli, et al., 2007). Therefore, it is no wonder that nutritional

quality based on vitamin C, carotenoid and polyphenol contents are now considered as breeding criteria in some citrus breeding projects (Alquézar, et al., 2009; Sdiri, et al., 2012).

1.3.2. Rationale of transgenic breeding

Conventional breeding by hybridization has important limitations. *Citrus* species have a complex reproductive biology (see paragraph “citrus biology: some clues on growth and development”). Most genotypes are facultative apomictic, and this feature seriously limits the recovery of sexual progeny populations in breeding programs. Some important genotypes have total or partial pollen and/or ovule sterility and cannot be used as parents in breeding programs; for example most Navel oranges are male sterile while satsuma mandarins and most Navel and Valencia oranges are female sterile. There are many cases of cross- and self-incompatibility. Clementines, grapefruits and certain important lemons are self-incompatible, and many hybrids between self-incompatible cultivars are also cross-incompatible. They have a long juvenile period and most species need at least 5 years to start flowering in subtropical areas, and usually several years more to achieve fully mature characteristics. Citrus have high heterozigosity, there is a lack of basic knowledge about how the most important horticultural traits are inherited, and they show quantitative inheritance of important characters, as many related to fruit quality and maturity time. All these features together with their large plant size have greatly impeded genetic improvement of citrus through conventional breeding methods (Peña, et al., 2008). Moreover, sources of efficient resistance against important pathogens such as *Candidatus* Liberibacter asiaticus (causal agent of Huanglongbing) have not been found in the citrus germplasm (NRC, 2010).

Genetic transformation offers excellent alternatives for genetic improvement of citrus because it is based in the introduction of specific traits into known genotypes without altering their genetic elite background. Therefore, theoretically, it should be possible to create desired phenotypes with greater precision and efficiency than with other breeding methods. Further, the transgene of interest could come from another *Citrus* species or relatives, from another plant species, or from another organism as a bacterium, an insect or a virus, widening the possibilities for genetic improvement. Moreover, genetic transformation allows overcoming the heterozygosis, inbreeding depression, linkage drag and genetic incompatibility barriers associated to hybridization. Facultative apomixis, in the context of genetic transformation, is an advantage because it could be possible to use vigorous juvenile material genetically identical to the elite mature germplasm as source of plant tissue for transformation. More important, sweet orange was the first fruit tree from which adult material was transformed (Cervera, et al., 1998) providing the only biotechnology-based system able to overcome the juvenility obstacle of citrus breeding.

1.3.3. Potential applications of genetic engineering in the improvement of citrus scions

The development of viable genetically-modified (GM) citrus varieties could be a slow, expensive, and time-consuming process, but its advantages are many. Although there are technical, economic, regulatory, and market hurdles in the use of genetic engineering in citrus culture, the potential is tremendous, particularly for generating disease- and insect/pest-resistant GM citrus varieties. In this sense, it would be of great interest to obtain HLB-resistant varieties because of the threat that this disease poses to the citrus industry worldwide, and the use of genetic transformation is one of the strategies proposed by the National Research Council to accomplish it (NRC, 2010). But, to date, as for defense against biotic stress is concerned, the most promising results are those achieved by Rodríguez, et al. (2011). In this study, D-limonene production, which represents up to 97% of total volatile organic compounds (VOCs) in orange fruit peel, has been successfully downregulated in mature sweet orange plants (*C. sinensis*, cv. Navelina) by overexpressing an antisense construct of a D-limonene synthase gene. Transgenic orange fruit peels with up to 85 times reduced D-limonene accumulation were less attractant to males of the citrus pest medfly (*Ceratitis capitata, Diptera:* Tephritidae) and strongly resistant to fungal and bacterial pathogens (in concrete to *Penicillium digitatum* and *Xanthomonas citri* subsp. *citri*). This work illustrates how fruit VOCs emissions can be manipulated in citrus cultivars providing novel strategies for pest and disease management without altering important agronomic traits (**Figure 1**). Soler, et al. (2012) have used RNAi to block the expression of the three silencing suppressor protein from *Citrus tristeza virus* and thus get strong resistance in Mexican lime transgenic scions.

Regarding fruit quality aspects, genetic transformation can be very helpful in improving existing fresh and processed citrus varieties in a number of ways. Recent developments in the fields of biotechnology, biochemistry, and molecular genetics have opened up avenues for creating genetically modified citrus cultivars with better organoleptic qualities (appearance, flavor, seedless, and firmness), higher nutritive value (vitamin content), and physiological benefits (reduced respiration rate or increased wax deposition for reduced water loss) (Koltunow, et al., 2000; Ikoma, 2001; Sanchez-Ballesta, et al., 2001; Wong, et al., 2001; Costa, et al., 2002; Li, et al., 2002, 2003; Alquézar, et al., 2008).

Seedlessness is one of the most important economic traits relating to fruit quality for fresh-fruit marketing oranges and mandarins, and it is also desirable for the juice industry because of the unfavourable aromatic compounds associated with the presence of seeds in the fruit (Ollitrault, et al., 2008). The presence of a large number of seeds in citrus fruits greatly decreases consumer acceptability, even in fruits with high organoleptic quality (Navarro, et al., 2005). Inducing parthenocarpy by genetic engineering and a seed-ablated strategy by expressing the cytotoxin gene (*Barnase*) are the major methods of molecular breeding of seedless citrus. Li, et al. (2002, 2003) reported the generation of 'Ponkan' and 'Valencia' sweet orange transgenic plants, respectively, through *Agrobacterium*-mediated transformation of

embryogenic calluses with a chimeric ribonuclease gene (*barnase*) derived from *Bacillus amyloliquefaciens* under the control of an anther tapetum-specific promoter (pTA29). The aim of the work was to produce pollen sterile transformants, and subsequently seedless fruit. More than 20 lines from each genotype were generated. Since transformants were juvenile, several years of cultivation are needed to evaluate possible male sterility. The same can be applied in part for (Koltunow, et al., 2000), who produced juvenile transgenic Mexican limes containing genes for decreased seed set. But, although the juvenile period of Mexican lime is one of the shortest among citrus types, to our knowledge, there are not published data on the phenotype of the mature plants and their fruits.

The quality of citrus fruits can also be substantially improved through the enhancement of levels of certain secondary metabolites, such as carotenoids. For many years, the interest of food researchers and biotechnologists in carotenoids resided largely in the fact that they imparted the yellow, orange or red colors of many foods and in the provitamin A activity exhibited by some of them. Recently, interest in these isoprenoid pigments has grown considerably because of their probable relation to the prevention and/or protection against serious human health disorders such as cancer, heart disease and macular degeneration, among others, which may be somehow linked to their probable antioxidant properties (Ziegler, 1989; Krinsky, 2001; Fraser and Bramley, 2004; Krinsky and Johnson, 2005; Meléndez-Martínez, et al., 2007). In addition, some of the apocarotenoids, which are products of the catabolism of carotenoids, contribute to the flavor and aroma of flowers and fruits (Auldridge, et al., 2006). The citrus fruits and their products in general are a complex source of carotenoid pigments, with the largest number of them reported for any fruit (Alquézar, et al., 2008). Orange juices undoubtedly stand out among them all for being one of the most globally accepted fruit products and because their consumption is increasing worldwide (Mouly, et al., 1999; Mouly, et al., 1999). Carotenoid engineering is expected to contribute to the development of better-colored citrus fruits/juices with increased nutri-functional attributes by purposely accumulating specific desirable carotenoid compounds (such as β-carotene and/or lycopene). In 2002, Costa, et al. introduced several carotenoid biosynthetic genes under the control of constitutively expressed promoters into juvenile Duncan grapefruit and few PCR-positive plants were obtained. Authors noticed that transgenic plants appeared to have increased pigmentation in the leaves compared to controls, but other than that plants were not analysed further. This work constitutes the only attempt to modify carotenoid content in citrus reported to date and no data has been published on fruit production from these plants. However, recent advances in the identification and isolation of the genes responsible for carotenogenesis in citrus fruits (Kato, et al., 2004; Alquézar, et al., 2008) and the development of genetic transformation procedures for this crop type (Peña, et al., 2008) enable the production of novel and improved carotenoid-enriched citrus fruits via metabolic engineering of carotenoid biosynthesis.

Resistance against abiotic stresses could also be addressed in citrus varieties by transgenic approaches. For example, studies to understand the molecular mechanisms and changes underlying resistance to chilling injury in some citrus fruits after certain treatments may

lead to developing genetically engineered, low temperature-resistant cultivars (Sanchez-Ballesta, et al., 2006). Similarly, the study of molecular changes and the mechanisms of maturation and senescence may lead to developing genetically modified cultivars with slower maturation and better qualities (Yang, et al., 2011). For example, the enzymes involved in depolymerization of cell wall components could be theorically modulated. Polygalacturonase (PG) is the major enzyme responsible for the depolymerization of cell walls and the softening of fruit tissues. Inhibition of the expression of an endogenous gene encoding PG, by antisense or RNAi mechanisms, in transgenic citrus might be useful for that.

Furthermore, some metabolic pathways can be modified to get rid of certain problems related to fruit flavor and/or physico-chemical properties of juices. Research on the creation of transgenic citrus trees that produce fruits free of the limonoid bitterness problem (Manners, 2007), and the attempts to preventing juice cloud separation by modifying the expression of a pectin methylesterase gene (*Cs-PME4*) (Guo, et al., 2005) are good examples to illustrate this.

These are only a few examples of potential applications of genetic engineering in improving citrus varieties. The potential is great but its realization depends on our understanding of these (and others) desirable traits at the biochemical and genetic level. The modern tools of molecular biology are expected to throw more light on the functions of enzymes, their pathways, and the genes controlling them, broadening the range of possibilities for improvement. Moreover, despite that a consumers' reluctance to buy genetically modified foods has been reported (Vardi, et al., 2008), recent market studies showed that a higher-quality fruit bringing tangible value to the consumer could improve the market acceptance of biotech citrus crops (Rommens, 2010; Cressey, 2013). The cost/benefit ratio will be the determining factor as to whether transgenic or GM citrus is going to be a commercial reality. Therefore, although there are no commercial GM citrus crops yet, genetic transformation is considered an essential tool in many current improvement programmes.

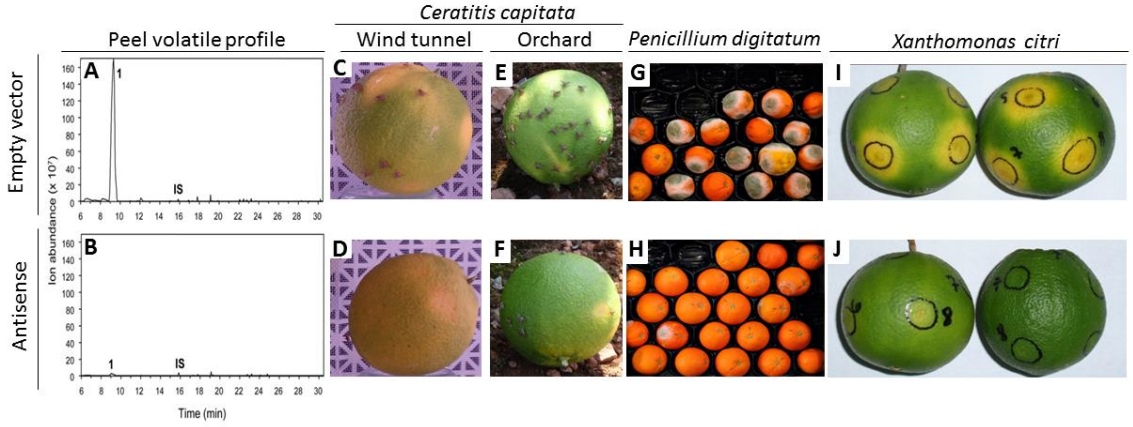

Figure 1. Incresed defense against biotic stresses achieved by D-limonene downregulation in transgenic orange fruits (AS) compared to the control oranges (EV) A, **B**: Representative total ion chromatograms of the volatile profile for orange fruit flavedo from EV (**A**) and AS transgenic plants (**B**). Peaks number one and IS correspond to limonene and the internal standard (2-octanol), respectively. **C-J**: Empty vector and antisense transgenic fruits challenged with medfly (**C-F**) in wind tunnel assays (**C**, **D**) and in the orchard (**E**, **F**), *Penicillium digitatum* (**G**, **H**) and *Xanthomonas citri* subsp. *citri* (**I**, **J**) infection. Rodríuez *et al*., 2011.

2. Risk and concerns related to the field-release and commercialization of GM trees

Although the commercial production of transgenic annual plants is a reality, commercialization of GM fruit trees is still uncommon (Petri and Burgos, 2005). Currently about 20 different fruit tree species have been modified through modern biotechnology, mainly through the insertion of transgenes, and have been introduced into the environment for field trials (Verwer, et al., 2010). However, only very few of these are the objects of commercially-relevant research and development. The majority of these GM fruit trees are commonly planted, commercial species, which were modified in an attempt to improve traits related to growth rates, flowering, resistance to pests and diseases, or abiotic stress tolerance. GM apples, citrus, and papaya make up most of the fruit trees approved for field trials (it should be mentioned that the papaya is a woody annual plant, not a tree in a strict sense, but many authors consider it as such due to similarities in many aspects) (Hanke and Flachowsky, 2010). Two diferent types of fruit trees, virus-resistant papaya and plum, have been approved for commercialization (in the United States - see http://www.isb.vt.edu/search-petition-data.aspx). GM papaya has been used for more than a decade in the USA, where it makes up approximately 90% of trees grown in Hawaii, the main producing region.

Despite the fact that the advances provided by genetically engineered fruit trees may be significant (Peña and Séguin, 2001), their release and commercialization still raises concerns about their safety and validity. On the one hand, some key issues of concern to biotechnologists such as transgene efficacy and its stability over the time, as well as presence of undesired alterations in the tree performance (other than those related with the target trait) must be addressed in GM trees to validate its commercialization. On the other hand, the potential environmental risks that GM trees may pose are also matter of concern. Of all biotechnology methods, genetic engineering has received the most attention and scrutiny by regulators and the general public (often unjustifiably). Consequently, GM trees (and all transgenic plants) are required to undergo thorough and rigorous safety and risk assessments before commercialization. Regulatory justifications for these assessments differ between countries, although they usually require similar tests. In the US, for example, the process is based on the determination of substantial equivalence, whereas Europe has passed regulations based more on certification of the process rather than of the product, and Canada regulates the product itself, irrespective of the process used to generate it. In the US and Europe no such formal assessment is required for products obtained with conventional methods. In Europe, transgenic plants are subject to special regulations including a horizontal directive (EC, 2001) that commences from research and development through release onto the market, and vertical rules governing specific areas including food safety and traceability (EU Regulation 1829/2003).

A risk can be defined as a function of the probability of a negative effect occurring and its seriousness (Burdon and Walter, 2004). A generally accepted methodology for biotechnology risk assessment has been outlined in several easily accessible documents including the

International Technical Guidelines for Safety in Biotechnology (UNEP, 1996), the Cartagena Protocol on Biosafety to the Convention on Biological Diversity, and EC Directive 2001/18/EEC (EC, 2001). Each of these include the following steps that, together, identify potential impacts and assess the risks: 1) Identify potential adverse effects on human health and/or the environment; 2) Estimate the likelihood of these adverse effects being realized; 3) Evaluate the consequences should the identified effects be realized (the risk); 4) Consider appropriate risk-management strategies; 5) Estimate the overall potential environmental impact, including a consideration of potential impacts that may be beneficial to human health or the environment. To accomplish the first step of the risk assessment process it is necessary to identify potential differences of transgenic plants with their non-engineered counterpart(s) by performing a comparative analysis (substantial equivalence). Then, the risk assessment process requires clear identification of any differences between the transgenic and non-transgenic crop(s), including management and usage, and is meant to focus on the significance and implications of any differences (EFSA, 2004).

Cultivation of fruit trees is in many aspects different from cultivation of crop species and such differences should be taken into consideration for risk assessment. Trees can be distinguished from annual crop plants by their long lifespan and delayed onset of reproduction. However, these characteristics are also shared with a number of non-trees such as perennial grasses and shrubs, which can also live for many years and may delay reproduction for one or several years. The juvenile phase of a tree, on which only vegetative propagation is possible, may extend from a few years to several decades. Due to their large size, trees often have high fecundity (in biology, fecundity is defined as the *potential* reproductive capacity of an individual or population, measured by the number of gametes (eggs), seed set, or asexual propagules).Trees from highly seasonal climates often show seed dormancy, and most trees, as well as many annual and perennial herbaceous species, can spread by vegetative reproduction. Cloning of trees through vegetative reproduction enables “instant domestication”, in that unique genotypes, hybrids, or mutants can be immediately grown on a large scale even if they have low sexual fertility (White, et al., 2007). With the exception of effectively sterile crops such as banana, all cultivated plant species, including fruit trees, can establish in both cultivated and wild environments either via pollination of wild relatives or naturalization of seedlings (Ellstrand, 2003).

Thus, in broad terms, the main risks and concerns related to GM fruit trees are their potential environmental impact of transgene dispersal, the long-time transgene stability and the occurrence of unintended effects of the transgene in the long period they are going to be grown in the field under variable environmental conditions (Wolfenbarger and Phifer, 2000). However, it is important to note that the specific risks may vary dramatically depending on the gene and trait inserted, the fruit tree species, and the environment the tree is living in, so field trials are essential for (case-by-case) evaluation of the value and environmental safety of GM trees.

2.1. Transgene dispersal

Although vegetative reproduction is the most common way of propagation of fruit trees in agriculture, these species are generally also able to reproduce and disseminate via pollen and seeds Trees often produce large amounts of pollen and seed per individual that are often designed to spread far and wide (e.g., Williams, 2010). All fruit tree species are entomophilous, and some studies on pollinator foraging range have reported the (ocasional) occurrence of long-distance flights. In addition, a number of fruit trees, as well as many species of annual and perennial plants, have developed seeds that are capable of remaining dormant for a very long period of time (Roloff, 2004). Seeds inside fruits may travel as commodities around the globe and be released at the place of consumption such as road margins, railways or touristic areas, as well as in farmers' fields and local gardens (OECD, 2010). However, movement of seedy fruits into natural areas (mediated by frugivores) is the most common way of seed dissemination and, whether soil and environmental conditions are the adequate, it may lead to the naturalization of the cultivated species (a phenomenon widely spread, mostly in fruit trees and shrubs).

For tree species relying on pollinators and frugivores as dispersal vectors, reported dispersal patterns are dominated by short-distance movements (Levin, 1981). However, pollinators and frugivores can remove large amounts of pollen and fruits, a fraction of which may be deposited several hundreds of meters away from the source tree (Handel, 1983; Godoy and Jordano, 2001; Jordano, et al., 2007; Pasquet, et al., 2008). In GM crops, such undesired long-distance gene flow has already caused legal problems in bentgrass, alfalfa, and sugar beets. In recent litigation involving alfalfa and sugar beets, courts have ruled that failure to intensively consider economic impacts associated with gene dispersal violates the National Environmental Policy Act (e.g., Endres and Redick (2008)). Thus, a precedent exists for similar controversies due to gene flow in annual GM crops. But, it is important to mention that in those specific cases there exist the risk of hybridization with wild species already present in the specific areas of cultivation of the GM crop, and this fact explains the specific concern and the special regulation.

According to the Cartagena Protocol, risks associated with GMOs or products thereof should be considered in the context of the risks posed by the non-modified recipients or parental organisms in the likely potential receiving environment. However, the identification and characterisation of likely potential receiving environment will be highly dependent on the species in question and its mechanisms for dispersal. As detailed below, some aspects of the reproductive biology of a given GM tree species are critical to determine the extent of dispersal of transgenes (e.g., fertility, cross-compatibility with sympatric tree species, degree of selfing, date and amount of bloom, viability and longevity of pollen and/or seeds, etc.) (Poppy and Wilkinson, 2005). Additionally, the level of dependence on human intervention for their survival heavily influences their invasive capacity. Regarding the mechanisms of dispersal, the different possibilities and points to consider in each case are detailed below.

2.1.1. By seeds

Many different dispersal agents of seeds exist depending on the fruit tree species in question. As the kind of dispersal agent greatly influences on the degree of transgene dispersal (understood as the maximum distance and frequency of dispersal) (Jordano, et al., 2007), it should be taken into account when designing appropriate containment measures.

With respect to the risk assessment of GM fruit trees, seed-mediated crop-to-crop transgene flow is not relevant. In the incidental case that transgenic seedlings could germinate in a commercial plantation, they would be removed by farmers (as it is usually done with any adventitious seedling/weed) before flowering because of the long juvenile period of trees. On the contrary, seed-mediated crop-to-wild transgene flow from GM fruit trees could be relevant in terms of environmental safety. The main risk is that a GM tree becomes naturalized as consequence of the successful establishment of volunteer populations in the wild. But, for this to happen, movement of transgenic seeds is not enough; seeds must remain viable, find a suitable environment to germinate and, later, to establish in an effective manner over other organisms that are already present in the invaded ecosystem.

Therefore, there are some points to consider regarding dispersal of seed when assessing risks of GM trees:

- Frugivores consuming the fruit and dispersing the seed
- Seed viability and seedling rusticity
- Surrounding biotic and abiotic environment

2.1.2. By pollen

There are several risks arising from pollen-mediated crop-to-crop transgene flow from GM fruit trees. On the one hand, the adventitious presence of transgenic seeds in the fruit of other commercial plantations (as a result of efective cross-pollination with the GM pollen source) poses an environmental hazard because it creates new "uncontrolled" GM material that could expand the possibility of dispersion of transgenes to the wild by seeds (if this were an issue; addressed in the previous section). On the other hand, in the specific case of fruit tree crops, the ocurrence of GM seeds in non-GM trees could cause problems related to consumer acceptance, and it may have implications on the marketability of the fruit, especially if organic fruit-growing orchards are exposed (Bock, et al., 2002).

Pollen-mediated crop-to-wild also posses an environmental risk. The possibility that transgenic plants would hybridize with wild-type plants, is one of the most frequently mentioned risks among genetically modified plants (Mathews and Campbell, 2000; Conner, et al., 2003). But, it should be noted that the pollen movement *per se* does not constitutes a gene flow. There must be a set of circumstances to hybridization occurs. A prerequisite is the existence of sympatric wild-relatives that are cross-compatible and flowering-synchronized. This condition is not always given, for example, in almost all citrus-production areas of the world there are

virtually no wild sympatric citrus species and relatives (Peña, et al., 2008). Moreover, there are many other different factors affecting pollen dispersal and cross-pollination, such as type of dispersal agent (e.g. animal vectors), size and density of pollen source and sink, environmental factors (weather, local environment, physical barriers), pollen viability and competitive ability, and level of outbreeding in the specific plant and its wild and cultivated relatives (Wilcock and Neiland, 2002; Poppy and Wilkinson, 2005). As all these parameters ultimately determine the extent of pollen-mediated gene flow, they should be considered when assessing risk of hybridization of GM trees with other fruit tree species/relatives in the wild.

2.1.3. Containement measures

The propensity for gene dispersal in fruit trees has prompted considerable effort to develop containment strategies on GM trees aimed at prevent the escape of transgenes into natural ecosystems and surrounding orchards from cross-compatible crops.

Risk management strategies designed for GM trees will vary significantly depending on whether the GM tree under consideration is a forest/plantation tree or a fruit tree. On the one hand, risk management for forest or plantation trees may rely on strategies for delaying or avoiding flowering (e.g. fast-growing trees for lumber production being cut before reaching the reproductive phase) and bioconfinement (e.g. induction of male sterility or flower ablation) where dispersal poses serious legal or ecological risks. On the other hand, fruit trees may have to rely on different strategies for confinement than those indicated, since flowering and pollination are crucial for fruit production. For fruit tree species, only crop management estrategies are posed for transgene containment, including careful site selection before planting, use of buffer tree rows or spacings with other crops, cultivation under screehouses, use of flower bags to avoid bee pollination, etc.. However, some of the containment strategies are unrealistic (Traynor, et al., 2002). They often lack scientific foundation and/or are impractical from an agronomic point of view (i. g., excesive safety distances). Therefore, in these cases, conducting field trials aimed at studying factors that influence transgene flow is essential to design suitable case-by-case containment measures.

Another important aspect to consider when designing containment strategies is the effect(s) of the introduced trait. The biological effect(s) of a given new trait (either target effects or unintented effects -theme developed in the next section-) influences the biological consequences of dispersal. For example, transgenes conferring resistance to abiotic or biotic (pests, diseases, and herbicides) stresses could provide enhanced fitness, survival and spread to the GM crop and hybrids, making them more prone to naturalization (Ellstrand, 2001; Strauss, et al., 2010). However, in this regard, it is important to note that the creation of new traits in crop plants is an inherent feature of plant breeding, irrespective of the method of improvement used. Nevertheless, unlike plants created by other improvement methods, transgenic crops are carefully assessed for biosafety, nutritional equivalence and environmental

impact prior to allowing field release on a commercial scale (EFSA, 2004; NRC, 2004 Rommens, 2010).

Finally, the value (benefits) of the introduced trait, and the characteristics of the test environment (e .g., proximity and weediness of wild relatives), are also important in decisions about regulation and data collection (Strauss, 2003).

2.2. Unintended effects of transgenes. Importance of pleiotropic effects

Genetic engineering introduces novel or modified traits that could have unintended effects. The occurrence of unintended effects is not a phenomenon specific to genetic engineering. In classical breeding programmes, extensive backcrossing procedures are applied in order to remove unintended effects. Multiple mutations with diverse pleiotropic (that is, collateral) effects can be induced by irradiation or chemical mutagenesis, providing ample opportunity for unexpected consequences to occur (NRC, 2004). Other intensive breeding methods that are routinely used, such as intervarietal hybrids, wide interspecies crosses, inbreeding, ploidy modification and tissue culture, produce abundant pleiotropic effects on gene structure and trait expression in plants (Ozcan, et al., 2001). In these cases, subsequent selection has been almost entirely made on the basis of phenotypic characteristics, generally without any knowledge of the underlying genomic changes causing the phenotype.

However the occurrence and implications of potential unintended effects of the transgene(s) are often cause for concern, and their study is an essential part of the risk assessment of GM fruit trees (and GM plants in general). According to their nature, unintended effects of transgenes can be classified into two main classes: locus-dependent (also called event-specific) and locus-independent (commonly known as pleiotropic).

2.2.1. Event-specific unintended effects: Position and insertion effects

During transformation, foreign DNA integrates randomly into the plant genome (Puchta and Hohn, 1996). Some insertions might inactivate or alter the expression of endogenous genes or interact with different genetic backgrounds (Taylor, 1997; Kappeli and Auberson, 1998), thereby resulting in unexpected consequences (phenomenon commonly known as insertion effect). In addition, different insertion events often vary in transgene expression levels, patterns or stability, which constitutes the so called position effect (Meyer, 1995; Kumar and Fladung, 2001; Schubert, 2004).

In a commercial transgenic variety development program, the event-specific unintended effects are routinely eliminated through phenotypical screening. During the development of transgenic plant varieties and for any given trait(s), a large number of transformants/clones that do not perform up to the required expectations will be discarded through assessment in the laboratory, glasshouse, and small scale field trials. In all cases, new cultivars produced by

genetic engineering are extensively tested and screened prior to commercial release. Evaluations of plant vigour, growth habit, yield, crop quality, and insect and disease susceptibility would be performed.

2.2.2. Pleiotropic effects

Pleiotropy, the condition in which the expression of a single gene affects multiple traits, can cause changes in plant characteristics that are, in most cases, difficult to predict. Pleiotropic changes in plant characteristics such as vegetative and flower development as a result of the transformation process have been reported in several studies (Elkind, et al., 1990; Ahuja and Fladung, 1996; Romero, et al., 1997; Donegan, et al., 1999; Gutiérrez-Campos, et al., 2001; Lemmetyinen, et al., 2004). The pleiotropic effects caused by a specific transgene are locus-independent, thus creating challenges to the risk assessment of genetically modified organisms. There are pleiotropic effects with relevance in terms of environmental impact, which are carefully considered in experimental field trials, as for example the effect of incorporated resistance to pests and diseases on non target organisms. Sometimes, the biological relevance of unintended effects resulting from genetic modification refers to the implications of these effects on the agronomic performance of the plant. Several examples exist showing that, following genetic modification, unintended effects can have an impact on potential agronomic performance (Cellini, et al., 2004). Some of those phenotypes are obviously detrimental to any further commercial development of the transgenic lines in question. For example, the capacity for fructan biosynthesis, when introduced into the cytoplasm of potato tuber cells, results in transformants with impaired carbohydrate transport and perturbed tuber development (Dueck, et al., 1998; Turk and Smeekens, 1999). Some other pleiotropic effects do not completely prevent the commercialization of the crop, but they reduce their marketability to some extent. For example, attempts to increase food quality through metabolic engineering often compromise the yield potential of the targeted crop (Tanaka and Ohmiya, 2008; Ufaz and Galili, 2008). In the case of GM trees, several detrimental pleiotropic effects with agronomic relevance have been reported. Constitutive expression in apple of genes encoding a strongly antifungal endochitinase from the mycoparasitic fungus *Trichoderma harzianum* resulted in some level of resistance against the fungus *Venturia inaequalis* (causal agent of apple scab), but also in a reduction in plant vigor/growth (Bolar, et al., 2000). Another study showed that the overexpression of floral homeotic genes from forest tree species, as a strategy to genetically induce male or female sterility, caused adverse pleiotropic alterations to vegetative development of transgenic trees (Rottmann, et al., 2000). Obviously, these traits would never enter the commercialization phase.

The possible ocurrence of pleiotropic effects with biological relevance (in terms of enviromental concerns or agronomic performance) must be taken into account when assessing risk and validity of the GM trees. Moreover, in some cases, pleiotropic effects are only manifested under determinated environmental conditions (Zeller, et al., 2010). Then, the only

way to study the possibility of unintended effects on the environment derived from GM plantings in the open, and evaluate transgenic trees under agronomic conditions, is through field trials.

2.3. Transgene stability over time

Some frequently mentioned issues of GM plants that are closely related to their validity on a commercial basis include the instability of transgene expression, especially in long-lived species (Van Frankenhuyzen and Beardmore, 2004). Several studies on transgenic trees have found that the transgenic traits can be less stable than originally thought (Petri and Burgos, 2005). Fluctuations in transgene expression in trees have been observed and often correspond to the metabolic state of the cells and tissues (Levée, et al., 1999; Cervera, et al., 2000). In many cases, transgene silencing has also been reported. For example, introduction of transgenes encoding caffeic acid *O*-methyltransferase in aspen and poplar plants sometimes resulted in a loss of expression of the transgene and the homologous endogenous gene by the silencing phenomenon of cosuppression (Jouanin, et al., 2000). Consequently, transgene instability including those causing gene silencing and variable expression levels during the long lifespan of trees an important consideration (Ahuja, 2009; Harfouche, et al., 2011). There is also evidence that gene/environment interactions play an important role for expression level of the transgenes (Strauss, et al., 2004), which stresses again the importance of assessing trait efficacy under field conditions similar to real cultivation.

Although some cases of transgene instability have been reported (Dominguez, et al., 2002), the level of molecular and phenotypic instability has been shown to be quite low in a number of multi-year studies with GM trees (reviewed by Walter, et al. (2010); Brunner, et al., (2007)); see also four publications (Li, et al., 2008; Li, et al., 2009). Recent studies have shown a high stability of the transgenes expression in trees during a short period of time (2 to 4 years) in plants cultivated *in vitro*, the greenhouse or in the field (Charity, et al., 2005; Flachowsky, et al., 2008). Short-term and mid-term studies with reduced lignin, herbicide and insect resistance GE trees, in particular poplars, have been encouraging with regard to stability of transgene expression under field conditions (Lachance, et al., 2007). Further, long-term transgene stability has also been reported in transgenic prunus (Maghuly, et al., 2006) and apple (Borejsza-Wysocka, et al., 2010) trees. In this last work, Borejsza-Wysocka et al. demonstrated the stable integration and expression of a transgene (*attacin E*) in apple for more than 12 years under orchard conditions. Expression of this gene resulted in an increase in resistance to fire blight throughout these years and had no effect on tree morphology, fruit morphology or internal fruit quality characteristics.

2.4. Conclusion and future prospects

Possible benefits of transgenic fruit trees are associated with increasing economic efficiency of agriculture, and they can also provide important benefits such as reduced use of

pesticides and improved quality of the fruit. The commercialization of GM trees is still in the distant future because the GM research has not progresses as far as that on crop plants, and there are a number of unresolved biosafety, environmental and regulatory obstacles (Jaffe, 2004; Hoenicka and Fladung, 2006; Finstad, et al., 2007; Groover, 2007; Sederoff, 2007). These concerns are based on the endogenous behavior of the transgene (stability, and interaction with other genes in the host genome in space and time), and exogenous effects of the transgene (dispersal of pollen and seed) on the ecosystem. These biosafety and regulatory concerns must be addressed before commercialization of GM trees. Moving beyond a greenhouse to outdoor studies is essential to understand the ecological impact and agricultural value of newly inserted genes and traits. In this regard, design of field trials and whether these would be conducted for a period that is long enough to reveal the differences in the GM trees as compared to the non-modified trees (e.g. after exposure to multiple biotic and abiotic stresses) are important considerations. These should logically follow the periods of time and practices of conventional breeding programs for the unmodified species.

3. The contribution of plants in promoting human health

3.1. Citrus and health: nutri-functional attributes of oranges

Besides for its pleasant flavor, citrus fruits are prized by consumers for its nutritional value and healthy properties. Citrus fruit or juice can be an excellent source of macro- and micro- nutrients, as well as other health-promoting substances (Economos and Clay, 1999). The amount of these compounds varies depending on the specific citrus variety. Other factors that influence citrus fruit composition are rootstock, fruit size, maturity, storage, horticultural conditions, and climate (Kefford and Chandler, 1970). In addition, different processing procedures with capabilities to adjust extraction and homogenization pressures as well as pasteurization affect juice composition (Betoret, et al., 2012). The recommended dietary allowance for average adults in terms of nutrients available in oranges is given in **Table 1**. From a nutritional standpoint, it is noteworthy the low content in fat (and in overall dietary energy) of orange fruit, in part due to its high water content. This is a major consideration given the increasing rate of obesity in developed countries in both adults and children. As well as being low in fat and energy, orange fruits are free of sodium and cholesterol, and contain a wide range of naturally occurring vitamins, minerals and non-nutrient phytochemicals that have been shown to have beneficial effects on health. Notable amongst these active ingredients are vitamin C, carotenoids, folate, potassium, calcium, fibre, polyphenols, coumarins and monoterpenes and, to a lesser extent, phytosterols (Liu, et al., 2012). These substances are necessary for proper functioning of the body but some may confer additional protection against chronic disease over and above basic nutrition (Silalahi, 2002; Knekt, et al., 2002; Liu, 2003; Yao, et al., 2004; Key, 2011).

Table 1. Nutrient content of orange fruit

Nutrient	Nutritional value per 100 g of edible portion	% RDA
Energy	197 kJ (47 kcal)	
Carbohydrates	11.75 g	
- Sugar	9.35 g	
- Dietary Fiber	2.4 g	
Total lipid (fat)	0.12 g	
Protein	0.94 g	
Water	86.75 g	
Vitamins		
Vitamin C	53.2 mg	60%
Thiamin (vit. B_1)	0.087 mg	8%
Riboflavin (vit. B_2)	0.04 mg	3%
Niacin (vit. B_3)	0.282 mg	2%
Pantothenic acid (B_5)	0.25 mg	5%
Vitamin B_6	0.06 mg	5%
Folate (vit. B_9)	30 µg	8%
Choline	8.4 mg	2%
Vitamin A equiv. (RAE)	11 µg	1%
β-carotene	71 µg	~
α-carotene	11 µg	~
β-cryptoxanthin	116 µg	~
Vitamin E	0.18 mg	1%
Minerals		
Calcium, Ca	40 mg	4%
Iron, Fe	0.1 mg	1%
Magnesium, Mg	10 mg	3%
Phosphorus, P	14 mg	2%
Potassium, K	181 mg	4%
Sodium, Na	0 mg	0%
Zinc, Zn	0.07 mg	1%
Manganese, Mn	0.025 mg	1%
Non-nutrient phytochemicals		
Carotenoids (non provit. A)		
Lutein + zeaxanthin	129 µg	~
Flavanones		
- Hesperetin	27.2 mg	~
- Naringenin	15.3 mg	~

Source: Nutrient data for this listing was provided by USDA SR-26 (Oranges raw, all commercial varieties). Percentages of RDA are roughly approximated using US recommendations for adults. Each "~" indicates a missing or incomplete value.

Indeed, the health benefits of fresh citrus fruit, including oranges, have been the subject of extensive research (epidemiological studies and other investigations) and it is well established that its consumption promotes health and protection against chronic and degenerative diseases (Franke, et al., 2005). In particular, the inhibition of breast cancer cell proliferation (So, et al., 1996), decrease of colon tumorigenesis (Miyagi, et al., 2000) and

antimutagenic properties (Franke, et al., 2006) have been evidenced in cell culture and animal models. Moreover, orange consumption has been associated with a lower risk of acute coronary events and stroke (Johnsen, et al., 2003; Dauchet, et al., 2004). Clinical results indicate that consumption of orange juice reduces oxidative DNA damage in blood cells (Guarnieri, et al., 2007) and improves plasma concentrations of markers of inflammation and oxidative stress (Johnston, et al., 2003; Sánchez-Moreno, et al., 2003; Ghanim, et al., 2010). Consumption of orange juice also improves lipemia in patients who have undergone coronary bypass surgery (Kurowska, et al., 2000). In addition, eating oranges can ward off age-related macular degeneration (Gale, et al., 2003) and cataracts (Taylor, et al., 2002; Zhou, et al., 2011).

The protective effects of citrus fruit have been mainly attributed to the presence of bioactive compounds which have antioxidant properties. The antioxidant and antiradical activities of citrus fruit are mainly due to the hydrosoluble fraction containing polyphenols and vitamin C and also to the apolar fraction including carotenoids, leading to their protective effects against chronic disorders, especially in at-risk populations with low antioxidant status (Gorinstein, et al., 2001; Franke, et al., 2005; Tripoli, et al., 2007). However, in some cases, the beneficial effects of orange intake were not explained by the sole presence of certain bioactive compounds alone (Guarnieri, et al., 2007). Then, it is likely that the different components of this particular food matrix influence their health-promoting qualities, either through synergistic interactions or through effects on bioavailability (Liu, 2003).

The main components of orange fruits and their nutritional and/or therapeutic value are detailed below:

Vitamins

Contrary to many other sources of vitamins, a fresh orange or a glass of freshly extracted orange juice provides many vitamins in abundance without any loss by cooking. Vitamin C (also known as L-ascorbic acid or ascorbate) is the most abundant nutrient found in citrus fruit, and it was this component that first raised the health profile of citrus. It is estimated that a glass of orange juice (177.4 ml) or a standard-size orange fruit (corresponding to 180-gram edible portion) provides about 100 percent of the recommended daily allowance of vitamin C to the average human diet (Araujo, 1977) (**Table 1**). Vitamin C, in addition to preventing scurvy (Kaur and Kapoor, 2001) and being an important component of human nutrition, is considered one of the most prevalent antioxidative components of fruits that exert substantial chemopreventive effects without apparent toxicity (Drake, et al., 1996; Lee, et al., 2003). Recent reports suggest that the chemopreventive effects of vitamin C are linked to its protective effects against epigenetic mechanisms such as the inflammation and inhibition of gap-junction intercellular communication as well as its antioxidant activities (Bowie and O'Neill, 2000; Lee, et al., 2002; Wu, et al., 2002).

Other vitamins present in fresh citrus fruits are compounds of the vitamin B complex. Folic acid, folate, and folacin are interchangeable forms of the chemical compound pteroyl glutamic acid; its deficiency is known to cause a type of anemia, being growing children and pregnant women the more sensitive. Folate also reduces neural tube birth defects by up to 75 percent when taken by pregnant women and has been associated with a reduced risk of heart disease by lowering blood serum homosystine levels (Widmer and Stinson, 2000). A standard-size orange fruit (180 g) provides more than 10 percent of the US RDA (United States Recommended Daily Allowances) of 400 µg (**Table 1**). Folacin contains at least one molecule of glutamic acid. As far as absorption is concerned, monoglutamate forms are more absorbable. Since citrus fruits contain monoglutamate forms, they are likely to provide a more absorbable vitamin species than other sources. Folic acid is prone to oxidation and is generally protected in fresh fruit because of the antioxidant property of ascorbic acid (Streiff, 1971). Thiamin (Vitamin B1) content in 100 g of orange fruit is 0.087, which constitutes about 8 percent of the US RDA (**Table 1**). Thiamin increases from about 0.5–0.6 g to about 0.75–0.8 g per gram of juice with maturity in oranges. Similarly, niacin content also increases (Ting, 1977). Citrus fruits are also a source of the B6 vitamins known as pyridoxal, pyridoxamine, and pyridoxine. These are interchangeable in the body. The coenzyme form of the vitamin, i.e. pyridoxal phosphate, is required for the metabolism of amino acids, proteins, and fats in the body. Some types of stomatitis and a type of anemia have been shown to be cured by the administration of pyridoxine. The B6 requirement varies depending on the amount of protein eaten, since this vitamin has a role in protein metabolism. The US RDA has been set at 1.3 mg per day, and orange intake (100 mg) provides about 5% of this nutritional requirement (**Table 1**).

Vitamin A is also present in orange fruits through pro-vitamin A carotenoids, though oranges are not considered a particularly rich source of this vitamin. Vitamin A (retinol, retinal, retinoic acid) has multiple functions: it is important for growth and development, for the maintenance of the immune system and good vision (Combs, 2008; Tanumihardjo, 2011). It can be ingested directly as retinol from animal food sources or in the form of carotenoids with provitamin A activity from plants. The carotenoids β-carotene, α-carotene, γ-carotene, and β-cryptoxanthin (all them containing beta-ionone rings), but no other carotenoids, function as provitamin A in herbivores and omnivore animals which possess the enzyme 15-15'-dioxygenase. This enzyme cleaves provitamin A-carotenoids in the intestinal mucosa and converts them to retinol (Biesalski, et al., 2007). The efficacy of this conversion *in vivo* has been established for each provitamin A carotenoid and it is expressed as Retinol Activity Equivalent (RAE) units. Each µg RAE corresponds to 1 µg retinol, 2 µg of β-carotene in oil, 12 µg of "dietary" beta-carotene, or 24 µg of the three other dietary provitamin-A carotenoids. Orange fruit contains (per 100 g) 71 µg of β–carotene, 11 µg of α-carotene, and 116 µg of β-cryptoxanthin, which is equivalent to 11 µg RAE in total. That amount of vitamin A barely covers 1% of the US RDA (**Table 1**).

Similarly, Vitamin E, or α–d–Tocopherol has been reported to be present in oranges but in small amounts (Newhall and Ting, 1965) (**Table 1**).

Minerals

Orange fruits have very high potassium content (181 mg per 100 g of fruit), while the sodium content is very low (virtually zero). The ratio of K and Na in oranges plays an important role in maintaining electrolyte balance. Sodium is understood to play a role in water retention and edema. Citrus juices also provide minerals that are part of the vital enzyme system of the human body. Calcium, magnesium, and phosphorus are supplied by oranges, providing about the 4%, 3% and 2% of de US RDA respectively (**Table 1**). Other important minerals such as Fe, Mn and Zn are also provided by oranges, but to a lesser extent. Despite the amounts of these compounds in citrus fruit are relatively low, ascorbic acid and citric acid, both present in large amounts in oranges, can increase the bioavailability of some of them. For example, the citric acid in orange juice may act as chelating agent and thus increase calcium absorption by preventing the formation of insoluble salts. Moreover, a study conducted by Ballot, et al. (1987) showed a close correlation between iron absorption and ascorbic acid content, and a weaker but still significant correlation with the citric acid content. Therefore, the presence of citrus fruit is expected to increase iron absorption markedly in diets low in iron (Nair and Iyengard, 2009).

Dietary Fiber and Pectin

Dietary fiber is the edible part of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine, with complete or partial fermentation in the large intestine. Dietary fibers promote beneficial physiologic effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation. An average-size orange (7–8 cm diameter) can provide 0.8 g of fiber in the diet. Drinking of a cup of fresh orange juice provides 0.3 g of fiber (Church and Church, 1970). Fiber has its own importance for the people of industrialized nations who eat high-fat, low-fiber diets full of highly refined and processed carbohydrates that move slowly through the intestines. In fresh citrus fruit, fiber contains cellulose, hemicellulose, lignin, and pectin – all found in citrus segments, membranes, and other parts of the albedo. Most of these are carbohydrates, except lignin, which is a complex polymer of aromatic compounds linked by propyl units. Cellulose, hemicellulose and lignin, which are water-insoluble fibers, prevent digestive disorders by easing the motion and rapid passage of food through the gastrointestinal tract (Anderson, 1990; Lattimer, 2010). Insoluble fiber is also associated with reduced diabetes risk, but the mechanism by which this occurs is unknown (Weickert, 2008). In addition, evidence exists that fermentable fiber sources improve absorption of minerals, especially calcium (Nishimura, et al., 1992). Lemons, grapefruits, tangerines, and oranges are rich in pectin content. This type of fiber dissolves in water to form a gel-like material that can help decrease blood cholesterol (McCready, 1977). Viscous soluble fibers may also attenuate the absorption of sugar, reduce sugar response after eating, normalize blood lipid levels and, once fermented in the colon, produce short-chain fatty acids as by-products with

wide-ranging beneficial physiological activities (Bolton, 1981; Roth, 2001; Bandera, 2007; Du, et al., 2010). Pectin has been found to significantly inhibit the binding of fibroblast growth factor (FGF-1) to fibroblast growth factor receptor (FGFR1) in the presence of 0.1 μg/ml heparin (Liu, 2001). Kinetic studies have revealed a competitive nature of pectin inhibition with heparin, which is a crucial component of the FGF signal transduction process. Thus, pectins can be effectively utilized as anti-growth factor agents in fibroblasts.

Other non-nutrient phytochemical/nutraceutical compounds: Carotenoids

Carotenoids are lipid-soluble pigments found in all photosynthetic organisms, responsible of the red, yellow and orange colors of a wide number of flowers and fruits of many different plant species (Hirschberg, 2001; DellaPenna and Pogson, 2006). Among the naturally occurring plant pigments, carotenoids (which are isoprenoid compounds) are widely distributed, with a high degree of structural diversity and large variations in biological functions (Schoefs, 2002). There are >600 carotenoids found in nature, with 40 dietary carotenoids regularly consumed in the human diet (Rao and Rao, 2007). The most common carotenoids in North American diets are α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene (USDA, 2008). As mentioned above, one of the most important physiological functions of (some) carotenoids in human nutrition is to act as vitamin A precursors. Apart of this nutritional role, other health benefits attributed to carotenoids include prevention of certain cancers (Seifried, et al., 2003; Tang, et al., 2005), cardiovascular diseases (Granado, et al., 2003) and age-related ocular diseases (Johnson, et al., 2000), as well as enhanced immune system functions (Hughes, 1999; Garcia, et al., 2003; Krinsky and Johnson, 2005). These beneficial effects of carotenoids mainly derive from their potent antioxidant activity, since carotenoids are known to function as free-radical scavengers (Yeum and Russell, 2002). Nevertheless, other non-antioxidant mechanisms (such as induction of gap junctional communication) have been linked to the biological effects of carotenoids (Hanusch, et al., 1997; Bertram, 1999).

Citrus fruits are, in general, a rich and complex source of carotenoids in which up to 110 different carotenes and xanthophylls have been reported, although many of them may be isomers (Meléndez-Martínez, et al., 2007). In citrus fruits, carotenoids are mainly associated with pulp and its particles extracted in the juice; hence too much filtration is likely to remove them from the juice. In the pulp of oranges, carotenoid content differs among cultivars, ranging from 4 to 38 μg/ g FW (Alquézar, et al., 2008). Fruit of most orange varieties accumulates mainly β,β-xanthophylls, being, within these compounds, 9-*cis*-violaxanthin the most abundant carotenoid present in the pulp of the mature fruit (Alquézar, et al., 2008). Multiple health benefits in the protection against eye disease and other chronic diseases have been attributed to this specific group of carotenoids (xanthophylls) (Tanaka, et al., 2000; Kohno, et al., 2001; Abdel-Aal, et al., 2013). However, other nutritionally important carotenoids are scarce in orange fruits: β-carotene content in oranges is low (about 71 μg) compared to many other food sources (see USDA page (USDA, 2008)), and lycopene accumulation is an unusual feature in orange fruits,

since it has only been reported in a few under-utilized mutants (Shara, Cara Cara, Hong Anliu, Mombuca, Puka, Pinhal, etc.).

Other non-nutrient phytochemical/nutraceutical compounds: Phenolics

Citrus fruits are also rich in phenolic compounds including flavonoids, and benzoic and hydroxycinnamic acids, with potential health-promoting properties. These components have been proposed as important contributors to the total antioxidant capacity (Rapisarda, et al., 1999; Burda and Oleszek, 2001). Flavonoids, the most abundant phenolics in citrus fruits (Nogata, et al., 2006), are shown to have many biological functions in antioxidative, anticarcinogenic, cardiovascular, and anti-inflammatory activities (Benavente-García and Castillo, 2008; Huang and Ho, 2010). Health benefits of these flavonoids are probably potentiated by combinations with other phytochemicals occurring in plant foods, particularly carotenoids (Kohno, et al., 2001; Tanaka, et al., 2000). The highest concentrations found in oranges and other citrus fruits correspond to flavanone glycosides, followed by flavones, flavonols and the fully polymethoxylated flavones (PMFs) (Kawaii, et al., 1999; Peterson, et al., 2006). Hesperidin, narirutin, naringin, eriocitrin and neohesperidin are the major flavanone glycosides (Mouly, et al., 1994). PMFs exist exclusively in the *Citrus* genus especially in the peels of mandarins, sweet and sour oranges (Gattuso, et al., 2007). Although citrus juice contains low concentrations of PMFs, these compounds exhibit high biological activity and have been reported as having anti-tumor and anticarcinogenic activity (Murakami, et al., 2000; Du and Chen, 2010). In addition to flavonoids, a major part of phenolic compounds of citrus fruits are benzoic and hydroxycinnamic acids. Previous studies have reported that hydroxycinnamic acids also possess significant antioxidant activity and chemoprotective effects, as shown by *in vitro* and *in vivo* studies (Natella, et al., 1999).

The anthocyanins, a class of flavonoids, which are the pigments of blood oranges (e. g. ‘Tarocco’, ‘Moro’ and ‘Sanguinelli’ sweet orange varieties) also have therapeutic value. It has been observed that the consumption of the juice of blood oranges (cultivar Moro) can modulate the permeability of the blood vessels and induce a protective effect on the gastric mucosa (Saija, et al., 1992). On the basis of studies conducted on rats, this juice is reported to elicit an immuno-stimulatory effect. The juice is desirable because it can act as co-adjuvant in the therapy of some circulatory system pathologies. It also increases the capability to react to unfavorable conditions (possibly infections) promptly. Finally, a recent study showed that dietary supplementation of Moro juice, but not Navelina one (blond orange) significantly reduced fat accumulation in mice. However, authors concluded that the anti-obesity effect observed could not be explained only by its anthocyanin content, suggesting that multiple components present in the Moro orange juice might act synergistically to inhibit fat accumulation (Titta, et al., 2010).

3.2. Metabolic engineering towards development of functional food

Role of phytonutrients with antioxidant capacity on health

Citrus is not the only crop to which health-promoting properties have been attributed. It is generally accepted that plant-derived foods exert some beneficial effects on human health beyond basic nutrition, particularly on defense against age-related diseases. It was the group of Peto (Doll and Peto, 1981; Peto, et al., 1981) who first proposed 30 years ago, based on epidemiological studies, that many of the cancers diagnosed in U.S. could be prevented with an adequate diet, based on the regular consumption of plant-foods rich in certain types of healthy compounds. Nowadays, although the relationship diet/food-health is not completely clarified, there is a wealth of information (based on numerous epidemiological and randomised intervention studies (Calder, et al., 1980; Lindeberg, et al., 2007; Osterdahl, et al., 2007; Frassetto, et al., 2009; Jonsson, et al., 2009) indicating that some of the global burden of chronic disease could be alleviated by adoption of diets containing greater amounts of plant foods. The US Healthy People 2020 (HP2020) objectives (USDA, 2010) recommend that citizens increase their consumption of fruit and vegetables to reduce the risk of morbidity and mortality from type 2 diabetes (Carter, et al., 2010), heart disease (Dauchet, et al., 2006), stroke (He, et al., 2006), obesity (Michimi and Wimberly, 2010; Ledoux, et al., 2011) and cancer (Valdés-Ramos and Benítez-Arciniega, 2007; Boggs, et al., 2010). The 2012 American Cancer Society guidelines recommend consumption of at least 2.5 cups of fruit and vegetables per day as a vital component of a healthy diet (Kushi, et al., 2012). But, although, overall, the literature indicates a protective effect of fruits/vegetables against a number of chronic diseases, the estimates of amounts of food required for benefit are imprecise and the relative importance of the individual components of the fruit/vegetable is not clear. Therefore, in the last decades, interest in the relationship between food and health goes beyond the preventive action of the nutrients in nutritional deficiencies, and the possibility that dietary intervention may significantly decrease incidence of diet-related diseases has catalysed scientific efforts to understand this relationship, which is fundamental to developing future strategies for stemming disease.

Oxidative damage is involved in many chronic diseases including the major causes of death in Western societies such as cardiovascular disorders and cancer (Cheeseman and Slater, 1993). Oxidized LDL contributes to the formation of atherosclerotic lesions and poses an additional oxidant stress that injures smooth muscle and endothelial cells (Berliner, 1996). Adipositiy leads to oxidant stress because intracellular triglycerides cause increased superoxide formation (Bakker, et al., 2000) and stimulate adipocytes or pre-adiposites to produce inflammatory cytokines (Coppack, 2001), which induce formation of various oxidative radicals (Fenster, et al., 2002). Mutagens and carcinogens may act through the generation of free radicals which initiate a series of degenerative processes related to cancer, heart disease, and aging. Antioxidants may prevent these degenerative processes by various mechanisms including scavenging of free radicals. The capacity of some plant-derived food to reduce the risk

of chronic diseases has been associated to the occurrence of many different functional metabolites with antioxidant activity. Essential micronutrients, which are either organic compounds (vitamins) or minerals required in amounts <1 mg/day, act as cofactors or metabolic precursors and are required for specific biological processes, such that insufficient intake results in characteristic deficiency diseases (Zhu, et al., 2007; Gómez-Galera, et al., 2010). But, as well as their requirement for particular metabolic processes, certain essential nutrients present in plant foods also act as antioxidants or promote the activity or availability of antioxidants. A key example of such a 'dual-purpose nutrient' is vitamin A, which is obtained in the diet either as esters of retinol from meat and dairy products or as pro-vitamin A carotenoids such as β–carotene from plants. Vitamin A is converted into the visual pigment rhodopsin (retinal), in the retina of the eye, and acts as a co-regulator of gene expression (retinoic acid); β-carotene is also an antioxidant, as are many other (non-essential) carotenoids. Similarly vitamin C (ascorbate) is an essential cofactor for several enzymes and vitamin E (tocochromanol family) is a regulator of protein kinase activity and gene expression. In all these cases, their potent antioxidant activities are arguably just as important as their essential and non-replaceable functions. Even metal ions, which are usually regarded as pro-oxidants, can be important to maintain antioxidant activity in humans, because they act as cofactors for certain antioxidant enzymes, for example iron is a cofactor for catalase. On the other hand, it is well known that many other non-essential molecules consumed in the diet are also antioxidants with health-promoting effects. These non-nutrient secondary plant metabolites are commonly known as phytochemicals or nutraceuticals. Phytochemicals that are present in the diet and have been associated to reducing the risk of chronic diseases include glucosinolates, sulphur-containing compounds of the Alliaceae, terpenoids (carotenoids, monoterpenes, and phytosterols), and various groups of polyphenols (anthocyanins, flavones, flavan-3-ols, isoflavones, stilbenoids, ellagic acid, etc.). Hence there is an overlap between essential nutrients and non-essential compounds that act as antioxidants. All the functional plant metabolites that have shown a protective effect against degenerative diseases, whether or not essential nutrients, are collectively known as phytonutrients and their bioactivity has been, to some extent, associated to their antioxidant properties. However, the view that such phytonutrients also act on the signaling pathways that respond to reactive oxygen and nitrogen species (RONS) independently of their antioxidant activities, and in this way impact inflammation and the inception of chronic disease, is gaining considerable ground (Traka, et al., 2008; Virgili and Marino, 2008).

Although a growing body of evidence supports the healthy properties of phytonutrients, in most cases, a concrete beneficial effect could not be attributed unequivocally to a particular phytonutrient. Many factors complicate the interpretation of epidemiological studies, which are one of the major sources of evidence for the role of dietary plant secondary metabolites in contributing to the prevention of chronic disease, and there is also a limitation to the extent of resolution that this type of studies can provide. On the other hand, a wide variation has been observed in preclinical and clinical studies on responsiveness to food components as modifiers

of human health (Traka and Mithen, 2011). This is because our food consists of complicated mixtures of nutrients and metabolites making it extremely difficult to identify and dissect out the contributions of any single component to nutrition and health. All nutrients are subject to metabolism by the enzymes of the GI tract and by the gut microbiotica, and the gut microflora may be highly personalized for individuals and yet change with time. In addition, phytonutrients may impact the composition of the gut microflora, which can, in turn, impact the risk of chronic disease, as it has been shown for the role of the microflora of the GI tract in relation to obesity (Gill, et al., 2006; Turnbaugh, et al., 2006). The sites of absorption of different nutrients within the GI tract vary, and the degree of absorption (bioavailability) may vary significantly for slightly different chemical species. Phytonutrients are usually further metabolized once absorbed. Finally, the efficacy of different phytonutrients in promoting health likely varies significantly as a result of the specificity with which such compounds and/or their metabolites impact different animal signalling pathways, although the meaning of such differences in efficacy currently remain ill defined.

All this renders the results of bioactivity of a given plant-compound (or plant extract / plant-based food) with possible beneficial health properties as generally inconclusive, and even contradictory, depending on how analyses have been performed. Thus, for example, antioxidant capacity estimated through *in vitro* tests (such as ORAC**,** FRAP, ABTS, DPPH and lipid peroxidation assays) is extensively used to define and claim the 'goodness' of some phytonutrients (alone or within a food matrix). However, there is an increasing consensus in considering that the *in vitro* antioxidant activity of a certain compound may not reflect its actual activity *in vivo*, especially in view of its *in vivo* transformation into metabolites and/or other derivatives which are the true bioactive compounds (Cerdá, et al., 2004; Larrosa, et al., 2006). This fact stresses the importance of testing functionality on *in vivo* systems. Moreover, preclinical experimental studies with cell and animal models, although serve an important role to gain insight into underlying mechanisms of the health-promoting and disease-preventing activity of particular foods and their chemical components, do not consider some key issues such as bioavailability, metabolism, tissue distribution, dose/response and toxicity of food bioactive compounds in humans. Hence, human intervention studies (clinical trials) with adequate experimental designs provides the most reliable evidence for health benefits and is the only source of data upon which health claims for functional food products can be substantiated. Furthermore, evidence suggests that phytonutrients have not the same effect when consumed as supplements than as part of a food matrix. For example, anthocyanins from various foods were only effective at reducing obesity in mice subjected to a fatty diet when provided in the form of food, either red fruits (Prior, et al., 2008) or blood orange juice (Titta, et al., 2010). Also, in several large human intervention studies, it was found that β-carotene, vitamins A, C and E, or selenium supplementation were not preventive of gastrointestinal cancers, and instead increased mortality was recorded (Bjelakovic, et al., 2008). Similarly, while yet another study concluded that β-carotene supplementation does decrease the risk of developing cancer (Gallicchio, et al., 2008), some other works reported that β-carotene may take on

circumstantially adverse properties when given in high dose under highly oxidative conditions (Young and Lowe, 2001; Shukla and Mattoo, 2009). It is important to note, in this context, that under certain conditions, a particular antioxidant may not be effective in neutralizing oxidative damage, and that the very nature of antioxidants can make them pro-oxidants (Halliwell, 2007; Halliwell, 2008). This may also be relevant in the context of the findings that a synergistic relationship among different antioxidants (phytonutrients), present in dietary vegetables and fruits, has been proposed to be the reason for their beneficial effects on human health (Halliwell, et al., 2005). These are only a few examples that evidenced the importance of assessing the health impact of a particular phytochemical compound when is provided within the food matrix and in the appropriate amount, rather than as supplement. In this regard, one of the important contributions that plant scientists could make to this field of study is to develop plant genotypes that have contrasting levels of bioactive compounds but are otherwise identical for the use in dietary intervention studies, as reviewed recently by Martin, et al. (2011).

In summary, with the information compiled to date it can be stated that phytonutrients have low potency as bioactive compounds when compared to pharmaceutical drugs, but when they are ingested regularly and in significant (but not excessive) amounts as part of the diet (rather than as dietary supplements), they may have a noticeable long-term physiological effect. Further human intervention studies with well-characterized plant-based foods and adequate experimental designs are need to clarify the true potency of phytonutrients on human health at all levels (mechanism of action, proper dose, bioavailability, interactions with other compounds found in the food matrix, etc.).

Plant metabolic engineering for crop biofortification: nutrition versus functionality

The important contribution of phytonutrients to the nutritional value and healthy properties of certain fruits and vegetables has led to attempts to induce or increase their levels in commonly consumed crops, a process commonly known as biofortification. Although conventional breeding is one means of achieving this goal, the genetic diversity available within sexually compatible species of any given crop will limit the extent of improvement. In addition, these breeding methods involve crosses and backcrosses for several generations which is a highly time consuming process and require high genetic variations of the trait and heritability (McGhie and Currie, 2008). Metabolic pathway engineering approaches have demonstrated the power of genetic manipulation in enhancing the content of phytonutrients beneficial for human health in transgenic crops. Several examples of crop biofortification by genetic engineering have received widespread coverage in the scientific literature as well as the general media, including rice and potato with enhanced β-carotene levels, lysine-rich corn, iron-rich lettuce, and lycopene-enhanced tomatoes (Davies, 2007; Newell-McGloughlin, 2008). In the same vein, suppression of key genes to inhibit production of allergenic proteins or toxins in crops and their derived products is highly sought (reviewed by Zhu, et al. (2013)). Nonetheless, the examples of crop biofortification by genetic engineering can be loosely grouped into two major classes

regarding their purpose. First, the biofortification attempts aimed at the prevention/alleviation of nutritional deficiency diseases. Second, examples of crop biofortification aimed at developing healthier crops or functional food.

Approximately 50% of the global population is thought to be malnourished but the vast majority of malnourished people are the rural poor in developing countries, where diets are based almost exclusively on a single starch-based crop (so-called staple crops, such as rice, maize, or cassava) lacking many essential nutrients and other health-promoting compounds (Farré, et al., 2011). The first attempts to enhance concentrations of beneficial phytonutrients in crop plants through metabolic engineering consisted on biofortify this type of food with some essential nutrient. These biofortification programs were aimed at the development of micronutrient-dense staple crops predominantly as a strategy to alleviate some kind of micronutrient deficiency in developing country settings, where supplements may not reach those needing them due to limitations in distribution (Mayer, et al., 2008). The classic example of the contribution of plant science to biofortification with this purpose is the production of Golden Rice. In this work, rice was enriched in β-carotene, a pro-vitamin A with high bioactivity, in order to alleviate vitamin A deficiency. The expression of either two or three genes (phytoene synthase from daffodil, phytoene desaturase from *Erwinia uredovora* and lycopene β-cyclase from daffodil), encoding enzymes required for β-carotene biosynthesis, resulted in rice that accumulated up to 1–2 mg β-carotene per gram in the endosperm (Ye, et al., 2000; Paine, et al., 2005). However, these levels were not adequate to provide the daily recommended allowance of provitamin A in a standard portion of rice. Consequently, considerable effort was invested in improving the efficiency of these enzymes to develop Golden Rice 2, which accumulates 37 mg provitamin A per gram of rice (31 mg per gram β-carotene), enough to provide the daily recommended allowance (DRA) in a 100 g serving of rice (Paine, et al., 2005). In Golden Rice 2, phytoene synthase gene came from corn, and it was demonstrated that, in combination with phytoene desaturase from *Erwinia uredovora*, it was much more efficient in increasing β-carotene accumulation in the rice endosperm. Golden phenotypes resulting from enhanced β-carotene content have also been achieved in other crops, such as potato (Diretto, et al., 2010), although since these are no longer staple crops providing the bulk or sole source of nutrients to population groups, it is less likely that these will be useful in terms of alleviating vitamin A deficiency. Works that have succeeded in enhancing the content of folate or iron in rice also belong to this group (Goto, et al., 1999; Lucca, et al., 2001; Storozhenko, 2007). A more recent advance in this field has been the development of transgenic multivitamin corn, through the introduction of four cDNAs encoding enzymes in the biosynthetic pathways of vitamins β-carotene, ascorbate and folate (Naqvi, et al., 2009). The results of these works clearly indicate the power of genetic engineering in markedly enhance intracellular concentrations of some of the beneficial nutrients, to levels that, in some cases, are close to the recommended daily allowance (RDA) threshold (Mattoo, et al., 2010). However, it is important to emphasize the difference between bioaccumulation (the amount of a particular nutrient that can be stored in plant tissues) and bioavailability (the amount that can be absorbed when the plant

tissue is consumed as food). Whereas most studies have focused on bioaccumulation, the bioavailability of nutrients in engineered crops is a more important indicator of its nutritional quality (Hirschi, 2008). The food matrix plays an important role in the bioavailability of organic and inorganic compounds. For example, 12 mg of β–carotene in a food matrix must be ingested to gain the same benefit as 1 mg of pure β-carotene dissolved in oil. Similarly, vitamin E absorption requires the presence of bile salts, pancreatic enzymes and oils or fats to promote solubility (Jeanes, et al., 2004), and the bioavailability of ascorbate is enhanced by co-presentation with proteins in the food matrix (Vinson and Bose, 1988; Gómez-Galera, et al., 2010). In the case of minerals, the presence of antinutritional compounds such as phytate and oxalate in vegetables can inhibit mineral absorption because they act as chelating agents, whereas nutritional enhancers such as inulin can promote mineral absorption by slowing down the movement of food through the gut (Gómez-Galera, et al., 2010). Reducing the quantities of antinutritional compounds and/or increasing the quantities of nutritional enhancers can therefore increase the bioavailability of nutrients. Bioavailability may also depend on the chemical form in which a nutrient is presented; for example selenium is absorbed more efficiently when presented in an organic form such as selenomethionine rather than as inorganic metal ions (Combs, 2001), and iron presented as a complex with ferritin is less susceptible to the effects of antinutritional compounds than nonheme iron (Lönnerdal, 2009). Therefore, bioavailability studies of the target metabolite are essential to guarantee the validity of biofortified foods in alleviating nutritional deficiencies. In these regard, it has been recently demonstrated that β-carotene in biofortified rice (Golden Rice) and maize has good bioavailability as a plant source of vitamin A in humans (Tang, et al., 2009; Li, et al., 2010; Muzhingi, et al., 2011).

The second major class of crop biofortification include numerous examples of development of transgenic crops with enhanced levels of beneficial phytonutrients, on which, unlike the previous examples, the purpose was not to offset the nutritional lack but to grant some extra health benefits to the biofortified crops. Some examples of biofortification gathered within this category are the tomatos enriched in specific carotenoids (reviewed by Shukla and Mattoo (2009)), resveratrol (Nicoletti, et al., 2007), or flavonoids (Muir, et al., 2001; Davuluri, et al., 2005), the piceid-enriched kiwi and papaya (Zhu, et al., 2004; Rühmann, et al., 2006), the soybean with increased levels of α- and β-tocopherol (Van Eenennaam, et al., 2003; Tavva, et al., 2006), and the sweet potatos enriched with linolenic acid (Wakita, et al., 2001). These works arise in response to the evergrowing interest in the functionality of food. One of the most pressing challenges for the next 50 years is to reduce the impact of chronic age-related diseases, which continue to expand as the human population lives longer, and are known to be related to dietary habits. However, despite the appreciation of the importance of plant foods, and the provision of theories as to why diets rich in fruit and vegetables reduce the risk of chronic disease (Eaton and Konnor, 1985), public information programs are not particularly effective at persuading people to change long-established habits and campaigns to increase adoption of diets rich in fruit and vegetables have met with very limited success (National Cancer Institute, 2000; Pomerleau, et al., 2004; Truhe, 2006; Crawley, 2009). At the same time,

consumers are becoming increasingly aware of their self-care and expect to reach or maintain their health and welfare through the foods they eat. All this has resulted in a steep increase in the development of functional foods (FF) in the last years, mainly for luxury markets in the industrialized world. FF are those that when consumed regularly exert a specific health-beneficial effect beyond their nutritional properties (i.e., a healthier status or a lower risk of disease) and this effect must be scientifically proven (International Life Science Institute; http://www.ilsi.org). Biofortification through engineering of crops to synthesize and/or accumulate essential micronutrients and other health-promoting compounds may be a sustainable strategy to develop FF, avoiding the need to fortify processed food products with additives (Gómez-Galera, et al., 2010). In these biofortification programs, the crops that are naturally rich in micronutrients and highly consumed in the diet are usually the preferred targets for enrichment, rather than a staple-food. This approach of biofortification seeks to take advantage of possible beneficial interactions with phytonutrients already present in the food matrix of the target crop and, thus, enhance their healthy qualities, resulting in the development of a novel FF.

Testing functionality of biofortified crops (importance of isogenic lines)

Enhancing the nutritional quality of plant-based foods through genetic modification is an attractive and potentially useful contribution to tackling the twin global health burdens of micronutrient deficiencies and diet-related non-communicable diseases (Martin, et al., 2011). Notwithstanding some differences, many of the same technical issues must be addressed in both environments, that is, the need to modulate endogenous plant metabolic pathways to ensure that flux is directed to the appropriate compounds, the need to ensure such compounds accumulate in the most appropriate tissues and the focus on bioavailability rather than bioaccumulation (Zhu, et al., 2013). Besides contend with the technical requirements referred, the concept of developing nutritionally functional food requires: (1) the understanding of the mechanisms of prevention and protection against a concrete diet-related disease; (2) the identification of the biologically active molecules and (3) the demonstrated efficacy of these molecules with human subjects. Accurate information is also highly desired to have in place about the RDA for effective phytonutrients, in order to design biotechnological strategies for manipulating their contents in vegetables and fruits. Still, even if all these issues were resolved, the functionality/biological activity of the final biofortified crop must also be scientifically corroborated to meet the definition of FF (forth above). The necessity of scientific support for health claims of these phytonutrient-enriched crops is also specifically indicated in the new regulation of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods (http://eur-lex.europa.eu/JOIndex.do?ihmlang=en; Official Journal of the European Journal, OJ L 404, 30/12/2006).

Plant-based approaches to alleviating micronutrient deficiencies have been widely advocated by international agencies, such as the Harvest Plus initiative, and well-defined

targets in addressing iron, zinc, and vitamin A deficiencies have been agreed upon (Hotz and McClafferty, 2007). By contrast, identifying plant metabolites whose manipulation in fresh and processed foods might have a significant effect in reducing the burden of chronic disease is more challenging. As mentioned above, this is, in part, due to the lack of well-characterized and contrasting plant foods required to test hypotheses for the health-promoting activity of specific plant metabolites (Traka and Mithen, 2011). Plant metabolic engineering is an important tool to reduce some of the complexity in the diet-health relationship particularly through the development of near isogenic genotypes of common foods that vary in specific phytochemicals that can be used within dietary intervention studies to ask specific questions concerning biological activity of different phytochemicals when provided not as supplements but within a common chemical and physical food matrix. Once isogenic food materials have been prepared, the impact of target phytonutrients on a range of different chronic diseases can be assessed using *in vivo* systems, and, if these isolines have been generated through genetic transformation, preclinical experiments with animal models become essential, prior to perform human intervention studies. The most notable approach to this is the manipulation of the phenylpropanoid and/or flavonoid pathways in crop plants to develop fruits that have contrasting flavonoid compositions, providing material that served as true matrix controls in experiments with animal models for specific diseases (Martin, et al., 2011). For example, an study demonstrated that consumption of a transgenic flavonoid-enriched tomato, at a dose achievable with a human diet, reduced C-reactive protein in human C-reactive protein transgenic mice expressing markers of cardiovascular risk more than wild-type tomato intake (Rein, et al., 2006). A similar approach using tomatoes genetically engineered to produce high levels of delphinidin and petunidin anthocyanins demonstrated that dietary consumption of high levels of anthocyanins can extend the life span of Trp532/2 (p53 knockout) cancer-prone mice by as much as 30% (Butelli, et al., 2008). A recent study used genetically engineered apples with increased flavonoids (through overexpression of the myeloblastis transcription factor 10 (MYB10) gene), compared to non-transformed apples, to investigate the effects of dietary flavonoids on inflammation and gut microbiota in mouse feeding trials. They concluded that high-flavonoid apple was associated with decreases in some inflammation markers and changes in gut microbiota when fed to healthy mice. These three studies constitute the only examples to date in the literature of transgenic crop biofortification whose functionality has been tested with animal models. The use of these plant materials (and other biofortified crops enriched with different potentially health-promoting phytonutrients) in short- and long-term intervention trials with humans would potentially make a major contribution to our understanding of the dietary role of the target compounds in a manner that is not feasible through epidemiological and animal studies. Therefore, plant metabolic engineering can contribute to understanding the benefits of specific fruit and vegetables (Martin, et al., 2011; Martin, 2012), at a fundamental level, and such biofortification can potentially contribute to improving diets without fundamental compositional changes.

2. OBJECTIVES

2. Objectives

The main aims of this thesis are (1) performing the first studies on environmental biosafety and field performance of genetically modified citrus trees, required to validate the use of the GM technology in citrus improvement programs (**Chapters 1** and **2**), and (2) addressing, for the first time, an improvement goal on the nutri-functional fruit quality of a citrus variety of commercial interest by metabolic engineering (**Chapter 3**). This thesis, therefore, consists of two different parts, and as a whole seeks to provide essential information for the future possibilities of modern biotechnology in citriculture.

The specific objectives are:

- To investigate the maximum frequency of transgene dispersal through pollen from GM citrus trees in an orchard that is grown under open pollination conditions and is embedded in a diverse floral neighbourhood of non-GM citrus trees, and to assess the relative contribution of the genetic, phenological and environmental factors involved in transgene dispersal (**Chapter 1**).

- To study the stability of the integration and expression of the *uidA* and *nptII* transgenes in GM citrus trees grown under agronomical, long-term culture conditions and the potential impact of transgenesis on the morphology, phenology and fruit quality of trees (**Chapter 2**).

- To generate transgenic orange plants flowering and fruiting fast, and producing fruits enriched in β-carotene (pro-vitamin A) by metabolic engineering. To evaluate subsequently the biological activity *in vivo* (antioxidant capacity) of these fruits in the model organism *Caenorhabditis elegans* (**Chapter 3**).

3. RESULTS: CHAPTER 1.

Pollen competition as a reproductive isolation barrier represses transgene flow between compatible and co-flowering citrus genotypes

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Abstract

Background/Objective: Despite potential benefits granted by genetically modified (GM) fruit trees, their release and commercialization raises concerns about their potential environmental impact, and the transfer via pollen of transgenes to cross-compatible cultivars is deemed to be the greatest source for environmental exposure. Information compiled from field trials on GM trees is essential to propose measures to minimize the transgene dispersal. We have conducted a field trial of seven consecutive years to investigate the maximum frequency of pollen-mediated crop-to-crop transgene flow in a citrus orchard, and its relation to the genetic, phenological and environmental factors involved.

Methodology/Principal Findings*:* Three different citrus genotypes carrying the *uidA* (GUS) tracer marker gene (pollen donors) and a non-GM self-incompatible contiguous citrus genotype (recipient) were used in conditions allowing natural entomophilous pollination to occur. The examination of 603 to 2990 seeds per year showed unexpectedly low frequencies (0.17-2.86%) of transgene flow. Paternity analyses of the progeny of subsets of recipient plants using 10 microsatellite (SSR) loci demonstrated a higher mating competence of trees from another non-GM pollen source population that greatly limited the mating chance of the contiguous cross-compatible and flowering-synchronized transgenic pollen source. This mating superiority could be explained by a much higher pollen competition capacity of the non-GM genotypes, as was confirmed through mixed-hand pollinations.

Conclusions/Significance*:* Pollen competition strongly contributed to transgene confinement. Based on this finding, suitable isolation measures are proposed for the first time to prevent transgene outflow between contiguous plantings of citrus types that may be extendible to other entomophilous transgenic fruit tree species.

Introduction

The progressive increase in the global area and number of GM crops has lead to numerous empirical studies on transgene flow in field trials aimed at developing containment strategies, which are required by regulators and policy makers to legislate, on a case-by-case basis, how deliberate releases should be performed. Containment could be important to protect the rights of the owner of the transgenic variety and of GM-free growers and to avoid the unintended release of certain transgenic traits to other cultivars or to wild relatives [1, 2]. Most of these investigations have so far been carried out in annual crops [3, 4], while research in perennial species is still scarce or is focused on contemporary gene flow based on the genetic structure of natural populations [5-8]. Thus, it is necessary to carry out transgene flow studies specifically in trees because their long life and complex reproductive biology may have significant effects on the extent of transgene dispersal.

Citrus is the most extensively produced fruit-tree crop in the world [9]. Commercial citrus genotypes are subjected to important biotic stresses, which are only partially controlled by the application of pesticides and, in many instances, limit the use of certain rootstocks and/or varieties. At the same time, markets demand fresh fruit and juice of increasing quality. In this context, the main focus of citrus breeding programs has been disease resistance plus fruit quality. However, improvement of citrus by conventional breeding is constrained by genetic crossing barriers, such as self and cross incompatibility, high heterozygosity, long juvenile periods, and facultative apomixis and sterility [10]. Genetic engineering (GE) could circumvent some of these limitations, especially by bypassing the long crossing cycles of tree breeding programs, without the complications of linkage drag. Moreover, it allows improvement of citrus varieties that are not amenable to breeding, like sweet oranges and grapefruits. Furthermore, GE is the only technology that enables gene transfer between unrelated organisms, even if they belong to widely divergent taxa, offering promising prospects in disease resistance approaches, especially when resistance sources are not present in reproductively compatible relatives. Thus, though there are no commercial GM citrus crops yet, genetic transformation is considered an essential tool in many current improvement programs, and experimental field trials are underway in several countries [11].

Cross-pollination in citrus is accomplished by insects, and honeybees are the most successful pollinators [12]. In insect-pollinated plants, pollen dispersal is generally the main component of gene flow [13]. The potential for pollen-based gene flow depends on the geographic distribution of the different compatible species (wild or crop) present in the area of study. In all citrus-production areas of the world, except East Asia, it is unlikely that transgenic plants could become feral populations because there are virtually no wild sympatric citrus species and relatives. However, cross-pollination between conventional citrus cultivars and transgenic citrus genotypes would be theoretically possible in many cases if they are grown in the same production areas. The presence of transgenic seeds in non-transgenic fruits as a result of effective cross-pollination could be a matter of concern. Although seeds in citrus are

never consumed deliberately, their adventitious presence in non-GM fruits could cause problems related to consumer acceptance, and it may have implications on the marketability of the fruit, especially if organic fruit-growing orchards are exposed [14]. For the specific case of self-incompatible, cross-compatible mandarins and mandarin hybrids, this problem is not contemplated because the presence of seeds in the fruit already represents a marketability problem, so different cultural strategies are commonly used to avoid cross-pollination with sympatric citrus cultivars. From an agronomic viewpoint, there is no concern over the adventitious propagation of GM citrus cultivars through escaped seeds because commercial citrus varieties are exclusively propagated by grafting adult vegetative buds onto juvenile rootstocks. In the incidental case that transgenic seedlings germinated in an orchard, they would be removed by farmers. Moreover, these seedlings would never flower before being removed because citrus seedlings need several years to start flowering [15]. Information about pollen-mediated crop-to-crop gene flow from a GM citrus cultivar is therefore required to estimate the likelihood of the adventitious presence of GM seeds in non-GM citrus varieties grown in the same area.

In entomophilous species, the physical distance between the pollen source and sink is one of the most important factors determining the distribution of frequency and maximum dispersal distances of gene flow [16]. In fact, it is well known that bees in fruit tree orchards restrict their activity to single or adjacent plants [17], resulting in increased pollination between neighboring trees, e.g., in lychees [18], avocado [19], apples [20], almonds [21], citrus [22] and other tree species [23]. In all of these species, the maximum frequency of gene flow was adjacent to the source and rapidly declined with distance, often describing a marked leptokurtic curve [24].

Based on this finding, we designed an experimental field trial that involved the release of GM citrus trees with the objective of measuring during seven consecutive years the frequency of pollen-mediated transgene flow (PMTF) from GM lines to contiguous recipient trees under open pollination (OP) conditions. Three different citrus genotypes (sweet orange, citrange and lime) carrying the β-glucuronidase gene (*uidA*), which served in this study as marker to track gene transfer, were used as pollen donors, and clementine, a self-incompatible mandarin type, was used as the recipient.

Although recent studies demonstrate that bees have the potential to move pollen over several kilometers, the probability of pollen movement is very low if patches are more than 50 m away [25], and these rare outcrossing events contribute little to adventitious GM presence in non-GM receptor crops. Therefore, field assessment of the 'extreme cases' in which GM and non-GM citrus are cultivated adjacently is an essential first step for a thorough evaluation of gene flow and its potential consequences. Additionally, the influence of the diverse floral neighborhood on transgene flow frequency between sexually compatible and flowering-synchronized species located in close proximity was also assessed. The role of the floral neighborhood as a possible isolation barrier between GM and non-GM crops is investigated here for the first time, providing valuable information for properly designing future field trials for

efficient GM containment. The study site where the experimental field is located represents a collection of genetic resources of citrus, such as various widely diverse cultivars and breeding materials, which allows estimating the frequency and range of gene flow from different pollen sources by paternity analysis of progeny from OP recipients with the assistance of specific molecular markers.

The objectives of this study were (1) to estimate the frequency of PMTF from three different GM citrus types to a non-GM citrus variety planted adjacently as an edge; (2) to assess the role of the surrounding flora as isolation barrier between co-flowering and compatible transgenic pollen donors and recipients through estimation of the mating success and gene flow patterns from different pollen sources within the study site; (3) to elucidate isolation mechanisms to explain how pollen donors showing higher mating success can limit PMTF; and (4) to propose containment strategies to repress transgene pollen dispersal from citrus (and other fruit) orchards.

Materials and Methods

Plant materials

Eight independent transgenic lines of three citrus genotypes with a different genetic background were used as potential pollen donors in this work: Pineapple sweet orange (*Citrus sinensis* L. Osb.; named P1 to P8), Carrizo citrange (*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.; named C1 to C8) and Mexican lime (*C. aurantifolia* (Christm.) Swing.; named L1 to L8). All transgenic lines carried the *35S::uidA::Nos* (GUSINT) and *Nos*::*nptII*::*Nos* marker transgenes, providing constitutive GUS expression and resistance to kanamycin, respectively. The *uidA* transgene was used as a marker to track gene flow. The transgenic lines used were selected based on their high-level transgene expression and low copy number of transgene insertions (ranging from 1 to 4, depending on the line) [26]. Three control lines (one per genotype, named PC, CC and LC) were also used in the current study as non-transgenic pollen donors. Trees of the self-incompatible and monoembryonic citrus genotype Clemenules clementine served as pollen recipients for monitoring PMTF.

Experimental field design

The gene flow experiment was conducted for seven production seasons (from 2001 to 2007) at an experimental field named T plot, located at the Instituto Valenciano de Investigaciones Agrarias, Spain (latitude $39^0$35”N, longitude $0^0$23”W and altitude 50 m; typical Mediterranean climate). The field study was designed to evaluate the short-distance PMTF from transgenic to non-transgenic citrus plants, that is, the maximum expected dispersal frequency. The T plot, with an extension of 1.638 m^2, contained 130 adult trees distributed in rows, as described in Fig. 1. The pollen-donor genotypes (transgenic and control lines) were planted at

the center, while 58 non-transgenic recipient clementine trees were planted on an external edge. All scion types were grafted onto Carrizo citrange rootstocks and grown in a loamy clay soil with drip irrigation. The field was managed as for normal citrus cultivation. No treatments were performed to control bees and pollinators in general. Visual surveys showed that the number of open flowers from pollen donors and recipient trees as well as the number of bees at the study site during the flowering periods greatly exceeded the amounts needed to ensure natural cross-pollination every year (Fig. S1).

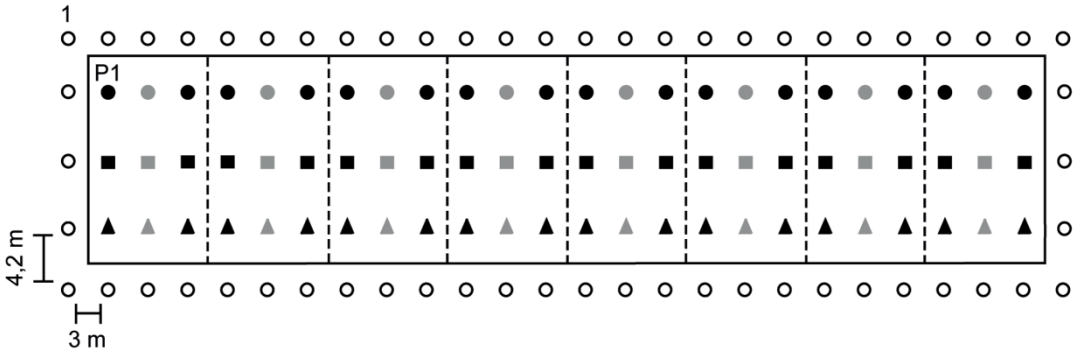

Figure 1. Schematic diagram of the experimental field trial. It consisted of 130 trees, planted in rows along the transgenic (T) plot, including 16 transgenic plants of Pineapple sweet orange (black circles), 16 transgenic plants of Carrizo citrange (black squares) and 16 transgenic plants of Mexican lime (black triangles) (2 plants each from 8 independent transgenic lines numbered from 1 to 8, from left to right). In addition, there were 8 non-transgenic control plants from each genotype individually interspersed between the two plants from each transgenic line and represented by grey figures. Fifty-eight non-transgenic Clemenules clementine trees planted along an external edge (white circles; numbered in increasing order going clockwise) were used as the pollen recipients to estimate transgene flow frequencies.

Determination of PMTF frequencies

Fruit samples of every open-pollinated (OP) recipient clementine tree were collected annually. At least 10 randomly selected fruits per tree were harvested when the fruits were fully mature. Seeds were extracted from fruits, counted and tested for GUS expression. A histochemical GUS assay was performed on seeds that were cut to provide substrate penetration. A sample of seeds from a transgenic citrus line was used as the positive control (Fig. S2A). The PMTF frequency was calculated annually as the percentage of GUS-positive (transgenic) seeds over the total number of seeds analyzed, and we assumed that this frequency was the maximum achievable for our experimental conditions due to the proximity of the recipient trees to the transgenic pollen source.

To validate the method used to determine the PMTF frequency, seedlings from seeds of an array of randomly selected OP recipient trees were tested for transgene expression and integration over 2 years (2005-2006). Seeds were sown on seedbeds containing steam-sterilized artificial soil mix suitable for growing citrus and under regular greenhouse conditions. The greenhouse-grown seedlings were assessed through histochemical GUS assays of the

leaves (Fig. S2B,C) and PCR analysis for the *uidA* transgene. For PCR analysis, DNA was extracted from 20 mg of leaves according to [27]. Standard PCR techniques were used to detect the *uidA* transgene. The primers used to amplify the transgenic DNA fragment were GUS-up (5'-ggtgggaaagcgcgttacaag-3') and GUS-down (5´-tggattccggcatagttaaa-3'). The reactions were performed in 30 cycles of 0.50 min at 95^0C, 0.50 min at 58^0C and 1 min at 72^0C. The PCR products were detected by electrophoresis using 1% agarose-ethidium bromide gels. The DNA was stored at -20°C for further microsatellite (SSR; Simple Sequence Repeat) analyses.

Flowering synchrony, pollen viability and cross-compatibility studies

To check the flowering synchrony between the pollen donor and recipient genotypes, the phenology of all trees in the T Plot was studied in 2005 and 2006 from the start of flowering to the initiation of fruit set. Phenological calendars were established for each genotype by weekly observation and recording of the predominant phenological stages of trees, following the BBCH codifications [28]. Mexican limes were excluded from this study because they tend to show sparse flowering over the year, which implies that throughout the year, almost all phenological stages can be found in a tree at the same time.

Pollen viability of all pollen donors of the T Plot (transgenic and control lines) was evaluated by estimating pollen germination rate *in vitro*. A minimum of ten flowers per genotype was collected from field-grown plants. Anthers were removed from flowers and placed in a desiccator. Pollen from fully dehisced anthers was distributed with a fine brush onto small Petri dishes (diameter: 5.5 cm) containing germination medium (Murashige and Skoog mineral medium with 3% sucrose and 0.8% agar, pH 5.7). These Petri dishes were placed inside larger Petri dishes (diameter: 9 cm) containing a moist piece of filter paper and incubated at 24°C in the dark for 24 h. Germination was quantified as the percentage of germinated pollen grains form a minimum of 600 grains counted.

The reproductive compatibility between the pollen donors and the recipient genotype in the T Plot were tested *in vivo* through directed hand crosses. The PC, P1, P7, CC, C1, LC and L8 lines were used as male parents in each single-pollination treatment. Hand pollinations were carried out in two years (2005 and 2006) by deposition of entire anthers on the stigmas of flowers from the clementine trees grown at the edge. The number of pollinated flowers per cross was 100. The fruits produced were collected at maturity and counted. Their seeds were extracted, counted and used in further analyses. For each pollination treatment, two measures of individual maternal fitness ("fruit set" and "seed set") were used to determine the reproductive compatibility between the crossed lines. Fruit set was defined as the percentage of mature fruits produced from the total number of pollinated flowers. Seed set was defined as the number of viable seeds per fruit averaged over each treatment.

Assessing the influence of other nearby pollen sources on PMTF frequencies

Potential pollen donor (PPD) genotypes in the neighboring plots

The role of the surrounding flora as an isolation barrier between transgenic pollen donors and recipients was examined through paternity analysis of the progeny from a subset of OP clementine trees for two years. For this purpose, surrounding citrus orchards were also taken into consideration as alternative pollen sources able to pollinate recipient plants in OP conditions. Thus, adult trees of any citrus genotype that was male fertile, cross compatible and synchronized in flowering with clementine at the study site (the T plot and neighboring plots within 100 m) were considered PPDs, as represented in Table 1. In the neighboring plots, named A and B, there were populations of adult citrus trees belonging to different breeding programs carried out at IVIA. Plot A consisted of a population of triploid hybrids as well as their diploid parental genotypes [29]. As triploid hybrids are sterile [30], only some of the diploid genotypes that are known to be cross-fertile with clementine mandarin were considered PPDs. Plot B was composed of a population of 477 hybrids belonging to a rootstock breeding program. These hybrids were randomly distributed within the plot, and all them were, in principle, potential pollinators of clementine.

Table 1. Potential pollen donor (PPD) genotypes present at the study site, including their abbreviation codes, population sizes (number of adult trees) and relative amounts.

Plot	PPD Genotype	code	Population size	Relative amount (%)
T	Pineapple sweet orange	P	24	3.80
	Carrizo citrange	C	24	3.80
	Mexican lime	L	24	3.80
A	Fortune mandarin	F	34	5.38
	Orlando tangelo	ORL	10	1.58
	Murcott mandarin	MU	7	1.11
	Nova tangor	N	6	0.95
	Ortanique tangor	ORT	6	0.95
	Willowleaf mandarin	MC	6	0.95
	Ellendale mandarin	E	6	0.95
	Kara mandarin	K	4	0.63
	Minneola tangelo	MI	4	0.63
B	King mandarin x *Poncirus trifoliata*	H1	202	31.96
	C. volkameriana x *Poncirus trifoliata*	H2	88	13.92
	Cleopatra mandarin x *Poncirus trifoliata*	H3	84	13.29
	Troyer citrange x Cleopatra mandarin	H4	77	12.18
	Troyer citrange x Willowleaf mandarin	H5	26	4.11

Molecular typing of progeny from OP recipients by microsatellite (SSR) analysis

Genomic DNA from progeny of a subset of OP recipient plants was subjected to SSR analysis to determine the pollen parentages of each hybrid seedling. Because there were no unique markers with total allelic differentiation among all PPD genotypes, we performed a multilocus paternity analysis. We chose 10 SSR markers that were highly polymorphic among PPD genotypes. These markers were *CI01G11, CIR07C07, CIR01E02* [31], *mest192* [32], *CIR01C06*, *CIR03C08* [33], *mest458*, *mest107*, *mest86* (Luro et *al.*, unpublished) and *CAC23* [34]. PCRs with wellRED oligonucleotides (Sigma®), which use cyanine-based fluorescent dyes at the 5'end, were performed as described by [35] with slight modifications. An Eppendorf® Mastercycler ep gradient S was used with a reaction volume 15 µl, composed as follows: 0.8 U Taq polymerase (N.E.E.D.®), reaction buffer – 750 mM Tris HCl (pH 9), 50 mM KCl, 200 mM $(NH_4)_2SO_4$, 0.001% BSA, 0.1 mM of each dNTP, 5 mM $MgCl_2$, 3 µM of each primer, and 30 ng DNA. The following PCR program was used: 5 min at 94°C; 40 cycles of 30 sec at 94°C, 1 min at 50-55°C and 30 sec at 72°C; final elongation 10 min at 72°C. After performing the PCR, genetic analysis was performed in a capillary-array sequencer CEQTM 800 System (Beckman Coulter_ Inc., Fullerton, CA), and the results were analyzed with Genome- LabTM GeXP Genetic Analysis System software.

Paternity assignment

Paternity analysis was performed based on SSR genotyping, using a simple exclusion approach [36]. When the paternal allele(s) at a locus could be inferred from the observed progeny and maternal genotype, then all PPDs that lacked the allele(s) were excluded. This process was repeated for each locus, until all PPDs could be excluded except one. In some cases, it was not feasible to assign a single PPD even after the hybrid was analyzed for all the 10 markers. In these cases, phenotypical traits, such as leaf morphology (trifoliate vs. monofoliate) and GUS expression, were considered for discriminating among different ambiguously assigned PPDs.

Pollen competition studies

To clarify the mechanisms of isolation by which other PPDs at the study site limited PMTF frequencies, the pollen competition capacity of one of the PPDs displaying higher mating success in OP conditions (H3 in Table 1) was compared to that of one transgenic PPD of plot T (P1) by mixed pollination treatments over two years (2006-2007). P1 was chosen as the competitor from plot T because it had displayed high cross-compatibility with clementine in single pollination treatments and had three copies of the *uidA* transgene [26], meaning that inheritance of this trait would be considerably high (theoretically 87.5%, assuming independency between loci). Mixed pollinations were carried out by depositing one entire anther from each genotype onto the stigmas of clementine flowers. Previously, to avoid the possible influence of pollen density effects [37], the number of pollen grains per anther for each genotype had been determined to ensure the deposition of approximately the same number of pollen

grains. Likewise, differences in pollen viability between both genotypes were estimated by determining the percentage of pollen germination *in vitro*, as described above.

One hundred flowers were pollinated per year. The fruits produced were collected at maturity and counted. Their seeds were extracted, counted and tested for GUS expression. The siring success of transgenic pollen (P1) in the mixed pollination treatment was inferred from the GUS-positive frequency achieved in the tested progeny. We compared this GUS expression rate to that obtained in the progeny of single-pollination control treatments performed with P1.

Data analyses

For the molecular validation of the PMTF assessment method, the χ^2-test was performed. The minimum sample sizes of progeny required for this purpose in both years were calculated according to [38].

In single pollination treatments, separate multifactor analyses of variance (ANOVA) were conducted to examine the effects of “Variety” and “Genetic Modification” of the pollinator and their interaction on the variables “Fruit set” and “Seed set”. LSD multiple range tests were performed for separation of means. Before performing the analyses, Box-Cox transformations [39] were applied on both variables to fit the data to a normal distribution.

Data obtained from paternity analysis were used (1) to estimate the maximum reproductive success of each plot, calculated as the total percentage of progeny assigned; (2) to provide a spatial overview of the pollen dispersal patterns from the different plots by performing radial graphs; (3) to examine the influence of the proximity of plot B in the mating chance of the rest of pollen sources by drawing pollen dispersal curves with the percentage of pollination events unambiguously assigned to each plot as the y-axis and the distance from plot B as the x-axis; (4) to assess the possible relationship between the relative abundance of each PPD in plot B and their maximum mating success achieved. Simple regression analyses were used to model the relationships between the variables for (3) and (4).

All statistical analyses were performed using STATGRAPHICS Plus 5.1.

Results

PMTF frequencies from three different citrus genotypes were unexpectedly very low in contiguous recipient trees

PMTF frequencies found at the study site showed that the percentage of transgenic seeds in self-incompatible clementine fruits was consistently very low (between 0.17% and 2.86%) (Table 2), taking into account the proximity of transgenic pollen donors to the recipient trees. As the numbers of flowers and bee pollinators were usually very high in the spring (Fig. S1), the average seed production in OP recipient trees was also high, as expected (Table 2). This high production allowed us to analyze many seeds (ranging from 603 to 2990) each year

by histochemical GUS assays. This high number of seeds analyzed, together with the seven consecutive years of assessment, provided strong confidence to our results.

Table 2. The pollen-mediated transgene flow (PMTF) frequencies for seven years as determined by testing seeds from open-pollinated recipient trees for GUS expression.

Year	**Number of seeds**			**PMTF (%)**
	per fruit (seed set mean ± SE)	tested	GUS positive	
2001	7.91 ± 0.63	2990	5	0.17
2002	1.21 ± 0.12	1359	13	0.96
2003	2.68 ± 0.25	2171	9	0.41
2004	0.80 ± 0.12	603	5	0.67
2005	4.68 ± 0.34	2619	75	2.86
2006	2.67 ± 0.20	1573	22	1.39
2007	3.43 ± 0.27	1398	29	2.18

Next, we decided to validate the method used and to investigate whether low/silenced GUS expression in seeds could be contributing to the low PMTF frequency observed. A total of 224 hybrid seedlings from 12 recipient trees in 2005 and 140 seedlings from 9 recipient trees in 2006 were tested for GUS expression in the leaves and *uidA* integration. Sample sizes used exceeded the minimum required to statistically represent the population at 95% confidence with an acquired precision error of ≤ 3%. The PMTF frequencies obtained from analyzing GUS expression in seedlings were 2.86% in 2005 and 1.39% in 2006 (Table 3). Moreover, PCR analyses confirmed, at the molecular level, the transgenic nature of all GUS-positive seedlings and dismissed the presence of transgene-silencing in GUS-negative seedlings without exception (Table 3). When comparing these results with those obtained previously for GUS expression in seeds in the same years (Table 1), a χ^2-test showed no statistically significant differences between the frequencies for either of the two years at the 95% confidence level, indicating that the hybrid seed identification system used during the seven years of assessment to determine PMTF frequency was reliable.

Table 3. Molecular validation of the pollen-mediated transgene flow (PMTF) assessment method by testing seedlings from a subset of open-pollinated recipient trees during two years (2005 and 2006).

Year	clementine number	Number of seedlings			PMTF (%)[2]	χ^2 value[3]
		Tested	Transgenic[1]	Trifoliated		
2005	2	21	0	13		
	8	21	1	0		
	14	3	0	1		
	20	30	1	6		
	25	6	0	1		
	27	15	4	9		
	29	11	0	0		
	35	5	0	2		
	42	15	1	4		
	48	36	0	9		
	53	27	0	4		
	55	34	0	6		
	Total	**224**	**7**	**55**	**3.12**	**0.024**
2006	2	6	0	0		
	6	11	0	2		
	20	2	0	0		
	27	15	2	1		
	30	18	0	1		
	36	18	0	0		
	42	30	0	2		
	50	34	0	1		
	55	6	0	5		
	Total	**140**	**2**	**12**	**1.42**	**0.001**

1. Number of transgenic seedlings was determined by GUS expression in leaves and confirmed by PCR analysis of the *uidA* transgene. False GUS negative seedlings were not found in any case.
2. PMTF frequency was calculated as the percentage of transgenic seedlings from the total number of seedlings analyzed per year.
3. For each year, χ^2 tests were performed to compare the PMTF frequencies obtained by this method with the PMTF frequencies obtained by testing GUS expression in seeds (Table 2). The critical value for 1 *df* at a 95% confidence level is 3.84.

Transgenic pollen donors and recipient trees showed flower synchrony and were cross compatible

To discard the idea that low PMTF was due to asynchrony in flowering times between the transgenic pollen donors and the recipient clementine trees, phenological calendars of flowering were assessed and compared. The extent of the full flowering stage varied among citrus types and was longer in clementine trees. This stage lasted 3 and 4 weeks for Pineapple sweet orange and Carrizo citrange, respectively, while it lasted up to 6 weeks for Clemenules clementine. However, the full flowering phase of both pollen donor genotypes, though shorter, fully coincided with that of the recipient plants (Fig. 2).

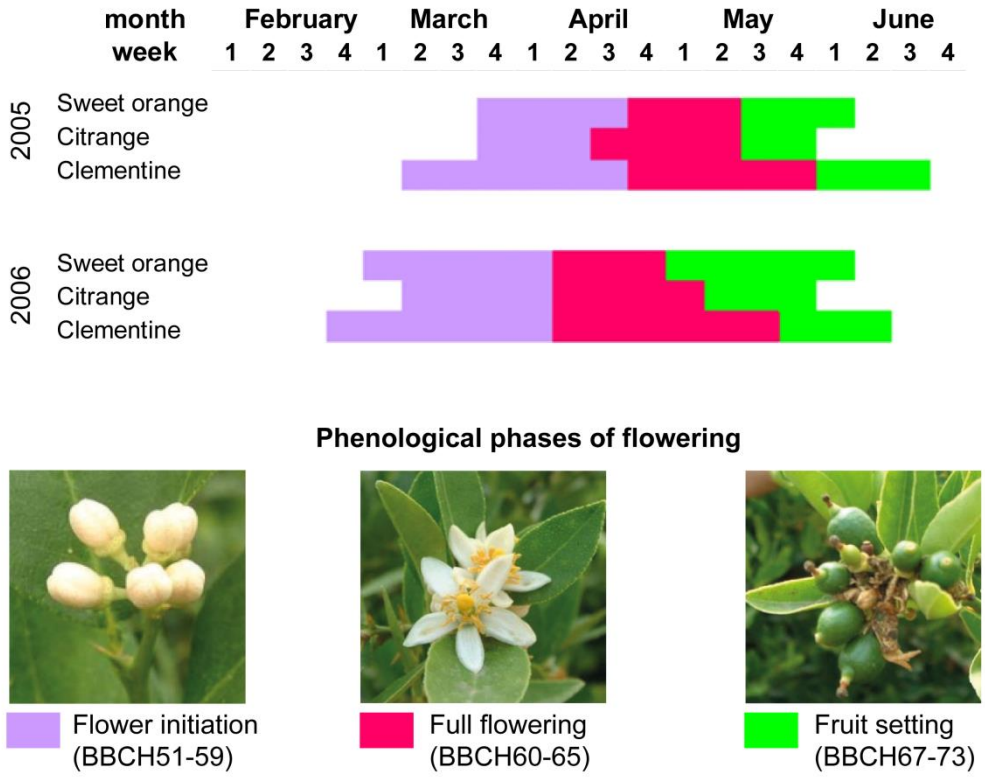

Figure 2. Phenological calendars of flowering for genotypes in plot T. Different phases of the bloom period for Pineapple sweet orange, Carrizo citrange and Clemenules clementine genotypes are represented by different colors. The overlap in the full-flowering phase (pink) determines the flowering synchrony between genotypes.

The viability and capacity of fertilization of transgenic pollen was studied and compared with those of controls using *in vitro* and *in vivo* systems. *In vitro* studies of pollen viability showed that 1) germination rates varied among citrus types, reaching considerable high levels for sweet orange and citrange lines (about 50% and 70% on average, respectively) and 2) for each citrus type, pollen germination rates from transgenic lines did not differ from those of the

correspondent controls (Fig S3). This demonstrates the absence of pleiotropic effects derived from the insertion of transgenes that affect negatively to pollen viability.

To check whether pollen donors from the T plot and recipient trees were cross compatible, hand pollinations were performed. As shown in Table S1, "Variety" was the most important factor determining cross-compatibility in directed crosses because it had effects on both variables (P-value = 0.0002 for fruit set; P-value = 0.0310 for seed set). Pineapple sweet orange and Carrizo citrange induced higher fruit set and seed set than Mexican lime (Fig. S4). The "GM" factor had no effect (P > 0.05) on the variables investigated, indicating that transgenic trees were as compatible with recipients as the corresponding controls for the same background variety (Fig. S4).

Influence of other nearby pollen sources on PMTF frequencies

Identification of specific, highly mating PPD types in the neighboring plots

The analysis of GUS expression and leaf morphology in seedlings from a subset of OP recipient trees showed the presence of many trifoliate but GUS-negative hybrids (Table 3). Because transgenic Carrizo citrange trees were as cross compatible with clementines as their non-transgenic counterparts (Fig. S4), these results suggested that (trifoliate) neighbor trees from other surrounding plots were competing with trees from the T plot for pollination of recipient trees and likely interfering with the PMTF frequencies obtained. To identify the pollen source(s) that competed with pollen donors from the T plot under OP conditions, the DNA of 191 seedlings from 12 recipient trees and of 140 seedlings from 9 recipient trees was subjected to paternity analysis in 2005 and 2006, respectively. To this aim, marker profiles for each PPD genotype (or candidate father) from plots T and A were assessed as well as for the recipient (mother) genotype (Table S2). Because the PPD genotypes from plot B (reported in Table 1 as H1, H2, H3, H4 and H5) were F1 hybrids from a rootstock breeding program, their marker profiles in Table S2 corresponded to the alleles that could potentially be found in each F1 progeny, which were inferred from the known profiles of their parents. Then, hybrid seedlings were classified according to the source plot of their assigned parents (Table 4; Table S3 and Table S4). In this way, the percentage of progeny unambiguously assigned to a given plot was very high, 82.19% in 2005 and 79.28% in 2006, especially considering the close genetic background of many PPDs from the 3 plots. Moreover, the percentage of progeny that could not be assigned to any PPD (because their pollen parents within the population could not be assigned) was very low, 1.57% and 7.14% in 2005 and 2006, respectively. Based on these results, the analysis showed that the pollen source that had the highest reproductive success with recipient clementine trees was Plot B (78.5% in 2005 and 63.6% in 2006), followed by Plot A (29.8% in 2005 and 36.4% in 2006). Plot T showed the lowest reproductive success (7.4% in 2005 and 3.6% in 2006) (Fig. 3).

Table 4. Results of paternity assignment in progeny from open-pollinated recipients harvested in 2005 and 2006.

Number of pollen donor(s) assigned	Source of the pollen donor(s) assigned (Plot)	Category Name	Number of progeny placed within the class		Percent of progeny placed within the class	
			Year 2005	Year 2006	Year 2005	Year 2006
0	-	Not assigned	3	10	1.57	7.14
1	T	Unambiguously assigned T	7	3	3.66	2.14
1	A	Unambiguously assigned A	26	28	13.61	20.00
1	B	Unambiguously assigned B	45	47	23.56	33.57
>1	A	Unambiguously assigned A	5	7	2.62	5.00
>1	B	Unambiguously assigned B	74	26	38.74	18.57
>1	T / A	Ambiguously assigned T/A	0	1	0.00	0.71
>1	T / B	Ambiguously assigned T/B	5	1	2.62	0.71
>1	A / B	Ambiguously assigned A/B	24	12	12.57	8.57
>1	T / A / B	Ambiguously assigned T/A/B	2	2	1.04	1.43

All pollination events were categorized by the origin of the pollen donor(s) assigned according to microsatellite (SSR) genotyping, GUS expression and leaf morphology (trifoliate character)

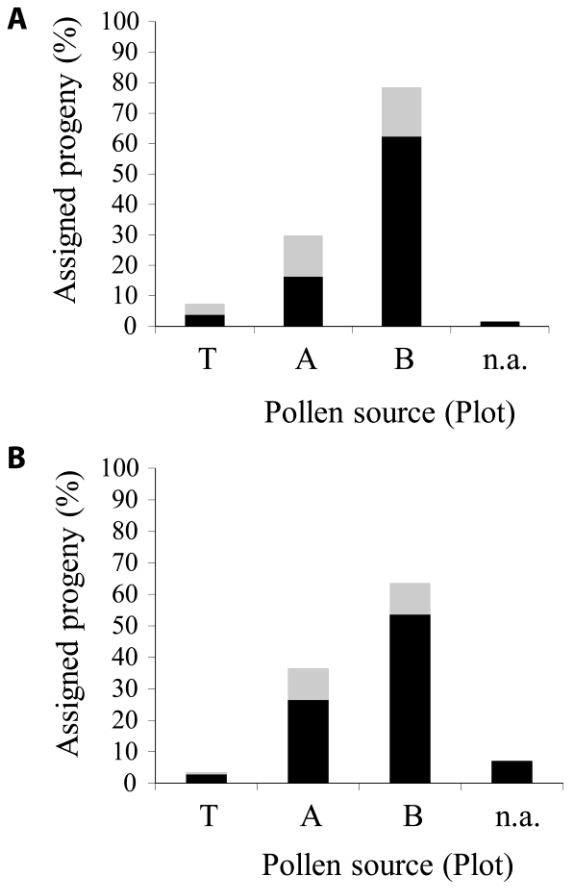

Figure 3. Maximum reproductive success assessed for each pollen source population in A) 2005 and B) 2006. Based on the classification of the pollination events made in Table 4, maximum reproductive success was estimated for each plot, as the percentage of pollination events unambiguously assigned (black color) plus the partial contributions of the percentages corresponding to pollination events ambiguously assigned (grey color). n.a., not assigned.

Distance effect

When considering the distance from recipient trees (Fig. 4), the frequency of mating events assigned to plot B was very high (almost 100%) in the progeny of recipient trees near that plot (see recipient numbers 2, 48, 53 and 55 for 2005 and recipient numbers 2, 6, 50 and 55 for 2006) and lower in recipients located at greater distances from the plot (see recipient numbers 20, 27 and 29 for 2005 and recipient numbers 27, 30 36, 42 for 2006), as expected. However, the extent of the mating capacity of plot B was considerably higher than that of competing plots because 50% of the mating events in the farthest recipient trees (see recipient numbers 20, 27 and 29 for 2005 and recipient numbers 27 and 30 for 2006) were clearly attributable to pollen from plot B (Fig. 4). Together, these results indicate that (1) the mating success of plot B was directly correlated with the distance to the recipient trees and (2) the mating capacity of plot B was able to explain (with 50% success) the parentage of hybrid seedlings from trees located at least 26 rows away.

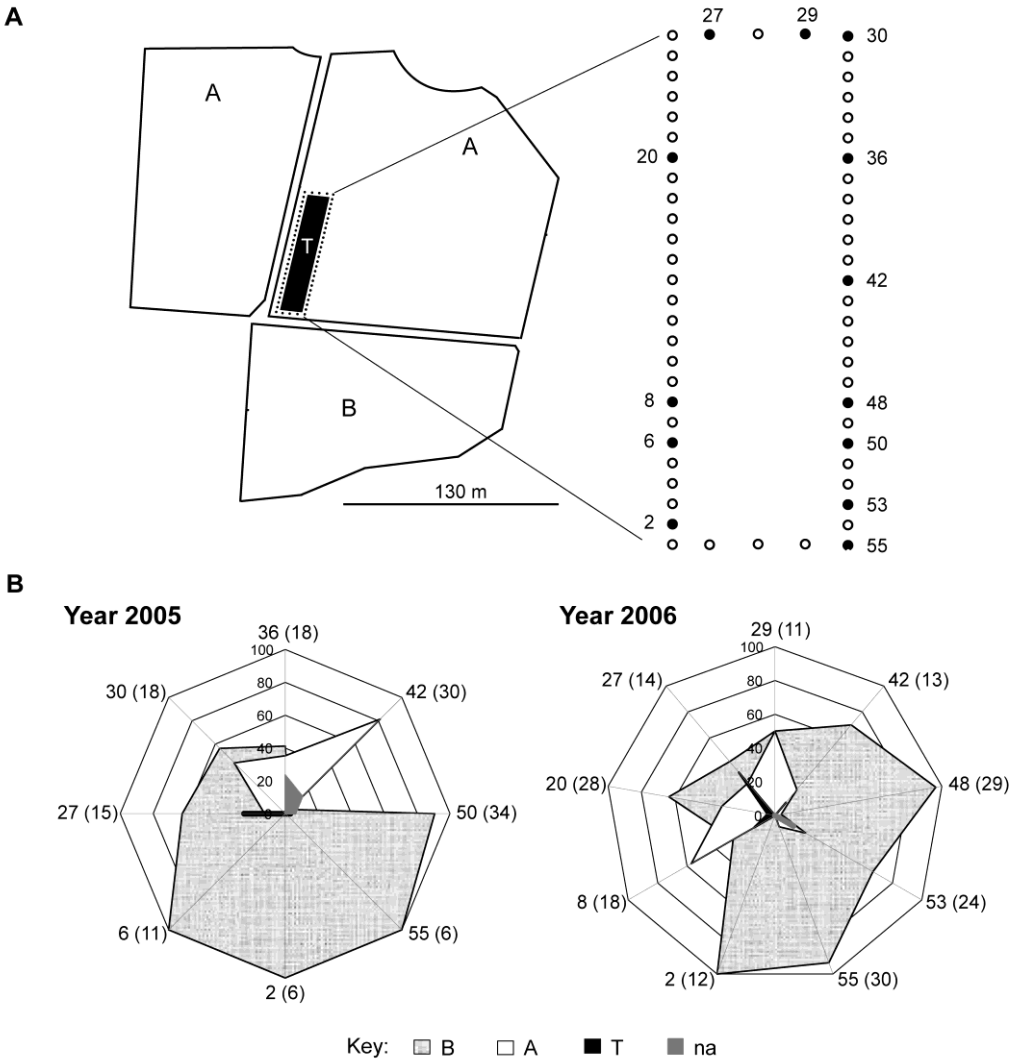

Figure 4 Schematic representation of pollen dispersal patterns at the study site. A) Map showing the relative location of recipients (dots) and pollen source populations (T, A and B plots). Recipient (mother) trees sampled in 2005 and/or 2006 whose progeny were analyzed for paternity assignment are represented by filled circles. **B)** Radial graphs represent the profiles of genotyped progeny from each mother tree. Numbers in the vertices indicate the recipient tree number followed by the total number of progeny seedlings analyzed from the mother tree (number in parentheses). The distribution of recipient trees in the vertices of the graph has been established according to their relative position in the field to accurately visualize the pollen dispersal patterns. The percentage of progeny from each corresponding recipient tree is represented on each radial axis by following categories: "B", progeny unambiguously assigned to B; "A", progeny unambiguously assigned to A; "T", progeny unambiguously assigned to T; and "na", progeny that could not be assigned to any PPD. Clementine plants producing an insufficient number of progeny seedlings were excluded from this study.

The frequency of mating events assigned to plots T or A was null or very low in recipients near plot B and progressively increased with distance from that plot. Therefore, PPDs from plot B strongly limited the mating opportunities of the rest of PPDs from the study site, including those of the contiguous plot T. These results were reliable and indicate a consistent trend in pollen dispersal under our experimental conditions because the patterns were very similar in 2005 and 2006 (Fig. 4), likely also explaining the very low PMTF frequencies obtained during the seven years of the study (Table 2).

Pollen dispersal curves were performed to confirm the influence of the distance from plot B in the mating opportunity of each pollen source population. The logarithmic-X regression model showed that mating chance of plot B was strong and negatively correlated with the distance to B (R^2 = 0.43; correlation coefficient = -0.66). For plot T, the linear regression model showed a relatively weak positive relationship between the variables (R^2 = 0.24; correlation coefficient = 0.495). The square root regression model showed that the mating chance of plot A was moderately strong and positively correlated with the distance to B (R^2 = 0.40; correlation coefficient = 0.638) (Fig. 5).

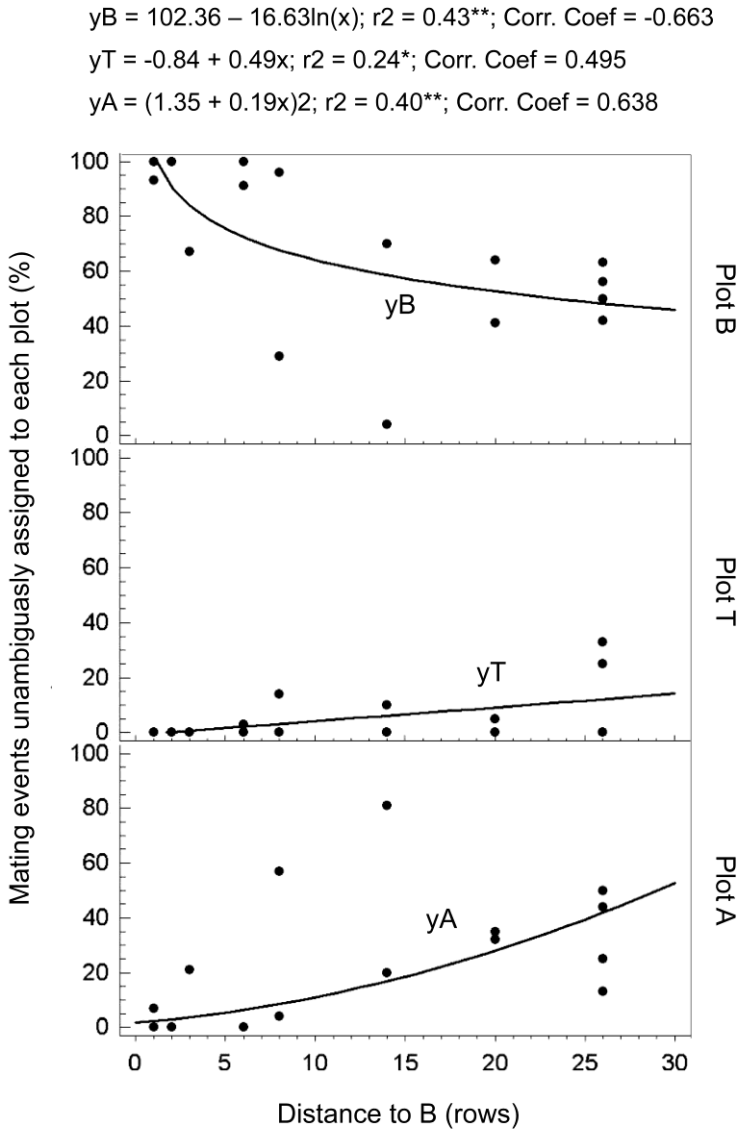

Figure 5. Pollen dispersal curves of each plot as a function of distance to plot B. Progeny from all recipient trees analyzed in 2005 and/or 2006 unambiguously assigned to each plot was divided into classes based on the distance between the (mother) recipient tree and plot B, measured in rows. Black dots represent the mating frequencies in each distance class as a proportion of all pollination events unambiguously assigned to this plot. Lines represent the curves fitted to regression models that best describe the relationship between mating frequencies and distance to Plot B for each pollen source population (*P < 0.05; **P < 0.01).

Density effect

We attempted to determine whether the relative abundance of each PPD from plot B correlated with its mating success. As shown in Fig. 6, there was no statistically significant relationship ($P > 0.1$) between these variables for any of the simple regression models fitted. Indeed, the most abundant PPDs, H1 and H2 (representing 31.96% and 13.92%, respectively, of the total number of PPDs at the study site) displayed low mating success compared to other less-abundant genotypes (such as H3, H4 and H5).

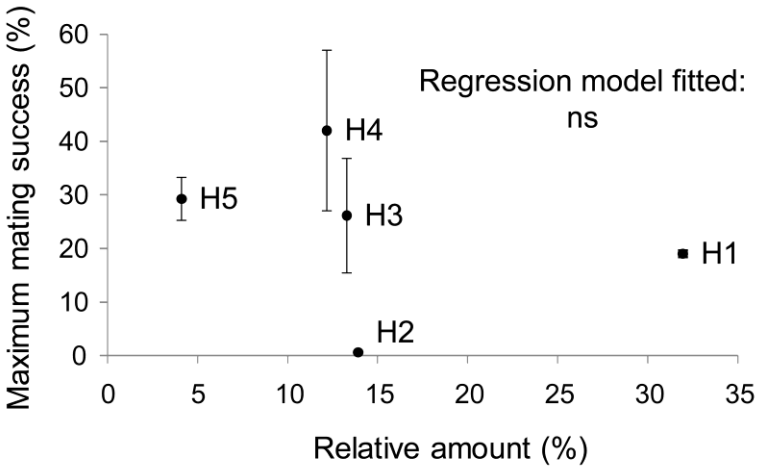

Figure 6. Density effect. Relationship between the maximum mating success achieved in 2005 and 2006 by each Potential Pollen Donor (PPD) of plot B (H1, H2, H3, H4 and H5) and its relative abundance at the study site (reported in Table 1). Black dots represent the proportion of mating events (unambiguously plus ambiguously) assigned to each PPD from plot B calculated over the total progeny unambiguously assigned to this plot and averaged between years. Bars represent standard errors. n.s., P > 0.1 (not significant).

Pollen competition capacity/ability

The pollen competition capacity of H3 (a specific PPD from plot B that showed high mating success in OP conditions) was compared to that of P1 (a transgenic pollen donor of plot T) by mixed pollination treatments, with the aim of clarifying the mechanisms of isolation whereby the surrounding flora limited PMTF. Single pollinations of clementine flowers with P1 and PC, performed as controls, resulted in similar fruit set and seed set for both pollen donors (Table 5), indicating that the transgenic character of P1 did not affect its mating success. Moreover, 86% of the progeny seedlings from that cross were GUS positive, which fit well with expected transgene inheritance. In mixed pollinations with H3+P1, the percentage of GUS-positive progeny seedlings was extraordinarily reduced (5%) with respect to the expected rate if the pollen competition capacity of the two pollen donors were similar (43.75%). Additionally, mixed pollination resulted in a higher seed set (13.5 $\pm$ 2.1) than single pollinations (6.8 $\pm$ 1.7 for P1 and 7.2 $\pm$ 1.9 for PC), indicating that (1) the H3 type strongly reduced the siring success of

P1 and (2) it was much more efficient in cross pollination of recipient clementine trees than P1 or PC.

Table 5. Results of mixed-pollination treatments performed in 2006 and 2007 in comparison with single-pollination control treatments with PC and P1, including fruit set, seed set, and the percentage of GUS-positive (GUS+) seedlings in progeny as a parameter determining the siring success of P1.

Pollen source in pollination treatments		**Fruit set (%)**	**Seed set (No. seeds/fruit)**	**GUS+ progeny (%)**	**Minimum no. of hybrid progeny analyzed per year**
Mixed:	H3+P1	80.9 ± 11.5	13.5 ± 2.1	5.0 ± 1.1	100
Single:	PC	78.5 ± 17.7	7.2 ± 1.9	0.0 ± 0.0	340
	P1	68.0 ± 12.7	6.8 ± 1.7	86.0 ± 3.4	222

Discussion

We report here the first experimental field trial performed with transgenic citrus trees to study maximum transgene flow frequencies. With this aim, eight independent transgenic lines from three genetically diverse citrus types were used as transgenic pollen donors, and a non-transgenic self-incompatible citrus type planted along a contiguous edge was used as the recipient. The choice of a recipient unable to self-fertilize ensured a maximum outcrossing rate and facilitated the monitoring of transgene dispersal [40].

Pollination in most fruit trees, including citrus, is entomophilous [41], and honeybees are the predominant dispersal agents. Bees have the capacity to travel long distances (up to 3 km), but such long-distance flights are extremely rare in high-density plantings [42]. Consequently, as pollen-mediated gene flow in these species may be largely driven by the availability and foraging behavior of the pollinators [43], many studies have demonstrated that the maximum frequency of pollen-mediated gene flow between compatible and co-flowering crops occurred adjacent to the pollen source and typically decreased as the distance between crops increased, drastically decreasing 3 rows away (approximately 15 m) in the case of citrus [22].

In our experimental field, the spatial design, together with the lack of treatments against bees, allowed the maximum PMTF estimable in recipient trees under open-pollinated conditions to be achieved. However, contrary to our expectations, the data compiled during 7 years of assessment indicated that the rate of transgenic seeds from the edge trees was consistently very low. We decided to determine the factor/s that could have contributed to such results with the objective of proposing suitable containment measures applicable to future field trials with GM citrus and possibly other fruit tree crops.

The PMTF monitoring method used in this work was based on the expression of a tracer marker (*uidA*) in seeds. Visual markers have been extensively used in field trials because they make it relatively easy to follow the stability of transgene expression after outcrossing and accurately estimate gene flow [44]. To discard the possibility that transgene silencing and/or

transgene loss in seeds from recipient trees could have masked the actual rate of transgene spread, we validated the monitoring method by analyzing transgene integration in hybrid seedlings during two consecutive years, and the results confirmed that only GUS-positive seeds carried the *uidA* transgene.

Next, we decided to examine isolation barriers that could have limited the mating opportunities between transgenic donors and recipients under our experimental conditions. Barriers to gene exchange between populations may arise through a variety of mechanisms. Pre-mating barriers, such as divergent flowering times and scarcity of flowers from the pollen source, could reduce opportunities for hybridization, thus limiting PMTF [45]. However, our phenological and visual surveys of flowering at the study site indicated that open flowers were highly abundant and synchronic in both transgenic pollen donor and recipient trees.

Reproductive barriers reduce gene flow between groups of organisms and act sequentially before and/or after mating [46]. It has been extensively reported that the potential gene flow from the transgenic pollen source to sympatric species is highly influenced by their reproductive compatibility, which can be measured by fruit set and seed set under controlled pollination conditions [47]. If the extent of reproductive compatibility between the transgenic source and overlapping genotypes were known in advance, it would represent an early 'tier' of risk assessment prior to the measurement of PMTF rates in experimental fields [48]. Single hand-pollination assays showed that the genetic background of the pollen source determined the extent of cross compatibility with the self-incompatible recipient. The importance of this factor has also been stressed in similar studies with other plant species, such as plum [49] and olive, [50] as well as in citrus [51]. As transgenic and control pollen donors produced viable pollen and were cross compatible with the recipient genotype in hand pollinations and the results were irrespective of the transgenic or non-transgenic nature of the pollen donor genotype, the very low rate of PMTF could not be attributed to low sexual compatibility between the source and sink nor to pleiotropic effects derived from expression of the transgenes.

Gene flow can also be influenced by the surrounding flora [52]. A diverse floral neighborhood may reduce conspecific pollen deposition by driving potential pollinators away or by increased heterospecific pollen deposition [53]. Therefore, a key factor that could greatly limit the gene flow between sexually compatible and flowering-synchronized species located at close proximity is the influence of the flowering environment, including conspecific and heterospecific co-flowering plants [54]. The presence of many seeds in fruits from self-incompatible OP recipient trees and the low PMTF obtained indicated effective pollen dispersal from other non-transgenic pollen source/s, most likely from citrus trees present in neighboring plots (A and B). Paternity analysis using molecular markers in the hybrid progeny from a subset of OP recipients confirmed the clear superiority in mating success for plot B. Moreover, the low mating success assigned to plot T (< 8%) coincided with the very low PMTF rates observed along the seven consecutive years of assessment.

Additionally, pollen dispersal curves showed that the pollination competence of trees from plot B was so high that it strongly limited the mating opportunities of the other pollen

sources within the study site, including those of the T plot, even when these were contiguous to the recipients. Furthermore, the mating competence of plot B decreased as the distance to the recipients increased, as expected based on the behavior of bees in citrus orchards [12].

Pollen dispersal curves of entomophilous plants are dependent on the foraging habits of the pollinators, which in turn are responsive to pollinator-linked pre-mating barriers, such as plant population size and density [43]. Bees are very sensitive to plant density and respond in a similar fashion regardless of the plant species involved. Density-dependent foraging distances and pollen dispersal may be a common feature for bees and bee-pollinated plants [41]. However, the relative abundances of each PPD from plot B did not correlate with their mating success efficiencies. Therefore, ecological or pollinator-linked pre-mating barriers were not sufficient to explain the results of the paternity analyses.

It has been suggested that reproductive barriers acting after pollination but before fertilization may play an important role in limiting gene flow [55]. If flowers receive more pollen grains from different pollen sources than the number of ovules they have, not every pollen grain will be able to sire a seed, and selection may occur during mating. This selection may involve discrimination between self and non-self pollen as well as discrimination among compatible donors, between too closely or too distantly related conspecifics, and among species [56]. Nonrandom mating among compatible mates at this level is of particular interest because it has the potential to produce sexual selection [57-59]. Such differential fertilization success often is stronger or exclusively observed when pollen from two species competes for fertilization [60-62]. Pollen competition is recognized as an important and common reproductive barrier [63, 64]. The mixed pollination treatments performed in this study demonstrated that a higher pollen competition capacity of H3 (a PPD from Plot B) compared to that of P1 (a pollen donor from Plot T) explained most of the mating superiority achieved by plot B in OP conditions (71.05% of hybrid progeny in OP conditions versus a maximum of 94.29% obtained in controlled hand pollinations), meaning that pollen competition may have greatly contributed to transgene confinement. Therefore, the presence of neighboring genotypes with very high pollen competition capacity is a crucial factor able to strongly limit PMTF between cross-compatible species when they have synchronized flowering and are planted at close proximity.

Based on these results, it is possible to propose transgene confinement measures that could be applicable to contiguous commercial plantings of citrus and may be extendible to other entomophilous fruit tree species, such as those from the genus *Malus*, *Pyrus*, *Cydonia*, *Eriobotrya* and *Prunus*:

(1) Careful site examination and selection before the release of the GM crop. An essential first step is to determine the extent of reproductive compatibility and flowering synchrony between the transgene source and sympatric crops present at close proximity. If there were not previous information about these issues for the species/genotypes involved, it would be necessary to assess them before the release by performing controlled hand pollinations and phenological studies.

(2) If the species involved were co-flowering and cross compatible, we propose the use of an external edge of trees from a non-GM pollen donor genotype showing pollen competition capacity clearly exceeding that of the transgenic pollen source. The use of a "strong pollinator" could serve as isolation barrier, acting as an alternative source for pollinators and/or as an effective competitor during the fertilization process with the transgenic pollen, and would make transgenic pollen escape practically nonexistent. The choice of the "strong pollinator" genotype would therefore depend on the species considered and could be based on the results obtained from mixed-pollination treatments carried out before the release.

(3) We also propose the use of an external edge of trees from another non-GM genotype as an alternative pollen sink, as has previously been used by others [40]. The genotype chosen for this purpose should have several characteristics: flower synchrony with the transgenic genotype/s and the "strong pollinator", production of high amounts of pollen to attract pollinators and male sterility or self-incompatibility. This edge of trees would facilitate estimating transgene flow frequencies over short distances.

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Supporting Information

Figure S1. Representative pictures of plot T during the flowering period. A) Picture showing the amount of flowers produced by transgenic pollen donor trees. **B)** Picture showing the presence of honeybees at the study site.

Figure S2. Detection of transgenic hybrids in progeny from open-pollinated recipient trees. (A) Seed progeny screened for GUS expression. **(B)** Seedling progeny cultivated on seedbeds in the greenhouse. **(C)** Seedling progeny screened for GUS expression in the leaves. GUS+, GUS-positive. The scale bar on pictures (A) and (C) represents 10 mm.

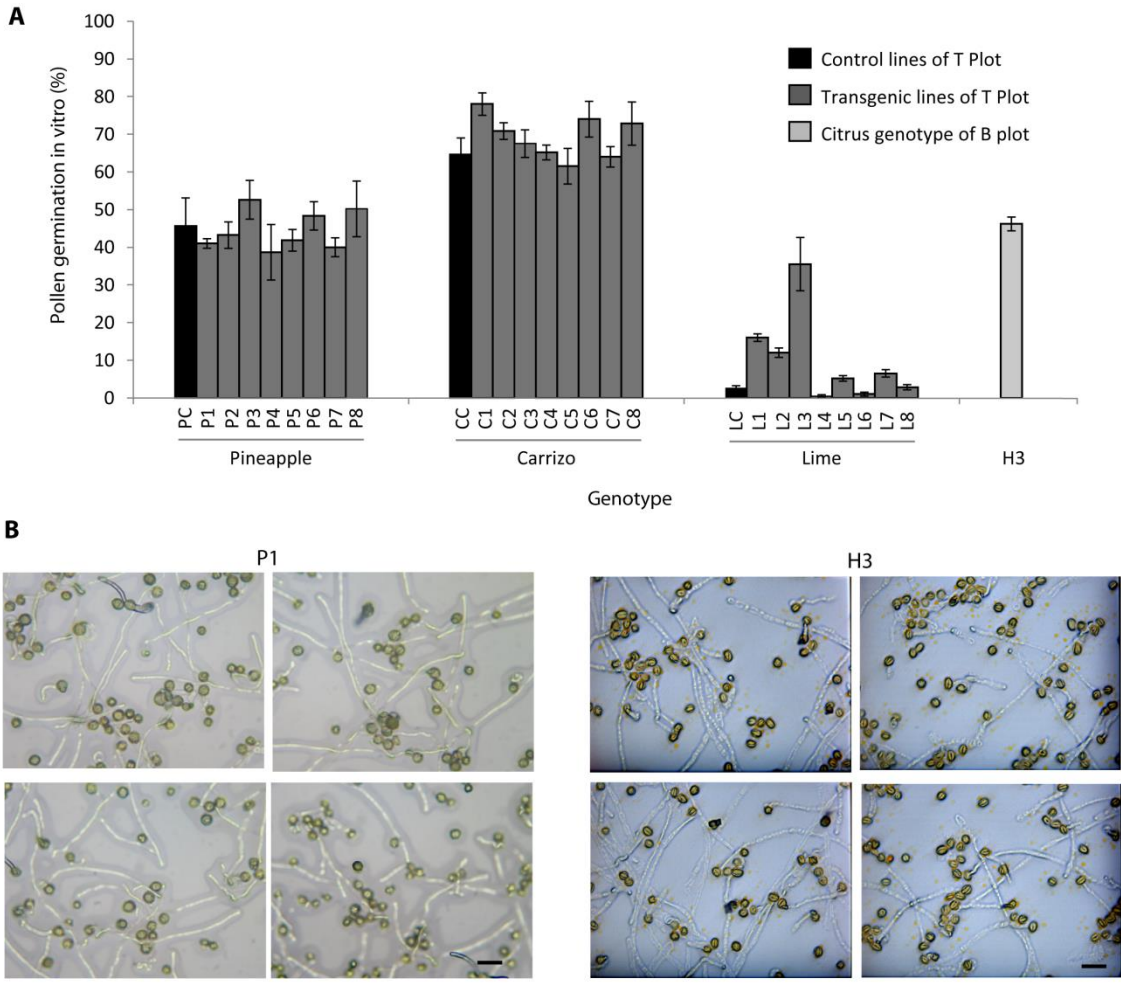

Figure S3. *In vitro* studies of pollen viability. (A) Effect of genotype on pollen germination rate. Bars represent means ± SE. **(B)** Photographic views of pollen germination and tube growth (at 24°C after 24 h incubation in germination medium) from the P1 and H3 genotypes, chosen as competitors in mixed pollination treatments. Scale bars: 100µm.

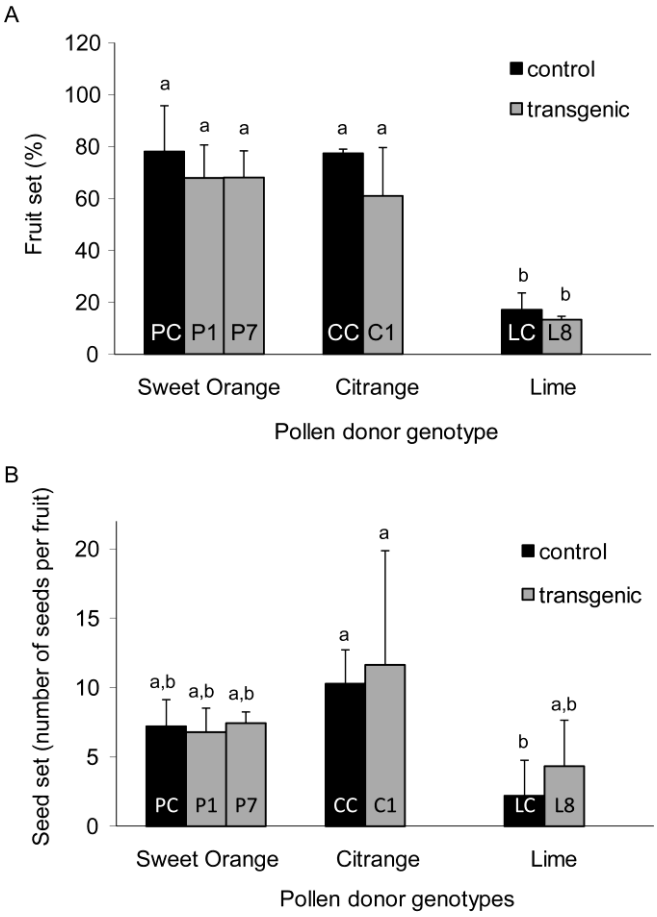

Figure S4. *In vivo* studies of cross-compatibility. The effect of different pollen donors on **A)** fruit set and **B)** seed set in directed crosses with recipient plants. The data are the means obtained in two years (2005 and 2006) ± standard error (SE) bars. The means with at least one common letter are not significantly different (P < 0.05; LSD test).

Table S1. ANOVA analysis for effects of Variety and Genetic Modification (GM) of the pollinator and their interaction on transformed versions of "Fruit set" and "Seed set" data obtained in single pollination treatments.

Variable	Source	df	MS	*F*-value	*P*-value
Fruit set	Variety	2	4283.35	31.12	0.0002
	GM	1	386.451	2.81	0.1323
	Variety x GM	2	51.3173	0.37	0.7001
Seed set	Variety	2	56.0141	5.54	0.0310
	GM	1	4.15599	0.41	0.5395
	Variety x GM	2	2.41639	0.24	0.7930

Table S2. Possible alleles of 10 SSR loci (markers) for each citrus genotype present at the study site and considered for paternity assignment, including clementine as known maternal genotype and all Potential Pollen Donors (PPD) as candidate fathers.

Parental genotypes	PPD code	*CIR01C06*	*CIR07C07*	*CIR01E02*	*CIR01G11*	*mest458*	*CAC23*	*mest107*	*mest86*	*CIR03C08*	*mest192*
Clemenules clementine (maternal genotype)	-	134 166	227 239	155 167	103/109	214	248 251	176 184	112 128	207 225	227
Pineapple sweet orange	P	134 160	227	155 171	103/109	214 217	248 251	176 184	120 128	212 207	222 227
Carrizo citrange	C	146 160	212	170 171	103/109	213 217	245 248	173 176	118 120	212	227
Mexican lime	L	148 170	227	159	100/106 103/109	212 231	245 260	176 182	112	199 241	211 227
Willowleaf mandarin	MC	134 166	227 239	155 167	103/109	208 214	248 251	176	112 120	207 225	222 226
Minneola tangelo	MI	134 160	227	161 165	103/109	214 226	248 251	176 184	128	207	216 226
Orlando tangelo	ORL	132 134	225	161 165	103/109	214 226	248 251	176 184	128	207	202 222
Ellendale mandarin	E	132 134	227 239	155	103/109	214	248 251	176 184	112 128	207 225	216 226
Murcot mandarin	MU	132 160	225 237	155 161	100/106 103/109	214 226	248 251	176	112	207 228	216 230
Ortanique tangor	ORT	132 160	227 239	171	103/109	214 217	248 251	176 184	128	212 225	216 232

Fortune mandarin	F	134 166	239	165 167	103/109	214	248 251	176	112 128	225	216 226
Kara mandarin	K	132 146	233 237	155 157	100/106	217 226	248 251	176 184	112 120	222 225	222
King mandarin x *Poncirus trifoliata*	H1	132 146 160	213 225 237	157 161 169 175	100/106 103/109	214 217 226	245 248 251	173 176 184	112 118 120	214 225 228	222 225 227 232
C. volkameriana x *Poncirus trifoliata*	H2	146 170	212 235	160 176	103/109	206 213	245	173 176	112 118	212 225	230 242
Cleopatra mandarin x *Poncirus trifoliata*	H3	132 146	212 237 241	161 170 176	100/106 103/109	208 213	245 248	173 176	112 118	214 222	222 225
Troyer citrange x Cleopatra mandarin	H4	132 146 160 134	212 241 237 227	161 170 171 155 176	100/106 103/109	208 213 214 217	248 245 251	176 173 184	112 118 120	212 222	222 227
Troyer citrange x Willowleaf mandarin	H5	146 166 134 160	227 212 239	155 170 171 176 167	103/109	208 217 213 214	248 251 245	173 176 184	112 118 120	212 207	222 226 227

Table S3. Results of Paternity assignment in progeny from open-pollinated (OP) recipients harvested in 2005, according to microsatellite (SSR) genotyping, GUS expression and leaf morphology (trifoliate character).

Number of pollen donor(s) assigned	Progeny of OP clementine (Seedling code)	Code(s) of pollen donor(s) assigned	Plot of pollen donor(s) assigned
0	53.2	-	Not assigned
0	53.9	-	Not assigned
0	53.10	-	Not assigned
1	8.14	P	T
1	20.3	P	T
1	42.2	C	T
1	27.2	C	T
1	27.5	P	T
1	27.12	P	T
1	27.14	C	T
1	20.16	H4	B
1	53.26	H4	B
1	48.5	H4	B
1	55.13	H4	B
1	8.19	H4	B
1	20.12	H4	B
1	42.3	H4	B
1	42.13	H4	B
1	48.9	H4	B
1	48.12	H4	B
1	48.17	H4	B
1	48.30	H4	B
1	48.32	H4	B
1	53.1	H4	B
1	53.19	H4	B
1	53.16	H4	B
1	55.18	H4	B
1	55.28	H4	B
1	8.10	H5	B
1	20.4	H5	B
1	20.5	H5	B
1	20.6	H5	B
1	20.22	H5	B
1	25.2	H5	B
1	25.3	H5	B
1	27.3	H5	B
1	27.4	H5	B
1	27.13	H5	B
1	27.15	H5	B
1	35.5	H5	B
1	42.6	H5	B
1	42.10	H5	B
1	42.14	H5	B
1	42.15	H5	B
1	48.8	H5	B
1	53.22	H5	B
1	55.30	H5	B
1	55.34	H5	B
1	2.1	H5	B
1	2.4	H5	B
1	2.6	H5	B
1	2.8	H5	B
1	2.9	H5	B
1	20.2	H1	B
1	27.1	H1	B
1	8.2	MI	A
1	8.11	F	A
1	8.20	F	A
1	8.21	F	A
1	14.1	F	A

1	20.8	F	A
1	53.17	N	A
1	20.14	N	A
1	53.4	N	A
1	20.24	F	A
1	20.25	F	A
1	29.1	F	A
1	29.4	F	A
1	29.5	F	A
1	29.11	F	A
1	35.1	F	A
1	35.3	F	A
1	42.9	F	A
1	42.12	F	A
1	55.29	F	A
1	53.5	F	A
1	20.27	ORL	A
1	20.10	ORL	A
1	27.9	MU	A
1	27.10	MU	A
1	27.11	MU	A
>1	55.3	H3, H4	B
>1	55.5	H3, H4	B
>1	14.2	H3, H4	B
>1	14.3	H3, H4	B
>1	20.9	H3, H4	B
>1	20.18	H3, H4	B
>1	20.26	H3, H4	B
>1	29.3	H3, H4	B
>1	48.7	H3, H4	B
>1	48.11	H3, H4	B
>1	48.18	H3, H4	B
>1	48.19	H3, H4	B
>1	48.23	H3, H4	B
>1	48.25	H3, H4	B
>1	48.29	H3, H4	B
>1	48.36	H3, H4	B
>1	53.3	H3, H4	B
>1	53.6	H3, H4	B
>1	53.15	H3, H4	B
>1	53.20	H3, H4	B
>1	53.25	H3, H4	B
>1	55.7	H3, H4	B
>1	55.9	H3, H4	B
>1	55.10	H3, H4	B
>1	55.14	H3, H4	B
>1	55.16	H3, H4	B
>1	55.17	H3, H4	B
>1	55.20	H3, H4	B
>1	55.24	H3, H4	B
>1	55.26	H3, H4	B
>1	55.27	H3, H4	B
>1	55.31	H3, H4	B
>1	55.32	H3, H4	B
>1	55.33	H3, H4	B
>1	2.2	H3, H4	B
>1	53.8	H3, H4, H1	B
>1	55.21	H3, H4, H1	B
>1	20.15	H3, H4, H1	B
>1	29.2	H3, H4, H1	B
>1	29.10	H3, H4, H1	B
>1	48.10	H3, H4, H1	B
>1	48.13	H3, H4, H1	B
>1	48.14	H3, H4, H1	B
>1	48.16	H3, H4, H1	B
>1	48.21	H3, H4, H1	B

>1	48.26	H3, H4, H1	B
>1	48.31	H3, H4, H1	B
>1	48.33	H3, H4, H1	B
>1	48.34	H3, H4, H1	B
>1	53.13	H3, H4, H1	B
>1	53.18	H3, H4, H1	B
>1	53.21	H3, H4, H1	B
>1	53.23	H3, H4, H1	B
>1	55.11	H3, H4, H1	B
>1	55.15	H3, H4, H1	B
>1	55.25	H3, H4, H1	B
>1	2.5	H3, H4, H1	B
>1	2.7	H3, H4, H1	B
>1	2.10	H3, H4, H1	B
>1	2.12	H3, H4, H5, H1	B
>1	48.2	H3, H4, H5	B
>1	48.22	H3, H4, H5	B
>1	55.12	H3, H4, H5	B
>1	55.4	H3, H2, H1	B
>1	29.9	H4, H1	B
>1	20.28	H4, H5	B
>1	2.11	H4, H5	B
>1	2.3	H4, H5	B
>1	20.11	H4, H5	B
>1	20.21	H4, H5	B
>1	35.2	H4, H5	B
>1	27.7	H4, H5	B
>1	42.1	H4, H5	B
>1	53.7	H4, H5	B
>1	48.3	E, ORT, N	A
>1	20.1	E, ORT, N	A
>1	53.12	E, ORT, N	A
>1	53.27	E, ORT, N	A
>1	55.2	E, ORT, N	A
>1	42.8	C, H4 ,H5, H1	T / B
>1	27.8	C, H4, H5	T / B
>1	48.4	C, H4, H5	T / B
>1	55.8	C, H3, H4, H5, H1	T / B
>1	8.6	P, H5	T / B
>1	20.7	H1, ORL,MU	A / B
>1	20.19	H1, ORL,MU	A / B
>1	20.20	H1, ORL,MU	A / B
>1	20.23	H1, ORL,MU	A / B
>1	20.29	H1, ORL,MU	A / B
>1	20.30	H1, ORL,MU	A / B
>1	48.20	H1, MU	A / B
>1	25.1	H5, MC, F, N	A / B
>1	25.5	H5, MC, F, N	A / B
>1	29.6	H5, MC, F, N	A / B
>1	29.8	H5, MC, F, N	A / B
>1	42.7	H5, MC, F, N	A / B
>1	55.6	H5, MC, F, N	A / B
>1	8.1	H5, MC F, N	A / B
>1	8.8	H5, MC, F, N	A / B
>1	8.13	H5, MC, E, F	A / B
>1	25.6	H5, MC	A / B
>1	8.12	H5, MC	A / B
>1	42.11	H5, MC	A / B
>1	8.4	H5, MC	A / B
>1	8.3	H4, H5, MC	A / B
>1	8.9	H4, H5, MC	A / B
>1	8.16	H4, H5, MC	A / B
>1	29.7	H4, H5, MC	A / B
>1	8.15	P, H4,H5, ORT	T / A / B
>1	8.5	P, H4,H5, ORT	T / A / B

Table S4. Results of paternity assignment in progeny from open-pollinated (OP) recipients harvested in 2006, according to microsatellite (SSR) genotyping, GUS expression and leaf morphology (trifoliate character).

Number of pollen donor(s) assigned	Progeny of OP clementine (Seedling code)	Code(s) of pollen donor(s) assigned	Plot of pollen donor(s) assigned
0	20.6	-	Not assigned
0	36.14	-	Not assigned
0	36.2	-	Not assigned
0	36.4	-	Not assigned
0	36.5	-	Not assigned
0	42.15	-	Not assigned
0	42.27	-	Not assigned
0	42.35	-	Not assigned
0	50.29	-	Not assigned
0	50.6	-	Not assigned
1	27.4	P	T
1	27.5	P	T
1	50.24	L	T
1	30.11	H1	B
1	36.8	H1	B
1	50.18	H1	B
1	50.37	H1	B
1	55.8	H1	B
1	6.7	H1	B
1	30.13	H2	B
1	2.2	H3	B
1	2.4	H3	B
1	2.3	H4	B
1	2.6	H4	B
1	20.1	H4	B
1	30.5	H4	B
1	36.9	H4	B
1	50.21	H4	B
1	50.28	H4	B
1	50.36	H4	B
1	50.38	H4	B
1	50.7	H4	B
1	50.8	H4	B
1	50.9	H4	B
1	6.11	H4	B
1	6.12	H4	B
1	6.16	H4	B
1	2.9	H5	B
1	27.11	H5	B
1	27.2	H5	B
1	27.8	H5	B
1	27.9	H5	B
1	30.1	H5	B
1	30.12	H5	B
1	30.15	H5	B
1	30.18	H5	B
1	36.1	H5	B
1	36.10	H5	B
1	36.18	H5	B
1	42.19	H5	B
1	50.15	H5	B
1	50.2	H5	B
1	50.23	H5	B
1	50.3	H5	B
1	50.30	H5	B
1	50.31	H5	B
1	55.10	H5	B
1	55.14	H5	B
1	55.4	H5	B
1	6.6	H5	B

1	6.8	H5	B
1	30.6	F	A
1	36.13	F	A
1	36.19	F	A
1	42.11	F	A
1	42.17	F	A
1	42.2	F	A
1	42.3	F	A
1	30.17	MI	A
1	30.7	MI	A
1	30.9	MI	A
1	36.17	MI	A
1	42.10	MI	A
1	42.14	MI	A
1	42.20	MI	A
1	42.28	MI	A
1	42.31	MI	A
1	42.7	MI	A
1	42.33	MI	A
1	30.21	N	A
1	36.11	N	A
1	36.20	N	A
1	42.18	N	A
1	42.21	N	A
1	42.24	N	A
1	42.32	N	A
1	42.8	N	A
1	36.12	N	A
1	42.26	N	A
1	27.6	ORL	A
>1	2.1	H1, H4	B
>1	50.1	H1, H4	B
>1	50.19	H1, H4	B
>1	50.26	H1, H4	B
>1	50.27	H1, H4	B
>1	6.13	H1, H4	B
>1	6.3	H1, H4	B
>1	30.23	H1, H4	B
>1	36.3	H1, H4	B
>1	50.4	H1, H4	B
>1	27.7	H1, H3, H4	B
>1	30.22	H1, H3, H4	B
>1	36.16	H1, H3, H4	B
>1	50.10	H1, H3, H4	B
>1	50.11	H1, H3, H4	B
>1	50.12	H1, H3, H4	B
>1	50.16	H1, H3, H4	B
>1	50.20	H1, H3, H4	B
>1	50.22	H1, H3, H4	B
>1	50.25	H1, H3, H4	B
>1	50.34	H1, H3, H4	B
>1	50.35	H1, H3, H4	B
>1	55.12	H1, H3, H4	B
>1	55.15	H1, H3, H4	B
>1	6.10	H1, H3, H4	B
>1	6.14	H1, H3, H4	B
>1	6.15	H1, H3, H4	B
>1	30.24	MI, F	A
>1	42.16	MI, F	A
>1	42.30	MI, F	A
>1	42.4	MI, F	A
>1	42.6	MI, F	A
>1	42.34	MI, ORL	A
>1	30.3	ORL, MU	A
>1	50.14	C, H4, H5	T / B
>1	50.17	P, MI, E, ORT	T / A

>1	30.2	H1, ORL, MU	A / B
>1	42.37	H4, N	A / B
>1	27.14	H5, MC	A / B
>1	27.15	H5, MC	A / B
>1	27.16	H5, MC	A / B
>1	27.3	H5, MC	A / B
>1	42.36	H5, MC, F	A / B
>1	27.13	H5, MC, F, N	A / B
>1	27.20	H5, MC, F, N	A / B
>1	30.14	H5, MC, F, N	A / B
>1	36.15	H5, MC, F, N	A / B
>1	42.5	H5, MC, N	A / B
>1	27.10	H5, P, MC	T / A / B
>1	42.9	H5, P, MC, E, F	T / A / B

4. RESULTS: CHAPTER 2.

Field performance of transgenic citrus trees: Assessment of the long-term expression of *uidA* and *nptII* transgenes and its impact on relevant agronomic and phenotypic characteristics

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Abstract

Background: The future of genetic transformation as a tool for the improvement of fruit trees depends on the development of proper systems for the assessment of unintended effects in field-grown GM lines. In this study, we used eight transgenic lines of two different citrus types (sweet orange and citrange) transformed with the marker genes β-glucuronidase (*uidA*) and neomycin phosphotransferase II (*nptII*) as model systems to study for the first time in citrus the long-term stability of transgene expression and whether transgene-derived pleiotropic effects occur with regard to the morphology, development and fruit quality of orchard-grown GM citrus trees.

Results: The stability of the integration and expression of the transgenes was confirmed in 7-year-old, orchard-grown transgenic lines by Southern blot analysis and enzymatic assays (GUS and ELISA NPTII), respectively. Little seasonal variation was detected in the expression levels between plants of the same transgenic line in different organs and over the 3 years of analysis, confirming the absence of rearrangements and/or silencing of the transgenes after transferring the plants to field conditions. Comparisons between the GM citrus lines with their non-GM counterparts across the study years showed that the expression of these transgenes did not cause alterations of the main phenotypic and agronomic plant and fruit characteristics. However, when comparisons were performed between diploid and tetraploid transgenic citrange trees and/or between juvenile and mature transgenic sweet orange trees, significant and consistent differences were detected, indicating that factors other than their transgenic nature induced a much higher phenotypic variability.

Conclusions: Our results indicate that transgene expression in GM citrus remains stable during long-term agricultural cultivation, without causing unexpected effects on crop characteristics. This study also shows that the transgenic citrus trees expressing the selectable marker genes that are most commonly used in citrus transformation were substantially equivalent to the non-transformed controls with regard to their overall agronomic performance, as based on the use of robust and powerful assessment techniques. Therefore, future studies of the possible pleiotropic effects induced by the integration and expression of transgenes in field-grown GM citrus may focus on the newly inserted trait(s) of biotechnological interest.

Background

Crop improvement via genetic modification (GM) remains controversial, with one of the major issues being the potential for unintended effects caused by the integration and expression of the transgene. Such unintended effects may occur as a result of interactions between the transgene or its regulatory elements and the plant genome at the site of insertion. The integration site could affect a transgenic plant in two ways: with regard to the functioning of the surrounding DNA sequences (insertion effect) and with regard to the expression of the transgene (position effect). The insertion effect can be of a mutagenic nature and could result in null, loss of function, gain of function or other possible phenotypes, depending on the specific DNA region that is randomly targeted by the insertion and the regulatory elements within the foreign DNA (T-DNA in the case of *Agrobacterium tumefaciens*-mediated transformation). With respect to the position effect, it is well known that the integration site and transgene architecture (i.e., transgene copy number) may influence the transgene expression level and stability (see [1,2] for reviews). All of these effects can vary according to the specific integration event and would, therefore, be unique to each independent transgenic line. Moreover, the full range of recurring locus-independent changes induced by the expression of a given transgene constitutes the so-called pleiotropic effects. Although some of these effects may be expected based on the intended trait, others may occur through unexpected interactions of the transgene products with plant cell metabolism [3].

Within the context of GM crops, the relevance of unintended effects is mainly related to their implications regarding agronomic performance [4]. There are examples showing that transgenesis may generate non-desirable phenotypic alterations as a consequence of pleiotropic changes in plant growth and development, compromising the preservation of the identity of the transformed genotype [5-9]. Although the existence of such unintended effects does not necessarily generate concerns in terms of safety (for human health and/or the environment), it is important to evaluate their extent to validate or discard the application of each genetic engineering product in agriculture [10]. Some studies have reported unintended pleiotropic effects generated by the expression of selectable marker genes [11-13], despite the fact that such transgenes are not generally believed to alter biological processes in plants [14-16]. These findings indicate that the pleiotropic effects associated with selectable marker genes also need to be assessed in a range of plants, particularly in those that are expected to remain in the field for many years and be subjected to highly variable environmental conditions.

The significance of unintended changes is negligible in most cases because most event-specific effects are routinely eliminated during the early screening stages [1]. However, even after selection, there are some reports of apparently normal transgenic plants exhibiting aberrant behavioral or biochemical characteristics upon further analysis (for reviews, see the references in [17-19]). Such studies often focus on the possibility that a transgene may not result in the desired phenotypic effect when GM plants are moved from a controlled glasshouse environment to more variable field conditions [20]. However, some studies have also reported potentially unintended phenotypic effects of transgenes in GM plants exposed to a range of realistic environmental

conditions. Examples of these unexpected traits include lower yields [21-23], an enhanced susceptibility to pathogens [24], altered insect resistance as a consequence of non-targeted changes in secondary metabolism [25] and an enhanced outcrossing ability of transgenic plants [26,27].

Therefore, it is important to investigate the substantial equivalence of transgenic crops through the assessment of phenotypic differences between GM lines and their non-GM counterparts in field trials [4]. The comparative analysis of physiological character ristics, such as agronomic-, morphology- and development-related traits, is an essential first step in identifying these differences [28]. Furthermore, an appropriate experimental design is required for the assessment of GM crops [29]. The guidelines described internationally for performing agronomic and phenotypic analyses of GM crops emphasize that choosing appropriate comparators and performing adequate field trials (e.g., number of growing seasons, replicates, selection of characteristics to be analyzed) are crucial to ensure confidence in the results. The experimental design should also be consistent with the intended method of statistical analysis [30].

Citrus is the most economically important and extensively grown fruit tree crop in the world [31]. Genetic transformation is considered an essential tool in some current citrus improvement programs and offers great opportunities to achieve the goals of interest, such as the resistance to devastating diseases or enhancement of health-promoting fruit qualities [32]. However, there are no available reports regarding the agronomic performance of transgenic citrus plants. Although there are some studies on the integration patterns, expression and inheritance of transgenes in citrus plants and their progeny [33,34], none of these investigations has addressed the impact of transgene integration and expression on agronomic characteristics. The aim of the present study was to estimate the effects of transgenesis on the performance of citrus trees grown in an orchard since 1997 and to study the stability of transgene integration and expression. The experiment involved the release of 8 independent transgenic lines of Carrizo citrange (*Citrus sinensis* L. Osb. X *Poncirus trifoliata* L. Raf.) and Pineapple sweet orange (*C. sinensis* L. Osb.) carrying the marker transgenes *nptII* and *uidA* (GUS). We also included non-transgenic regenerants obtained from the transformation experiments, which were used as the non-GM controls. Making use of comparative analyses of fruit quality, tree morphology and phenology conducted over several years, the present work evaluates the substantial equivalence of field-grown transgenic citrus plants relative to their non-GM counterparts. Furthermore, to validate the evaluation techniques applied in this work, the effects of genetic and physiological factors (other than 'transgene') were also investigated. For this purpose, some transgenic lines of each citrus type that could be distinguished by an additional trait, either their ploidy level (diploid vs. tetraploid, in the case of Carrizo citrange) or developmental stage (juvenile vs. mature, in the case of Pineapple orange), served as comparators to test the effects of 'ploidy' and 'ontogeny' on the parameters studied for the citrange and sweet orange lines, respectively. This is the first detailed study demonstrating the substantial equivalence of field-grown GM and non-GM citrus trees reported thus far and could represent a model for investigating the performance of GM fruit trees under field conditions through the use of appropriate controls and comparators.

Results

All of the citrus plants used in the field trial (T plot) were generated previously in our laboratory, and their main characteristics are summarized in Figure 1. The Pineapple sweet orange plants were obtained from the experiments described in Cervera *et al*., 1998a [35]. For the field release, we selected six independent transgenic lines (designated P3 to P8) and one non-GM regenerant (PCA) derived from adult plant material. Moreover, to address the 'ontogeny' effect in the sweet orange lines, we also included two independent transgenic lines (designated P1 and P2) and a non-GM control (designated PCJ) derived from juvenile material in the experimental orchard. "Juvenile" transformants flowered in 2002 and set fruits in 2003 for the first time. Although they could not be considered strictly juvenile from that moment on, these plants passed through a transition phase [36] characterized for tree vigorous growth, thorniness, alternate bearing and reduced yield, which prolonged at least for the 3 years of study. Conversely, mature transformants set fruits soon after being grafted in the field and they showed typical features of true-to-type Pineapple sweet orange trees bearing regular fruits. The Carrizo citrange plants were generated in experiments described in Peña *et al*., 1995 [37] and Cervera *et al*., 1998b [38], and six independent transformants were selected for the field release (designated C1, C3, C4, C5, C6 and C8) in addition to one non-GM regenerant. All of these lines were diploid and presented a normal appearance in a preliminary screen under greenhouse conditions [33]. We also decided to include two unintentionally obtained off-type transgenic tetraploid lines (designated C2 and C7) in the field trial to assess the 'ploidy' effect in the citrange lines. We did not include a non-GM tetraploid control line because none was spontaneously generated during the course of the original experiments [33]. Mexican lime (*C. aurantifolia* (Christm.) Swing.) plants present in the orchard were obtained from experiments described in [39]. Although our original intention was to conduct the same analyses with these transgenic lime trees, they were excluded from further analysis because they suffered severe symptoms from frost during successive winters.

In 2004, seven years after planting in the orchard (Figure 1B), when all of the transgenic and control lines had experienced several cycles of fruit production, the molecular and phenotypic analyses of each plant were initiated.

Long-term stability of transgene integration and expression

To demonstrate the long-term stable integration and expression of *nptII* and *uidA* gene cassettes, analyses of genomic DNA were performed on 7-year-old, orchard-grown transgenic citrus trees, and the results were compared to the results previously reported by our group [33,35,38]. Southern blot analysis confirmed the presence of stably integrated transgene cassettes into the plant genomes of all of the transgenic trees. Digestion with either *Hind*III or *Dra*I + *Cla*I resulted in the generation of internal fragments of the *uidA* and *nptII* cassettes, with the expected sizes of 2.8 and 2.0 kb, respectively. The corresponding non-transgenic controls showed no hybridization signals (results not shown). The T-DNA of the binary vector used has unique restriction sites for *Eco*RI and *Dra*I at

A

citrus type	Line	GM	Ontogeny	Ploidy level	Nº of plants
Sweet orange	P1	T	J	2n	2
	P2	T	J	2n	2
	P3	T	A	2n	2
	P4	T	A	2n	2
	P5	T	A	2n	2
	P6	T	A	2n	2
	P7	T	A	2n	2
	P8	T	A	2n	2
	PCJ	C	J	2n	2
	PCA	C	A	2n	6
citrange Carrizo	C1	T	J	2n	2
	C2	T	J	4n	2
	C3	T	J	2n	2
	C4	T	J	2n	2
	C5	T	J	2n	2
	C6	T	J	2n	2
	C7	T	J	4n	2
	C8	T	J	2n	2
	CC	C	J	2n	8
Mexican lime	L1	T	J	2n	2
	L2	T	J	2n	2
	L3	T	J	2n	2
	L4	T	J	2n	2
	L5	T	J	2n	2
	L6	T	J	2n	2
	L7	T	J	2n	2
	L8	T	J	2n	2
	LC	C	J	2n	8

B

C

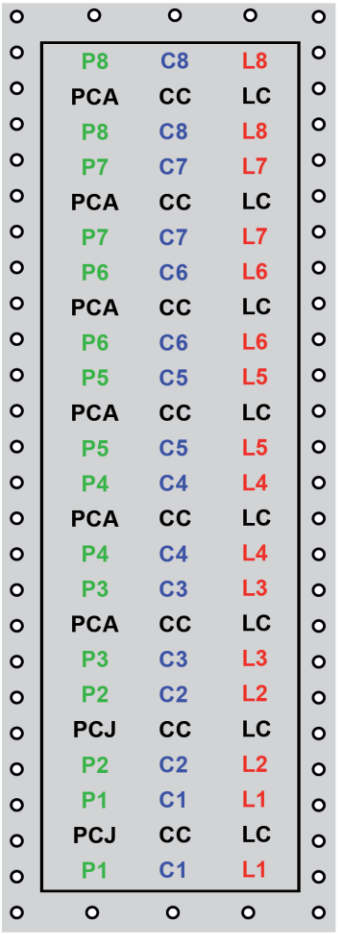

Figure 1. Experimental field trial (T plot). A) Description of all of the citrus lines selected for release in the experimental orchard, including the citrus type, genetic modification (GM), developmental stage (ontogeny), ploidy level and number of plants of each line. T, transgenic; C, control; J, juvenile; A, adult; 2n, diploid; 4n, tetraploid. **B)** Images showing the T plot in 1998 (left) and in 2004 (right). **C)** Schematic diagram of the T plot showing the arrangement of the 130 trees, including 16 transgenic plants of Pineapple sweet orange (green), 16 transgenic plants of Carrizo citrange (blue) and 16 transgenic plants of Mexican lime (red). In addition, there were 8 non-transgenic control plants from each citrus type interspersed individually between the two plants from each transgenic line (black). Fifty-eight non-transgenic Clemenules clementine trees planted along an external edge (white circles) were used as a buffer to prevent transgene flow through pollen dispersal.

the left and right borders of the sequence, respectively, and digestion of the DNA with either of these enzymes generated unique fragments between the T-DNA and plant DNA. A different number of insertions and integration patterns were revealed in the different transgenic lines following hybridization with the *uidA* or *nptII* probes, as summarized in Table 1. All of the transgenic plants exhibited the long-term stable integration of both the *uidA* and *nptII* genes, with different hybridization patterns being detected among independent transgenic lines. As shown in Table 1, the estimated number of copies of each transgene was identical to that shown previously by our group (when the transformants were generated).

Table 1. Long-term stability of the integration of transgenes in transgenic sweet orange and citrange lines determined by Southern blot analysis

Line	Copy number determined by Southern blot			
	In previous analyses[1]		In 2004[2]	
	uidA	*nptII*	*uidA*	*nptII*
P1	nd	nd	4	4
P2	nd	nd	1	1
P3	2	1	2	1
P4	1	2	1	2
P5	1	3	1	3
P6	1	1	1	1
P7	4	4	4	4
P8	1	1	1	1
C1	2	2	2	2
C2	1	1	1	1
C3	nd	nd	2	2
C4	2	2	2	2
C5	2	1	2	1
C6	2	1	2	1
C7	1	1	1	1
C8	1	1	1	1

[1]Analyses performed prior to the release in the experimental orchard in 1997, as described in [35] [38] and [33]
[2]Analyses performed in this work.
nd, not determined

GUS analyses of different organs of the transgenic trees were performed periodically beginning in 2004. All of the transgenic samples showed blue staining in histochemical assays during the 3 consecutive years of the study (Figure 2A), whereas no coloration was visible in the control samples (Figure 2A, left column of the image). In spite of some detectable variation in the expression levels among the different transgenic lines, GUS expression remained relatively high in all of the tissues analyzed for all of the transgenic lines. Moreover, the transgenic plants showed similar conserved patterns of GUS expression throughout the study period, and no drastic decreases or increases in transgene activity were observed within any tree or between trees of the same line.

To estimate the enzyme activities, fluorimetric GUS assays and NPTII ELISAs were performed on leaf samples from all of the plants every 3 months over a period of one year (2007). The measurements were performed using different plants of the same line and at different time points to ensure a reliable representation of the temporal intraline transgene expression. The results regarding the fluorimetric GUS activity and immunological quantification of NPTII accumulation are shown in Figure 2B. All of the transgenic lines displayed both NPTII and GUS activity, with the expression levels varying from 2.8 to 26.6 ng NPTII per mg total protein and from 20.4 to 191.8 pmol MU per min per µg total protein, respectively, in the transgenic samples. These ranges in activity were similar to those obtained in the initial populations of transformants from which we propagated the sweet orange and citrange plants under investigation [33,35]. The data shown in Figure 2B are the average annual values per line. The relatively low SE bars indicate little variation in the expression levels between the plants of the same line and a lack of considerable seasonal fluctuations.

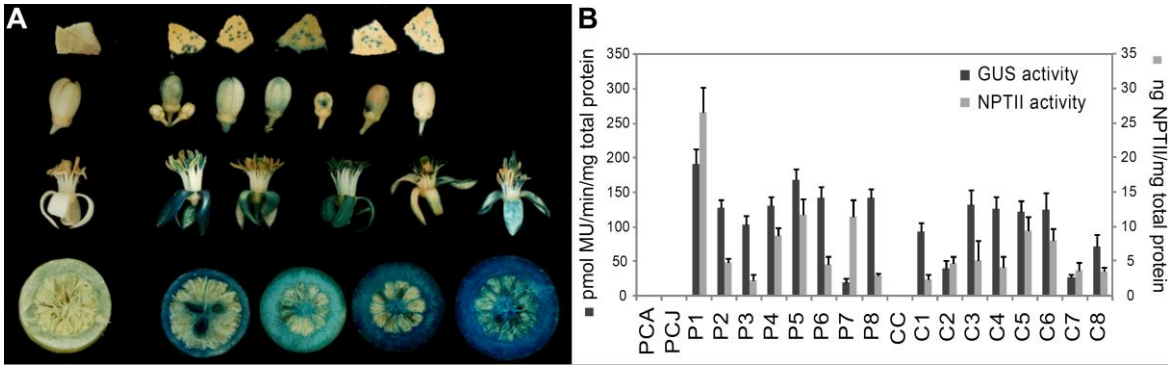

Figure 2 Characterization of 7-year-old, orchard-grown transgenic citrus trees: analyses of GUS and NPTII protein activities. A) Histochemical GUS analysis of different organs (row 1, leaves; row 2, flowers in pre-anthesis; row 3, flowers in post-anthesis; row 4, transverse sections of immature fruit) from the transgenic citrus plants. Representative image showing the staining patterns exhibited by the different transgenic lines under investigation. Left column, control samples showing no coloration; all of the leaves were punched to facilitate substrate infiltration. After the reaction, the organs were cleared of chlorophyll by means of an ethanol series. **B)** GUS and NPTII activities in the leaf samples from all of the sweet orange and citrange lines grown in the experimental orchard. Data represent the average values ± SEM from the different plants of each line, assayed at four time points (seasonally) over the course of one year

Morphological and phenological analyses revealed the normal appearance and development of 7-year-old orchard-grown transgenic trees

We performed morphological and phenological analyses of the transgenic trees in comparison with their respective non-GM counterparts to study the influence of transgenesis on the main phenotypic characteristics of the plants (i.e., the 'transgene' effect). For this purpose, each citrus genotype was analyzed separately.

Based on an initial visual scrutiny, noticeable differences were detected among the sweet orange lines with respect to the size of the tree, as the juvenile lines showed a greater size than the adult lines (Figure 3A). No other morphological differences were observed among the lines. Indeed, the transgenic trees could not be visually distinguished from their respective non-transgenic controls at any time during the growing season or after fruit harvesting (Figure 1B). To confirm these observations, two morphological variables related to tree size, tree height (TH) and tree canopy volume (TCV), were measured in two consecutive years (2004 and 2005) for each line, and the data were analyzed statistically. Differences among the sweet orange lines were confirmed using the Kruskal-Wallis test ($p < 0.001$) for the variables TH and TCV. Notched box-and-whisker plots showed that the median values of both variables were always higher for the juvenile lines (PCJ, P1 and P2) than for the adult lines (PCA, P3, P4, P5, P6, P7 and P8), indicating that the factor 'ontogeny' (developmental stage) had a marked effect on the parameters. Mann–Whitney tests confirmed the highly significant differences in these variables between 'ontogeny' classes (juvenile versus adult lines). However, no significant differences ($p < 0.05$) in these variables were detected between the transgenic and control lines (Figure 3B). These results indicated that the juvenile plants continued to display the morphological features typical of juvenility (faster growth behavior than adults), even after

entering the fruit production stage. In contrast, transgenesis did not affect any of the morphological traits.

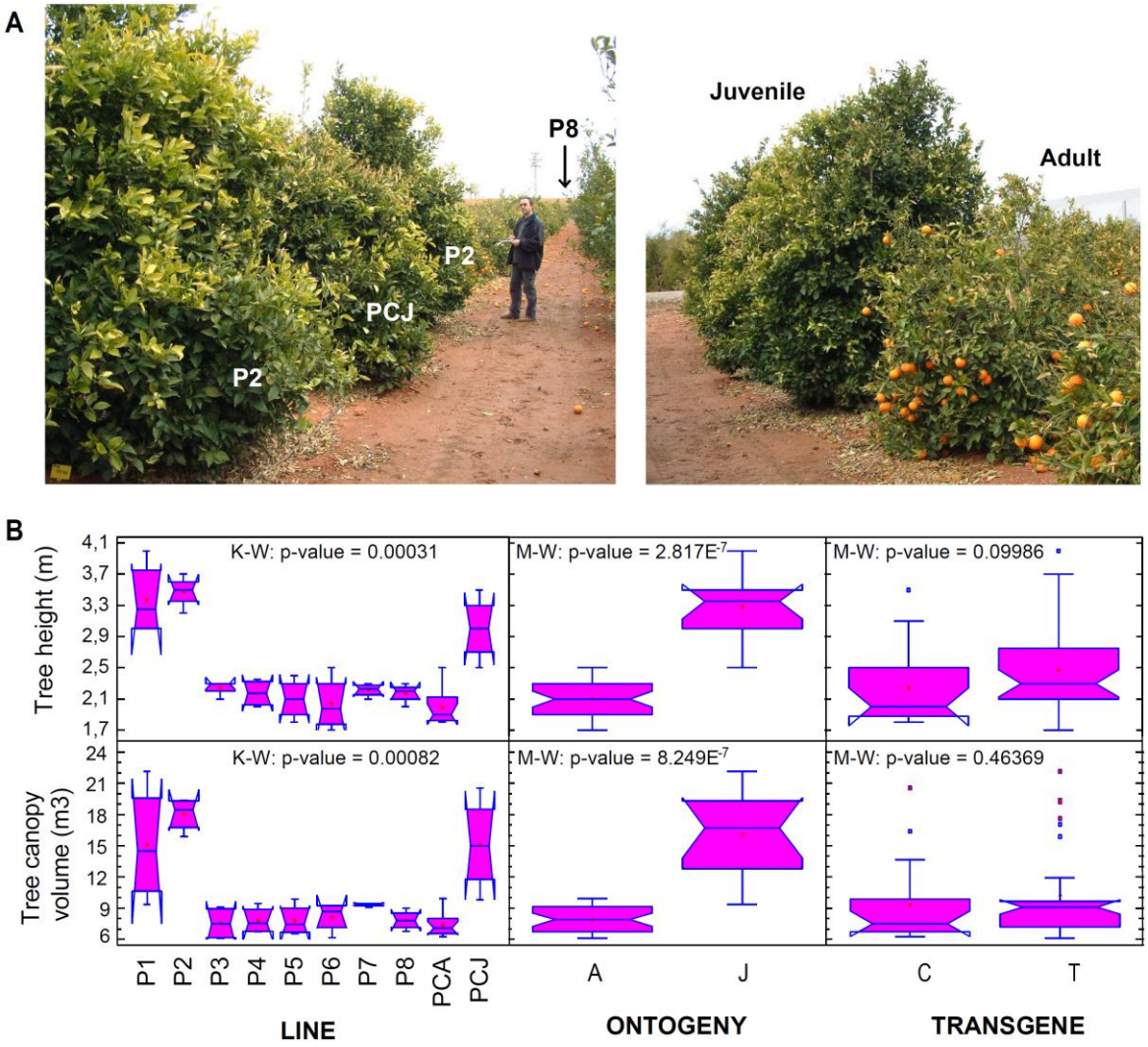

Figure 3 Morphological analysis of sweet orange trees. A) Images showing the appearance of the sweet orange trees in the experimental orchard. Image on the left: general view of the sweet orange trees (from the P2 to P8 lines) distributed in a row in the orchard; image on the right: size comparison between juvenile and adult trees. **B)** Effects of the 'line', 'transgene' and 'ontogeny' factors on the morphological variables Tree height and Tree canopy volume. The data represented in the notched box-and-whisker plots were calculated from measurements recorded over the course of two years (2004 and 2005) at the end of the growing season. K-W, Kruskal-Wallis test (n = 48); M-W, Mann–Whitney tests; A, all adult lines (n = 36); J, all juvenile lines (n = 12); C, all control lines (n = 16); T, all transgenic lines (n = 32).

In the citrange population, several obvious differences were visually detected only in lines C2 and C7. These tetraploid lines developed thicker and broader leaves, having a darker green color, and larger flowers and showed a slightly smaller tree size and leaf density in comparison with the rest of the citrange lines (all diploid) (Figure 4A). A Kruskal-Wallis test confirmed significant differences (at the 95 % confidence level) among the medians of the variables TH, TCV, leaf fresh weight (LFW) and leaf area (LA) for the citrange lines. Subsequently, Mann–Whitney tests detected a statistically significant 'ploidy' effect for the TH and TCV variables ($p < 0.01$) and for the LFW and LA variables at

higher levels of significance ($p < 0.0001$), whereas the 'transgene' factor had no effect on any of these variables ($p < 0.05$).

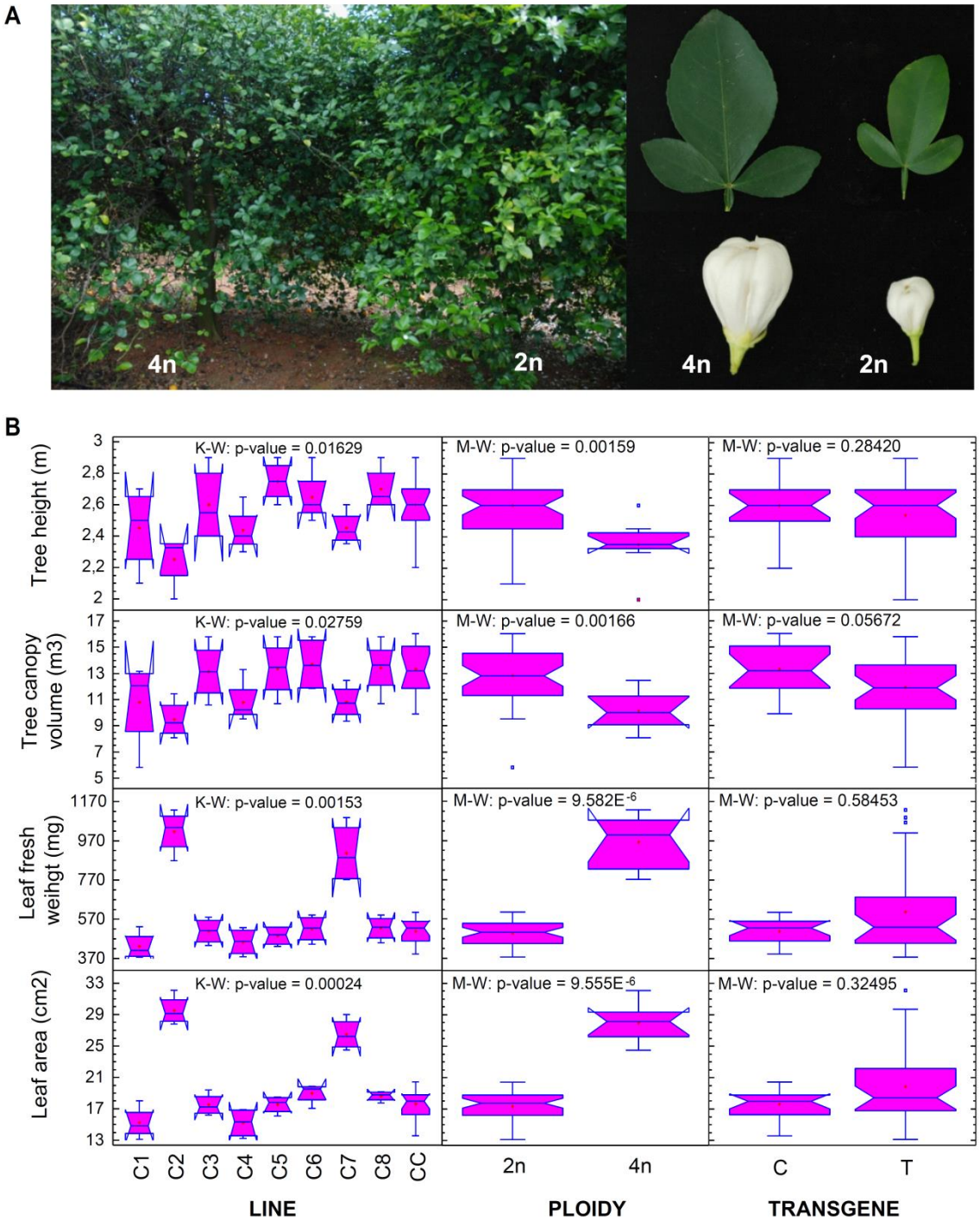

Figure 4 Morphological analysis of citrange trees. A) Images showing morphological differences observed between tetraploid (4n) and diploid (2n) lines. Image on the left: differences in the coloration and leaf density of the trees; image on the right: differences in the morphology of their leaves and flowers. **B)** The effects of the 'line', 'transgene' and 'ploidy' factors on the morphological variables Tree height, Tree canopy volume, Leaf fresh weight and Leaf area. The data represented in the notched box-and-whisker plots were calculated from measurements recorded over the course of two years (2004 and 2005) at the end of the growing season. K-W, Kruskal-Wallis test (n = 48); M-W, Mann–Whitney tests; 2n, all diploid lines (n = 40); 4n, all tetraploid lines (n = 8); C, control line CC (n = 16); T, all transgenic lines, except tetraploids C2 and C7 (n = 24).

Phenological calenders showed no differences in the transgenic trees when compared with the non-GM controls for either of the two genotypes studied. Marked differences were not detected due to either 'ploidy' (in the citrange lines) or 'ontogeny' (in the sweet orange lines). As expected, the most notable differences were observed when the phenological cycles of the two citrus genotypes under study were compared (Figure 5). Therefore, transgenesis *per se* did not affect the morphological appearance or phenological cycle of the trees, whereas other factors, such as the developmental stage (for the sweet orange plants) or the ploidy level (for the citrange plants), had a

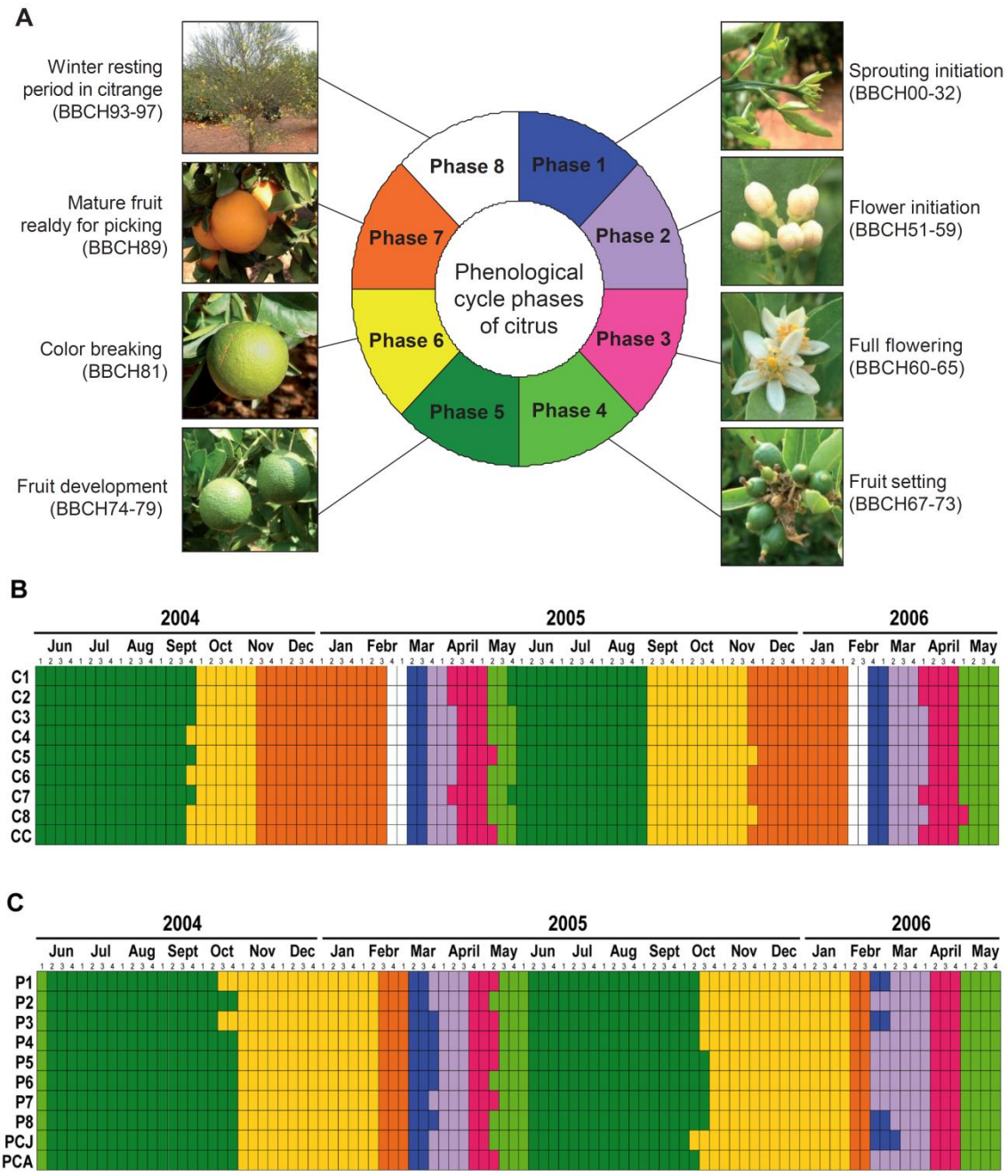

Figure 5 Phenological assessment. A) Schematic representation of the phenological cycle of the citrus lines. The main phases of development are shown using different colors (key legend), which were used to draw the phenological calender of **B)** the Carrizo citrange lines and **C)** the Pineapple sweet orange lines. Phenological stages were recorded weekly according to the BBCH codification for citrus and grouped into 8 phases stressing flower and fruit developmental stages

highly significant impact on the morphological variables.

The 'transgene' effect did not influence fruit quality, whereas 'ontogeny' and 'ploidy' did alter many quality parameters

To assess whether transgenesis affected the agronomic performance of the transgenic citrus trees, the typical parameters commonly used to define the quality of citrus fruit [40] were evaluated in the fruit samples from the orchard-grown transgenic citrus lines and from their respective non-GM controls. The parameters evaluated for all of the sweet orange and citrange lines in the 2004, 2005 and 2006 seasons (S1 to S3) were as follows: fruit weight (W), fruit volume (V), caliber, the color index (CI), juice content (JC), total soluble solids (TSS), titratable acidity (TA) and maturity index (MI). The fruit of the citrange lines was analyzed for an additional season (2007; S4). The data for each citrus type and season was analyzed separately using an ANOVA procedure to test the effect of 'line' on each fruit quality parameter. The 'transgene' effect was assessed by performing *a posteriori* contrasts in which each transgenic line was compared with its respective non-GM control. Moreover, as it is known that the developmental stage and the ploidy level of citrus plants may affect the quality of their fruit, the effects of 'ontogeny' and 'ploidy' were also evaluated in the sweet orange and citrange lines, respectively, by performing the pertinent planned (or *a priori*) contrasts.

A summary of the quality characteristics of the fruit from the sweet orange trees is presented in Additional file 1. We observed visually marked variations in yield among the sweet orange trees, depending on the year of analysis. This phenomenon, known as alternancy, is common in such citrus cultivars as Pineapple sweet orange and may affect fruit quality [41]. The lines in which the reduction of the yield was particularly drastic (less than 30 fruits per tree) were PCJ, P1 and P2 for S2 and PCA, P3 and P4 for S3 (shown in bold in Additional file 1). These productivity data were taken into account when drawing conclusions in the analysis of the fruit. The effects of the 'line', 'transgene' and 'ontogeny' factors on the fruit quality of the sweet orange lines are represented in Table 2. The ANOVA results showed that the 'line' factor had a significant effect on the variables TA and MI in all of the seasons analyzed. For TA, these effects were always highly significant ($p < 0.0001$). The 'line' factor also had an effect on the parameters caliber, CI and JC but only in one of the three years tested and at a lower significance level ($p < 0.01$). The 'line' factor had no effect on any other variable. The results of the contrasts performed to test the 'transgene' effect showed that no significant differences ($p < 0.01$) were found for any fruit quality parameter evaluated when each transgenic line was compared with its corresponding non-GM control. The only exceptions were the significant differences found for the variable MI detected in the contrasts "P3 vs. PCA" and "P4 vs. PCA" for S3. These results could be explained by the poor yield of the P3 and P4 trees in that particular season (see Additional file 1). Therefore, 'transgene' did not induce any detectable difference in fruit quality in the sweet orange lines. Table 2 also shows the results from contrasts performed to test the 'ontogeny' effect on the fruit quality parameters in the sweet orange lines. Highly significant differences were detected between the juvenile and adult lines, irrespective of their transgenic nature, for the variables TA and MI in at least two of the three seasons analyzed. As presented in Figure 6A, the juvenile lines.

Table 2. The effects of the 'line', 'transgene' and 'ontogeny' factors on the fruit quality in the sweet orange lines

Source	Fruit quality parameter (dependent variable)																							
	Weight			Volume			Caliber			Color Index			Juice content[1]			TA			TSS			MI		
	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3	S1	S2	S3
Factor / ANOVA[2]																								
Line	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	*	NS	*	NS	NS	***	***	***	NS	NS	NS	*	*	***
Plant (Line)	***	***	***	***	***	***	***	***	***	***	***	***	*	*	NS	***	***	***	***	***	***	***	***	NS
Contrasts to test 'transgene' effect[3]																								
P3A vs PCA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	*
P4A vs PCA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	**
P5A vs PCA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P6A vs PCA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P7A vs PCA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
P8A vs PCA	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Contrasts to test 'ontogeny' effect[4]																								
J vs A	NS	*	NS	NS	**	NS	NS	**	NS	NS	**	NS	NS	NS	NS	***	***	***	NS	NS	NS	**	**	***
PCJ vs PCA	NS	*	NS	NS	*	NS	NS	*	NS	NS	**	NS	NS	NS	NS	***	*	***	NS	NS	NS	**	NS	***
TJ vs TA	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	*	NS	NS	NS	NS	*	***	***	NS	NS	NS	NS	**	***

[1]The "juice content" variable was log-transformed prior to the analyses to fit the data to a normal distribution

[2]ANOVA to test the effects of Line on each fruit quality variable. Independent statistical analyses were performed for each fruit quality parameter and season.

[3]Contrasts to test for significant differences between each adult transgenic line and their respective control line (PCA) using Dunnett's test

[4]Planned comparisons to test for significant differences between juvenile and adult lines. J, average of all juvenile lines; A, average of all adult lines; TJ, average of all juvenile and transgenic lines; TA, average of all adult and transgenic lines

TA, titratable acidity; TSS, total soluble solids; MI, maturity index (TSS/TA); S1, season 2004; S2, season 2005; S3, season 2006

*p < 0.01; **p < 0.001; ***p < 0.0001; NS, not significant

showed higher TA (at a $p < 0.0001$ significance level) and lower MI (at a $p < 0.001$ significance level) values than the adult lines in all three of the seasons. This result was somewhat expected, taking into account that the differences in the TSS were not detected when comparing juvenile and adult lines (Table 2). In contrast, for W, V, caliber and CI, a significant 'ontogeny' effect was only detected for S2 (Table 2), which could be explained by the low yield in all of the juvenile lines in that particular year (see the sampling data in Additional file 1). Thus, 'transgene' had no impact on any fruit quality parameter evaluated, whereas a significant and consistent 'ontogeny' effect was detected for certain variables. Juvenile transformants were not producing regular fruits five years after flowering for the first time. This should be taken into account when using juvenile instead of mature tissues as starting material for genetic engineering.

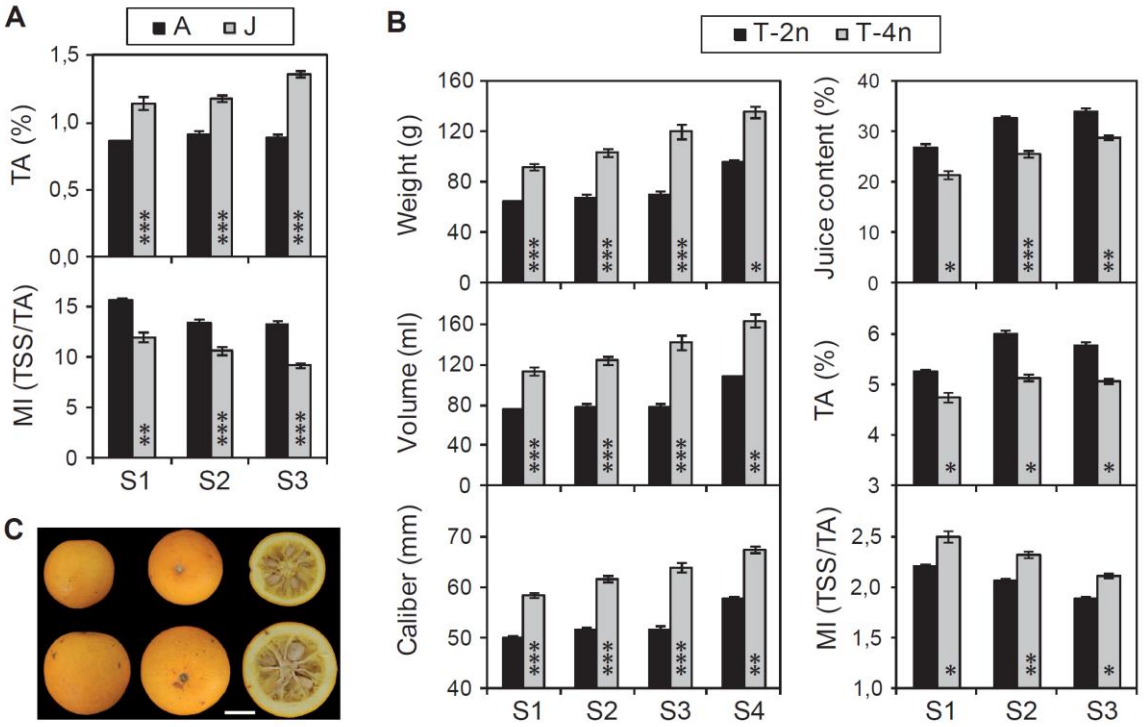

Figure 6 Graphic representation of the significant and consistent effects detected in the analysis of fruit quality in the sweet orange and citrange lines. A) 'ontogeny' effect detected in sweet orange lines. **B)** 'Ploidy' effect detected in citrange lines. **C)** Images showing the representative appearance of mature diploid (2n) and tetraploid (4n) fruit from Carrizo citrange trees. The scale bar represents 2 cm. Level of significance achieved in the planned contrasts: *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$. Seasons analyzed: S1, season 2004; S2, season 2005; S3, season 2006; S4, season 2007. Average ± SE from contrasts showing significant differences ($p < 0.01$) in at least two seasons are represented

A summary of the fruit quality characteristics of the citrange lines is presented in Additional file 2. There were no noticeable differences in yield among the seasons analyzed. The effects of 'line', 'transgene' and 'ploidy' on the fruit quality of the citrange lines are presented in Table 3. The ANOVA results showed that the effect of 'line' on all of the fruit quality variables was significant for at least two of the four seasons analyzed, at $p < 0.01$, with the exception of the variable CI. Regarding the

'transgene' effect, Table 3 also shows that no significant differences were found for more than one season for any of the quality parameters evaluated when each transgenic line was compared with its corresponding non-GM control. For most of the variables (V, W, caliber and JC), significant differences were found exclusively for the first season analyzed, and these differences decreased in the following seasons, ceasing to be significant ($p < 0.01$) in all cases. This result may indicate that the citrange trees were not fully mature in the first year of assessment (S1). Thus, it was necessary to evaluate these parameters in an additional (fourth) year (S4) to confirm that the highly significant differences found for S1 were not repeated and, therefore, could not be attributed to the 'transgene' effect. Table 3 shows that 'ploidy' had a significant effect on W, V, caliber and JC in all of the four seasons analyzed. Moreover, for these variables, the differences between the diploid (T-2n) and tetraploid (T-4n) transgenic lines were highly significant ($p < 0.001$) for at least two of the four seasons. 'Ploidy' also had a significant effect on the MI in S1, S2 and S3, although at a lower significance level than the other variables tested (Figure 6). The tetraploid lines showed higher W, V, and caliber and lower JC values than the diploid lines (Figure 6B), indicating that the higher weight and size of the tetraploid fruit were due to a greater peel thickness and not to a higher juice percentage (Figure 6C). For these variables, the trend of the compared means within the contrasts was consistent over the seasons, meaning that these differences between the diploid and tetraploid lines, in addition to being highly significant, were consistent, regardless of the season/environmental conditions. The tetraploid lines also showed higher MI values than the diploid lines. This less-pronounced but consistent 'ploidy' effect was due to a lower TA in the tetraploid lines compared to the diploid lines.

In summary, the results from the analysis of fruit quality indicated that (1) no significant 'transgene' effect was detected for any fruit quality parameter evaluated, and (2) both the methods of evaluation and the statistical analyses performed to study the influence of transgenesis on the fruit quality of the different citrus genotypes were robust and sufficiently powerful to detect differences due to other physiological and genetic factors.

Table 3. The effects of the 'line', 'transgene' and 'ploidy' factors on the fruit quality in the citrange lines

	Fruit quality parameter (dependent variable)																							
	Weight				Volume				Caliber				Color Index				Juice Content[1]				MI (TSS/TA)			
Source	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**	**S1**	**S2**	**S3**	**S4**
Factor /ANOVA[2]																								
Line	***	*	**	NS	***	*	***	NS	***	*	***	NS	NS	NS	NS	–	**	***	*	-	*	*	NS	-
Plant(Line)	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	–	*	NS	***	-	***	***	*	-
Contrasts to test 'transgene' effect[3]																								
C1 vs CC	***	NS	NS	NS	***	NS	NS	NS	***	NS	NS	NS	NS	NS	NS	–	*	NS	NS	–	NS	NS	NS	–
C3 vs CC	*	NS	NS	NS	NS	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	–	*	NS	NS	–	NS	NS	NS	–
C4 vs CC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	–	NS	NS	NS	–	NS	*	NS	–
C5 vs CC	**	NS	NS	NS	**	NS	NS	NS	**	NS	NS	NS	NS	NS	NS	–	NS	NS	*	–	NS	NS	NS	–
C6 vs CC	**	NS	NS	NS	*	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	–	*	NS	NS	–	NS	NS	NS	–
C8 vs CC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	–	NS	NS	NS	–	NS	NS	NS	–
Contrasts to test 'ploidy' effect[4]																								
T-2n vs T-4n	***	***	***	*	***	***	***	**	***	***	***	**	NS	NS	NS	–	*	***	**	-	*	**	*	-

[1]The "juice content" variable was transformed prior to the analyses to fit the data to a normal distribution

[2]ANOVA to test the effects of Line on each fruit quality variable. Independent statistical analyses were performed for each fruit quality parameter and season

[3]Contrasts to test for significant differences between each transgenic diploid line and their respective control line (CC) using Dunnett's test. In the absence of a tetraploid control line, transgenic lines C2 and C7 (tetraploids) were excluded from this analysis to avoid confounding effects

[4]Planned comparisons to test for significant differences between the average of all transgenic diploid lines (T-2n) and the average of all transgenic tetraploid lines (T-4n). Because only one control line (CC) was available for the Carrizo citrange plants, which was diploid, the data from this line were excluded from this analysis to avoid confounding effects

MI, maturity index; TSS, total soluble solids; TA, titratable acidity; S1, season 2004; S2, season 2005; S3, season 2006; S4, season 2007

-, Not measured; *p < 0.01; **p < 0.001; ***p < 0.0001; NS, not significant

Discussions

For such long-lived and vegetatively propagated crops with complex genetic and reproductive characteristics as fruit trees, genetic modification offers an important potential for crop improvement. Genetic engineering allows desirable traits to be transferred into mature tissues of selected genotypes, bypassing the long crossing cycles required in tree breeding programs. Moreover, genetic engineering overrides incompatibility barriers and permits gene transfer not only between unrelated tree species, but also between widely divergent taxa. Additionally, the potentially undesirable effects of linked alleles, which could be inadvertently introduced into the progeny in conventional breeding programs, can be avoided. However, the future prospects for commercial plantations of GM trees are controversial and remain uncertain, as certain biological and regulatory issues still need to be satisfactorily resolved [42-46]. The modification of crops *via* genetic engineering is a subject of public concern. A question that is often asked is "do genetically modified crops differ significantly from their non-modified equivalents?" The term 'substantial equivalence' has been used in the fields of food safety and biotechnology to describe the relationship between components produced from the same source using either novel or conventional methodologies: if the resulting components are indistinguishable, they can be considered equivalent [47]. Substantial equivalence in the context of this work is used to describe the relationship between the phenotype and agronomic performance of the GM citrus plants and their non-GM counterparts.

We report here that several independent transgenic sweet orange and citrange lines stably carrying and expressing *uidA* and *nptII* transgenes showed a similar phenotype (at morphological, phenological and agronomic levels) to their non-transgenic comparators when both were grown under orchard conditions for a long period of time (> 7 years). We intentionally used transgenes with a well-characterized function to simplify the analysis of substantial equivalence and because this was the first release of transgenic citrus plants into the field. The evaluated parameters allowed the assessment of the outcomes of numerous metabolic pathways that would tentatively result in a distinguishable phenotype in the modified plants, as recommended in the Guidance Document (Section III, D7) described by the EFSA GMO Panel [29]. Moreover, some aspects regarding the design of the experimental orchard contributed to the validation of our study. The relatively high number of independent transgenic lines (eight) of each citrus type used allowed the minimization of event-specific unintended effects derived from transgene integration. The availability of more than one plant per line permitted investigating the intraline variability and discarding possible chimeric events, which frequently occur during the genetic transformation of citrus [48]. The homogeneous distribution of the non-transgenic control trees within the orchard contributed to reducing the possible environmental effects caused by the position of the trees in the field. Lastly, the inclusion of some off-type lines from each citrus type (juvenile sweet orange and tetraploid citrange lines) allowed assessing the influence of other (genetic and physiological) factors on the parameters studied. Thus, by performing the comparisons "juvenile versus adult" for the sweet orange lines and "diploid

versus tetraploid" for the citrange lines, we also addressed the effects of 'ontogeny' and 'ploidy' on the phenotypic variables.

There are reported cases of transgenic trees in which the expression of transgenes was silenced at some point during development [34,49]. There are also instances of T-DNA loss, such as in transgenic apples [50], which are likely due to chimerism rather than T-DNA instability. In general, a high stability of transgene integration and expression have been observed in trees over 3 to 4 years of culture *in vitro* in either the greenhouse or in the field [51,52]. However, there is limited information available about the stability of transgene expression over the many years that trees remain in the field, where they are subjected to highly variable environmental conditions. The results of our molecular analyses confirmed the long-term stability of transgene insertion and expression over 7 years for all of the transgenic citrus lines examined. Moreover, little seasonal variation in the expression levels was detected between plants of the same transgenic line in different organs and over the duration of the study, confirming the absence of rearrangements and/or silencing of the transgenes after transferring the plants to the orchard conditions. The long-term stability of *attacin E* transgene expression has also been recently shown in orchard-grown apples trees over a 12-year period [53].

The monitoring of commercial transgenic crop varieties in the field has allowed the observation of unintended traits. Verified examples of such traits include stem splitting and decreased yields in transgenic soybean plants [54] and a 67-fold reduction in beta-carotene content in a transgenic squash variety engineered for virus resistance (USDA Application # 95-352-01). Therefore, it is important to test whether the stable expression of transgenes in different organs affects morphological, phenological and fruit quality parameters, especially in perennial crops. While investigating apple, Ruhmann et al. [55] have shown that the expression of a stilbene synthase transgene did not affect the leaf shape, flower morphology and color, or fruit shape and size when compared to control plants and fruit. *Attacin E* overexpression also did not affect the fruit characteristics of transgenic apple fruit of trees grown in the field over a period of 7 years [53]. No significant differences in the morphological variables or fruit quality parameters have been found between the transgenic and non-transformed controls of the two citrus genotypes tested in our study. Furthermore, the evaluation methods and statistical analyses used for this purpose were robust and sufficiently powerful to detect significant differences when comparing trees at different developmental stages (for sweet orange) or with different ploidy levels (for citrange). These results indicated that the modification of the citrus genome via conventional breeding (with the subsequent generation of -juvenile- seedlings) or via ploidy manipulation (i.e., through polyploidization processes) generates much more genetic and phenotypic variability in terms of morphology and fruit quality than is induced by genetic engineering. Therefore, transgenesis can be considered to be a more precise method for altering genotypes, without (or minimally) affecting phenotypes in comparison with other breeding methods commonly used in citriculture.

The goal of genetic engineering in crop improvement programs generally involves the modification of metabolic pathways in a manner that may alter plant development and/or fitness under real agricultural conditions at much more complex levels than those described here. However, particular attention should be paid to the selectable marker genes used, as they usually remain linked to the transgenes of interest, at least in vegetatively propagated crops. The detailed pleiotropic effects of selectable marker genes need to be understood, as they may influence the interpretation of scientific results when co-transforming genes of interest are being examined in transgenic plants [3]. Our research has shown that *nptII* and *uidA* did not induce pleiotropic effects on the main phenotypic plant characteristics of transgenic citrus trees.

Conclusions

We have demonstrated that the stable integration and expression of *uidA* and *nptII* transgenes for more than 7 years under orchard conditions has minimal effects on the main agronomic plant characteristics (tree morphology, phenology and fruit quality) of transgenic citrus lines compared to appropriate controls. Therefore, transgenic sweet orange and citrange lines carrying the selectable marker genes that are most commonly used in citrus transformation are substantially equivalent to the non-transformed controls during long-term agricultural cultivation. This information is essential to be able to focus mainly on the pleiotropic effects that may be induced by the insertion of gene(s) of interest in future experiments with GM citrus.

Methods

Plant materials and experimental field design

The citrus transformants and controls used in this work (see Figure 1A) were generated previously in our laboratory. *A. tumefaciens* EHA 105 containing the binary plasmid p35SGUSINT was used in the different experiments as a vector for the transformation of plant materials from three citrus types: Pineapple sweet orange (*C. sinensis* L. Osb.) [35]; Carrizo citrange (*Citrus sinensis* L. Osb. X *Poncirus trifoliata* L. Raf.) [37,38] and Mexican lime (*C. aurantifolia* (Christm.) Swing.) [39]. Two gene cassettes in the T-DNA, 35 S-*uidA*(GUSINT)-35 S and NOS-*nptII*-NOS, served as the reporter and selectable marker genes, respectively. Six independent sweet orange transgenic lines derived from adult plant material (designated P3 to P8) and two derived from juvenile material (designated P1 and P2) were selected for the release. Non-GM regenerants obtained from these transformation experiments served as the control adult (PCA) and juvenile (PCJ) sweet orange lines. For the release, we also selected six independent transgenic citrange lines (designated C1, C3, C4, C5, C6 and C8) and one non-GM regenerant, which was used as a control line (CC). Moreover, we included two off-type transgenic tetraploid lines (designated C2 and C7) in the orchard that were unintentionally obtained during the course of the experiments [33]. The Mexican lime plants included in the field

trial (named L1 to L8 and LC) were excluded from the study because they suffered severe symptoms from frost in several consecutive winters.

The transgenic lines were chosen based on their high level of transgene expression and low copy number of transgene insertions. The plants were transferred to the orchard conditions in 1997, together with their respective non-GM controls. The experimental orchard, designated the T plot, was located at the Instituto Valenciano de Investigaciones Agrarias, Spain (latitude 39º35”N, longitude 0º23”W and elevation of 50 m; typical Mediterranean climate), and was approved by the Spanish Ministry of Environment (permit Nr. B/ES/96/15). All of the scion types were grafted onto Carrizo citrange rootstock and grown in a loamy clay soil using drip irrigation. The orchard was managed as for normal citrus cultivation. The T plot, which covered an area of 1.638 m2, contained 130 trees distributed in rows, as described in Figure 1C. Non-transgenic Clemenules clementine (*C. clementina* ex. Hort. Tan.) trees planted along an external edge were used as a buffer to prevent transgene flow through pollen dispersal [56]. It was designed to study long-term transgene integration/expression and the influence of transgenesis (the ‘transgene’ effect) on the main phenotypic plant characteristics.

Molecular characterization

Southern blot analysis

Genomic DNA was isolated from leaves according to Dellaporta et al. (1983) [57]. The Southern blot analysis was performed using 20 µg of *Eco*RI-, *Dra*I-, *Hind*III- and *Dra*I + *Cla*I-digested samples, which were separated on 1 % (w/v) agarose gels and blotted onto nylon membranes (Hybond-N+, Amersham,, Buckinghamshire, UK). The filters were probed with a digoxigenin (Boehringer-Mannheim, East Sussex, UK)-labeled fragment corresponding to the coding region of the *uidA* or the *nptII* gene prepared by PCR following the supplier’s instructions.

Histochemical and fluorimetric GUS assays and NPTII ELISA

The histochemical GUS activity of the transgenic plants was analyzed as described in [37]. The GUS activity in the leaf samples was estimated by measuring the fluorescence emitted at 445 nm during the hydrolysis of 4-MUG to 4-MU [58]. The NPTII activity in leaf samples was quantitated using a commercial Patho Screen NPTII ELISA kit (Agdia Inc., Indiana, USA). The GUS and NPTII analyses were performed using crude protein samples extracted from the fully expanded leaves from each plant. The total protein was quantified using the Bradford assay.

Phenotypic characterization

Morphology

To analyze the size of the trees, measurements of the height and average diameter for each tree were recorded at the end of the growing season. We defined the tree height (TH) as the highest point of the plant measured from the soil. The average diameter was calculated from

two independent measurements of the diameter of the tree obtained at different points. The tree canopy volume (TCV) was calculated by applying the volume formula for the ellipsoid, as follows: V = 0.524 h d2, where "h" is the TH and "d" is the average diameter of the tree. To study leaf morphology, the average leaf fresh weight (LFW) and average leaf area (LA) parameters were calculated for each tree. Measurements were performed using 30 adult leaves located in the intermediate zone of spring shoots. The area was measured using a LiCor 3100 C device (Nebraska, USA).

Statistical analyses were performed using STATGRAPHICS Plus software, version 5.0. Each citrus genotype was analyzed separately. The data for each morphological variable (TH, TCV, LFW and LA) were analyzed using the Kruskal-Wallis non-parametric test to determine whether differences in the median values existed among the lines [59]. The effects of the 'transgene', 'ontogeny' and 'ploidy' factors were tested by performing pertinent planned comparisons using the Mann–Whitney non-parametric test. We chose these tests because the data did not show clear normality or equal variances. Moreover, Kruskal-Wallis is a recommended as an alternative to parametric analysis of variance (ANOVA) for populations containing uneven sample sizes [60], as was the case in the present study. The Kruskal-Wallis test compares the medians instead of the means; therefore, we report the medians and interquartile ranges instead of the means and standard deviations for these variables.

Phenology

The phenological cycle of every tree in the orchard was evaluated through weekly observations and the recording of the predominant phenological stage of development according to BBCH codifications [61]. A visual representation of the phenological cycle of each line was produced by generating phenological calenders.

Analysis of fruit quality

The assessment of fruit quality for the sweet orange and citrange lines was performed for 3 and 4 consecutive seasons, respectively, starting in the 2004 production season in both cases. Measurements of quality parameters were performed based on fruit samples from every citrus tree in the T plot. A total of 30 fruits (six samples of 5 fruits each) per tree were harvested annually when the fruit was fully mature. The following fruit quality parameters were measured and averaged for each sample: fruit weight (W), fruit volume (V), caliber, the color index (CI), juice content (JC), total soluble solids (TSS), titratable acidity (TA) and maturity index (MI). The V was estimated via the water displacement method. To estimate the caliber, the equatorial diameter of the fruit was measured using MITUTOYO digital calipers (Ilinois, USA). The CI was determined according to the method described by Jiménez-Cuesta et al. (1981) [62]. The L (0–100, black to white), a (± yellow/blue), and b (± red/green) parameters of the color system were measured using a Minolta CR-200 Chroma Meter (Osaka, Japan). The juice was extracted from the fruit and weighed, and the JC was expressed as a percentage on the basis of weight. Immediately after the extraction of the juice, the TSS was determined in terms of Brix degrees

using a refractometer (Atago PR-101 model 0-45 %, Tokyo, Japan). The TA of the juice was determined by titration with 0.1 mol L-1 NaOH and expressed as the percentage of anhydrous citric acid by weight, using phenolphthalein as a visual endpoint indicator, according to AOAC methods (AOAC. 1980. Official Methods of Analysis, 13th ed. N°46024 and N° 22061. Association of Official Analytical Chemists, Washington. DC). The MI was estimated as the TSS/TA ratio. The MI was estimated as the TSS/TA ratio.

Prior to the statistical analysis, the quality variables were checked for normality, and those that deviated were transformed *via* log transformation. A double hierarchical analysis of variance was conducted using the General Linear Models procedure (GLM, for ANOVA with unbalanced data) to assess the influence of 'line' (independent variable) on the variance of each fruit quality parameter measured (dependent variable). The analysis was performed separately for each citrus type and season, and the model used was as follows: xij= μ+ linei + plant (line)j(i) + errork(ij). The main factor, 'line', included the C1, C2, C3, C4, C5, C6, C7, C8, and CC treatments for the citrange samples and the P1, P2, P3, P4, P5, P6, P7, P8, PCJ, and PCA treatments for the sweet orange samples. The hierarchical factor, 'plant', included the plant treatments within each level of 'line'. The 'plant' effect was considered random, and it was used as the source of error for the 'line' effect. We used the restricted maximum-likelihood estimation technique to avoid negative estimates of variance. *A posteriori*, we used Dunnett's test to address the effect of 'transgene' (each transgenic line vs. control) on each fruit quality variable. Additionally, the effects of 'ploidy' (2n vs. 4n) and 'ontogeny' (juvenile vs. adult) were also addressed in the citrange and sweet orange lines, respectively, by performing the corresponding planned (or *a priori*) contrasts/comparisons. The statistical analyses were all performed using the software package SAS version 8.02 (SAS Institute Inc., Cary, NC, USA), and a significance level (α) of 0.01 was taken into consideration to protect against Type I errors.

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Additional files

Additional file 1. Summary of the analysis of fruit quality for the transgenic sweet orange lines

		Sampling				Fruit quality parameter							
Season	**Line**	trees/ line	samples/tree	*n*1	*n*2	Weight (g)	Volume (ml)	Caliber (mm)	Color Index	JC (%)	TSS (%)	TA (%)	MI (TSS/TA)
S1	PCJ	2	6	12	12	149.83 ± 3.08	182.25 ± 3.89	68.74 ± 0.53	12.23 ± 0.27	45.59 ± 0.92	13.09 ± 0.30	1.34 ± 0.03	9.77 ± 0.23
(2004)	P1	2	6	12	12	149.18 ± 5.03	171.18 ± 6.87	68.14 ± 0.90	14.88 ± 0.24	43.47 ± 1.19	13.13 ± 0.24	1.32 ± 0.01	9.98 ± 0.18
	P2	2	6	12	12	119.50 ± 2.14	138.75 ± 3.28	62.91 ± 0.38	11.98 ± 0.17	40.12 ± 0.46	12.80 ± 0.10	0.79 ± 0.02	16.25 ± 0.40
	PCA	6	6	36	36	150.22 ± 4.17	170.33 ± 5.24	67.72 ± 0.63	14.10 ± 0.25	42.10 ± 0.52	13.39 ± 0.08	0.81 ± 0.02	16.86 ± 0.42
	P3	2	6	12	12	155.33 ± 5.21	178.33 ± 6.08	69.39 ± 0.83	14.34 ± 0.39	46.44 ± 0.83	13.33 ± 0.13	0.87 ± 0.02	15.57 ± 0.52
	P4	2	6	12	12	154.08 ± 3.06	177.17 ± 4.17	69.16 ± 0.52	15.23 ± 0.19	41.61 ± 0.78	12.90 ± 0.07	0.97 ± 0.01	13.28 ± 0.17
	P5	2	6	12	12	121.92 ± 3.16	136.00 ± 3.43	61.90 ± 0.57	13.11 ± 0.35	47.04 ± 0.53	13.87 ± 0.09	0.86 ± 0.02	16.21 ± 0.39
	P6	2	6	12	12	128.42 ± 2.26	139.67 ± 3.86	63.49 ± 0.41	12.96 ± 0.16	43.13 ± 0.70	13.38 ± 0.09	0.94 ± 0.02	14.33 ± 0.25
	P7	2	6	12	12	137.00 ± 3.21	149.83 ± 3.41	65.76 ± 0.60	14.62 ± 0.21	41.88 ± 0.42	13.10 ± 0.09	0.91 ± 0.01	14.44 ± 0.18
	P8	2	6	12	12	144.58 ± 2.43	155.17 ± 2.78	66.61 ± 0.40	13.26 ± 0.21	44.05 ± 0.79	13.44 ± 0.10	0.81 ± 0.03	16.82 ± 0.63
S2	PCJ	2	6	12	**8**	239.38 ± 20.75	284.75 ± 40.11	80.60 ± 2.75	10.59 ± 0.16	41.76 ± 0.90	11.90 ± 0.32	1.15 ± 0.05	10.46 ± 0.45
(2005)	P1	2	6	12	**8**	230.00 ± 4.19	297.25 ± 10.45	81.62 ± 0.58	12.60 ± 0.18	43.84 ± 1.06	12.66 ± 0.13	1.43 ± 0.02	8.87 ± 0.18
	P2	2	6	12	**8**	166.88 ± 6.76	193.63 ± 18.14	68.73 ± 1.43	10.14 ± 0.32	39.67 ± 0.83	12.84 ± 0.27	0.99 ± 0.01	13.00 ± 0.28
	PCA	6	6	36	36	147.67 ± 4.67	162.61 ± 5.58	67.44 ± 0.70	14.02 ± 0.19	41.36 ± 0.67	12.01 ± 0.06	0.92 ± 0.01	13.12 ± 0.18
	P3	2	6	12	12	150.42 ± 9.53	161.00 ± 9.86	64.82 ± 1.90	12.43 ± 0.15	41.81 ± 0.49	12.09 ± 0.11	0.95 ± 0.03	12.82 ± 0.38
	P4	2	6	12	12	166.42 ± 14.17	186.08 ± 16.90	70.17 ± 2.07	13.01 ± 0.43	42.05 ± 0.89	12.13 ± 0.06	0.98 ± 0.01	12.42 ± 0.20
	P5	2	6	12	12	120.00 ± 2.51	127.17 ± 2.64	61.78 ± 0.52	12.90 ± 0.28	44.81 ± 0.67	12.78 ± 0.09	0.82 ± 0.03	15.84 ± 0.55
	P6	2	6	12	12	154.83 ± 3.96	167.17 ± 3.75	68.43 ± 0.63	13.76 ± 0.32	41.10 ± 0.72	12.37 ± 0.20	0.88 ± 0.02	14.01 ± 0.20
	P7	2	6	12	12	125.33 ± 5.20	134.17 ± 5.57	63.06 ± 0.97	14.43 ± 0.15	40.47 ± 0.83	12.50 ± 0.09	0.93 ± 0.01	13.49 ± 0.17
	P8	2	6	12	12	144.42 ± 4.84	156.50 ± 5.61	66.45 ± 0.85	12.81 ± 0.32	41.34 ± 0.91	12.54 ± 0.21	0.87 ± 0.01	14.53 ± 0.36
S3	PCJ	2	6	12	12	176.58 ± 3.95	199.00 ± 3.94	73.11 ± 0.64	12.36 ± 0.53	46.68 ± 0.43	12.13 ± 0.09	1.46 ± 0.02	8.33 ± 0.10
(2006)	P1	2	6	12	12	197.50 ± 10.49	221.17 ± 11.91	74.16 ± 1.24	13.71 ± 0.25	44.97 ± 0.64	12.15 ± 0.14	1.53 ± 0.03	7.97 ± 0.18
	P2	2	6	12	12	144.67 ± 2.65	161.17 ± 3.24	67.08 ± 0.52	11.89 ± 0.40	45.64 ± 0.33	12.29 ± 0.22	1.00 ± 0.03	12.33 ± 0.21
	PCA	6	6	36	**24**	216.54 ± 3.85	242.63 ± 4.68	76.22 ± 0.45	13.15 ± 0.17	44.39 ± 0.70	11.91 ± 0.11	0.85 ± 0.01	14.03 ± 0.16
	P3	2	6	12	**6**	196.00 ± 2.56	228.33 ± 5.76	73.97 ± 0.47	13.79 ± 0.34	46.63 ± 0.78	11.47 ± 0.19	0.99 ± 0.03	11.68 ± 0.32
	P4	2	6	12	**8**	237.75 ± 14.74	277.13 ± 18.83	79.27 ± 1.50	12.35 ± 0.23	43.35 ± 0.66	11.40 ± 0.20	1.05 ± 0.03	10.86 ± 0.18
	P5	2	6	12	12	159.58 ± 3.91	173.67 ± 5.08	68.48 ± 0.63	11.58 ± 0.31	45.78 ± 0.64	11.94 ± 0.07	0.88 ± 0.01	13.60 ± 0.23
	P6	2	6	12	12	170.25 ± 3.43	193.58 ± 4.03	71.15 ± 0.54	13.59 ± 0.36	43.16 ± 0.46	11.88 ± 0.07	0.94 ± 0.02	12.70 ± 0.27
	P7	2	6	12	12	176.50 ± 2.11	199.83 ± 2.40	72.18 ± 0.44	13.01 ± 0.35	44.73 ± 0.61	11.27 ± 0.11	0.90 ± 0.01	12.49 ± 0.13
	P8	2	6	12	12	177.25 ± 5.43	197.92 ± 6.70	71.72 ± 0.73	11.85 ± 0.40	44.23 ± 0.67	11.85 ± 0.09	0.88 ± 0.02	13.44 ± 0.19

*n*1, theorical/planned sampling; *n*2, sampling carried out. The cases where yield was very scarce (*n*2 < *n*1) are shown in bold.

Each value represents the average ± SE of the *n2* samples analyzed per line and year.

JC, juice content; TSS, total soluble solids; TA, titratable acidity; MI, maturity index

Additional file 2. Summary of the analysis of fruit quality for the transgenic citrange lines. Data are the average ± SE of the *n* samples analyzed per line and year. -, Not measured

		Sampling			Fruit quality parameter							
Season	**Line**	trees/ line	samples/ tree	*n*	Weight (g)	Volume (ml)	Caliber (mm)	Color Index	JC (%)	TSS (%)	TA (%)	MI (TSS/TA)
S1	CC	8	6	48	82.63 ± 1.03	96.67 ± 1.54	54.53 ± 0.29	8.61 ± 0.16	29.75 ± 0.54	11.10 ± 0.09	5.26 ± 0.05	2.12 ± 0.02
(2004)	C1	2	6	12	46.75 ± 1.97	56.50 ± 3.20	45.06 ± 0.73	8.16 ± 0.20	24.29 ± 1.15	12.27 ± 0.15	5.22 ± 0.11	2.36 ± 0.07
	C2	2	6	12	101.63 ± 4.78	126.50 ± 4.63	59.45 ± 0.92	8.13 ± 0.72	22.28 ± 1.61	11.79 ± 0.23	4.91 ± 0.24	2.43 ± 0.10
	C3	2	6	12	64.75 ± 2.35	77.50 ± 3.05	49.54 ± 0.46	7.73 ± 0.27	23.54 ± 1.27	11.69 ± 0.22	5.11 ± 0.11	2.30 ± 0.05
	C4	2	6	12	76.42 ± 2.32	86.58 ± 3.29	53.04 ± 0.55	8.28 ± 0.17	26.44 ± 0.68	12.13 ± 0.20	5.69 ± 0.06	2.13 ± 0.04
	C5	2	6	12	60.58 ± 2.15	67.83 ± 2.99	48.24 ± 0.66	8.82 ± 0.44	34.06 ± 1.30	10.70 ± 0.19	5.19 ± 0.09	2.07 ± 0.03
	C6	2	6	12	60.08 ± 2.04	72.17 ± 2.98	49.76 ± 0.55	8.26 ± 0.23	23.71 ± 1.48	11.69 ± 0.15	5.55 ± 0.12	2.11 ± 0.04
	C7	2	6	12	84.92 ± 2.39	104.83 ± 3.58	57.75 ± 0.66	9.27 ± 0.26	20.84 ± 0.69	11.78 ± 0.12	4.63 ± 0.08	2.56 ± 0.05
	C8	2	6	12	78.17 ± 1.77	93.00 ± 2.21	54.36 ± 0.43	9.76 ± 0.28	29.96 ± 1.22	11.28 ± 0.14	4.92 ± 0.07	2.30 ± 0.04
S2	CC	8	6	48	83.46 ± 1.79	96.58 ± 2.16	55.70 ± 0.44	2.08 ± 1.24	33.82 ± 0.35	11.83 ± 0.12	5.61 ± 0.08	2.12 ± 0.02
(2005)	C1	2	6	12	60.75 ± 1.88	74.75 ± 4.35	49.63 ± 0.57	-3.64 ± 1.37	31.75 ± 1.23	11.60 ± 0.17	5.34 ± 0.07	2.18 ± 0.04
	C2	2	6	12	102.67 ± 5.91	128.75 ± 7.70	62.30 ± 1.31	-1.82 ± 1.33	25.94 ± 1.11	11.49 ± 0.08	5.16 ± 0.08	2.23 ± 0.03
	C3	2	6	12	72.00 ± 4.06	81.50 ± 4.53	52.77 ± 0.94	1.57 ± 0.68	33.06 ± 0.80	11.70 ± 0.13	6.14 ± 0.08	1.91 ± 0.03
	C4	2	6	12	58.27 ± 0.96	67.64 ± 1.48	49.61 ± 0.33	8.57 ± 0.31	30.07 ± 0.64	13.40 ± 0.06	7.12 ± 0.08	1.88 ± 0.01
	C5	2	6	12	62.17 ± 2.57	71.33 ± 2.85	50.49 ± 0.72	8.35 ± 0.32	37.17 ± 0.68	12.38 ± 0.06	5.99 ± 0.07	2.07 ± 0.02
	C6	2	6	12	64.42 ± 2.83	73.50 ± 2.92	51.17 ± 0.76	7.84 ± 0.17	31.53 ± 0.57	13.43 ± 0.15	6.20 ± 0.11	2.17 ± 0.03
	C7	2	6	12	105.08 ± 1.80	121.58 ± 1.89	61.29 ± 0.39	9.27 ± 0.30	25.10 ± 0.59	12.38 ± 0.13	5.15 ± 0.12	2.41 ± 0.05
	C8	2	6	12	85.58 ± 2.01	95.92 ± 2.41	56.24 ± 0.49	8.60 ± 0.23	33.54 ± 0.59	11.59 ± 0.08	5.36 ± 0.05	2.17 ± 0.03
S3	CC	8	6	48	77.10 ± 1.24	86.71 ± 1.27	53.80 ± 0.31	4.50 ± 0.17	34.46 ± 0.51	10.66 ± 0.06	5.74 ± 0.08	1.88 ± 0.04
(2006)	C1	2	6	12	72.33 ± 1.97	82.00 ± 2.12	52.11 ± 0.57	4.53 ± 0.48	33.96 ± 1.07	10.62 ± 0.13	5.88 ± 0.11	1.81 ± 0.02
	C2	2	6	12	141.17 ± 7.93	168.08 ± 9.47	66.97 ± 1.40	2.71 ± 0.51	29.98 ± 0.78	10.57 ± 0.07	5.15 ± 0.08	2.06 ± 0.04
	C3	2	6	12	78.00 ± 0.96	87.58 ± 1.11	54.00 ± 0.23	3.74 ± 0.25	30.67 ± 0.68	10.63 ± 0.08	5.76 ± 0.05	1.85 ± 0.02
	C4	2	6	12	74.92 ± 3.42	82.50 ± 3.69	52.87 ± 0.96	4.64 ± 0.20	31.81 ± 1.14	11.17 ± 0.19	5.93 ± 0.12	1.89 ± 0.04
	C5	2	6	12	59.75 ± 2.35	67.00 ± 2.52	49.24 ± 0.72	5.60 ± 0.35	40.42 ± 0.90	11.55 ± 0.13	5.91 ± 0.10	1.96 ± 0.04
	C6	2	6	12	63.00 ± 0.99	71.33 ± 1.08	50.06 ± 0.26	4.53 ± 0.47	33.32 ± 0.79	11.03 ± 0.19	5.67 ± 0.12	1.95 ± 0.02
	C7	2	6	12	100.33 ± 3.03	117.67 ± 3.27	61.23 ± 0.62	5.68 ± 0.26	27.82 ± 0.65	10.87 ± 0.17	5.02 ± 0.06	2.17 ± 0.02
	C8	2	6	12	71.92 ± 2.07	81.33 ± 2.37	52.55 ± 0.62	4.45 ± 0.41	34.66 ± 0.94	10.39 ± 0.10	5.58 ± 0.08	1.87 ± 0.03
S4	CC	8	6	48	109.63 ± 2.86	123.29 ± 3.38	60.62 ± 0.58	-	-	-	-	-
(2007)	C1	2	6	12	80.33 ± 4.00	89.92 ± 4.79	54.16 ± 0.90	-	-	-	-	-
	C2	2	6	12	133.92 ± 4.72	160.50 ± 6.25	67.90 ± 0.88	-	-	-	-	-
	C3	2	6	12	102.33 ± 4.11	116.58 ± 4.70	59.58 ± 0.88	-	-	-	-	-
	C4	2	6	12	101.17 ± 3.33	114.75 ± 3.20	59.39 ± 0.62	-	-	-	-	-
	C5	2	6	12	75.92 ± 1.83	86.42 ± 2.62	53.38 ± 0.48	-	-	-	-	-
	C6	2	6	12	110.08 ± 5.18	119.92 ± 6.03	60.25 ± 1.10	-	-	-	-	-
	C7	2	6	12	139.00 ± 7.40	168.25 ± 9.65	67.49 ± 1.30	-	-	-	-	-
	C8	2	6	12	109.00 ± 2.35	122.25 ± 2.76	59.99 ± 0.52	-	-	-	-	-

5. RESULTS: CHAPTER 3.

Metabolic engineering of β-carotene in orange fruit increases its *in vivo* antioxidant properties

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Summary

Oranges are a major crop and an important source of health-promoting bioactive compounds. Increasing the levels of specific antioxidants in orange fruit through metabolic engineering could strengthen the fruit's health benefits. In this work, we have afforded enhancing the β-carotene content of orange fruit through blocking by RNA interference the expression of an endogenous β-carotene hydroxylase gene (Csβ-CHX) that is involved in the conversion of β-carotene into xanthophylls. Additionally, we have simultaneously overexpressed a key regulator gene of flowering transition, the *FLOWERING LOCUS T* from sweet orange (*CsFT*), in the transgenic juvenile plants, which allowed us to obtain fruit in an extremely short period time. Silencing the Csβ-CHX gene resulted in oranges with a deep yellow (''golden'') phenotype and significant increases (up to 36-fold) in β-carotene content in the pulp. The capacity of β-carotene-enriched oranges for protection against oxidative stress *in vivo* was assessed by using *Caenorhabditis elegans* as experimental animal model. Golden oranges induced a 20% higher antioxidant effect than the isogenic control. This is the first example of the successful metabolic engineering of the β-carotene content (or the content of any other phytonutrient) in oranges and demonstrates the potential of genetic engineering for the nutritional enhancement of fruit tree crops.

Introduction

Plants are a source of many phytonutrients, including nutrients (such as vitamins) and phytochemicals to which a beneficial physiological function has been directly or indirectly attributed (such as folates, carotenoids, flavonoids, isothiocyanates, glucosinolates, polyphenols, and glutathione). Phytonutrients are essential for human nutrition and contribute to the promotion of good health (Beecher, 1999; Block et al., 1992; Lampe, 1999; Nagura et al., 2009). The bioactivity of phytonutrients has been, to a certain extent, associated with their antioxidant properties, such as the capacity to scavenge free radicals, which are involved in the onset and development of many chronic degenerative diseases (e.g., low density lipoprotein oxidation and atheroma plaque development, DNA oxidation and cancer, oxidation and aging) (Dröge, 2002). Moreover, evidence suggests that the beneficial effects of these bioactive molecules on human health at a nutritional and/or pharmacological level are higher when the phytonutrients are ingested regularly and in specific amounts as part of the diet rather than as dietary supplements (Asplund, 2002; Cooper, 2004; Guarnieri et al., 2007). Unfortunately, due to either low access to fruits and vegetables or consumer ignorance about the types and quantities of the right foods required for benefit, optimal levels of these substances are not always reached in the diet. The biofortification of crops can foster significant progress at a fundamental level by facilitating the elucidation of the relationship between diet and health and at an applied level by improving diets and reducing the risk of chronic diseases (reviewed by Martin et al. (2011)). In this context, genetic engineering has emerged as a powerful tool to introduce favorable changes in the metabolic pathways of plants to improve the quantity and bioavailability of phytonutrients, particularly antioxidants (Martin, 2012; Shukla and Mattoo, 2009).

Citrus is the most extensively produced and economically important fruit tree crop in the world and, among the 10.9 million tons (valued at $9.3 billion) of citrus products traded in 2009, sweet orange (*Citrus sinensis* L. Osbeck) accounted for approximately 60% of citrus production (FAO statistics, http://faostat.fao.org/default.aspx). Oranges contain an array of potent antioxidants, including carotenoids, vitamin C, and certain phytochemicals (i.e., flavonoids and phenolics), with potential health-promoting properties (Franke et al., 2005; Guarnieri et al., 2007; Miyagi et al., 2000; So et al., 1996). Carotenoids are the main pigments responsible for the color of the peel and pulp of citrus fruits and greatly contribute to the fruit's nutritional and antioxidant value. Although citrus fruits are a rich and complex source of carotenoids, the fruit of most orange varieties predominantly accumulates β,β-xanthophylls, which may represent more than 90% of the total carotenoids, with 9-Z-violaxanthin being the main carotenoid in the pulp of mature fruits (Alquézar et al., 2008b; Kato et al., 2004). However, the levels of other nutritionally important carotenoids (such as β-carotene) are considered suboptimal in these varieties. In addition to being the most potent dietary precursor of vitamin A, a large body of epidemiological and laboratory (*in vitro*, animal, and cell culture) studies suggest that β-carotene offers protection against certain age-related degenerative diseases, such as various cancers

(predominantly of the aero-digestive tract) (Bertram and Bortkiewicz, 1995; Chew et al., 1999; IARC, 1998; Mathews-Roth, 1982; van Poppel, 1996), type 2 diabetes (Abahusain et al., 1999; Montonen et al., 2005), and coronary heart disease (Gey et al., 1993; Shaish et al., 1995). These health-promoting effects are independent of pro-vitamin A activity and have most frequently been linked to the high antioxidant activity of β-carotene, which is one of the most efficient carotenoid singlet oxygen quenchers (Cantrell et al., 2003).

Recent advances in the identification and isolation of the genes responsible for carotenogenesis in citrus fruits (Alquézar et al., 2008b; Kato et al., 2004) and the development of genetic transformation procedures for this crop type (Peña et al., 2008) enable the production of increased β-carotene levels in orange fruits via metabolic engineering of carotenoid biosynthesis. Specifically, in this work we sought to block by RNA interference (RNAi) in transgenic sweet orange plants the expression of an endogenous β-carotene hydroxylase gene (*Csβ-CHX*) involved in the conversion of β-carotene into xanthophylls (Figure S1). However, improving the nutritional quality of citrus fruits can be time consuming, laborious, and expensive because the plants' long juvenile phase delays regular fruit production for years; most citrus types need 5-15 years to begin flowering and fruiting (Peña et al., 2008). Alternatively, early flowering has been achieved in transgenic trees, including citrus plants, by constitutively overexpressing flower meristem identity genes (Bohlenius et al., 2006; Endo et al., 2005; Peña et al., 2001; Weigel and Nilsson, 1995). Then, the fructification of *β-CHX*-transgenic plants has been accelerated by simultaneously overexpressing the *FLOWERING LOCUS T* gene from sweet orange (*CsFT*), a key regulator of the flowering transition.

Analyzing the antioxidant ability of a fortified food would be the first step in studying a food's effectiveness in exerting a specific health benefit (i.e., improved overall health or a lower risk of disease) (Shukla and Mattoo, 2009). However, the *in vitro* antioxidant capacity, which is often used as a claim, can be irrelevant to *in vivo* antioxidant effects because critical factors such as the bioavailability, metabolism, tissue distribution, dose/response, and toxicity of food bioactive compounds affect the true health benefits of engineered crops (Espín et al., 2007). Thus, preclinical animal studies have become an essential first step in testing the *in vivo* functionality of genetically engineered food. Here, we present a strategy to induce early fruit production and increase the β-carotene level in the pulp of sweet oranges by metabolic engineering. We also confirm the increased capacity of the enriched orange juice to enhance *in vivo* protection against oxidative stress by approximately 20% in an animal model.

Results

To increase β-carotene levels and simultaneously induce early fruit production in sweet orange plants, we constructed a binary vector, named HRP, containing both an intron-spliced hairpin (ihp) β-CHX RNAi cassette and an FT overexpression cassette (Figure 1a). We used this vector to transform seedlings via *Agrobacterium tumefaciens*-mediated T-DNA transfer. The

binary plasmid pROK2-*CsFT*, which was previously generated in our laboratory (for details see Data S1), served as the vector system for transforming control plants in this work (Figure 1a).

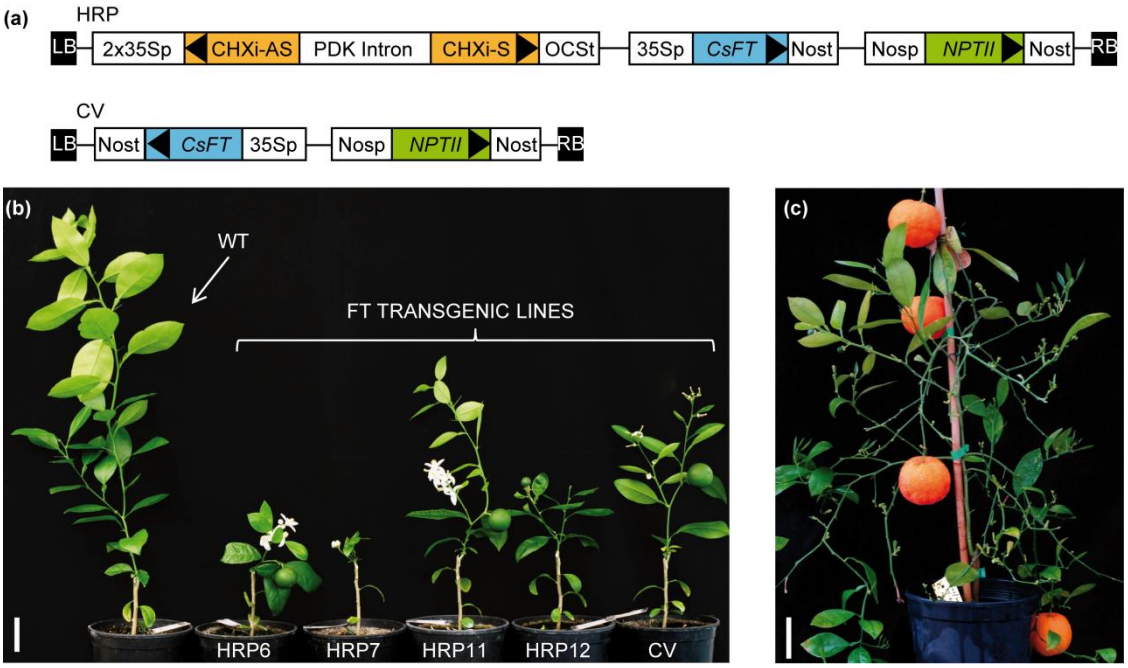


Figure 1. Induction of early flowering in transgenic sweet orange (cv. Pineapple) plants containing a sweet orange *FLOWERING LOCUS T* (*CsFT*) overexpression cassette. (a) Schematic diagram of the T-DNA region of the HRP and control (CV) vectors used for plant transformation. LB and RB, left and right T-DNA borders, respectively; 35Sp, CaMV 35S promoter; CHXi-AS and CHXi-S, antisense- and sense-oriented sequences, respectively, designed to silence the expression of the *Csβ-CHX* gene; PDK intron, pyruvate dehydrogenase kinase intron; OCSt, terminator region of octopine synthase gene; *CsFT*, *FLOWERING LOCUS T* from sweet orange; *NPTII*, *neomycin phosphotransferase II* selectable marker gene conferring kanamycin resistance; NOSt and NOSp, nopaline synthase terminator and promoter sequences, respectively. The transcription orientation for each cassette is indicated by black triangles. (b) Four selected HRP lines and one CV line, all carrying the *CsFT* transgene, exhibiting an early-flowering and fruiting phenotype compared with the WT control. All of the plants were obtained from seedling plant material, and the photograph was taken one year after grafting in the greenhouse. (c) Representative fruits from CV plants at the full-color stage after 18 months of cultivation in the greenhouse.

The FT overexpression system induces an extremely early fruiting phenotype and two fruiting cycles per year in sweet orange plants

Kanamycin-resistant regenerants obtained after performing transformation experiments with either the HRP vector or the pROK2-*CsFT* control vector (CV) were screened by PCR using primers specific to the *CsFT* transgene. Putatively transformed (PCR-positive) plants, designated as HRP and CV lines, respectively, were grafted onto vigorous non-GM citrus rootstocks in a greenhouse in April 2008 and subjected to phenotypic observation for three consecutive years. Whereas the wild type (WT) seedlings remained in the non-reproductive vegetative growth phase for the entire study period, the FT lines (either HRP or CV) flowered for

the first time in June 2009 or, at the latest, in June 2010. The FT lines not only produced fruits in an extremely short period of time (approximately one year after being grafted in the greenhouse) but also had two effective fruiting cycles per year instead of one. None of the FT transformants exhibited morphological features typical of juvenility (i.e., vigorous growth and thorniness). Indeed, they remained stunted, showing smaller leaves than WT seedlings (Figure 1b). Early flowering and fruiting confirmed the effective integration and expression of the FT cassette in all of the HRP and CV transformants. Moreover, the FT transgenic fruits developed normally (Figure 1c).

Transgenic HRP lines harboring intact copies of the β-CHX RNAi cassette produce fruits with a golden coloration

The presence and integrity of the β-CHX RNAi cassette, as well as the number of transgene DNA loci integrations, were assayed in the HRP lines by Southern blot analysis (Data S1 and Figure S2). The HRP lines that did not flower the first year after grafting (2009) or did not set enough number of mature fruit were excluded from the analysis. DNA restriction with either *Not*I or *Cla*I, followed by Southern blot hybridization with a 35S promoter-specific probe revealed that plant lines HRP6, HRP11, and HRP12 contained one or two non-truncated copies of the β-CHX RNAi cassette (Figure S2b). This result was confirmed by digestion with either *Not*I or *Kpn*I, followed by hybridization with a CHXi-specific probe (Figure S2c). Then, based on their low loci number and whole-transgene integrity (Figure S2b,c), plant lines HRP6, HRP11, and HRP12 were selected for propagation and investigated in detail in successive seasons.

During the first stages of development (immature green, mature green, and breaker), the fruits from the selected HRP6, HRP11, and HRP12 lines were visually indistinguishable from fruits transformed with the CV. However, at the full-color stage, the HRP fruits developed an orange-yellow color, whereas the CV fruits exhibited the bright orange coloration typical of sweet oranges. The difference in the coloration of the fully mature fruits from the HRP lines was easily distinguishable in the flavedo (outer colored part of the peel), pulp (internal juice vesicles), and juice (Figure 2a). There were no substantial color differences between the three HRP lines, and this golden phenotype remained stable in all three fruiting seasons and after propagation by grafting onto different rootstocks. Significant differences in the external coloration ($P < 0.01$) of the HRP and CV fruits were confirmed by measuring the flavedo color index (CI) at maturity (Figure 2b). To ensure complete fruit maturity at the time of measurement, all fruits were sampled when fully colored, and the internal maturity index (MI) was assayed. Because no significant differences in the MI were detected in the HRP and CV fruits (Figure 2b), the differences in coloration cannot be attributed to an incomplete maturation of the HRP fruits. Rather, changes in the carotenoid content and/or profile are the most likely explanation.

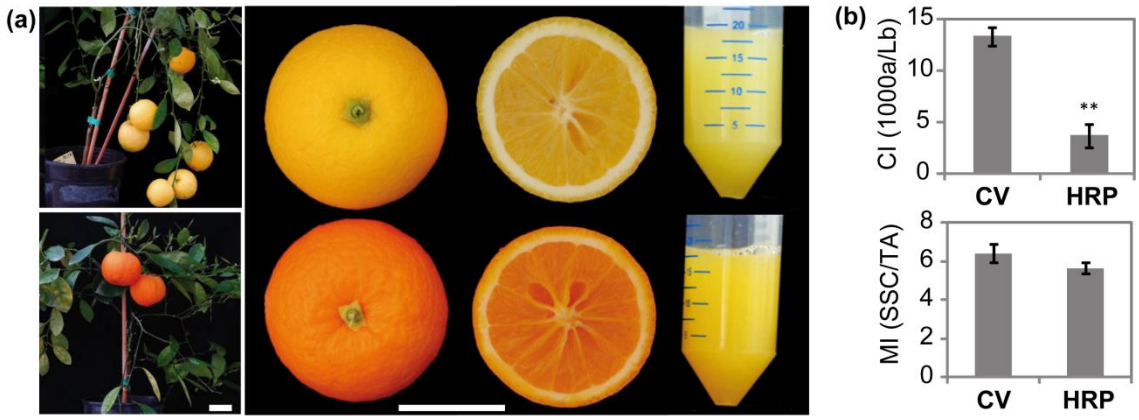


Figure 2. Golden phenotype of fruits from transgenic sweet orange (cv. Pineapple) plants carrying an ihp β-CHX RNAi cassette (HRP). (a) Phenotype of fruits from the selected HRP6, HRP11, and HRP12 lines, which display a golden color at the full-color stage. Representative HRP (upper row) and CV (lower row) sweet orange plants (left), whole and cross-sectioned fruits (middle), and juice (right). All scale bars, 5 cm. (b) Flavedo color index (CI) and internal maturity index (MI) of fruits from the three HRP and CV transgenic lines at the full-color stage. The data represent mean values ± SEM and are derived from at least three fruits from two independent plants per line analyzed in three different fruiting seasons. A statistical analysis of differences between average HRP fruits and average CV fruits was conducted using Student's *t*-test, and the significance of the differences are indicated (**, $P < 0.01$). *L*, *a*, *b*, Hunter color values; SSC, solid soluble content; TA, titratable acidity.

Silencing of Csβ-CHX results in increased β-carotene in the pulp

The golden phenotype of fruits from the transgenic lines containing the RNAi construct suggests that *β-CHX* gene expression is suppressed in the HRP fruits. We therefore examined endogenous *Csβ-CH*X mRNA abundance in fully mature fruits from the transgenic lines HRP6, HRP11, and HRP12 in comparison with the mature fruits of the CV lines by quantitative reverse transcription PCR (qRT-PCR). While the target carotenoid biosynthetic gene was readily expressed in the flavedo and pulp of the CV plants, *Csβ-CHX* transcript accumulation was highly reduced (up to 39-fold) in both tissues of the HRP lines (Figure 3a).

To estimate carotenoid accumulation in the edible part of the transgenic fruits, we conducted comparative profiling of the carotenoid content and composition of pulp samples collected at the full-color stage by high-performance liquid chromatography (HPLC). The results revealed that, consistent with the silencing of the *Csβ-CHX* gene, the levels of β-carotene were significantly increased (up to 36-fold) in fruits from the three HRP lines compared with the CV fruits (Figure 3b; Table 1). In the best-performing HRP line, the pulp accumulated 114.0 ng β-carotene/g fresh weight (FW) on average, while this carotenoid was barely detectable in the CV lines (Table 1). A similar trend was observed for α-carotene, whose content increased (up to 45-fold) in the HRP lines but reached lower absolute amounts compared with β-carotene (47.2 ng/g of FW in the best-performing line). By contrast, a slight reduction in xanthophyll content was detected in the pulp of the HRP lines compared with the CV lines, particularly for β,β-branch xanthophylls (which comprise β-cryptoxanthin, zeaxanthin, antheraxanthin, violaxanthin, and neoxanthin) (Figure S1). Despite this decrease, the β,β-xanthophylls remained the major

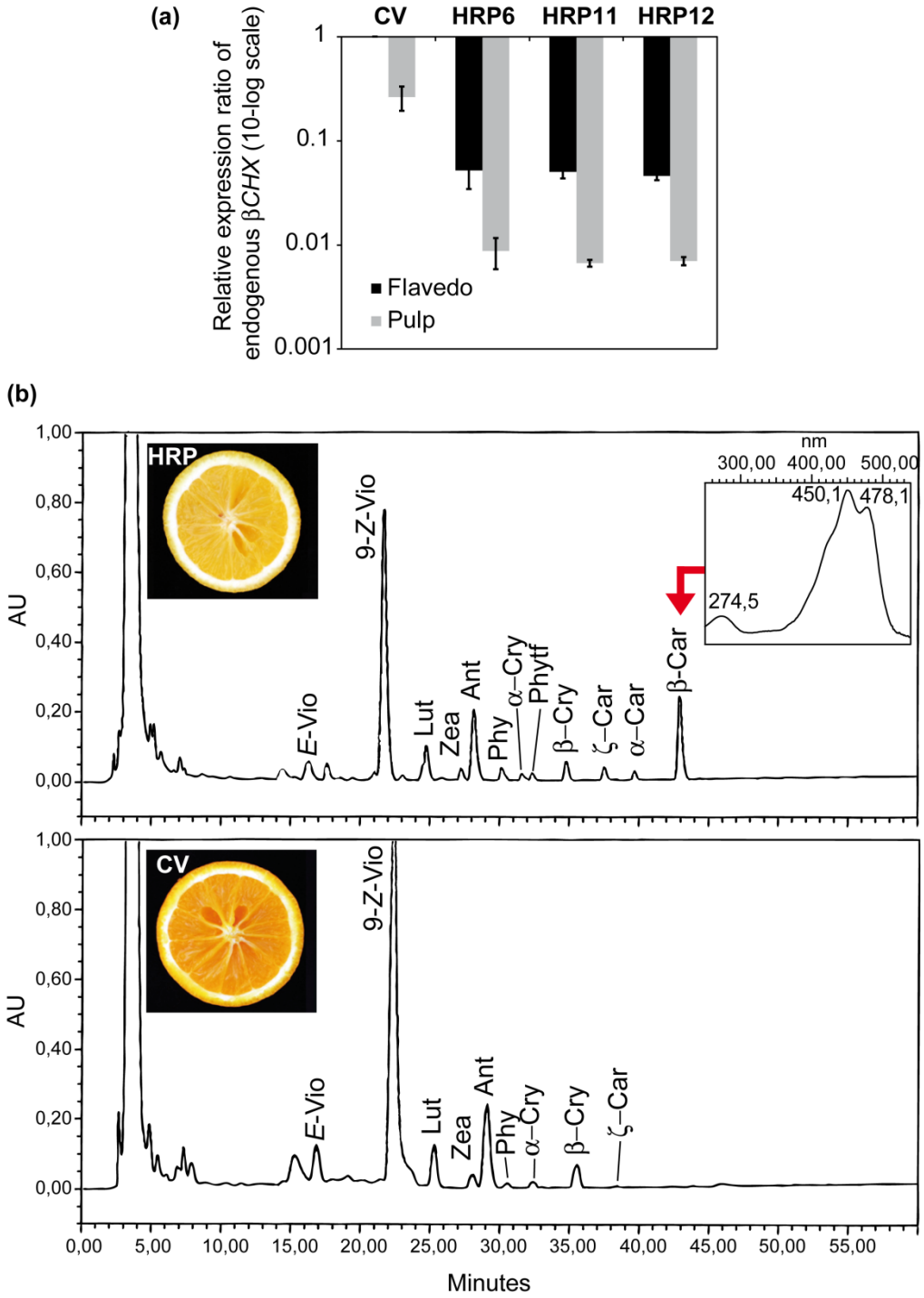


Figure 3. Quantitation of *Csβ-CHX* transcript levels and HPLC analysis of pulp carotenoids in transgenic fruits. (a) qRT-PCR analysis of *Csβ-CHX* expression in the flavedo (black bars) and pulp (gray bars) of full-colored fruits from the CV and HRP6, HRP11, and HRP12 lines. The *Csβ-CHX* transcript accumulation, normalized to the *CsACT* levels, is expressed relative to accumulation in the flavedo of CV fruits and was analyzed in at least six independent technical replicates (using two different 96-well plates). The data for the CV represent the average of data for two independent CV lines. (b) Representative HPLC chromatograms of the carotenoid profiles of orange pulp from HRP11 and CV transgenic plants, with the retention time shown on the x-axis and the intensity shown on the y-axis. *E*-Vio, all *E*-violaxanthin; 9*Z*-Vio, 9-*Z*-violaxanthin; Lut, lutein; Zea, zeaxanthin; Ant, antheraxanthin; Phy, phytoene; α-Cry, α-cryptoxanthin; Phytf, phytofluene; β-Crypt, β-cryptoxanthin; ζ-Car, ζ-carotene; α-Car, α-carotene; β-Car, β-carotene.

carotenoids in the pulp of the HRP lines (mainly 9-*Z*-violaxanthin), accounting for approximately 80% of the total carotenoids, whereas in the CV lines, the β,β-xanthophylls represented more than 90% of the total carotenoids. Consistent with the reduction in xanthophyll content (the major carotenoid compounds in pulp), a reduction in total carotenoids was observed in the HRP lines (Table 1). The levels of the colorless linear carotenes at the early steps of the biosynthetic pathway (phytoene, phytofluene, and ζ- carotene) (Figure S1) were not significantly different from the levels achieved in the CV pulp (Table 1). In summary, the distribution of carotenoid species indicated that the β-CHX RNAi construct promoted the silencing of the *Csβ-CHX* gene in the sweet orange pulp, resulting in the accumulation of significant amounts of β-carotene and α-carotene accompanied by a mild general decrease in the downstream products of β-hydroxylation (xanthophylls).

Table 1 Comparative analysis of carotenoid content and composition in pulp samples from transgenic lines.

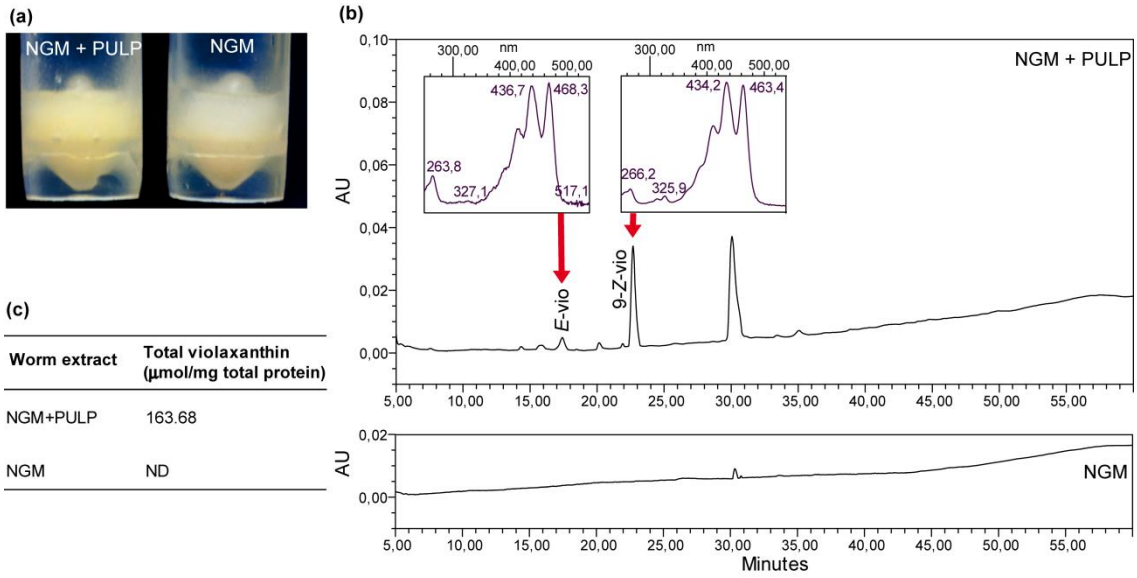
	Total carotenoids	**Lineal carotenes**	**β-carotene**	**α-carotene**	**β.β-xanthophylls**	**ε.β-xanthophylls**
CV	12060.4 ± 1412.1	97.5 ± 23.3	3.1 ± 1.9	1.0 ± 0.7	11015.6 ± 1327.0	943.1 ± 113.6
%	100.0	0.8	0.0	0.0	91.3	7.8
HRP6	6509.2 ± 944.0	135.6 ± 64.5	98.3 ± 7.5	47.2 ± 3.8	5363.3 ± 991.5	864.7 ± 76.8
%	100.0	2.1	1.5	0.7	82.4	13.3
Fold variation	**-1.9**	**1.4**	**31.4*** **	**45.2*** **	**-2.1**	**-1.1**
HRP11	4187.3 ± 493.6	38.8 ± 15.2	114.0 ± 2.1	27.1 ± 7.5	3511.8 ± 453.4	495.7 ± 73.6
%	100.0	0.9	2.7	0.6	83.9	11.8
Fold variation	**-2.9***	**-2.5**	**36.4*** **	**26.0*** **	**-3.1***	**-1.9**
HRP12	1885.5 ± 646.0	33.6 ± 17.8	60.4 ± 12.7	11.3 ± 7.6	1505.9 ± 611.8	274.3 ± 47.5
%	100.0	1.8	3.2	0.6	79.9	14.5
Fold variation	**-6.4****	**-2.9**	**19.3*** **	**10.8***	**-7.3****	**-3.4***

The values are the means ± SEM of at least three independent measurements and are given in ng/g of FW. For each line, the percentage of each carotenoid or group of carotenoids was calculated on the total carotenoid content. The total content of carotenoids was assessed as the sum of the content of individual pigments. The fold variation with respect to the CV is reported for each carotenoid or group of carotenoids and for each HRP line. The asterisks indicate the significance of the fold variation according to Student's *t*-test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Establishment of a *C. elegans* system to evaluate the *in vivo* antioxidant effect of orange juice

C. elegans has been widely used as a model to study the *in vivo* antioxidant capacity of different pure compounds and certain plant extracts (Artal-Sanz et al., 2006; Martorell et al., 2011; Martorell et al., 2012; Van Raamsdonk and Hekimi, 2010) but not the juice from fruits or vegetables. Therefore, it was necessary to optimize certain essential aspects concerning the experimental method prior to performing the bioassays with the orange fruit. First, based on preliminary dose-response experiments performed with commercial pasteurized orange juice (data not shown), a range of 1-2% (by vol.) was chosen as the optimal range for supplementation of the nematode growth medium (NGM) to test antioxidant effect. At higher doses, antioxidant effect was also observed but reaching levels close to saturation. Subsequently, when including the food matrix under study (not-sterilized pulp powder samples obtained from orange fruits) at a concentration of 2% in the NGM, microbial contamination was detected in the supplemented medium during the course of experiments. Therefore, it was necessary to establish a sample sterilization system prior to the supplementation of the NMG with pulp (for details, see Data S1 and Figure S3).

Another important task was to confirm the intake of the orange-pulp extracts by the nematodes during the optimized culture protocol. Therefore, intake confirmation experiments were performed by feeding worm populations with WT pulp extracts and using the NGM without supplementation as a control (see Experimental procedures). As the only purpose of this experiment was confirming the intake of pulp (and not the biological response of nematodes), we decided to use a high dose of supplementation (20%), which greatly facilitated the detection of carotenoids in the worm extracts. The two conditions yielded worm pellets of differing color; the nematodes fed with the pulp extract were slightly orange (Figure 4a). Afterward, HPLC analysis of the lysed worm pellets indicated the presence of violaxanthin in the nematodes fed with WT pulp extracts, whereas no carotenoid was detected in the control sample (worms fed with NGM) (Figure 4b,c), thus confirming the intake and bioassimilation of pulp extract by *C. elegans*.



(c)

Worm extract	Total violaxanthin (µmol/mg total protein)
NGM+PULP	163.68
NGM	ND

Figure 4. Bio-assimilation of pulp extracts by *C. elegans*. (a) Comparison of worm pellets (not disrupted) obtained after feeding with wild type pulp extracts (20%) (NGM + PULP) or a standard diet (NGM), showing significant increases in the levels of colored carotenoids in worms fed with the citrus pulp. (b) HPLC analysis of carotenoids in disrupted worms previously fed with NGM + PULP or NGM, with the retention time shown on the x-axis and the intensity shown on the y-axis. (c) Total violaxanthin levels as measured by HPLC analysis of worms fed with NGM + PULP or NGM and normalized to the total protein content in the worm samples. *E*-vio, *E*-violaxanthin; 9-*Z*-vio, 9-*Z*-violaxanthin; ND, not detected.

β-carotene-enriched (HRP) orange juice exerts a much higher antioxidant effect than control (CV) juice in *C. elegans*

To investigate whether the levels of β-carotene achieved were sufficient to offer antioxidant properties in a dietary context, we tested diets supplemented with orange pulp in *C. elegans*. The two samples compared in the bioassay (pulp extracts from the HRP or CV fruits) were processed according to the method described in Data S1. After processing and measuring the content of carotenoids and vitamin C (Data S1; Table S1), the samples were added to the NGM agar plates at a final concentration of either 1% or 2% (by vol.). In our trials, we also included two additional feeding conditions that served as internal experimental controls: NGM without supplementation (negative control) and NGM supplemented with vitamin C (a well-known antioxidant compound) at 0.1 µg/mL. As shown in Figure 5, both doses of supplementation (1% and 2%) demonstrated the positive effect of the citrus pulp extracts (and, in particular, the HRP pulp extract) on resistance against oxidative stress in *C. elegans*. However, 2% was chosen as the optimal dose for supplementation because better protection was observed (Figure 5b). The animals fed with CV pulp extract had a survival rate of 52%, which was significantly ($P < 0.01$) higher than the rate obtained in the negative control condition (34%) and similar to the rate obtained for the worms fed with vitamin C (46.13%). More interestingly, the animals fed with the HRP pulp extract had a survival rate of 71.67%, which

represented a significant ($P < 0.01$) increase of approximately 20% compared with the survival rate observed in the CV pulp extract-fed worms (Figure 5b). These results demonstrate that the worms fed with the β-carotene-enriched orange pulp were more resistant to the oxidative stressor hydrogen peroxide than worms fed with the control orange pulp.

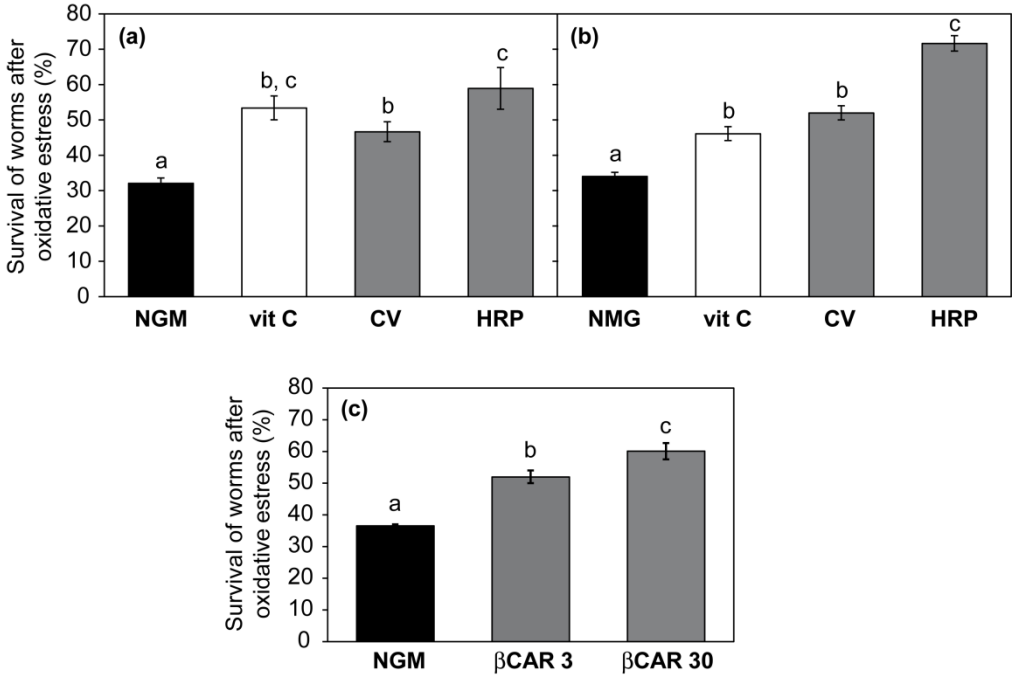


Figure 5. Antioxidant activity of orange pulp extracts and pure β-carotene in *C. elegans*. (a and b) Survival of *C. elegans* treated with 2 mM H_2O_2 on NGM agar plates, with β-carotene-enriched (HRP) or control (CV) pulp extract supplementation at 1% (a) or 2% (b). The HRP pulp extract tested in the bioassays was obtained from mixtures of pulp from the HRP6, HRP11, and HRP12 lines. The standard diet (NGM) and a diet supplemented with vitamin C (0.1 µg/mL) were used as negative and positive control treatments, respectively. (c) Survival of *C. elegans* treated with 2 mM H_2O_2 on NGM agar plates, with or without β-carotene supplementation. Doses of supplementation with pure β-carotene used were 3 µg/mL (βCAR 3) or 30 µg/mL (βCAR 30). The trials were performed in triplicate (100 worms scored per condition). In each experiment, the mean values ± SEM are presented, and treatments labeled with different letters are significantly different at $P < 0.01$ using Fisher's Protected LSD test.

Finally, to study whether the observed antioxidant effect of HRP pulp was related to the β-carotene, we performed oxidative stress response assays in *C. elegans* with exogenous pure β-carotene at a dose equivalent to the amount of β-carotene present in the HRP pulp extract (3µg/mL). We also included in the bioassays a 10-fold higher dose of β-carotene supplementation (30µg/mL) and the standard nematode diet as control. Results showed a significantly higher ($p < 0.01$) antioxidant effect of β-carotene at a dose of 3µg/mL compared to the NGM control condition; at a 10-fold higher dose, greater effect was observed (Figure 5c). These results confirmed the antioxidant capacity of β-carotene. However, the fact that the survival rate obtained by feeding worms with the HRP pulp extract (71.67% worm survival, Figure 5b) was higher than that achieved with pure β-carotene at 3µg/mL (52% worm survival,

Figure 5c) suggests that this particular background food matrix (orange pulp) enhances the antioxidant effect of increased β-carotene.

Discussion

The possibility that dietary intervention via nutrition-enriched food may significantly decrease the incidence of certain chronic degenerative diseases, in conjunction with increased public awareness of the nutritional benefits of antioxidants for human health, has catalyzed scientific efforts to increase many bioactive constituents in fruits and vegetables (Davies, 2007; Hossain and Onyango, 2004; Shukla and Mattoo, 2009). Although conventional breeding is one means of achieving this goal (Nestel et al., 2006; Mayer et al., 2008), the genetic diversity available within the sexually compatible species of any given crop limits the extent of improvement. In this regard, genetic engineering has become a refined tool to increase the antioxidant and nutrient capacity of economically important crops, not only to achieve levels favorable for highly nutritional diets but also to enable in-depth studies on the relationships between diet, genetics, and metabolism (Christou and Twyman, 2004). Moreover, a Fast-Track system, as the one used here based on the ectopic overexpression of *CsFT* in juvenile plants, greatly facilitates addressing metabolic engineering strategies aimed at improving fruit quality in plant species requiring many years to begin to flower and set fruits. To our knowledge, this is the first report in which this Fast-Track system has been successfully used for that purpose, opening the possibility for rapid characterization of fruit quality traits achieved by transgenic approaches in citrus and other fruit tree crops.

The important contribution of carotenoids to the nutritional value and healthy properties of certain fruits and vegetables has led to attempts to induce or increase carotenoid levels in foods, particularly β-carotene, through metabolic engineering of carotenoid biosynthesis (e.g., tomato, maize, rice, potato, and canola seeds; reviewed in (Botella-Pavía and Rodríguez-Concepción, 2006; Della Penna and Pogson, 2006)). Sweet orange fruit is an excellent candidate for the transgenic enhancement of β-carotene content. Increased levels of β-carotene (a lipophilic antioxidant) would complement vitamin C (a hydrophilic antioxidant highly abundant in oranges) because it is generally thought that foods rich in both soluble and membrane-associated antioxidants offer the best protection against disease (Yeum et al., 2004). Additionally, other factors, such as the low complexity of the food matrix, would potentially enhance the absorption and bioavailability of the increased β-carotene in oranges (de Pee et al., 1998). In this work, we have shown that RNAi-mediated silencing of *Csβ-CHX*, which regulates an important step in orange carotenogenesis, induces the accumulation of high levels of β-carotene in oranges (up to 36.4-fold with respect to control fruits). This increase was accompanied by a general, mild decrease in the accumulation of downstream xanthophylls. This result is consistent with the findings of other studies, in which increases in one carotenoid were found to occur at the expense of others (Fraser et al., 2002), suggesting feedback

inhibition or rate-limiting steps within the carotenoid biosynthetic pathway and/or possible saturation of the carotenoid storage capacities within citrus fruits (Lu et al., 2006). The unexpectedly slight decrease of xanthophyll concentration in β-carotene-enriched oranges may be explained by the presence of a second putative *β-CHX* in the sweet orange genome (http://citrus.hzau.edu.cn/orange/, http://www.phytozome.net/) that would not be silenced by the RNAi strategy used here because its transcripts do not show enough sequence homology with *Csβ-CHX* RNA targets. Therefore, activity of the second putative *β-CHX* could likely counterbalance, at least in part, the very low *Csβ-CHX* transcript levels found in golden orange fruits.

Various vegetable and fruit crops have been transformed with the objective of enhancing the concentration of health-promoting phytonutrients, with special attention to antioxidants (Davies, 2007; Newell-McGloughlin, 2008; Shukla and Mattoo, 2009). Although most studies have successfully increased the amount of the target metabolite(s), only a few studies have also evaluated the antioxidant capacity of the enriched foods (Butelli et al., 2008; Rein et al., 2006). In this study, we have developed a straightforward experimental system that permits the characterization of the biological activity of transgenic citrus fruit *in vivo* in an inexpensive manner using *C. elegans* as a model organism for the functional analysis of orange juice.

We have engineered an increased level of β-carotene in sweet oranges, and *C. elegans* studies indicate that this level is sufficient to impart a substantial protective effect against oxidative damage when orange juice is included as part of the regular diet. The biofortified oranges exerted a higher protective effect than control oranges despite its slightly lower content of xanthophylls (oxygenated carotenoids also described as dietary antioxidants (Haegele et al., 2000)), which supports the strong antioxidant effect of dietary β-carotene reported in previous studies (Jialal et al., 1991; Meydani et al., 1994; Nakagawa et al., 1996). The effect of dietary β-carotene against oxidative stress achieved in this work (70% worm survival after hydrogen peroxide treatment) is similar to the effect reported for cocoa polyphenols (Martorell et al., 2011) and Tonalin (a conjugated linoleic acid commercial mixture) (Martorell et al., 2012). The mechanism of the antioxidant activity of β-carotene is related to this compound's hydrophobic character and ability to quench singlet oxygen and deactivate free radicals (Burton and Ingold, 1984; Rice-Evans et al., 1997). There is evidence indicating that the efficacy of β-carotene (as well as the efficacy of other phytonutrients) is heavily influenced by nutritional context (Hadad and Levy, 2012; Palozza and Krinsky, 1992; Shaish et al., 1995). Consistent with this finding, we observed that the level of resistance to oxidative stress achieved with exogenous pure β-carotene was lower than the levels of protection reached with the biofortified oranges, though both feeding conditions supplied diets with equivalent concentrations of β-carotene. This result indicates that the orange juice nutritional context has a substantial influence on the impact of dietary β-carotene, either through synergistic interactions with other constituents of the food matrix or through effects on bioavailability.

Experimental procedures

Generation of citrus transformants

A binary vector (HRP) was constructed that contained both an ihp β-CHX RNAi cassette and an FT overexpression cassette. The details of the construction are provided in the Data S1. After the HRP construct was confirmed by restriction mapping and DNA sequence analysis, the plasmid vector was transferred to *A. tumefaciens* strain EHA105 by electroporation and used to transform sweet orange plants (cv. Pineapple). The binary plasmid pROK2-*CsFT*, which contains a *CsFT* overexpression cassette, was used as the vector system for transforming control plants (Data S1). In both cases, the transformation of epicotyl explants from citrus seedlings was performed as previously described (Peña et al., 2001). The regenerated shoots obtained after kanamycin selection were screened by PCR using primers specific for the *CsFT* chimeric cassette. To avoid nonspecific amplification of the endogenous *FT* gene(s), the primers 35Sfinal-F (5'-CACAATCCCACTATCCTTCG-3') and FTCs2 (5'-GGGATTGATCATCGTCTGAC-3'), which amplified the region encompassing the end of the 35S promoter and the entire *CsFT* transgene, were used to screen the transformants. The PCR program used was 95 ºC for 5 min, 30 cycles of 95 ºC for 30 s, 58 ºC for 30 s, and 72 ºC for 1 min, followed by 72 ºC for 10 min. All PCR-positive shoots were shoot-tip grafted onto Troyer citrange (*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.) seedlings growing *in vitro* (Peña et al., 2008). Three to five weeks after shoot-tip grafting, the plantlets were grafted again in a greenhouse onto 5-month-old Carrizo citrange seedlings. The putatively transformed Pineapple sweet orange plants were maintained in greenhouses for 3-4 years and grafted onto different citrus rootstocks for further analyses.

Plant material, color index and internal maturity index

Fully ripened fruits of the transgenic HRP and CV lines were harvested during three consecutive seasons. The Color index (CI) of each fruit was measured with a Minolta colorimeter (model CR-200; Minolta Co. Ltd., Osaka, Japan) by taking three measurements in the equatorial zone of each fruit. The mean values of the lightness (*L*), red-green (*a*), and yellow-blue (*b*) Hunter parameters were calculated for each fruit, and presented as previously described (CI = 1000*a*/*Lb*) (Jiménez-Cuesta et al., 1981).

After color measurement, part of the flavedo and pulp tissues was separated with a scalpel, frozen in liquid nitrogen, ground to a fine powder, and stored at -80 ºC until analysis. Juice was extracted from the remaining pulp of each fruit and immediately analyzed. The titratable acidity (TA) of the juice was determined by titration with 0.1 N NaOH solution using phenolphthalein as an indicator and expressed as grams of citric acid per 100 mL of juice. The soluble solids content (SSC) was determined by measuring the refractive index of the juice

(Atago Digital Refractometer PR-101 model 0-45%; Atago Co., Ltd., Tokyo, Japan), and the data were expressed as ºBrix. The Maturity Index (MI) was estimated for each fruit from the SSC/TA ratio.

qRT-PCR

RNA extractions were performed from flavedo and pulp samples using the RNAeasy Plant Mini Kit (Qiagen, Hilden, Germany). The total RNA preparations were treated with recombinant DNase I (RNase-Free DNase Set; Qiagen) for complete genomic DNA removal, and the resultant RNA was accurately quantified in triplicate using a NanoDrop®ND-1000 (NanoDrop products, Wilmington, DE, USA) spectrophotometer. Gene expression analysis was performed by a two-step real-time qRT-PCR method. First-strand cDNA was synthesized from 2 µg of each DNase-treated RNA in 20 µL using oligo(dT)18 and a SuperScript™ II Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. After synthesis, the cDNA was subjected to a 20-fold dilution with RNase-free water (Sigma-Aldrich, St. Louis, MO, USA). Subsequent qPCR reactions were performed with a LightCycler®480 Instrument (Roche), and fluorescence was analyzed using LightCycler®480 Software. The primer pair and reaction conditions used for *Csβ-CHX* target gene amplification were obtained from Alquézar et al. (2009). Normalization was performed using the expression levels of the *ACTIN* gene from *C. sinensis* (*CsACT*) (Romero et al., 2012). Fluorescence intensity data were acquired during the 72 ºC extension step, and the specificity of the reactions was verified by analyzing the post-amplification dissociation curves. Melting curve analysis confirmed the presence of a single PCR product from all samples with no primer-dimers. The relative expression of the target gene (*Csβ-CHX*) normalized to the expression of the housekeeping gene (*CsACT*) was calculated following the mathematical model described by Pfaffl (2001). cDNA from the flavedo of the CV lines at the full-colored stage was used as a calibrator sample, and the rest of the values were expressed relative to this sample's value. The PCR efficiency values for *Csβ-CHX* and *CsACT* were approximately equal and were calculated by generating respective standard curves using cDNA serial dilutions. The values reported are the mean ± SEM of at least two independent assays. Each assay included (in triplicate) a standard curve, a no-template control, and 1 µL of each test and calibrator cDNA.

Carotenoid extraction and analysis

The extraction of carotenoids from the pulp of the sweet oranges followed a previously described protocol (Alquézar et al., 2008b). The extracts were dried by rotary evaporation and stored under a nitrogen atmosphere at -20 ºC until HPLC analysis. Carotenoid extracts were prepared for HPLC analysis by dissolution in 30 µL of chloroform:MeOH:acetone (5:3:2 by vol.), and a 25-µL aliquot was immediately injected. The HPLC analysis method was described previously (Alquézar et al., 2008b). Carotenoids were identified by their retention time,

absorption, and fine spectra (Britton, 1998). The carotenoid peaks were integrated at their individual maximum wavelengths, and the peaks' content was calculated using calibration curves of β-carotene (Sigma) for α- and β-carotene; β-cryptoxanthin (Extrasynthese, Lyon, France) for α- and β-cryptoxanthin; zeaxanthin (Sigma) for zeaxanthin; and antheraxanthin and lutein (Sigma) for lutein, violaxanthin, and neoxanthin isomers. Phytoene and phytofluene standards for quantification were obtained from flavedo extracts of Pinalate sweet oranges, which accumulate large amounts of these compounds (Rodrigo et al., 2003), and were then purified by TLC (Pascual et al., 1993). Quantification was performed using Empower chromatography software (Waters Corp., Milford, MA, USA). Carotenoids were measured for a minimum of three different fruits from two different plants per line and three consecutive fruiting seasons.

Worm feeding studies

Strains and maintenance conditions: The *C. elegans* strain used in this study (WT Bristol N2) and *Escherichia coli* OP50 were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. The worms were maintained at 20 ºC on NGM (3 g/L NaCl, 2.5 g/L peptone, 5 g/L cholesterol, 1 M MgSO4, and 1 M KPO4, pH 6.0) agar plates on a lawn of *E. coli* OP50.

Intake confirmation experiments: A sample processing system was established suitable for including citrus pulp in the nematode growth medium (NGM) (for details see Data S1). The intake confirmation experiments were performed with synchronized populations of the *C. elegans* WT strain Bristol N2. The nematodes were cultured on NGM or NGM supplemented with WT pulp extract (20%), and eggs were recovered in 50 plates per condition. Embryos were incubated at 20 ºC until reaching the young-adult stage (3 days old). The worms were then recovered with M9 buffer and washed three times to eliminate the E. coli OP50 present in the media. An additional 2 h of incubation in M9 buffer was performed to facilitate the removal of gut microbiota from the nematodes. Once the supernatant was discarded, the worm pellets (containing approximately 12,500 worms per condition) were recovered in Eppendorf tubes and disrupted by sonication (three pulses, 10 W, 20 s/pulse). The evacuated and washed worms were rotated gently for 3 min at 4 °C to form a loose pellet, and the supernatant was carefully removed with a pipette. The worm pellets were ground to a powder with a micropestle (Eppendorf, Hamburg, Germany) and liquid N2. Acetone was then added to the powder (0.5 mL), followed by vortex-stirring for 1 min and centrifugation for 2 min at 13,000 rpm (4 °C). Upon centrifugation, the acetone extracts were recovered, and the pellet was re-extracted with acetone. The colorless pellets were stored at -20 °C until subsequent measurements of protein content. Pooled acetone extracts were dried under a nitrogen atmosphere at 30 °C until reducing their volume to approximately 0.5 mL, after which the extracts were sequentially washed with two nonpolar organic solvents (ether and chloroform, 0.5 mL each) to remove any traces of water and impurities. The extracts were dried with nitrogen and stored under a

nitrogen atmosphere at -20 ºC until performing HPLC analysis of the carotenoids, following the protocol above described. Finally, the protein content of the stored worm pellets was measured essentially as previously described (Lamitina et al., 2004), and the content value was used to normalize the carotenoid levels. Briefly, the pellets resulting from the carotenoid extraction were treated with 0.5 mL 10% perchloric acid (PCA) to precipitate proteins. After centrifugation (10,000 rpm, 30 min, 4 ºC), the acidic supernatant was removed, and the PCA-precipitated pellets were solubilized with 0.1 N NaOH (200 µL). The protein concentration of these solutions was quantified according to Bradford (1976) using Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as a standard.

Hydrogen peroxide-induced oxidative stress assays: To measure *C. elegans* survival rates after exposure to oxidative stress, we employed synchronized eggs hatched in NGM on agar plates containing the *E. coli* OP50 strain and in the presence of either the β-carotene-enriched (HRP) or the control (CV) pulp extracts at a final concentration of either 1% or 2% (by vol.). The HRP pulp extract was obtained from mixtures of pulp from the HRP6, HRP11, and HRP12 lines. The trials were performed with fruits from two different fruiting seasons. Ascorbic acid (0.1 µg/ mL, Sigma-Aldrich, St. Louis, MO, USA) was used as antioxidant positive control. After 7 days of growth at 20 ºC, the worms were transferred to NGM plates containing 2 mM H_2O_2 and incubated for 5 h. The animals were then washed, and their viability was measured. Worms were considered dead when they no longer responded to prodding. The experiments were performed in triplicate. The proportion surviving after treatment with H_2O_2 was used to estimate the antioxidant capacity resulting from each feeding condition. The data on the survival rates were subjected to ANOVA using Statgraphics v.5.1 software (Manugistics Inc.), and Fisher's Protected LSD test ($P < 0.01$) was used to separate the means.

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Supporting information

Data S1. Supplemental experimental procedures

(a) Plasmid construction

The binary vector pROK2-*CsFT* was previously constructed using standard restriction and ligation DNA techniques (Rodríguez et al., unpublished results). This vector contains the *FLOWERING LOCUS T* gene from sweet orange (*CsFT*), which is 100% identical to the *Citrus unshiu CiFT2* homolog (GenBank accession number AB301934.1), in sense orientation under the control of the CaMV 35S promoter and the NOS terminator. The T-DNA of this binary vector also includes the *neomycin phosphotransferase II* gene (*NPTII*) driven by the NOS promoter and terminator sequences. The binary plasmid pROK2-*CsFT* was used in the present work as the vector system for transforming control plants. The HRP vector was constructed in two steps. First, the β-CHX RNAi cassette was generated using the Gateway System (Invitrogen). The 399-bp fragment corresponding to the sequence used in the hairpin (CHXi; nucleotide positions 357-756 of the 936-bp complete coding sequence) was PCR-amplified from a cDNA clone of a *β-CHX* gene isolated from the fruit of *C. sinensis* (GenBank accession number DQ228870) (Inoue et al., 2006) using the gene-specific primers 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGACTCTCCG-GAAATAAGGCACGTC-3' and 5'-GGGGACCACTTTGTACAAGAAAGCTG-GGTTTATCTCGTTGCTGCCGTCATGTC-3', flanked by attB recombinase sites (underlined). The gel-purified PCR product was recombined into the Gateway donor vector pDONR211 (Invitrogen) following the manufacturer's protocol for BP recombination. After the pDONR construct was confirmed by restriction digestion, Gateway LR recombination was performed with the pHellsgate8 (pHG8) destination vector (Helliwell et al., 2002), generating the ihp vector pHG8-CHXi. The T-DNA of this binary vector also includes the *NPTII* gene, which confers kanamycin resistance, under the control of the NOS promoter and terminator sequences. Next, the FT overexpression cassette was cloned into pH8-CHXi using standard restriction and ligation DNA techniques. The complete 1780-bp cassette was PCR-amplified from pROK2-*CsFT* with AccuPrime Pfx DNA polymerase (Invitrogen, Carlsbad, CA) using the primers FT-NheI-up (5'-TGGCGTAATCATGGTGCTAGCTG-TTT-3') and FT-NheI-down (5'-GTTTTGCTAGCCACG-ACGTTGTAAAA-3'), which contain the *Nhe*I restriction sites (underlined). The resulting PCR product was gel-purified and cloned into the unique *Nhe*I site of pHG8-CHXi, generating the final HRP vector.

(b) Southern blot analysis.

Genomic DNA was isolated from leaves according to Dellaporta et al. (1983). Southern blot analysis was performed using 20 µg of either *Not*I-, *Cla*I- or *Kpn*I-digested samples, which were separated on 1% (w/v) agarose gels, blotted onto nylon membranes (Hybond-N+, Amersham Pharmacia, London, UK), and fixed by UV irradiation. The filters were probed with a digoxigenin (DIG-11-dUTP; Roche Diagnostics Corporation, Indianapolis, IN)-labeled fragment of either the 35S promoter or the coding region of the *βCHX* gene used in the hairpin construct

(CHXi), prepared by PCR following the supplier's instructions (Boehringer Mannheim GmbH, Mannheim, Germany), and detected with the chemiluminescent CSPD substrate (Roche Diagnostics).

(c) Processing and biochemical analysis of pulp extracts/worm food

NGM supplementation with citrus pulp extracts: A protocol was developed for the processing and sterilization of pulp samples prior to addition to the NGM using oranges from WT plants. Pulp powder samples stored at -80 ºC were thawed and homogenized in a Polytron (on ice) until a near-liquid consistency was obtained. For sterilization, the homogenized samples were pretreated overnight with 7 mM Velcorin® (DMDC, Lanxess), a compound widely used in the food industry for the sterilization of drinks. This pretreatment partially reduced the initial contamination of the pulp and was completely innocuous to the worms. Higher doses of Velcorin® (10, 15, 20, and 25 mM) did not improve the sterilization of the pulp extracts. Subsequently, because microbial contamination was still present on the NGM agar plates supplemented with the Velcorin®-treated samples (Figure S3a), which precluded the completion of the experiments, we applied a heat treatment system for proper sterilization. A volume of each pulp sample (10 mL) was subjected to different periods of heat at 90 ºC: 0.5, 2, 5, 10, 15, or 30 min. Afterward, the pulp extracts were added to the NGM plates at 2% to determine the presence of contaminant microbiota. In parallel, the carotenoid profile and vitamin C content of the treated pulp extracts were characterized by HPLC. After testing the different heat treatment times, 15 min was selected as the effective time for sterilization of the WT pulp because this time prevented contamination while minimally altering pulp composition. As shown in Figure S3, any contamination in the NGM agar plates supplemented with pulp extracts was completely removed (Figure S3a). The carotenoid losses from the samples were acceptable; that is, no losses were observed for β-carotene and other important carotenoids compared with the untreated sample, and the xanthophylls antheraxanthin and violaxanthin were the only carotenoids whose content was substantially reduced (Figure S3b). Furthermore, the loss of vitamin C from the pulp extract after 15 min of heat treatment at 90 °C was relatively low (approximately 15%; Figure S3c).

Quantification of carotenoid content: Carotenoids were extracted from the pulp extracts as previously described by Stinco et al. (2012), with slight modifications. Briefly, 1 mL of each pulp extract was centrifuged, and the aqueous phase was removed. Acetone was then added to the pellet (2.5 mL), followed by stirring for 5 min and centrifugation for 5 min at 18,000 g. Upon centrifugation, the acetone extracts containing the carotenoid pigments were recovered. The pellet was re-extracted with acetone until it was colorless. To obtain the saponified carotenoids, the pooled acetone extracts were treated with 5 mL of methanolic KOH (10% w/v) for 1 h under dim light at room temperature. The saponified carotenoids were subsequently re-extracted with dichloromethane (10 mL), after which the material was washed three times with water to remove any traces of base and impurities. The colored dichloromethane extracts were dried by rotary evaporation and stored under a nitrogen atmosphere at -20 ºC until HPLC analysis. HPLC

analysis of carotenoids was performed as described in the “Carotenoid Extraction and Analysis” section (in Experimental Procedures).

Determination of vitamin C content: The vitamin C content (ascorbic acid (AA)) was determined according to the method described by Sdiri et al. (2012), with slight modifications. Pulp extract samples (3-4 mL) were centrifuged at 14,000 rpm (4 °C, 5 min) to remove the pulp and coarse cloud particles. Aliquots (1 mL) of supernatant were added to 9 mL of 2.5% meta-phosphoric acid (MPA). The diluted samples were filtered through a 0.45-µm nylon filter and injected into an HPLC system (LachromElite, Merck Hitachi, Germany) equipped with a diode array detector (L-2450), column oven (L-2300), and auto-sampler (L-2200). Separation was performed on a Lichospher 100 RP-18 column (4 mm x 250 mm), preceded by a precolumn (4 x 4 mm) with a particle diameter of 5 µm. KH2PO4 (0.2 M, adjusted to pH 2.3 with phosphoric acid) was used as the mobile phase at a flow rate of 1 mL/min and with UV detection at 243 nm. The total elution time was 10 min, and the injection volume was 20 µL. The analyses were performed in triplicate.

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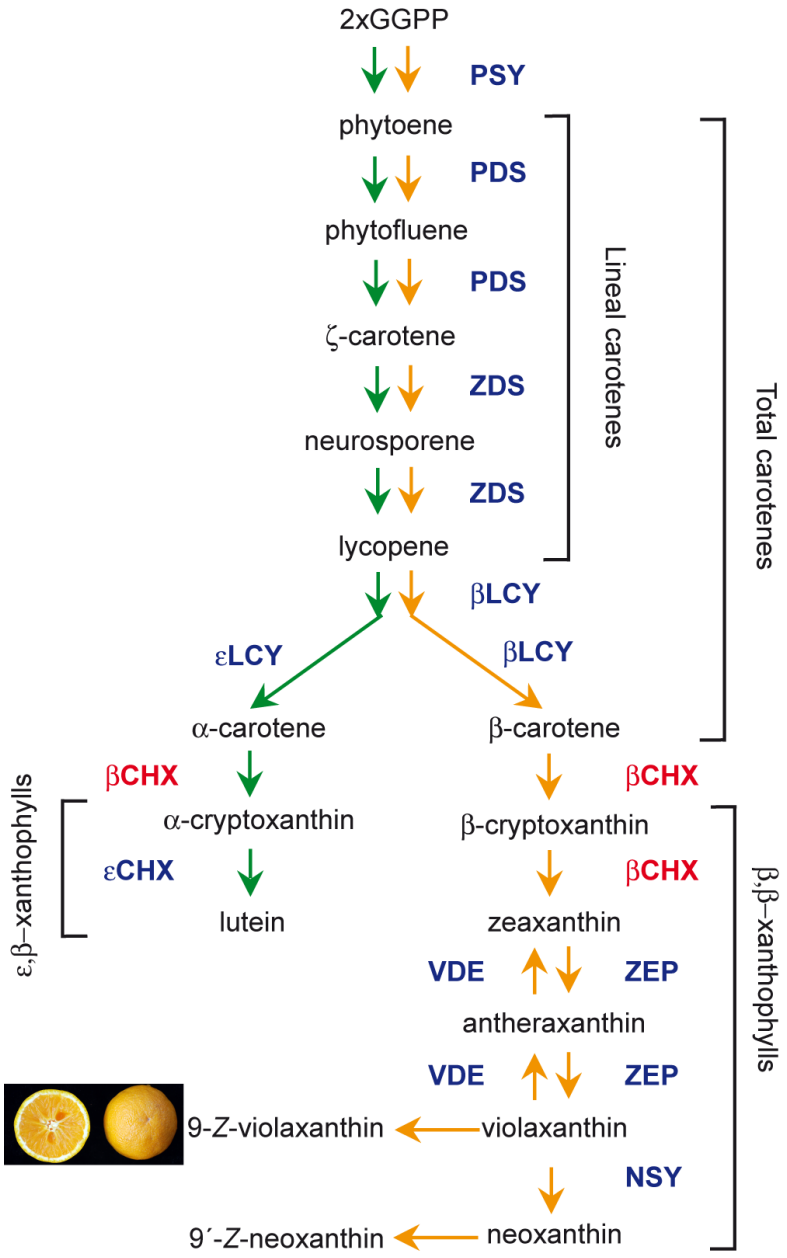

Figure S1. Carotenoid biosynthesis in sweet oranges. Green and orange arrows indicate the branch of the pathway predominantly active in the immature green and full-color stages of fruit development, respectively. A photograph of the fully mature fruit from the Pineapple sweet orange cultivar used in this study is presented besides 9-*Z*-violaxanthin, which is the major carotenoid in the pulp of full-colored fruits. The key step of the pathway that we sought to knock down in this work as a strategy for accumulating β-carotene is shown in red. The rest of the enzymes are shown in blue. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; ε-LCY, lycopene ε-cyclase; β-LCY, lycopene β-cyclase; β-CHX, β-carotene hydroxylase; ε-CHX, ε-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase.

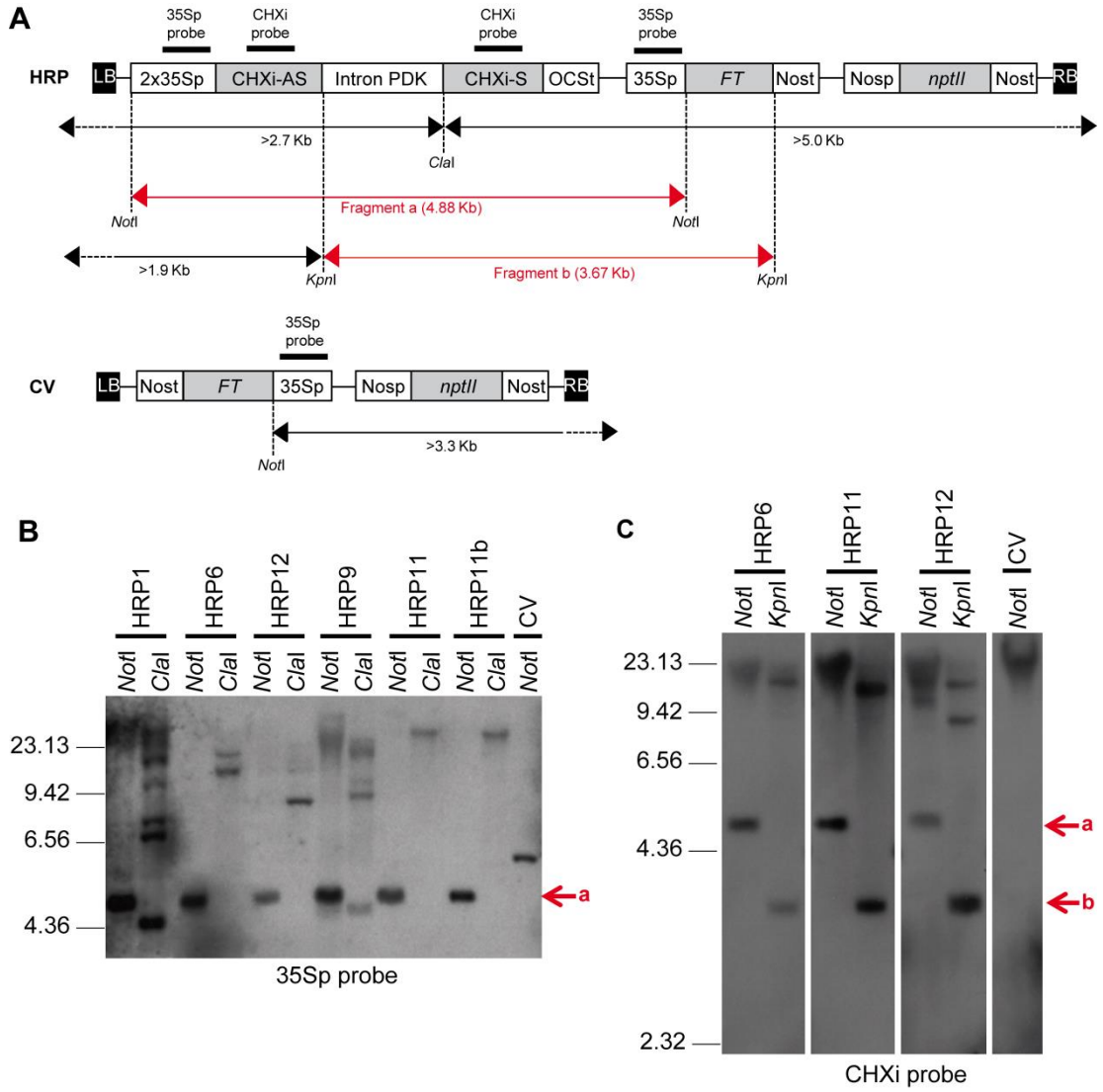

Figure S2. Southern blot analysis of Pineapple sweet orange plants transformed with the β-CHX RNAi silencing plasmid. (a) Schematic representation of the T-DNAs used for citrus transformation, showing the restriction sites for the enzymes *Not*I, *Cla*I, and *Kpn*I, the 35S and CHXi probes used, and the expected sizes of the hybridization products. (b) Southern blot analysis of DNA preparations from a set of sweet orange plants transformed with the HRP construct (lines HRP1, 6, 9, 11, and 12), or with the control vector (CV). Hybridization was performed with a DIG-labeled DNA probe of the 35S promoter. The DNA restrictions with *Not*I revealed that all the HRP transgenic lines contained at least one intact copy of the entire β-CHX RNAi cassette, because a band of 4.88 kb (fragment a) was detected in all cases. The detection of additional bands higher than 4.88 kb in lanes 1 and 7 suggests truncated insertions of the RNAi cassette in the transgenic HRP1 and HRP9 lines. The other lines containing non-truncated insertions (HRP6, 11 and 12) showed low T-DNA loci number, according to the digestion pattern observed with *Cla*I. HRP11 and HRP11b were considered to be plant clones because their restriction profiles were identical. (c) Confirmation of whole-transgene integrity and low T-DNA loci number in plant lines HRP6, HRP11, and HRP12 by restriction analysis with either *Not*I or *Kpn*I, and Southern blot hybridization with a CHXi-specific probe. Digestion with *Not*I confirmed the presence and integrity of the β-CHX RNAi cassette because a 4.88 kb band was detected in all the three HRP lines and not in the CV line. Additional bands at the top of all *Not*I-lanes suggested hybridization with endogenous sweet orange *β-CHX* gene/s. Digestions with *Kpn*I released a 3.67 kb band (fragment b), plus a number of bands that varied depending on the number of T-DNA loci integrations. The size of the DNA marker used is indicated at the left in kilobases.

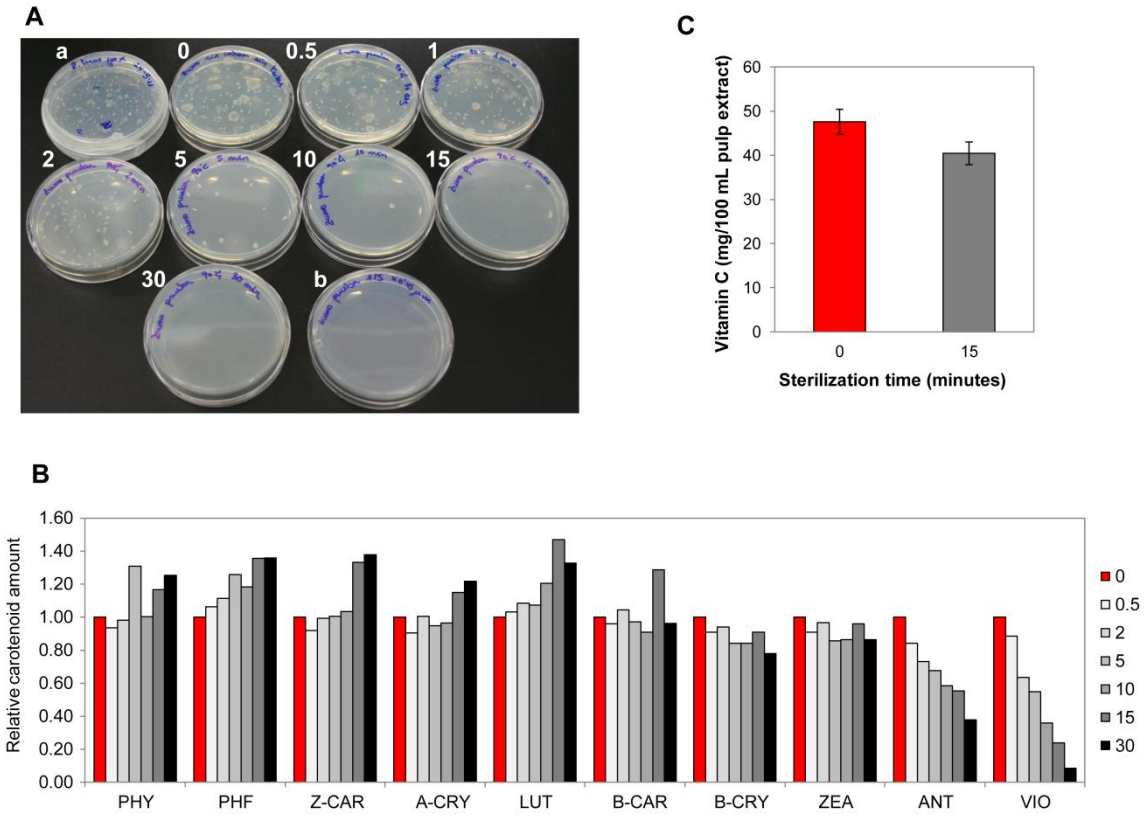

Figure S3. Effect of heat treatment (90 ºC) of the wild type (WT) pulp extracts on microbial contamination, carotenoid profile, and vitamin C content. (a) Plate count in NGM supplemented with 2% of WT pulp extracts previously treated with Velcorin® and heated at 90 ºC for 0.5, 1, 2, 5, 10, 15, or 30 min. a, Plate count in NGM supplemented with 2% of the non-heat-treated HRP pulp extracts. b, Plate count in NGM without supplementation. (b) HPLC analysis of carotenoid accumulation in WT pulp samples treated at 90 °C for different lengths of time (key legend in minutes), presented as the amount relative to the untreated sample (t = 0). (c) Quantification of the loss of vitamin C from the pulp extracts after 15 min of heat treatment. The values are the means ± SE (n = 3). PHY, phytoene; PHF, phytofluene; Z-CAR, ζ-carotene; A-CRY, α-cryptoxanthin; LUT, lutein; B-CAR, β-carotene; B-CRY, β-cryptoxanthin; ZEA, zeaxanthin; ANT, antheraxanthin; VIO, all *E*-violaxanthin and 9-*Z*-violaxanthin.

Table S1. Carotenoid levels (ng/mL) and vitamin C content (mg AA/100 mL) in the pulp extracts used for nematode diet supplementation. HRP and CV pulp extracts were analyzed after sterilization (15 min at 90 ºC). For each pulp extract, the percentage of each carotenoid or group of carotenoids was calculated on the total carotenoid content. AA, ascorbic acid. The HRP pulp extract was obtained from mixtures of pulp from the HRP6, HRP11, and HRP12 lines.

	CV pulp extract		**HRP pulp extract**	
Carotenoids	**ng/mL**	**%**	**ng/mL**	**%**
Lineal carotenes	270.4	**6.7**	64.4	**4.9**
β-Carotene	41.6	**1.0**	194.7	**15.0**
α-Carotene	42.3	**1.1**	59.5	**4.6**
Total carotenes	354.4	**8.8**	318.5	**24.5**
β,β-xanthophylls	1963.4	**48.7**	465.3	**35.8**
ε,β-xanthophylls	1714.4	**42.5**	517.0	**39.7**
Total xanthophylls	3677.8	**91.2**	982.2	**75.5**
Total carotenoids	4032.2	**100.0**	1300.7	**100.0**
Vitamin C (mg AA/100 ml)	29.53 ± 2.64		33.04 ± 1.72	

6. GENERAL DISCUSSION AND OUTLOOK

6. General discussion and outlook

In this thesis, it has been investigated in detail the first field trial with GM citrus trees performed in the world. The results of this field trial have provided crucial information on factors influencing pollen dispersal from transgenic citrus to non-transgenic genetically diverse surrounding citrus trees under field conditions, data that were non-existent to date. It has been found out that pollen-mediated transgene flow (PMTF), which is the main component of transgene dispersal to the environment in entomophilous plants (Ennos, 1994), could be greatly limited by the presence of neighboring sympatric citrus genotypes with high pollen competition capacity (superior to that of the GM citrus genotypes) (**Chapter 1**). Such information has had a direct practical application as it has served to draft part of the White Paper that contains the guidelines on how releases of GM sweet orange trees should be performed in Brazil, the main producer country of citrus for processing. The release of GM plants into the environment for either research or commercial purposes should follow the guidelines and applicable regulations of the country where the field test are being conducted. Legislation at this respect is generally made case-by-case, and the White Paper is the document on which protocols and safeguards are proposed, including the containment measures that should be adopted for each crop to minimize transgene dispersal. Such measures must be duly justified, and preferably based on empirical studies on transgene flow for the crop under study. Following the approval of the White Paper document by the relevant regulatory authority (the correspondent National Biosafety Committee of each country), the proposed measures become mandatory regulations.

The non-profit association FUNDECITRUS (FUNDO DE DEFESA DA CITRICULTURA), in consensus with other institutions that also work in the field of plant biotechnology and plan to make deliberate releases of GM citrus in Brazil, proposed in 2012 a White Paper for the release of GM citrus and presented it to the Comissão Técnica Nacional of Biossegurança (CTNBio). The measures contemplated in that document basically consisted on the use of trees from non-GM citrus genotypes with high pollen competition ability (or "strong pollinators") as buffer rows surrounding the transgenic trees. It was also proposed the use of other external edge of trees from a monoembryonic and self-incompatible citrus genotype that would serve to track PMTF (because the only seeds/seedlings they could produce would come from cross-pollination). These containment measures have been approved in October 2013 (see **Annex 1**) and are much more realistic and viable than others previously proposed and approved for the same crop in Brazil. For example, the biotechnological company Allelyx proposed in 2007 containment measures that consisted on the use of empty spaces (8 m) as isolation barriers plus avoiding flowering of GM citrus by performing the field tests when trees were juvenile and removing the flowers in the case they would flower (Allelyx, 2007). In such assays, citrus trees were not allowed to produce fruits. A very similar approach has been recently approved in Florida to allow release of transgenic sweet orange trees to test disease resistance. Unlike those cases, the White Paper approved in Brazil permits flowering and fruiting to occur. The only pending work is (1) to specify the citrus genotypes suitable for using as "strong pollinators", which will

vary depending on the transgenic varieties intended to be released and should be selected based on the results of mixed-pollination treatments carried out before the release, and (2) testing the effectiveness of the proposed measures using the selected genotypes, under actual field conditions for each country. FUNDECITRUS is currently conducting a large-scale experiment in Brazil for these purposes.

To fully validate the use of the genetic transformation technology on a commercial basis, it requires the ability to adequately grow and test the transgenic trees in the field. This needs legislative action to provide appropriate protocols and safeguards, when none currently exist. Without this ability, the scope of the efforts and resources used to develop these trees are greatly reduced. Where no legislation exists or while legislation is being developed to field test transgenic trees, collaborative field plantings should be encouraged between research institutions from countries that have proper regulatory systems approved and those that do not. Acceptance of this White Paper by the CTNBio may have a major impact because it allows the possibility of addressing some of the challenges that are currently threatening the Brazilian (and global) citrus industry through the use of genetic transformation as a modern tool for improvement.

The field trial with GM citrus trees conducted in this thesis has further implications. On the one hand, it has served as model to study for the first time in citrus the long-term stability of the transgene expression. Moreover, it represents the first approach to propose how field trials with GM citrus should be performed to detect transgene-derived unintended effects with regard to the main crop characteristics (tree morphology, phenology and fruit quality) (**Chapter 2**). Regarding this, it is particularly noteworthy the importance of performing control comparisons (using suitable comparators to be able to detect statistically significant differences), besides those obvious comparisons of "GM *versus* non-GM" required to assess substantial equivalence. Thus, the robustness of the system used to detect differences (that is, the experimental design and statistical analysis) is verified, giving more reliability and confidence to the results obtained.

The comparisons performed in **Chapter 2** revealed a most noticeable and significant effect of other factors than transgenesis (as plant ontogeny and ploidy level) on the fruit quality parameters and morphological variables analysed. Similar results were shown in research performed with other crops. After a large number of studies afforded to find out unintended effects of transgenesis in different plants, no differences between the GMs and their conventionally bred counterparts have been found beyond natural variability, whether using targeted (Cellini, et al., 2004) or non-targeted approaches (that is "omic" assessments or large-scale profiling techniques) (Ricroch, et al., 2011) to detect them. Indeed, the most pronounced differences were consistently found among the various conventional varieties, a trend linked to the genetic diversity maintained or created by plant breeders. This should be put in perspective, taking into account that conventional breeding is generally regarded as safe, despite the fact that the nature of the genetic changes in new conventional cultivars is usually unknown (Parrott, et al., 2010). Other intensive breeding methods that are routinely used, such as mutational breeding, intervarietal hybrids, wide interspecies crosses, inbreeding, ploidy modification and

tissue culture multiplication, produced abundant pleiotropic effects on gene structure and trait expression in some plants (Ozcan, et al., 2001). Likewise, a number of environmental factors (field location, sampling time during the season or at different seasons, mineral nutrition) have also been shown, consistently, to exert a greater influence on quality than transgenesis (Pilate, et al., 2002; Tilston, et al., 2004; Halpin, et al., 20007; Ricroch, et al., 2011). Although the generation of new unintended effects is an often cited fear of plant genetic modification (Filipecki and Malepszy, 2006), no significant differences attributable to the transgenic nature of a crop have been reported so far (Shepherd, et al., 2006), and none of the published omic assessments has raised new safety concerns about marketed GM cultivars (Lehesranta, et al., 2005; Abdeen, et al., 2010; Rommens, 2010; Zhao, et al., 2013).

The next generation of GM crops is likely to include those with improved nutritional properties which are more prone to affect metabolic pathways, and thus introduce an increased complexity to the genetic modification process. The incorporation of new biosynthetic pathways in plants as well as genetic modifications targeting key enzymes in primary and secondary metabolism could result in metabolic perturbations not explicable based on our current knowledge of plant biology and metabolic pathway networks. In these cases, it is encouraged the use of non-targeted approaches such as transcriptomics, proteomics and metabolomics analyses to evaluate substantial equivalence (Cellini, et al., 2004). Results from these analyzes could provide an idea on the aspects of plant development and quality on which to focus during further field evaluations.

On the other hand, the field study carried out in the **Chapter 2** of this thesis demonstrated the innocuousness of marker transgenes *uidA* and *nptII* at phenotypic level as well as their long-term stability in transgenic citrus trees grown under agronomic conditions. The absence of unintended effects associated with these marker genes has been demonstrated in other plants, at different levels (food safety, environmental risks, agronomical performance) and with different techniques (transcriptomics, metabolomics, animal toxicity testing model, etc.) (Dale and McPartlan, 1992; Nap, et al., 1992; Redenbaugh, et al., 1992; El Ouakfaoui and Miki, 2005; Hopkins, et al., 2007; Miki, et al., 2009). Recently, the safety of *nptII* has been studied in detail. The product of the *nptII* gene (providing resistance to kanamycin and related antibiotics) was classified as Generally Recognized as Safe (GRAS) during deregulation of the Flavr Savr tomato (Redenbaugh, et al., 1992; Fuchs, et al., 1993). A working group of the British Society for Antimicrobial Chemotherapy made a strong general argument for the safety of virtually all antibiotic resistance genes in plants (Bennett, 2004): “The Working Party finds that there are no objective scientific grounds to believe that bacterial AR [antibiotic resistance] genes will migrate from GM [genetically modified] plants to bacteria to create new clinical problems. Use of these genes in GM plant development cannot be seen as a serious or credible threat to human or animal health or to the environment.” This view largely echoes that of (Flavell, et al., 1992) and the US Food and Drug Administration in their “Guidance for Industry” issued in 1998 (FDA, 1998). Strong arguments have been made for the safety of the β-glucuronidase reporter gene (Gilissen, et al., 1998), which was present in commercially released transgenic papaya

(Gonsalves, 1998). Nevertheless, due to the reported reluctance of people to consume food that has been transformed with bacterial genes (Rommens, 2010), the last trend is to limit their use as far as possible. To do this, alternatives to the use of selectable marker genes as well as systems for marker gene removal when it is no longer needed in the plant, such as the CRE/lox and similar marker transgene excision systems are currently being developed for many crops (Klaus, et al., 2004; Wang, et al., 2005; Fladung and Becker, 2010). These technologies are not yet applicable for citrus at a large scale, and the current protocols to generate transgenic plants are still largely dependent on the use of screenable and selectable genes, and among them, *uidA* and *nptII* are the most commonly used. Hence, information provided by this field trial (in the **Chapter 2**) is relevant and must be taken into consideration when interpreting their effects and safety when co-transforming them with genes of interest in future studies with GM citrus.

In summary, studies performed in this field trial constitute the first attempt to address concerns of biotechnologists, regulators and general public about using GM citrus. It is well known that there exist certain public disquiet over GM foods and crops, especially in some EU countries, and this is, in part, because consumers simply find no reason to support a new technology that does not provide any benefits to them. In this sense, and as demonstrated in some market surveys (http://ageconsearch.umn.edu/bitstream/6407/2/469580 a.pdf) (Hossain and Onyango, 2004; Onyango and Nayga, 2004; Costa-Font, et al., 2008), it is expected that the use of the GM technology could be much more accepted when the objective is to develop a functional food (FF), and even more if the functionality of this new food is proven using *in vivo* systems. For this reason, enrichment of crops with health-promoting phytonutrients has become one of the main improvement goals addressed by genetic transformation during the last decade (Cressey, 2013).

Orange fruits (which are the most consumed citrus types worldwide) are already very healthy, but in **Chapter 3** of the present thesis we have proposed to enrich them with a phytonutrient through metabolic engineering, with the aim of increasing their *in vivo* antioxidant properties. To address this challenge, the first technical difficulties we encountered were (1) excessive periods of time to see the results due to the long juvenile phase of citrus, (2) need of a lot of space due to the large size of trees, and (3) the animal model systems available to test the *in vivo* functionality of the FF are very expensive and require lots of fruit. These issues were resolved in **Chapter 3** by (1) developing a new experimental system that combines the use of a fast track system (over-expression of the *CsFT* gene in juvenile plants) to accelerate the production of the transgenic fruit and (2) through the use of *C. elegans* as a model organism to test the *in vivo* functionality of such enhanced fruit. This system brings the possibility of performing early-tests on the effectiveness of this (and any other) goal of nutri-functional improvement of citrus fruits, in a remarkably short period of time and in an inexpensive manner.

The next challenge we faced was to select the right phytonutrient and the proper strategy to increase it. In our case, the chosen target metabolite was β-carotene for the following reasons: it has known benefits on human health; its content in the orange pulp is very low; currently, there is an ample knowledge about the regulation of carotenogenesis in the

orange fruit and most genes from the pathway have been cloned. Lycopene (another carotenoid with well-recognized health-promoting properties (Rao and Agarwal, 1999; Karppi, et al., 2009) and absent in most orange cultivars), or anthocyanins (a specific class of flavonoid compounds, whose health-promoting qualities are supported by extensive literature, and that are present in blood oranges only under very strict and particular environmental conditions) are examples of other phytonutrients that would be interesting targets for enrichment in the orange fruit. Our laboratory is currently focused on work over these targets with the aim of enhancing the health-promoting qualities of sweet orange fruits and juices. Very promising results are already available, only needing confirmation in coming fruiting seasons (Alquézar *et al*.; Pons *et al*., unpublished results) (**Annex 2**). As for the strategy used in this thesis, we opted for silencing the *βCHX* gene (involved in the conversion of β-carotene into xanthophylls) by RNAi. A similar strategy was conducted to successfully increase the β-carotene content in other crops, (e.g. in potato (Diretto, et al., 2007)). Other attempts to increase specific carotenoid contents in several other crops by using carotenoid pathway genes are well reviewed (Botella-Pavía and Rodríguez-Concepción, 2006; Giuliano, et al., 2008). Alternative to those, though not incompatible with them, it is also possible to use more elaborated strategies, such as overexpressing specific transcription factors, or the *Or* gene (which represents a novel regulatory gene in mediating carotenoid accumulation by inducing chromoplast biogenesis) (Lu, et al., 2006; Lu and Li, 2008). Irrespective of the target metabolite and the chosen strategy for improvement, nowadays, tissue-specific strategies are desirable to avoid unintended effects derived from the transgene expression in non-target tissues. In the case of citrus, there was not available any fruit-specific promoter when this work was initiated, which led us to use a constitutive promoter in this thesis. In this sense, one of the priority lines of research being conducted today in our laboratory is the development of a fruit-specific promoter for citrus to be used for biotechnological purposes. The use of such a promoter, besides reducing the possibility of generating unintended effects, represents a critical step towards the development of cisgenics and intragenics strategies, which are highly desirable due to their greater acceptance by consumers and likely lower regulatory requirements (Rommens, 2004; Schouten, et al., 2006; Conner, et al., 2007; Rommens, et al., 2007).

In **Chapter 3** of this thesis, it has been achieved an increase (of about 37 fold) of β-carotene content in the orange pulp, which results in a 20% increase of its *in vivo* antioxidant capacity, as measured in the animal model *C. elegans*. Although these enriched oranges have served, together with control oranges (transformed with the empty vector) as isogenic food material, to be tested in the bioassays, they could not be used commercially because they contain the *CsFT* transgene, which produces developmental alterations in the plants. For this reason, the next step performed in our laboratory was to silence the *βCHX* gene in adult sweet orange varieties that flower and fruit normally about two years after transformation. After confirming that the transgenic adult orange trees produce β-carotene-enhanced fruit and grow normally, we are currently submitting a proposal to CTNBio to get the appropriate permits to plant them in Brazil at large scale. The aim is to carry out in depth studies of these plants and

their fruits at all levels (fruit quality, field performance, metabolomics, etc.), including research on their potential health benefits. Compared with the control fruits, golden oranges provide highly characterized, isogenic material with enhanced antioxidant capacity to evaluate the protective effects of dietary β-carotene on animal models of chronic diseases, such as cancer, cardiovascular disease and the metabolic syndrome, for which there are strong correlative data for protective effects of β-carotene. Furthermore, β-carotene-enriched orange fruits could contribute substantially to the antioxidant levels of human diets and might, as foods, be more widely adopted in preventive medicine strategies by healthy consumers than antioxidant supplements, such as vitamins, which are often viewed in the same way as conventional medicines. Such oranges with higher antioxidant capacity would create a niche market of specialty produce, more so if their usefulness in improving the health benefits, including protection against a particular disease, is demonstrated through clinical assays.

7. CONCLUSIONS

7. Conclusions

1. It has been demonstrated, for the first time in citrus, the efficiency of a pollen mediated transgene flow (PMTF) monitoring method consisting on testing the expression of a tracer marker gene (*uidA*) in seeds from a self-incompatible and monoembryonic citrus genotype (Clemenules clementine), used as pollen recipient.

2. Unexpectedly low frequencies (from 0.17% to 2.86%) of PMTF were found during 7 consecutive years in a field trial that involved the release of three different citrus genotypes carrying the *uidA* (GUS) tracer marker gene (pollen donors), as estimated by measuring the percentage of transgenic seeds in non-GM clementine trees (pollen recipient), planted along a contiguous edge in conditions allowing natural entomophilous pollination to occur. Phenological studies and hand pollination treatments demonstrated that transgenic pollen donors and recipient trees showed flower synchrony and were cross compatible.

3. Paternity analyses of the progeny of subsets of open pollinated recipient plants using 10 microsatellite (SSR) loci demonstrated a higher mating competence of trees from another non-GM pollen source population that greatly limited the mating chance of the contiguous cross-compatible and flowering-synchronized transgenic pollen source. This mating superiority could be explained by a much higher pollen competition capacity of the non-GM genotypes, as was confirmed through mixed-hand pollinations.

4. Then, presence of neighboring genotypes with very high pollen competition capacity is a crucial factor able to strongly limit PMTF between cross-compatible species when they have synchronized flowering and are planted at close proximity. Based on this finding, suitable confinement measures are proposed for the first time to minimize transgene outflow between contiguous plantings of citrus types that may be extendible to other entomophilous transgenic fruit tree species.

5. The stability of the integration and expression of the transgenes *uidA* and *nptII* was confirmed in 7-year-old, orchard-grown transgenic lines from two distinct citrus types (Pineapple sweet orange [*Citrus sinensis* L. Osb.] and Carrizo citrange [*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.]) by Southern blot analysis and enzymatic assays, respectively.

6. Comparisons between such GM citrus lines with their non-GM counterparts across the study years showed that the integration and expression of these transgenes (*uidA* and *nptII*) did not cause alterations of the main phenotypic and agronomic plant and fruit characteristics. However, when comparisons were performed between diploid and

tetraploid transgenic citrange trees and between juvenile and mature transgenic sweet orange trees, significant and consistent differences were detected, indicating that factors other than their transgenic nature induced a much higher phenotypic variability.

7. These results establish the principle that it is possible to produce transgenic citrus trees that are substantially equivalent to the control non-transformed lines, with regard to their overall agronomic performance, based on the use of robust and powerful assessment techniques. Therefore, future studies of the possible pleiotropic effects induced by the integration and expression of transgenes in field-grown GM citrus may focus on the newly inserted trait(s) of biotechnological interest.

8. It has been developed a fast-track system based on the ectopic overexpression of the *FLOWERING LOCUS T* gene from sweet orange (*CsFT*) in juvenile plants that induces an extremely early fruiting phenotype (less than 1 year after being grafted in the greenhouse) and two fruiting cycles per year in sweet orange. This system opens the possibility for rapid characterization of the fruit quality traits achieved by transgenic approaches in citrus and other fruit tree crops usually requiring many years to begin to flower and set fruits.

9. It has been established a straightforward experimental system that permits the characterization of the biological activity of transgenic citrus fruit *in vivo* in an inexpensive manner using *Caenorhabditis elegans* as a model organism for the functional analysis of orange juice. As necessary requirement of the system, an orange pulp processing method was set up and the intake of orange pulp extracts by the nematodes was confirmed.

10. It has been proven that RNAi-mediated silencing of a β-carotene hydroxylase gene from sweet orange (*Csβ-CHX*), which regulates an important step in orange carotenogenesis, induces the accumulation of high levels of β-carotene in oranges (up to 36.4-fold with respect to control fruits).

11. Levels of pro-vitamin A that accumulate in oranges are sufficient to impart a substantial protective effect against oxidative damage in *C. elegans* (20% higher antioxidant effect than the isogenic control), when included as part of their regular diet. This effect is presumably due to the synergistic interaction between β-carotene and orange juice phytochemicals.

8. LITERATURE CITED IN INTRODUCTION AND GENERAL DISCUSSION

8. Literature cited in Introduction and General Discussion

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ANNEX

ANNEX 1

Normative Resolution No. 10, published in the DOU on 02 October 2013, establishing the conditions of isolation for the planned release of genetically modified sweet orange (*Citrus sinensis* (L.) Osbeck) plants to the environment

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tecnologias da informação devidos a título de contrapartidas em razão da fruição dos incentivos fiscais, seja pela incorporada, seja por ela, incorporadora, resolvem:

Art. 1º Fica transferida a titularidade da Portaria Interministerial MCT/MDIC/MF nº 235, de 13 de maio 2003, publicada no DOU de 14.5.2003, da empresa Metrocable Indústria e Comércio Ltda., CNPJ nº 04.183.611/0003-35, para a empresa Furukawa Industrial S.A. Produtos Elétricos, CNPJ nº 51.775.690/0018-30.

Art. 2º Esta Portaria entra em vigor na data de sua publicação, ficando convalidados todos os atos praticados pela empresa Furukawa Industrial S.A. Produtos Elétricos, CNPJ nº 51.775.690/0018-30, em decorrência da sucessão, desde a data em que esta se operou.

MARCO ANTONIO RAUPP
Ministro de Estado da Ciência,Tecnologia e Inovação

FERNANDO DAMATA PIMENTEL
Ministro de Estado do Desenvolvimento, Indústria e Comércio Exterior

PORTARIA INTERMINISTERIAL Nº 1.025, DE 2 DE OUTUBRO DE 2013

OS MINISTROS DE ESTADO DA CIÊNCIA, TECNOLOGIA E INOVAÇÃO e DO DESENVOLVIMENTO, INDÚSTRIA E COMÉRCIO EXTERIOR, no uso das atribuições que lhes confere o § 2º do art. 22 c/c o art. 50 do Decreto nº 5.906, de 26 de setembro de 2006, tendo em vista o contido no Processo MCTI nº 01200.003672/2011-44, de 14/10/2011, e

Considerando que a empresa Atos Automação Industrial Ltda., inscrita no Cadastro Nacional da Pessoa Jurídica do Ministério da Fazenda - CNPJ sob o nº 44.020.535/0001-08, é titular da Portaria Interministerial MCT/MDIC/MF nº 801, de 13 de dezembro de 2001, publicada no Diário Oficial da União, de 17 de dezembro 2001, que lhe concedeu habilitação à fruição dos incentivos fiscais previstos no Decreto nº 3.800, de 20 de abril de 2001, atualmente regulamentados pelo Decreto nº 5.906, de 26 de setembro de 2006;

Considerando que a Portaria Interministerial MCT/MDIC/MF nº 446, de 16 de junho de 2009, de 17 de junho de 2009, transferiu a titularidade da Portaria Interministerial MCT/MDIC/MF nº 801, de 13 de dezembro de 2001, da empresa Atos Automação Industrial Ltda., inscrita no CNPJ sob o nº 44.020.535/0001-08, para o estabelecimento matriz da empresa Schneider Electric Brasil Ltda., inscrita no Cadastro Nacional da Pessoa Jurídica do Ministério da Fazenda - CNPJ sob o nº 82.743.287/0001-04;

Considerando que, posteriormente, a matriz, CNPJ sob o nº 82.743.287/0001-04, mudou seu endereço, mas manteve o estabelecimento Industrial no antigo endereço, localizada na Avenida das Nações Unidas, nº 23.223, Vila Almeida, CEP 04795-907, Estado de São Paulo, com a criação de uma filial da empresa Schneider Electric Brasil Ltda., inscrita no CNPJ sob o nº 82.743.287/0027-43, que deu prosseguimento às atividades da matriz, sem solução de continuidade, conforme consta da documentação juntada ao Processo acima referido, que foi devidamente registrada nos órgãos próprios, resolvem:

Art. 1º Fica transferida a titularidade da Portaria Interministerial MCT/MDIC/MF nº 446, de 16 de junho de 2009 e Portaria Interministerial MCT/MDIC/MF nº 801, de 13 de dezembro de 2001, do estabelecimento matriz da empresa Schneider Electric Brasil Ltda., CNPJ sob o nº 82.743.287/0001-04, para o estabelecimento filial, CNPJ sob o nº 82.743.287/0027-43.

Art. 2º Esta Portaria entra em vigor na data de sua publicação, ficando convalidados todos os atos praticados pelo estabelecimento filial da empresa Schneider Electric Brasil Ltda., CNPJ sob o nº 82.743.287/0027-43, em decorrência da transferência de titularidade, desde a data em que esta se operou.

MARCO ANTONIO RAUPP
Ministro de Estado da Ciência,Tecnologia e Inovação

FERNANDO DAMATA PIMENTEL
Ministro de Estado do Desenvolvimento, Indústria e Comércio Exterior

PORTARIA Nº 1.019, DE 2 DE OUTUBRO DE 2013

O MINISTRO DE ESTADO DA CIÊNCIA, TECNOLOGIA E INOVAÇÃO, no uso das atribuições que lhe confere o art. 2º do Decreto nº 98.830, de 15 de janeiro de 1990, resolve:

Art. 1º. Fica o Dr. RICARDO DIEGO TORRES, contraparte brasileira, na condição de representante do Programa de Pós Graduação em Engenharia Mecânica (PPGEM) da Pontifícia Universidade Católica do Paraná - PUC/PR, autorizado a coordenar, no âmbito do Processo CNPq nº 001717/2013-8, o projeto de pesquisa científica intitulado "Desenvolvimento de Revestimentos Protetivos Contra a Carbonetação de Superfícies Metálicas", a ser realizado nas dependências do PPGEM/PUC/PR, de interesse da pesquisadora estrangeira, natural do México, OLIMPIA SALAS MARTINEZ, vinculada ao Instituto Tecnológico de Estudos Superiores de Monterrey - ITESM (MEX), representado pelo Dr. PEDRO LUIS GRASA SOLER, natural do México, pelo prazo de um ano, contado a partir da publicação desta Portaria no Diário Oficial da União,

Parágrafo único. A presente autorização não compreende a realização de trabalhos de coleta em campo no território brasileiro.

Art. 2º. Esta Portaria entra em vigor na data de sua publicação

MARCO ANTONIO RAUPP

PORTARIA Nº 1.024, DE 2 DE OUTUBRO DE 2013

O MINISTRO DE ESTADO DA CIÊNCIA, TECNOLOGIA E INOVAÇÃO, no uso das atribuições que lhe confere o art. 87, parágrafo único, incisos II e IV, da Constituição Federal, e tendo em vista o disposto nos arts. 3º da Lei nº 8.248, de 23 de outubro de 1991, e 7º do Decreto nº 5.906, de 26 de setembro de 2006, resolve:

Art. 1º Reconhecer, conforme consta do processo MCTI nº 01200.003164/2013-28, de 23 de julho de 2013, que os produtos e respectivos modelos descritos abaixo, desenvolvidos pela empresa UPSAI Sistemas de Energia Ltda., inscrita no Cadastro Nacional da Pessoa Jurídica do Ministério da Fazenda - CNPJ/MF sob o nº 02.258.188/0001-06, atendem às condições de bens de informática e automação, desenvolvidos no País, nos termos e para os fins estabelecidos na Portaria MCT nº 950, de 12 de dezembro de 2006:

Produto 1: Equipamento de alimentação ininterrupta de energia microprocessado ("UPS" ou "No Break").

Modelos: Flash II; ProSaver II.

Produto 2: Regulador/estabilizador eletrônico de voltagem, baseado em técnica digital.

Modelos: Pró Micro IV; Pró Gel; EWA; RVE; ACR 1100; ACR 2200; ACR 3100 D; ACF 1300; ACF 1600; ACF 2300; ACF 3100.

Art. 2º Esta Portaria entra em vigor na data de sua publicação.

MARCO ANTONIO RAUPP

CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL

RESOLUÇÃO NORMATIVA Nº 14, DE 2 DE OUTUBRO DE 2013

Dispõe sobre a situação das instituições que não solicitaram seu credenciamento no CONCEA, as quais utilizam animais para fins científicos ou didáticos.

O PRESIDENTE DO CONSELHO NACIONAL DE CONTROLE DE EXPERIMENTAÇÃO ANIMAL - CONCEA, no uso das atribuições que lhe confere o art. 5º, inciso VII, e no art. 10, incisos III e I, da Lei nº 11.794, de 8 de outubro de 2008, e, tendo em vista o disposto nos incisos I e II do art. 41 do Decreto nº 6.899, 15 de julho de 2009, bem como no caput do art. 1º, no caput, no § 1º, no inciso VI e no § 2º do art. 8º da Lei nº 12.527, de 18 de novembro de 2011,

Considerando que compete ao CONCEA credenciar instituições para criação ou utilização de com finalidade de ensino ou pesquisa científica;

Considerando que qualquer instituição legalmente estabelecida no território nacional que crie ou utilize animais para ensino ou pesquisa científica deverá constituir uma CEUA para requerer seu credenciamento no CONCEA;

Considerando que a criação ou a utilização de animais para pesquisa e ensino ficam restritas, exclusivamente, às instituições credenciadas no CONCEA;

Considerando ter o CONCEA editado a Resolução Normativa nº 3, de 14 de dezembro de 2011, que instituiu o Credenciamento Institucional para Atividades com Animais em Ensino ou Pesquisa (CIAEP) e estabelece os critérios e procedimentos para requerimento, emissão, revisão, extensão, suspensão e cancelamento do credenciamento das instituições que criam, mantêm ou utilizam animais em ensino ou pesquisa científica;

Considerando caber ao CONCEA aplicar as sanções previstas nos arts. 17 e 18 da Lei nº 11.794, de 2008, reproduzidas nos arts. 49 e 50 do seu Decreto nº 6.899, de 2009, encontrando-se prevista, dentre elas, a criação ou utilização de animais em atividades de ensino e pesquisa científica sem estar credenciado no CONCEA ou em desacordo com as normas por ele expedidas;

Considerando ter o CONCEA deliberado no decorrer de sua 20ª Reunião Ordinária que a ausência de pleito de credenciamento, de conformidade com as disposições previstas na referida Resolução Normativa nº 3, de 2011, configura a ocorrência de uma infração de natureza grave, no uso da competência prevista o art. 50 do Decreto nº 6.899, de 2009, que faculta ao Colegiado graduar as sanções administrativas, segundo os critérios previstos nos incisos I a V do parágrafo único do mesmo art. 50, resolve:

Art. 1º. Ficam interditadas temporariamente as instituições que fazem uso de animais para fins científicos ou didáticos no País e que não solicitaram seu credenciamento no CONCEA, de conformidade com as disposições previstas na Resolução Normativa nº 3, de 2011, nos termos do art. 20 da Lei nº 11.794, de 2008, e de acordo com a letra "c" do inciso I e do parágrafo único do art. 49 c/c o art. 50 do Decreto nº 6.899, de 2009.

Parágrafo único. A listagem das instituições credenciadas no CONCEA, bem como daquelas que se encontram com processo de solicitação de credenciamento em andamento estão disponíveis no sítio eletrônico do CONCEA em http://concea.mct.gov.br.

Art. 2º. As instituições que criam ou utilizam animais para fins científicos ou didáticos e que quiserem se regularizar perante o CONCEA podem solicitar seu credenciamento, que ocorre em fluxo contínuo por meio do endereço eletrônico do Cadastro de Instituições de Uso Científico de Animais - CIUCA em http://ciuca.mct.gov.br/

Art. 3º. Esta Resolução entra em vigor na data de sua publicação.

MARCO ANTONIO RAUPP

COMISSÃO TÉCNICA NACIONAL DE BIOSSEGURANÇA

RESOLUÇÃO NORMATIVA Nº 10, DE 2 DE OUTUBRO DE 2013

Estabelece condições de isolamento para a Liberação Planejada no Meio Ambiente de laranja doce (Citrus Sinensis (L.) OSBECK) geneticamente modificada.

A Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições legais e regulamentares, e tendo em vista o disposto no inciso II do art. 14 da Lei n.º 11.105, de 24 de março de 2005, resolve:

Art. 1º. Na liberação planejada de citros geneticamente modificados no meio ambiente deverá ser observada a estratégia de competição de pólen, mediante a introdução de três tipos de bordaduras, compondo, no mínimo, seis linhas de plantas cítricas, observadas, ainda, as seguintes condições:

I - para áreas experimentais inseridas em plantios comerciais de citros:

a) dispor, ao redor da área que contenha laranja doce geneticamente modificada, uma bordadura composta por duas linhas de cultivo (filas de árvores) de um genótipo polinizador não geneticamente modificado, nos termos do Anexo I desta Resolução;

b) alocar uma segunda bordadura ao redor da bordadura apresentada na letra "a" deste item, composta por duas linhas de cultivo de um genótipo não modificado geneticamente receptor de pólen, autoincompatível e monoembrionário, nos termos do Anexo II; e

c) dispor a terceira bordadura ao redor das bordaduras anteriores, nos termos das letras "a" e "b" deste item, compostas por, no mínimo, duas linhas de cultivo de uma variedade de laranja doce (Citrus sinensis (L.) Osbeck), nos termos do Anexo III.

II - para áreas experimentais fora de plantios comerciais de citros, a bordadura citada na letra "c" do inciso I deste artigo deverá possuir, no mínimo, quatro linhas de cultivo de uma variedade de laranja doce (Citrus sinensis (L.) Osbeck), nos termos do Anexo III.

Art. 2º. Fica estabelecida a distância mínima de 3 km (três quilômetros) em relação às colmeias destinadas à apicultura comercial ou doméstica pré-existentes à época da instalação do experimento.

Parágrafo único. Após a instalação do experimento, os apicultores interessados em instalar colmeias comerciais deverão ser informados de que deverão respeitar a distância mínima de 1 km (um quilômetro) entre a área experimental e o apiário.

Art. 3º. Para a obtenção de porta-enxertos cítricos de viveiros comerciais, deverá ser observada a distância mínima de 1 km (um quilômetro) em relação às plantas cítricas fonte de sementes (sementeiras).

Art. 4º. Na instalação do experimento de que trata esta Resolução Normativa deverá ser respeitada a distância de, pelo menos, 100 (cem) metros de áreas de preservação natural.

Art. 5º. Deverá ser realizado monitoramento de um raio de 100 (cem) metros em torno da área experimental, a partir da última linha da bordadura, visando à eliminação de plantas cítricas espontâneas.

Art. 6º. Os preceitos estabelecidos nesta Resolução Normativa não se aplicam quando a planta cítrica for formada pelo porta-enxerto transgênico enxertado, com uma copa não transgênica.

Art. 7º. Esta Resolução Normativa entra em vigor na data de sua publicação.

FLÁVIO FINARDI FILHO

ANEXO I

Genótipo polinizador de citros. Citrandarins, híbridos de:
1.Tangerina Cleópatra (Citrus reshni hort. Ex Tanaka) X Poncirus trifoliata (L.) Raf.;
2.Tangerina Sunki (Citrus reshni hort. Ex Tanaka) X Poncirus trifoliata (L.) Raf.;
3.Tangerina Cleópatra (Citrus reshni hort. Ex Tanaka) X Citrange (Citrus sinensis L. Osb. X Poncirus trifoliata (L.) Raf.);
4.Mexerica tardia (Citrus deliciosa Ten.) X Citrange (Citrus sinensis L. Osb. X Poncirus trifoliata (L.) Raf.).

ANEXO II

Genótipos receptores de citros, monoembriônicos e autoincompatíveis
1. Tangerina Clementina (Citrus clementina hort. ex. Tau)
2. Tangerina Imperial (Citrus reticulata Blanco)
3. Tangerina Ellendale (Citrus reticulata Blanco X Citrus sinensis L. Osb.)
4. Pomelo Sukega (Citrus paradisi Macf.)
5. Toranja Siamesa (Citrus maxima (Burm.) Merril)

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ANEXO III

Variedades de laranja doce (Citrus sinensis L. Osb.)
1.Folha Murcha
2.Hamlin
3.Natal
4.Pera
5.Pineapple
6.Rubi
7.Seleta
8.Valência
9.Westin

EXTRATO DE PARECER TÉCNICO Nº 3.772/2013

O Presidente da Comissão Técnica Nacional de Biossegurança - CTNBio, no uso de suas atribuições e de acordo com o artigo 14, inciso XIX, da Lei 11.105/05 e do Art. 5º, inciso XIX do Decreto 5.591/05, torna público que na 165ª Reunião Ordinária, ocorrida em 19 de setembro de 2013, a CTNBio apreciou e emitiu parecer técnico para o seguinte processo:

Processo nº: 01200.001516/2013- 19
Requerente: Monsanto do Brasil Ltda.
CNPJ: 64.858.525/0001 45
Endereço: Avenida das Nações Unidas, 12901, Torre Norte 7º Andar, São Paulo SP.
Assunto: Liberação planejada no meio ambiente, importação de sementes e exportação.
Extrato Prévio: 3.557/2013
Decisão: Deferido

A CTNBio, após apreciação do pedido de liberação planejada no meio ambiente, importação e exportação de soja geneticamente modificada, concluiu pelo seu DEFERIMENTO, nos termos deste parecer técnico. A Monsanto do Brasil Ltda., detentora do Certificado de Qualidade - CQB - 003/96, solicita autorização para liberação planejada no meio ambiente, importação e exportação de soja MON 87427. Os experimentos serão realizados em Porto Nacional (TO) e ocuparão uma área total de 0,74 hectares e a área com OGM será de 0,4 hectares.

Fica autorizada a importação de 30,0 Kg de sementes de soja geneticamente modificada da Monsanto Company, EUA. O local de desembarque será Brasília - DF e a estação quarentenária, CENARGEN/EMBRAPA. O destino do material será a Estação Experimental da Monsanto do Brasil Ltda. localizada em Morrinhos - GO e Porto Nacional (TO); e a exportação de amostras de soja MON 87701 × MON 89788 × MON 87708 × A841661 que Serão coletadas e exportadas para os Centros de Pesquisa da Monsanto Company na Argentina, Paraguai, Uruguai, Canadá e Estados Unidos aproximadamente 200 amostras de grãos totalizando 50 kg de grãos.

No âmbito das competências do art. 14 da Lei 11.105/05, a CTNBio considerou que as medidas de biossegurança propostas atendem às normas e à legislação pertinente que visam garantir a biossegurança do meio ambiente, agricultura, saúde humana e animal.

A CTNBio esclarece que este extrato não exime a requerente do cumprimento das demais legislações vigentes no país, aplicáveis ao objeto do requerimento.

A íntegra deste Parecer Técnico consta do processo arquivado na CTNBio. Informações complementares ou solicitações de maiores informações sobre o processo acima listado deverão ser encaminhadas por escrito à Secretaria Executiva da CTNBio.

FLÁVIO FINARDI FILHO

ANNEX 2

Phenotype of fruits from transgenic sweet orange (cv. Pineapple) plants carrying RNAi-inductive constructions targeted to block the expression of key carotenogenic genes, resulting in the accumulation of (**A**) lycopene and (**B**) β-carotene. EV, control oranges that were transformed with the empty vector.