Tesis Doctoral

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Carotenoides y tocoferoles en frutos cítricos: implicación en la tolerancia a los daños por frío durante la conservación refrigerada

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TABLA DE CONTENIDO

La conservación a bajas temperaturas es una de las tecnologías más ampliamente utilizadas para preservar la calidad postcosecha de los frutos. Sin embargo, los frutos de ciertas especies y variedades de cítricos son sensibles a desarrollar daños por frío (DF) a temperaturas inferiores a 5 °C, que deprecian su calidad y comercialización. La sensibilidad al DF varía entre especies y variedades, y también está influenciada por distintos factores ambientales, prácticas de manejo, etc. Debido a que el desarrollo del DF está asociado a un incremento del estrés oxidativo, uno de los factores que puede influir en la diferente sensibilidad al DF entre especies/variedades es el contenido de compuestos antioxidantes en el flavedo (parte externa coloreada de la piel). Por ello, el objetivo principal de esta Tesis Doctoral fue profundizar en la implicación de moléculas con potente capacidad antioxidante, principalmente carotenoides y tocoferoles, en la sensibilidad al DF durante la conservación refrigerada de frutos cítricos. Para ello, en primer lugar, se estudió el papel de dos de los principales antioxidantes en los cítricos, los carotenoides y la vitamina C, y su relación con la capacidad antioxidante, en la diferente sensibilidad natural al DF de frutos de tres variedades de mandarina (Capítulo 1). En segundo lugar, se analizó el papel de los tocoferoles, como otro potente antioxidante liposoluble, en la diferente tolerancia natural al DF entre variedades de mandarina (Capítulo 2), y en la tolerancia inducida en frutos de pomelo mediante el tapado de frutos (Capítulo 3). Este análisis se complementó con el estudio del efecto de las bajas temperaturas y la ausencia de luz sobre la expresión de los genes de la ruta de biosíntesis de tocoferoles. Por último, se realizó por primera vez una caracterización bioquímica y molecular de la acumulación de tocoferoles durante la maduración de frutos de las principales especies cultivadas de *Citrus*: pomelo, limón, naranja y mandarina (Capítulo 4).

Los resultados de los capítulos 1 y 2 en frutos de mandarina con diferente tolerancia natural al DF, revelaron una relación positiva entre el contenido de carotenoides y tocoferoles en la cosecha y la tolerancia al DF durante la conservación a bajas temperaturas. A su vez, estos compuestos, especialmente los carotenoides, se correlacionaron positivamente con la capacidad antioxidante SOAC, lo que indica que su potencial papel protector frente al DF parece estar asociado a su eficiencia como secuestradores de oxígeno singlete. Por el contrario, se observó una relación inversa entre la tolerancia de los frutos de mandarina al DF y los contenidos de vitamina C en el flavedo. De forma similar, la actividad antioxidante total DPPH• y FRAP reflejó los contenidos de vitamina C y se correlacionó negativamente con la

tolerancia al DF. Estos resultados indican que el ácido ascórbico y la capacidad antioxidante total no parecen estar implicadas en la tolerancia natural al DF en estos frutos.

A diferencia de lo observado en frutos de mandarina, la tolerancia al DF inducida mediante el tapado de frutos de pomelo Star Ruby fue independiente de los niveles de tocoferoles en el momento de la cosecha o durante la conservación en frío. Sin embargo, entre los frutos de mandarina y pomelo, y también entre frutos sensibles y tolerantes, se detectaron cambios similares en la expresión de genes implicados en la síntesis de tocoferoles en respuesta a las bajas temperaturas, indicando una respuesta común a las bajas temperaturas de conservación entre especies, independiente de la tolerancia al DF. Además, en los frutos de pomelo se detectó que la ausencia de luz durante las últimas etapas del desarrollo de los frutos reduce la acumulación de γ-tocoferol, sin afectar el contenido de α-tocoferol, y este efecto parece estar asociado a la represión de los genes *GGDR*, *VTE1*, *VTE2*, *VTE3a* y *VTE4*.

Por último, el estudio de la acumulación de tocoferoles y su regulación durante la maduración en frutos de las principales especies cítricas reveló diferencias entre el flavedo y la pulpa, tanto durante la maduración como entre genotipos. Los contenidos de tocoferoles fueron superiores en el flavedo y, mientras que durante la maduración aumentaron en el flavedo, en la pulpa disminuyeron o se mantuvieron constantes. Además, parecen existir distintos mecanismos de regulación que controlan la acumulación de tocoferoles en ambos tejidos. Así, en el flavedo, el incremento en tocoferoles durante la maduración estuvo acompañado principalmente por una inducción de los genes *TAT1* y *VTE4*, que regulan la disponibilidad de HGA y la conversión de γ- a α-tocoferol, respectivamente; mientras que, en la pulpa, los contenidos reflejaron los cambios en la expresión de los genes *VTE6*, *DXS2* y *GGDR*, que regulan la disponibilidad de PPP. Entre los distintos genotipos analizados, los frutos maduros de limón acumularon las mayores concentraciones de tocoferoles tanto en el flavedo como en la pulpa, seguidos de los frutos de pomelo, naranja y mandarina.

ABSTRACT

Storage at low temperatures is one of the most widely used technologies to maintain fruit quality during postharvest. Nonetheless, fruit of certain citrus species and cultivars are prone to develop chilling injuries (CI) at temperatures below 5 °C, which reduces their quality and marketability. CI sensitivity varies among species and cultivars and is also influenced by different environmental factors, cultural practices, etc. Because CI development is associated to an increase in oxidative stress, it has been proposed that the different concentration of compounds with antioxidant capacity in the flavedo (external colored part of the peel) may influence the different sensitivity to CI among species/cultivars. Therefore, the main objective of this Doctoral Thesis was to study the role of different molecules with antioxidant capacity, mainly carotenoids and tocopherols, on CI sensitivity of citrus fruit during cold storage. To this end, the role of two major antioxidants in citrus, carotenoids and vitamin C, and their relation to the antioxidant capacity of the flavedo, on CI sensitivity was studied among mandarin fruit (Chapter 1). Secondly, the role of tocopherols, as another potent lipophilic antioxidant, on the different natural tolerance of mandarin fruit to CI (Chapter 2) and in the tolerance induced in grapefruit by fruit bagging was analyzed (Chapter 3). This analysis was further complemented with the evaluation of molecular changes in the expression of tocopherol biosynthetic genes in response to low temperatures and light deprivation. Lastly, a biochemical and molecular characterization of tocopherol accumulation during fruit ripening was initiated in fruit of the main cultivated *Citrus* species: grapefruit, lemon, orange and mandarin (Chapter 4).

Results in Chapter 1 and 2 in mandarin fruit with different natural tolerance to CI revealed a positive relationship between carotenoid and tocopherol content at harvest and CI tolerance during postharvest cold storage. Moreover, concentrations of these compounds, especially carotenoids, were positively correlated with SOAC antioxidant capacity, indicating that their protecting role against CI is associated with their efficiency as singlet oxygen quenchers. On the other hand, CI tolerance of mandarin fruit was negatively correlated with vitamin C contents in the flavedo. Similarly, total antioxidant activity (DPPH• and FRAP) reflected vitamin C contents and was negatively correlated with CI tolerance. These results indicate that vitamin C and the total antioxidant activity do not seem to be implicated in the natural tolerance to CI of mandarin fruit.

In contrast to what was observed in mandarin fruit, CI tolerance induced by on-tree fruit bagging of Star Ruby grapefruit was independent of tocopherol levels at harvest or during cold storage. Nonetheless, between mandarin and grapefruit, and also between tolerant and sensitive fruit, similar changes in the expression of genes involved in tocopherol synthesis were detected in response to low temperatures, which indicates a common molecular response to cold between species, independent of CI tolerance. Furthermore, light deprivation during the last stages of fruit development in grapefruit reduced the accumulation of γ-tocopherol, without altering α-tocopherol levels, and this effect was associated to a repression of genes *GGDR*, *VTE1*, *VTE2*, *VTE3a* y *VTE4* under darkness.

The analysis of tocopherol accumulation and its regulation during ripening of fruit of the main citrus species revealed differences between the flavedo and the pulp, and among maturation stages and genotypes. Tocopherol contents were higher in the flavedo and, while contents increased in the flavedo during ripening, contents in the pulp decreased or remained constant. Moreover, different regulation mechanisms seem to regulate tocopherol accumulation in both tissues. In the flavedo, the increase in tocopherols during maturation paralleled the induction of genes *TAT1* and *VTE4*, which regulate HGA availability and the conversion of γ- to α-tocopherol, respectively; whereas in the pulp, contents reflected changes in the expression of genes *VTE6*, *DXS2* and *GGDR*, which regulate PPP availability. Among the different genotypes analyzed, mature fruit of lemon accumulated the highest concentrations in both the flavedo and the pulp, followed by grapefruit, orange and mandarin fruit.

1-MCP	1-Metilciclopropeno
$^1O_2$	Oxígeno singlete
ABA	Ácido abscísico
ABTS	Ácido 2,2'-azinobis (3 etilbenzotiazolín)-6-sulfónico
APX	Ascorbato peroxidasa
AsA	Ácido ascórbico
β-CHX	β-Caroteno hidroxilasa
β-LCY	β-Licopeno ciclasa
CAT	Catalasa
CCDs	Dioxigenasas de corte de carotenoides
CPTA	Hidrocloruro de 2-(4-tioclorofenil) trietilamina
DF	Daño por frío
DMAPP	Dimetilalil difosfato
DMPBQ	2,3-dimetil-5-fitilbenzoquinol
DPPH	2,2-difenil-1-picrilhidrazilo
DXP	Dioxixilulosa-5-fosfato
DXR	Dioxixilulosa-5-fosfato reducto isomerasa
DXS	Dioxixilulosa-5-fosfato sintasa
ε-CHX	ε-Caroteno hidroxilasa
ε-LCY	ε-Licopeno ciclasa
FAD	Desaturasas de ácidos grasos
Fitil-P	Fitil fosfato
FRAP	Poder reductor antioxidante férrico ("*Ferric Reducing Antioxidant Power*")
γ-TMT	γ-Tocoferol metiltransferasa
G3P	Gliceraldehído-3-fosfato
GGDR	Geranilgeranil difosfato reductasa
GGPP	Geranilgeranil difosfato
GGPPS	Geranilgeranil difosfato sintasa
GPAT	Glicerol-3-fosfato aciltransferasa
GR	Glutatión reductasa
HDR	Hidroximetil-butenil difosfato reductasa
HGA	Ácido homogentísico o homogentisato
HMBPP	Hidroximetil-butenil difosfato
HPP	p-Hidroxifenilpiruvato
HPPD	p-Hidroxifenilpiruvato dioxigenasa
HPT	Homogentisato fitiltransferasa
IPP	Isopentenil difosfato
L-tyr	L-tirosina
MEP	Metileritritol fosfato
MPBQ	2-metil-6-fitil-1,4-benzoquinol

MPBQ-MT	2-metil-6-fitil-1,4-benzoquinol metiltransferasa
MVA	Ácido mevalónico
NSY	Neoxantina sintasa
ORAC	Capacidad de absorción de radicales de oxígeno ("*Oxygen Radical Absorbance Capacity*")
PAL	Fenilalanina amonio liasa
PDS	Fitoeno desaturasa
PPP	Fitil difosfato
PSY	Fitoeno sintasa
ROS	Especies reactivas de oxígeno
SK	Ácido siquímico
SOAC	Capacidad de secuentrar oxígeno singlete ("*Singlet Oxygen Absorption Capacity*")
SOD	Superóxido dismutasa
TATs	Tirosina aminotransferasas
TC	Tocoferol ciclasa
VDE	Violaxantina de-epoxidasa
VTE1	Tocoferol ciclasa
VTE2	Homogentisato fitiltransferasa
VTE3	2-Metil-6-fitil-1,4-benzoquinol metiltransferasa
VTE4	γ-Tocoferol metiltransferasa
VTE5	Fitol quinasa
VTE6	Fitil fosfato quinasa
ZDS	ζ-Caroteno desaturasa
ZEP	Zeaxantina epoxidasa
ZISO	ζ-Caroteno isomerasa

1.1. Daño por frío durante la conservación postcosecha de frutos cítricos

1.1.1. Generalidades de la conservación postcosecha de frutos cítricos

Los frutos cítricos son uno de los principales cultivos frutales del mundo, con una producción anual alrededor de 157 millones de toneladas en el año 2019 (FAOSTAT, 2020). Dentro de los países productores de cítricos, España ocupa el sexto lugar en el ranking mundial, con una producción de aproximadamente 6 millones de toneladas en el año 2019 (FAOSTAT, 2020). A diferencia de los mayores productores mundiales, como Estados Unidos o Brasil, en los que la mayor parte de la producción se destina al procesado industrial de zumo, el destino principal de la producción española es para consumo de frutos en fresco. Además, en el mercado de frutos en fresco, la mayor proporción (60-70%) se destina a la exportación a terceros países, lo que convierte a España en el primer exportador mundial de frutos cítricos en fresco (FAOSTAT, 2020). Esta situación hace que en la citricultura española se impongan elevados niveles de exigencia en cuanto a la calidad interna y externa de los frutos, y que sean especialmente críticas todas las etapas de la cadena postcosecha, que abarcan desde la recolección, la manipulación y tratamiento en los almacenes, hasta el transporte y la conservación, a fin de evitar pérdidas, asegurar la calidad, cumplir los requerimientos de los mercados y suministrar frutos no solo con una adecuada presentación comercial, sino también con alta calidad organoléptica y nutricional.

A lo largo de la cadena productiva existen diversas causas que pueden deteriorar la calidad de los frutos generando importantes pérdidas en su comercialización (Porat, Lichter, Terry, Harker, & Buzby, 2018). Las alteraciones de la calidad causadas por factores abióticos se conocen como desórdenes fisiológicos y pueden darse tanto durante la precosecha, causadas por factores climáticos o del cultivo como deficiencias nutricionales, hídricas etc., como durante el manejo postcosecha de los frutos (Lado, Cronje, Rodrigo, & Zacarías, 2019). Los desórdenes fisiológicos más comunes que ocurren durante la postcosecha de frutos cítricos pueden clasificarse en dos grupos: (i) aquellos relacionados con el daño por frío (DF), que ocurren por la exposición a muy bajas temperaturas de conservación (< 5 °C), y (ii) aquellos que se producen a temperatura ambiente o por encima de 10 °C (Lado, Cronje, et al., 2019; Zacarias, Cronje, & Palou, 2020).

El almacenamiento a bajas temperaturas (1-5 °C) es una de las tecnologías postcosecha más utilizadas para preservar la calidad y extender la vida postcosecha de los frutos cítricos durante su transporte y comercialización, ya que ralentiza el metabolismo celular retrasando la maduración y senescencia de los frutos (Lado, Cronje, et al., 2019; Zacarias et al., 2020).

Además, para la exportación de frutos a ciertos países como Estados Unidos, Japón o China, se exigen tratamientos cuarentenarios a temperaturas próximas a la congelación (1-2 °C) como medidas preventivas para la eliminación de plagas, como es el caso de la mosca del Mediterráneo *Ceratitis capitata* (Lado, Cronje, et al., 2019). Estas condiciones de transporte y conservación a bajas temperaturas pueden prolongarse por períodos superiores a dos semanas, y suponen un reto para frutos de especies de origen tropical o subtropical, como los cítricos, que son sensibles a las bajas temperaturas y pueden desarrollar lesiones de diferente sintomatología durante la exposición prolongada a estas condiciones (Lafuente & Zacarías, 2006; Zacarias et al., 2020). El conjunto de estas lesiones es conocido como "daño por frío" (DF), y es un desorden de gran importancia en postcosecha, ya que disminuye la calidad externa de los frutos y, por consiguiente, su comercialización, además de predisponer al fruto a la infección de patógenos.

1.1.2. Sintomatología de los daños por frío en los frutos cítricos

La sintomatología de los DF en los frutos cítricos es diversa, pero uno de los síntomas macroscópicos más comunes es el picado de la piel (*"peel pitting"* en inglés). Este síntoma se caracteriza por la aparición de depresiones pequeñas en el flavedo (parte externa coloreada de la piel) de color marrón, que con la exposición al frío se van haciendo más oscuras y marcadas, y se extienden hasta cubrir gran parte de la superficie del fruto (Figura 1) (Lado, Cronje, et al., 2019; Lafuente & Zacarías, 2006). Mientras que los síntomas de picado en la piel son típicos en frutos de mandarina y pomelo (Figura 1A y B), en frutos de naranja los DF se suelen manifestar como un manchado superficial llamado "bronceado" (*"scalding"* en inglés) (Figura 1C) y en limones aparecen como hendiduras individuales distribuidos por la superficie, que se conoce como "peteca" (Figura 1D).

A pesar de las diferencias en la sintomatología del DF entre las especies de cítricos, a nivel microscópico los cambios estructurales que provocan los DF son similares entre ellas (Kratsch & Wise, 2000). Estos cambios incluyen, en general, el colapso de las células de la epidermis de los frutos, que presentan menor tamaño, forma irregular, organelos más pequeños y mal definidos, y las vacuolas llenas de un material denso y oscuro, mientras que las células de frutos o zonas del fruto sin daño permanecen intactas, con sus estructuras y organelos bien definidos (Lado, Rodrigo, Cronje, & Zacarías, 2015). Además, en las células del tejido dañado se observan espacios entre la pared y membrana celular, y las paredes son más finas (Lado, Rodrigo, Cronje, et al., 2015).

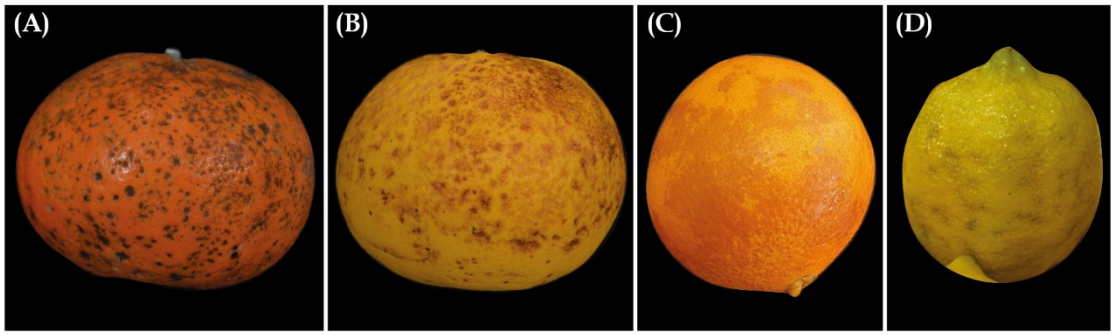

Figura 1. Fotografías mostrando los síntomas característicos de daño por frío en frutos de mandarina (A), pomelo (B), naranja (C), limón (D) conservados durante diferentes períodos de tiempo a 2 °C (Fotos tomadas de experimentos realizados por el grupo de postcosecha del IATA).

1.1.3. Respuestas fisiológicas, bioquímicas y moleculares al daño por frío en los frutos cítricos

A nivel molecular, una de las primeras hipótesis que se propusieron para explicar la respuesta primaria de los tejidos vegetales a la exposición a bajas temperaturas fue la pérdida de permeabilidad de las membranas celulares debido a la alteración en la organización y composición de los lípidos que las conforman (Sevillano, Sanchez-Ballesta, Romojaro, & Flores, 2009). Así, se establece que el frío puede provocar una serie de cambios en la composición de lípidos que disminuyen la fluidez de la membrana y, finalmente, su funcionalidad (Lyons, 1973). Estas modificaciones incluyen procesos de peroxidación de lípidos, aumento de la saturación de los ácidos grasos y la degradación de fosfolípidos y galactolípidos, con un aumento de la proporción esteroles/fosfolípidos. Entre ellos, el grado de saturación de los lípidos ha sido un parámetro tradicionalmente asociado a la funcionalidad de las membranas y a la capacidad de los organismos de tolerar el frío (Sevillano et al., 2009). Se ha sugerido que uno de los mecanismos de adaptación de los organismos a las bajas temperaturas es mediante el incremento de la proporción de ácidos grasos insaturados, lo que implicaría una activación de enzimas desaturasas de ácidos grasos (FAD, por sus siglas en inglés "*fatty acids desaturases*") e isoformas de glicerol-3-fosfato aciltransferasa (GPAT, por sus siglas en inglés "*glycerol-3-phosphate acyltransferase*") (Sevillano et al., 2009). En este sentido, estudios transcriptómicos en frutos de pomelo Star Ruby acondicionados a 16 °C previamente a la conservación en frío (2 °C), observaron que la reducción del DF inducida por el acondicionamiento inducía una serie de respuestas que incluían la activación de enzimas relacionadas a la saturación de lípidos de membranas como las FAD (Sapitnitskaya et al., 2006). Sin embargo, estudios similares en frutos de mandarina Fortuna, una variedad muy sensible al DF, detectaron que el frío favorecía procesos de degradación de lípidos en el flavedo y que el acondicionamiento a altas temperaturas (37 °C), que evita los DF, reprimía un conjunto

de genes involucrados en la degradación de lípidos (ej. fosfolipasas), sin tener un efecto marcado sobre genes relacionados con la instauración de ácidos grasos (Lafuente, Establés-Ortíz, & González-Candelas, 2017). De forma similar, en frutos de pomelo Marsh, también muy sensibles al DF, se ha observado que las bajas temperaturas alteran la expresión de genes relacionados con la síntesis de ácidos grasos y fosfolípidos, y enzimas de degradación de lípidos (Maul, McCollum, Popp, Guy, & Porat, 2008). A pesar de estos avances, el efecto del frío sobre el metabolismo de lípidos a nivel molecular requiere ser estudiado con mayor profundidad en distintas variedades de cítricos con diferente susceptibilidad al DF, ya que existen diferencias entre variedades en la respuesta a los distintos mecanismos para conferir resistencia al frío.

A su vez, como respuesta secundaria a la exposición a bajas temperaturas y al DF, se generan condiciones de estrés oxidativo debido a la acumulación de especies reactivas de oxígeno (ROS), como el oxígeno singlete ($^1O_2$), peróxido de hidrógeno, radicales libres de hidroxilo y anión superóxido, que al tener una elevada reactividad oxidan los componentes celulares, generando alteraciones en el metabolismo y daños estructurales (Decros et al., 2019; Toivonen, 2004). Normalmente las ROS se mantienen a niveles bajos en las células vegetales, pero distintas condiciones de estrés inducen su producción y cuando las células son incapaces de contrarrestarlas o eliminarlas se acumulan y provocan daños oxidativos (Decros et al., 2019). Para protegerse frente a la propagación de las ROS, las plantas cuentan con un complejo sistema antioxidante que incluye tanto enzimas con actividad antioxidante, como la superóxido dismutasa (SOD), ascorbato peroxidasa (APX), catalasa (CAT) y glutatión reductasa (GR), como compuestos con capacidad antioxidante como el ácido ascórbico, glutatión, tocoferoles, carotenoides y polifenoles, entre otros (Decros et al., 2019; Huang et al., 2019). En los frutos cítricos, el desarrollo de DF se ha asociado a un incremento del estrés oxidativo, evidenciado por un mayor nivel de peroxidación lipídica (contenido de malondialdehído) (Lado, Rodrigo, López-Climent, Gómez-Cadenas, & Zacarías, 2016) y daños estructurales de los componentes celulares (Lado, Rodrigo, Cronje, et al., 2015). A su vez, en frutos de mandarina se ha observado que el estrés oxidativo causado por los DF parece estar relacionado con la actividad de las enzimas responsables de eliminar ROS, y que las variedades con mayor tolerancia natural al DF presentan un sistema enzimático antioxidante más eficiente que las sensibles (Sala, 1998). La enzima CAT, específicamente, parece desempeñar un papel importante en la tolerancia al DF, ya que se ha detectado una mayor actividad de esta enzima en frutos tolerantes al DF (Lado, Rodrigo, et al., 2016; Lo'ay & Dawood, 2019; Sala, 1998). A su vez, tratamientos de acondicionamiento térmico que reducen

la sensibilidad al DF en mandarina Fortuna y naranja Navel inducen la actividad CAT (Bassal & El-Hamahmy, 2011; Sala & Lafuente, 2000), mientras que tratamientos con inhibidores de esta actividad enzimática eliminan el efecto protector de los tratamientos de acondicionamiento y favorecen el desarrollo de DF (Sala & Lafuente, 2000). Por tanto, estos resultados indican que la tolerancia natural de ciertas variedades o la inducida por tratamientos de acondicionamiento podría estar relacionada en parte con la capacidad del fruto de reducir ROS a través del sistema enzimático antioxidante. El papel de otros compuestos con capacidad antioxidante, como los carotenoides y tocoferoles, en la susceptibilidad al DF de frutos cítricos, y que han sido objeto de estudio en esta Tesis Doctoral, se discute más adelante.

Por otro lado, una respuesta común de los frutos cítricos durante el almacenamiento a bajas temperaturas es el aumento en la producción de etileno (McCollum, 1991). El etileno es una hormona vegetal involucrada en un amplio número de procesos en las plantas, y está estrechamente asociada a procesos de maduración y senescencia de frutos, así como en las respuestas a situaciones de estrés (Alós, Rodrigo, & Zacarias, 2018). En frutos cítricos sensibles al DF, como los de pomelo o de mandarina Fortuna, se ha observado un aumento moderado en la producción de etileno durante la conservación en frío coincidiendo con la aparición de síntomas de DF (Ghasemnezhad, Marsh, Shilton, Babalar, & Woolf, 2008; Lado, Rodrigo, & Zacarías, 2015; Lafuente, Zacarias, Martínez-Téllez, Sanchez-Ballesta, & Granell, 2003; Martínez-Téllez & Lafuente, 1997). Esta mayor producción de etileno está asociada a la inducción en la expresión de los genes involucrados en su síntesis, que ocurre en paralelo al desarrollo de DF (Lado, Rodrigo, Cronje, et al., 2015; Lado, Rodrigo, & Zacarías, 2015; Maul, McCollum, Guy, & Porat, 2011; Maul et al., 2008). Estos estudios indicarían que la estimulación de la síntesis de etileno durante la conservación en frío parece ser una respuesta al desarrollo de DF y no al frío en sí. Sin embargo, el papel del etileno en la respuesta de los frutos cítricos al estrés por bajas temperaturas no está claramente elucidado, ya que en algunas especies parece tener un papel protector mientras que en otras parece promover el desarrollo de DF (Lafuente, Martínez-Téllez, & Zacarías, 1997; Lafuente & Zacarías, 2006; Sevillano et al., 2009). En una misma variedad el efecto del etileno puede ser distinto y variar de acuerdo a la concentración o momento de aplicación. En este sentido, aplicaciones exógenas de etileno previo a la conservación refrigerada en frutos sensibles de mandarina Fortuna incrementaron el DF (Lafuente et al., 1997), mientras que aplicaciones continuas a bajas concentraciones durante el almacenamiento en frío redujeron la incidencia de los daños (Lafuente, Sala, & Zacarias, 2004). De manera similar, la aplicación de un inhibidor de la acción de etileno (1-

MCP, 1-metilciclopropeno), en frutos de Fortuna aceleró el desarrollo de DF durante su conservación (Lafuente, Zacarias, Martínez-Téllez, Sanchez-Ballesta, & Dupille, 2001), apoyando la hipótesis de que los frutos cítricos requieren niveles bajos de etileno durante la postcosecha para mantener la calidad de la fruta y estimular las respuestas de defensa frente a las bajas temperaturas (Porat et al., 1999). No obstante, la respuesta al tratamiento con 1-MCP no parece ser igual en las diferentes especies y variedades de cítricos (Lado, Rodrigo, & Zacarías, 2015), y tiende a ser muy dependiente de la variedad y también de las concentraciones utilizadas (Dou, Jones, & Ritenour, 2005). Es interesante destacar que el reacondicionamiento de los frutos a temperatura ambiente después de la conservación refrigerada (o simulación de vida comercial) provoca un aumento masivo de la producción de etileno, asociado a un incremento en la expresión de genes de su biosíntesis durante la conservación, que a temperaturas óptimas aumentan rápidamente su actividad enzimática incrementando la producción de etileno (Lado, Rodrigo, & Zacarías, 2015).

Por último, la alteración del metabolismo de fenilpropanoides es también una respuesta importante de los cítricos a los daños DF. En este sentido, la actividad de la enzima fenilalanina amonio liasa (PAL) es clave en la respuesta al estrés por frío en los frutos cítricos. La expresión del gen PAL y su actividad enzimática se induce durante la conservación en frío, tanto en frutos sensibles como resistentes, pero más marcadamente y en paralelo al desarrollo del DF en frutos sensibles (Lafuente et al., 2001, 2003; Sanchez-Ballesta, Zacarias, Granell, & Lafuente, 2000). Además, se ha detectado una mayor actividad de esta enzima en frutos más tolerantes al DF, sugiriendo un posible papel de esta enzima en la tolerancia al DF (Lafuente et al., 2001; Sanchez-Ballesta et al., 2000).

1.1.4. Factores que afectan la susceptibilidad al daño por frío

Uno de los principales problemas asociados a los DF es que no se conoce en profundidad los factores implicados en la susceptibilidad o tolerancia a los DF. Parece evidente que, en primer lugar, existe un componente o factor genético que determina la susceptibilidad al DF entre los frutos de las diferentes especies y variedades de cítricos. En este sentido, los limones y pomelos están descritos como las especies con mayor susceptibilidad al DF, seguidas por los híbridos, mandarinas, y por último las naranjas (Zacarias et al., 2020). A su vez, dentro de una misma especie, la susceptibilidad al DF difiere según la variedad. Así, los frutos de pomelo Marsh son más sensibles al DF que los frutos de Star Ruby o Red Ruby (Dou et al., 2005; Lado, Rodrigo, Cronje, et al., 2015), mientras que los híbridos de mandarina Fortuna, Nova y Murcott presentan una mayor susceptibilidad al DF que la mandarina Clementina o su mutación

Clementina de Nules (Arras & Usai, 1991; Sala, 1998). La susceptibilidad a las bajas temperaturas de otras variedades de comercialización más recientes, como Nadorcott, no ha sido ampliamente evaluada, pero parece ser una variedad resistente al DF, aunque se han descrito diferencias entre los distintos países de cultivo (Cronjé, 2013; Zacarias et al., 2020).

A su vez, otros factores ambientales o de manejo, como el momento de recolección, el patrón utilizado o las condiciones ambientales previas a la cosecha también influyen en la sensibilidad del fruto al DF. El momento de recolección y, por lo tanto, el estado de madurez del fruto, es un factor que influye en la tolerancia de los frutos al DF, pero cuyo efecto es variable según la especie y variedad. En este sentido, los frutos de pomelo recolectados al principio y final de la temporada son más susceptibles al DF (Dou, 2005; Schirra et al., 2000), mientras que frutos de mandarina Fortuna lo son a mediados de la estación (Lafuente et al., 1997, 2003). Dentro de los factores ambientales que pueden estar influyendo en la sensibilidad al DF, la temperatura y la luz son los más relevantes. En frutos de mandarina se ha observado una relación entre las temperaturas en el campo previas a la cosecha y la posterior susceptibilidad al DF durante la postcosecha, siendo más susceptibles aquellos frutos recolectados durante los meses más fríos, como enero y febrero en el hemisferio norte (Gonzalez-Aguilar, Zacarias, Perez-Amador, Carbonell, & Lafuente, 2000). En cuanto a la luz, su posible efecto sobre la susceptibilidad al DF parece estar relacionado con su influencia en la pigmentación y la síntesis de otros compuestos en el flavedo de los frutos. Tradicionalmente se ha descrito que los frutos del interior del árbol, con menor exposición a la luz solar, presentan menor coloración y tonalidades más pálidas, y son más susceptibles a desarrollar alteraciones fisiológicas en el flavedo durante la conservación en comparación con los provenientes del exterior, con una mayor incidencia de luz y coloración del flavedo más intensa (Cronje, Barry, & Huysamer, 2011, 2013). Sin embargo, en frutos de pomelo Star Ruby la ausencia de luz durante el desarrollo resulta en frutos con zonas de coloración roja, debido a la acumulación del carotenoide licopeno, y mayor tolerancia al DF durante el almacenamiento a bajas temperaturas (Lado, Cronje, Alquézar, Page, et al., 2015). A su vez, la luz tiene un efecto sobre la acumulación de otros metabolitos, como el ácido ascórbico o carbohidratos, que también pueden tener una función en la sensibilidad de los frutos a desórdenes durante la postcosecha (Cronje et al., 2013; Lado, Alós, Rodrigo, & Zacarías, 2015; Lado, Rodrigo, et al., 2016).

1.2. Carotenoides

1.2.1. Características generales de los carotenoides

Los carotenoides son compuestos isoprenoides liposolubles sintetizados principalmente por organismos fotosintéticos, como plantas, algas y cianobacterias, y son los principales pigmentos responsables de las coloraciones amarillas, naranjas y rojizas de los frutos de numerosas especies vegetales, incluidos los frutos cítricos (Figura 2) (Lado, Zacarías, & Rodrigo, 2016; Rodrigo, Alquézar, Alós, Lado, & Zacarías, 2013; Rodríguez-Concepción et al., 2018). A pesar de que algunos artrópodos y otros organismos no fotosintéticos (bacterias, hongos, arqueas) también son capaces de sintetizarlos, la mayoría de los animales no los sintetizan y deben ingerirlos a través de la dieta, teniendo una gran relevancia en la nutrición y salud humana por ser algunos de ellos los precursores de la vitamina A y esenciales para numerosas funciones como la visión, respuesta inmune y reproducción, entre otras (Rodríguez-Concepción et al., 2018). Además, como antioxidantes su ingesta regular se ha asociado con una reducción del riesgo de padecer determinadas enfermedades degenerativas o tipos de cáncer. Por otro lado, algunos carotenoides forman parte del pigmento macular protegiendo frente a la degeneración macular (Meléndez-Martínez, 2019). .

La estructura básica de los carotenoides es una cadena poliénica de 40 átomos de carbono (C40), formada a partir de la condensación de 8 unidades de isopreno (C5), con doble enlaces conjugados. Aunque los carotenoides más abundantes en la naturaleza son C40, también existen estructuras más cortas, denominados apocarotenoides (C30), o más largas (C45, C50). El sistema de doble enlaces conjugados conforma el cromóforo, que es la estructura que permite absorber luz en el espectro visible y brindar las coloraciones del amarillo al rojo típicas de estos pigmentos. El número de doble enlaces conjugados varia de 3-11, según el carotenoide y determina, además de su color, otras propiedades químicas y físicas, como su capacidad antioxidante (Britton, 1995). Además de diferenciarse según el número de carbonos y doble enlaces, los carotenoides pueden clasificarse según otras características como, por ejemplo, la ciclación de los extremos de su estructura, que determina que los carotenoides sean cíclicos o acíclicos (lineales) (Figura 2). A su vez, según su composición los carotenoides pueden clasificarse en dos grandes grupos: los carotenos, que son aquellos formados exclusivamente por átomos de carbono e hidrógeno; y las xantofilas, que son aquellos que contienen moléculas de oxígeno en su estructura (Figura 2) (Rodríguez-Concepción et al., 2018). Por último, debido a la presencia de dobles enlaces, algunos carotenoides también pueden hallarse en distintas conformaciones dando lugar a diferentes isómeros geométricos *cis (Z)* y *trans (E)* (Figura 2).

En general, la mayoría de los carotenoides se encuentran en su conformación *trans*, aunque existen excepciones en los que los isómeros *cis* son los más abundantes de forma natural, como es el caso del 15-Z-fitoeno (Meléndez-Martínez, Paulino, Stinco, Mapelli-Brahm, & Wang, 2014).

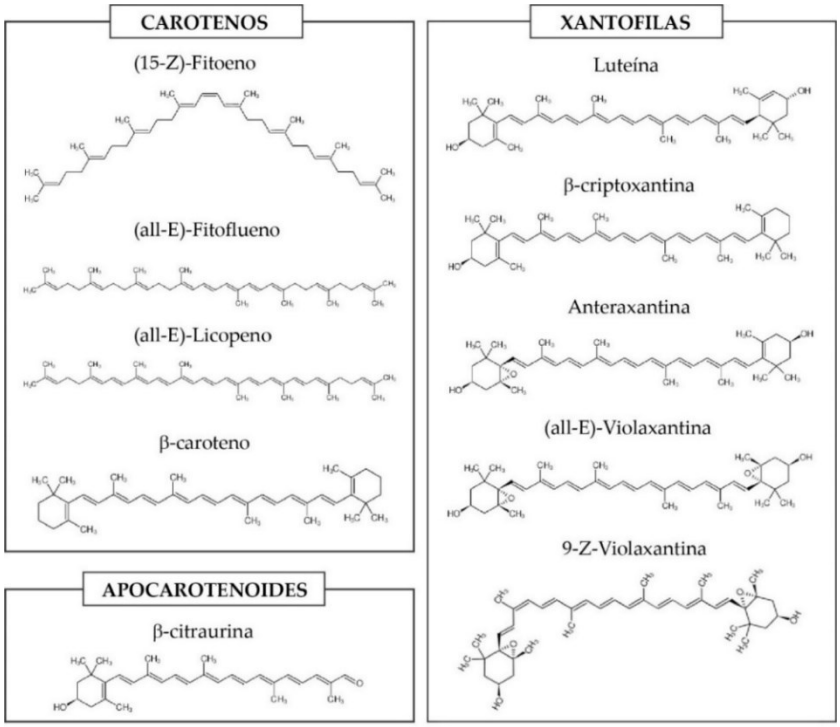

Figura 2. Estructura química de los principales carotenos, xantofilas y apocarotenoides presentes en los frutos cítricos.

1.2.2. Biosíntesis de carotenoides y su regulación

La biosíntesis de carotenoides (Figura 3) ocurre en plastidios, principalmente en cromoplastos y cloroplastos, y su regulación ocurre a diferentes niveles, siendo la regulación transcripcional de las enzimas de la ruta uno de los mecanismos más relevantes y ampliamente estudiados (Hermanns, Zhou, Xu, Tadmor, & Li, 2020; Nisar, Li, Lu, Khin, & Pogson, 2015; Rodríguez-Concepción et al., 2018; Ruiz-Sola & Rodríguez-Concepción, 2012; Torres-Montilla & Rodriguez-Concepcion, 2021). El primer paso específico de la ruta de biosíntesis de carotenoides es la condensación de dos moléculas de geranilgeranil difosfato (GGPP), mediante la enzima fitoeno sintasa (PSY), para formar fitoeno (caroteno incoloro). Posteriormente el fitoeno es desaturado por la enzima fitoeno desaturasa (PDS), dando lugar al fitoflueno (caroteno incoloro), que es transformado en ζ-caroteno (caroteno amarillo pálido) por esta misma enzima. Mediante la acción de ζ-caroteno isomerasa (ZISO) y ζ-caroteno desaturasa (ZDS) el ζ-caroteno forma licopeno (caroteno rojo), que es el último carotenoide lineal de la ruta (Rodríguez-Concepción et al., 2018). A continuación, la ruta se divide en dos

ramas de acuerdo a las reacciones de ciclación que sufre el licopeno. Mediante la acción de la enzima β-licopeno ciclasa (β-LCY), se añaden anillos β-ionona en los extremos de la molécula de licopeno formando el β-caroteno (caroteno naranja), mientras que la acción conjunta de β-LCY y ε-licopeno ciclasa (ε-LCY) forman α-caroteno (caroteno naranja). Hidroxilaciones secuenciales del α-caroteno por la β- y ε-caroteno hidroxilasa (β-CHX y ε-CHX) dan lugar a la formación de luteína (xantofila amarilla), que tiene lugar principalmente en tejidos cloroplásticos. La hidroxilación de β-caroteno por la β-CHX forma β-criptoxantina (xantofila naranja), que posteriormente sufre una segunda hidroxilación mediante la misma enzima a zeaxantina (xantofila amarilla). La zeaxantina epoxidasa (ZEP) es la enzima responsable de la conversión de zeaxantina en violaxantina (xantofila amarilla) que en ciertas condiciones puede volver a convertirse en zeaxantina mediante una reacción reversible catalizada por la violaxantina de-epoxidasa (VDE). El último paso es la conversión de violaxantina a neoxantina por la neoxantina sintasa (NSY) (Nisar et al., 2015; Rodríguez-Concepción et al., 2018).

De los pasos previamente mencionados, la formación de fitoeno por la enzima PSY es uno de los pasos claves y limitantes de la síntesis de carotenoides en la mayoría de situaciones y tejidos, ya que controla el flujo de entrada a la ruta (Fraser et al., 2007; Rodríguez-Concepción et al., 2018; Welsch, Beyer, Hugueney, Kleinig, & Von Lintig, 2000). La importancia de esta enzima en la acumulación de carotenoides ha sido también corroborada en los frutos cítricos (Ikoma et al., 2001; Rodrigo, Marcos, & Zacarías, 2004; Tao et al., 2007). Por otro lado, la ciclación del licopeno también es un punto clave en la ruta, ya que controla la ramificación hacia la síntesis de β,ε-xantofilas o β,β-xantofilas (Figura 3), y actúa como un cuello de botella favoreciendo la acumulación de licopeno en frutos como tomate o variedades de cítricos que acumulan este caroteno (Alquézar et al., 2013; Lado, Cronje, Alquézar, Page, et al., 2015; Liu et al., 2015).

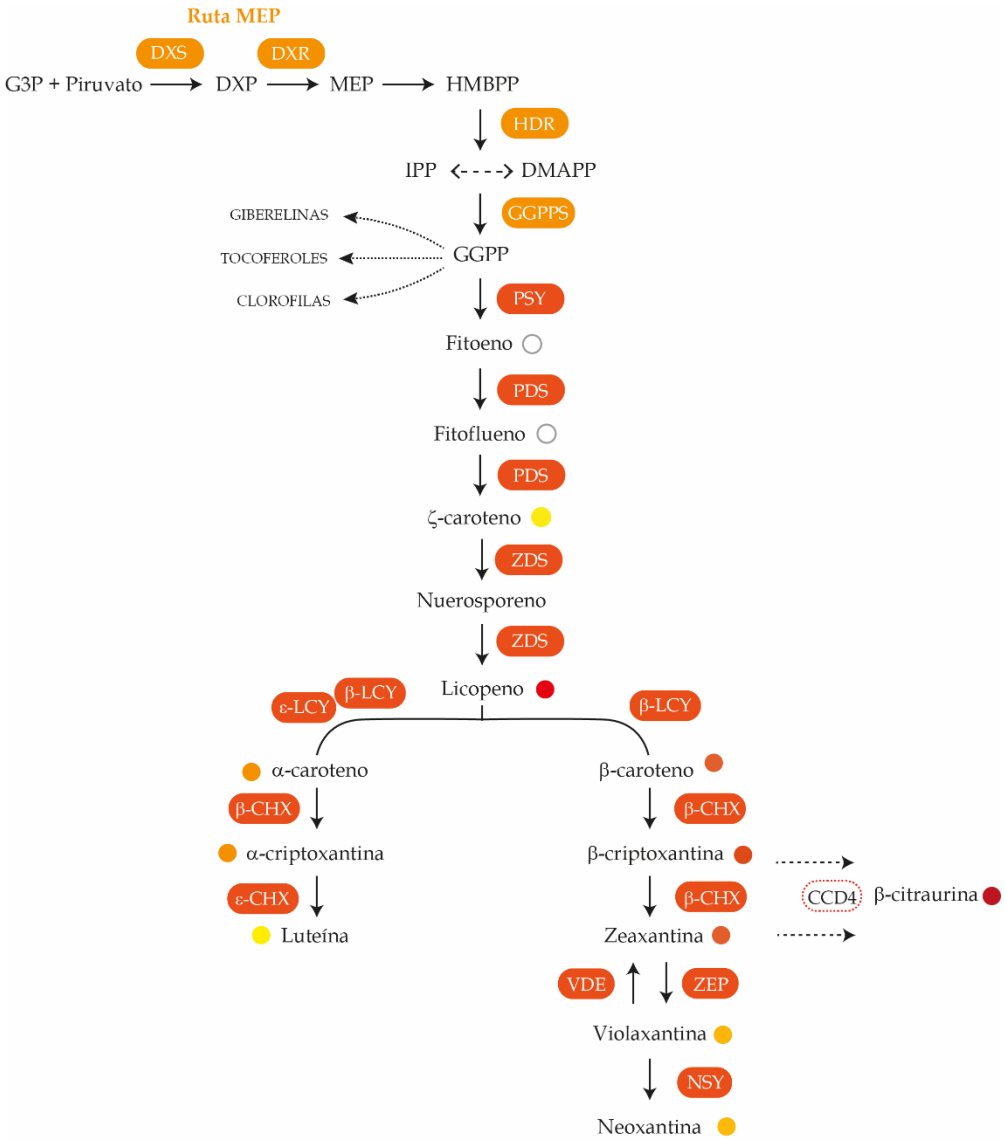

Figura 3. Esquema de la ruta de biosíntesis de los carotenoides en los frutos cítricos. Abreviaturas de los substratos: G3P, Gliceraldehído 3-fosfato; DXP, dioxixilulosa-5-fosfato; MEP, metil eritritol fosfato; HMBPP, hidroximetil-butenil difosfato; IPP, isopentenil difosfato; DMAPP, dimetilalil difosfato; GGPP, geranilgeranil difosfato. Abreviaturas de las enzimas: DXS, DXP sintasa; DXR, DXP reducto isomerasa; HDR, HMBPP reductasa; GGPPS, GGPP sintasa; PSY, fitoeno sintasa; PDS, fitoeno desaturasa; ZDS, ζ-caroteno desaturasa; β-LCY, β-licopeno ciclasa; ε-LYC, ε-licopeno ciclasa; β-CHX, β-caroteno hidroxilasa; ε-CHX, ε-caroteno hidroxilasa; ZEP, zeaxantina epoxidasa; VDE, violaxantina de-epoxidasa; NSY, neoxantina sintasa; CCD4, dioxigenasa de corte de carotenoides. Los círculos al lado de los carotenoides representan el color de cada uno de ellos.

En los frutos cítricos, la acumulación de carotenoides está altamente regulada por la expresión de los genes mencionados, y cambios en la expresión de los mismos durante la maduración pueden explicar parcialmente los cambios en los contenidos y perfil de carotenoides que ocurren durante el cambio de color de los frutos (Ikoma, Matsumoto, & Kato, 2016; Kato, 2012; Lado, Cronje, Alquézar, et al., 2015; Rodrigo, Alquézar, Alós, Lado, et al., 2013; Zhang et al., 2012). En este sentido, en frutos cítricos inmaduros de color verde la

composición de carotenoides es típica de tejidos cloroplásticos, donde predomina la acumulación de luteína, α-caroteno, β-caroteno y all-*E*-violaxantina, asociado a la expresión de ε-*LCY* y baja o moderada expresión relativa de *PSY*, *ZDS* y *β-LCYs* (Alquézar, Rodrigo, & Zacarías, 2008b; Kato et al., 2004; Lado, Alós, et al., 2019; Rodrigo et al., 2004). La expresión de ε-*LCY* indicaría el predominio del flujo metabólico hacia la ruta β,ε y, por tanto, la acumulación de β,ε-carotenoides, como α-caroteno y, principalmente, luteína. Durante el cambio de color, estos carotenoides disminuyen y se incrementa la acumulación de β,β-xantofilas, entre las que predominan la *9-Z*-violaxantina y la β–criptoxantina (Kato et al., 2004; Lado, Alós, et al., 2019; Matsumoto et al., 2007; Rodrigo et al., 2004). Está acumulación masiva de β,β-xantofilas durante el cambio de color está asociada a un aumento en la expresión de los genes *PSY*, *PDS*, *ZDS*, *β-LCY* y *CHX*, y una disminución de la expresión de ε-*LCY*, lo que favorece el cambio hacia la rama β,β de la ruta (Kato et al., 2004; Lado, Alós, et al., 2019; Rodrigo et al., 2004). La expresión relativa de diferentes isoenzimas *β-LCYs* es clave en el aumento masivo de β,β-xantofilas durante la maduración y, por ejemplo, en ciertas variedades de pomelo, naranja y limón su inhibición durante la maduración, en combinación con otros genes de la ruta, conducen a un bloqueo parcial de la síntesis de β,β-xantofilas y a la acumulación de licopeno en la pulpa y/o flavedo (Alquézar et al., 2008b; Lado, Cronje, Alquézar, Page, et al., 2015; Lana et al., 2020).

Por otro lado, en los frutos del género *Citrus* existen apocarotenoides C30 específicos, siendo los más abundantes: β-citraurina, apo-8′-β-carotenal y β-citraurineno. Los apocarotenoides se forman por degradación oxidativa a través de enzimas dioxigenasas de corte de carotenoides (CCD, por sus siglas en inglés), y en cítricos tanto la β-citraurina, apo-8′-β-carotenal y β-citraurineno se forman por la acción de la enzima CCD4b, que fragmenta asimétricamente la β-criptoxantina y zeaxantina en las posiciones 7,8/7′,8′ (Ma et al., 2013; Rodrigo, Alquézar, Alós, Medina, et al., 2013; Zheng et al., 2019). La acumulación de β-citraurina imparte un color naranja-rojizo en la piel de los cítricos, y su acumulación se limita solo al flavedo de algunas especies, como las mandarinas y sus híbridos y, en menor concentración, en las naranjas (Rodrigo, Alquézar, Alós, Lado, et al., 2013). Su acumulación en la piel está fuertemente regulada por la expresión del gen *CCD4b*, como también por la disponibilidad de sus precursores β-criptoxantina y zeaxantina (Ma et al., 2013; Rodrigo, Alquézar, Alós, Medina, et al., 2013).

Otro factor limitante en la acumulación de carotenoides en tejidos vegetales es la disponibilidad de su precursor GGPP y, por lo tanto, la regulación de su biosíntesis también

tiene un impacto en la síntesis de carotenoides (Rodríguez-Concepción, 2010). El GGPP se forma a partir de la condensación de isopentenil difosfato (IPP) y dimetilalil difosfato (DMAPP), que puede ser sintetizado en las plantas por dos rutas: la ruta del ácido mevalónico (MVA) y la ruta del metileritritol fosfato (MEP). Sin embargo, la producción de carotenoides en plantas ocurre principalmente a partir de los precursores originados en plastidios a través de la ruta MEP (Rodríguez-Concepción, 2010; Ruiz-Sola & Rodríguez-Concepción, 2012). Esta ruta se inicia con la condensación de gliceraldehído-3-fosfato (G3P) con piruvato, catalizada por la enzima dioxixilulosa-5-fosfato sintasa (DXS), que resulta en la formación de dioxixilulosa-5-fosfato (DXP). Esta etapa es considerada un punto clave en la regulación de la entrada a la ruta y en la síntesis de los isoprenoides derivados de la ruta MEP, como los carotenoides, los tocoferoles y las giberelinas, entre otros (Estévez, Cantero, Reindl, Reichler, & León, 2001; Rodríguez-Concepción & Boronat, 2015). En los frutos cítricos, la inducción de los genes *DXS* y *PSY* al inicio de la maduración del fruto es paralela a la acumulación de carotenoides en la pulpa (Fanciullino et al., 2008; Peng et al., 2013). Posteriormente, el DXP es reducido a MEP por la enzima DXP reducto isomerasa (DXR) y tras cuatro reacciones sucesivas se obtiene el hidroximetil-butenil difosfato (HMBPP), para formar finalmente los isómeros IPP y DMAPP por la acción de la enzima HMBPP reductasa (HDR). Esta enzima también puede tener un papel limítate en la regulación del flujo de la ruta MEP, y su inducción acompaña la acumulación de carotenoides, mientras que la enzima DXR no parece tener un papel tan limitante (Botella-Pavía et al., 2004; Rodríguez-Concepción & Boronat, 2015). Finalmente, la adición de tres moléculas de IPP a una de DMAPP origina el GGPP en una reacción catalizada por la enzima GGPP sintasa (GGPPS). Esta enzima es codificada por una familia compleja de genes, de hasta 12 isoformas en *Arabidopsis thaliana*, de las cuales solo una (*AtGGPPS11*) parece ser la responsable de producir el GGPP utilizado para la síntesis de carotenoides y otros isoprenoides en plastidios (Ruiz-Sola et al., 2016). De manera similar, en tomate se han descrito recientemente tres isoformas de GGPPS que están localizadas en plastidios y presentan actividad *in vitro*, de las cuales solo dos (*SlGGPPS2* y *SlGGPPS3*) parecen ser las responsables de producir el GGPP necesario para la síntesis de carotenoides en los cloroplastos y cromoplastos (Barja et al., 2021). Un aspecto importante a considerar sobre la regulación de la ruta MEP es que el GGPP es el precursor directo no sólo de los carotenoides, sino también de las clorofilas, tocoferoles, plastoquinonas, giberelinas y diterpenos, entre otros, por lo que puede existir competencia por este precursor entre las distintas rutas metabólicas (Rodríguez-Concepción, 2006; Rodríguez-Concepción & Boronat, 2015). En este sentido, en plantas transgénicas de arroz que sobre-expresan *GGPPS* se ha detectado un

incremento en el contenido de clorofilas, junto con un descenso en la concentración de carotenoides y giberelinas, demostrando la competencia por el sustrato entre las rutas de biosíntesis de los diferentes isoprenoides (Zhou et al., 2017).

1.2.3. Carotenoides en los frutos cítricos

Los carotenoides en los frutos cítricos se acumulan tanto en la piel como en la pulpa, y su contenido y composición puede sufrir importantes modificaciones por distintos factores tanto endógenos como exógenos (Alquézar, Rodrigo, & Zacarías, 2008a; Rodrigo, Alquézar, Alós, Lado, et al., 2013). Tanto los contenidos como el perfil de carotenoides están determinados por el genotipo de las diferentes especies y variedades, pero, además, las condiciones ambientales (exposición a la luz, temperatura, etc.) y prácticas de cultivo pueden tener un importante efecto en la composición de carotenoides en el fruto (Kato, 2012; Rodrigo, Alquézar, Alós, Lado, et al., 2013).

En general, los frutos maduros de mandarina y sus híbridos son las especies que pueden alcanzar mayor contenido de carotenoides (25-300 μg g^{-1} en el flavedo), seguidos por los frutos de naranja (40-120 μg g^{-1} en el flavedo), y por último los frutos de limón y pomelo, en los que los contenidos suelen ser bajos (1-60 μg g^{-1} en el flavedo) (Alquézar et al., 2008a; Rodrigo, Alquézar, Alós, Lado, et al., 2013; Tadeo, Terol, Rodrigo, Licciardello, & Sadka, 2020). La composición de carotenoides de los frutos cítricos es muy compleja (Fanciullino et al., 2006; Matsumoto et al., 2007), pudiéndose encontrar más de 20 carotenoides en el flavedo de variedades de mandarinas y sus híbridos (Kato, 2012; Rodrigo, Alquézar, Alós, Lado, et al., 2013). Matsumoto et al. (2007) caracterizaron 39 genotipos de *Citrus* según el contenido y composición de carotenoides en el flavedo y zumo, y propusieron que las especies cítricas se pueden categorizar en distintos grupos de acuerdo a su composición, siendo el contenido de violaxantina y β-criptoxantina los carotenoides más determinantes en esta clasificación. En este sentido, los frutos maduros de mandarina se caracterizan por tener altos contenidos de carotenoides, en los que predomina la acumulación de violaxantina y β-criptoxantina (y otras β,β-xantofilas) (Matsumoto et al., 2007) y, además, en el flavedo acumulan β-citraurina, que junto a la β-criptoxantina brindan la coloración naranja intensa característica de estos frutos (Rodrigo, Alquézar, Alós, Medina, et al., 2013). Por otro lado, los frutos de naranja presentan un perfil similar al de las mandarinas, donde predomina la acumulación de violaxantina, pero con un menor contenido de β-criptoxantina y β-citraurina, lo que resulta en frutos con una coloración naranja más pálida (Rodrigo, Alquézar, Alós, Lado, et al., 2013). Por último, los frutos de limón, pummelo y pomelo de piel amarilla se agrupan en un mismo grupo, y se

caracterizan por presentar bajos contenidos totales de carotenoides y una composición muy simple, donde predomina la acumulación de carotenoides incoloros, como los carotenos lineares fitoeno y fitoflueno (Alquézar et al., 2013; Kato et al., 2004; Rodrigo, Alquézar, Alós, Lado, et al., 2013). Los contenidos de carotenoides en la pulpa suelen ser menores (2-40 $\mu g\ g^{-1}$) que en el flavedo (Alquézar et al., 2008a; Kato et al., 2004; Tadeo et al., 2020). Las diferencias entre genotipos se mantienen y la pulpa de los frutos de mandarina presenta mayores contenidos de carotenoides, seguidos por los frutos de naranja y, por último, los frutos de limón, lima, pomelo y pummelo (Fanciullino et al., 2006; Matsumoto et al., 2007).

Por último, la acumulación de licopeno en los frutos cítricos, que confiere una coloración rosa o roja, es una característica inusual y suele limitarse a la pulpa de ciertas variedades de pummelo y pomelo rojo, como Star Ruby (Lado, Zacarías, Gurrea, Page, et al., 2015), o variedades mutantes de naranja, como Cara-Cara y Valencia Ruby (Alquézar et al., 2008b; Zacarías-García, Rey, Gil, Rodrigo, & Zacarías, 2020), y limón Pink (Lana et al., 2020). A pesar de que la acumulación de licopeno suele restringirse a la pulpa, en ciertas variedades de pomelo y pummelo rojo/rosado también se acumula licopeno en el flavedo (Xu et al., 2006). Además, su acumulación se incrementa de forma muy significativa en el flavedo de frutos de pomelo Star Ruby cuando se evita la incidencia de luz durante el desarrollo y maduración del fruto (Lado, Cronje, Alquézar, Page, et al., 2015). Esta anormal acumulación de carotenoides en el flavedo de frutos tapados de esta variedad de pomelo se asoció a una diferenciación acelerada de cloroplastos en cromoplastos, así como a una alteración en la expresión de ciertos genes de la ruta y de isoformas de *β-LCYs*, lo permitió una mayor capacidad de acumulación de carotenoides. De manera similar, se ha demostrado que la aplicación de un inhibidor de la actividad β-LCY, (CPTA, hidrocloruro de 2-(4-tioclorofenil) trietilamina) induce la acumulación de licopeno en el flavedo de pomelo Marsh (Lado, Rodrigo, Cronje, et al., 2015). Estas evidencias indican que la acumulación de licopeno en esta especie de cítricos parece estar asociada a alteraciones en la actividad y/o expresión génica de β-licopeno ciclasas.

1.2.4. Funciones de los carotenoides en plantas y su posible papel en la tolerancia al daño por frío

Los carotenoides desempeñan múltiples funciones en plantas, teniendo un papel esencial en la fotoprotección y fotomorfogénesis, formando parte del sistema fotosintético y siendo precursores de fitohormonas como el ácido abscísico (ABA) y estrigolactonas, o de moléculas señalizadoras y reguladoras del crecimiento vegetal (Havaux, 2020; Rodríguez-Concepción et al., 2018). Además, cumplen funciones como metabolitos secundarios, siendo los principales pigmentos responsables del color de muchos frutos, flores y otros tejidos, atrayendo a

polinizadores y a la dispersión de las semillas. De manera similar, los carotenoides son los sustratos para la formación de otros derivados volátiles, como los norisoprenoides, que median en la interacción de las plantas con animales u otras plantas (Cazzonelli, 2011; Rodríguez-Concepción et al., 2018).

Una de las funciones biológicas más relevantes de los carotenoides es su papel como fotoprotectores, siendo capaces de disipar el exceso de energía causado por la alta incidencia de luz o altas temperaturas, en forma de calor (Cazzonelli, 2011; Havaux, 1998). El mecanismo de protección de la maquinaria fotosintética por los carotenoides está estrechamente relacionado con el ciclo de las xantofilas, en especial el de violaxantina-anteraxantina-zeaxantina, que reduce el exceso de energía que llega a los centros de reacción fotosintéticos (Cazzonelli, 2011; Namitha & Negi, 2010). Además, los carotenoides forman parte del fotosistema II, y como parte de los complejos antena de los cloroplastos captan energía en un amplio rango de longitudes de onda y participan en la transferencia de electrones a las clorofilas (Hashimoto, Uragami, & Cogdell, 2016). Por último, los carotenoides también tienen un papel como estabilizadores de las membranas tilacoidales (Havaux, 1998).

Por otro lado, los carotenoides constituyen una de las primeras líneas de defensa de las plantas frente a ROS, especialmente frente al $^1O_2$, ya que son potentes antioxidantes capaces de inactivar estos radicales que se producen durante diferentes procesos metabólicos, como en la fotosíntesis, o en respuesta a condiciones de estrés (Hossain, Nouri, & Komatsu, 2012). La capacidad de los diferentes carotenoides para eliminar ROS está asociada a su estructura química y determinada principalmente por el número de dobles enlaces conjugados que poseen, pero también por la concentración y su localización en las células (Stahl & Sies, 2003). En este sentido, el licopeno (11 enlaces conjugados) es el carotenoide con mayor capacidad antioxidante (mayor eficiencia en secuestrar $^1O_2$), seguido por el β-caroteno (9 enlaces conjugados más dos en los anillo β), luteína y α-caroteno (9 más uno en el anillo β), y las xantofilas violaxantina y neoxantina (9 enlaces) (Di Mascio, Kaiser, & Sies, 1989; Ouchi et al., 2010).

Como se ha mencionado previamente, una de las respuestas al estrés por frío es un incremento de los ROS, que si no es contrarrestada eficientemente pueden provocar daños oxidativos en los componentes celulares (Sevillano et al., 2009). Los carotenoides forman parte del sistema antioxidante no enzimático de las plantas y su posible papel protector frente al DF se ha asociado a su función como antioxidantes liposolubles capaces de eliminar ROS (Decros et al., 2019). La relación entre el contenido de carotenoides en el flavedo y la susceptibilidad a

desarrollar DF durante la postcosecha ha sido estudiada principalmente en frutos de pomelo con diferente pigmentación (Dou, 2005; Lado, Rodrigo, Cronje, et al., 2015; Lado, Rodrigo, et al., 2016). Así, los frutos de pomelo Marsh, con coloración amarilla y bajo contenido de carotenoides, presentan una mayor susceptibilidad al DF que frutos de pomelo rojo como Star Ruby o Ruby Red, con una coloración más intensa y mayor contenido de carotenoides (Dou, 2005; Lado, Rodrigo, Cronje, et al., 2015). De manera similar, en frutos de Star Ruby, que pueden desarrollar distinto grado de coloración roja en el flavedo, se ha comprobado que el desarrollo del DF se restringe a las zonas amarillas del flavedo, mientras que las zonas rojas, que acumulan licopeno, permanecen prácticamente intactas y sin lesiones (Lado, Rodrigo, Cronje, et al., 2015). Estas observaciones indican que los frutos de esta variedad no tienen diferente sensibilidad genética a los DF, sino que la acumulación de licopeno es la que confiere la tolerancia al estrés por bajas temperaturas (Lado, Rodrigo, Cronje, et al., 2015). Posteriormente el papel del licopeno en la tolerancia al DF fue corroborado al demostrar que en los frutos de pomelo Star Ruby, en los que la ausencia de luz (tapado de frutos) durante el desarrollo y maduración confería una coloración roja homogénea, se acumulaban altos contenidos de licopeno en la piel y se inducía una tolerancia a los DF durante la exposición posterior a la conservación a 2 °C. Además, el flavedo rojo de los frutos con alto contenido en licopeno tenía una mayor actividad antioxidante SOAC (capacidad para secuestrar específicamente el radical $^1O_2$), mientras que la actividad antioxidante total o la concentración de ácido ascórbico (AsA) estaban reducidas. Además, la aplicación de un inhibidor de la actividad de β-LCY provocó la acumulación de licopeno en el flavedo de frutos de pomelo Marsh (sensibles al DF), reduciendo la incidencia del DF durante la conservación refrigerada (Lado, Rodrigo, Cronje, et al., 2015). En conjunto, estas observaciones indican que el licopeno actúa reduciendo los efectos nocivos generados por este radical, que posiblemente es uno de los principales desencadenantes de los DF en los frutos cítricos, y que el incremento en la actividad antioxidante total o en AsA serían respuestas frente a los DF (Lado, Rodrigo, Cronje, et al., 2015; Lado, Rodrigo, et al., 2016).

En frutos de otras variedades de cítricos, como las naranjas y mandarinas, no se ha establecido una relación directa entre el contenido y composición de carotenoides y la tolerancia/sensibilidad a los DF durante la conservación a bajas temperaturas. Sin embargo, es interesante mencionar la idea ampliamente distribuida entre los productores y otros sectores de la citricultura de que los frutos situados en el exterior de la copa de los árboles, y expuestos a la radiación luminosa directa, desarrollan una coloración naranja más intensa que los situados en el interior, con una menor incidencia luminosa, y clásicamente se les ha

considerado más resistentes a desarrollar DF que los internos. Resultados obtenidos en frutos de la mandarina Clementina de Nules cultivados en Sudáfrica indican que los frutos cosechados del interior de la copa del árbol presentan una menor coloración y contenido de carotenoides, y son más susceptibles a desarrollar desórdenes fisiológicos durante la conservación postcosecha (Cronje et al., 2011, 2013). Sin embargo, estas observaciones no siempre son extrapolables para una misma variedad, pero en condiciones de cultivo y climáticas diferentes, ya que los DF en los frutos de mandarina ocurren de forma distinta y en diferentes condiciones postcosecha en Sudáfrica y en los países de la cuenca mediterránea.

1.3. Tocoferoles

1.3.1. Características generales de los tocoferoles

Los tocoferoles son compuestos isoprenoides liposolubles sintetizados principalmente por organismos fotosintéticos, como plantas, algas y cianobacterias (Fritsche, Wang, & Jung, 2017; Mène-Saffrané, 2017). Pertenecen al grupo de los tococromanoles, dentro del cual también están incluidos los tocotrienoles, los tocomonoenoles y el plastocromanol 8. La estructura básica de todos los tococromanoles consiste en un anillo cromanol (hidrosoluble) unido a una cadena lateral hidrocarbonada (hidrofóbica). El anillo cromanol deriva del ácido homogentísico/homogentisato (HGA), que es el precursor común para la síntesis de todos los tococromanoles, mientras que la cadena isoprenoide se origina a partir de un sustrato poliprenol y varía según el tipo de tococromanol. En el caso de los tocoferoles, el sustrato poliprenol es fitil difosfato (PPP) y la cadena hidrocarbonada está totalmente saturada. A su vez, de acuerdo al número y posición del grupo metilo en el anillo cromanol, existen cuatro isoformas distintas de tocoferoles: δ-tocoferol (un grupo metilo), β- and γ-tocoferol (dos grupos metilo pero en diferente posición) y α-tocoferol (tres grupos metilos) (Figura 4**¡Error! No se encuentra el origen de la referencia.**). Además de cumplir diversas funciones en plantas, los tocoferoles son de gran importancia junto con los tocotrienoles, porque son los únicos compuestos naturales con actividad de vitamina E en las células animales y, por tanto, son nutrientes esenciales en la dieta humana (Traber & Sies, 1996; Weiser, Riss, & Kormann, 1996).

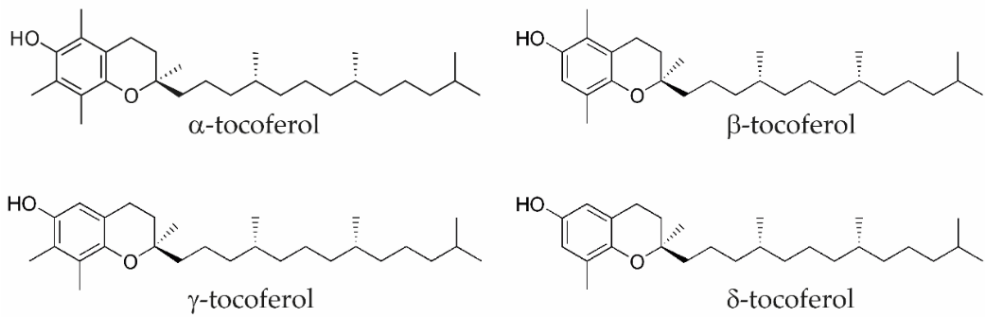

Figura 4. Estructura química de las distintas isoformas de los tocoferoles.

1.3.2. Biosíntesis de tocoferoles y su regulación

La ruta de biosíntesis de tocoferoles ha sido dilucidada en los últimos años, identificando todos los genes que codifican las enzimas involucrados en los principales pasos de la ruta (genes VTE) (Figura 5) (Fritsche et al., 2017; Mène-Saffrané, 2017; Muñoz & Munné-Bosch, 2019). El primer paso de la ruta de biosíntesis de tocoferoles *per se* es la condensación de HGA con PPP mediante la enzima homogentisato fitiltransferasa (HPT; *VTE2*). Esta reacción resulta en la formación de 2-metil-6-fitil-1,4-benzoquinol (MPBQ), que a continuación puede sufrir dos tipos de reacciones diferentes que determinan que la ruta continúe hacia la síntesis de δ- y β-tocoferol o hacia la de γ- y α-tocoferol. Por un lado, MPBQ puede formar δ-tocoferol tras sufrir una ciclación de su anillo cromanol mediante la enzima tocoferol ciclasa (TC; *VTE1*), y posteriormente β-tocoferol tras una metilación por la acción de la enzima γ-tocoferol metiltransferasa (γ-TMT; *VTE4*). Por otro lado, MPBQ puede sufrir primero una metilación mediante la MPBQ metiltransferasa (MPBQ-MT; *VTE3*), formando 2,3-dimetil-5-fitilbenzoquinol (DMPBQ), que mediante la acción de la misma TC (*VTE1*) y γ-TMT (*VTE4*) forma γ-tocoferol primero y α-tocoferol, por último. Este conjunto de pasos es conocido como la síntesis específica de tocoferoles ("tocopherol-core pathway" en inglés), aunque de todas las enzimas involucradas solo la HPT (*VTE2*) es exclusiva de la síntesis de tocoferoles, mientras que MPBQ-MT (*VTE3*), TC (*VTE1*) y γ-TMT (*VTE4*) participan también en la síntesis de los otros tipos de tococromanoles. Sin embargo, a pesar de que la enzima HPT es considerada exclusiva y presenta mayor afinidad por el sustrato PPP, también se ha observado que es capaz de catalizar la condensación de HGA con GGPP, lo que da lugar a la síntesis de tocotrienoles (Zhang et al., 2013).

Estudios sobre los mecanismos moleculares que regulan la acumulación de tocoferoles en mutantes de *Arabidopsis thaliana* y otras plantas modelo han concluido que la actividad de HPT (*VTE2*) y, por lo tanto, la condensación entre HGA y PPP, es un paso limitante en la síntesis de tocoferoles en semillas y hojas (Collakova & DellaPenna, 2003a; Savidge et al., 2002). La

enzima TC (*VTE1*) también es clave en la regulación de la acumulación de tocoferoles, ya que los mutantes *vte1* son deficientes en todas las formas de tocoferoles y acumulan mayores contenidos de DMPBQ/MPBQ, los precursores de γ- y δ-tocoferol, respectivamente (Kanwischer, Porfirova, Bergmüller, & Dörmann, 2005; Porfirova, Bergmuller, Tropf, Lemke, & Dormann, 2002). Por otro lado, los genes *VTE3* y *VTE4* parecen no influir directamente en el contenido final de tocoferoles, si no que regulan la composición de los mismos (Bergmüller, Porfirova, & Dörmann, 2003; Cheng et al., 2003; Collakova & DellaPenna, 2003b). No obstante, estudios en distintas especies como tomate y oliva han demostrado que la acumulación de tocoferoles en frutos no parece estar limitada por la expresión de *VTE2* (Georgiadou et al., 2019; Quadrana et al., 2013) y que en frutos maduros el gen *VTE3* parece tener un papel más relevante en determinar los niveles de tocoferoles (Quadrana et al., 2013).

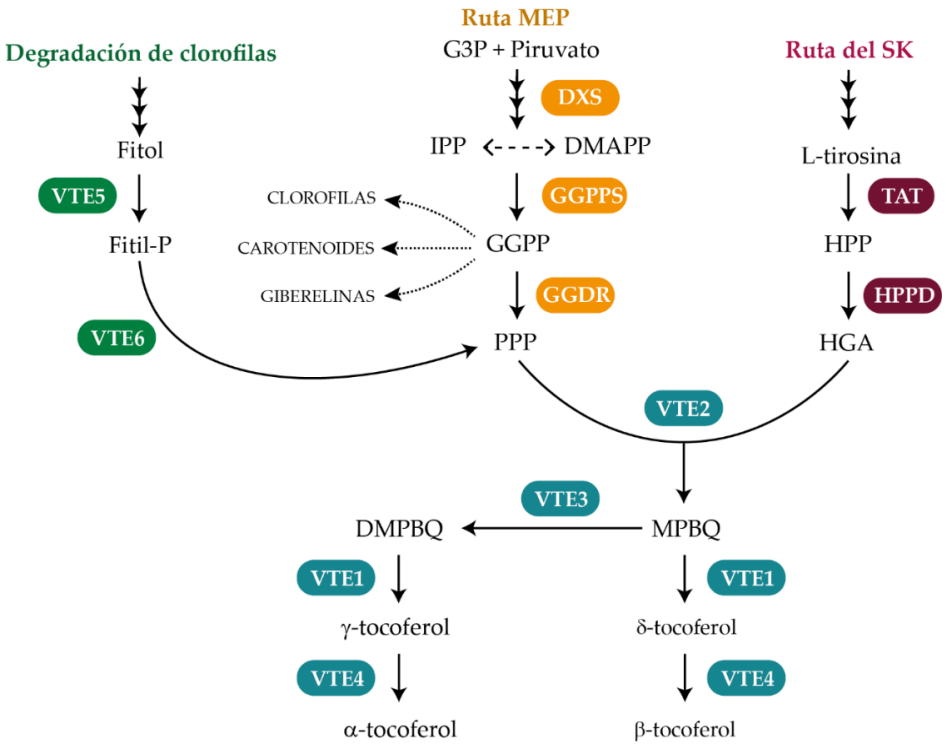

Figura 5. Esquema de la ruta de biosíntesis de tocoferoles en plantas. Abreviaturas: Fitil-P, fitil fosfato; G3P, Gliceraldehído 3-fosfato; IPP, isopentenil difosfato; DMAPP, dimetilalil difosfato; GGPP, geranilgeranil difosfato; PPP, fitil difosfato; HPP, p-hidroxifenilpiruvato; HGA, homogentisato; MPBQ, 2-metil-6-fitil-1,4-benzoquinol; DMPBQ, 2,3-dimetil-5-fitilbenzoquinol; VTE5, fitol quinasa; VTE6, fitil fosfato quinasa; DXS, dioxixilulosa-5-fosfato sintasa; GGPPS, GGPP sintasa; GGDR, geranilgeranil difosfato reductasa; TAT, tirosina aminotransferasa; HPPD, p-hidroxifenilpiruvato dioxigenasa; VTE2, homogentisato fitiltransferasa (HPT); VTE3, MPBQ metiltransferasa; VTE1, tocoferol ciclasa (TC); VTE4, γ-tocoferol metiltransferasa (γ-TMT).

La acumulación de tocoferoles en tejidos vegetales también depende de la disponibilidad de los precursores HGA y PPP y, por tanto, de los mecanismos de regulación de las rutas

metabólicas que originan estos precursores (Mène-Saffrané, 2017; Pellaud & Mène-Saffrané, 2017). En plantas, el precursor HGA es sintetizado a partir de la degradación del aminoácido L-tirosina (L-tyr), que se forma en la ruta del ácido siquímico (SK), y dos enzimas claves regulan su síntesis: tirosina aminotransferasa (TATs) y p-hidroxifenilpiruvato dioxigenasa (HPPD). La enzima TAT cataliza la conversión de L-tyr a p-hidroxifenilpiruvato (HPP), mientras que HPPD controla la conversión de HPP a HGA. Se han identificado varios genes que codifican las enzimas TATs, pero la actividad enzimática solo se ha probado para *TAT1* and *TAT2* (Prabhu & Hudson, 2010; Riewe et al., 2012). El gen *TAT1* tiene mayor afinidad por L-tyr (Wang, Toda, & Maeda, 2016), y la pérdida de función de este gen en mutantes de Arabidopsis resulta en la reducción de la actividad de TAT y de los contenidos de tocoferoles en hojas (Riewe et al., 2012). En cuanto a la enzima HPPD, los mutantes *pds1* no presentan actividad enzimática de HPPD y son deficientes en tocoferoles (Norris, Shen, & Della Penna, 1998), mientras que la sobre-expresión de este gen solo produjo un incremento modesto de los tocoferoles totales en hojas y semillas de Arabidopsis y soja (Karunanandaa et al., 2005; Tsegaye, Shintani, & DellaPenna, 2002). Resultados similares se han observado en frutos de tomate, en donde la manipulación genética de la ruta del corismato-tirosina para incrementar la producción de HPP, por la sobre-expresión de *HPPD* resultó en un incremento masivo de la acumulación de tocotrienoles, pero solo moderado en la de tocoferoles (Burgos et al., 2021). Sin embargo, en frutos de tomate y mango la mayor acumulación de transcriptos de *HPPD* se produce en los genotipos con mayores niveles de tocoferoles (Quadrana et al., 2013; Singh, Chaurasia, Bari, & Sane, 2017). Por último, es interesante destacar que, a diferencia de las otras enzimas involucradas en la síntesis de tocoferoles, y del precursor PPP, que se localizan en las membranas plasmáticas, TAT y HPPD se localizan en el citoplasma (Joyard et al., 2009; van Wijk & Kessler, 2017; Wang et al., 2016). Esto supone la existencia de transportadores de membrana que permitan el intercambio de L-tyr/HPP y HGA entre el citoplasma y los plástidios, e implica otro nivel adicional de regulación de la síntesis de tocoferoles (Mène-Saffrané, 2017).

El otro precursor necesario para la síntesis de tocoferoles es el PPP, que puede ser sintetizado directamente a partir de GGPP a través de la ruta MEP o alternativamente a través de reciclaje de fitol generado durante la degradación de clorofilas (Pellaud & Mène-Saffrané, 2017). El suministro de PPP mediante el reciclaje de fitol involucra la acción de dos enzimas: fitol quinasa (*VTE5*) y fitil fosfato quinasa (*VTE6*) (Valentin et al., 2006; vom Dorp et al., 2015). En Arabidopsis se ha sugerido que entre el 65% y 80% del PPP utilizado para la síntesis de tocoferoles en hojas y semillas, respectivamente, proviene del reciclaje de fitol (Valentin et al.,

2006), y experimentos en los que se ha añadido fitol a tejidos celulares de plantas han resultado en un incremento significativo de los contenidos de tocoferoles (Furuya, Yoshikawa, Kimura, & Kaneko, 1987; Karunanandaa et al., 2005). El papel del gen *VTE5* en la regulación de la acumulación de tocoferoles también se ha comprobado en frutos de tomate y oliva, donde su disminución durante el desarrollo del fruto limita la disponibilidad de PPP para la síntesis de tocoferoles (Almeida et al., 2016; Georgiadou et al., 2015). Sin embargo, en frutos maduros de tomate se ha observado que la expresión de *VTE5* no parece ser tan limitante, y la expresión de genes de la ruta MEP tienen una mayor implicación, siendo capaces de compensar la disminución de PPP a través del reciclaje de fitol (Almeida et al., 2015; Gramegna et al., 2019; Quadrana et al., 2013). En este sentido, dos genes relacionados con la ruta MEP parecen jugar un papel importante en la regulación de los contenidos de tocoferoles: *DXS* y geranilgeranil difosfato reductasa (*GGDR*) (Almeida et al., 2015; Gramegna et al., 2019; Quadrana et al., 2013). La enzima DXS, como se mencionó en la sección *1.2.2.*, controla el flujo hacia la ruta MEP y cambios en su expresión resultan en una reducción o aumento de la síntesis de varios isoprenoides, incluidos los tocoferoles (Estévez et al., 2001). Por otro lado, la enzima GGDR controla la reducción final de GGPP a PPP, y parece limitar la disponibilidad de este precursor a través de la ruta MEP, sobre todo en frutos maduros (Almeida et al., 2015; Quadrana et al., 2013). En frutos de tomate se ha detectado una inducción del gen *DXS* y una disminución de la expresión de *GGDR* durante la maduración, pero sin una repercusión en el contenido de tocoferoles, que se mantienen constantes. Esto sugiere, por un lado, que la mayor disponibilidad de intermediarios por la inducción de *DXS* se canalizan hacia la síntesis de otros isoprenoides, como por ejemplo los carotenoides y, por otro lado, que existe un mecanismo de compensación en la formación de PPP a través del reciclaje de fitol que determina que los tocoferoles no disminuyan (Almeida et al., 2015; Quadrana et al., 2013). La importancia de la interacción entre la expresión de *GGDR* y el suministro de PPP, a través del reciclaje de fitol, también se ha corroborado en frutos albinos de tomate, que no contienen clorofilas, y en donde la disminución de la expresión de *GGDR* durante la maduración provoca una reducción en los contenidos de tocoferoles (Almeida et al., 2015). De manera similar, en frutos maduros de tomate que retienen clorofilas se ha detectado una inducción en la expresión de *GGDR* durante la maduración, y estos niveles parecen ser suficientes para mantener los niveles necesarios de PPP para la síntesis de tocoferoles (Almeida et al., 2015).

1.3.3. Tocoferoles en frutos

En plantas, la acumulación de tocoferoles ha sido estudiada principalmente en hojas y semillas, pero también se han detectado en frutos, tallos, raíces y flores (Horvath, Wessjohann, Bigirimana, Jansen, et al., 2006; Mène-Saffrané & DellaPenna, 2010). En general, el α-tocoferol es la forma predominante en los tejidos vegetales, con la excepción de semillas de ciertas especies que acumulan principalmente γ-tocoferol (Horvath, Wessjohann, Bigirimana, Jansen, et al., 2006). En frutos, el contenido y composición de tocoferoles parece variar según la especie y variedad, y también durante el desarrollo y maduración del fruto (Almeida et al., 2015; Georgiadou et al., 2019, 2015; Osuna-García, Wall, & Waddell, 1998; Quadrana et al., 2013). Mientras que el α-tocoferol es la forma predominante en frutos de tomate, pimiento, mango, uva, olivas y aguacate (Georgiadou et al., 2019; Horvath, Wessjohann, Bigirimana, Monica, et al., 2006; Koch, Arango, Mock, & Heise, 2002; Quadrana et al., 2013; Singh et al., 2017), mayores concentraciones de γ-tocoferol se han detectado en calabacín y frambuesa (Carvalho, Fraser, & Martens, 2013; Rodov et al., 2020).

A pesar de que los contenidos de tocoferoles se han evaluado en frutos maduros de varias especies (Chun, Lee, Ye, Exler, & Eitenmiller, 2006), existe poca información respecto a los mecanismos moleculares que regulan la biosíntesis de tocoferoles en frutos, aunque en los últimos años se han hecho avances en frutos de tomate (Almeida et al., 2015, 2016, 2011; Burgos et al., 2021; Gramegna et al., 2019; Quadrana et al., 2013), pimiento (Arango & Heise, 1998; Koch et al., 2002), mango (Singh et al., 2017) y oliva (Georgiadou et al., 2016, 2019, 2015). Estos estudios han concluido que la acumulación de tocoferoles en frutos está regulada a nivel transcripcional y que la modulación de los genes varía durante el desarrollo y maduración, además de estar influenciada por el genotipo y factores ambientales (Almeida et al., 2015; Georgiadou et al., 2016, 2019; Gramegna et al., 2019; Quadrana et al., 2013). También se ha sugerido que la acumulación de tocoferoles durante la maduración del fruto depende de la naturaleza climatérica y no climatérica de los frutos. De esta manera, en los frutos no climatéricos, como uva y oliva, los contenidos tienden a disminuir con la maduración (Georgiadou et al., 2016; Horvath, Wessjohann, Bigirimana, Monica, et al., 2006), mientras que en los frutos climatéricos, como mango, aumentan (Singh, Ali, Nath, & Sane, 2011). No obstante, resultados en otras especies no parecen apoyar esta idea, ya que, por ejemplo, en frutos no climatéricos como pimiento y cereza los niveles de α-tocoferol se incrementan con la maduración (Koch et al., 2002; Osuna-García et al., 1998; Tijero, Teribia, Muñoz, & Munné-

Bosch, 2016), mientras que en tomate, fruto climatérico, los contenidos se mantienen constantes (Quadrana et al., 2013).

En frutos cítricos, la información sobre la acumulación de tocoferoles es muy limitada, pero se han detectado tocoferoles en el flavedo de naranja, mandarina, limón y otras especies cítricas menos cultivadas (Assefa, Saini, & Keum, 2017; Mathaba, Bower, & Bertling, 2014), y en la pulpa de naranja, mandarina y pomelo (Cardeñosa et al., 2015; Chun et al., 2006). En la pulpa de frutos maduros, los contenidos varían entre 1.7-2.5 $\mu g\ g^{-1}$ (FW), siendo el α-tocoferol la forma predominante (Chun et al., 2006). Estos contenidos son similares a frutos de otras especies como cerezas, manzanas y melocotones (2-2.5 $\mu g\ g^{-1}$), y menores a los detectado en frutos de olivo, mora, frambuesa y aguacate (~30-38 $\mu g\ g^{-1}$) (Chun et al., 2006). El análisis de los tocoferoles en la pulpa de frutos de distintas variedades de naranja detectaron pocas variaciones entre las mismas, pero los contenidos tienden a descender durante la maduración del fruto (desde enero a abril) (Cardeñosa et al., 2015). Los contenidos reportados en el flavedo son superiores a los de la pulpa y varían entre 65-131 $\mu g\ g^{-1}$ de peso seco, siendo el γ- y α-tocoferol las únicas formas detectadas, y cuyo predominio depende de la especie (Assefa et al., 2017).

1.3.4. Funciones de los tocoferoles en plantas y su posible papel en la tolerancia al daño por frío

Los tocoferoles desempeñan numerosas funciones en las plantas, de las que su papel como antioxidantes es uno de los más destacables y mejor estudiados (Asensi-Fabado & Munné-Bosch, 2010; Falk & Munné-Bosch, 2010; Kamal-Eldin & Appelqvist, 1996). Sin embargo, se han descrito otras funciones biológicas, como su implicación en la germinación y viabilidad de semillas, transporte de fotoasimilados y metabolismo de carbohidratos, señalización celular, y en la respuesta de las plantas al estrés abiótico y biótico (Falk & Munné-Bosch, 2010; Ma et al., 2020; Muñoz & Munné-Bosch, 2019).

Como antioxidantes, los tocoferoles son altamente eficientes debido a que son capaces de desactivar químicamente radicales lipídicos, disminuyendo la peroxidación lipídica y protegiendo a las membranas celulares y, además, pueden desactivar química y físicamente el $^1O_2$, protegiendo los lípidos y otras macromoléculas (Gruszka, Pawlak, & Kruk, 2008; Kamal-Eldin & Appelqvist, 1996; Lieblers, Kling, & Reed, 1986). La habilidad de los tocoferoles para neutralizar radicales lipídicos se debe a la capacidad de su anillo cromanol de donar el hidrógeno fenólico a los radicales libres, lo que resulta en la formación de radicales de tocoferoles que son más estables (Kamal-Eldin & Appelqvist, 1996). Además, los radicales de

tocoferoles pueden ser reconvertidos a tocoferoles nuevamente mediante su interacción con otros antioxidantes, como el AsA y glutatión (Szarka, Tomasskovics, & Bánhegyi, 2012), lo que les permite participar en varias reacciones de desactivación de radicales libres. Por otro lado, la desactivación química de $^1O_2$ resulta en la degradación de tocoferoles y formación de quinonas y otros derivados (Gruszka et al., 2008), que también se ha sugerido que poseen propiedades antioxidantes y que pueden ser reconvertidos en tocoferoles nuevamente (Kobayashi & DellaPenna, 2008).

Las distintas estructuras químicas de las isoformas de tocoferoles determinan la habilidad de donar hidrógeno fenólico y, por tanto, de neutralizar radicales lipídicos. En este sentido, se ha descrito que la actividad antioxidante entre las isoformas varía en el siguiente orden: $\alpha > \beta \geq \gamma > \delta$, aunque distintas condiciones físicas y químicas pueden afectar este orden (Kamal-Eldin & Appelqvist, 1996). En cuanto a la capacidad de secuestrar $^1O_2$, la efectividad está influenciada por el grupo metilo en la posición C6 del anillo cromanol, y es en el siguiente orden: $\alpha \geq \beta > \gamma > \delta$ (Gruszka et al., 2008; Kaiser, Di Mascio, Murphy, & Sies, 1990). A pesar de estas diferencias en cuanto a la actividad antioxidante, en plantas se ha visto que las distintas formas de tocoferol pueden compensar la deficiencia de una isoforma u otra. Por ejemplo, los mutantes de Arabidopsis *vte4* no acumulan α-tocoferol, pero tienen un mayor contenido de γ-tocoferol y en condiciones de estrés estas plantas no presentan un fenotipo diferencial al control o mayores signos de oxidación, demostrando que el γ-tocoferol puede reemplazar el papel protector del α-tocoferol (Bergmüller et al., 2003; Maeda, Song, Sage, & DellaPenna, 2006). Es más, en mutantes de Arabidopsis *vte1*, que no acumulan ninguna forma de tocoferoles pero tienen un mayor contenido del precursor DMPBQ, solo se observó un fenotipo diferencial en condiciones de estrés muy severo, indicando que este precursor también tiene capacidad antioxidante y puede compensar la falta de tocoferoles (Havaux, Eymery, Porfirova, Rey, & Dörmann, 2005; Maeda et al., 2006; Porfirova et al., 2002).

Los tocoferoles han sido descritos como parte de la respuesta de las plantas frente a distintas situaciones de estrés abiótico y, en general, las especies más tolerantes a condiciones de estrés contienen un mayor contenido de tocoferoles que las sensibles (Gabruk, Habina, Kruk, Dłużewska, & Szymańska, 2016; Munné-Bosch & Alegre, 2002; Sadiq, Akram, Ashraf, Al-Qurainy, & Ahmad, 2019). En este sentido, se han detectado incrementos en la acumulación de tocoferoles en respuesta a distintos tipos de estrés como el exceso de luz, deficiencia nutricional, estrés hídrico y bajas temperaturas (Allu, Simancas, Balazadeh, & Munné-Bosch,

2017; Bergmüller et al., 2003; Collakova & DellaPenna, 2003b; Havaux et al., 2005; Maeda et al., 2006; Spicher et al., 2017; Wang et al., 2017).

El efecto del estrés por frío en la acumulación de tocoferoles ha sido estudiado principalmente en tejidos vegetativos de plantas modelos como Arabidopsis, en donde se ha visto que temperaturas de 4-7 °C producen un incremento en los niveles de tocoferoles en hojas en comparación a plantas cultivadas en condiciones óptimas (Bergmüller et al., 2003; Maeda et al., 2006). Además, la importancia de los tocoferoles en la tolerancia a las bajas temperaturas se apoya en observaciones realizadas en plantas mutantes de Arabidopsis y de arroz deficientes en tocoferoles. Estas plantas exhibieron un fenotipo hipersensible frente a las bajas temperaturas (Maeda et al., 2006; Wang et al., 2017), mientras que la exposición de las mismas a otras condiciones de estrés (alta luminosidad, alta salinidad y sequía) no provocaron diferencias fenotípicas con respecto a las plantas silvestres (Maeda et al., 2006). Además, en estos estudios los parámetros indicadores de daño oxidativo no fueron superiores en los frutos con un fenotipo sensible al frío, sugiriendo que la protección de los tocoferoles frente al estrés por frío no está únicamente relacionada a sus propiedades como antioxidantes (Maeda et al., 2006; Wang et al., 2017).

En frutos, el efecto de las bajas temperaturas en la síntesis de tocoferoles o su posible papel frente al estrés por frío no ha sido estudiado en profundidad. En frutos de aguacate, el almacenamiento a 4 °C durante 4 horas incrementó los niveles de γ-tocoferol, pero no de α- y β-tocoferol, mientras que el almacenamiento durante 10 días provocó una reducción del 20% en los contenidos de α-tocoferol, sin afectar significativamente las otras formas (Vincent, Mesa, & Munné-Bosch, 2020). Por otro lado, en frutos de cereza se observó un aumento transitorio en los tocoferoles totales después de 3 días de almacenamiento a 4 °C, (Tijero et al., 2016). En cuanto a la posible relación del DF y los niveles de tocoferoles en frutos, hasta la fecha solo se ha estudiado en frutos de calabacín. En esta especie se observó una relación inversa entre el contenido de tocoferoles en la cosecha y la susceptibilidad a desarrollar DF durante la postcosecha, siendo los frutos con mayores concentraciones de tocoferoles en el exocarpo más resistentes al DF durante el almacenamiento a 4-5 °C y 8-9 °C durante 2 semanas (Rodov et al., 2020).

1.4. Implicación del ácido ascórbico y la capacidad antioxidante en la sensibilidad de los frutos a los daños por frío

1.4.1. Ácido ascórbico

El AsA es un ácido orgánico hidrosoluble sintetizado principalmente por las plantas y algunas especies de animales (Smirnoff, 2018). Es uno de los antioxidantes más abundantes en las plantas y está involucrado en numerosos procesos biológicos, como la fotosíntesis, la división celular, la regulación del crecimiento, desarrollo y senescencia (Davey et al., 2000; Smirnoff, 2011), como también en la respuesta de las plantas frente a condiciones de estrés (Akram, Shafiq, & Ashraf, 2017). Además, el AsA junto al ácido dehidroascórbico (DHA), que resulta de la oxidación de AsA, son moléculas que presentan actividad de vitamina C en los organismos y deben ser ingeridos a través de la dieta, debido a que los humanos y otros grupos de mamíferos han perdido la capacidad de sintetizarlos (Smirnoff, 2018; Strobbe, De Lepeleire, & Van Der Straeten, 2018).

Los frutos cítricos son una de las fuentes más relevantes de AsA entre las diferentes especies vegetales, con contenidos entre 20 y 70 mg/100 g de PF en la pulpa (Bermejo & Cano, 2012; Martí, Mena, Cánovas, Micol, & Saura, 2009) y 100-250 mg/100 g de PF en el flavedo (Alós, Rodrigo, & Zacarías, 2014; Lado, Alós, Rodrigo, & Zacarías, 2015). La acumulación de AsA parece estar influenciada por factores genéticos ya que los contenidos varían entre las diferentes especies y variedades de *Citrus* y, en este sentido, los frutos de naranja suelen acumular niveles de AsA más altos, seguidos por los limones y pomelos, y por último las mandarinas (Cano, Medina, & Bermejo, 2008; Martí et al., 2009). A su vez, varios factores externos influyen en los contenidos de AsA en los tejidos vegetales, como las condiciones ambientales o prácticas de manejo durante la precosecha o postcosecha (Lee & Kader, 2000; Magwaza, Mditshwa, Tesfay, & Opara, 2017). La luz, dentro de los factores ambientales, parece desempeñar un papel clave en la acumulación de AsA en los frutos, principalmente a través de su efecto sobre la regulación transcripcional de los genes su la biosíntesis (Fenech, Amaya, Valpuesta, & Botella, 2019; Magwaza et al., 2017). En este sentido, experimentos de tapado de frutos de kiwi y manzana provocaron una reducción en la acumulación de AsA, asociada a una menor expresión de genes de la ruta de la L-galactosa, principal ruta de biosíntesis de AsA en este fruto (Li, Ma, Liu, & Li, 2010; Li et al., 2009). De manera similar, el tapado de frutos cítricos durante el desarrollo y la maduración redujo en casi un 50% los contenidos de AsA en el flavedo, asociado principalmente a la represión de genes de la ruta alternativa de biosíntesis del ácido L-galactúronico (Lado, Alós, Rodrigo, & Zacarías, 2015).

Una de las principales funciones del AsA en plantas es como antioxidante hidrosoluble, siendo uno de los más abundantes y potentes en las células vegetales. En este sentido, el AsA es un antioxidante muy eficiente ya que forma parte tanto del sistema antioxidante enzimático como no enzimático, siendo capaz de neutralizar ROS directamente o como cofactor de la enzima APX (Davey et al., 2000; Smirnoff, 2018). A su vez, mediante su capacidad de donar/aceptar electrones el AsA es capaz de neutralizar radicales libres y es importante destacar que de esta manera participa en el reciclaje de tocoferol al donar electrones a radicales de tocoferol inestables (Szarka et al., 2012). Por otro lado, el AsA es cofactor de otras enzimas entre las que se destaca la VDE, mediante la cual el AsA participa del ciclo de las xantofilas, como también de enzimas implicadas en las síntesis de hormonas vegetales como ácido abscísico y giberelinas (Davey et al., 2000).

Debido a su actividad biológica como antioxidante y cofactor de varias enzimas, el AsA modula el metabolismo oxidativo y está implicado en la defensa de las plantas frente a condiciones de estrés, como la alta salinidad, la sequía y las altas temperaturas (Akram et al., 2017; Smirnoff, 2011). El papel del AsA frente al estrés por bajas temperaturas en frutos no ha sido ampliamente estudiado, y los estudios se han enfocado principalmente en el efecto protector del AsA en tejidos vegetativos. No obstante, en frutos de manzana, pera y tomate, se ha observado una relación positiva entre los contenidos de AsA y la reducción de la incidencia de desórdenes fisiológicos durante la postcosecha (Cascia et al., 2013; Mellidou et al., 2014; Stevens et al., 2008). En el caso de los frutos cítricos, pocos estudios han analizado la relación entre el contenido de AsA y la tolerancia al DF, y la mayoría de ellos se han limitado a cuantificar su contenido en el zumo, como parámetro de calidad interna durante la conservación refrigerada. En este sentido, los contenidos de AsA en el zumo suelen mantenerse constantes o disminuyen ligeramente durante la conservación refrigerada, y esta respuesta a las bajas temperaturas es similar en las distintas especies y variedades de cítricos (Magwaza et al., 2017). Sin embargo, la relación de la vitamina C y el DF parece depender de la especie y también de la variedad. Por ejemplo, en frutos de naranja Navel y Valencia se detectaron mayores concentraciones de AsA en la pulpa de frutos tolerantes al DF (Bassal & El-Hamahmy, 2011), mientras que en frutos de mandarina Clementina se detectaron menores contenidos en los frutos con menor incidencia de DF. Por otro lado, en frutos de pomelo Star Ruby, mandarina Fortuna o naranjas sanguínas no se encontró una relación entre el DF y los contenidos de AsA (Chaudhary, Jayaprakasha, Porat, & Patil, 2014; Lafuente, Ballester, Calejero, & González-Candelas, 2011; Magwaza et al., 2017; Schirra, 1993). Sin embargo, y como se mencionó anteriormente, estos trabajos se centraron en estudiar los contenidos de

AsA en el zumo/pulpa y no en el flavedo, que es el tejido donde se desarrollan los daños, por lo que la relevancia de resultados en los DF debe ser tomada con reservas. En este sentido resaltar que Lado et al. (2016) analizaron los contenidos de AsA en el flavedo de frutos de pomelo Star Ruby tolerantes y sensibles al DF, y encontraron menores contendido en los sensibles. Esta relación inversa entre la concentración de AsA y los DF en los frutos de pomelo, tanto en la cosecha como durante el almacenamiento, sugiriere que el mayor contenido en frutos sensibles puede ser una respuesta a la mayor demanda de antioxidantes frente a las bajas temperaturas en situaciones de sensibilidad y desarrollo de los daños, y no estar directamente relacionados con la resistencia a los DF. Ya que la resistencia a los DF se produce en las zonas rojas de los frutos con alto contenido de licopeno, podría especularse que la presencia de este carotenoide con alta capacidad antioxidante evitaría o reduciría las respuestas habituales de los frutos a las bajas temperaturas, entre ellas el AsA. Finalmente, mencionar que Lo'ay y Dawood (2019) encontraron mayores niveles de AsA en el flavedo de frutos de limón Baladi cosechados en estado verde, que fueron más resistentes a los DF, que en frutos verde-amarillo.

1.4.2. Relación entre la capacidad antioxidante y el daño por frío en frutos cítricos

El papel de compuestos como los carotenoides, tocoferoles o AsA en la tolerancia al DF se ha atribuido tradicionalmente a su contribución a la capacidad antioxidante de los frutos (Decros et al., 2019; Hodges, Lester, Munro, & Toivonen, 2004). La capacidad antioxidante es un indicador de la habilidad de un extracto de neutralizar radicales libres y ROS y, por tanto, de mantener la integridad de las células frente a condiciones de estrés y prevenir el daño oxidativo (Decros et al., 2019; Zou, Wanpeng, Yan, Chao, & Zhiqin, 2016). La composición química y contenido de compuestos antioxidantes de un determinado tejido determinan su capacidad antioxidante total, a pesar de que no todos los compuestos contribuyen de la misma manera, si no que depende de sus propiedades antioxidantes, como su eficiencia para neutralizar radicales libres y ROS o su naturaleza hidrosoluble y liposoluble. En este sentido, el flavedo de los frutos cítricos contiene una mezcla compleja de compuestos con variadas funciones antioxidantes, entre los que destacan la vitamina C, polifenoles, tocoferoles y carotenoides, entre otros (Zou et al., 2016). A su vez, un estudio comparativo de frutos de diferentes especies de cítricos con distinta coloración y contenido de carotenoides y AsA indicó que cada grupo de compuestos contribuye de forma diferente a la capacidad antioxidante hidro- y liposoluble (Zacarías-García et al., 2020). Además, otro aspecto a tener en cuenta es que estos compuestos pueden tener una acción sinérgica frente al estrés oxidativo, de manera

que la protección que brinden en conjunto sea superior a la de cada uno individualmente (Niki, Noguchi, Tsuchihashi, & Gotoh, 1995; Szarka et al., 2012).

Existen diferentes metodologías para determinar la capacidad antioxidante, pero convencionalmente los métodos más utilizados para evaluar la capacidad antioxidante de alimentos y extractos vegetales han sido el DPPH, ABTS, FRAP y ORAC (Huang, Ou, & Prior, 2005). Mientras que los tres primeros métodos se basan en reacciones de transferencia de electrones, y miden la capacidad de un extracto para reducir un oxidante que cambia de color cuando se reduce, el método ORAC mide la capacidad de un antioxidante de neutralizar una mezcla de radicales libres (principalmente radicales peroxilo y superóxido (Huang et al., 2005). Lado et al. (2016) analizaron la capacidad antioxidante total en el flavedo de frutos de pomelo tolerantes y sensibles, mediante los métodos ABTS, DPPH y ORAC, y detectaron una mayor capacidad en las muestras de frutos sensibles al DF que en los frutos tolerantes, tanto en el momento de la cosecha como durante la conservación. Estas diferencias en la capacidad antioxidante se asociaron a los mayores contenidos de vitamina C, reforzando la idea que los frutos no tapados presentan una mayor demanda por antioxidantes. A su vez, la capacidad antioxidante medida por estos métodos aumentó durante la conservación en frío tanto en frutos sensibles como tolerantes, indicando que la estimulación del sistema antioxidante parece ser una respuesta al frío, no relacionada con el desarrollo de los DF. En frutos de otras variedades, como mandarina Fortuna o Satsuma, y también en frutos de limón Fino, la capacidad antioxidante DPPH, FRAP y ABTS se mantuvo constante durante el almacenamiento (Lafuente et al., 2011; Serna-Escolano et al., 2021; Shen et al., 2013) y, en el caso de Fortuna, no se detectaron diferencias entre frutos sensibles y tolerantes al DF (Lafuente et al., 2011). Por el contrario, en frutos de naranja sanguina con tolerancia al DF inducida por tratamientos químicos, se detectó un aumento en la capacidad antioxidante total, mientras que en los frutos control la capacidad fue menor y se redujo durante la conservación (Habibi, Serrano, Zacarías, Valero, & Guillén, 2021).

No obstante, estos métodos convencionales presentan una importante limitación técnica, ya que el efecto antioxidante se evalúa sobre radicales no fisiológicos (a excepción de ORAC) y, por tanto, son métodos con escasa relevancia biológica. En 2010 Ouchi et al. propusieron una nueva metodología denominada SOAC (por sus siglas en inglés "*Singlet Oxygen Absorption Capacity*"), que permite evaluar la capacidad de un extracto de secuestrar el oxígeno singlete, una de las ROS más tóxicas en sistemas celulares (Ouchi et al., 2010). Una de las ventajas de este método para evaluar la capacidad antioxidante en cítricos, u otros alimentos

o extractos ricos en carotenoides, es que utiliza solventes orgánicos que permiten extraer de la matriz o tejido los compuestos lipofílicos, evaluando su efecto sobre el $^1O_2$, que es una de las principales funciones de los carotenoides como antioxidantes (Stahl & Sies, 2003). El análisis de la capacidad antioxidante SOAC en el flavedo de frutos de pomelo Star Ruby tolerantes y sensibles al DF reveló que lo frutos tolerantes, que acumulaban licopeno, presentaban una mayor capacidad SOAC que aquellos sensibles, indicando que la tolerancia inducida por el licopeno se relaciona con una mayor capacidad de secuestrar $^1O_2$.

Teniendo en cuenta los antecedentes anteriormente descritos, el objetivo de esta Tesis Doctoral se enmarca dentro del proyecto genérico "profundizar en las bases bioquímicas y moleculares implicadas en la sensibilidad a los daños por frío durante la conservación postcosecha en frutos de diferentes especies y variedades de frutos cítricos". En concreto, el objetivo general de esta Tesis Doctoral fue **estudiar la implicación de los carotenoides y de los tocoferoles, como dos de los principales compuestos liposolubles con potente actividad antioxidante en las plantas, en la tolerancia a los daños por frío en frutos de distintas variedades de mandarina y pomelo durante la conservación a bajas temperaturas**. Para abordar este objetivo se plantearon los siguientes objetivos específicos:

1. Estudiar el papel de los carotenoides y el ácido ascórbico, y su relación con la capacidad antioxidante, en la susceptibilidad/tolerancia de frutos de mandarina a desarrollar daños por frío durante la conservación a bajas temperaturas. Para ello se utilizaron frutos de tres variedades de mandarina (Fortune, Nova y Nadorcott) con diferente intensidad de pigmentación de la piel y tolerancia a desarrollar daños por frío durante la conservación a 2 °C.

2. Estudiar el papel de los tocoferoles en la susceptibilidad de los frutos cítricos a desarrollar daños por frío durante la conservación a bajas temperaturas. Para ello se llevaron a cabo las siguientes actividades:

    2.1 Identificación de los genes de las principales rutas biosintéticas de los tocoferoles en los frutos cítricos.

    2.2 Análisis de los cambios en el contenido de tocoferoles y en la expresión de los genes de su biosíntesis en el flavedo de frutos de tres variedades de mandarina (Fortune, Nova y Nadorcott) con diferente tolerancia a desarrollar daños por frío durante la conservación a 2 °C.

    2.3 Análisis de los cambios en el contenido de tocoferoles y en la expresión de los de genes de su biosíntesis en el flavedo de frutos del pomelo Star Ruby en los que se ha inducido la tolerancia a los daños por frío durante la conservación a 2 °C.

3. Estudiar la regulación del metabolismo de tocoferoles a lo largo del desarrollo y maduración del fruto en las principales especies cultivadas de cítricos: naranja, mandarina, limón y pomelo. Para ello, se analizaron los cambios en el contenido de tocoferoles y en la expresión de genes de su biosíntesis en la piel y la pulpa de los frutos de las especies seleccionadas.

3.1. CAPÍTULO 1

Carotenoids, vitamin C, and antioxidant capacity in the peel of mandarin fruit in relation to the susceptibility to chilling injury during postharvest cold storage.

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Abstract

Chilling injury (CI) is a postharvest disorder occurring in fruit of cold-sensitive *Citrus* species during storage at low temperature. This study investigated the involvement of carotenoids and vitamin C, two major antioxidants of citrus peel, and the antioxidant capacity, in the CI susceptibility of mandarin fruit. To that end, fruit of three commercial varieties, Fortune, Nova and Nadorcott, with significant differences in CI susceptibility were selected. By on-tree fruit bagging, carotenoids and vitamin C contents were modified, and a differential effect of each cultivar on CI was observed. Carotenoid analysis in the peel revealed a strong negative correlation between total carotenoid concentration (TCC) at harvest, and specifically of β-cryptoxanthin and violaxanthin, and CI index at the end of storage. In contrast, vitamin C content was significantly and positively correlated with CI susceptibility. The antioxidant activity assessed by the DPPH• and FRAP reflected the contribution of vitamin C to the antioxidant system, while the SOAC assay correlated positively with TTC, β-cryptoxanthin and violaxanthin. Collectively, the antioxidant capacity of carotenoids at harvest, as efficient singlet oxygen quenchers, suggests a protective role against the development of CI in mandarin fruit, while vitamin C is not likely playing a critical role.

Keywords: antioxidant capacity; chilling injury; citrus fruit; carotenoids; mandarin; vitamin C

1. Introduction

Storage at low temperatures is the technology most widely used to maintain fruit quality and extend postharvest life. However, prolonged storage at low temperature may cause physiological disorders in many species of tropical and subtropical origin, such as citrus fruit, that are prone to develop external injuries during cold storage, depreciating their quality and increasing postharvest losses. Furthermore, some countries enforce quarantine measures on citrus fruit imports, requiring storage during transport at 1-2 °C. Damage developed during storage at low non-freezing temperatures is commonly known as chilling injury (CI) and, even though macroscopic symptoms in *Citrus* fruit vary between species and varieties, they are generally manifested as brown pit-like depressions in the flavedo (outer layer of fruit peel) that expand progressively over the peel surface and become darker under prolonged exposure to low temperatures [1–3].

Many pre- and postharvest factors have been described as influencing the susceptibility of citrus fruit to CI during postharvest storage. The genotype of the species/variety is an intrinsic genetic factor determining CI sensitivity or tolerance. Among *Citrus* species, lemon and grapefruit are more sensitive to CI than orange and mandarin, which are generally more tolerant [3]. Nonetheless, within mandarins and their hybrids, there is great variability in susceptibility to CI, with cultivars ranging from tolerant, such as those of the Clementine group, to highly sensitive, such as the hybrid Fortune [2,3]. Environmental factors and agronomic practices, such as temperature before harvest, maturity stage or canopy position, among others, can affect the biochemical profiling of the fruit peel and their response to postharvest cold storage and sensitivity to CI [3–5]. In line with this, higher carbohydrate content has been associated with higher tolerance to disorders during cold storage of Nules Clementine [6,7] and Pinalate sweet orange [8], although no relationship between changes in carbohydrate content and CI could be established in Fortune mandarin [9]. Other peel properties, such as rind coloration, are also influenced by environmental and endogenous factors and may be involved in the differential sensitivity to CI among *Citrus* cultivars [5,6].

The coloration of mature citrus fruit is mainly due to the accumulation of carotenoids [10,11]. Light is one of the most crucial environmental factors influencing carotenoid biosynthesis [12–14] and, in general, stimulates carotenoid accumulation by up-regulating key genes of their biosynthetic pathway [14–16]. In citrus fruit, light can have the opposite effect according to the tissue or species. For instance, the fruit of Nules Clementine mandarin and Navelina orange, which were bagged on the tree or harvested from inside of the tree canopy

(reduced light incidence), developed a paler rind color than those exposed to normal light incidence, which had an intense orange coloration and accumulated higher contents of total carotenoids [4,6,7,14]. The opposite effect was found in fruit of Star Ruby grapefruit, where avoiding light during fruit development on the tree promoted the accumulation of carotenes, in particular the red carotene lycopene, resulting in fruit with an intense red coloration of the peel in comparison with the pale-yellow peel of those exposed to direct sunshine [17].

The influence of carotenoid content and composition on the tolerance of citrus fruit to CI has been studied in grapefruit, where white cultivars accumulating very low levels of carotenoids in the peel were more prone to develop CI than red grapefruit with moderate to high levels [5]. Moreover, symptoms of CI in red grapefruit, such as the Star Ruby, were restricted to the yellow areas of the peel, whereas the red areas accumulating high amounts of lycopene were undamaged [5,17]. In mandarin fruit, the involvement of carotenoids on CI has not been studied in depth. However, in Clementine mandarin under South African growing conditions, it has been detected that fruit borne inside the tree canopy are less colored, contain less carotenoid content, and are more prone to develop rind-breakdown when stored at low temperature than fruits from outside the canopy [6,7].

The potential role of carotenoids in conferring higher tolerance to CI has been associated with their properties as antioxidants. Oxidative stress is thought to be a secondary response of plant cells to CI caused by an increase in the production of reactive oxygen species (ROS), due to low temperature stress and the plant's inability to counteract this proliferation of ROS [3,18]. Among carotenoids, lycopene is reported as an efficient singlet oxygen quencher [19,20]. Accumulation of this carotenoid in the flavedo of CI-tolerant red grapefruit was associated with lower oxidative damage (membrane structure and lipid peroxidation), higher singlet oxygen scavenging capacity (SOAC), and higher catalase activity [21]. Nonetheless, the potential role of other carotenoids as antioxidants, which accumulate at important concentrations in the peel of mandarins, in the tolerance/sensitivity of these fruits to CI has not been explored in detail yet.

Ascorbic acid (vitamin C) is another major antioxidant compound of the citrus fruit peel, which contributes to the antioxidant capacity of plant cells by detoxifying ROS and free radicals [22–24]. Vitamin C content in citrus varies among different species and cultivars and is widely affected by many pre-harvest factors, of which light is one of the most noteworthy [25–27]. Light avoidance produced a dramatic reduction in the content of vitamin C in the peel of orange, mandarin, and grapefruit, and downregulated the expression of key genes involved

in vitamin C synthesis and recycling [7,17,28]. The relation between vitamin C content and tolerance to CI in citrus fruit has been explored with controversial results. While a direct link between CI incidence and high vitamin C concentration was observed in the Navel orange [29], an indirect relationship was reported in Clementine mandarins [30], and no relation could be established in the Valencia orange [31] or Star Ruby grapefruit [32]. However, higher levels of ascorbic acid were found in the peel of CI-sensitive Star Ruby grapefruit than in CI-tolerant, at harvest and after storage at 2 °C [21]. It has been hypothesized that abiotic stress conditions, inducing ROS production, increase the requirements and response of plant tissues for antioxidants compounds, and this could explain the rise in ascorbic acid contents in CI-sensitive fruit [21,33].

Despite previous evidence indicating a relationship between sensitivity to CI and antioxidant compounds in fruits of different *Citrus* species and varieties, systematic research of this potential relation in mandarin fruit is still lacking. Therefore, the aim of the current study was to investigate the involvement of carotenoids and vitamin C, and the changes in antioxidant activity/capacity, in the susceptibility of mandarin fruit to CI. To that end, we used fruit of three commercial varieties: Fortune, Nova and Nadorcott, with differences in peel pigmentation and in the susceptibility to develop CI during postharvest cold storage.

2. Materials and Methods

2.1. Plant material and storage conditions

Fruit from 3 mandarin cultivars were used in this work: Fortune (*Citrus clementina* Hort. Ex Tan. × *Citrus reticulata* Blanco), Nova (*Citrus clementina* Hort. ex Tanaka × (*Citrus paradisi* Mc-Fadyen × *Citrus reticulata* Blanco)) and Nadorcott (*Citrus reticulata* Blanco). Fruits were harvested from the Citrus Germplasm Bank (Instituto Valenciano de Investigaciones Agrarias, IVIA, Valencia, Spain) and commercial orchards located in Valencia (Spain) under similar environmental conditions and agronomic practices. In the 3 cultivars, fruit bagging experiments were also performed, and light incidence was avoided during fruit ripening by covering the fruits with black polyethylene bags at the mature green stage (covered fruit, C), following the methodology previously described by Lado et al. [14]. Control fruit (non-covered, NC) were located outside of the canopy and exposed to direct natural light and normal photoperiodic conditions. At least 50 fruit were used for each treatment. At commercial maturity, both NC and C fruit were harvested and stored at 2 °C and 85% RH for up to 8 weeks. At harvest and periodically through cold storage, samples of flavedo (outer

colored layer of citrus fruit peel) were excised, frozen in liquid nitrogen, ground to a fine powder and stored at −80 °C until analysis.

2.2. Chilling injury evaluation

Fruit from each cultivar were inspected for CI symptoms throughout cold storage. Chilling injury in mandarin fruit generally manifested as small brown pit-like depressions in the flavedo ('pitting'), which become darker and sunken and expanded over the fruit surfaced with increase exposure to low temperatures [2,3]. According to the severity and extension of CI symptoms, mandarin fruits were assessed visually and classified using the following scale: 0 = no pitting; 1 = small pits covering <25% of the fruit surface; 2 = darker pits covering between 25 and 50% of the surface; and 3 = severe damage covering 50 to 100% of the surface (Figure S1). Results were expressed as CI index, which was calculated by adding the product of the number of fruit in each category multiplied by the score for each category and afterward dividing this amount by the total number of fruit evaluated [34]. For CI evaluation, at least 30 fruit per treatment and cultivar were used.

2.3. External peel color evaluation

Peel color was measured during cold storage using a Minolta CR-400 colorimeter (Minolta, Osaka, Japan) on 3 areas of the equatorial plane of the fruit and expressed as the *a/b* Hunter ratio [35]. The *a/b* ratio was negative for green fruit, around zero for yellow fruit at color break, and positive for orange and red colorations. For color evaluation, at least 10 fruit per treatment and cultivar were used.

2.4. Carotenoid extraction and analysis

Carotenoids were extracted from the flavedo as described previously in Rodrigo et al. [35], with slight modifications. Briefly, 0.5 g of flavedo tissue were extracted with 3 mL of methanol (HPLC grade, Sharlau, Barcelona, Spain), 1.5 mL Tris buffer (50 mM Tris-HCl pH 7.5 with 1 M NaCl) and 4 mL of chloroform (HPLC grade, Sharlau, Barcelona, Spain) in a mortar and pestle with sea sand (PanReac, AppliChem, Barcelona, Spain) as an abrasive. The homogenate was recovered in a polypropylene tube and sonicated in a XUBA3 ultrasonic water bath (Grant Instruments, Cambridge, England) for 5 min at room temperature. After this, samples were centrifuged at 4500× *g* for 10 min at 4 °C for liquid-phase separation. The hypophase was recovered in a new tube and the aqueous phase was re-extracted with chloroform until it was colorless. The pooled chloroform extracts were concentrated on a rotatory evaporator at 30 °C, and saponified overnight using a 10% methanolic:KOH solution under N_2 atmosphere.

Saponified carotenoids were recovered from the upper phase after adding water and petroleum ether:diethyl ether (9:1, *v:v*) to the mixture. Extracts were dried under a nitrogen stream and kept at −20 °C until further analysis.

Carotenoid composition was analyzed by high-performance liquid chromatography (HPLC) with a Waters liquid chromatography system equipped with a 600E pump, coupled to a 2998 photodiode array detector (PAD) and Empower3 software (Waters, Barcelona, Spain). A C30 carotenoid column (250 × 4.6 mm, 5 µm) coupled to a C30 guard column (20 × 4.0 mm, 5 µm) (YMC, Tecknochroma, Barcelona, Spain) was used. Samples were prepared for HPLC by dissolving the dried carotenoid extracts in chloroform:methanol:acetone (3:2:1, *v:v:v*). A ternary gradient elution with methanol, wáter, and methyl tert-butyl ether was used for carotenoid separation as reported in previous work [35]. The PAD was set to scan from 250 to 540 nm throughout the elution profile. For each elution, a Maxplot chromatogram was obtained, which integrated each carotenoid peak at its corresponding maximum absorbance wavelength. Individual carotenoid content was calculated using previously described calibration curves [14,36]. A characteristic chromatogram is displayed in Figure S2. The limit of detection and working linear range for carotenoids quantified is shown in Table S1. All operations were carried out on ice under dim light to prevent photodegradation, isomerization, and structural changes of carotenoids. At least 2 independent flavedo extracts were obtained for each sample and each extract was injected twice on HPLC system. Results are the mean of at least two replicates from each extract (mean ± SD). Total carotenoids (TCC) were calculated as the sum of all the individual carotenoids quantified. Standard deviations were calculated from the mean of the total carotenoids value of each replicate. The concentrations are expressed as µg per g of fresh weight (FW).

2.5. Vitamin C determination

Ascorbic acid (AsA) and dehydroascorbic acid (DHA) contents were determined as described by Alós et al. [26]. Briefly, 0.5 g of flavedo tissue was homogenized for 1 min using a homogenizer (Polytron, Eschbach, Germany) with 0.1% metaphosphoric acid (4 mL) at maximum speed. The homogenate was centrifuged for 10 min at 4500× *g* at 4 °C. Then, the supernatant was filtered through a C18 cartridge (SepPak, Waters, Barcelona, Spain) previously activated with 4 mL of methanol, 4 mL of water and 4 mL of 0.1% metaphosphoric acid. The filtered extract was re-filtered through a 0.45 µm nylon filter (25 mm diameter, Análisis Vínicos, Tomelloso, Spain), and the filtrate was injected in the HPLC-PAD system for AsA determination. DHA content was calculated from the difference between total vitamin C

and the AsA content. To determine total vitamin C, an adaptation of the protocol described by Davey et al. [37] was used. Thus, 200 µL aliquot of the above-mentioned filtrate was incubated for 15 min at room temperature with 100 µL 200 mM DTT in 400 mM Tris-base, which generated a final pH of 6–6.8. Then, the reaction was stopped by acidification with 100 µL of 8.5% orthophosphoric acid.

For AsA and total vitamin C determination, 10 µL of the above mentioned extracts were injected in a Dionex HPLC system with a PAD and Chromeleon software (Dionex, Thermo Fischer Scientific, Barcelona, Spain), an Ultrabase C18 column (100 × 4.6 mm, 2.5 µm) with a mobile phase of methanol:water pH 2.5 (adjusted with metaphosphoric acid, 15:85, *v:v*) at 0.2 mL min^{-1} flux. The temperature of the column was set at 35 °C. The PAD was set to monitor the spectrum from 200 to 450 nm, and the peak area at 248 nm (wavelength of maximum absorption for AsA) was used for quantification. A characteristic chromatogram was displayed in Figure S3. The method was calibrated daily with a curve of an AsA standard solution in 0.1% metaphosphoric acid. Results are expressed as mg per 100 g of sample (FW).

2.6. Antioxidants assays

2.6.1. DPPH and FRAP assays

Two different assays were performed to measure antioxidant activity in the flavedo: (i) Reaction with 2,2-diphenyl-1-picrylhydrazyl (DPPH• assay) and (ii) ferric-reducing antioxidant power in aqueous solution (FRAP assay). The same extraction method was carried out for both assays. Briefly, 0.15 g of frozen flavedo tissue was extracted in 3 mL of 80% methanol using a pre-chilled mortar and pestle on an ice bath. The homogenate was centrifuged for 5 min at 4500× *g* (4 °C), and the collected supernatant was immediately used for analysis. Each assay was replicated twice, and a curve of AsA solution in methanol 80% was used as a standard in both assays.

Determination of the DPPH• assay was performed as described in Parra-Rivero et al. [38] with slight modifications. In a 96-well microplate, 10 µL of the sample extract were mixed with 290 µL DPPH• methanolic solution (100 µM) and allowed to stand for 30 min at room temperature in complete darkness. Each sample was replicated in 3 wells within one microplate, and methanol (80%) was used as a control (10 µL methanol 80% + 290 µL of DPPH•). Changes in the absorbance were measured at 512 nm in a UV/Vis microplate spectrophotometer (Multiskan FC, Thermo Fisher Scientific, Barcelona, Spain) and compared to the control. DPPH• scavenging capacity was expressed as inhibition percentages by the Formula (1):

$$\%\ DPPH\bullet\ scavenging\ capacity = [(^{515nm}Acontrol - {}^{515nm}Asample)/\ {}^{515nm}Acontrol] \times 100 \quad (1)$$

For the FRAP assay the procedure described by Parra-Rivero et al. [38] adapted for flavedo tissue was followed. First, a reagent was prepared with 2,4,6-tri(2-pyridyl)-1,3,5-triazine (10 mM), acetate buffer (300 mM) and a ferric chloride solution (20 mM) (1:10:1, *v:v:v*). Then, 40 µL sample extract were mixed with 260 µL of the FRAP reagent on a microplate well, allowing the microplate to stand at 37 °C for 30 min after loading. Each sample was replicated in 3 wells within 1 microplate and methanol (80%) was used for control (40 µL methanol 80% + 260 µL of FRAP). Absorbance was measured at 593 nm in a UV/Vis microplate spectrophotometer (Multiskan FC, Thermo Fisher Scientific, Barcelona, Spain). To calculate FRAP, the antioxidant activity sample absorbance was first corrected by the absorbance of control. In both assays, results are the mean of the replicates in the 2 microplates (mean ± SE).

2.6.2. Singlet oxygen absorption capacity (SOAC)

The SOAC determination was done following previous procedures [21,39,40] with slight modifications. Briefly, 0.3 g of frozen flavedo tissue was extracted in 6 mL of cooled ethanol:chloroform:water (50:50:1, *v:v:v*) using a pre-chilled mortar and pestle on an ice bath with sea sand (PanReac, AppliChem, Barcelona, Spain) as an abrasive. Then, the homogenate was centrifuged for 5 min at 4500× *g* at 4 °C and the collected supernatant was immediately used for analysis.

To measure the extracts $^1O_2$ quenching capacity a competition reaction was carried out using endoperoxide (EP, Invitrotech, Kyoto, Japan) as a singlet oxygen generator and 2,5-diphenyl- 3,4-benzofuran (DPBF, Sigma–Aldrich, Barcelona, Spain) as an UV-Vis absorption probe in a 96-well quartz glass microplate. In each well, 15 µL of the peel extract was mixed with 150 µL of DPBF (0.8 mM solution) and 75 µL of EP (1 mM). The microplate was loaded in dim light and on an ice bath. Absorbance changes of DPBF at 413 nm were monitored during a 90 min reaction at 35 °C using a UV-Vis spectrophotometer microplate reader (SPECTROstar® Omega, BMG Labtech, Offenburg, Germany). α-tocopherol (Sigma–Aldrich, Barcelona, Spain) was used as a standard compound and ethanol:chloroform:water (50:50:1, *v:v:v*) as a blank. The relative SOAC value for each sample was calculated with the following Formula (2):

$$(t1/2\ sample - t1/2\ blank) / (t1/2\ \alpha\text{-toc} - t1/2\ blank) \times ([\alpha\text{-toc, g L}^{-1}] / [sample, g L^{-1}]) \quad (2)$$

Each sample was replicated in 3 wells, and the assay was replicated twice. Results were the mean of the replicates in the 2 microplates (mean ± SE).

2.7. Statistical analyses

Results were expressed as the mean ± standard error (SE). Unpaired Student's t-test was used to determine the mean differences statistically between NC and C fruit, within each cultivar and sample date (significance level at $p \leq 0.05$ or $p \leq 0.10$). Additionally, a one-way ANOVA was carried out and Tukey's test (significance level at $p \leq 0.05$) was used for mean comparisons among dates. The correlations between variables were examined by Pearson's correlation. Analysis were made using the XLSTAT Software version 2019.3.2 (Addinsoft, France) and SigmaPlot version 14.0 (Systat Software, USA).

3. Results

3.1. Incidence of chilling injury and changes in peel pigmentation of Fortune, Nova and Nadorcott mandarins during cold storage

The mandarins Fortune, Nova and Nadorcott were selected on their reported different sensitivity to CI and peel color intensity. Fortune and to a lower extent Nova fruit are prone to develop CI during storage at low temperatures [34,41], while Nadorcott appears to be more resistant to CI, even though it is susceptible to other postharvest blemishes [2,42]. Nadorcott is a late maturing cultivar characterized by a deep reddish-orange rind and pinkish albedo, and excellent internal quality [43].

Sensitivity to CI was evaluated in NC and C fruit of Fortune, Nova, and Nadorcott during storage at 2 °C for up to 8 weeks (Figure 1 and 2). Symptoms of CI appeared earlier in Fortune than in Nova mandarins, but in both cultivars, symptoms manifested as the typical peel pitting, with small shrunken darks pits spread over the surface that progressively expanded creating large necrotic and depressed areas, depreciating external fruit quality (Figure 1 and Figure S1). In fruit of the Nadorcott mandarin, CI symptoms were almost absent in control fruits, and only few pits, in general around the stylar zone, were observed after prolonged cold storage (Figure 1 and Figure S4).

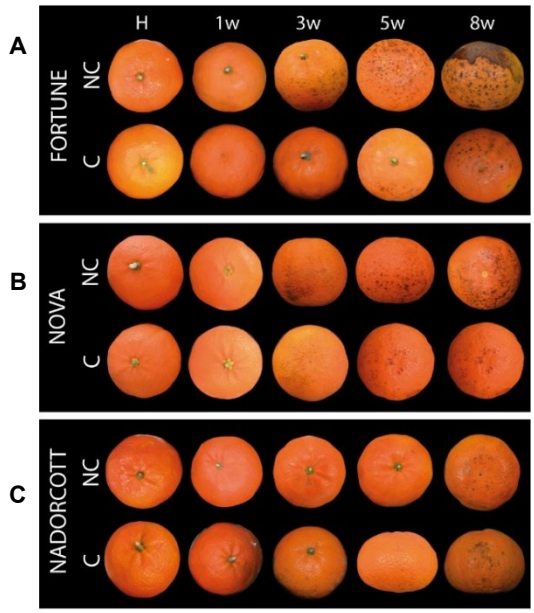

Figure 1. External appearance of fruit of Fortune (**A**), Nova (**B**) and Nadorcott (**C**) at harvest (H) and evolution of chilling injury (CI) symptoms during storage at 2 °C of non-covered (NC) and covered (C) fruit for up to 8 weeks.

The CI index increased markedly in NC fruits of Fortune after 2 weeks of storage, reaching values of 2 by week 3 and about 3 after 5 weeks, and almost all fruit exhibited severe symptoms at the end of storage (Figure 2A). In Nova mandarin (NC fruit), CI increased steadily to reach at the end of the storage period an index of 2 (Figure 2B). NC fruits of Nadorcott mandarin were resistant to CI, developing an index of 0.3 at the end of the storage period (Figure 2C). Since light avoidance during the last stage of fruit development has been shown to induce cold-tolerance in Star Ruby grapefruit during postharvest storage [21], mature-green fruits of the three cultivars were covered, and the incidence on CI during cold storage evaluated. Covered fruit of Fortune and Nova mandarin exhibited a similar response during cold storage, with a delay in the development of symptoms and a reduction in CI at the end of the storage period with respect to NC fruits (Figure 2A,B). In contrast, light avoidance produced the opposite effect on fruit of the Nadorcott mandarin, as the CI index was higher in NC fruit than in C after 3 weeks, being about 4-times higher at the end of the cold storage (Figure 2C).

Rind color (*a/b* Hunter ratio) was monitored in NC and C fruit of the three cultivars at harvest and during cold storage (Figure 2D–F). At harvest, NC fruits of the Nadorcott mandarin had a more intense coloration and higher *a/b* ratio than Fortune and Nova mandarins. Only C fruit of Nadorcott was affected by light avoidance, resulting in less colored fruit and lower *a/b* ratio than NC fruit, whereas no significant differences were detected in Fortune and Nova. In general, during cold storage, the color index remained steady or

decreased slightly and, although not always significant, C fruit were paler and had a lower *a/b* ratio than NC fruit (Figure 2D–F).

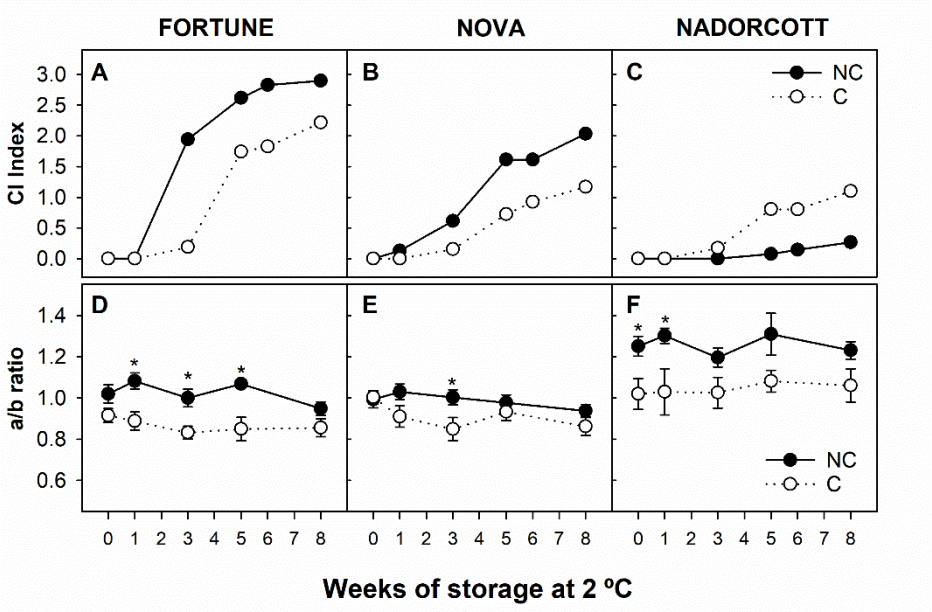

Figure 2. Development of chilling damage expressed as the CI index of non-covered (NC) and covered (C) fruit of Fortune (A), Nova (B) and Nadorcott (C); and rind color expressed as a/b ratio of non-covered (NC) and covered (C) fruit of Fortune (D), Nova (E) and Nadorcott (F), at harvest (0) and during storage at 2 °C. Asterisks indicate significant differences between NC and C fruit for each cultivar and sample date ($p \leq 0.05$, t-test).

3.2. Changes in carotenoids and vitamin C in the peel of Fortune, Nova and Nadorcott mandarins during cold storage

3.2.1. Total carotenoid content and carotenoid composition

Carotenoid content and composition were analyzed in NC and C fruit of Fortune, Nova and Nadorcott at harvest and after 3 and 8 weeks of cold storage (Tables 1–3). At harvest, the highest TCC was detected in the flavedo of Nadorcott NC fruit (582.20 ± 22.85 μg g^{-1} FW), which was 3.1- and 1.3-times higher than that of Fortune and Nova, respectively. Fruit bagging had a differential effect on TCC depending on the cultivar. In the peel of Fortune mandarin, TCC was lower than in the other two cultivars and was not affected by the absence of light. During cold storage, TCC increased in NC fruit and remained constant in C fruit of Fortune (Table 1). Light avoidance reduced by about 30% TCC in Nadorcott and Nova mandarins with respect to NC fruit exposed to natural photoperiodic conditions (Tables 2 and 3). Cold storage did not affect TCC in NC and C fruit of Nova mandarin (Table 2), but provoked a reduction in NC fruit of Nadorcott (Table 3). Nonetheless, TCC at the end of cold storage remained higher in NC than in C fruits of these two mandarins (Tables 2 and 3).

Table 1. Individual and total carotenoids (µg g-1 FW) detected in the peel of non-covered (NC) and covered (C) fruit of Fortune mandarin at harvest (0) and after 3 and 8 weeks of storage at 2 °C.

Carotenoids (µg g^{-1} FW)	FORTUNE											
	0				3				8			
	NC		C		NC		C		NC		C	
Phytoene	6.34±0.40	**c**	17.02±2.53**	**A**	12.61±2.09*	**b**	7.64±0.20	**B**	19.62±1.24**	**a**	5.09±2.20	**B**
Phytofluene	1.95±0.28	**a**	4.31±1.03*	**A**	1.51±0.19	**a**	1.44±0.17	**B**	2.59±0.87	**a**	1.00±0.23	**B**
β-cryptoxanthin	3.44±1.02	**a**	2.23±0.30	**A**	3.56±2.27	**a**	5.29±2.63	**A**	1.96±0.43	**a**	8.23±3.19*	**A**
β-citraurin	41.83±8.39	**a**	29.79±5.48	**A**	31.68±4.79	**a**	23.06±1.23	**A**	39.89±0.50**	**a**	24.50±3.16	**A**
Antheraxanthin	14.45±1.34	**b**	14.25±1.13	**B**	24.49±1.64**	**a**	7.91±0.24	**C**	31.14±2.61	**a**	24.74±1.69	**B**
Luteoxanthin	2.74±0.68	**b**	2.61±0.25	**B**	2.55±0.11*	**b**	1.87±0.22	**B**	4.75±0.10	**a**	5.73±0.98	**A**
Violaxanthin	99.37±2.61	**a**	86.56±8.96	**A**	100.35±6.97	**a**	97.50±14.53	**A**	119.28±4.17	**a**	120.79±2.84	**A**
Lutein	0.58±0.34	**a**	0.88±0.40	**A**	1.19±0.01	**a**	nd		2.07±0.57	**a**	1.07±0.20	**A**
Other β.β-xanthophylls	12.99±2.58	**b**	25.62±5.65*	**A**	23.66±3.24*	**a**	8.96±4.39	**A**	29.52±0.96**	**a**	10.05±3.82	**A**
Total	**185.90±17.86**	**b**	**189.53±13.67**	**A**	**204.03±15.33***	**ab**	**157.28±14.24**	**A**	**256.32±9.12****	**a**	**201.88±3.75**	**A**

Asterisks indicate significant differences between NC and C fruit for each cultivar and sample date (***p ≤ 0.05*; **p ≤ 0.10, t-test*). Bold lowercase letters indicate significant differences between dates in NC fruit and bold capital letters indicate significant differences between dates in C fruit (p ≤ 0.05, *Tukey test*). *nd*: non-detected.

Table 2. Individual and total carotenoids (µg g^{-1} FW) detected in the peel of non-covered (NC) and covered (C) fruit of Nova mandarin at harvest (0) and after 3 and 8 weeks of storage at 2 °C.

Carotenoids (µg g^{-1} FW)	NOVA											
	0				3				8			
	NC		C		NC		C		NC		C	
Phytoene	98.12±8.60**	**a**	41.19±4.01	**C**	120.23±4.37**	**a**	81.11±4.83	**A**	103.43±9.12**	**a**	59.65±3.30	**B**
Phytofluene	24.17±2.40**	**a**	12.71±0.76	**C**	36.26±0.48**	**a**	28.34±1.40	**A**	32.32±4.38*	**a**	18.56±1.18	**B**
β-cryptoxanthin	25.36±1.43*	**a**	19.47±1.43	**A**	44.54±0.95	**b**	27.24±14.12	**A**	24.09±4.18*	**b**	12.54±2.02	**A**
β-citraurin	37.77±2.97	**a**	58.69±5.68**	**A**	23.32±0.55	**b**	39.22±11.51	**A**	36.26±0.59	**a**	36.29±0.43	**A**
Antheraxanthin	29.70±2.15**	**a**	8.22±2.27	**B**	34.90±4.01*	**a**	22.85±0.86	**A**	34.93±0.80**	**a**	17.55±0.46	**A**
Luteoxanthin	5.35±2.64	**a**	2.44±0.02	**A**	4.15±0.19**	**a**	1.83±0.40	**A**	5.65±0.25**	**a**	2.41±0.39	**A**
Violaxanthin	169.09±4.00**	**a**	133.12±0.56	**A**	159.17±6.42**	**a**	116.84±8.21	**A**	168.07±1.13**	**a**	134.73±2.20	**A**
Lutein	7.56±0.09*	**a**	2.59±1.95	**A**	9.06±1.10**	**a**	4.11±0.96	**A**	7.67±1.03*	**a**	3.22±0.03	**A**
Other β.β-xanthophylls	39.52±10.13	**a**	25.00±14.30	**A**	32.96±6.58**	**a**	7.38±0.66	**A**	38.09±5.56*	**a**	14.83±6.96	**A**
Total	**444.36±1.59****	**a**	**308.85±17.79**	**A**	**478.06±16.59****	**a**	**338.44±41.35**	**A**	**463.61±15.52****	**a**	**304.30±9.26**	**A**

Asterisks indicate significant differences between NC and C fruit for each cultivar and sample date (***p ≤ 0.05*; **p ≤ 0.10, t-test*). Bold lowercase letters indicate significant differences between dates in NC fruit and bold capital letters indicate significant differences between dates in C fruit (p ≤ 0.05, *Tukey test*).

Table 3. Individual and total carotenoids (μg g^{-1} FW) detected in the peel of non-covered (NC) and covered (C) fruit of Nadorcott mandarin at harvest (0) and after 3 and 8 weeks of storage at 2 °C.

Carotenoids (μg g^{-1} FW)	NADORCOTT											
	0				3				8			
	NC		C		NC		C		NC		C	
Phytoene	59.90±1.18**	**a**	29.75±1.07	**A**	48.06±0.21**	**b**	24.43±0.84	**A**	21.06±3.87*	**c**	12.29±1.77	**B**
Phytofluene	17.30±2.21**	**a**	7.70±0.30	**A**	12.88±1.44**	**a**	3.69±0.63	**B**	11.28±0.36**	**a**	5.51±0.49	**B**
β-cryptoxanthin	61.46±8.05	**ab**	68.87±16.53	**A**	84.87±5.54**	**a**	66.98±0.83	**A**	54.15±4.73	**b**	68.38±2.46*	**A**
β-citraurin	56.75±9.55	**a**	46.22±6.36	**A**	32.69±1.26	**a**	30.13±5.38	**A**	48.52±5.48**	**a**	31.15±1.02	**A**
Antheraxanthin	42.74±2.15**	**b**	26.37±2.22	**B**	58.21±1.70**	**a**	42.56±0.64	**A**	50.96±4.52**	**ab**	33.08±1.59	**B**
Luteoxanthin	7.54±3.36	**a**	2.85±1.00	**A**	3.87±2.97	**a**	5.41±0.63	**A**	5.06±1.37	**a**	2.05±0.15	**A**
Violaxanthin	264.06±16.51**	**a**	209.95±2.14	**A**	242.69±3.51**	**ab**	225.78±1.06	**A**	218.18±3.71	**b**	202.19±19.83	**A**
Lutein	9.82±1.82	**a**	8.30±0.65	**B**	17.41±2.54	**a**	13.33±0.03	**A**	11.23±2.28	**a**	12.19±1.19	**A**
Other β.β-xanthophylls	51.10±5.83**	**a**	18.72±2.21	**A**	54.06±9.67**	**a**	13.65±0.94	**AB**	34.14±5.62*	**a**	12.58±0.08	**B**
Total	**582.20±22.85****	**a**	**424.99±16.80**	**AB**	**565.05±9.10****	**a**	**430.16±2.50**	**A**	**479.82±14.39****	**b**	**381.69±25.44**	**B**

Asterisks indicate significant differences between NC and C fruit for each cultivar and sample date (**p ≤ 0.05; *p ≤ 0.10, *t-test*). Bold lowercase letters indicate significant differences between dates in NC fruit and bold capital letters indicate significant differences between dates in C fruit (p ≤ 0.05, *Tukey test*).

By HPLC-PDA analysis a total of 22 carotenoid-like peaks were identified in the flavedo of the three mandarins (Table S2). Comparing the spectra and retention times of these peaks to authentic standards or those described in other works using similar chromatographic conditions [14,35,44], we were able to unambiguously identify 13 carotenoids (Table S2), which represented more than 90% of TCC (Tables 1–3). The flavedo of the three mandarin cultivars displayed a carotenoid profile characteristic of mature mandarin, accumulating predominantly β,β-xanthophylls, which represented 60-80% of TCC. The β,β-xanthophyll violaxanthin and, in particular, its 9-Z isomer, was the main carotenoid accounting for 40-60% of TCC. The content of individual carotenoids differed among the three cultivars, and, in general, the highest concentrations for all carotenoids were detected in Nadorcott. However, Nova showed the highest content of the colorless carotenes phytoene and phytofluene in all samples. In the three cultivars, high concentrations of the *Citrus*-specific C30 apocarotenoid β-citraurin, which confers an orange-reddish coloration, were quantified. At harvest, β-citraurin contents ranged from nearly 30 μg g^{-1} in Fortune to around 58 μg g^{-1} FW in Nova (Table 1–3). The concentration of β-cryptoxanthin, the characteristic xanthophyll of mandarins, which also confers a deeper orange coloration to the peel, was extremely high in Nadorcott (~65 μg g^{-1}) when compared to the other cultivars. In Nova, β-cryptoxanthin content was also high (between 20-25 μg g^{-1}), while in Fortune, moderate to low amounts (below 5 μg g^{-1}) were detected.

When the carotenoids profile between NC and C fruit is compared in Nova and Nadorcott, the differences in TCC are explained by a higher accumulation of almost all carotenoids in NC fruits at harvest, which remained during storage (Tables 2 and 3). The concentration of phytoene was two-fold higher in NC fruit compared to C fruit in most samples of Nova and Nadorcott. Violaxanthin content was 20% higher in NC than in C fruit of Nova and Nadorcott at harvest and, while differences in Nova persisted during storage, in Nadorcott, they shortened and no significant differences were detected after 8 weeks. The concentration of antheraxanthin was also higher in NC than C fruit of both Nova (2–3 times higher) and Nadorcott (about 1.5-fold). At harvest, the levels of β-cryptoxanthin were not significantly different between NC and C fruit of Nadorcott but were higher in NC fruit of Nova. Regarding β-citraurin, an apocarotenoid with high impact in the peel color, the content at harvest in Nova was higher in C than in NC fruit, whereas no differences were observed in Nadorcott. After 8 weeks of storage, β-citraurin levels were the same between NC and C fruit of Nova, and significantly higher in NC fruit of Nadorcott than in C fruit. In Fortune mandarin, differences between NC and C fruit and changes in carotenoid levels during storage differed from those described in the other two cultivars. At harvest, the concentration of phytoene and phytofluene was significantly higher in C fruit. However, after 8 weeks of cold storage, the opposite effect occurred, and NC fruit accumulated higher phytoene. Moreover, differences in individual carotenoids between NC and C Fortune fruit during cold storage were less evident than for Nova and Nadorcott, being remarkable the lower concentration of phytoene, β-cryptoxanthin, β-citraurin, and other β,β-xanthophylls in C fruits after 8 weeks of storage (Table 1).

3.2.2. Vitamin C content

Vitamin C (AsA + DHA) content was determined in the flavedo of NC and C fruit of the three mandarins at harvest and after 3 and 8 weeks of cold storage (Figure 3). Among NC fruit, Fortune accumulated the highest amount of vitamin C (~240 mg 100 g^{-1}), which was twice the content detected in Nova and Nadorcott, both with similar levels (~120–150 mg 100 g^{-1}). Light deprivation produced a significant reduction in vitamin C content in the flavedo of all varieties (around 50% in Nadorcott and 30% in Fortune and Nova). During cold storage, vitamin C remained nearly constant and the differences between NC and C fruit were maintained.

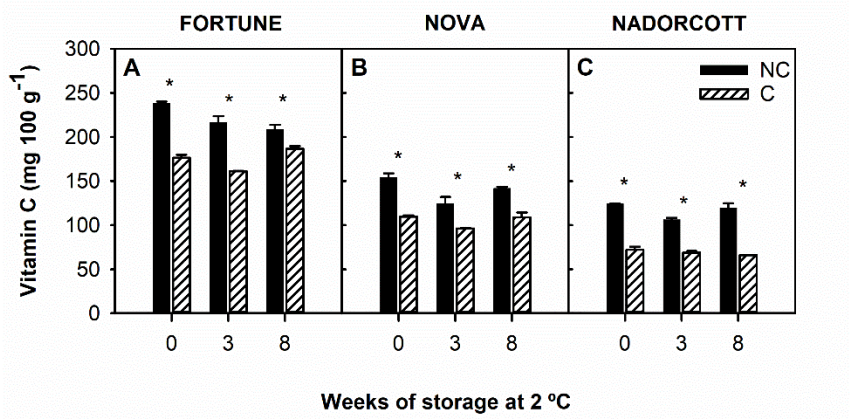

Figure 3. Total vitamin C content (mg 100 g^{-1}) at harvest (0) and during storage at 2 °C in the peel of non-covered (NC) and covered (C) fruit of Fortune (A), Nova (B) and Nadorcott (C). Asterisk indicates significant differences between NC and C fruit for each cultivar and sample date ($p \leq 0.05$, t-test).

3.3. Antioxidant activity and singlet oxygen absorption capacity in the peel of Fortune, Nova and Nadorcott mandarins during cold storage

The antioxidant activity of the flavedo of the three mandarin cultivars was determined in hydrophilic extracts by DPPH• (radical scavenging activity) and FRAP (reducing activity) at harvest and after 8 weeks of storage in both NC and C fruit (Figure 4). Results obtained by both DPPH• and FRAP methods were similar. At harvest, the DPPH• and FRAP antioxidant activity of Fortune and Nova mandarin were comparable and about twice that of Nadorcott mandarin. Light avoidance produced a slight reduction in the antioxidant activity of Fortune and Nadorcott mandarins, and reduced between 35–45% that of Nova fruit. The DPPH• and FRAP antioxidant activity of the extracts remained fairly stable after 8 weeks of cold storage in both NC and C fruit of the three cultivars (Figure 4). Extracts from Fortune fruit (NC and C) and Nova NC fruit showed the highest relative values of DPPH• and FRAP antioxidant activity in comparison to Nadorcott (NC and C) and Nova C fruit, which showed 50 to 20% lower values, respectively (Figure 4).

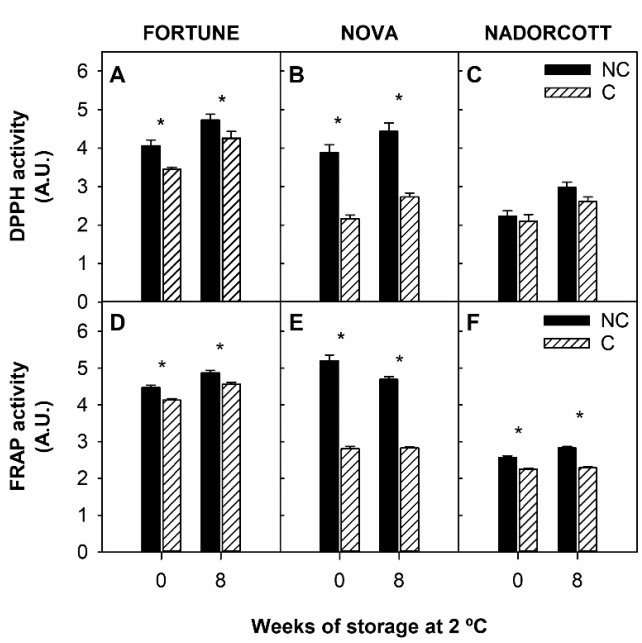

Figure 4. DPPH• antioxidant activity in the peel of non-covered (NC) and covered (C) fruit of Fortune (A), Nova (B) and Nadorcott (C); FRAP antioxidant activity in the peel of non-covered (NC) and covered (C) fruit of Fortune (D), Nova (E) and Nadorcott (F), at harvest (0) and after 8 weeks of storage at 2 °C. Asterisk indicates significant differences between NC and C fruit for each cultivar and sample date ($p \leq 0.05$, t-test).

The antioxidant potential of the flavedo of the three cultivars at harvest and after 8 weeks of cold storage was also determined by the SOAC assay, which measures the singlet oxygen scavenging capacity of a lipophilic extract. In general, SOAC values were higher in Nadorcott fruit than in the other cultivars. At harvest, the SOAC capacity of NC Nadorcott fruit extract was 62% and 40% higher than that of Fortune and Nova, respectively, and these differences were slightly reduced after 8 weeks of storage (Figure 5). SOAC was not significantly affected by light deprivation, at harvest or after cold storage, with the exception of Nova after 8 weeks of storage when the SOAC was 1.8 times higher in NC than in C fruit (Figure 5B).

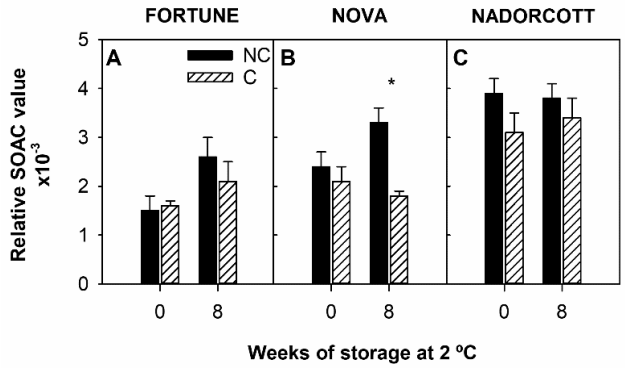

Figure 5. Relative SOAC value at harvest (0) and after 8 weeks of storage at 2 °C in the peel of non-covered (NC) and covered (C) fruit of Fortune (A), Nova (B) and Nadorcott (C). Asterisks indicate significant differences between NC and C fruit for each cultivar and sample date ($p \leq 0.05$, t-test).

3.4. Correlation analysis between chilling injury index, carotenoids, vitamin C and DPPH•, FRAP and SOAC antioxidant activity/capacity in the peel of Fortune, Nova and Nadorcott during cold storage

The correlation between CI index after 8 weeks of cold storage, carotenoid content (total and individual), vitamin C and DPPH•, FRAP and SOAC antioxidant activity/capacity at harvest was analyzed. The analysis integrated data from the three cultivars in both NC and C conditions. According to the data in Table 4, CI index after cold storage was significantly and negatively correlated with TCC (r^2 = –0.81), β-cryptoxanthin (r^2 = –0.82), violaxanthin (r^2 = –0.85) and SOAC capacity (r^2 = –0.88) at harvest. On the other hand, CI showed a significant positive correlation with vitamin C content (r^2 = 0.82), DPPH• (r^2 = 0.89) (sample´s reducing activity) and FRAP (r^2 = 0.81) (sample`s radical scavenging activity). Also, the correlation analysis shows that the DPPH• method reflects the vitamin C contribution to the antioxidant system (r^2 = 0.87), and that SOAC is highly influenced by TCC (r^2 = 0.96) and by the individual xanthophylls violaxanthin (r^2 = 0.99) and β-cryptoxanthin (r^2 = 0.92).

Table 4. Pearson's correlation coefficients (r^2) among CI at the end of storage (8 weeks) and total carotenoid content (TCC), individual carotenoids (β-cryptoxanthin, β-citraurin and Violaxanthin), vitamin C and antioxidant capacity/activity (SOAC, DPPH• and FRAP) at harvest.

	CI	TCC	β-cryptoxanthin	β-citraurin	Violaxanthin	Vitamin C	SOAC	DPPH•	FRAP
CI	1	-0.81*	-0.82*	-0.74	-0.85*	0.82*	-0.88*	0.89*	0.81*
TCC	-0.81*	1	0.85*	0.52	0.97*	-0.62	0.96*	-0.52	-0.41
β-cryptoxanthin	-0.82*	0.85*	1	0.51	0.93*	-0.78	0.92*	-0.73	-0.71
β-citraurin	-0.74	0.52	0.51	1	0.57	-0.51	0.55	-0.75	-0.71
Violaxanthin	-0.85*	0.97*	0.93*	0.57	1	-0.64	0.99*	-0.62	-0.57
Vitamin C	0.82*	-0.62	-0.78	-0.51	-0.64	1	-0.66	0.87*	0.75
SOAC	-0.88*	0.96*	0.92*	0.55	0.99*	-0.66	1	-0.65	-0.60
DPPH•	0.89*	-0.52	-0.73	-0.75	-0.62	0.87*	-0.65	1	0.96*
FRAP	0.81*	-0.41	-0.71	-0.71	-0.57	0.75	-0.60	0.96*	1

Asterisk indicates significant Pearson's correlation coefficient at level $p \leq 0.05$.

4. Discussion

Exposure of *Citrus* fruit to chilling temperatures during storage supposes a challenge in species sensitive to the development of CI symptoms, which depreciates their external quality and increases postharvest losses. Fruit conditions at harvest, including peel color, can influence cold sensitivity during postharvest [2,3]. The relationship between peel pigmentation and sensitivity to CI has been explored in grapefruit cultivars, showing that those with red-colored peel display higher tolerance to CI than yellow-colored ones [5,21]. In

mandarin fruit, it has been traditionally considered that pale fruits from inside the tree canopy develop less external coloration and are more prone to postharvest blemishes, in particular CI, than fruits borne outside of the tree canopy with higher pigmentation [4,7,45]. However, solid evidence linking CI and carotenoids, or other antioxidants as vitamin C, in relation to their antioxidant activities, is still lacking. To provide insights into this question, we selected fruit of three mandarin cultivars (Fortune, Nova and Nadorcott) with marked differences in peel pigmentation and susceptibility to develop CI during cold storage. Since carotenoids and vitamin C contents in the peel of citrus fruit are largely influenced by light during ripening [14,17,25], pre-harvest bagging experiments were performed in order to modify the content of these compounds in the peel, and subsequently to study their effects on CI during storage.

Susceptibility to CI of the three mandarin cultivars during storage at 2 °C was markedly different (Figure 1 and 2A–C and Figure S1). Fortune mandarin, as expected, was the most sensitive to CI. Nova mandarin was also chilling-sensitive but developed chilling symptoms at a lower rate than Fortune, in accordance with previous studies [3,41]. Nadorcott is considered tolerant to CI, but information about its response to low temperatures is scarce because of its recent commercialization. Under Mediterranean growing conditions, the fruit of Nadorcott was tolerant to CI, developing only minor symptoms in a low percentage of fruits even after prolonged exposure to cold (Figure 1 and 2C). In agreement with our results, peel quality of Nadorcott fruit stored at 5 °C, and even at lower temperatures (~0°), remained in good quality throughout storage, developing only slight pitting at the end of storage [46]. Fruit bagging before the natural development of peel color had a differential effect on the incidence of CI in each mandarin cultivar during cold storage (Figure 2A–C). In the CI-sensitive Fortune and Nova, chilling symptoms were delayed, whereas in the CI-tolerant Nadorcott they were enhanced (Figure 2A–C). However, the development of CI in sensitive cultivars was still high, and only a moderated reduction was observed after 8 weeks of storage. Furthermore, the effect of fruit bagging on CI, in Fortune and Nova, was not associated with an enhanced peel pigmentation at harvest and during postharvest storage (Figure 2D,E), in contrast to previous results in Star Ruby grapefruit in which the peel of shaded fruits turned red [5]. These results suggest that light avoidance has a differential effect on peel pigmentation in grapefruit and mandarin fruit, and also in the response of fruit to postharvest cold storage.

The CI tolerance in the red peel of Star Ruby grapefruit has been directly related to carotenoid accumulation [5,21]. It was hypothesized that high TCC or specific individual carotenoids in the peel of mandarins could be related to the different susceptibility to CI. To

explore this hypothesis, the profile and content of carotenoids were analyzed in the flavedo of the selected mandarins. TCC and carotenoid composition were markedly different between NC fruit of the three cultivars (Table 1–3). At harvest, Nadorcott fruit accumulated the highest concentration of carotenoids (~580 μg g^{-1} FW), followed by Nova (~445 μg g^{-1} FW) and Fortune (~185 μg g^{-1} FW), in an inverse relationship with their susceptibility to CI (Figure 2A–C). Noteworthy is the extremely high TCC in the flavedo of Nadorcott mandarin, in accordance with the intense external orange coloration, making it one of the richest sources of carotenoids described so far for citrus fruit [47,48]. The qualitative carotenoid composition was similar among cultivars and displayed the characteristic profile found in mandarin peel [10,11]. Levels of β-citraurin, which provides intense orange-reddish color to the peel [49–51], were similar among cultivars. The concentration of β-cryptoxanthin, a carotenoid which also highly influences the characteristic intense orange color of mandarin peel [11,52], was about 2.5 and 20 times higher in Nadorcott than in Nova and Fortune, respectively (Table 1–3). Thus, the combination of β-citraurin and β-cryptoxanthin contents in Nadorcott could explain the higher color index in this mandarin in comparison to the other cultivars. Moreover, β-citraurin levels in Fortune were high and represented about 25% of total carotenoids whereas it accounted for 8% in Nova, which could explain why the color index was comparable between both cultivars when TCC was markedly lower in Fortune. High levels of this apocarotenoid have been previously reported in Fortune and related to a higher expression of the carotenoid-cleavage dioxygenase gene involved in β-citraurin synthesis [49]. Regarding the influence of carotenoids contents on CI, a strong negative correlation was found between TCC at harvest and CI index after prolonged cold storage (Table 4). Moreover, β-cryptoxanthin and violaxanthin contents highly influenced CI (Table 4), while other carotenoids (data not shown) and β-citraurin were not significantly correlated with CI (Table 4), indicating that not all carotenoids seem to play a role in the response of the fruit to cold stress.

Light avoidance has been shown to modify carotenoid content in citrus fruit [14,17] and we have used this effect to investigate the relationship between carotenoids and CI in mandarin fruits. Studies in Satsuma and Clemenules mandarin have reported that fruit exposed to lower sunlight, either grown inside of the tree canopy or bagged, accumulated lower concentrations of carotenoids compared to fruit grown under normal light incidence [4,7,14,45]. In Nadorcott and Nova mandarin, fruit bagging reduced TCC and the concentration of almost all carotenoids at harvest, but not the contents of the apocarotenoid β-citraurin (Tables 2 and 3). The reduction of carotenoids in dark-grown citrus fruit has been associated with a down-regulation of main genes involved in carotenoid biosynthesis, however, the expression of the

carotenoid-cleavage dioxygenase involved in β-citraurin biosynthetic was not affected by light conditions which may explain the absence of an effect on this apocarotenoid [14]. Interestingly, during cold storage the levels of β-citraurin remained high (>20 μg g^{-1} FW) in C fruits of the three mandarin cultivars which may explain the maintenance of color index in these fruits (Tables 1–3). It is noteworthy that in Fortune, the effect of light avoidance on carotenoids was different to that occurring in the other two cultivars (Table 1). In dark-grown Fortune fruit most of the carotenoids were similar to NC fruit, suggesting that Fortune fruit has a different regulatory mechanism of carotenoid biosynthesis and accumulation to the other mandarin cultivars, which may delineate an altered response to light avoidance.

The effect of storage at low temperatures (<5 °C) on peel color and carotenoids in mandarin cultivars has not been extensively investigated, and seems to be highly dependent on the cultivar and fruit ripening stage at harvest [2]. Cold storage in Or and Odem mandarins has been reported to reduce peel coloration [53] while in Satsuma, a slight increase in carotenoid content has been reported [54]. It is interesting to note that, at the end of storage (8 weeks), TCC and specific individual carotenoids (as phytoene, phytofluene and violaxanthin) in the peel of the three mandarins showed a differential response in accordance with their respective changes in the CI index (Figure 2A–C; Table 1–3). In the fruit of the CI-sensitive Fortune, an important increase (38%) in TCC was observed in highly damage NC fruit after 8 weeks of storage, which was reduced (7%) by bagging associated with a delay in the development of CI. No relevant changes were detected in fruit with moderate CI index, such as Nova, and a decrease was detected in CI-tolerant Nadorcott fruit. These results suggest that the increase of carotenoids in injured mandarin may be part of the fruit's response to cope against cold-damage. This, together with the high and inverse correlation between TCC and CI (Table 4), suggests that fruit of varieties or from environmental conditions with large carotenoid content at harvest may have better ability/tolerance to withstand cold damage during postharvest storage.

Vitamin C is a potent antioxidant metabolite in the peel of citrus fruit [27], and its water soluble nature allows it to scavenge aqueous radicals efficiently [23]. The role of this compound in the development of CI in citrus fruit is still controversial, and while in certain species a positive relation has been found [29], in others, higher contents were detected in CI-sensitive species [30,47]. In fruits of the three mandarin varieties studied in the current work, vitamin C content at harvest was directly related to CI sensitivity and the levels did not substantially change during cold storage (Figure 3). Covering the fruit had a detrimental effect

on vitamin C, as it reduced its concentration over 60–70% with respect to light-exposed fruit (Figure 3). Similar results have been previously observed in Navelina orange, Star Ruby grapefruit, and Satsuma and Nules Clementine mandarins, indicating that stimulation of AsA synthesis and accumulation by light is a general feature in *Citrus* species [7,25]. It has been proposed that in citrus fruit grown under dark or shaded conditions, the synthesis of vitamin C is impaired by a reduction of the expression of genes involved in the L-galacturonic acid pathway [25] –one of the four pathways regulating AsA biosynthesis in plants. A significant positive correlation (r^2= 0.82) was found between vitamin C content in fruit and CI index after cold storage (Table 4). This agrees with results in other *Citrus* species where it has been hypothesized that higher vitamin C content in sensitive fruit could be due to a higher demand for antioxidants as a defense mechanism [21,33], rather than an initial protective mechanism under cold stress. By contrast, in the fruit of other species, such as tomato, mango or cucumber, a lower incidence of cold damage during storage has been associated with high levels of vitamin C [55–57]. Therefore, it is likely that the association between fruit vitamin C content and the susceptibility to develop CI is largely dependent on each species and the storage conditions.

Chilling damage in citrus fruit has been linked to harmful oxidative processes triggered by cold-induced ROS [2,3]. Thus, the antioxidant capacity of the fruit peel may be related to their cold tolerance. To explore this hypothesis, the antioxidant potential of the fruit peel of the three mandarins was assessed by three different methods (Figure 4 and 5). The DPPH• and FRAP assays are based on electron transfer reactions and measure the ability of a hydrophilic extract to reduce a specific oxidant [58], while the SOAC assay assesses singlet oxygen absorption capacity of compounds present in a lipophilic extract, and has been previously used to determine the carotenoid antioxidant ability in carotenoid rich tissues, as citrus fruit extracts [21,39]. In general, higher DPPH• and FRAP antioxidant activity was detected in extracts from CI-sensitive varieties than in extracts of the CI-tolerant Nadorcott (Figure 4), showing a significant positive correlation between CI and the DPPH• and FRAP antioxidant activity in the peel extracts of mandarin fruit (Figure 4; Table 4). Similarly, in chilling-sensitive fruit of Star Ruby grapefruit higher DPPH•, ABTS and ORAC antioxidant activity was detected [21]. In the three mandarin cultivars, the total antioxidant activity assessed by DPPH• and FRAP followed the same trend observed in vitamin C content (Figure 3), implying that these methods are appropriate to measure the contribution of hydrophilic compounds to the antioxidant system. Our correlation analysis also corroborated this conclusion as a strong positive correlation between vitamin C content and DPPH• and FRAP antioxidant activity was found

(Table 4). By contrast, the relative SOAC values in the lipophilic extracts of the mandarins followed the same pattern as TCC and, interestingly, were negatively correlated with CI sensitivity (Figure 5; Table 4). In the peel of red grapefruit, a negative correlation was also reported between CI development and SOAC values, and also with TCC [21]. It was then proposed that high lycopene concentrations in CI-tolerant fruit [5] provides a superior ability to scavenge singlet oxygen, suggesting a protective role of this carotene in the development of CI in the peel of citrus fruit [21]. Mandarins are devoid of lycopene but contain significant amounts of other carotenoids, which have been associated with high antioxidant capacity in citrus [59]. Interestingly, the correlation analysis also showed a significant and strong positive relationship between β-cryptoxanthin and violaxanthin contents, and SOAC values (Table 4). Therefore, these carotenoids, particularly abundant in the peel of mandarins, may play a protective role against cold stress by quenching singlet oxygen. Furthermore, these results support previous reports indicating that SOAC antioxidant capacity is a reliable and convenient system to assess the antioxidant capacity related to carotenoids as singlet oxygen quenchers, one of the main properties of these compounds [60] and a good indicator of CI tolerance.

Nonetheless, it is important to mention that the antioxidant lipophilic and hydrophilic capacity of citrus fruits extracts is provided by complex mixtures of many compounds, including carotenoids, vitamin C and also tocopherols or polyphenols, among others [61]. Tocopherols and polyphenols are efficient ROS scavengers, and their relative abundance in extracts of citrus fruits may affect the antioxidant activities [62]. However, the particular contribution of tocopherols and polyphenols to the development of CI in citrus fruits is still unclear. In lemon, a lower concentration of tocopherols and flavonoids have been detected in CI-sensitive fruit [63]. In mandarins, an increase in phenylamonia-lyase activity, the rate controlling enzyme of the phenylpropanoids pathway, has been associated with the development of CI during cold storage [64,65]. Regarding the enzymes scavenging active oxygen species, it has been suggested that catalase activity may be involved in the induction of chilling injury tolerance in mandarin and red grapefruit [21,66]. Then, it is likely that the antioxidant activity of the extracts used in the current study may be due to the presence not only of carotenoids and ascorbic acid, but also other lipo- and hydro-soluble antioxidant components [67,68]. In this work, we have focused our attention on the potential contribution of carotenoids and vitamin C to the chilling tolerance, which has not been yet explored in mandarins. Carotenoids have been suggested to be the first line of defense against singlet oxygen toxicity in plants [69,70]. Their efficiency in quenching singlet oxygen has been proved

for different carotenoids including violaxanthin and β-cryptoxanthin [70,71]. In particular, β-cryptoxanthin has a potent *in vitro* antioxidant capacity and can also act as a scavenger of free radicals, preventing the oxidative damage of biomolecules and protecting against oxidative stress in *in vivo* systems [72–74]. Moreover, an elevated concentration of this carotenoid in orange juice samples was associated with a higher antioxidant capacity in cultivars not necessarily rich in TCC [75]. Therefore, considering the antioxidant properties of carotenoids and the results of the correlation analysis of the current work (Table 4), it is envisaged that β-cryptoxanthin and violaxanthin are major contributors to the SOAC capacity and to the CI susceptibility in mandarin fruits, since highly significant positive and negative relationships, respectively, between these parameters were established.

5. Conclusions

Fruit of three mandarin cultivars with different susceptibility to develop CI (Fortune > Nova > Nadorcott) during cold storage were selected to investigate the potential relationship between CI, carotenoids, vitamin C and antioxidant activity/capacity in hydrophilic and lipophilic peel extracts. Carotenoids and vitamin C content in the peel was manipulated by pre-harvest fruit bagging of the fruits. A strong negative correlation between TCC at harvest, specifically of β-cryptoxanthin and violaxanthin, and CI index at the end of storage period was found, while vitamin C content was positively correlated with CI. The three methods used for the determination of antioxidant capacity showed that, DPPH• and FRAP methods reflect the contribution of vitamin C to the antioxidant system, while SOAC correlated with TCC. Collectively, the antioxidant capacity of carotenoids at harvest, as efficient singlet oxygen quenchers, suggest a protective role for carotenoids, specifically β-cryptoxanthin and violaxanthin, against development of CI in mandarin fruit during cold storage while the correlation analysis indicates that vitamin C is not likely playing a critical role.

Supplementary Materials: The following are available online at www.mdpi.com/2076-3921/9/12/1296/s1, Figure S1: Pictures of the scale used to rate the severity of CI symptoms during postharvest cold storage; Figure S2: MaxPlot chromatogram of the saponified carotenoid extract of the Nadorcott flavedo at harvest; Figure S3: Chromatogram at 248 nm showing ascorbic acid peak; Figure S4: Pictures illustrating CI symptoms severity and magnification of CI development in fruit of Fortune, Nova and Nadorcott; Table S1: Carotenoid limits of detection and working linear range; Table S2: Chromatographic and spectroscopic characteristics of carotenoids found in the flavedo of the three mandarin cultivars.

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3.2. CAPÍTULO 2

Accumulation of tocopherols and transcriptional regulation of their biosynthesis during cold storage of mandarin fruit.

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Abstract

Tocopherols are plant-derived isoprenoids with potent antioxidant activity, which have been implicated in the tolerance of plants to different stresses. However, tocopherol accumulation and biosynthesis in fruit, and their potential implication in postharvest chilling injury (CI), has been scarcely studied. Therefore, in this work, we have investigated tocopherol accumulation and biosynthesis in the peel of mandarin fruit of three cultivars with contrasting susceptibility to CI during storage at 2 °C ('Fortune' > 'Nova' > 'Nadorcott'). α- and γ-tocopherol were the isoforms detected in the flavedo of the fruit, and a direct relationship between tocopherols content and CI-tolerance was found, since the CI-tolerant fruit accumulated the highest tocopherol content whereas the CI-sensitive fruit the lowest. Moreover, the transcriptional profiling of 14 genes related to the specific steps of tocopherol biosynthesis, and to their precursor's synthesis, were analyzed. Upstream genes *DXS1* and *DXS2* (1-deoxy-D-xylulose-5-phosphate synthase) and *GGDR* (geranylgeranyl diphosphate reductase), involved in the supply of phytyl pyrophosphate, and the *VTE3* (2-methyl-6-phytyl-1,4-benzoquinol methyltransferase) isoforms appear to be key for the differences in total tocopherol content among the cultivars at harvest. During cold storage, most genes involved in the precursors supply were up-regulated, whereas genes of the tocopherol-core pathway were in general repressed. Changes in *VTE4* during cold storage may account for the differences in γ-tocopherol among cultivars. Collectively, results suggest that the concentration of tocopherols at harvest may play a function in the natural tolerance of mandarin fruit to CI, and that changes in the expression of genes during storage appear to be cold-regulated responses, rather than involved in CI tolerance.

Keywords: antioxidant, chilling injury, gene expression, mandarin, tocopherols

1. Introduction

A recurring problem during the commercialization of citrus fruit is the appearance of peel damage due to the exposure to low temperatures during postharvest transport and storage, which depreciates their commercial value. These damages and blemishes caused by low non-freezing temperatures are referred to as chilling injury (CI), which is usually manifested as small depressions in the flavedo that expand, sink and darken with the continuous exposure to cold (Lado, Cronje, et al., 2019; Zacarias et al., 2020). Although mandarin fruit is considered more resistant to CI than fruit of other *Citrus* species, contrasting predisposition to CI can be observed among different cultivars (Rey et al., 2020; Sala, 1998).

Exposure to cold temperatures has a direct impact on the integrity of cell membranes (Lyons, 1973), and accelerates changes in their composition and conformation which negatively affects their functionality (Lafuente et al., 2017; Sevillano et al., 2009). Furthermore, the stability and functionality of membranes is affected by oxidative processes caused by a cold-induced overproduction and accumulation of ROS (Suzuki et al., 2012). Experimental evidence indicates that CI in *Citrus* fruit is associated with a boost in oxidative stress processes, exemplified by an enhancement of the expression of genes and enzymatic activities of the antioxidant system, and a higher singlet oxygen quenching capacity (Lado et al., 2016; Lafuente et al., 2017).

Accumulation of bioactive compounds with antioxidant properties, might play an important role in the tolerance of fruit to CI. The peel of citrus fruit contains compounds such as carotenoids, vitamin C and polyphenols which are part of the non-enzymatic defense mechanism against oxidative stress in plant tissues (Zou et al., 2016). Carotenoids are potent lipid-soluble antioxidants and have been associated with the tolerance of grapefruit (Lado et al., 2015, 2016) and mandarin to CI (Rey et al., 2020). On the other hand, changes in the concentration of vitamin C in the peel of citrus fruit appears to be a response to low temperatures, but its involvement in CI tolerance is still controversial (Lado et al., 2016; Rey et al., 2020). Tocopherols are highly efficient lipophilic antioxidants (Falk and Munné-Bosch, 2010; Munné-Bosch and Alegre, 2002) and have been detected in the flavedo of citrus fruit (Assefa et al., 2017; Mathaba et al., 2014), but their role in the sensibility/tolerance of citrus fruit to CI is currently unknown.

Tocopherols are plant isoprenoids belonging to the chemical family of tocochromanols, which also includes tocotrienols, tocomonoenols and plastochromanol 8. All tocochromanols

are formed by a polar chromanol ring and a hydrophobic polyprenyl side chain that varies according to the specific tocochromanol, and is fully saturated in the case of tocopherols. In addition, four natural sub-forms of tocopherols exist according to the degree of methylation and position of the chromanol ring: δ-, β-, γ- and α-tocopherol. They participate in a variety of plant metabolic processes (Falk and Munné-Bosch, 2010; Munné-Bosch and Alegre, 2002), and tocopherols, together with tocotrienols, are the only natural compounds that exhibit vitamin E activity in animal cells, with α-tocopherol being the most potent vitamin E form (Traber and Sies, 1996).

Tocopherols can scavenge and quench lipid peroxyl radicals and ROS, such as singlet oxygen, protecting plant tissues from lipid peroxidation and oxidative damage (Foyer and Noctor, 2005; Havaux et al., 2005; Mène-Saffrané et al., 2010; Sies and Stahl, 1995). Increases in tocopherol content have been detected in plant tissues in response to different abiotic stresses, such as high light, salinity, drought, nutrient deficiency and cold (Bergmüller et al., 2003; Collakova and DellaPenna, 2003a; Havaux et al., 2005; Maeda et al., 2006; Spicher et al., 2016). They seem to play a crucial role in the adaptation of plants to low temperatures, and mutants impaired in tocopherol synthesis have shown a cold-sensitive phenotype, with retarded growth, early senescence and reduced seed germination under cold stress (Havaux et al., 2005; Maeda et al., 2006; Wang et al., 2017). However, the involvement of tocopherol metabolism in the response of fruit to cold stress during postharvest storage has been scarcely studied.

Although the tocopherol biosynthetic pathway was elucidated several years ago, key genes encoding enzymes of the main biosynthetic steps have been recently identified (Fritsche et al., 2017; Mène-Saffrané, 2017; Muñoz and Munné-Bosch, 2019; Pellaud and Mène-Saffrané, 2017). The first committed step of the tocopherol-core pathway is the condensation of homogentisate (HGA) with phytyl pyrophosphate (PPP) (Fig. 1). While HGA is the common precursor of all tocochromanols, PPP is the specific polyprenyl donor substrate for tocopherols. This reaction is catalyzed by homogentisate phytyl transferase (HPT), encoded by the *VTE2* gene, and results in the formation of 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) (Collakova and DellaPenna, 2003b). Later, MPBQ can either be directly converted into δ-tocopherol first and then into β-tocopherol by tocopherol cyclase (TC, encoded by *VTE1*) and γ-tocopherol methyltransferase (γ-TMT, encoded by *VTE4*); or, it can be first methylated by MPBQ methyltransferase (MPBQ-MT, encoded by *VTE3*) resulting in 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ). DMPBQ can be subsequently cyclized into γ-tocopherol and later methylated into α-tocopherol by the same TC and γ-TMT mentioned before. The activity of

HPT, and expression of *VTE2*, has been proven to limit tocopherol synthesis in leaves and seeds of *Arabidopsis thaliana* (Collakova and DellaPenna, 2003b; Savidge et al., 2002), while expression of *VTE3* and *VTE4* appears to shape tocopherol composition rather than affecting their content (Bergmüller et al., 2003; Cheng et al., 2003; Collakova and DellaPenna, 2003b).

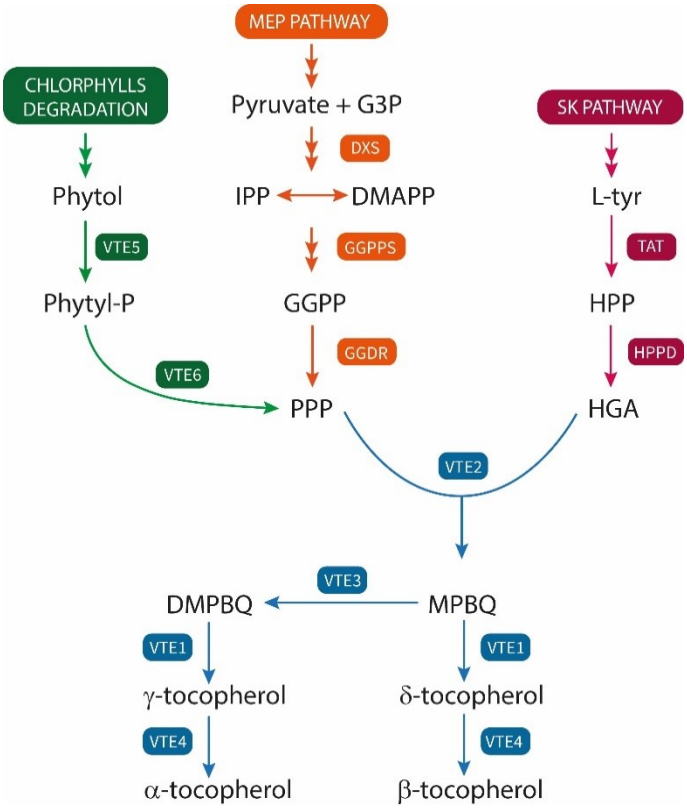

Figure 1. Schematic representation of the tocopherol biosynthetic pathway. The intermediates and enzymes catalyzing the reactions are: Phytyl-P, phytyl phosphate; G3P, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; PPP, phytyl pyrophosphate; L-tyr, amino acid L-tyrosine; HPP, 4-hydroxyphenylpyruvate; HGA, homogentisate; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; *VTE5*, phytol kinase; *VTE6*, phytyl-P kinase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; GGPPS, GGPP synthase; GGDR, GGPP reductase; TAT, tyrosine aminotransferase; HPPD, HPP dioxygenase; *VTE2*, homogentisate phytyltransferase (HPT); *VTE3*, MPBQ methyltransferase (MPBQ-MT); *VTE1*, tocopherol cyclase (TC); *VTE4*, tocopherol methyltransferase (γ-TMT).

Tocopherols precursors HGA and PPP are formed in independent metabolic pathways and their availability have a strong impact in the final amount of tocopherol accumulated in a tissue (Karunanandaa et al., 2005). The precursor HGA, which confers the polar chromanol ring, derives from 4-hydroxyphenylpyruvate (HPP) formed by the degradation of the amino acid L-tyrosine (L-tyr) produced by the shikimate (SK) pathway. The conversion of L-tyr into HPP is catalyzed by tyrosine aminotransferases (TATs), and the loss of function of *TAT1* in Arabidopsis resulted in a reduction of TAT activity and tocopherol levels in leaves (Riewe et

al., 2012). The synthesis of HGA from HPP is catalyzed by HPP dioxygenase (HPPD), and null *HPPD* Arabidopsis mutants were deficient in tocopherols (Norris et al., 1998), while its overexpression only generated a modest increase of total tocopherols in leaves and seeds of Arabidopsis and other species (Karunanandaa et al., 2005; Tsegaye et al., 2002).

The specific polyprenyl substrate for tocopherol synthesis PPP can either be derived from geranylgeranyl diphosphate (GGPP) (MEP pathway) or from free phytol (formed during chlorophyll degradation). Several studies have suggested that the majority of PPP used for tocopherol synthesis comes from the recycling of phytol (Karunanandaa et al., 2005; Valentin et al., 2006). Phytol is phosphorylated to phytyl phosphate by a phytol kinase (*VTE5)*, and later forms PPP by a phytyl phosphate kinase (*VTE6*). In leaves and seeds of Arabidopsis, it has been estimated that most of the PPP used for tocopherol synthesis is derived from the *VTE5*-dependent recycling of phytol (Valentin et al., 2006). Similar results have been observed in fruit and leaves of tomato, where the down-regulation of *VTE5* strongly impaired tocopherol production (Almeida et al., 2016). Changes in the expression of *VTE6* also have a direct impact in tocopherol accumulation (vom Dorp et al., 2015), indicating that the supply of PPP through the recycling of phytol formed during chlorophyll degradation has an important participation in the synthesis of tocopherols in plants. Moreover, genes of the MEP pathway, such as DXS, also influence tocopherol concentrations in plant tissues (Estévez et al., 2001; Rodríguez-Concepción and Boronat, 2015).

In *Citrus*, information about tocopherol content and biosynthesis is very limited and only a few studies have assessed their accumulation in different tissues. Tocopherols have been detected in the peel of orange, lemon and other less known cultivars, mainly in the form of α- and γ-tocopherol, and content and proportion of each form varies depending on the specie (Assefa et al., 2017; Mathaba et al., 2014). However, the identification of the main tocopherol biosynthetic genes, and how they are regulated in citrus fruit in response to abiotic stress conditions is currently unknown. Since tocopherols are widely recognized by their antioxidant capacity, our working hypothesis envisages the potential participation of these compounds in the responses of citrus fruit to postharvest cold storage. To unravel this objective, tocopherol content and expression of 14 genes involved in tocopherol biosynthesis were analyzed in the peel of fruit of three mandarin cultivars differing in their sensitivity to CI ('Fortune' > 'Nova' > 'Nadorcott') stored at 2 °C for up to 8 weeks.

2. Materials and methods

2.1. Plant material and storage conditions

Fruit of three mandarin cultivars with contrasting susceptibility to develop chilling symptoms during postharvest cold stored were selected: 'Fortune' (*Citrus clementina* Hort. Ex Tanaka x *Citrus reticulata* Blanco), high sensitivity; 'Nova' (*Citrus clementina* Hort. ex Tanaka x (*Citrus paradisi* McFadyen x *Citrus reticulata* Blanco)), medium sensitivity; and 'Nadorcott' (*Citrus reticulate,* Blanco), low sensitivity (Rey et al., 2020). Fruit were harvested at commercial maturity from adult trees growing under standard agronomical conditions, located in a commercial orchard (Lliria, Valencia, Spain) or in The Citrus Germplasm Bank at the Instituto Valenciano de Investigaciones Agrarias (IVIA, Moncada, Valencia, Spain). Immediately after harvest, fruit were delivered to the laboratory, inspected for size uniformity, and fruit free of visible peel damage and defects were stored at 2 °C and 80-85 % RH for up to 8 weeks. At harvest and after 1, 3, 5 and 8 weeks of cold storage, flavedo tissue (colored part of the rind) was excised with a scalpel, frozen in liquid nitrogen, ground to a fine powder and stored at -80 °C until analysis.

2.2. Chilling injury evaluation

Mandarin fruit of each cultivar was visually inspected for CI symptoms during the storage period. Fruit were scored in a scale from 0 to 3, according to the intensity and extension of the symptoms, as previously described (Rey et al., 2020). Results are expressed as the percentage of fruit within each CI category. Three replicate samples of 20 fruit each were used for CI evaluation.

2.3. Tocopherol extraction, identification and quantification

Tocopherols in the flavedo were extracted following the procedure described by Fraser et al. (2000) with some modifications. A sample of 200 mg of flavedo was extracted with 2 mL of methanol (HPLC grade, Scharlau, Barcelona, Spain), 1 mL of Tris buffer (50 mM Tris pH 7.5 with 1 M NaCl) and 4 mL of dichloromethane (HPLC grade, Scharlau, Barcelona, Spain) with a mortar and pestle with sea sand as an abrasive. After vortex-mixing, samples were sonicated for 5 min and then centrifuged for 10 min at 3000 *g* and 4 °C. After this, the dichloromethane phase was recovered and the methanol phase and flavedo pellet were re-extracted with 2 mL of dichloromethane. Extracts were dried under a nitrogen stream and re-suspended in ethyl acetate (HPLC grade, Merck, Madrid, Spain) for chromatography analyses. Tocopherol

content was determined using a Waters HPLC system (Acquity® Arc™, Waters, Barcelona, Spain) coupled with a fluorescence detector (2475 FLR Detector, Waters, Barcelona, Spain). Separation of tocopherols was carried out with a YMC C30 column (150 x 4.6 mm, 3 µm) (Teknokroma, Barcelona, Spain), maintained at room temperature, and a ternary gradient elution (Table S1) with methanol, methyl tert-butyl ether and water, at a flow rate of 1 mL min^{-1}. Compounds were detected by fluorescence with excitation and emission wavelength at 296 and 340 nm, respectively. Identification and quantification of the different tocopherols was achieved by comparison with the retention times and peak areas of authentic standards of δ-, γ- and α-tocopherol (Sigma-Aldrich, Barcelona, Spain). All procedures were carried out on ice and under dim light to prevent degradation. Total tocopherol content was calculated as the sum of the different tocopherol isoforms. Concentrations are expressed as mg kg^{-1} of fresh weight. Samples were extracted twice and results are the mean of two replicates (mean ± standard deviation).

2.4. Identification of the genes involved in tocopherol biosynthesis in Citrus and primers design

In order to identify *Citrus* genomic sequences for the main enzymatic steps involved in the tocopherol biosynthesis pathway, and in HGA and PPP synthesis, peptide sequences of previously described genes (Fig. 1) from *Arabidopsis thaliana* and *Solanum lycopersicum* were obtained from the TAIR10 and iTAG2.4 databases (Table 1). These Arabidopsis and tomato protein sequences were used to perform a TBLASTN search against the *Citrus sinensis* (*C. sinensis*) genome databases Phytozome (JGI Phytozome v12; Wu et al., 2014) and Huazhong Agricultural University (http://citrus.hzau.edu.cn/orange; Xu et al., 2013). Protein sequence identity between the closest orthologous genes was performed using the DNAman software (Lynnon Biosoft, Quebec, Canada), and genes were selected based on the percentage of identity at the amino acid level (Table 1). A BLAST search against the *C. sinensis* genome with the nucleotide sequences of the selected *C. sinensis* genes was performed to corroborate homology within the genome that might interfere in primer design. Genes with high similarity were taken into account in the design of primers to assure specificity. Primer pairs were design manually or using the online software PCR Efficiency calculator (http://130.60.24.89/efficiency.html), and specificity was corroborated by sequencing the PCR products.

2.5. RNA extraction and cDNA synthesis

Total RNA was isolated from flavedo tissue using the RNeasy Plant Mini Kit (Qiagen, Madrid, Spain). Total RNA was treated with DNase I (DNA free, DNase treatment & removal, Ambion, Barcelona, Spain), and RNA concentration and quality was measured by spectrophotometric analysis (Nanodrop, Thermo Fisher Scientific, Barcelona, Spain). After quantification, the quality of the RNA was verified by 1 % agarose gel electrophoresis with GoodView™ Nucleic Acid Stain (SBS Genetech, Beijing, China). For cDNA synthesis, 5 µg of total RNA were reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Barcelona, Spain) in a total volume of 20 µL, following the manufacturer's procedure. First-strand cDNA samples were diluted 1:10 to a final concentration of approximately 100 ng μL^{-1} for each amplification reaction.

2.6. Gene expression analysis by quantitative real time PCR

Quantitative real-time PCR was performed on a LightCycler 480 instrument (Roche, Madrid, Spain), using the LightCycler 480 SYBRGreen I Master kit (Roche, Madrid, Spain) and following the manufacturer's instructions. Primer pairs sequences of the genes related to tocopherols biosynthesis used for the amplification of each gene are listed in Table S2. The cycling protocol, for all genes analyzed, consisted of 10 min at 95 °C for pre-incubation, 40 cycles of 10 s at 95 °C for denaturation, 10 s at 59 °C for annealing and 10 s at 72 °C for extension. Fluorescent intensity data was acquired during the extension time. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve performed after the amplification steps. For expression measurements, we used the LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche, Madrid, Spain) and calculated expression levels relative to values of a reference sample using the Relative Expression Software Tool (Pfaffl et al., 2002). The housekeeping gene used for normalization was *ACTIN* (Alós et al., 2014). For all cultivars and dates analyzed, the reference sample used to calculate relative expression was the value of each gene in the flavedo of 'Fortune' fruit at harvest (time 0), which was set at 1.

2.7. Statistical and principal components analysis

To determine if mean differences were significant among storage dates in each mandarin cultivar, data for γ- and α-tocopherol content was subjected to a one-way analysis of variance (ANOVA) followed by Tukey's test (significance level at $p \leq 0.05$), using the InfoStat software (version 2018, Grupo InfoStat, Córdoba, Argentina). Principal component analysis (PCA) was built using data of tocopherol content and relative gene expression at harvest and at the four

storage dates analyzed for the three mandarin cultivars, using the packages 'FactoMineR' (version 2.4) and 'factoextra' (version 1.0.7) in RStudio (version 1.3.1093, RStudio Team, PBC, Boston, MA, United States).

3. Results

3.1. Differences in the susceptibility to chilling injury of mandarin fruit during cold storage

To investigate the role of tocopherols in the susceptibility/tolerance of citrus fruit to CI during postharvest cold storage, we selected fruit of three mandarin cultivars with contrasting differences in their susceptibility to CI (Rey et al., 2020). 'Fortune' mandarin fruit were very prone to develop CI and, after 3 weeks of storage at 2 °C, CI affected almost 90 % of the fruit (40 % of fruit had severe symptoms). The severity of CI increased with storage time and, after 5 and 8 weeks of storage, 62 % and 90 % of the fruit, respectively, developed severe CI (Fig. 2). Fruit of 'Nova' mandarin developed CI symptoms more slowly than those of 'Fortune' and the proportion of fruit with high and severe damage was 48 % and 74 % after 5 and 8 weeks of storage, respectively. By contrast, fruit of 'Nadorcott' proved to be highly resistant to CI. After 5 weeks at 2 °C only 6 % of fruit developed slight symptoms, and by week 8, 74 % of the fruit was still healthy (Fig. 2).

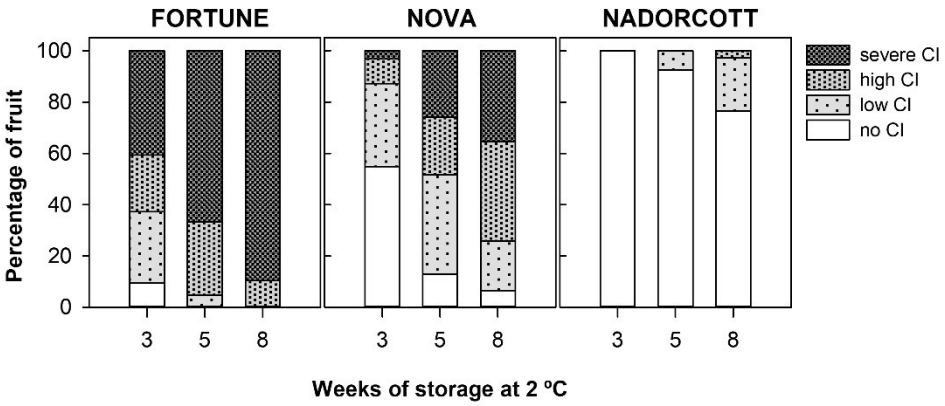

Figure 2. Chilling injury (CI) incidence (% of fruit) according to severity of symptoms in fruit of 'Fortune', 'Nova' and 'Nadorcott' mandarins stored at 2 °C for 3, 5 and 8 weeks.

3.2. Tocopherol content in the flavedo of mandarin fruit during cold storage

Accumulation of tocopherols was determined in the flavedo of fruit of the three mandarin cultivars during storage at 2 °C (Fig. 3). Of the four naturally occurring tocopherol forms (α, β, δ and γ), only α- and γ-tocopherol were detected in the flavedo of mandarin fruit. In all the samples analyzed, α-tocopherol was the predominant form, accounting for 78.4 %, 86.0 % and 94.7 %, on average, of total tocopherols in 'Nova', 'Nadorcott' and 'Fortune', respectively.

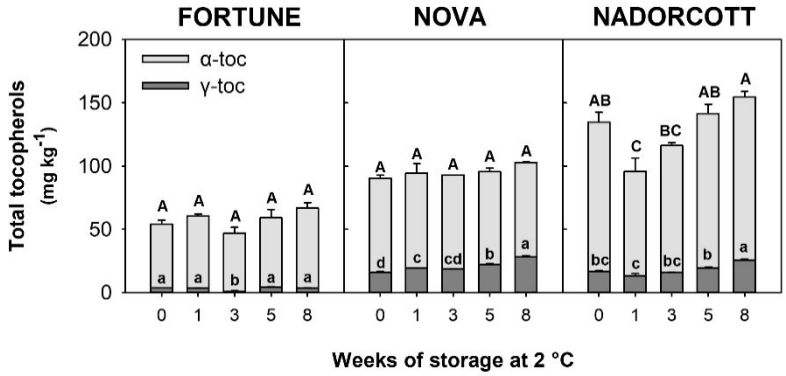

Figure 3. Total tocopherols content (mg kg^{-1}, as the sum of α- and γ-tocopherol) in the flavedo of 'Fortune', 'Nova' and 'Nadorcott' mandarins stored at 2 °C for up to 8 weeks. Capital letters indicate significant differences in α-tocopherol, while lowercase letters indicate significant differences in γ-tocopherol content among storage dates for each cultivar ($p \leq 0.05$, Tukey test).

Total tocopherols at harvest, calculated as the sum of γ- and α-tocopherol, were higher in the flavedo of 'Nadorcott' (135 mg kg^{-1}) than in 'Nova' (90 mg kg^{-1}) and 'Fortune' (54 mg kg^{-1}) (Fig. 3). These differences in total tocopherols among cultivars were mainly due to the low accumulation of α-tocopherol in 'Nova' and 'Fortune' (40 and 60 % lower, respectively) in comparison with 'Nadorcott' mandarin. The concentration of γ-tocopherol was similar between 'Nadorcott' and 'Nova' mandarins, and about 4-times higher than that detected in 'Fortune'. During storage at 2 °C, total tocopherols and α-tocopherol remained constant in 'Fortune' and 'Nova', while in 'Nadorcott' they experienced an initial reduction after 1 week of storage, increasing gradually to reach concentrations similar to the initial levels at the end of the storage (Fig 3). The concentration of γ-tocopherol during cold storage remained constant in fruit of 'Fortune', but significantly increased in 'Nova' (74.5 %) and in 'Nadorcott' (55.1 %) mandarins. Despite these fluctuations, the differences in tocopherol concentrations among mandarin cultivars were maintained during the whole cold storage period (Fig 3).

3.3. Selection of genes involved in tocopherol biosynthesis in Citrus

With the aim of understanding the transcriptional regulation of tocopherol biosynthesis during postharvest cold storage of citrus fruit, a search of genes corresponding to key enzymatic steps of the different pathways involved in tocopherol production was performed in the *Citrus sinensis* genome, based on genes previously identified in *Arabidopsis thaliana* and *Solanum lycopersicum* (Table 1). Tocopherol accumulation in plant tissues has been shown to be dependent not only on the transcriptional changes of specific genes of the tocopherol core-pathway, but also of the genes involved in the availability of the precursors PPP and HGA (Mène-Saffrané, 2017; Muñoz and Munné-Bosch, 2019). HGA is formed *via* the SK pathway,

whereas PPP can be formed *de novo* from GGPP (MEP pathway) or by the recycling of free phytol (formed during chlorophyll degradation) (Pellaud and Mène-Saffrané, 2017). Therefore, a total of 14 genes were selected for further expression analysis (Fig. 1), corresponding to 5 genes of the MEP pathway: 2 isoforms of 1-deoxy-D-xylulose-5-phosphate synthase (*DXS1* and *DXS2*), 2 isoforms of geranylgeranyl pyrophosphate synthase (*GGPPS1* and *GGPPS6*) and geranylgeranyl diphosphate reductase (*GGDR*); 2 genes involved in phytol catabolism, phytol kinase (*VTE5*) and phytol-P kinase (*VTE6*); 2 genes of the SK pathway, tyrosine aminotransferase (*TAT1*) and 4-hydroxyphenylpyruvate dioxygenase (*HPPD*); and 5 genes specifically involved in the tocopherol core-pathway: homogentisate phytyl transferase (*VTE2*), 2 isoforms of 2-methyl-6-phytyl-benzoquinol methyl transferase (*VTE3a* and *VTE3b*), tocopherol cyclase (*VTE1*) and γ-tocopherol methyl transferase (*VTE4*). Protein length and percentage of identity of the sequences selected with the corresponding *Arabidopsis thaliana* and *Solanum lycopersicum* orthologous are summarized in Table 1. Identity of the protein sequences ranged between 59 and 87 % in comparison with the corresponding sequences of Arabidopsis and between 62 and 90 % with those of tomato (Table 1).

Table 1. Identification of homologous genes and enzymes involved in tocopherol synthesis in the *Citrus sinensis* genome.

Gene	Enzyme	*C. sinensis*	Protein length (aa) [c]	*A. thaliana* [d]	% Id *Ath* [e]	*S. lycopersicum* [f]	% Id *Slyc* [g]
DXS1	1-deoxy-D-xylulose-5-phosphate synthase	orange1.1g003948 [a]	784	AT4G15560	71	Solyc11g010850	81
DXS2	1-deoxy-D-xylulose-5-phosphate synthase	orange1.1g004923 [a]	723		82	Solyc01g067890	86
GGPPS1	Geranylgeranyl pyrophosphate synthase	orange1.1g019903 [a]	334	AT4G38460	73	Solyc09g008920	78
GGPPS6	Geranylgeranyl pyrophosphate synthase	orange1.1g037716 [a]	354	AT4G36810	59	Solyc11g011240	72
GGDR	Geranylgeranyl diphosphate reductase	orange1.1g012488 [a]	462	AT1G74470	84	Solyc03g115980	83
VTE5	Phytol kinase	orange1.1g022218 [a]	301	AT5G04490	69	Solyc03g071720	62
VTE6	Phytol-P kinase	orange1.1g020374 [a]	327	AT1G78620	73	Solyc07g062180	84
TAT1	Tyrosine aminotransferase	orange1.1g014936 [a]	415	AT5G53970	75	Solyc10g007110	72
HPPD	4-hydroxyphenylpyruvate dioxygenase	orange1.1g013898 [a]	434	AT1G06570	77	Solyc07g045050	78
VTE2	Homogentisate phytyl transferase (HPT)	Cs4g03670.1 [b]	411	AT2G18950	68	Solyc07g017770	65
VTE3a	Dimethyl-phytylquinol methyl transferase	orange1.1g019479 [a]	340	AT3G63410	87	Solyc03g005230	88
VTE3b	Dimethyl-phytylquinol methyl transferase	orange1.1g019684 [a]	337		76	Solyc09g065730	90
VTE1	Tocopherol cyclase (TC)	orange1.1g011258 [a]	490	AT4G32770	68	Solyc08g068570	76
VTE4	γ-tocopherol methyl transferase (γ-TMT)	orange1.1g016921 [a]	380	AT1G64970	67	Solyc08g076360	72

[a] Phytozome v12.1, *Citrus sinensis* v1.1 (Sweet orange) genome database
[b] Huazhong Agricultural University, *Citrus sinensis* (Sweet orange) genome database
[c] Predicted protein size in Phytozome v12.1 (aa, amino acids)
[d] TAIR10, *Arabidopsis thaliana* locus
[e] % Id *Ath*: percentage of identity with the corresponding *Arabidopsis thaliana* protein sequence
[f] Phytozome v12.1, *Solanum lycopersicum* iTAG2.4 (Tomato) genome database
[g] % Id *S.lyc*: percentage of identity with the corresponding *Solanum lycopersicum* protein sequence

3.4. Comparison of the expression of genes involved in tocopherol biosynthesis in the flavedo of mandarin fruit during cold storage

The transcriptional profiling of 14 genes involved in tocopherol synthesis (Table 1) was analyzed in the flavedo of 'Fortune', 'Nova' and 'Nadorcott' mandarin stored for up to 8 weeks at 2 °C, in order to understand whether the expression patterns of these genes are related to the differences in tocopherol content and CI susceptibility. Figs. 4 and 5 show the changes in the expression of genes of the chlorophyll degradation and MEP pathways, respectively, involved in the formation of the metabolic precursors PPP (Fig. 1). In the flavedo of fruit at harvest, when the differences in total tocopherols among cultivars were already evident, major differences were found in genes of the MEP pathway, but not in those involved in the recycling of phytol. Accumulation of *VTE5* and *VTE6* transcripts were similar in fruit of the three cultivars at harvest and increased with the storage period. In general, these transcripts accumulated to lower levels in the flavedo of the high-accumulating tocopherol cultivar 'Nadorcott', than in 'Nova' or 'Fortune' mandarin (Fig. 4).

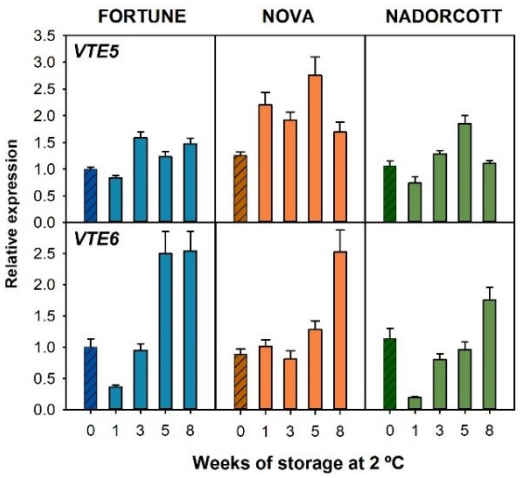

Figure 4. Relative expression of the genes of the chlorophyll degradation pathway involved in the synthesis of PPP (phytyl pyrophosphate) in the flavedo of 'Fortune', 'Nova' and 'Nadorcott' mandarins stored at 2 °C for up to 8 weeks. The genes analyzed were *VTE5* (phytol kinase) and *VTE6* (phytyl-P kinase). An expression value of 1 was arbitrarily assigned to the values obtained in the flavedo of 'Fortune' fruit at harvest. The data are mean ± S.E of at least three replicates.

With the exception of *GGPPS1*, whose mRNA was lower in fruit of 'Nadorcott' at harvest than in the other two cultivars, expression of other genes of the MEP pathway analyzed (*DXS1*, *DXS2, GGPPS6* and *GGDR*) were between 2 and 3-times higher in the flavedo of 'Nova' and 'Nadorcott' than in 'Fortune' mandarin (Fig. 5). Cold storage up-regulated the expression of most genes (except *GGDR*), although differences in the timing of induction were found among cultivars (Fig. 5). In general, accumulation of most transcripts after 8 weeks of storage were

higher than at harvest. Moreover, *GGDR* was the only gene displaying a contrasting expression pattern in 'Fortune' mandarin than in 'Nova' and 'Nadorcott', with a progressive up-regulation in the former and a decline in the peel of the other two mandarins (Fig. 5).

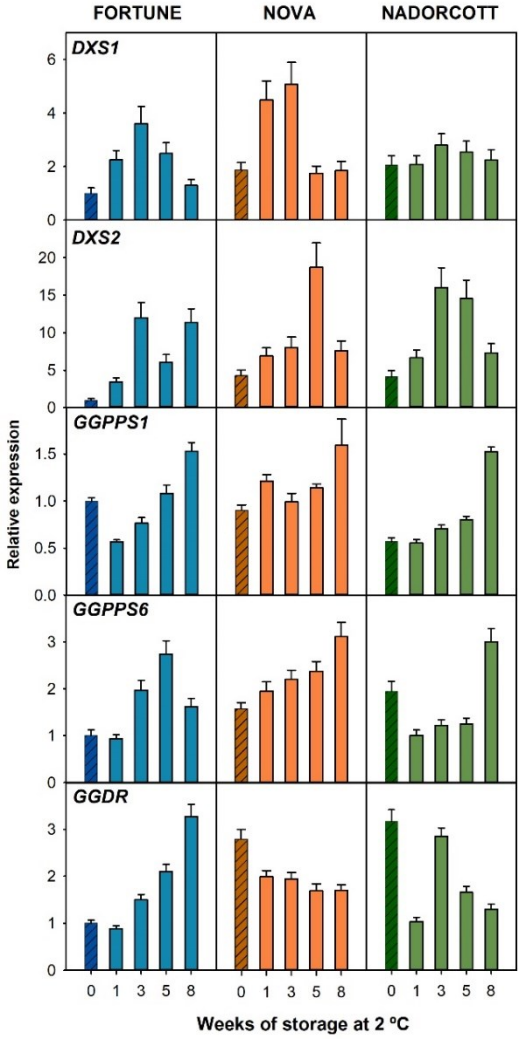

Figure 5. Relative expression of genes of the MEP pathway involved in the synthesis of PPP (phytyl pyrophosphate) in the flavedo of 'Fortune', 'Nova' and 'Nadorcott' mandarins stored at 2 °C for up to 8 weeks. The genes analyzed were *DXS1* and *DXS2* (1-deoxy-D-xylulose-5-phosphate synthase 1 and 2), *GGPPS1* and *GGPPS6* (geranylgeranyl pyrophosphate synthase 1 and 6) and *GGDR* (geranylgeranyl diphosphate reductase). An expression value of 1 was arbitrarily assigned to the values obtained in the flavedo of 'Fortune' fruit at harvest. The data are mean ± S.E of at least three replicates.

The expression pattern of the two genes belonging to the SK pathway analyzed, *TAT1* and *HPPD*, also showed differences among cultivars (Fig. 6). At harvest, accumulation of both transcripts was slightly higher in the peel of 'Nadorcott' and 'Nova' than in 'Fortune'. Cold storage produced a transient reduction in the accumulation of *TAT1* transcript, which was recovered after 5 or 8 weeks of storage. By contrast, *HPPD* gene was markedly up-regulated during cold storage, reaching levels 4-, 2- and 6.5-times higher after 5 weeks than initial ones

in the peel of 'Fortune', 'Nova' and 'Nadorcott', respectively. Transcripts levels declined after 8 weeks of storage in 'Nova' and 'Nadorcott' (Fig. 6).

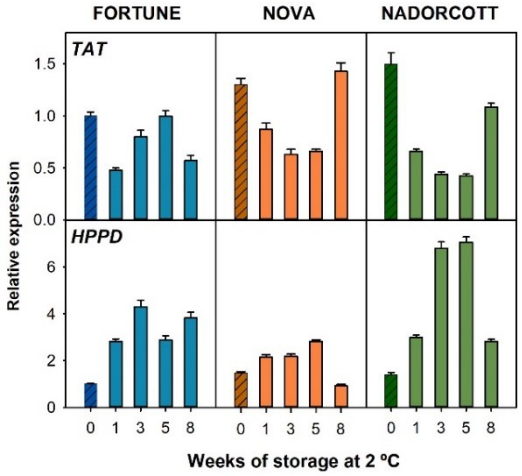

Figure 6. Relative expression of genes of the SK pathway involved in the synthesis of HGA (homogentisate) in the flavedo of 'Fortune', 'Nova' and 'Nadorcott' mandarins stored at 2 °C for up to 8 weeks. The genes analyzed were *TAT1* (tyrosine aminotransferase) and *HPPD* (4-hydroxyphenylpyruvate dioxygenase). An expression value of 1 was arbitrarily assigned to the values obtained in the flavedo of 'Fortune' fruit at harvest. The data are mean ± S.E of at least three replicates.

Genes of the specific tocopherol biosynthetic pathway also exhibited differences in their expression pattern among cultivars at harvest and after cold storage (Fig. 7). At harvest, expression of these genes tended to be higher in the flavedo of 'Nadorcott' and 'Nova', which accumulated higher tocopherol content. Expression of *VTE3a* was 4- and 5-times higher in 'Nadorcott' and 'Nova', respectively, than in 'Fortune', whereas *VTE4* was about 30 % higher in these two cultivars. By contrast, transcripts corresponding to *VTE3b* and *VTE1* were higher in 'Nadorcott' than in 'Nova' and 'Fortune', and *VTE2* was the only gene with higher expression levels in 'Nova' than in the other cultivars (Fig. 7). Analysis of the expression of these genes during storage at 2 °C revealed two patterns: a down-regulation for *VTE3b* and *VTE4*, and an initial decline only to increase again at the end of the storage for *VTE2*, *VTE3a* and *VTE1*. After 8 week of storage, relevant differences among cultivars were detected for *VTE2* and *V*TE3a, with expression levels being highest in 'Nova' mandarin (Fig. 7).

A global comparison of the changes in gene expression among cultivars revealed that the relative expression of the majority of genes (with the exception of *GGPPS1* and *VTE2*) involved in the different tocopherol synthetic pathways were always lower in the peel of 'Fortune' (low tocopherol content) than in the other cultivars with high tocopherol content.

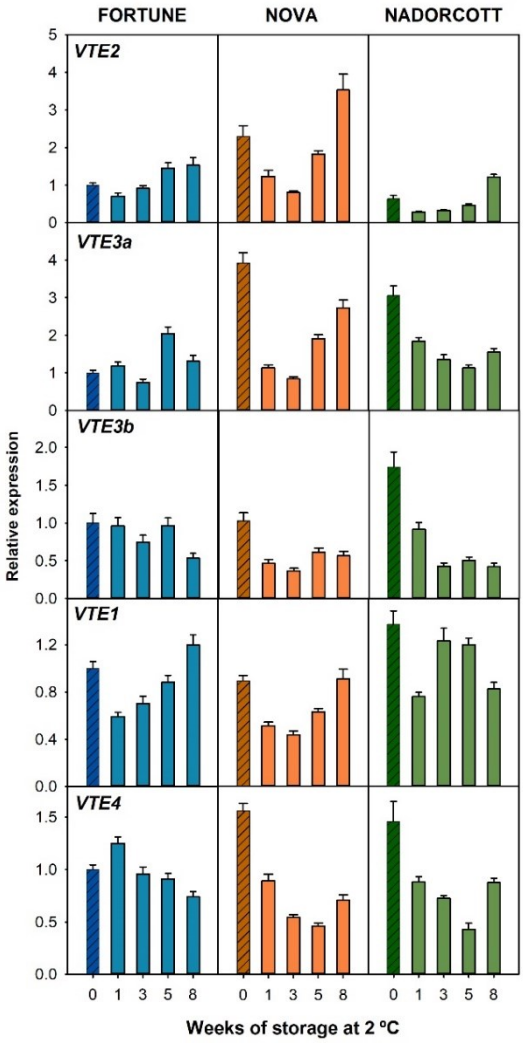

Figure 7. Relative expression of genes of the tocopherol-core pathway in the flavedo of 'Fortune', 'Nova' and 'Nadorcott' mandarins stored at 2 °C for up to 8 weeks. The genes analyzed were *VTE2* (HPT, homogentisate phytyl transferase), *VTE3a* and *VTE3b* (MPBQ-MT, 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase a and b), *VTE1* (TC, tocopherol cyclase) and *VTE4* (γ-TMT, γ-tocopherol methyltransferase). An expression value of 1 was arbitrarily assigned to the values obtained in the flavedo of 'Fortune' fruit at harvest. The data are mean ± S.E of at least three replicates.

3.5. Multivariate analysis of tocopherol accumulation and changes in gene expression in the flavedo of mandarin fruit during cold storage

A principal component analysis (PCA) was carried out to explore the relationship between tocopherol accumulation and gene expression in response to cold storage among the different cultivars. The first three principal components (PCs) explained for 70 % of the total variance of the data (PC1 = 26.4 %, PC2 = 25.1 % and PC3 = 17.8 %). The score and variable plots for PC1 *vs* PC2 (Fig. 8A and B) and PC1 *vs* PC3 (Fig. 8C and D) are presented. The two first PC tended to separate samples according to the storage dates rather than the different cultivars (Fig. 8A). Variable plot (Fig. 8B) revealed that the expression of genes *VTE3a*, *VTE3b*, *VTE4*

and *TAT1* positively contributed to PC1, while *HPPD* and *DXS2* contributed negatively. The first genes tended to be repressed by cold and their relative expression was highest at harvest (Fig. 8A and B), while *HPPD* and *DXS2* exhibited a sharp induction by cold and were highest during mid-storage (3w). Variables contributing to PC2 were mainly *GGPPS1, GGPPS6, VTE2, VTE6* and Gtoc (γ-tocopherol). These variables were grouped together and were genes up-regulated (gradual or sharply) towards the end of cold storage. The high correlation of γ-tocopherol with *GGPPS1*, *GGPPS6*, *VTE6* and *VTE2* suggests that the induction of these genes could explain the increase detected in γ-tocopherol content in 'Nova' and 'Nadorcott' at the end of the storage. When the PC3 was plotted against PC1 (Fig. 8C), 'Nadorcott' differentiated from the other two cultivars, which grouped together. The main variables contributing to PC3 were Ttoc (total tocopherols) and Atoc (α-tocopherol), and to a lesser extent the expression of *VTE1*, highlighting the differences in total and α-tocopherol content between 'Nadorcott' and the other cultivars. Moreover, the poor representation of *GGDR* in the selected PCs is likely due to the contrasting tendency of its expression in response to cold among the cultivars.

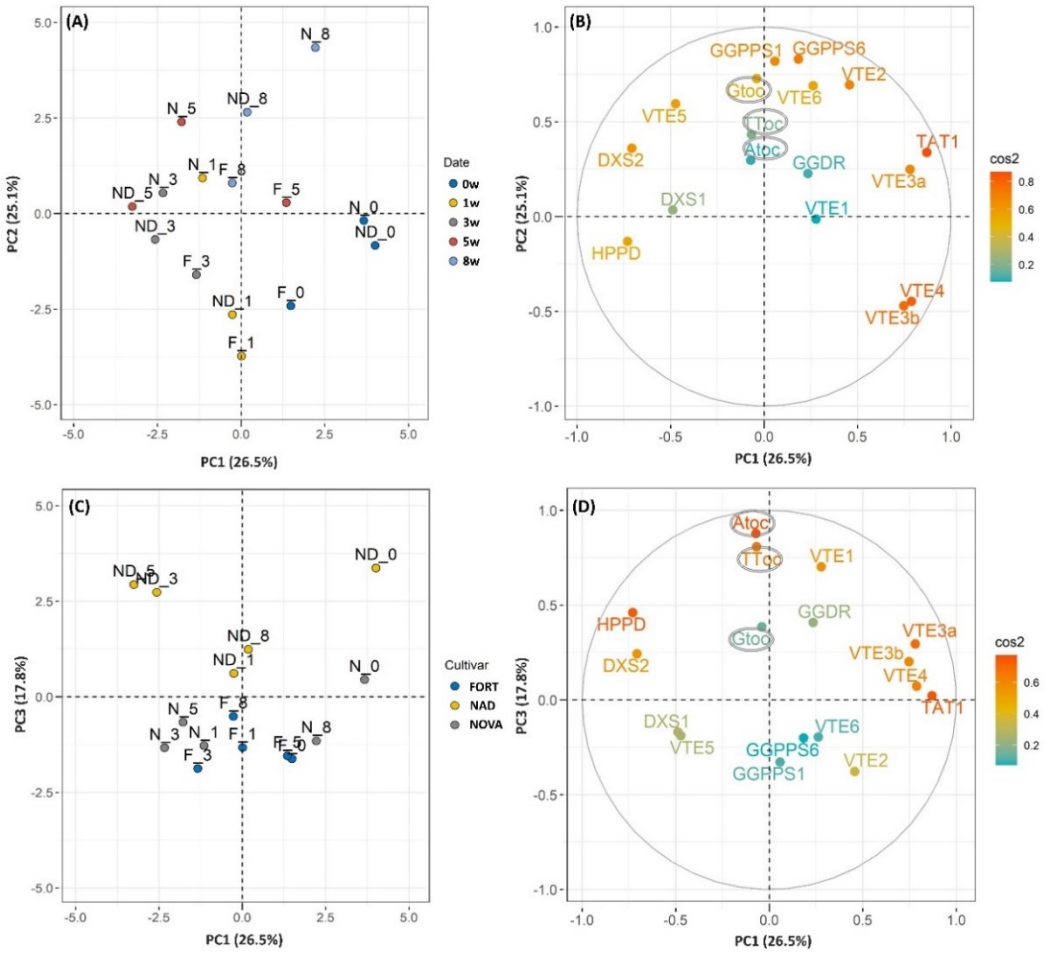

Figure 8. Principal components analysis (PCA) score plot (A) and variables plot (B) for PC1 *vs* PC2, and score plot (C) and variables plot (D) for PC1 *vs* PC3 of the variables analyzed (tocopherol content

(Cont. Figure 8) and relative gene expression) in 'Fortune' (F), 'Nova' (N) and 'Nadorcott' (ND) mandarin. Variables are colored in a gradient from blue to orange according to the square cosine (cos2), which reflects the representation quality of a variable in the selected PC axes (blue: low cos2, bad representation; orange: high cos2, good representation). Total tocopherol (Ttoc), α-tocopherol (Atoc) and γ-tocopherol (Gtoc) content are highlighted in circles.

4. Discussion

Tocopherols are plastid-derived isoprenoids that play important physiological functions in plants, of which their role as antioxidants is one of the most relevant (Falk and Munné-Bosch, 2010; Fritsche et al., 2017; Muñoz and Munné-Bosch, 2019). Tocopherols can either scavenge peroxyl radicals or quench singlet oxygen, being highly efficient in protecting plant tissues against lipid peroxidation and oxidative damage (Falk and Munné-Bosch, 2010). Experimental evidences indicate that CI development in *Citrus* fruit is an event associated with a boost in oxidative processes (Lado et al., 2016; Sala, 1998), and that the selective accumulation of certain antioxidant compounds, such as specific carotenoids, may be involved in the tolerance to this type of stress (Lado et al., 2015, 2016; Rey et al., 2020). To gain further insights into the implication of other antioxidants in the susceptibility of *Citrus* fruit to CI, in this work we have focused on the involvement of tocopherols, as highly efficient lipid-soluble antioxidants, in the tolerance of mandarin fruit to CI during storage at 2 °C. To that end, we analyzed changes in tocopherol content and in the expression of 14 genes involved in the synthesis of tocopherol precursors and in the specific steps shaping tocopherols composition in the peel of mandarin fruit with contrasting susceptibility to CI. 'Fortune' mandarin was highly sensitive to CI, whereas 'Nova' and 'Nadorcott' mandarins displayed moderated and low CI, respectively (Fig. 2). To our knowledge, this is first physiological and molecular characterization of tocopherols biosynthesis in *Citrus* fruit in an attempt to understand their involvement in the postharvest tolerance to cold stress.

Analysis of tocopherol content revealed that only the forms α- and γ-tocopherol accumulated in the flavedo of the three mandarin cultivars at harvest and during cold storage (Fig. 3). Moreover, α-tocopherol was the main form, accounting for 92.8 %, 82.3 % and 87.8 % of total tocopherols at harvest in 'Fortune', 'Nova' and 'Nadorcott' mandarin, respectively, and these proportions remained relatively stable or slightly decreased during 2 months of storage at 2 °C (Fig. 3). Information related to tocopherol accumulation in citrus fruit is limited, but in fruit of other *Citrus* species, α- and γ-tocopherol have been the detected forms (Assefa et al., 2017; Mathaba et al., 2014). In orange and tangerine grown in Korea, α-tocopherol represented 40 % and 36 % of total tocopherols, respectively (Assefa et al., 2017), which is

significantly lower than those determined in this work. If these discrepancies represent genotypic, environmental or growing conditions differences remain to be determined. Composition of tocopherols varies among different plant species but, in general, α-tocopherol is the major form in most plant tissues, with the exception of seeds of certain species which accumulate mainly γ-tocopherol (Horvath et al., 2006; Munné-Bosch and Alegre, 2002). In fleshly fruit, such as tomato (Quadrana et al., 2013), red pepper (Koch et al., 2002), avocado (Vincent et al., 2020), cherry (Tijero et al., 2016) or olive (Georgiadou et al., 2019), α-tocopherol was also the major form accumulated.

Analysis of total tocopherol content in the peel of mandarin at harvest revealed marked differences among cultivars. 'Nadorcott' accumulated the highest tocopherol content (135 g kg^{-1}), which was about 1.5- and 2.5-times higher than that of 'Nova' and 'Fortune', respectively (Fig. 3). These concentrations of total tocopherols were higher than those previously reported by Assefa et al. (2017) in fruit of other *Citrus* species (70-131 g kg^{-1} of dry weight). As with other bioactive molecules (Alós et al., 2014; Alquézar et al., 2008; Assefa et al., 2017), tocopherol contents in the peel were much higher to those reported in the pulp (Chun et al., 2006). Interestingly, we have recently reported that the concentration of total carotenoids in the flavedo of 'Nadorcott' mandarin is substantially higher than that of 'Fortune' and 'Nova' (Rey et al., 2020), and also of other traditional mandarin (Lado, Alós, et al., 2019), suggesting an enhanced synthesis and accumulation of isoprenoids-derived compounds in fruit of this mandarin cultivar.

Comparison of tocopherol concentration at harvest and during cold storage (Fig. 3) in the peel of the three mandarin cultivars revealed an inverse relationship between tocopherol content and the susceptibility to CI (Fig. 2). 'Fortune' mandarin fruit were extremely susceptible to CI and after 3 weeks at 2 °C, 90 % of the fruit exhibited chilling symptoms (40 % severe symptoms). At the same storage time, 40 % of 'Nova' fruit developed CI (3 % severe) while no damage was detected in 'Nadorcott' (Fig. 3). Since total tocopherols were 60 % and 30 % lower in the chilling-sensitive fruit of 'Fortune' and 'Nova', respectively, than in the CI-tolerant 'Nadorcott', it is tempting to speculate that high levels of tocopherols at the beginning of cold storage may play a protective role against chilling-induced damage. A similar inverse relationship between CI sensitivity and tocopherol levels has been observed in the exocarp of cold stored zucchini fruit, in which higher concentrations were detected in tolerant genotypes (Rodov et al., 2020). These results are in agreement with the general concept of tocopherols in plants under stress conditions, establishing that stress-tolerant species contain higher levels of

tocopherols than sensitive species (Munné-Bosch and Alegre, 2002; Sadiq et al., 2019). Moreover, although both γ- and α-tocopherol were lower in the peel of 'Fortune' fruit, differences between 'Nova' and 'Nadorcott' were mainly in α-tocopherol, as γ-tocopherol contents were very similar. α-tocopherol has been reported as the most efficient singlet oxygen quencher tocopherol sub-form (Gruszka et al., 2008), and it is likely that the differences in this form among the mandarin genotypes may play a role in their contrasting tolerance to CI.

During cold storage, total tocopherol and α-tocopherol content did not change in fruit of the CI-sensitive mandarins, but declined after the first week in the CI-resistant cultivar, only to recover to similar initial levels at the end of storage. In contrast, contents of γ-tocopherol significantly increased (almost 2-fold) in 'Nova' and 'Nadorcott' after 8 weeks of storage (Fig. 3). The effect of cold stress on tocopherol accumulation has been mainly studied in vegetative tissues of model plants, such as Arabidopsis (Bergmüller et al., 2003; Gabruk et al., 2016; Maeda et al., 2006; Sadiq et al., 2019), and only a few studies have evaluated the influence of this abiotic stress on tocopherol synthesis in fruit. In avocado, a short cold-shock increased γ- but not α- and β-tocopherol levels, whereas after 10 d under cold conditions α-tocopherol content decreased by a 20 % (Vincent et al., 2020). In cherry fruit, a transient increase in total tocopherol levels was detected after 3 d of storage (Tijero et al., 2016). The decrease in α-tocopherol after a 1 week of cold storage in 'Nadorcott' mandarin is similar to that observed in avocado and may indicate a primary cold-shock response in this tolerant genotype, but the potential influence of this change on CI-tolerance remains to be determined. Despite these fluctuations, changes in total tocopherols in fruit of mandarin under low temperature storage are similar to those occurring in other antioxidants compounds, such as ascorbate and glutathione, which experienced minor variations and remained almost unchanged during cold storage (Lado et al., 2016; Rey et al., 2020). These results may indicate that the pool of these compounds appears to be metabolically stable during postharvest cold storage or alternatively indicative of a rapid turnover. Therefore, it seems that in the peel of mandarin fruit the concentration of tocopherols, and in particular of α-tocopherol, at harvest and during the storage period may be an important factor for the fruit to cope with postharvest cold stress and in the tolerance to CI development.

Tocopherol biosynthesis in plants is very complex and involves the convergence of different metabolic pathways, which are also connected and provide intermediates for the synthesis of other relevant compounds (Mène-Saffrané, 2017; Muñoz and Munné-Bosch, 2019). In this study, we have analyzed the transcriptional profiling of 14 genes involved in the synthesis of

the metabolic precursors PPP and HGA, and in the main steps of the tocopherol-core pathway, at harvest and during postharvest storage of mandarin fruit. The *Citrus* genes were selected on the basis of sequence homology with previously identified genes from Arabidopsis and tomato (Almeida et al., 2011; Mène-Saffrané, 2017; Muñoz and Munné-Bosch, 2019). All these genes were expressed in the flavedo of the three cultivars at harvest and during storage, and changes in their transcriptional profile revealed key genes regulating tocopherols synthesis in the flavedo of fruit of the different mandarin genotypes.

The precursor HGA, which is common for all tocochromanols, is supplied by the SK pathway, while PPP can either be formed directly from GGPP (produced in the MEP pathway), or from the recycling of phytol formed in the degradation of chlorophylls. The expression analysis of the genes involved in the SK pathway (*TAT1* and HPPD) and the recycling of phytol (*VTE5* and *VTE6*) (Fig. 1) did not reveal important differences among mandarin genotypes nor with the differences in tocopherol content (Fig. 4 and 6), suggesting that the expression of these genes does not appear to limit tocopherol concentration in the peel of mandarin fruit. By contrast, the expression of most genes of the MEP pathway at harvest, with the exception of *GGPPS1*, was significantly lower in the low-tocopherol accumulating cultivar (Fig. 5). These differences were remarkable for the expression of *DXS1*, *DXS2* and *GGDR*, which were 2- to 3-times higher in fruit of 'Nadorcott' and 'Nova' than in 'Fortune' mandarin. DXS regulates the amount of IPP produced and controls the influx into the MEP pathway (Estévez et al., 2001), while GGDR is the enzyme limiting the reduction of GGPP into precursor PPP. Therefore, if a higher expression of *DXS* and *GGDR* genes correlate to higher enzymatic activity, these enzymatic steps supplying the precursor PPP for condensation with HGA may be important key steps regulating the concentration of tocopherols in mandarin. In vegetative tissues, a down-regulation of the *DXS* gene has been associated with a lower production of plants isoprenoids, including tocopherols (Estévez et al., 2001; Rodríguez-Concepción and Boronat, 2015). Moreover, in tomato fruit a higher expression of both *DXS* and *GGDR* was detected in genotypes accumulating high concentrations of tocopherols (Almeida et al., 2011; Quadrana et al., 2013). Nonetheless, differences in the expression of *DXS* and *GGDR* do not explain the higher tocopherol content in 'Nadorcott' than in 'Nova' (Fig. 3), suggesting the occurrence of other downstream metabolic steps or post-transcriptional mechanisms (Hemmerlin, 2013) regulating tocopherol concentrations in mandarin fruit.

The condensation of PPP with HGA to form MPBQ, catalyzed by HPT (*VTE2*) (Fig. 1), determines the entrance into the tocopherol-core pathway, which after subsequently

methylation and cyclization define the final content and composition of tocopherols (Fritsche et al., 2017; Mène-Saffrané, 2017). The expression of these genes, and in particular of *VTE3* isoforms, tended to be higher in the peel of fruit accumulating higher concentration of tocopherols at harvest ('Nadorcott' and 'Nova') than in 'Fortune' mandarin (Fig. 7). Although the gene *VTE2* is a limiting step in tocopherol synthesis in leaves and seeds of Arabidopsis, rice and soybean, among others (Collakova and DellaPenna, 2003b; Maeda et al., 2006; Wang et al., 2017), in mandarin fruit *VTE2* did not seem to restrict tocopherol synthesis since its expression was low in the cultivar with higher total tocopherols. Our results are in agreement with those of tomato and olive fruit (Georgiadou et al., 2019; Quadrana et al., 2013), in which *VTE2* appears not to be limiting for tocopherol accumulation. Interestingly, the expression of both isoforms of *VTE3* were lower in the peel of 'Fortune' (low tocopherol content), suggesting that the expression of these genes may be relevant for the accumulation of γ- and α-tocopherol in mandarin fruit peel. Likewise, a positive correlation between transcript levels of *VTE3* and tocopherol content and composition was detected in tomato fruit (Quadrana et al., 2013). Moreover, the last steps of the pathway controlling the final balance between the different isoforms of tocopherols are catalyzed by TC (*VTE1*) and γ-TMT (*VTE4*) (Bergmüller et al., 2003; Porfirova et al., 2002). Expression of both genes at harvest were slightly higher in fruit of 'Nadorcott' mandarin, but differences were minor among cultivars and may not explain tocopherol content. Collectively, these results suggest that the differences in tocopherol concentration in the fruit peel of the different mandarin cultivars at harvest appear to be dependent of the upstream genes involved in the precursors supply, in particular *DXS* and *GGDR*, in combination with the different isoforms of the gene *VTE3*.

The effect of cold storage on the transcriptional profiling of the genes of the different pathways involved in tocopherol synthesis was very variable and dependent on each gene and mandarin cultivar (Figs. 4-7). In general, genes involved in the supply of precursors PPP and HGA, with the exception of *GGDR* and *TAT1*, were up-regulated by low temperatures, although to a different extent and with variable patterns for each gene. The expression of *TAT1* experienced an early repression by cold, but increased again at 5-8 weeks of storage (Fig. 6). An induction of *HPPD*, the other gene involved in HGA synthesis, in response to different abiotic stresses has been previously described in different plant species, such as barley, alfalfa and Arabidopsis (Ma et al., 2020). Moreover, genes regulating PPP availability, either by the recycling of phytol (*VTE5* and *VTE6*) (Fig. 4) or through the MEP pathway (*DXS1, DXS2, GGPPS1* and *GGPPS6*) (Fig. 6), were stimulated during exposure to 2 °C. Nonetheless, since the induction of these genes occurred in fruit of the three mandarin genotypes, it is likely that

it represents a cold-response not directly related to the varietal susceptibility to CI. The only gene differentially affected among cultivars was *GGDR*, which expression appeared to be related to the genotypic differences in tocopherols content at harvest. *GGDR* was up-regulated in fruit with low tocopherol content ('Fortune') and repressed in the cultivars with higher content ('Nova' and 'Nadorcott') (Fig. 5). Since *GGDR* regulates the final step supplying PPP through the MEP pathway, it is tempting to speculate that its up-regulation in the CI-sensitive mandarin may be a CI response. Genes of the MEP pathway, and in particular *DXS* and *DXR*, have been shown to be up-regulated in response to different abiotic and biotic stresses (Xu et al., 2019), probably as part of the mechanisms to provide precursors for the synthesis of plastidial isoprenoids that may act as antioxidants under stress conditions. However, in Arabidopsis *GGDR* was not stimulated in response to stress (Collakova and DellaPenna, 2003a), similarly to what occurred in 'Nova' and 'Nadorcott' mandarins.

Exposure to postharvest cold temperatures had a different effect on the expression of the genes of the tocopherol-core pathway, than that observed in genes of the precursor's pathways. In general, these genes experienced an early repression that was progressively recovered towards the end of the storage (Fig. 7). This pattern of expression was similar in fruit of the three cultivars, suggesting that they may be cold-regulated responses rather than associated to the development of CI. Previous studies in Arabidopsis and other model plants have demonstrated that *VTE2*, *VTE3* and *VTE1* genes are essential for the maintenance of tocopherol levels and the tolerance of plants to different abiotic stresses, including low temperature (Ma et al., 2020; Maeda et al., 2006; Wang et al., 2017). Moreover, stimulation of these genes is a common response of plants in response to several stress conditions (Collakova and DellaPenna, 2003a; Ma et al., 2020). In citrus fruit peel, however, exposure to low temperatures did not up-regulate the expression of these genes in either CI-tolerant nor CI-sensitive cultivars. Since tocopherol levels remained nearly constant during cold storage, it is likely that the up-regulation of upstream genes, as *HPPD*, *VTE6*, *DXS1* and *GGPPS1*, may compensate the down-regulation of most genes of the tocopherol-core pathway. The idea that changes in gene expression are cold-mediated responses was supported by the PCA, in which the first two components tended to separate individuals according to the pattern of expression during cold storage, rather than according to the mandarin cultivar (Fig 8A and B). A third component differentiated 'Nadorcott' from the other cultivars, mainly the difference in total and α-tocopherol content (Fig. 8C and D).

It is interesting to mention that the changes in the relative abundance of *VTE4* transcripts during storage may account for the differences detected in γ-tocopherol among cultivars. In the tolerant 'Nova' and 'Nadorcott' mandarins, but not in the CI-sensitive 'Fortune', γ-tocopherol increased during cold storage (Fig. 3). Expression of the *VTE4* gene, responsible of the conversion of γ- to α-tocopherol, was markedly repressed in 'Nova' and 'Nadorcott' mandarins (70 % in both genotypes after 5 weeks) but not in 'Fortune' (4 %) (Fig. 7). Furthermore, the expression of upstream genes involved in the supply of PPP (*GGPPS1*, *GGPPS6*, *VTE6*) was correlated with γ-tocopherol content in the PCA (Fig. 8B), as they were up-regulated concomitantly with the increase in γ-tocopherol in 'Nova' and 'Nadorcott'. Then, the up-regulation of upstream genes together with the down-regulation of *VTE4*, would favor a higher influx into the tocopherol-core pathway, first, and a lower conversion of γ- into α-tocopherol, secondly, leading to a higher accumulation of γ-tocopherol in 'Nova' and 'Nadorcott'.

In summary, our results indicate a positive relationship between total tocopherol content, particularly α-tocopherol, in the peel of mandarin fruit and the tolerance to develop CI during storage at 2 °C. Higher tocopherol content appear to be important for the tolerance of mandarin fruit against cold stress. Expression of 14 genes involved in tocopherol synthesis revealed that the differences in tocopherol content among the cultivars are likely related to a higher relative expression of upstream *DXS1*, *DXS2* and *GGDR* genes, of the MEP pathway, and the gene *VTE3b* of the tocopherol-core pathway. While genes of the MEP pathway control the supply of PPP, and thus the influx into the tocopherol-core pathway, the methyltransferase encoded by *VTE3b* regulates the shift towards γ- and α-tocopherols synthesis. Moreover, the relative balance between the expression of upstream genes and *VTE4* may also influence the accumulation of the different tocopherol sub-forms. Finally, cold storage stimulated the expression of most genes of the pathways supplying tocopherol precursors, and down-regulated those of the tocopherol-core pathway, but these changes appeared to be cold-related responses rather than CI responses.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2021.111594.

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3.3. CAPÍTULO 3

Effect of fruit shading and cold storage on tocopherol biosynthesis and its involvement in the susceptibility of Star Ruby grapefruit to chilling injury.

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Abstract

The aim of this study was to investigate the role of tocopherols in the susceptibility of Star Ruby grapefruit to postharvest chilling injury (CI). Fruit exposed to normal sunlight (NC, non-covered) and deprived of light (C, covered) in the last stages of development were used. Tocopherol contents increased in the flavedo of both NC and C fruit during development, concomitantly with the up-regulation of *TAT1* and most genes of the tocopherol-core pathway. Fruit shading reduced total contents by repressing γ-tocopherol accumulation, associated to a down-regulation of *GGDR* and *VTE1* and, to a lesser extent, of *VTE2*, *VTE3a* and *VTE4*. During cold storage, total and α-tocopherol contents increased in NC and C fruit, and no direct relationship between tocopherol accumulation and CI tolerance was found. Cold stress up-regulated most genes involved in the synthesis of tocopherol precursors and down-regulated those of the tocopherol-core pathway, but changes seemed to be cold-mediated and not related to CI development.

Keywords: tocopherol, grapefruit, chilling injury, fruit shading, postharvest storage, Citrus

1. Introduction

Chilling injury (CI) is an economically important postharvest disorder that reduces external fruit quality, marketability and consumer acceptance, culminating in important economic losses. Because of their subtropical origin, fruit of many species and cultivars of *Citrus* are prone to develop peel injuries during storage at low temperatures (Lado, Cronje, Rodrigo, & Zacarías, 2019; Zacarias, Cronje, & Palou, 2020). Fruit of grapefruit cultivars are highly susceptible to CI when stored at temperatures below 10 °C (Lado, Rodrigo, Cronje, & Zacarías, 2015; Schirra, 1993). CI in the peel of grapefruit is manifested as a series of symptoms, referred to as peel pitting, which initiates as small brown pit-like depressions that expand forming large necrotic and depressed areas after prolonged cold storage (Lado et al., 2019; Lado, Rodrigo, et al., 2015). Susceptibility to CI is influenced by many factors, including the *Citrus* genotype and environmental and agronomical conditions, such as the harvest season, maturity, growing conditions, rootstock, fruit position on the tree canopy and peel pigmentation, among others, that markedly define the initiation and development of CI during postharvest cold storage (Lado et al., 2019; Zacarias et al., 2020).

Oxidative stress is a primary mechanism in the response of *Citrus* fruit against stress caused by low temperature, and/or by the damage induced under these conditions (Toivonen, 2004). The capability of certain species and cultivars to counteract these processes appears to be essential to determine fruit tolerance or susceptibility to CI under cold stress conditions (Lado et al., 2019; Zacarias et al., 2020). Antioxidant defense mechanisms in plants include detoxifying enzymes and low molecular weight compounds such as ascorbic acid, glutathione, carotenoids and tocopherols (Decros et al., 2019; Toivonen, 2004). Enhanced activity of enzymes of the antioxidant system has been associated with the natural tolerance to CI of several mandarin cultivars (Sala, 1998) and also with the tolerance induced by heat-conditioning treatments (Lafuente, Establés-Ortíz, & González-Candelas, 2017). Among them, a protective role of catalase against chilling has been proposed, as higher catalase activity and increased catalase transcripts have been detected in the peel of tolerant fruit of mandarin and grapefruit (Lado, Rodrigo, López-Climent, Gómez-Cadenas, & Zacarías, 2016; Maul, McCollum, Guy, & Porat, 2011; Sala, 1998). Ascorbic acid (vitamin C) and glutathione are potent water-soluble antioxidants (Decros et al., 2019) but their relation to CI in *Citrus* fruit is controversial, as contents in grapefruit and mandarin fruit did not support a direct relationship with CI tolerance (Lado et al., 2016; Rey, Zacarías, & Rodrigo, 2020). Carotenoids, on the other hand, are considered to be the first line of defense against singlet oxygen (Decros

et al., 2019) and have been associated with higher tolerance to CI and other postharvest disorders (Cronje, Barry, & Huysamer, 2011; Lado, Rodrigo, et al., 2015; Rey et al., 2020). In Star Ruby grapefruit, it has been shown that light avoidance at later stages of fruit development led to the accumulation of the red carotene lycopene, which results in fruit with a uniform red coloration of the peel and markedly induced fruit tolerance to CI after subsequent storage at low temperature (Lado, Rodrigo, et al., 2015; Lado et al., 2016). Interestingly, in the red peel of shaded grapefruit, the capacity to quench singlet oxygen was 2-3 times higher than in fruit exposed to light, which were susceptible to CI (Lado et al., 2016). Similarly, in fruit of mandarin cultivars with contrasting susceptibility to CI (Fortune, Nova and Nadorcott), a higher β-cryptoxanthin and violaxanthin content and capacity to quench singlet oxygen was detected in the CI-tolerant cultivars (Rey et al., 2020). Therefore, increased concentrations of carotenoids with enhanced antioxidant capacity may induce tolerance to CI in *Citrus* fruit. However, little is known about the role of tocopherols, which are potent lipid-soluble antioxidants, on the susceptibility of *Citrus* fruit to CI.

Tocopherols are plant-derived isoprenoids belonging to the chemical group of tocochromanols, which also includes tocotrienols, plastocromanol 8 and tocomonoenols. Their structure consists of a polar chromanol ring and a lipophilic polyprenyl side chain and, according to the position and degree of methylation of the chromanol ring, four isoforms exist: α-, β-, γ- and δ-tocopherols. Tocopherols are mainly produced in photosynthetic organisms and, together with tocotrienols, are the only organic molecules with vitamin E activity in animals (Mène-Saffrané, 2017; Muñoz & Munné-Bosch, 2019). These compounds have diverse physiological functions in plants, of which their role as antioxidants is among the most relevant, with the capability to scavenge free radicals and quench singlet oxygen, protecting cell membranes from lipid peroxidation and reducing oxidation of cellular components (Falk & Munné-Bosch, 2010). Additionally, the disposition and mobility of tocopherols within cell membranes helps to maintain stability and conformation of membrane's structure (Boonnoy, Karttunen, & Wong-Ekkabut, 2018). Since chilling in plants is associated with oxidative processes and the loss of cell membranes stability/fluidity (Lado et al., 2019; Zacarias et al., 2020), tocopherols could play a role in the protection of fruit against cold stress during postharvest storage by protecting cells against oxidative processes (Boonnoy et al., 2018; Falk & Munné-Bosch, 2010; Ma et al., 2020).

The synthesis of tocopherols in plants occurs mainly in plastids and can be divided into two stages. The first stage consists in the independent synthesis of the precursors

homogentisate (HGA) and phytyl pyrophosphate (PPP), while the second stage involves the specific steps and modifications leading to tocopherol formation (Mène-Saffrané, 2017; Muñoz & Munné-Bosch, 2019). The chromanol ring of all tocochromanols derives from the precursor HGA, which results from the degradation of L-tyrosine formed in the Shikimate (SK) pathway (Riewe et al., 2012; Tsegaye, Shintani, & DellaPenna, 2002). The precursor PPP is specific for tocopherol synthesis, and can be formed directly from GGPP (geranylgeranyl diphosphate) (Ruiz-Sola et al., 2016), produced in the methylerythritol 4-phosphate (MEP) pathway, or alternatively from the recycling of free phytol (Mène-Saffrané, 2017; Pellaud & Mène-Saffrané, 2017). The regulation of key enzymes involved in these steps define HGA and PPP availability and have a direct impact in the final amount of tocopherols accumulated (Pellaud & Mène-Saffrané, 2017). The specific tocopherol pathway, known as the tocopherol-core pathway, starts with the condensation of HGA and PPP, catalyzed by homogentisate phytyl transferase (HPT; *VTE2*), which forms 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ). MPBQ can produce δ-tocopherol by the action of tocopherol cyclase (TC; *VTE1*) and later β-tocopherol by the tocopherol methyl transferase (γ-TMT; *VTE4*); or, it can be methylated into 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) by a MPBQ methyltransferase (MPBQ-MT; *VTE3*). DMPBQ then produces γ-tocopherol and α-tocopherol by the same TC and γ-TMT. Tocopherol accumulation in vegetative tissues is limited by *VTE2* (Collakova & DellaPenna, 2003; Maeda, Song, Sage, & DellaPenna, 2006), and also highly influenced by the pathways supplying the precursors HGA and PPP controlling the influx into the tocopherol core-pathway (Muñoz & Munné-Bosch, 2019; Pellaud & Mène-Saffrané, 2017). Nonetheless, other studies have observed that the accumulation of tocopherols in fruit is not markedly influenced by the expression of the *VTE2* gene, and that *VTE3* seems to play a more pivotal role controlling tocopherol contents (Quadrana et al., 2013).

Accumulation of tocopherols and transcriptional changes of their biosynthetic genes in response to different abiotic stresses have been widely studied in many plant species (Ma et al., 2020; Sadiq, Akram, Ashraf, Al-Qurainy, & Ahmad, 2019). A role of tocopherols in the tolerance of plant species to low temperatures has been suggested, as increases in tocopherol contents have been detected in vegetative tissues in response to low temperatures, and plants lacking tocopherols exhibit hypersensitive phenotypes under cold stress (Bergmüller, Porfirova, & Dörmann, 2003; Ma et al., 2020; Maeda et al., 2006). The involvement of tocopherols in the response of fruit to postharvest cold storage has received little attention, and only few studies have addressed such relationship in cherry (Tijero, Teribia, Muñoz, & Munné-Bosch, 2016), avocado (Vincent, Mesa, & Munné-Bosch, 2020) and squash fruit (Rodov

et al., 2020). In *Citrus* fruit, we have recently observed that the natural tolerance of mandarin fruit to postharvest CI is associated with increased tocopherol contents (Rey, Rodrigo, & Zacarias, 2021), and that changes during storage appears to be independent of the development of CI. In order to gain further insights into the potential function of tocopherols in the response of other *Citrus* fruit to postharvest cold temperatures, we took advantage of the chilling tolerance of Star Ruby grapefruit induced by fruit shading during the last stages of development (Lado, Rodrigo, et al., 2015; Lado et al., 2016). Using such system, the objective of the present work was to study the effects of shading and storage at 2 °C on tocopherol concentration, and on the expression of genes of the different tocopherol biosynthetic pathways in Star Ruby grapefruits.

2. Materials and Methods

2.1. Plant material and storage conditions

Fruit of Star Ruby grapefruit (*Citrus paradisi* Macf.) were used throughout this study. Fruit of this cultivar are characterized by their red-colored flesh (due to lycopene accumulation), and they may also develop red coloration in areas of the peel. Accumulation of lycopene in the peel (external red coloration) is stimulated in this genotype by covering fruit and avoiding direct light exposure in the last stages of fruit development, which results in fruit tolerant to the development of CI, in contrast to CI-sensitive control fruit (Lado, Rodrigo, et al., 2015; Lado et al., 2016). Therefore, in this work we used non-covered (NC) and covered (C) fruit of Star Ruby to explore the involvement of tocopherols in the susceptibility/tolerance to CI. For this purpose, 100 fruits of Star Ruby grapefruit located outside of the tree canopy from four adult trees were covered with black polyethylene plastic bags 5 months before harvest at the immature-green stage (last week of July), leaving the bottom open to allow air circulation as described by Lado et al. (2015). Uncovered control fruit (NC) were located outside the canopy of the same trees, adjacent to C fruit and exposed to direct sunlight. Orchards were located in the Citrus Germplasm Bank at the Instituto de Investigaciones Agrarias (IVIA, Moncada, Valencia, Spain) and grown under normal agro-ecological conditions. NC and C fruit were harvested at commercial maturity in the first week of January, being the average for maximum and minimum field temperature for the four weeks previous to harvest of 16.8 ± 0.4 °C and 4.3 ± 0.6 °C, respectively. Fruit was delivered to the lab, inspected for color uniformity and external damages, and fruit free of any defect or damage, of both NC and C grapefruits, were randomly separated into two lots and stored at 2 °C and 85% RH for up to 8 weeks. The first lot of fruit was used for collecting samples of flavedo tissue, while the second lot, was divided

in three replicates of ten fruit (for each NC or C condition) and used for the periodic evaluation of CI. Flavedo samples were collected from the equatorial section of 6-8 fruits at the covering time (CT), harvest (H) and periodically throughout storage (1, 3, 5, 8 weeks). Flavedo tissue was excised with a scalpel, frozen in liquid nitrogen and ground to a fine powder. Samples were stored at -80 °C until analysis. At each date, peel color was measured using a Minolta CR-330 colorimeter (Minolta, Osaka, Japan) on three locations around the equatorial plane of the fruit, using three replicates of 10 fruit for each condition. Color was expressed as the *a/b* Hunter ratio, which is negative for green fruit, around zero for yellow fruit at color break, and positive for orange and red colored fruit.

2.2. Evaluation of chilling injury

During cold storage, CI was evaluated by inspecting and scoring fruit on a scale according to the extension and severity of the peel damage: 0 (no CI), 1 (low CI, <25% peel surface), 2 (high CI, 25-50% peel surface) and 3 (severe CI >50% peel surface). Results were expressed as CI index, which was calculated by adding the product of the number of fruit in each category multiplied by the score for each category and afterwards dividing it by the number of total fruits evaluated (Sala, 1998). CI was evaluated in 3 replicates of 10 fruit for each NC and C condition.

2.3. Tocopherol extraction and quantification

Tocopherols were extracted from the flavedo tissue following the protocol described in Fraser, Pinto, Holloway, & Bramley (2000). Briefly, 200 mg of flavedo tissue was extracted with methanol (MeOH, HPLC grade, Scharlau, Barcelona, Spain), Tris buffer plus NaCl (50 mM Tris-HCl pH 7.5/1 M NaCl) and dichloromethane (HPLC grade, Scharlau, Barcelona, Spain). Samples were grounded with a mortar and pestle with sea sand (PanReac AppliChem, Barcelona, Spain) as an abrasive, and the mixture was sonicated in a XUBA3 ultrasonic water bath (Grant Instruments, Cambridge, England) for 5 min. The homogenized sample was centrifuged for 10 min at 3,000 *g* and 4 °C for phase separation. The dichloromethane phase was recovered in a glass tube and the methanol phase and sediment were re-extracted with dichloromethane. The pooled dichloromethane extracts were dried under nitrogen gas and stored at -20 °C in a free O_2 atmosphere until analysis. For tocopherol quantification, dry extracts were re-suspended in 500-700 µl of ethyl acetate (HPLC grade, Merck, Madrid, Spain) and an aliquot of 20 µl was injected directly in a HPLC system (Acquity® Arc™, Waters, Barcelona, Spain) coupled with a fluorescence detector (2475 FLR Detector, Waters, Barcelona, Spain), and a YMC C30 column (150 × 4.6 mm, 3 µm) (Teknokroma, Barcelona, Spain) at 25 °C.

A ternary gradient elution at a flow rate of 1 ml/min was used for tocopherol separation. The initial solvent composition consisted of 90% of MeOH, 5% water and 5% methyl *tert*-butyl ether (MTBE, HPLC grade, Scharlau, Barcelona, Spain). After 12 min at these conditions, the solvent composition changed linearly to 95% MeOH and 5% MTBE, and after 2 min to 50% MeOH and 50% MTBE. These conditions were maintained for 5 min, after which the solvent composition was gradually re-established to initial conditions (min 20), and equilibrated for 10 min before the next injection. Compounds were detected by fluorescence with excitation at 296 nm and emission at 340 nm, and standards for δ-, γ- and α-tocopherol (SigmaAldrich, Barcelona, Spain) were used for the identification and quantification of tocopherols. All procedures were carried out on ice and under dim light to prevent degradation. Total tocopherol content was calculated as the sum of the tocopherol isoforms detected, and concentrations are expressed as μg/g of fresh weight. Samples were extracted twice and results are the mean of the replicates (mean ± standard error).

2.4. Total RNA extraction, cDNA synthesis and q-PCR conditions.

Total RNA was extracted from flavedo tissue using the RNeasy Plant Mini Kit (Qiagen, Madrid, Spain), following manufacturer's instructions. For cDNA synthesis, total RNA was treated with DNase I (DNA free, DNase treatment & removal, Ambion, Barcelona, Spain) and RNA concentration was measured by spectrophotometric analysis (Nanodrop, Thermo Fisher Scientific, Barcelona, Spain). The quality of the RNA was verified by agarose gel electrophoresis with GoodView™ Nucleic Acid Stain (SBS Genetech, Beijing, China). Five μg of total RNA were reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Barcelona, Spain) in a total volume of 20 μl, following the manufacturer's procedure. First-strand cDNA samples were diluted 1:5 to a final concentration of approximately 100 ng/μl for each amplification reaction.

Quantitative real-time PCR was performed using a LightCycler 480 instrument (Roche, Madrid, Spain), using the LightCycler 480 SYBRGreen I Master kit (Roche, Madrid, Spain) and following the manufacturer's instructions. The relative expression was measured for 14 genes related to: i) the SK pathway supplying the tocopherol precursor HGA (*TAT1* and *HPPD*); ii) the MEP pathway supplying the precursor PPP (*DXS1* and *DXS2*, *GGPPS1* and *GGPPS6 and GGDR*); iii) the recycling of free phytol into PPP (*VTE5* and *VTE6*); iv) and to the tocopherol-core pathway (*VTE2*, *VTE3a* and *VTE3b*, *VTE1* and *VTE4*). These genes were selected on the basis of their putative function and the activity of the corresponding enzyme in the metabolic pathway of tocopherol biosynthesis in plants (Mène-Saffrané, 2017; Pellaud & Mène-Saffrané,

2017; Quadrana et al., 2013). Moreover, the selected genes were previously identified in the *Citrus* genome based on homology to *Arabidopsis thaliana* and *Solanum lycopersicum* protein sequences and the primers designed for each gene were successfully used for analysis of gene expression in the peel of mandarin fruits (Rey et al., 2021). Details of the genes and primers sequences used are listed in Table S1. The cycling protocol, for all genes analyzed, consisted of 10 min at 95 °C for pre-incubation, 40 cycles of 10 s at 95 °C for denaturation, 10 s at 59 °C for annealing and 10 s at 72 °C for extension. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve performed after the amplification steps. For expression measurements, we used the LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche, Madrid, Spain) and calculated expression levels relative to values of a reference sample using the Relative Expression Software Tool (Pfaffl, Horgan, & Dempfle, 2002). For all genes and all dates analyzed the reference sample used to calculate relative expression was the expression value for covered fruit of Star Ruby at harvest, which was set as 1. The housekeeping gene used for normalization was *ACTIN* (Alós, Rodrigo, & Zacarías, 2014). The reference sample used for all genes was the expression values for covered fruit of Star Ruby at harvest.

2.5. Statistical analyses

Unpaired Student's t-test was used to determine statistically mean differences between NC and C fruit in each sample date (significance level at $p \leq 0.05$). Additionally, a one-way ANOVA was carried out and Tukey's test (significance level at $p \leq 0.05$) was used for mean comparisons among dates for each fruit condition (NC or C fruit). Analyses were performed using SigmaPlot version 14.0 (Systat Software, USA).

3. Results and Discussion

3.1. Fruit shading and cold storage influenced tocopherol accumulation in the flavedo of Star Ruby grapefruit

As a first step to understand the involvement of tocopherols, as potent lipid-soluble antioxidants, in the tolerance of *Citrus* fruit to CI, the concentration of tocopherols was determined in the flavedo of NC and C Star Ruby grapefruits, which displayed contrasting susceptibility to CI. For that purpose, immature fruit of Star Ruby grapefruit with an external green coloration were covered (C) in July with black bags to avoid light exposure during fruit ripening, whereas non-covered (NC) fruit were located outside of the fruit canopy and exposed to direct sunlight. Both fruit were harvested at the first week of January and were

similar in size but with different internal maturity. Total soluble solids and acidity were higher in the juice of NC fruit than in C, resulting in a lower internal maturity index (5.48 and 7.14, respectively (Table S2). These results may be indicative of an accelerated internal maturation in the pulp in covered conditions, similarly to the effect of shading on accelerating peel degreening, triggering the earlier degradation of chlorophylls and the accumulation of carotenoids (Lado, Cronje, et al., 2015). Moreover, at harvest, marked differences in peel coloration (Fig. 1A and 1B) between NC and C fruit were detected. C fruit developed a uniform red peel coloration, whereas peel color of NC fruit was pale-yellow with pale red shades in some areas of the fruit surface less exposed to sunlight (Fig. 1A). This effect was clearly reflected by the *a/b* Hunter ratio, which was around 1 for C fruit and 0.2, typical for yellow coloration, for NC fruit (Fig. 1B). During storage at 2 °C, peel coloration remained nearly constant in both NC and C fruit (Fig. 1B). NC fruit were highly sensitive to CI, with symptoms appearing after 1 week and increasing substantially during storage, reaching a CI index near 2.5 (severe damage) after 8 weeks at 2 °C. On the other hand, and as observed in previous studies (Lado, Rodrigo, et al., 2015; Lado et al., 2016), C fruit developed chilling symptoms at a much lower rate and, after 8 weeks, CI index was less than 1 (slight damage) (Fig. 1A and C).

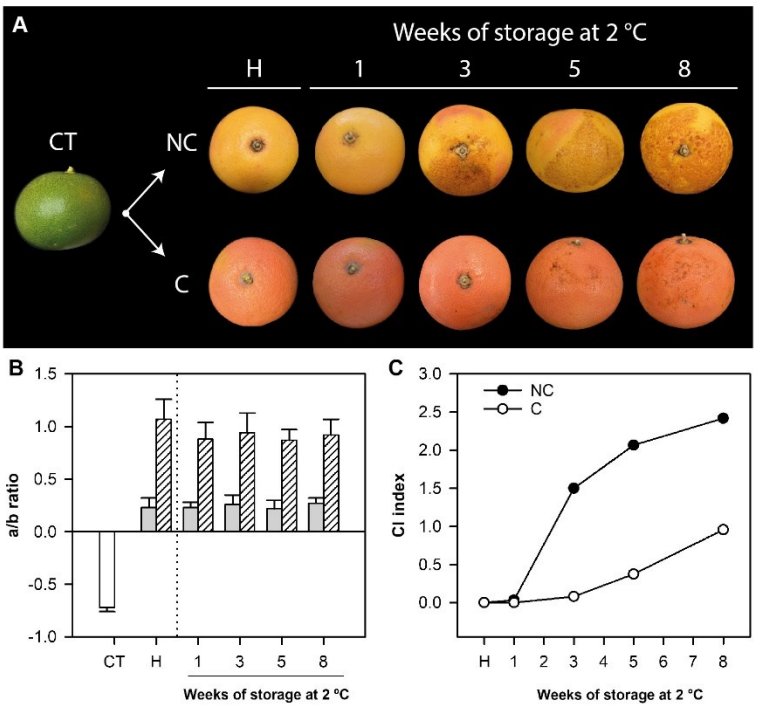

Figure 1. External appearance (**A**), rind color expressed as *a/b* ratio (**B**) and development of CI expressed as CI index (**C**) of immature-green fruit at the covering time (CT), and of non-covered (NC) and covered (C) fruits of Star Ruby grapefruit at harvest (H) and during postharvest storage at 2 °C for up to 8 weeks.

The HPLC-FLR analysis of tocopherols revealed that only the isoforms α- and γ-tocopherol were accumulated in the flavedo of NC and C Star Ruby grapefruit, and that α-tocopherol was the predominant form accumulated (Fig. 2). In other *Citrus* species, such as mandarin (Rey et al., 2021) and orange (Assefa, Saini, & Keum, 2017), α-tocopherol has been reported as the main tocopherol isoform, although the predominance of γ-tocopherol has also been described in the peel of fruit of other less common *Citrus* species (Assefa et al., 2017). Fruit shading significantly reduced total tocopherol content at harvest (Fig. 2A). In immature-green fruit, just before covering, total tocopherol was 81 μg/g, with α-tocopherol accounting for 76% and γ-tocopherol for 24% of total contents. During fruit development and maturation, α-tocopherol increased to a similar extent in NC and C (Fig. 2C), but fruit shading drastically impaired the increment of γ-tocopherol in flavedo of mature fruit, which was 4-times lower in C fruit (Fig. 2B). As a result of these changes, total tocopherols were lower in the flavedo of C than in NC fruit at harvest, and the proportion of α- and γ-tocopherol was substantially altered, with α-tocopherol accounting for 67-76% of the total in NC fruit in comparison to 89-95% in C fruit (Fig. 2). These results clearly indicate a differential effect of light on the accumulation of the different tocopherols isoforms in the peel of Star Ruby grapefruit. Previous experiments in Star Ruby revealed that shading altered the accumulation of carotenoids and vitamin C, and also influenced the expression of key genes of the biosynthetic pathways of these compounds (Lado, Cronje, et al., 2015; Lado, Rodrigo, et al., 2015; Lado et al., 2016). A similar effect of light on tocopherol synthesis has been recently reported in tomato fruit, in which the accumulation of tocopherols was reduced in fruit grown under darkness, associated with a down-regulation of several genes of their synthesis (Gramegna et al., 2019). Then, the concentration of tocopherols increased in the peel of grapefruit during the transition from fruit development to maturation, and it is likely that light avoidance affects metabolic steps regulating the accumulation of γ-tocopherol.

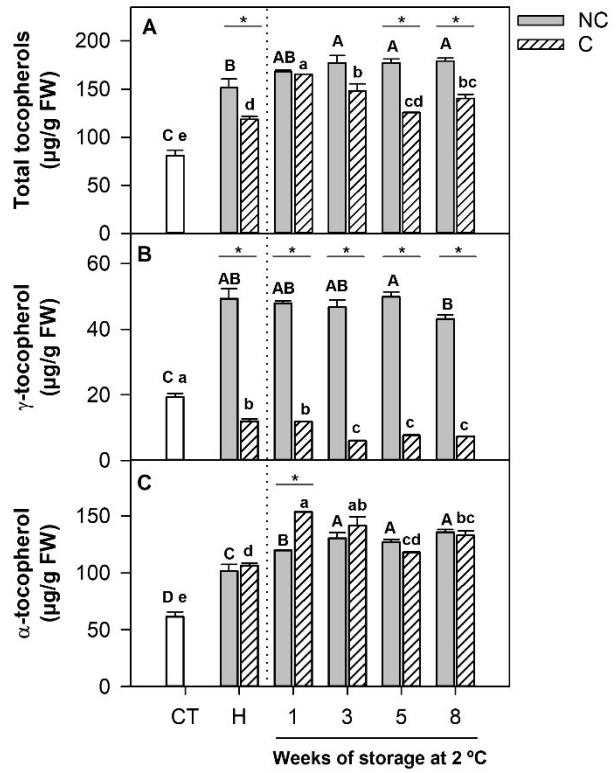

Figure 2. Total tocopherol (A), γ-tocopherol (B) and α-tocopherol content (C) in the flavedo of non-covered (NC) and covered (C) fruits of Star Ruby grapefruit, at the covering time (CT), harvest (H) and during postharvest storage at 2 °C for up to 8 weeks. Letters indicate significant differences in tocopherol contents among dates for each fruit condition, capital letters for NC fruit and lowercase letters for C fruit ($p \leq 0.05$, Tukey test). Asterisks indicate significant differences between NC and C fruit ($p \leq 0.05$, t-test). Results are expressed as µg/g of fresh weight.

Exposure of Star Ruby grapefruits to cold storage provoked a moderate increase in total tocopherol content in both NC and C fruit (Fig. 2A), although the effects on α- and γ-tocopherol accumulation were different. The concentration of γ-tocopherol remained nearly constant in NC fruit while the low content in C fruit decreased during storage (50% after 3 weeks) (Fig. 2B). α-Tocopherol increased progressively during the whole storage period in both NC and C (30% after 3 weeks), but with minor differences between both conditions (Fig. 2C). Because of these changes, total tocopherol content increased during cold storage in both conditions, but were still higher in NC than in C fruit (Fig. 2A). Increases in tocopherol content in response to low temperatures have been observed in vegetative tissues (Bergmüller et al., 2003; Ma et al., 2020; Maeda et al., 2006), but information in fruit is scarce and variable. While cold storage increased α-tocopherol content in cherries (Tijero et al., 2016) it decreased it in fruit of different avocado cultivars (Vincent et al., 2020).

Our results in Star Ruby grapefruits indicate that: (i) fruit shading reduced total tocopherols in the peel of C fruit, which were more tolerant to CI than NC fruit; (ii) this effect was more notable in γ-tocopherol contents, which were 4- to 8-times lower in C fruit; and (iii) changes in

α- and total tocopherol contents during storage were similar in both conditions. Together, it appears that the changes in tocopherols during cold storage are a response of the fruit to low temperature stress, and that the positive relation between shading and the tolerance to CI of Star Ruby grapefruit appears to be independent of tocopherol levels at harvest and during the storage period. In mandarin fruit, a direct association between tocopherol content at harvest and the tolerance to CI during cold storage has been observed (Rey et al., 2021). Similarly, in squash fruit stored at 4 °C, a higher resistance to CI was detected in genotypes accumulating higher tocopherol contents (Rodov et al., 2020). A possible explanation for the differences observed among *Citrus* species would take into account a differential function of tocopherols in the response of fruit to CI or, alternatively, that differences in tocopherol concentrations may be associated with the natural tolerance to CI (such as in mandarin varieties) but not with that induced by environmental stimuli, as light avoidance in Star Ruby grapefruits. It is important to remark that light deprivation in Star Ruby grapefruit triggers the accumulation of the carotene lycopene, which is detected at trace amounts in the peel of control fruit (Lado, Rodrigo, et al., 2015). Lycopene shares similar antioxidant properties with tocopherols and both compounds are considered highly efficient singlet oxygen quenchers (Decros et al., 2019). Therefore, the tolerance of Star Ruby C fruit to CI is primarily related to the differential accumulation of this carotene (Lado, Rodrigo, et al., 2015; Lado et al., 2016) rather than to the tocopherol content.

3.2. Transcriptional regulation of tocopherol biosynthesis in the flavedo of Star Ruby grapefruit

In order to understand the effect of fruit shading during development and ripening and of cold storage on the regulation of tocopherol synthesis, and whether the alterations in gene expression are associated with the changes in tocopherol concentration, the expression of 14 genes involved in different steps of tocopherol synthesis were analyzed in the flavedo of NC and C grapefruits. Tocopherol biosynthesis requires the condensation of the precursors PPP and HGA, and the steps controlling their availability have been described to be as important as the specific steps of tocopherol synthesis in determining the final tocopherol content (Mène-Saffrané, 2017; Pellaud & Mène-Saffrané, 2017). Therefore, we analyzed the expression of genes regulating HGA supply through the SK pathway (*TAT1* and *HPPD*), genes regulating PPP supply through the recycling of phytol (*VTE5* and *VTE6*) and through the MEP pathway (*DXS1*, *DXS2*, *GGPPS1*, *GGPPS6* and *GGDR*), and genes belonging to the tocopherol-core pathway (*VTE2*, *VTE3a*, *VTE3b*, *VTE1* and *VTE4*) regulating the final concentration and composition of tocopherols.

3.2.1 Effect of fruit shading

Fruit shading did not affect the expression of the two genes involved in the supply of HGA, but these genes were differentially expressed during fruit ripening (Fig. 3A). Transcripts of *TAT1* experienced a 15-fold increase from immature (CT) to ripe fruit, whereas those of *HPPD* remained nearly constant. Interestingly, these changes were similar in both NC and C, indicating that the regulation of these genes was independent of light exposure. On the other hand, the expression of *VTE5* and *VTE6* genes, which are involved in the supply of PPP by the recycling of phytol, were slightly or markedly down-regulated during development and ripening, respectively, but transcript levels were higher in fruit kept in the darkness indicating that light may play a negative effect on the expression of these genes (Fig. 3B). It is interesting that, of these genes, only *TAT1* was enhanced during fruit development and maturation with minor differences in NC and C fruit, suggesting that this step may contribute to the increase in total tocopherols observed at harvest in both conditions (Fig. 2A). Transcriptional levels and enzymatic activity of TAT have been related to tocopherol content in leaves of *Arabidopsis* (Riewe et al., 2012), and also appear to be involved in tocopherol accumulation in tomato fruit (Quadrana et al., 2013).

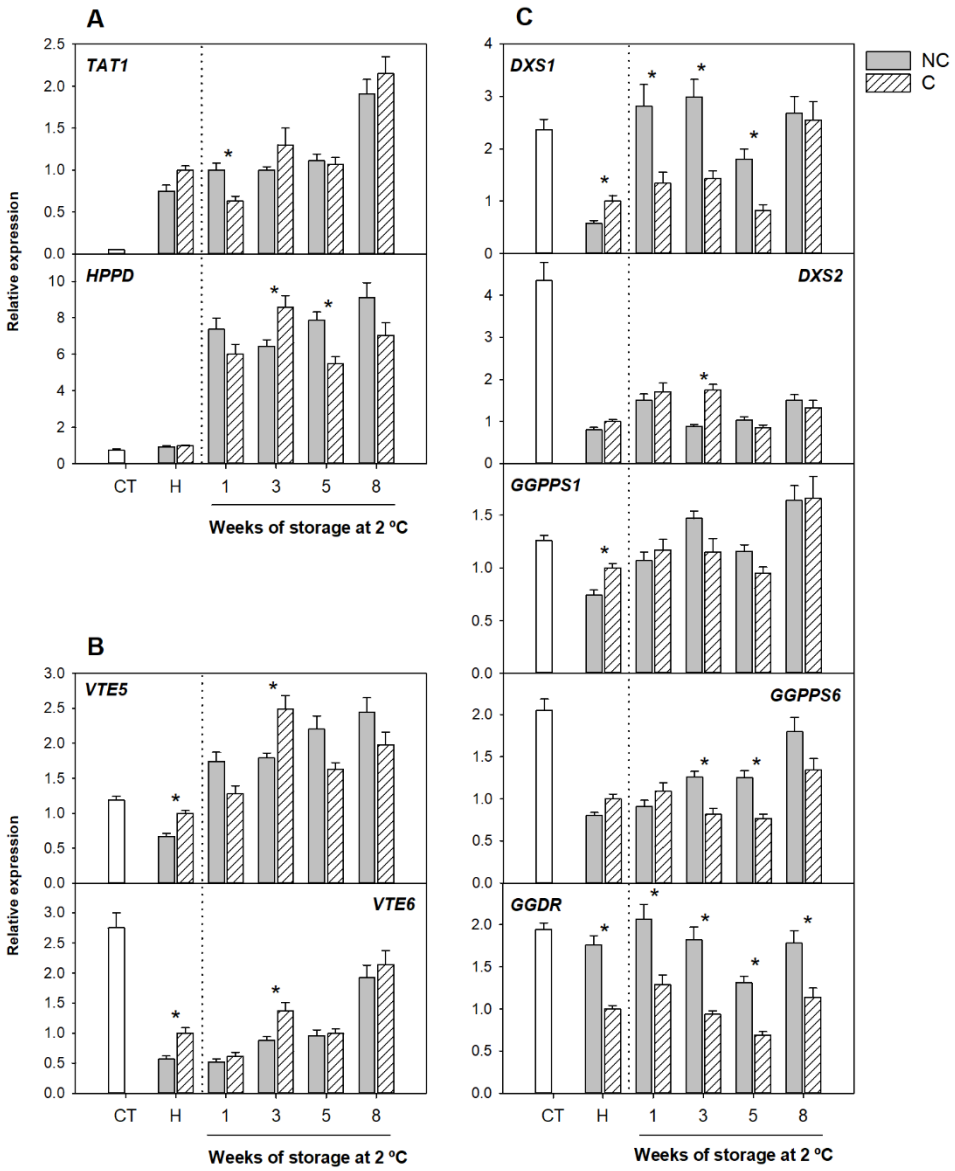

Figure 3. Relative expression of the genes involved in the synthesis of PPP (phytyl pyrophosphate) through the MEP pathway (A) and the recycling of free phytol (B), and of genes of the SK pathway (C) involved in the synthesis of HGA (homogentisate) in the flavedo of non-covered (NC) and covered (C) fruits of Star Ruby grapefruit, at the covering time (CT), harvest (0) and during postharvest storage at 2 °C for up to 8 weeks. The genes analyzed were *DXS1* and *DXS2* (1-deoxy-D-xylulose-5-phosphate synthase 1 and 2), *GGPPS1* and *GGPPS6* (geranylgeranyl pyrophosphate synthase 1 and 6), *GGDR* (geranylgeranyl diphosphate reductase), *VTE5* (phytol kinase), *VTE6* (phytyl-P kinase), and *TAT1* (tyrosine aminotransferase) and *HPPD* (4-hydroxyphenylpyruvate dioxygenase) of the SK pathway. An expression value of 1 was arbitrarily assigned to the values obtained in the flavedo of C fruit at harvest. The data are mean ± S.E of at least three replicates.

Most genes involved in the MEP pathway, the other source of PPP, were substantially down-regulated during fruit development, and *GGDR* was the only gene strongly repressed by light deprivation (Fig. 3C). From immature to ripe fruit, *DXS1* and *DXS2* experienced a dramatic reduction in their expression (2.5- and 5-times, respectively), and interestingly,

transcripts accumulation of *DXS1* and *GGPPS1* were significantly higher in the flavedo of C than in NC fruit at harvest. The enzyme DXS limits the influx into the MEP pathway by determining the amount of IPP available (Estévez, Cantero, Reindl, Reichler, & León, 2001; Rodríguez-Concepción & Boronat, 2015), while GGPPS regulates the synthesis of GGPP necessary for PPP (Ruiz-Sola et al., 2016). Many *GGPPS* paralogues have been identified in *Arabidopsis* and tomato, but *AtGGPPS11* (the orthologous of *GGPPS6* in *Citrus*), and *SlGGPPS1, 2* and *3* seem to be the main forms responsible for the synthesis of GGPP (Barja et al., 2021; Ruiz-Sola et al., 2016). Genes of MEP pathway are subject to multiple levels of regulation, including several environmental signals, and light has been demonstrated to be an essential promotor of their transcriptions (Rodríguez-Concepción & Boronat, 2015). Therefore, the higher expression of *DXS1* and *GGPPS1* in the flavedo of C fruit was an unexpected result. Previous experiments have shown an early repression of *DXS* and *GGPPS* genes in bagged fruit of grapefruit, although differences were not maintained in ripe fruit (Lado, Cronje, et al., 2015). Nonetheless, it is important to highlight that many intermediates of the MEP pathway are metabolic precursors for the synthesis of other plant isoprenoids (Rodríguez-Concepción & Boronat, 2015), and that many genes of this pathway are post-transcriptionally regulated (Hemmerlin, 2013). Thus, it is reasonable to assume that the down-regulation of genes of the MEP pathway during maturation does not necessarily implicate a reduction in tocopherol concentration, or that the higher expression of *DXS1* and *GGPPS1* in C fruit directly translates in higher contents.

The most remarkable effect of light deprivation during development and maturation on the genes of the MEP pathway was the reduction of *GGDR* (Fig. 3C). This gene controls the reduction of GGPP into PPP and thus, directly regulates the availability of PPP for tocopherol synthesis (Pellaud & Mène-Saffrané, 2017). A recent study in tomato fruit found a substantial down-regulation of this gene in plants grown under darkness (Gramegna et al., 2019). This effect was associated with the interaction of a phytochrome-interacting factor with the promoter region of *GGDR* in the absence of light, which negatively regulates *GGDR* expression in tomato fruit (Gramegna et al., 2019). Our results indicate that the expression of *GGDR* is also light-regulated in the peel of Star Ruby grapefruit and correlated with tocopherol accumulation, supporting the notion that expression of *GGDR* gene may be a key mechanism regulating tocopherol contents in the peel of *Citrus* fruit (Rey et al., 2021).

Regarding the genes of the tocopherol-core pathway, their expressions were in general up-regulated in the flavedo of NC grapefruit during maturation and, with the exception of *VTE3b*,

accumulation of the transcripts was significantly lower in C fruit than in NC (Fig. 4). These results indicate that the genes of the tocopherol-core pathway are stimulated during development and ripening of grapefruit under normal environmental conditions, in accordance with the increase in tocopherol content (Fig. 2). Interestingly, the absence of light impaired the enhancement of *VTE2*, *VTE3a*, *VTE1* and *VTE4* genes in C fruit, as expression levels were similar to those before fruit bagging. Likewise, in tomato, a down-regulation of the genes *VTE2*, *VTE1* and *VTE4* has also been observed in dark-grown fruit (Gramegna et al., 2019).

Tocopherol accumulation is highly dependent on the availability of the precursors PPP and HGA, and the up-regulation of the genes involved in their biosynthetic pathways usually translates in higher availability of precursors and hence higher tocopherol contents (Pellaud & Mène-Saffrané, 2017). In vegetative tissues, the tocopherol-core genes (with the exception of *VTE2 and VTE1*) are thought to play a role in shaping tocopherol composition rather than defining the contents, but transcriptional studies in tomato fruit have revealed the importance of *VTE3* in regulating tocopherol accumulation in fruit tissues (Quadrana et al., 2013). Our results in grapefruits suggest that the upsurge in total tocopherols in both NC and C fruit during maturation could be due to the combined up-regulation of *TAT1* (Fig. 3A), involved in HGA synthesis, and most of the genes of the tocopherol-core pathway (Fig. 4).

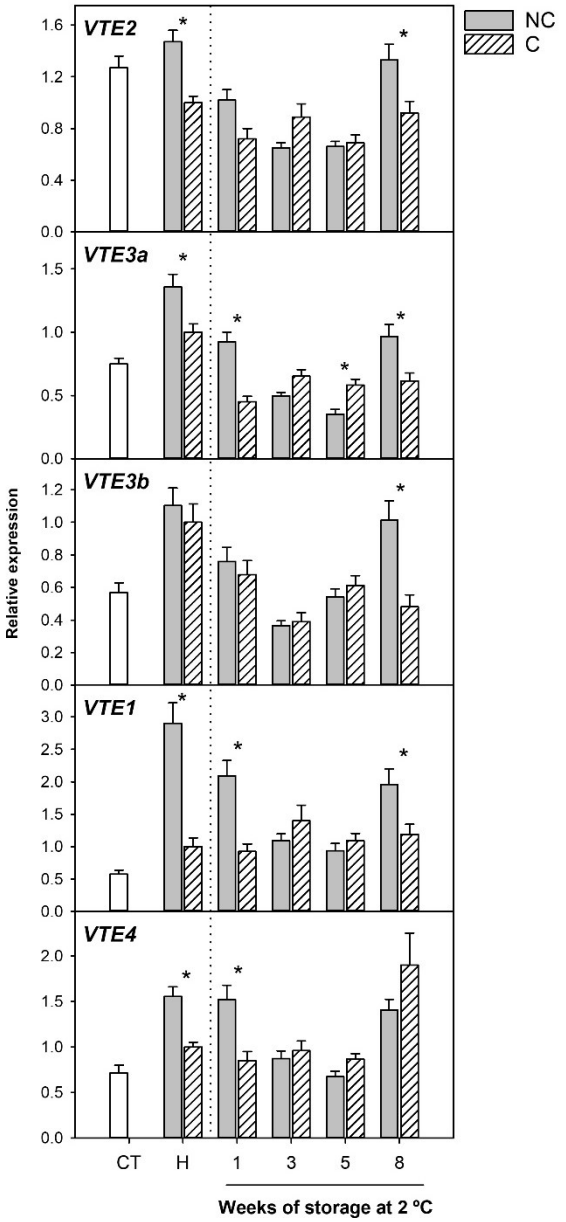

Figure 4. Relative expression of the genes of the tocopherol-core pathway in the flavedo of non-covered (NC) and covered (C) fruits of Star Ruby grapefruit, at the covering time (CT), harvest (0) and during postharvest storage at 2 °C for up to 8 weeks. The genes analyzed were *VTE2* (homogentisate phytyl transferase), *VTE3a* and *VTE3b* (2-methyl-6-phytyl-1,4-benzoquinol methyltransferase a and b), *VTE1* (tocopherol cyclase) and *VTE4* (γ-TMT, γ-tocopherol methyltransferase. An expression value of 1 was arbitrarily assigned to the values obtained in the flavedo of C fruit at harvest. The data are mean ± S.E of at least three replicates.

One of the most remarkable effects of light avoidance in Star Ruby grapefruits was the reduction of γ-tocopherol during development and maturation, as the content in C fruit was 4-times lower than in NC fruit (Fig 2B). Consequently, a moderated reduction in total tocopherols was also detected (Fig 2A). These reductions in contents under dark conditions could be related to the reduced expression of *GGDR*, *VTE1*, *VTE4, VTE3a* and *VTE2* in C fruit (Fig. 3C and 4). Similarly, Gramegna et al. (2019) concluded that the down-regulation of the

upstream gene *GGDR* and downstream genes *VTE2*, *VTE1* and *VTE4* explained the low levels of tocopherols accumulating in dark-grown tomato fruit. Although the expression of many genes (*DXS1*, *GGPPS1*, *VTE5* and *VTE6*) involved in the formation of PPP were significantly higher in C fruit (Fig. 3B and C), this was not translated into a higher tocopherol content. This suggests that the reduction in the levels of *GGDR* transcripts by light avoidance play a pivotal role controlling the supply of the precursor PPP for condensation with HGA. Therefore, the enzyme *GGDR* is revealed as a limiting step in tocopherol accumulation in grapefruit peel, reinforcing its role in tocopherol accumulation in fruit tissues of different species (Georgiadou et al., 2019; Gramegna et al., 2019; Quadrana et al., 2013; Rey et al., 2021). It is noteworthy that, besides the shade-induced repression of *GGDR*, four of the five genes of the tocopherol-core pathway analyzed were also affected by light avoidance. This effect was more prominent for *VTE1* (transcript accumulation was 3-times lower in C than in NC fruit) than in any other gene of this pathway (Fig. 4). The gene *VTE1* encodes for the enzyme tocopherol cyclase, which catalyzes the conversion of DMPBQ into γ-tocopherol, and *vte1* mutant plants lack all tocopherol isoforms and accumulate the intermediate DMPBQ (Porfirova, Bergmuller, Tropf, Lemke, & Dormann, 2002). A reasonable hypothesis to explain the reduction of γ-tocopherol provoked by fruit shading would take into account the down-regulation of *GGDR*, and to a lesser extent of *VTE2* and *VTE3a*, and the marked impairment of *VTE1* induction during ripening (Fig. 2-4). Then, a reduction in the precursor supply and in the conversion of DMPBQ to γ-tocopherol, and a non-limiting rate of conversion into α-tocopherol by *VTE4*, may lead to a lower γ-tocopherol pool in C grapefruits.

3.2.2 Effect of cold storage

The expression profiling of the genes involved in the different pathways of tocopherol biosynthesis were analyzed in the peel of NC and C fruit stored at 2 °C for up to 8 weeks. In general, cold temperatures induced an up-regulation of the genes involved in the synthesis of tocopherol precursors in the flavedo of both NC and C fruit of SR grapefruit (Fig. 3).

The most marked effect of cold storage was in the expression of the *HPPD* gene, with a near 7-fold increment only 1 week after storage at 2 °C in both NC and C fruit. Levels of *HPPD* transcripts remained with minor variations after subsequent storage. The other gene of this pathway, *TAT1*, also increased but only at the end of the storage (Fig. 3A). Similarly, an induction of *HPPD* by cold storage has also observed in other species, like alfalfa and lettuce, in response to other abiotic stresses, suggesting the involvement of *HPPD* in plant response to different stresses (Ma et al., 2020). Overexpression of this gene has increased tocopherol

content in seeds and leaves of *Arabidopsis* and oilseed crops (Tsegaye et al., 2002), and thus could be contributing to the increased tocopherol contents in both NC and C fruit. However, it is important to indicate that the induction of *HPPD* under cold temperatures does not necessarily indicate a higher HGA availability for tocopherol production, since HGA is a common precursor for all tocochromanols. In relation to this, plastochromanol-8 and its precursor plastoquinone-9 are potent singlet oxygen quenchers and have also been induced in response to abiotic stresses (Liu & Lu, 2016), but their potential role under cold stress is still unclear.

The genes *VTE5* and *VTE6,* involved in the supply of PPP through the recycling of phytol formed during chlorophyll degradation, were also cold-responsive. Both genes were stimulated by cold, at early stages of the storage period for *VTE5* and at the end for *VTE6* (Fig. 3B). In general, the effect of cold was similar in NC and C fruit, indicating that fruit shading does not affect PPP supply through this pathway during cold storage.

Genes of the MEP pathway were in general stimulated by postharvest cold storage, although each gene was induced at a different stage of storage. *DXS1* was the only gene of this pathway that was more stimulated by cold in NC than in C fruit. Other genes, like *GGPPS6* showed differences between both conditions after 3 weeks of storage. Interestingly, the differences in transcript levels of *GGDR* induced by shading were maintained during cold storage, and transcript accumulation was higher in NC than in C fruit (Fig. 3C), similarly to the effect of cold stress on these genes in other species (Xu et al., 2019). Since this metabolic pathway, which provides the precursor PPP in combination with the supply through the recycling of phytol, is enhanced during storage, it indicates that the metabolic precursor for tocopherol synthesis appears to be ensured during prolonged cold storage. These results reinforce the motion that in *Citrus* fruit the transcriptional changes in the genes of this pathway appear to be cold-responses rather than being associated with the susceptibility to CI (Rey et al., 2021).

Cold storage, in general, produced a similar effect in most genes of the tocopherol-core pathway in NC fruit: a decline at the middle of the storage period and a later increase by week 8 (Fig. 4). In C fruit, where transcripts levels were lower than in NC before storage, low temperature reduced (*VTE3a*, *VTE3b*) or maintained (*VTE2*, *VTE1 and VTE4*) accumulation of the corresponding mRNAs, but differences between both conditions were in general maintained. In green tissues under high-light stress, drought or nutrient deficiency, changes

in *VTE2*, *VTE1* and *VTE4* transcripts have been detected, supporting their role in the response of plants to stress conditions (Collakova & DellaPenna, 2003; Ma et al., 2020).

Collectively, the changes in gene expression during storage of NC and C grapefruits at low temperature indicated that the up-regulation of most genes involved in the synthesis of tocopherol precursor's (Fig. 3) may explain the slight increase in total and α-tocopherol contents during cold storage (Fig. 2A and C). Moreover, the down regulation or maintenance of the tocopherol-core genes was not limiting for tocopherol accumulation. Finally, most of the changes in gene expression during cold storage appeared to be cold-mediated responses not related to the tolerance of Star Ruby grapefruits to CI.

4. Conclusions

Fruit shading of immature green Star Ruby grapefruit was effective in conferring tolerance to CI during cold storage. Compared to immature grapefruit, total, α- and γ-tocopherol increased in mature Star Ruby fruit, but light avoidance during ripening repressed the accumulation of γ-tocopherol and reduced total content. The effect of light deprivation during maturation and cold storage on the transcriptional profile of genes of tocopherol biosynthesis are summarized in Fig 5. During development and maturation, expression of *TAT1* increased, while the expression of genes involved in PPP supply, either through the recycling of phytol or the MEP pathway, declined. On the other hand, genes of the tocopherol-core pathway were stimulated, and this was more significantly in light-exposed fruit. Genes specifically repressed in darkness (or light-stimulated) were *GGDR*, *VTE1*, *VTE4*, *VTE3a*, and *VTE2*, which appear to be key for tocopherol synthesis and accumulation. Interestingly, in both NC and C fruit cold enhanced the expression of genes involved in the synthesis of precursors, particularly *HPPD*, which were in general repressed during maturation (except for *TAT1* and *HPPD*). By contrast, genes of the core-pathway were down-regulated by cold when they had been stimulated during development. To our knowledge, this is the first report describing the synthesis and accumulation of tocopherol in grapefruits during fruit ripening and during cold storage, and exemplified the complexity of the regulatory network and signals modulating tocopherol biosynthesis in the peel of grapefruits, with specific and common responses to fruit shading and cold stress.

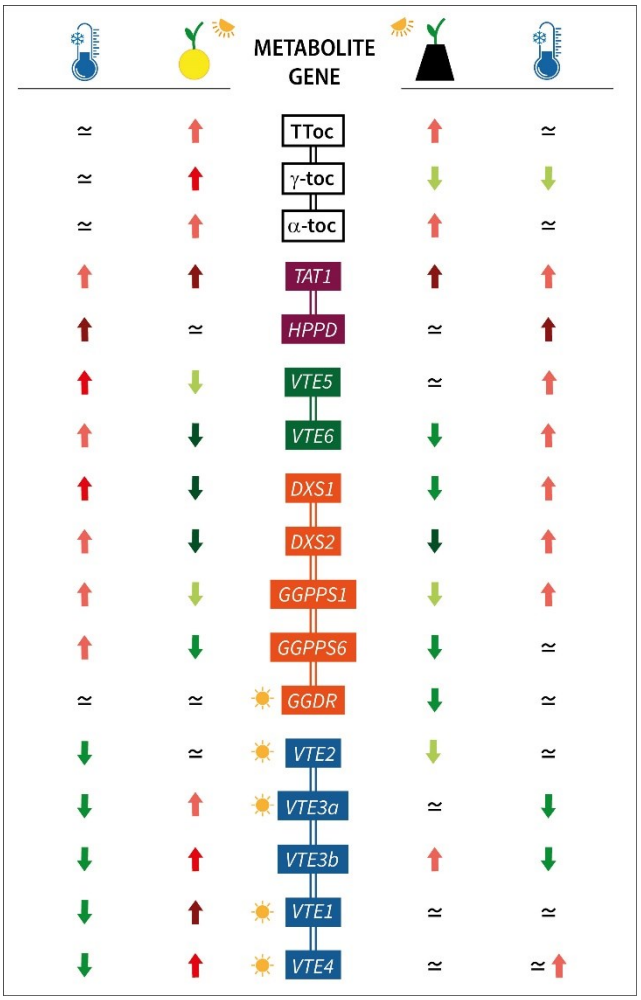

Figure 5. Representation summarizing the changes in tocopherol contents (Ttoc, total tocopherols; α-toc, α-tocopherol; γ-toc, γ-tocopherol) and gene expression during fruit ripening (covering time *vs.* harvest) and cold storage (harvest *vs.* storage), in non-covered (left) and covered fruits (right) of Star Ruby grapefruits. Red arrows indicate up-regulation, while green arrows indicate down-regulation, and the color intensity of the arrows represents the magnitude of the change. The symbol ≃ indicates similar expression values. Genes particularly affected by light deprivation are highlighted with a sun symbol.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochms.2021.100037.

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3.4. CAPÍTULO 4

Regulation of tocopherol biosynthesis during fruit maturation of different *Citrus* species.

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Abstract

Tocopherols are plant-derived isoprenoids with vitamin E activity, which are involved in diverse physiological processes in plants. Although their biosynthesis has been extensively investigated in model plants, their synthesis in important fruit crops as *Citrus* has scarcely been studied. Therefore, the aim of this work was to initiate a physiological and molecular characterization of tocopherol synthesis and accumulation in *Citrus* fruits during maturation. For that purpose, we selected fruit of the four main commercial species: grapefruit (*Citrus paradisi*), lemon (*Citrus limon*), sweet orange (*Citrus sinensis*) and mandarin (*Citrus clementina*), and we analyzed tocopherol content and the expression profile of 14 genes involved in tocopherol production during fruit maturation in both the peel and pulp. The selected genes covered the pathways supplying the precursors HGA (*TAT1* and *HPPD*) and PPP (*VTE5, VTE6, DXS1* and 2, *GGPPS1* and *6*, and *GGDR*) and the tocopherol-core pathway (*VTE2, VTE3a, VTE3b, VTE1* and *VTE4*). Tocopherols accumulated mainly as α- and γ-tocopherol, and α-tocopherol was the predominant form in both tissues. Moreover, differences were detected between tissues and among maturation stages and genotypes. Contents were higher in the peel than in the pulp during maturation, and while they increased in the peel they decreased or were maintained in the pulp. Among genotypes, mature fruit of lemon accumulated the highest tocopherol content in both the peel and the pulp, whereas mandarin fruit accumulated the lowest concentrations, and grapefruit and orange had intermediate levels. Higher concentrations in the peel were associated with a higher expression of all the genes evaluated, and different candidate genes seemed to explain the temporal changes in each tissue: i) in the peel, the increase in tocopherols was concomitant with the up-regulation of *TAT1* and *VTE4*, involved in the supply of HGA and the shift of γ- into α-tocopherol, respectively; ii) in the pulp, changes paralleled the expression of *VTE6, DXS2* and *GGDR*, which regulate PPP availability. Also, certain genes (i.e. *VTE6, DXS2* and *GGDR*) seemed to be co-regulated and shared a similar pattern during maturation in both tissues, suggesting they are developmentally modulated.

Keywords: tocopherol, vitamin E, Citrus, fruit, ripening, tocopherol gene expression

1. Introduction

Tocopherols are lipid-soluble isoprenoids of the tocochromanol family which are mainly synthesized in photosynthetic organisms (Fritsche et al., 2017; Mène-Saffrané, 2017). Their chemical structure consists of a polar chromanol ring, originated from homogentisate (HGA), and a lipophilic isoprenoid side chain derived from a specific prenyl pyrophosphate donor. While HGA is the common precursor for all tocochromanols, the polyprenyl precursor varies according to the type of tocochromanol and is phytyl pyrophosphate (PPP) for tocopherol synthesis (Mène-Saffrané, 2017). Additionally, according to the position and degree of methylation of the chromanol ring, four natural sub-forms can exist: δ- (one methyl group), β- and γ- (two methyl groups) and α-tocopherol (three methyl groups). Tocopherols are of great importance because, together with tocotrienols, they are the only natural compounds exhibiting vitamin E activity in animal cells and are essential as dietary nutrients (Traber and Sies, 1996). In plants, tocopherols play diverse physiological functions, of which their role as antioxidants, either scavenging peroxyl radicals or quenching reactive oxygen species, is probable the most notable (Havaux et al., 2005; Mène-Saffrané et al., 2010). Nonetheless, other functions have been recently described for tocopherols in plants, including their involvement in photo-assimilate transport, carbohydrate metabolism, cellular signaling and plant's response to biotic and abiotic stresses (Falk and Munné-Bosch, 2010; Muñoz and Munné-Bosch, 2019; Ma et al., 2020b).

The tocopherol biosynthesis pathway (Figure 1) has been well-characterized in the last decades, with all the vitamin E biosynthetic genes (VTE genes) encoding the enzymes catalyzing the core steps of tocopherol synthesis identified (Fritsche et al., 2017; Mène-Saffrané, 2017; Muñoz and Munné-Bosch, 2019). Tocopherol synthesis is initiated by the condensation of HGA with PPP, a reaction regulated by homogentisate phytyl transferase (HPT; *VTE2*) that results in the formation of 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) (Collakova and DellaPenna, 2001). After this step, the pathway can split into two branches depending on the subsequent reaction of MPBQ, leading towards the synthesis of δ- and β-tocopherol or γ- and α-tocopherol. Then, MPBQ can either be directly cyclized into δ-tocopherol, by the tocopherol cyclase (TC; *VTE1*), or it can be first methylated into 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ), by a MPBQ methyltransferase (MPBQ-MT; *VTE3*). The resulting product DMPBQ is then converted into γ-tocopherol by the same TC (*VTE1*) mentioned before. The final step of tocopherol synthesis is the methylation of δ- and γ-tocopherol into β- and α-tocopherol, respectively, which is catalyzed by the γ-tocopherol methyltransferase (γ-TMT; *VTE4*). These

steps represent the tocopherol-core pathway, and of these enzymes only HPT (*VTE2*) is considered exclusive to tocopherol synthesis, while MPBQ-MT (*VTE3*), TC (*VTE1*) and γ-TMT (*VTE4*) are also involved in the synthesis of the other tocochromanols (Fritsche et al., 2017; Mène-Saffrané, 2017). *VTE2* has been reported to be a limiting step in tocopherol synthesis in seeds and leaves of Arabidopsis and other model plants (Savidge et al., 2002; Collakova and DellaPenna, 2003a). However, it does not seem to limit tocopherol synthesis in fruit of species such as tomato, olive and mandarin (Quadrana et al., 2013; Georgiadou et al., 2019; Rey et al., 2021a), where *VTE3* appears to play a more important role regulating tocopherol content (Quadrana et al., 2013; Rey et al., 2021a).

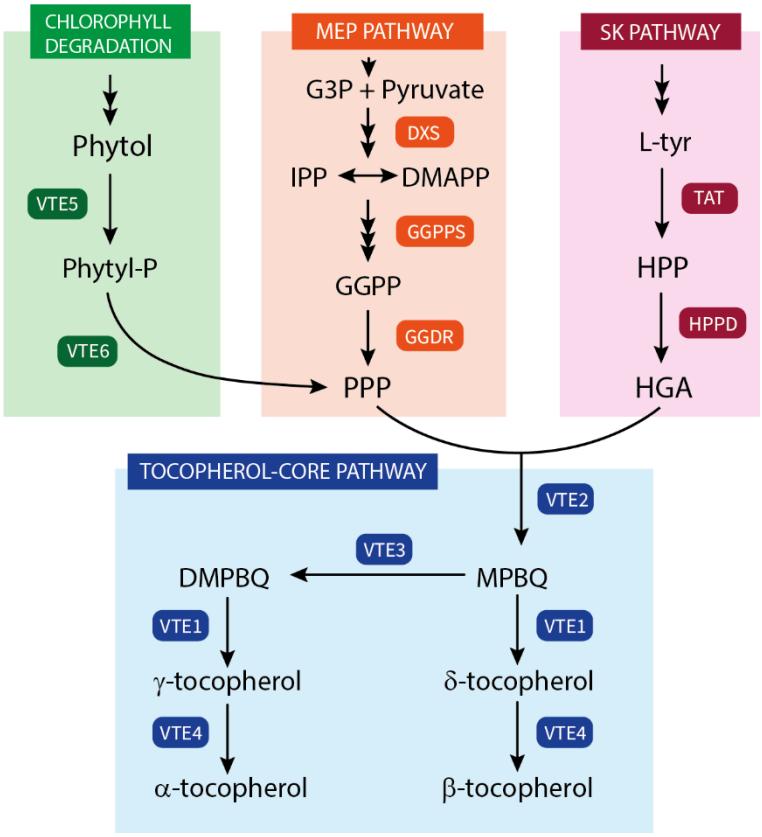

Figure 1. Schematic representation of tocopherol biosynthetic pathway in plants. Abbreviations: Phytyl-P, phytyl phosphate; G3P, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; PPP, phytyl pyrophosphate; L-tyr, amino acid L-tyrosine; HPP, 4-hydroxyphenylpyruvate; HGA, homogentisate; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; VTE5, phytol kinase; VTE6, phytyl-P kinase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; GGPPS, GGPP synthase; GGDR, GGPP reductase; TAT, tyrosine aminotransferase; HPPD, HPP dioxygenase; VTE2, homogentisate phytyltransferase (HPT); VTE3, MPBQ methyltransferase (MPBQ-MT); VTE1, tocopherol cyclase (TC); VTE4, tocopherol methyltransferase (γ-TMT).

Tocopherol content in plant cells is also highly dependent on the availability of the precursors HGA and PPP (Mène-Saffrané, 2017; Pellaud and Mène-Saffrané, 2017). In plants, the precursor HGA originates in a two-step reaction from the degradation of the amino acid

L-tyrosine (L-tyr) synthetized in the shikimate (SK) pathway. L-Tyr is converted into 4-hydroxyphenylpyruvate (HPP) by a tyrosine aminotransferase (TATs), and then transformed into HGA by a HPP dioxygenase (HPPD) (Figure 1). The loss of function of either *TAT* or *HPPD* in Arabidopsis has resulted in a reduction of tocopherol levels (Norris et al., 1998; Riewe et al., 2012), while the overexpression of these genes only modestly increased total tocopherols in leaves and seeds (Tsegaye et al., 2002; Karunanandaa et al., 2005). In fleshy fruit, such as tomato and mango, the role of *HPPD* in tocopherol accumulation has been reinforced, as higher accumulation of *HPPD* transcripts has been associated with genotypes containing high tocopherols levels (Quadrana et al., 2013; Singh et al., 2017). Recently, it has been shown that engineering the chorismate–tyrosine pathway in tomato fruit to produce HPP in combination with the overexpression of Arabidopsis *HPPD* resulted in a moderate increment in tocopherols (Burgos et al., 2021). The other precursor necessary for tocopherol synthesis, PPP, can be derived from geranylgeranyl pyrophosphate (GGPP), produced in the MEP pathway, or alternatively from the recycling of phytol formed in the degradation of chlorophylls (Figure 1). Two enzymes are involved in the supply of PPP via the recycling of phytol: phytol kinase (*VTE5*) and phytyl phosphate kinase (*VTE6*) (Valentin et al., 2006; vom Dorp et al., 2015). VTE5 appears to be a key enzyme regulating tocopherol content in fruits of tomato and olive, controlling the supply of PPP towards the tocopherol-core pathway (Georgiadou et al., 2015; Almeida et al., 2016). Nonetheless, in ripe tomato and mandarin fruits, tocopherol accumulation appears to be more influenced by the up-regulation of genes of the MEP pathway, such as *DXS* and *GGDR* (Quadrana et al., 2013; Almeida et al., 2015; Gramegna et al., 2019; Rey et al., 2021a). While the gene *DXS* regulates the influx into the MEP pathway, *GGDR* controls the final reduction of GGPP into PPP, and therefore its final availability for condensation with HGA (Estévez et al., 2001; Pellaud and Mène-Saffrané, 2017).

Tocopherol accumulation has been mainly studied in leaves and seeds, but they have also been detected in fruits, stems, roots, flowers and other plant tissues (Horvath et al., 2006a; Mène-Saffrané and DellaPenna, 2010). In general, α-tocopherol is the main form found in leaves, while γ-tocopherol is the predominant in seeds of most species (Horvath et al., 2006a). In fruits, α-tocopherol seems to be the main isoform accumulated (Chun et al., 2006; Horvath et al., 2006a), with variable contents depending on the species and through fruit ripening (Osuna-García et al., 1998; Almeida et al., 2011; Quadrana et al., 2013; Georgiadou et al., 2015, 2019). In recent years, great advances have been made into the regulation of tocopherol biosynthesis in fruit of different species, such as tomato (Almeida et al., 2011, 2016; Quadrana et al., 2013; Gramegna et al., 2019; Burgos et al., 2021), pepper (Arango and Heise, 1998; Koch

et al., 2002), olive (Georgiadou et al., 2015, 2016, 2019) and mango (Singh et al., 2017). These studies concluded that tocopherol accumulation in fruit is mainly transcriptionally regulated, and that tocopherol biosynthetic genes are modulated in a temporal manner, and also influenced by environmental factors (Almeida et al., 2011, 2020; Quadrana et al., 2013; Georgiadou et al., 2016, 2019; Singh et al., 2017; Gramegna et al., 2019).

Citrus is one of the world's most important fruit crops, being highly demanded for fresh consumption and juice processing (Spreen et al., 2020). This genus is characterized for its genotypic and phenotypic diversity (Wu et al., 2018), with a vast range of fruits with a different composition of nutrients and bioactive compounds (Rodrigo and Zacarías, 2006; Cano et al., 2008; Ma et al., 2020a). Current information about tocopherol contents in *Citrus* fruit is very limited. Tocopherols accumulate in the peel of mandarin, orange, lemon, grapefruit and other less known species in the range of 65 to 130 μg g^{-1}, and mainly in the forms of α- and γ-tocopherol, with composition varying according to the specie (Assefa et al., 2017; Rey et al., 2021a). In the pulp, tocopherols have also been detected in fruit of mandarin, grapefruit and orange in the form of α-tocopherol and at concentrations lower than in the flavedo (1.6 to 25 μg g^{-1}) (Chun et al., 2006; Cardeñosa et al., 2015). Recently, genes involved in the tocopherol-core pathway as well as genes regulating the supply of the precursors HGA and PPP have been identified in the *Citrus* genome. Analysis of their transcriptional profiling in the peel of mandarins and grapefruits in response to cold stress suggested candidate genes and mechanisms regulating tocopherol accumulation in each specie (Rey et al., 2021a, 2021b). However, the temporal changes of tocopherols and the regulation of their synthesis during maturation of *Citrus* fruit have not been explored yet. Therefore, the aim of the present work was to perform a comparative study of tocopherol accumulation during on-tree fruit maturation in different *Citrus* genotypes belonging to the main horticultural *Citrus* groups: orange, mandarin, lemon and grapefruit. The relationship between tocopherol accumulation and the expression of tocopherol-biosynthetic genes in the peel and pulp of fruit from the selected species and ripening stages was also investigated.

2. Materials and methods

2.1. Plant material

Fruit of four different genotypes belonging to the main horticultural groups of *Citrus* species: grapefruit (*Citrus paradisi* Macfad.) cv. 'Marsh', lemon (*Citrus limon* (L.) Burm. F) cv. 'Fino', sweet orange (*Citrus sinensis* (L.) Osbeck) cv. 'Washington Navel' and mandarin (*Citrus clementina* Hort. ex Tanaka) cv. 'Clemenules', were selected in this study. Fruit were harvested

at four maturation stages: immature green (IG), mature green (MG), breaker (Br) and mature fruit (M). The specific harvest dates for each genotype are detailed in Table S1. At each date, the flavedo (external colored layer of fruit peel) and pulp (juice vesicles) were excised with a scalpel, frozen in liquid nitrogen, ground to a fine powder using an electric grinder with liquid nitrogen, and stored at -80 °C until analysis.

2.2. Tocopherol extraction and quantification

Tocopherol extraction of the flavedo and pulp, and quantification by HPLC coupled to fluorescence detector, was carried out following the procedure described in Rey et al. (2021a). In summary, flavedo and pulp material (200 mg and 500 mg, respectively) were extracted with methanol, Tris buffer (50 mM Tris pH 7.5) with 1 M NaCl, and dichloromethane in a mortar and pestle with sea sand as an abrasive. After vortex-mixing, samples were sonicated for 5 min and centrifuged for 10 min at 3000 *g* and 4 °C. The dichloromethane phase was recovered in a glass tube, and the methanol phase was re-extracted with dichloromethane. The pooled dichloromethane extracts were then dried under nitrogen gas and stored at -20 °C until HPLC analysis. For quantification, dried extracts were re-suspended in ethyl acetate (500-700 µl) and a dilution (1:15 for flavedo extracts and 1:2 for pulps) was carried out. For analysis, 20 µl of the diluted extract were injected in a Waters HPLC system (Acquity® Arc™, Waters) coupled with a fluorescence detector (2475 FLR Detector, Waters). Tocopherol separation was done using a C30 column, 150 x 4.6 mm, 3 µm, (YMC, Teknokroma, Spain) and a ternary gradient elution with methanol, water and methyl *tert*-butyl ether at 1 ml min^{-1} flow (Rey et al., 2021a). Elution of tocopherols was monitored by fluorescence at an excitation wavelength of 296 nm and emission wavelength of 340 nm. Identification and quantification of the different tocopherols was achieved by comparison with the retention times and calibration curves for tocopherol standards (Sigma-Aldrich). All procedures were carried out on ice and under dim light to prevent photo-degradation. Total tocopherol content was calculated as the sum of the tocopherol isoforms, and concentrations are expressed as µg per g of fresh weight (FW). Samples were extracted twice and results are the mean of two replicates (mean ± SD).

2.3. RNA extraction and cDNA synthesis

Extraction of total RNA was different according to the fruit tissue. Total RNA was isolated from flavedo tissue using the RNeasy Plant Mini Kit (Qiagen), while the extraction from the pulp was done using the protocol described in Rodrigo et al. (2004). Once total RNA was isolated, DNA traces were removed by treating RNA with DNase I (DNA free, DNase

treatment & removal, Ambion) following the manufacturer's instructions. RNA concentration was later quantified by spectrophotometric analysis (Nanodrop, Thermo Fisher Scientific) and RNA quality was verified by agarose-gel electrophoresis with GoodView™ Nucleic Acid Stain (SBS Genetech). For cDNA synthesis, 5 µg of total RNA were reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen) in a final volume of 20 µl, following the manufacturer's procedure.

2.4. Gene expression analysis by quantitative real-time PCR

Gene expression was evaluated by quantitative real-time PCR in a LightCycler 480 instrument (Roche), using the LightCycler 480 SYBRGreen I Master kit (Roche) and following the manufacturer's instructions. Previously published oligonucleotides primers were used for the amplification of the following *Citrus sinensis* genes related to tocopherol synthesis: *DXS1, DXS2, GGPPS1, GGPPS6, GGDR, VTE5, VTE6, TAT1, HPPD, VTE2, VTE3a, VTE3b, VTE1* and *VTE4* (Rey et al., 2021a). For all the genes analyzed, RT-qPCR conditions consisted of an initial pre-incubation at 95 °C for 10min, followed by 40 cycles of 10 s at 95 °C for denaturation, 10 s at 59 °C for annealing and 10 s at 72 °C for extension. For each amplification reaction one microliter of 10 times diluted first-strand cDNA, containing approximately 100 ng of cDNA, was used. Specificity of the PCR reaction in the different *Citrus* species was assessed by the melting point analysis (Tm) after the amplification steps. For expression measurements, we used the LightCycler 480 Software release 1.5.0, version 1.5.0.39 (Roche) and calculated relative expression levels using the Relative Expression Software Tool (REST) (Pfaffl et al., 2002) with the same reference sample for peel and pulp analysis. Normalization was performed using *ACTIN* as a housekeeping gene (Alós et al., 2014; Zacarías-García et al., 2021).

2.5. Correlation matrix and networks

The correlation matrices were built with results from a Pearson's correlation analysis for the fold-change of all the variables studied: tocopherol contents and gene expression levels. All species and maturation stages were taken into account in the analysis. Subsequently, each correlation matrix was transformed into a correlation network to highlight the different connections between tocopherol contents and gene expression in the different tissues, and also possible co-regulation among genes. The correlation network was displayed in a graph illustrating all-versus-all correlations among the variables analyzed and it is composed of nodes, which represented tocopherol contents and gene expression levels, and edge lines which represented the links between those nodes. The Pearson's correlation analysis and

matrix was built in RStudio (version 1.3.1093, RStudio Team, PBC, Boston, MA, USA) using the package '*ggplot2*'. For the construction of the correlation networks only significant correlations were taken into account (*p-value* ≤ 0.05), following the recommendations by Cline et al. (2007), and the networks were built using Cytoscape version 3.8.2 (National Institute of General Medical Sciences, Bethesda, MD, USA).

3. Results

3.1. Changes in tocopherol content during fruit maturation of four Citrus species: grapefruit (C. paradisi), lemon (C. limon), sweet orange (C. sinensis) and mandarin (C. clementine) during ripening

Tocopherols were detected in the flavedo and pulp of the four *Citrus* genotypes selected at the four successive stages of fruit maturation (Figure 2 and 3). The isoforms α- and γ-tocopherol were identified in all samples, while the presence of δ-tocopherol was only detected in a few samples at concentrations below 10 and 6 ng g^{-1} FW in the flavedo and pulp, respectively. α-Tocopherol was the predominant form in all species and stages, accounting on average for 85% of total tocopherols in the flavedo and 99% in the pulp. In the flavedo of all species, tocopherol content was higher than in the pulp and contents gradually increased with maturation (Figure 2). By contrast, total tocopherols in the pulp decreased sharply after the immature green (IG) stage and, at the mature (M) stage, the content was between 3 and 8-times lower than in IG, with the exception of lemon where levels remained nearly constant during ripening (Figure 3). As a result, differences between tissues became greater during maturation, with contents being 2-7 times higher in the flavedo than in the pulp at the IG stage and more than 20-50 times higher at full maturity (M) (Figure 2 and 3).

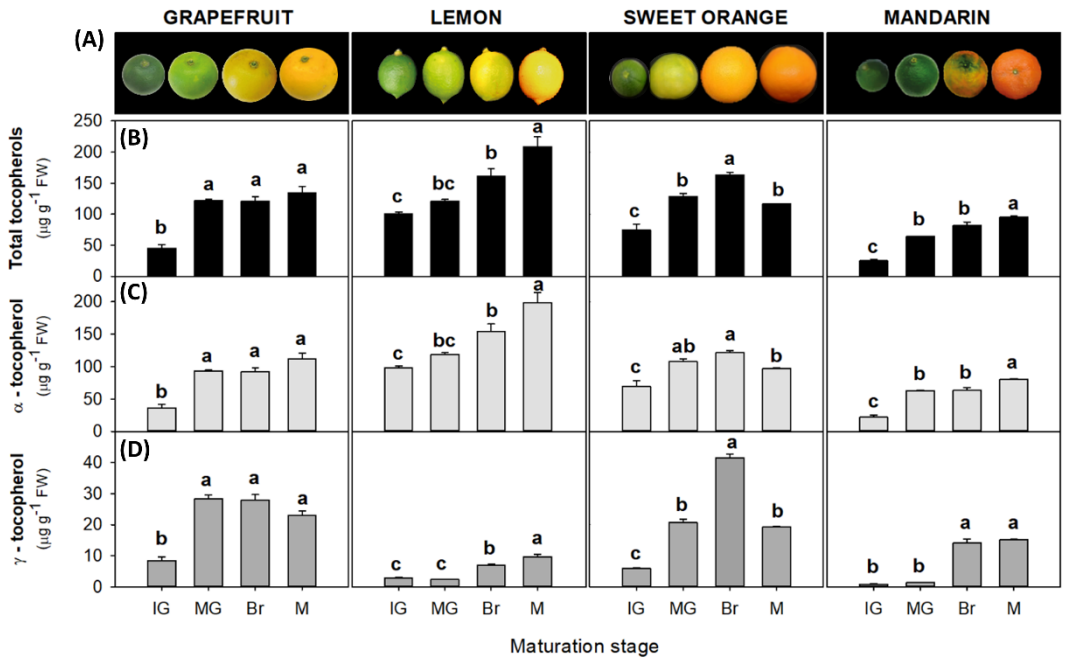

Figure 2. Fruit external appearance **(A)** and total tocopherol **(B)**, α-tocopherol **(C)** and γ-tocopherol content **(D)** in the peel during fruit maturation of grapefruit (*C. paradisi*), lemon (*C. limon*), sweet orange (*C. sinensis*) and mandarin (*C. clementine*). Contents are expressed as µg g^{-1} of fresh weight. Maturation stages correspond to immature green (IG), mature green (MG), breaker (Br) and mature (M).

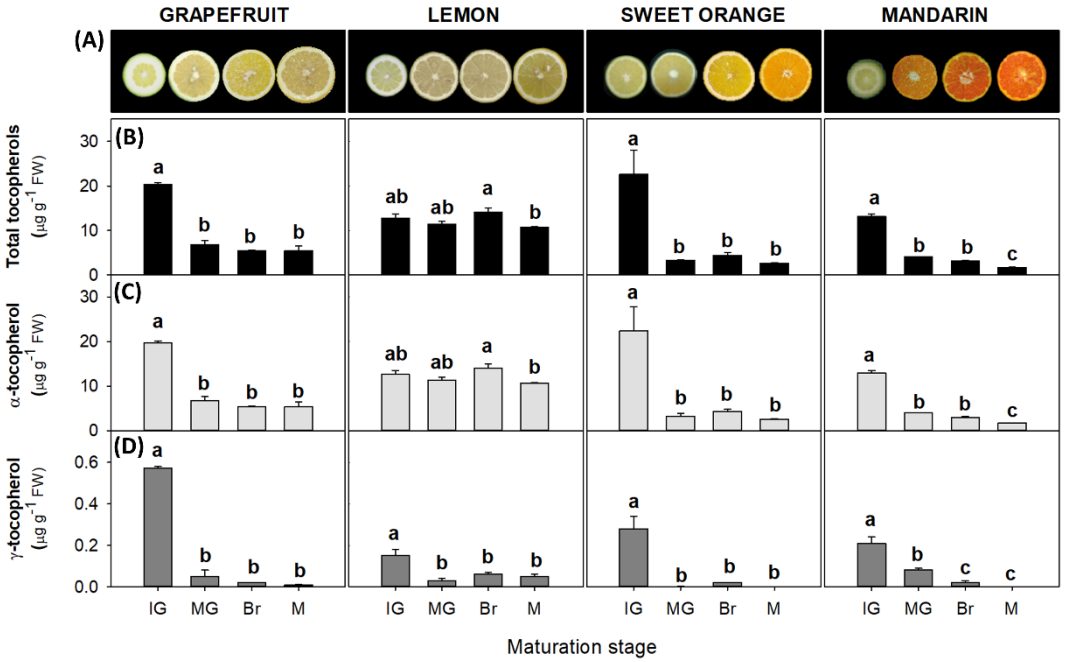

Figure 3. Fruit internal appearance **(A)** and total tocopherol **(B)**, α-tocopherol **(C)** and γ-tocopherol content **(D)** in the pulp during fruit maturation of grapefruit (*C. paradisi*), lemon (*C. limon*), sweet orange (*C. sinensis*) and mandarin (*C. clementine*). Contents are expressed as µg g^{-1} of fresh weight. Maturation stages correspond to immature green (IG), mature green (MG), breaker (Br) and mature (M).

Differences in tocopherol content in the flavedo and pulp were observed among genotypes (Figure 2 and 3). At the IG stage, total tocopherol contents (as the sum of α- and γ-tocopherol)

were higher in the flavedo of lemon (~100 μg g^{-1} FW), followed by sweet orange (~74 μg g^{-1} FW), and almost 2- and 4-times lower in the flavedo of grapefruit (~44 μg g^{-1} FW) and mandarin (~22 μg g^{-1} FW), respectively (Figure 2). During maturation, the magnitude of the increase in tocopherol content also varied among species. At the M stage, higher concentrations of tocopherols were detected in the flavedo of lemon (~208 μg g^{-1} FW), followed by grapefruit (~134 μg g^{-1} FW), sweet orange (~116 μg g^{-1} FW) and mandarin (~95 μg g^{-1} FW). In contrast to the other species, contents in sweet orange increased to a maximum at the breaker (Br) stage (~160 μg g^{-1} FW) and then slightly decreased towards M fruit (Figure 2B). Contents of α-tocopherol reflected the differences among species described for total tocopherol. In IG fruit, α-tocopherol levels ranged from 22-97 μg g^{-1} FW and increased to levels between 80-200 μg g^{-1} FW at the M stage, with mandarin accumulating the lowest content and lemon the highest (Figure 2C). γ-Tocopherol ranged from 0.75 to 8.3 μg g^{-1} FW at the IG stage, with lemon and mandarin accumulating the lowest content (<3 μg) (Figure 2D). This tocopherol isoform increased during maturation in the flavedo of all species, reaching maximum values at the Br or M stages, between 10 μg g^{-1} in lemon and 42 μg g^{-1} in sweet orange. It should be noticed that in all the ripening stages, the flavedo of lemon and mandarin showed lower concentrations of γ-tocopherol in comparison to sweet orange and grapefruit (Figure 2D).

The pulp of sweet orange, mandarin and grapefruit at the IG stage showed the highest levels of total tocopherol (~10-20 μg g^{-1} FW), and decreased sharply at the MG stage (more than 4-fold) (Figure 3). In lemon, no important changes occurred during ripening and levels remained almost constant (~10 μg g^{-1} FW). As in the flavedo, α-tocopherol contents reflected the main differences in total tocopherols among species and during maturation (Figure 3C). Contents in the pulp ranged from 12 to 22 μg g^{-1} FW at IG stage, and 2 to 10 μg g^{-1} FW at M fruit. The levels of γ-tocopherol in the pulp of the four species were below 1 μg g^{-1} FW at IG stage and decreased during maturation (Figure 3D).

3.2. Expression profile of genes involved in tocopherol synthesis in the flavedo during fruit maturation of four Citrus species: grapefruit (C. paradisi), lemon (C. limon), sweet orange (C. sinensis) and mandarin (C. clementine).

The relative expression of genes related to tocopherol precursors production: *VTE5*, *VTE6*, *DXS1*, *DXS2*, *GGPPS1*, *GGPPS6*, *GGDR*, *TAT1* and *HPPD* (Figure 4), and to the tocopherol-core pathway: *VTE2*, *VTE3a*, *VTE3b*, *VTE1* and *VTE4* (Figure 5) were evaluated in the flavedo of the four selected *Citrus* species during fruit maturation. The expression of the genes

involved in the synthesis of the precursor PPP, which includes genes involved in chlorophyll degradation (Figure 1 and 4A) and of the MEP pathway (Figure 1 and 4B), was in general down-regulated during fruit ripening, although changes were gene- and specie-dependent. Of the genes involved in the recycling of free phytol to form PPP (Figure 4A), a clear down-regulation of *VTE6* during fruit ripening (2- to 3-fold decrease) was detected in fruits of the four species. *VTE5* expression also decreased during maturation in sweet orange and lemon, although with a transient induction at the MG stage in lemon, but remained relatively constant in grapefruit and mandarin. Regarding genes of the MEP pathway (Figure 4B), a marked decrease in the expression of *DXS2* and *GGDR* was detected during fruit ripening, with the exception of *GGDR* in the peel of grapefruit which remained relatively constant. No clear common trend was found in the expression of *DXS1* and the two *GGPPS* paralogous during fruit maturation among the four species (Figure 4B). By contrast, the genes involved in the synthesis of the precursor HGA, *TAT1* and *HPPD*, were induced during maturation (Figure 4C). The expression of *TAT1* was markedly up-regulated in the four species, showing levels 6-28 fold higher at Br and M stages than at IG. The relative expression of *HPPD* gradually increased in grapefruit and at the last stage of maturation in sweet orange and mandarin, but remained fairly constant in lemon.

Expression of the tocopherol-core pathway genes during maturation varied among species, but was in general induced or maintained during ripening (Figure 5). The gene *VTE2*, which controls the condensation of PPP with HGA, was induced almost 2-times in lemon and displayed a transient down-regulation between the MG and Br stage in the other three genotypes. The two *VTE3* isoforms shared a similar expression profile during maturation, with slight variations or transient decrease in grapefruit, mandarin and sweet orange. In lemon, a transient increase at the MG stage was detected in the expression of both isoforms. The gene *VTE1* displayed an induction in grapefruit and mandarin (2-fold increase), while its expression was maintained with slight variations in sweet orange. In lemon, *VTE1* showed a similar pattern of expression to the *VTE3* isoforms, with a sharp and transient peak at the MG stage. Finally, *VTE4* displayed the most consistent expression pattern among the four species, with an up-regulation (2 and 3-times) during maturation.

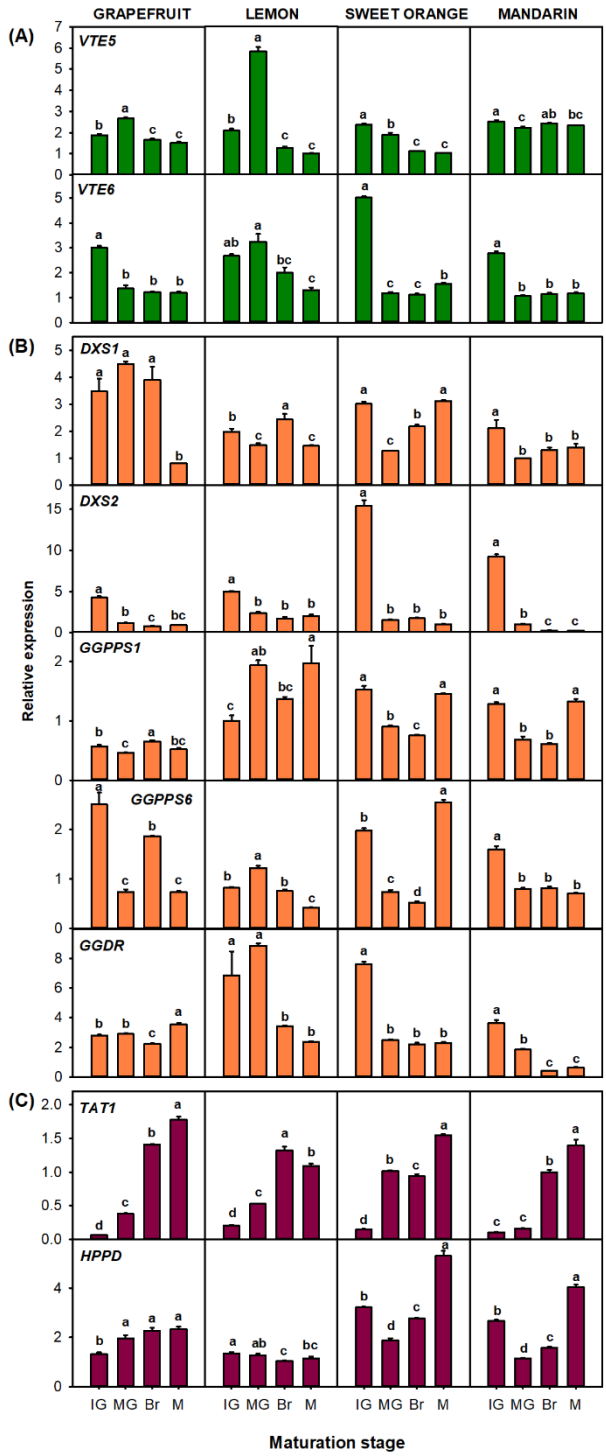

Figure 4. Relative expression of genes involved in the synthesis of precursor PPP (phytyl pyrophosphate), through the recycling of free phytol **(A)** and MEP pathway **(B),** and of precursor HGA (homogentisate) through the SK pathway **(C),** in the peel during fruit maturation of grapefruit (*C. paradisi*), lemon (*C. limon*), sweet orange (*C. sinensis*) and mandarin (*C. clementine*). The genes analyzed were *VTE5* (phytol kinase), *VTE6* (phytyl-P kinase), *DXS1* and *DXS2* (1-deoxy-D-xylulose-5-phosphate synthase 1 and 2), *GGPPS1* and *GGPPS6* (geranylgeranyl pyrophosphate synthase 1 and 6), *GGDR* (geranylgeranyl diphosphate reductase), *TAT1* (tyrosine aminotransferase) and *HPPD* (4-hydroxyphenylpyruvate dioxygenase). Maturation stages correspond to immature green (IG), mature green (MG), breaker (Br) and mature (M). The data are mean ± S.E of at least three replicates.

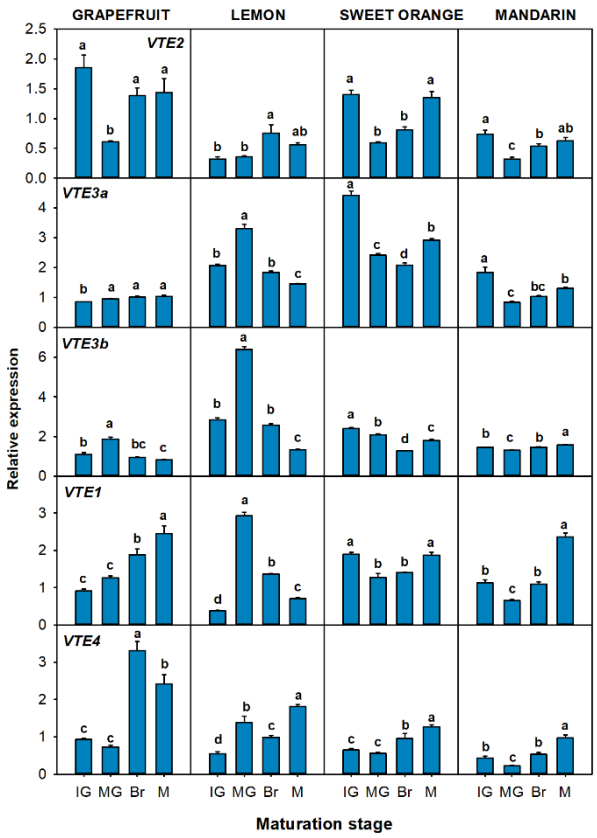

Figure 5. Relative expression of genes of the tocopherol-core pathway in the flavedo during fruit maturation of grapefruit (*C. paradisi*), lemon (*C. limon*), sweet orange (*C. sinensis*) and mandarin (*C. clementine*). The genes analyzed were *VTE2* (HPT, homogentisate phytyl transferase), *VTE3a* and *VTE3b* (MPBQ-MT, 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase a and b), *VTE1* (TC, tocopherol cyclase) and *VTE4* (γ-TMT, γ-tocopherol methyltransferase). Maturation stages correspond to immature green (IG), mature green (MG), breaker (Br) and mature (M). The data are mean ± S.E of at least three replicates.

3.3. Expression profile of genes involved in tocopherol synthesis in the pulp during fruit maturation of four Citrus species: grapefruit (C. paradisi), lemon (C. limon), sweet orange (C. sinensis) and mandarin (C. clementine).

The relative expression of the genes related to the production of tocopherol precursors: *VTE5*, *VTE6*, *DXS1*, *DXS2*, *GGPPS1*, *GGPPS6*, *GGDR*, *TAT1* and *HPPD* (Figure 6), and to the tocopherol-core pathway: *VTE2*, *VTE3a*, *VTE3b*, *VTE1* and *VTE4* (Figure 7), were analyzed in the pulp of fruit from the selected genotypes. In general, no consistent trend in the expression of genes involved in PPP synthesis was found among the pulp of the different species, with temporal changes dependent on the gene and specie. Concerning genes of chlorophyll degradation, *VTE5* transcripts were slightly up-regulated in the four species during ripening, while the expression of *VTE6* remained constant in lemon but was down-regulated in the pulp of fruit of the other three species (Figure 6A). Of the MEP pathway genes, *DXS1* and *GGPPS1* were induced in sweet orange and mandarin (more than 2-fold) but remained unaltered in

grapefruit and lemon (Figure 6B). Interestingly, expression of *DXS2* was induced in lemon but down-regulated in the pulp of the other *Citrus* species. No consistent change was detected for *GGPPS6,* with levels remaining relatively constant. The expression of *GGDR* decreased during maturation in the four species, but the reduction was more marked in sweet orange and mandarin than in the other species. On the other hand, the expression of both *TAT1* and *HPPD* genes, involved in HGA synthesis, was up-regulated during ripening in the pulp of the four citrus fruits.

In relation with the genes of the tocopherol-core pathway, a common expression trend among genotypes was only detected for the gene *VTE4*, which showed an up-regulation during maturation (Figure 7). Interestingly, a similar tendency in the expression of *VTE2*, *VTE3a*, *VTE3b* and *VTE1* was detected in the pulp of grapefruit, sweet orange and mandarin but not in lemon. It should be noticed that in the pulp of lemon, with the exception of *VTE4* and to a lesser extent of *VTE3a*, the expression of the tocopherol-core pathway genes was relatively constant and lower than the other citrus fruits. In mandarin, sweet orange and grapefruit *VTE2* was down-regulated during fruit maturation, while no significant variations were detected in the expression of *VTE3a* and *VTE3b*. Expression of *VTE1* showed minor alterations in the pulp of grapefruit and sweet orange, but was 3 fold increased in mandarin.

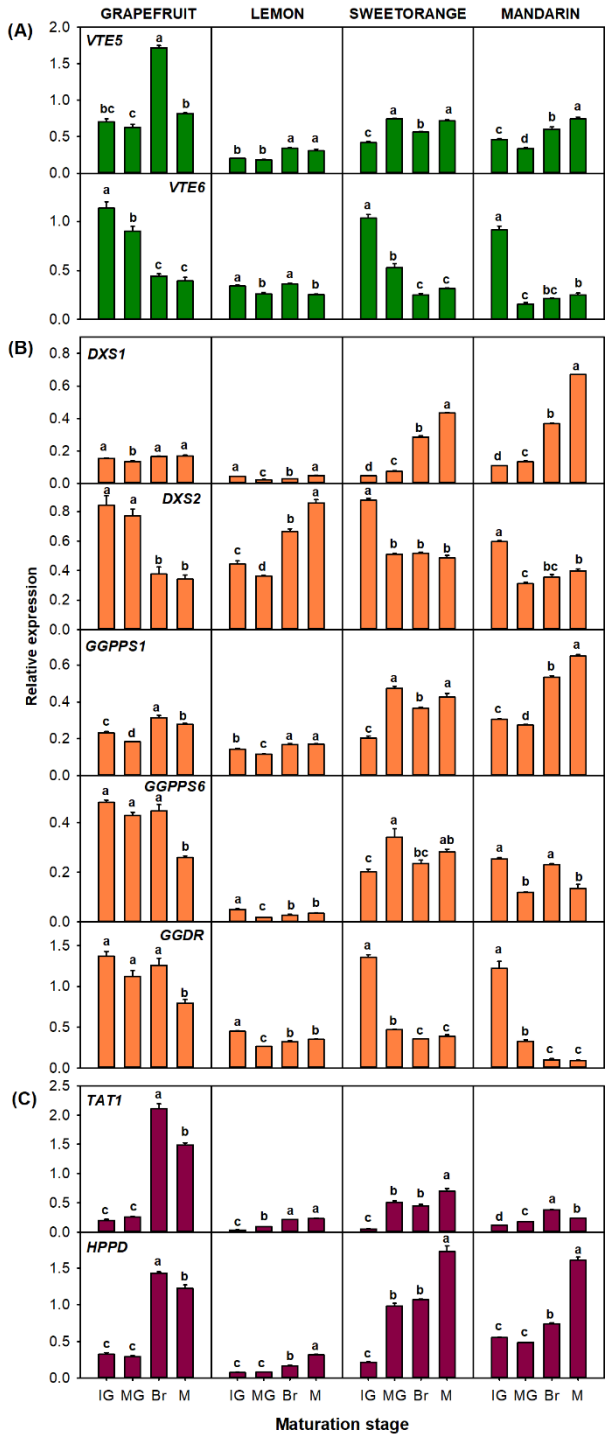

Figure 6. Relative expression of genes involved in the synthesis of precursor PPP (phytyl pyrophosphate), through the recycling of free phytol **(A)** and MEP pathway **(B),** and of precursor HGA (homogentisate) through the SK pathway **(C),** in the pulp during fruit maturation of grapefruit (*C. paradisi*), lemon (*C. limon*), sweet orange (*C. sinensis*) and mandarin (*C. clementine*). The genes analyzed were *VTE5* (phytol kinase), *VTE6* (phytyl-P kinase), *DXS1* and *DXS2* (1-deoxy-D-xylulose-5-phosphate synthase 1 and 2), *GGPPS1* and *GGPPS6* (geranylgeranyl pyrophosphate synthase 1 and 6), *GGDR* (geranylgeranyl diphosphate reductase), *TAT1* (tyrosine aminotransferase) and *HPPD* (4-hydroxyphenylpyruvate dioxygenase). Maturation stages correspond to immature green (IG), mature green (MG), breaker (Br) and mature (M). The data are mean ± S.E of at least three replicates.

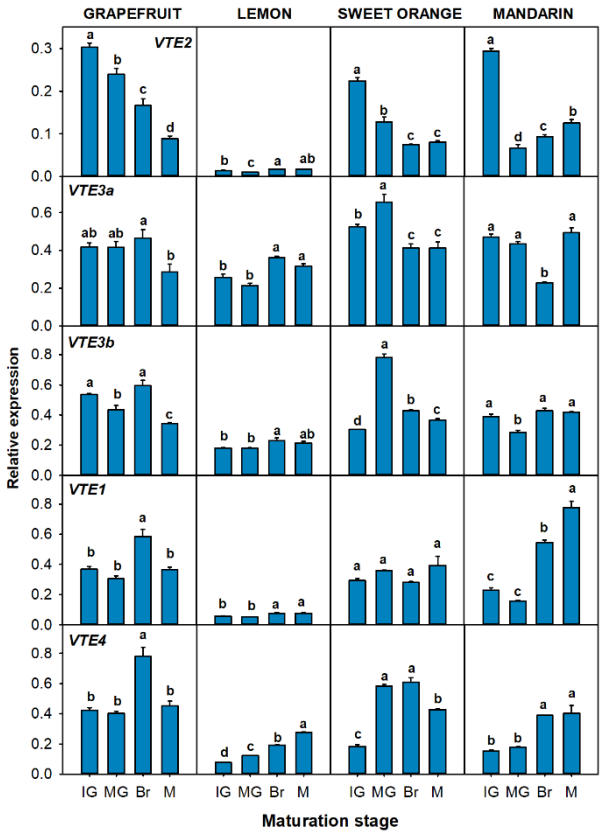

Figure 7. Relative expression of genes of the tocopherol-core pathway in the pulp during fruit maturation of grapefruit (*C. paradisi*), lemon (*C. limon*), sweet orange (*C. sinensis*) and mandarin (*C. clementine*). The genes analyzed were *VTE2* (HPT, homogentisate phytyl transferase), *VTE3a* and *VTE3b* (MPBQ-MT, 2-methyl-6-phytyl-1,4-benzoquinol methyltransferase a and b), *VTE1* (TC, tocopherol cyclase) and *VTE4* (γ-TMT, γ-tocopherol methyltransferase). Maturation stages correspond to immature green (IG), mature green (MG), breaker (Br) and mature (M). The data are mean ± S.E of at least three replicates.

3.4. Correlation and network analysis of tocopherol contents and relative expression of the genes involved in tocopherol synthesis in the flavedo and pulp during fruit maturation of four Citrus species: grapefruit (C. paradisi), lemon (C. limon), sweet orange (C. sinensis) and mandarin (C. clementine).

To better understand the relationship between gene expression and the accumulation of tocopherols, a correlation matrix (Figure 8A, C) and network analysis (Figure 8B, D) was built independently for the flavedo and pulp, using data of the four species at the four maturation stages. The network analysis revealed that tocopherols (total, α- and γ- forms) and all genes analyzed were present in both the flavedo and pulp networks, arranged in an interconnected group in each tissue (Figure 8). However, the number of interconnections in the networks was different between tissues, with a media of 4 edges in the flavedo and 9 in the pulp, and the number of negative links between metabolite nodes and the expression of genes was higher in the pulp than in the flavedo (Figure 8B, D).

In the flavedo, *VTE6, TAT1, VTE3b* and *VTE4* were the most interconnected genes (6-7 edges), but 4 more genes also had links above the median (*DXS2, GGPPS6, GGDR* and *VTE3a*) (Figure 8B). Not surprisingly, α-tocopherol and γ-tocopherol showed a strong and positive relationship with total tocopherols, but the correlation between α- and γ-tocopherol was not significant (Figure 8A, B). Moreover, tocopherols were positively correlated with the genes *TAT1*, of the SK pathway, and *VTE4*, of the tocopherol core pathway, suggesting that both *TAT1* and *VTE4* are key genes modulating tocopherol accumulation in the flavedo (Figure 8A, B). On the other hand, total tocopherols and α-tocopherol were negatively correlated with the gene *GGPPS6* of the MEP pathway, while γ-tocopherol displayed a negative correlation with the genes *VTE6*, *VTE5* and *DXS2* (Figure 8A). Interestingly, the gene *VTE6* seemed highly connected to genes of the MEP pathway (*DXS2*, *GGDR* and *GGPPS6*) and with both isoforms of *VTE3* (Figure 8B), suggesting that they are co-regulated in the flavedo during ripening.

In the pulp, 10 genes: *DXS1*, *GGPPS1*, *GGPPS6*, *VTE5*, *TAT1*, *HPPD*, *VTE2*, *VTE3b*, *VTE1* and *VTE4,* showed a number of links above the median, being *GGPPS1*, *HPPD*, *VTE1* and *VTE4* the most interconnected genes (12 edges) (Figure 8C, D). A strong positive correlation was detected between α- and γ-tocopherol in the pulp (Figure 8C, D). The network analysis in the pulp also revealed a positive correlation of the metabolite nodes with the genes *VTE6*, *DXS2* and *GGDR* (Figure 8C, D), suggesting that these genes play an important role in the regulation of tocopherol synthesis in the pulp. Tocopherols were negatively correlated with *GGPPS1*, *TAT1*, *HPPD* and *VTE4* and, in the case of total tocopherols and α-tocopherol, also with *DXS1* and *VTE1* (Figure 8C, D). Similar to what was observed in the flavedo, a connection between *VTE6* and the genes *DXS2* and *GGDR* of the MEP pathway was detected, reinforcing the idea of a possible co-regulation of these genes during fruit ripening also in the pulp. Furthermore, other genes were positively co-regulated in the pulp, including genes of the MEP pathway (*GGPPS6*, *GGPPS1*, *DXS1*), involved in phytol recycling (*VTE5*), of the SK pathway (*HPPD* and *TAT1*) and of the tocopherol-core pathway (*VTE2*, *VTE3b*, *VTE1* and *VTE4*).

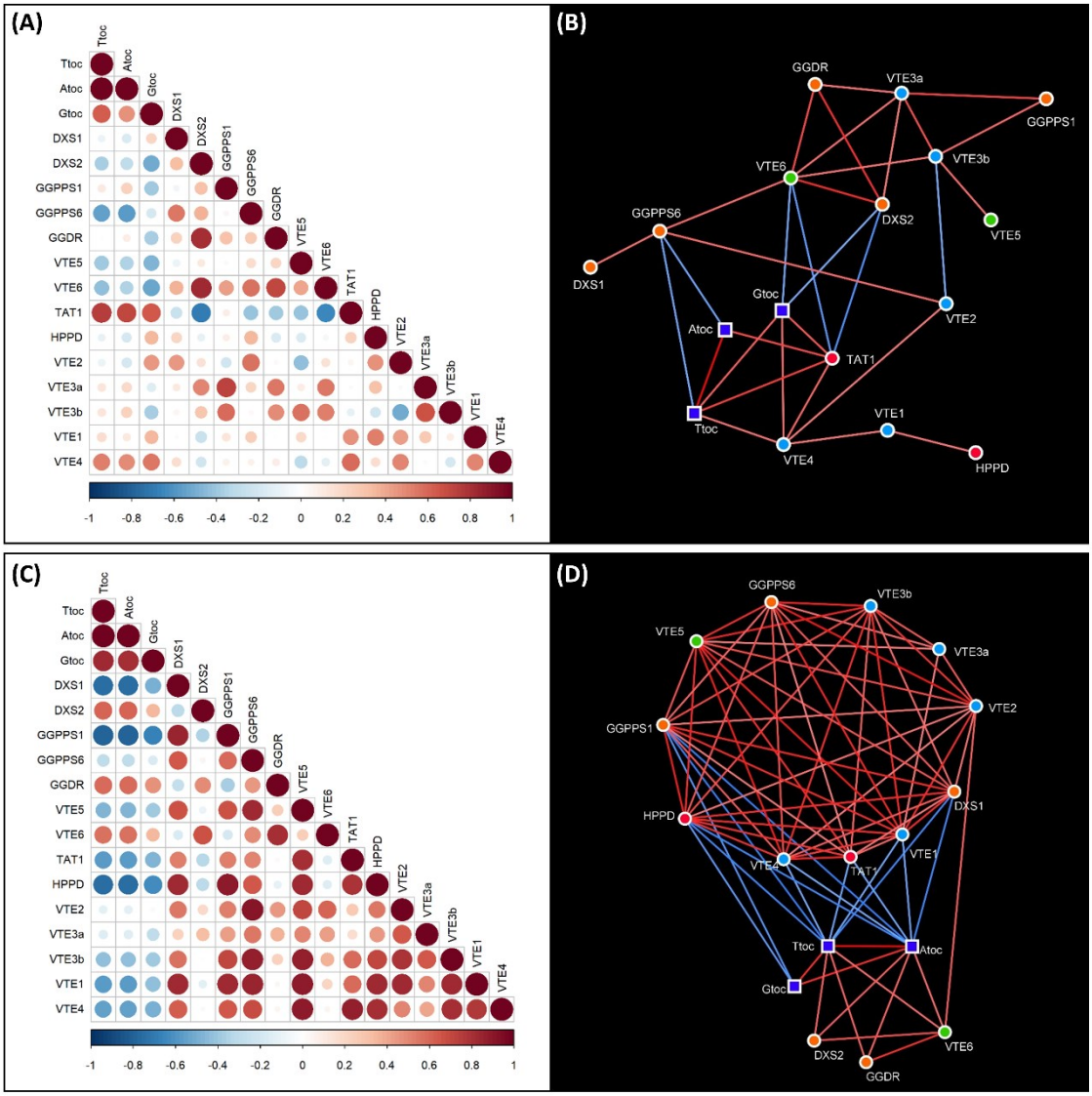

Figure 8. Correlation matrices (A, C) and networks (B, D) of tocopherol contents and expression of genes involved in tocopherol synthesis in the peel (A, B) and pulp (C, D) during fruit maturation of grapefruit (*C. paradisi*), lemon (*C. limon*), sweet orange (*C. sinensis*) and mandarin (*C. clementine*). Positive and negative correlations in the matrices are shown in different shades of red and blue, respectively, with the size of the circle and color intensity indicating the magnitude of Pearson's correlation coefficient. In the correlation networks, square nodes represent tocopherol contents (Ttoc, total tocopherols; Atoc, α-tocopherol; Gtoc, γ-tocopherol) and circle nodes represent genes (green, genes involved in the recycling of phytol; orange, genes of the MEP pathway; red, genes of the SK pathway; blue, genes of the tocopherol-core pathway). Lines joining the nodes represent correlations (edges); positive correlations are shown in red, while negative correlations are in blue, and the color intensity represents the strength of the correlation (absolute value of the Pearson's correlation coefficient). Only significant correlations (*p-value* ≤ 0.05) were taken into account for constructing the correlation networks.

4. Discussion

Citrus fruit are a significant source of nutrients and phytochemicals with positive impact in human nutrition and health (Cano et al., 2008; Ma et al., 2020a). Tocopherols, one of the most

powerful plant antioxidants with vitamin E activity (Fritsche et al., 2017), have been detected in the peel and pulp of mature fruit from the main *Citrus* species (Chun et al., 2006; Cardeñosa et al., 2015; Assefa et al., 2017; Rey et al., 2021a, 2021b), but how tocopherol accumulation is regulated during ripening has not yet been addressed. Therefore, taking advantage of the genetic and phenotypical diversity of the genus *Citrus*, the aim of this work was to investigate the changes in tocopherol contents and their regulation during maturation of fruit of four *Citrus* species belonging to the main horticultural groups: grapefruit, lemon, sweet orange and mandarin.

Tocopherols were identified in all the selected *Citrus* species throughout maturation, with contents varying between tissues, maturation stages and genotypes (Figure 2 and 3). The tocopherol profile was in agreement with previous reports, with α- and γ-tocopherol being the main forms detected in *Citrus* fruit (Assefa et al., 2017; Rey et al., 2021a, 2021b). In our experimental conditions, β-tocopherol was not detected, and δ-tocopherol was only identified in some samples but at levels below the limit of quantification, indicating that the δ-/β-tocopherol branch may not have a significant contribution to the tocopherol pool in citrus fruit. α-Tocopherol was the predominant form in both peel and pulp, and in all the species and maturation stages, representing 70-95% of total tocopherols in the peel and 97-99% in the pulp (Figure 2 and 3). A major accumulation of α-tocopherol has been previously detected in the peel of grapefruit and mandarins fruit (Rey et al., 2021a, 2021b), but predominance of one form or another seems to be dependent on the *Citrus* specie, as similar or higher γ-tocopherol contents have been detected in less common Korean *Citrus* genotypes (Assefa et al., 2017). In fruit of other species, the prevalence of α- or γ-tocopherol is also specie-specific and, while α-tocopherol is the main form in tomato, pepper, mango, grape, olive and avocado (Koch et al., 2002; Horvath et al., 2006b; Quadrana et al., 2013; Singh et al., 2017; Georgiadou et al., 2019; Vincent et al., 2020), γ-tocopherol accumulates at higher concentrations in zucchini and raspberry fruit (Carvalho et al., 2013; Rodov et al., 2020).

Analysis of tocopherol concentrations revealed that tocopherol contents were between 2 and 50 times higher in the peel than in the pulp throughout fruit maturation in the selected *Citrus* species (Figure 2 and 3). The peel and pulp of citrus fruits are two morphologically distinct and autonomous tissues with specific anatomical and physiological characteristics in which most of the metabolic processes are independently regulated (Tadeo et al., 2020). As it is described here for tocopherols, higher concentrations of other bioactive compounds or vitamins such as carotenoids, flavonoids and ascorbic acid, have been reported in the peel than

in the pulp of citrus (Alquézar et al., 2008, 2013; Alós et al., 2014; Assefa et al., 2017). The fruit peel is directly exposed to light, UV radiation and other environmental stresses, which could lead to a higher demand for antioxidants synthesis to cope with these adverse conditions. In relation to this, light could play a relevant role either as a stress factor or by its direct impact in the regulation of tocopherol biosynthesis. Tocopherols are believed to play a role in photo-protection (Spicher et al., 2017; Ma et al., 2020b), and increases in tocopherol contents have been reported in response to high light stress (Collakova and DellaPenna, 2003b; Havaux et al., 2005). Furthermore, a positive role of light in the transcriptional regulation of tocopherol biosynthetic genes has been recently proposed (Gramegna et al., 2019), which is also in agreement with the results of this work. Similarly, light deprivation at the last stages of fruit development negatively affected the expression of *GGDR* and genes of the tocopherol-core pathway in grapefruit (Rey et al. 2021a, 2021b). Interestingly, the differences detected in this study between citrus fruit tissues were not only quantitative but also in the pattern of tocopherol accumulation during maturation. In the peel, contents increased gradually in the four species towards M fruit (Figure 2), while contents in the pulp decreased sharply after the IG stage, with the exception of lemon where they remained constant (Figure 3). These results suggest that different mechanisms may operate in the regulation of tocopherol synthesis in the flavedo and pulp of *Citrus* fruit.

Another factor that might influence the differential accumulation of tocopherols between the peel and pulp is the chlorophyll content in each tissue. An alternative source of PPP, the prenyl-donor needed for tocopherol synthesis, is through the recycling of free phytol formed during the degradation of chlorophylls (Figure 1) (Valentin et al., 2006; Georgiadou et al., 2015; vom Dorp et al., 2015; Almeida et al., 2016). Chlorophyll content in the pulp of *Citrus* fruit is negligible or only detected in immature green fruit, while in the peel concentrations are high throughout development and decrease notably with the transition of chloroplasts to chromoplasts (Alquézar et al., 2008, 2013; Lado et al., 2015, 2019). Therefore, the higher concentration of chlorophylls in the peel at immature stages and their degradation as ripening progresses could lead to a higher availability of free phytol to form PPP, and higher influx into the tocopherol-core pathway resulting in an enhancement of tocopherol contents in the peel. The accumulation of tocopherols concomitantly with chlorophylls breakdown during color change has been previously observed in fruit of other species like pepper (Osuna-García et al., 1998; Koch et al., 2002) and olive (Georgiadou et al., 2016). Furthermore, in albino tomato fruits which lack chlorophylls at the ripe stage, due to an accelerated degradation in early

maturation stages, a reduction in tocopherol contents has been observed, associated with the absence of phytol derived from chlorophyll breakdown (Almeida et al., 2015).

The transcriptional analysis of tocopherol biosynthetic genes revealed that differences in tocopherol concentrations between tissues were associated with a generalized higher expression of the genes in the peel than in the pulp (Figure 4-7), which reinforces the hypothesis of the differential regulation of tocopherols accumulation between tissues. The molecular regulation of tocopherol biosynthesis during fruit maturation seemed to be modulated by specific candidate genes in each tissue (Figure 4-7). In the peel, the gradual increase in tocopherol content detected in the four species (Figure 2) was accompanied by a marked induction of the genes *TAT1* and *VTE4* (Figure 4C and 5), suggesting the importance of these genes in regulating tocopherol accumulation in this tissue. This idea is supported by the correlation matrix and network analysis, where *TAT1* and *VTE4* showed a significant positive correlation to total, α- and γ-tocopherol contents in the peel (Figure 8A, B). *TAT1* plays a major role in tocopherol synthesis by regulating HGA availability (Riewe et al., 2012), and an induction of this gene during senescence has been previously reported in *Arabidopsis* leaves, and associated with an increase in α- and γ-tocopherol content (Holländer-Czytko et al., 2005). On the other hand, the gene *VTE4*, which encodes for γ-TMT, seems to play a role in shaping tocopherol composition rather than increasing tocopherol contents, as it controls the shift from γ- and δ- into α- and β-tocopherol accumulation, respectively (Bergmüller et al., 2003). Modifications in the expression of *VTE4* have been successful in increasing α-tocopherol but in detriment of γ-tocopherol contents, and thus not altering total contents (Bergmüller et al., 2003; Collakova and DellaPenna, 2003a; Maeda et al., 2006). Therefore, in the peel of citrus fruit the combined up-regulation of *TAT1* and *VTE4* during maturation could indicate a higher influx into the tocopherol core-pathway, due to a higher availability of HGA, which afterwards is mostly converted into α-tocopherol by the increase in downstream *VTE4*. Still, it is important to keep in mind that many substrates and enzymes involved in tocopherol synthesis also participate in other metabolic pathways, and therefore the possible channeling to other pathways should be considered. Additionally, other genes were also induced during ripening in specific citrus species and may also contribute to the increase of tocopherols, although they were not significantly linked to tocopherol contents in the network analysis (Figure 8B). For instance, the induction of *HPPD* in grapefruit, orange and mandarin (only at the M stage in the later species) could also contribute to HGA availability (Figure 4C), while the up-regulation of *GGPPS1* in lemon could increase the precursor PPP availability (Figure 4B), but these changes seemed to be specie-specific.

The expression of most genes involved in the regulation of PPP production tended to decrease in the peel during maturation, but with no apparent effect on tocopherol contents (Figure 4A, B). A marked down-regulation of *VTE6* and *DXS2* was detected in the four species during the MG or Br stages, and expression of *GGPPS6* and *GGDR* also declined in most of the *Citrus* species. Mutant plants of *Arabidopsis* in these genes (*vte6*, *dxs* and *ggpps11*) have resulted in a reduction in tocopherol levels (Estévez et al., 2001; vom Dorp et al., 2015; Ruiz-Sola et al., 2016). Nonetheless, the down-regulation of these genes in the peel of citrus fruits during ripening was not reflected in a reduction of tocopherol levels and the network analysis also revealed a negative correlation between *VTE6, DXS2* and *GGPPS6* transcript levels and tocopherol content. However, these negative connections were only significant between *VTE6* and *DXS2* with γ-tocopherol, and *GGPPS6*, the citrus orthologous gene of Arabidopsis *GGPPS11*, with total and α-tocopherol levels (Figure 8B). A down-regulation of *DXS* after the MG stage has been previously reported in the peel of grapefruit, orange and mandarin (Alós et al., 2006; Alquézar et al., 2008, 2013; Lado et al., 2015, 2019), and appears to be linked to the onset of chlorophylls degradation at color break, rather than to the accumulation of other MEP-derived isoprenoids such as carotenoids and ABA during ripening. Collectively, these results suggest that the supply of PPP by the recycling of free phytol or by *de novo* synthesis through the MEP pathway does not seem to be limiting tocopherol synthesis in the peel of the selected *Citrus* genotypes during maturation. It is likely that the PPP production is reduced, but its content is still sufficient and may not constrain tocopherol synthesis.

In the pulp, most of the genes exhibited a similar expression profiling to that of the flavedo (Figure 4-7), although tocopherol concentrations were lower and followed contrasting temporal patterns in both tissues (Figure 2 and 3). Differences in the expression of genes in the pulp among genotypes were not consistent for all genes, but the pulp of lemon exhibited a distinct quantitative and qualitative expression patterns compared to other species. Expression of most genes was lower and remained relatively constant in the pulp of lemon during maturation, whereas transcript levels in grapefruit, orange and mandarin were more similar among them and experienced more fluctuations (Figure 6 and 7). A similar pattern of expression among genotypes was only detected for the genes *TAT1*, *HPPD* and *VTE4* (Figure 6C and 7), whereas the other genes seemed to follow a distinct profile in lemon (Figure 6A, B and 7). Interestingly, expression of *TAT1*, *HPPD* and *VTE4* was induced during maturation in the four species, similarly to the changes detected in the peel (Figure 4C and 5), but negatively correlated to tocopherol contents in the pulp (Figure 2 and 3). An induction of genes of the SK pathway (*TAT1*, *HPPD)* has also been observed with maturation in tomato and olive fruits but,

as in the citrus pulp, did not mirror the changes in tocopherol contents during maturation (Quadrana et al., 2013; Georgiadou et al., 2015). This absence of correlation may indicate that these genes are developmentally modulated, and likely related to the synthesis of other tocochromanols (Szymańska and Kruk, 2010; Kruk et al., 2014; Burgos et al., 2021). In relation to the other genes involved in tocopherol synthesis, their expression during maturation was relatively constant in the pulp of lemon, in accordance with the constant tocopherol contents in this specie (Figure 3). Interestingly, the decrease in tocopherol contents observed in the pulp of grapefruit, orange and mandarin was synchronized with the down-regulation of the genes *VTE6*, *DXS2*, *GGDR* and *VTE2* (Figure 6 and 7). These results suggest that transcriptional regulation of these genes play an important role determining tocopherol content in the pulp, which was supported by the correlation network (Figure 8C, D). These genes, in particular those involved in the supply of PPP, have been previously proposed as limiting steps in tocopherol accumulation in the flesh of tomato at later ripening stages (Quadrana et al., 2013; Almeida et al., 2015). Moreover, in this tissue the correlation network showed that besides *TAT1*, *HPPD* and *VTE4*, other genes like *DXS1*, *GGPPS1* and *VTE1* were also negatively correlated to tocopherol contents or a specific tocopherol form (Figure 8C, D). Then, it seems that in the pulp, the down-regulation of genes involved in regulating PPP availability (*VTE6*, *DXS2* and *GGDR*) constrains tocopherol synthesis, and that the up-regulation of other genes of the SK pathway (*TAT1* and *HPPD)* and tocopherol-core pathway (*VTE1* and *VTE4*) is not enough to compensate this limitation.

Furthermore, the network analyses in the peel and pulp also revealed genes that are co-regulated in both tissues during fruit maturation (Figure 8B, D). The genes *VTE6*, *DXS2* and *GGDR*, all involved in the supply of PPP, were positively linked together and shared a similar trend during maturation in both tissues. Other genes that seemed to be co-expressed in both tissues were those of the SK pathway with the genes regulating the last steps of the tocopherol-core pathway (Figure 8). It is noteworthy that in the pulp a higher number of genes were co-regulated than in the flavedo and, besides *VTE6*, *DXS2* and *GGDR*, other genes were linked together, such as genes involved in PPP synthesis (*VTE5*, *DXS1*, *GGPPS1* and *GGPPS6*) with those involved in HGA synthesis (*TAT1* and *HPPD*) and in the tocopherol-core pathway (*VTE2*, *VTE3b*, *VTE1* and *VTE4*).

Finally, the differences in the accumulation of tocopherols detected among genotypes in each tissue (Figure 2 and 3) were not clearly related to the expression of any gene (Figure 4-7), although some trends were observed in the flavedo. Focusing on differences in gene

expression at early maturation stages, when differences among genotypes were already evident, a higher expression of *GGDR* and *VTE3b* was detected in the peel of lemon and orange than in the other genotypes, which could explain the higher contents in these two species.

5. Conclusion

This study addresses for the first time a comparative analysis of tocopherols accumulation and transcriptional regulation of their biosynthetic genes during fruit maturation of four *Citrus* species. Differences in tocopherol contents were detected between the flavedo and pulp, and also during maturation and genotypes. Concentration of tocopherols in the flavedo were between 2-50 times higher than in the pulp, and this was associated with higher expression levels of most genes involved in the precursors PPP and HGA synthesis, and the tocopherol core-pathway in the flavedo. Moreover, tocopherols increased in the flavedo with maturation while they tended to decrease in the pulp, and different candidate genes appeared to regulate tocopherol accumulation in each tissue. In the flavedo, the increase in tocopherol contents mirrored a marked up-regulation of *TAT1* and *VTE4*, while contents in the pulp seemed to be limited by the expression of *VTE6*, *DXS2* and *GGDR*, regulating PPP availability. Furthermore, the genes *TAT1* and *VTE4*, *HPPD* and *VTE1*, and *VTE6*, *DXS2* and *GGDR*, seemed to be co-regulated and shared a similar pattern during maturation in both tissues, suggesting they are developmentally modulated. Finally, lemon fruit was the specie accumulating the highest tocopherol content in both tissues, whereas mandarin fruit accumulated the lowest, and grapefruit and orange had intermediate contents. However, the differences among genotypes were not clearly correlated with the expression of specific genes, suggesting the involvement of other regulatory mechanisms modulating differences among species.

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Supplementary Material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.743993/full#supplementary-material.

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El DF es un desorden fisiológico que ocurre durante la conservación postcosecha de frutos a bajas temperaturas (inferiores a 5 °C), y supone un problema en frutos de muchas especies y variedades de cítricos sensibles, ya que deprecia su calidad externa, la aceptación por los consumidores y su comercialización (Zacarias et al., 2020). Una de las principales problemáticas de este desorden es la variabilidad en su incidencia y la diversidad de factores que pueden influir en la susceptibilidad/tolerancia de los frutos al DF, muchos de los cuales no se conocen en profundidad. La incidencia en los frutos de las distintas especies y variedades es muy variable e impredecible, por lo que en muchas ocasiones es complicado realizar un adecuado manejo tecnológico postcosecha que asegure la ausencia de daños (Lado, Cronje, et al., 2019). Las bases fisiológicas, bioquímicas y moleculares implicadas en la respuesta postcosecha de los frutos cítricos a las bajas temperaturas de conservación, así como los diferentes factores que indicen en las mismas, y los sistemas y tratamientos de manipulación comercial, han sido objeto de revisiones exhaustivas en los últimos años (Lado, Cronje, et al., 2019; Zacarias et al., 2020).

El objetivo principal planteado en este trabajo de Tesis Doctoral fue profundizar en la implicación de dos grupos de compuestos con potente actividad antioxidante en los vegetales, los carotenoides y tocoferoles, en las respuestas de los frutos cítricos a las bajas temperaturas de conservación y su posible relación con la susceptibilidad/tolerancia al desarrollo del DF. Este objetivo se complementa con el estudio de la contribución del AsA y de la capacidad antioxidante asociada a estos compuestos en la respuesta a la conservación en frío durante la postcosecha. Para ello, se plantearon diferentes objetivos específicos, que se distribuyen en los respectivos capítulos de esta Tesis, donde se ha hecho uso de la diversidad genética de los frutos cítricos, que ha permitido comparar variedades de mandarinas y de pomelos con diferencias marcadas en la pigmentación de la piel y en su sensibilidad al DF durante la conservación refrigerada. Finalmente, esta diversidad genética se ha utilizado para analizar el metabolismo de tocoferoles durante la maduración de frutos de variedades representativas de las cuatro principales especies cultivadas de cítricos (naranja, mandarina, limón y pomelos), con el objetivo de caracterizar la acumulación y regulación transcripcional de estos compuestos en la piel y la pulpa de estos frutos.

4.1. Carotenoides y AsA en la susceptibilidad/tolerancia de los frutos cítricos a los DF

Numerosas evidencias experimentales indican la implicación del estrés oxidativo, la generación de ROS, y el incremento en otros procesos oxidativos, en la respuesta de los frutos a las bajas temperaturas de conservación postcosecha y con los daños que se producen en esas

condiciones (Sevillano et al., 2009; Toivonen, 2004), incluidos los frutos cítricos (Zacarias et al., 2020). Por lo tanto, la concentración de diferentes compuestos con capacidad antioxidante en el flavedo, así como la actividad de enzimas antioxidantes, pueden ser una primera barrera de defensa frente a las bajas temperaturas o a las lesiones que éstas inducen, y desempeñar un papel esencial en la susceptibilidad o tolerancia de los frutos cítricos a desarrollar DF durante la conservación a bajas temperaturas (Lado, Rodrigo, et al., 2016; Sala, 1998). En este sentido, los carotenoides y la vitamina C son dos de los principales antioxidantes presentes en el flavedo de los frutos cítricos con capacidad de neutralizar radicales libres y ROS (Alós et al., 2014; Lado, Rodrigo, et al., 2016; Rodrigo, Alquézar, Alós, Lado, et al., 2013). El papel de la vitamina C en la incidencia y desarrollo de los DF en los frutos cítricos no está totalmente esclarecido, ya que se han obtenido resultados en ocasiones contradictorios (Lado, Rodrigo, et al., 2016; Mditshwa, Magwaza, Tesfay, & Opara, 2017). De forma similar, el papel de los carotenoides, principales pigmentos responsables de la coloración de los frutos cítricos (Rodrigo, Alquézar, Alós, Lado, et al., 2013), es todavía objeto de debate. En frutos de la mandarina Clemenules cultivados en Sudáfrica se ha observado que aquellos frutos recolectados del interior del árbol, con una menor incidencia de luz, desarrollan menor coloración externa y presentan mayor susceptibilidad a desarrollar desórdenes postcosecha durante el la conservación en frío, en comparación con los frutos del exterior de la copa con mayor coloración (Cronje et al., 2011; Magwaza, Opara, Cronje, Landahl, & Terry, 2013). Estos resultados corroboran la observación tradicional entre los productores y agricultores de cítricos de que los frutos de naranjas y mandarinas del interior del árbol expuestos a una menor incidencia luminosa, desarrollan menor coloración y son más propensos a desarrollar DF que los frutos de color intenso ubicados en el exterior. Por otro lado, estudios en pomelo Star Ruby, que pueden presentar coloración rojiza en la piel, han permitido obtener resultados más concluyentes sobre la implicación de carotenoides en los DF. Estos estudios demostraron que la acumulación de licopeno en el flavedo, inducida mediante el tapado de los frutos en campo en las últimas etapas de desarrollo o mediante la aplicación postcosecha de inhibidores de la actividad licopeno ciclasa (β-LYC), resultaba en frutos de coloración roja en los que se inducía tolerancia al DF durante el almacenamiento en frío (Lado, Rodrigo, Cronje, et al., 2015; Lado, Rodrigo, et al., 2016). A pesar de estas evidencias, el posible rol de los carotenoides y otros compuestos antioxidantes como la vitamina C en la tolerancia al DF necesita ser confirmado y extendido a frutos de diferentes variedades de cítricos, así como entender el posible modo de acción de ambos antioxidantes en la susceptibilidad/tolerancia a los DF, y ha consistido en el primer objetivo de esta Tesis Doctoral (Capítulo 1). Para ello se utilizaron frutos de tres

variedades de mandarina con diferente coloración en la piel y grado de susceptibilidad al DF: Fortune, Nova y Nadorcott, que se almacenaron a 2 °C durante 8 semanas. Además, teniendo en cuenta el efecto de la luz sobre la acumulación de carotenoides y vitamina C en la piel de frutos cítricos (Lado, Alós, et al., 2019; Lado, Alós, Rodrigo, & Zacarías, 2015; Lado, Cronje, Alquézar, Page, et al., 2015), los frutos de las tres variedades se taparon en el campo previo al cambio de color, con el objetivo de manipular los contenidos de estos compuestos y poder evaluar su influencia sobre el desarrollo de DF.

La sensibilidad de los frutos al DF durante el almacenamiento a 2 °C fue diferente según la variedad, confirmando que el sistema experimental seleccionado era adecuado para el estudio comparativo planteado (Figura 1 y 2A, Capítulo 1). Los frutos de Fortune fueron altamente sensibles al DF, mientras que los de Nova fueron moderadamente sensibles, desarrollando menos síntomas y de menor intensidad que los de Fortune, corroborando las observaciones previas descritas en estas variedades (Lado, Cronje, et al., 2019; Sala, 1998). Sin embargo, los frutos de Nadorcott presentaron una baja incidencia de DF, confirmando la alta tolerancia de estos frutos frente al DF. A pesar de que los resultados de este trabajo confirman la tolerancia de los frutos de Nadorcott frente a los DF, su comportamiento aún no estaba ampliamente demostrado debido a su reciente introducción en el mercado. A su vez, el análisis de carotenoides reveló que en el flavedo de frutos de mandarina existe una correlación inversa entre el contenido de carotenoides totales en el flavedo en el momento de la cosecha y la susceptibilidad al DF al final de la conservación refrigerada (Tabla 4, Capítulo 1). De esta manera, los frutos más sensibles al DF (Fortune) fueron aquellos con un menor contenido de carotenoides en el flavedo al inicio de la conservación, mientras que los frutos resistentes (Nadorcott) presentaron un alto contenido de carotenoides (Figura 2A y Tabla 1-3, Capítulo 1). Por otro lado, el análisis de la composición de carotenoides reveló que no todos los carotenoides parecen estar implicados en la tolerancia al DF, ya que solo β-criptoxantina y violaxantina se correlacionaron negativamente con el índice de DF final, mientras que la β-citraurina y otros carotenoides presentes en menores concentraciones no mostraron una buena correlación, lo que parece indicar que no tuvieron un papel tan evidente (Tabla 4, Capítulo 1). Estos resultados son similares a los observados en el flavedo de frutos tolerantes de pomelo Star Ruby, en donde se detectaron mayores contenidos de carotenoides totales, y en particular del caroteno lineal licopeno (Lado, Rodrigo, Cronje, et al., 2015).

La comparación de los contenidos de vitamina C en la piel de los frutos de las tres mandarinas en el momento de la cosecha y durante la conservación se correlacionaron

positivamente con el índice de DF al final de la conservación, contrariamente a los carotenoides, indicando que la acumulación de este antioxidante hidrosoluble no parece jugar un papel protector frente al DF en frutos de mandarina. Los contenidos en la cosecha fueron mayores en los frutos más sensibles, Fortune, y no se detectaron cambios importantes durante la conservación en frío (Figura 3, Capítulo 1). Lado et al. (2016) tampoco encontraron diferencias en los contenidos de vitamina C entre frutos de pomelo tolerantes y sensibles al DF.

En cuanto al tapado de los frutos, éste tuvo un efecto diferencial sobre el desarrollo de DF según la variedad de mandarina. En las variedades sensibles de Fortune y Nova disminuyó el índice de DF, mientras que en Nadorcott lo incrementó (Figura 1 y 2A, Capítulo 1). Sin embargo, la disminución del DF en Fortune y Nova fue moderada, y los frutos tapados también alcanzaron niveles altos de DF al final de la conservación. Estos resultados son diferentes a lo previamente observado en frutos de pomelo, en donde los frutos tapados presentaron una marcada tolerancia al DF (Lado, Rodrigo, Cronje, et al., 2015), sugiriendo que la ausencia de luz en las últimas etapas del desarrollo tiene un efecto diferencial sobre la tolerancia al DF, según la especie. Es importante resaltar que el tapado de frutos disminuyó significativamente el contenido de carotenoides totales y la mayoría de los carotenoides individuales en el flavedo de Nova y Nadorcott, pero no en Fortune, sugiriendo que diferentes mecanismos de regulación de la acumulación de carotenoides parecen operar en ausencia de luz en esta variedad. También es importante destacar que los contenidos de β-citraurina, que brinda un color naranja intenso a la piel de los frutos, no se afectaron de forma significativa por el tapado de frutos, lo que puede explicar en parte el índice de color similar de los frutos tapados y no tapados (Figura 2B, Capítulo 1). Los resultados en Nova y Nadorcott son similares a los detectados en frutos de otras variedades de mandarina, como Satsuma y Clementina, en donde la ausencia de luz redujo los contenidos de carotenoides totales (Cronje et al., 2011, 2013; Lado, Alós, et al., 2019). Además, el tapado también produjo una disminución de los contenidos de vitamina C en las tres variedades, de acuerdo con lo previamente descrito en frutos de otras variedades de mandarina, naranja y pomelo, donde la ausencia de luz produjo una reducción de la expresión de determinados genes de la síntesis de AsA y sus contenidos (Lado, Alós, Rodrigo, & Zacarías, 2015).

Por otro lado, en el objetivo de este primer trabajo también se planteó evaluar la capacidad antioxidante de la piel de los frutos, que puede estar asociada en parte con los contenidos en los compuestos anteriores, y su relación con la susceptibilidad de los frutos de las tres

variedades a los DF. Esta actividad se evaluó mediante los métodos de DPPH• y FRAP, que miden la actividad antioxidante en extractos hidrosolubles (D. Huang et al., 2005), y el método SOAC, que mide la capacidad de secuestrar oxigeno singlete de un extracto liposoluble (Ouchi et al., 2010). Las actividades antioxidantes, mediante los ensayos DPPH• y FRAP, fueron mayores en el flavedo de los frutos sensibles de Fortune y Nova que en los frutos tolerantes de Nadorcott, y se correlacionaron positivamente con el índice final de DF (Figura 4 y Tabla 4, Capítulo 1). Estos resultados fueron similares a los detectados en frutos sensibles de pomelo Star Ruby, donde se detectó una mayor actividad antioxidante que en los frutos tolerantes (Lado, Rodrigo, et al., 2016). Además, se detectó una alta correlación entre la actividad, medida por DPPH• y FRAP, y los contenidos de vitamina C, lo que indicaría que son métodos apropiados para medir la contribución de la vitamina C al sistema antioxidante hidrosoluble total, de acuerdo a lo detectado por otros autores en la pulpa de distintas variedades de cítricos (Zacarías-García et al., 2020). Sin embargo, la correlación negativa con el contenido de carotenoides indicaría que estos sistemas no son adecuados para evaluar la capacidad antioxidante asociada a los mismos, al ser de naturaleza liposoluble. Por el contrario, la capacidad antioxidante medida por el método SOAC, se correlacionó positivamente con el contenido total de carotenoides, de β-criptoxantina y de violaxantina, y negativamente con el DF al final de la conservación. De manera similar a los resultados del Capítulo 1, también se ha detectado una correlación positiva entre los valores de SOAC, el contenido de licopeno (y carotenoides totales) y la tolerancia a los DF en frutos de pomelo Star Ruby (Lado, Rodrigo, et al., 2016), sugiriendo que la protección que confiere este carotenoide está relacionada a su capacidad para neutralizar el radical $^1O_2$ generado durante la exposición de los frutos a bajas temperaturas. Los carotenoides son considerados la primera barrera frente al daño por $^1O_2$ en plantas, y la eficiencia de distintos carotenoides en secuestrar $^1O_2$, incluidos la violaxantina y la β-criptoxantina, ha sido probada *in vitro* (Conn, Schalch, & Truscott, 1991; Triantaphylidès & Havaux, 2009). En particular, la β-criptoxantina tiene una potente capacidad antioxidante *in vitro* (Di Mascio et al., 1989; Ouchi et al., 2010), y se ha demostrado que puede proteger frente al daño oxidativo en sistemas *in vivo* (Llopis et al., 2019). Por tanto, los resultados alcanzados en este primer capítulo sugieren que los contenidos en la concentración de carotenoides en la cosecha, principalmente de las xantofilas β-criptoxantina y violaxantina, pueden tener un papel protector frente al desarrollo del DF en frutos de mandarina, y las diferencias en el flavedo de las tres variedades explica la diferente tolerancia a los DF. Este efecto de los carotenoides estaría asociado principalmente a su capacidad de secuestrar $^1O_2$, mientras que los contenidos de vitamina C no parecen tener un rol en la tolerancia al daño por frío.

4.2. Tocoferoles en la susceptibilidad/tolerancia de los frutos cítricos a los DF

A pesar de que los carotenoides y la vitamina C son compuestos antioxidantes importantes en los cítricos, la capacidad antioxidante de un tejido es el resultado de la actuación de una mezcla compleja de diferentes sistemas enzimáticos y moléculas, entre las que se incluyen los polifenoles y tocoferoles (Sevillano et al., 2009; Zou et al., 2016). Por tanto, a partir de un análisis metabolómico realizado en frutos de pomelo tolerantes y sensibles al DF (realizado en colaboración con el Dr. Gianfranco Diretto, ENEA, Roma) en el que se encontraron diferencias significativas en los niveles de tocoferoles en el flavedo de ambos tipo de frutos (datos no presentados), se planteó un nuevo objetivo en esta Tesis Doctoral: explorar la influencia de los tocoferoles en la sensibilidad de frutos de diferentes especies/variedades de frutos cítricos a desarrollar DF durante la conservación postcosecha a bajas temperaturas.

Los tocoferoles cumplen numerosas funciones en las plantas, entre las que destaca su papel como antioxidantes liposolubles, siendo capaces de secuestrar ROS y radicales libres (Falk & Munné-Bosch, 2010). Además, estos compuestos han sido asociados a la respuesta de las plantas frente a condiciones de estrés, incluido el estrés por bajas temperaturas (Bergmüller et al., 2003; Collakova & DellaPenna, 2003b; J. Ma et al., 2020; Maeda et al., 2006). Para llevar a cabo este estudio se utilizaron dos sistemas experimentales: i) por un lado, se utilizaron frutos de las tres variedades de mandarinas utilizadas en el capítulo 1 (Fortune, Nova y Nadorcott), en las cuales se había confirmado la diferente susceptibilidad al DF y, ii) se utilizaron frutos de pomelo Star Ruby expuestos a condiciones normales de luz durante su maduración (sensibles al DF) y frutos en los que se evitó la incidencia de luz mediante el embolsado de los mismos (tolerantes al DF), aprovechando la metodología desarrollada por Lado, Rodrigo, Cronje, et al. (2015). Este último apartado, que corresponde al capítulo 3, no solo permitió estudiar la acumulación de tocoferoles y los DF, si no también evaluar el efecto de la luz (o su ausencia) sobre la síntesis de estos compuestos en el flavedo de los frutos durante su maduración. Debido a que la regulación de la biosíntesis de tocoferoles en frutos cítricos no había sido estudiada anteriormente, en ambas líneas de trabajo se planteó como objetivo llevar a cabo un estudio detallado de la regulación transcripcional de los principales genes involucrados en la síntesis de tocoferoles en el flavedo de los frutos cítricos.

Los resultados obtenidos en el Capítulo 2 mostraron que el perfil de tocoferoles en el flavedo de frutos de mandarina consiste principalmente en las formas de α- y γ-tocoferol, siendo el α-tocoferol la forma mayoritaria (Figura 3, Capítulo 2). Estos resultados coinciden con lo reportado anteriormente para este género, aunque también se ha detectado γ-tocoferol

como la forma mayoritaria en algunas especies (Assefa et al., 2017; Mathaba et al., 2014). Sin embargo, α-tocoferol es la forma predominante en la mayoría de los tejidos vegetales, y es la forma principal en frutos de otras especies, como tomate, pimiento rojo, aceitunas, aguacate y cerezas (Georgiadou et al., 2019; Koch et al., 2002; Quadrana et al., 2013; Tijero et al., 2016; Vincent et al., 2020).

El análisis de tocoferoles también reveló diferencias marcadas en los contenidos entre las distintas variedades, y una relación inversa con la incidencia de DF. Así, se detectaron mayores concentraciones en el momento de la cosecha en el flavedo de los frutos tolerantes de Nadorcott, que fueron entre 1.5 y 2.5 veces superiores a las encontradas en el flavedo de los frutos sensibles de Nova y Fortune, respectivamente (Figura 2 y 3, Capítulo 2). Estos resultados sugieren que los mayores contenidos de tocoferoles al inicio de la conservación en frío pueden tener un papel protector frente al desarrollo de DF durante el almacenamiento. A pesar de que la información sobre la función de los tocoferoles en el DF de frutos es muy limitada, una relación similar se ha descrito en frutos de calabacín, donde el exocarpo de los genotipos más tolerantes al DF contenían mayores concentraciones de tocoferoles (Rodov et al., 2020). Es interesante destacar que, aunque el contenido de ambas formas fue inferior en el flavedo de Fortune, las diferencias entre Nova y Nadorcott fueron principalmente en los contenidos de α-tocoferol, mientras que los niveles de γ-tocoferol fueron similares. El α-tocoferol es considerado la forma más eficiente para secuestrar $^1O_2$ (Gruszka et al., 2008), y los resultados sugieren que las diferencias en esta forma específica explicarían las diferencias en la susceptibilidad al DF entre los tres genotipos de mandarina.

Durante la conservación en frío los contenidos de α-tocoferol se mantuvieron constantes en las especies sensibles al DF, mientras que en Nadorcott se observó una disminución después de una semana a 2 °C. Sin embargo, esta disminución fue transitoria y los contenidos incrementaron hacia el final de la conservación, alcanzando valores similares al inicio. Por el contrario, los contenidos de γ-tocoferol aumentaron tanto en Nova como en Nadorcott. Existe poca información acerca del efecto del frío sobre los contenidos de tocoferoles en frutos y, mientras que en algunos frutos como aguacate parecen disminuir (Vincent et al., 2020), en otras especies como cerezas se ha detectado un aumento (Tijero et al., 2016). A pesar de las fluctuaciones en los contenidos durante la conservación, las diferencias entre variedades se mantuvieron, y las concentraciones de tocoferoles en el flavedo, sobre todo la de α-tocoferol, tanto al inicio como durante la conservación parece que son factores importantes en la tolerancia de los frutos de mandarina frente al DF.

Con el fin de integrar los resultados obtenidos en el Capítulo 1 y 2 sobre la relación de distintos compuestos antioxidantes con el DF en las tres variedades de mandarina, se realizó un análisis de correlación similar al presentado en el capítulo 1 pero incluyendo también los contenidos de tocoferoles (Figura 6). Este análisis reveló una correlación negativa entre el índice de DF final y el contenido total de carotenoides (β-criptoxantina y violaxantina) y tocoferoles (α-tocoferol), y positiva con la vitamina C. Además, al igual que con los carotenoides, los tocoferoles tuvieron una alta correlación con la capacidad de secuestrar oxígeno singlete (SOAC). Por tanto, estos resultados indicarían que un mayor contenido de antioxidantes liposolubles, como carotenoides y tocoferoles, brindan una mayor tolerancia al DF en frutos de mandarina debido principalmente a su capacidad para secuestrar $^1O_2$. Además, apoyarían la hipótesis de que los carotenoides y tocoferoles, presentes principalmente en plástidos, representan una primera barrera contra la propagación de ROS en células y una mayor capacidad de proteger frente a procesos oxidativos, que finalmente resultan en la aparición de los síntomas visibles de DF.

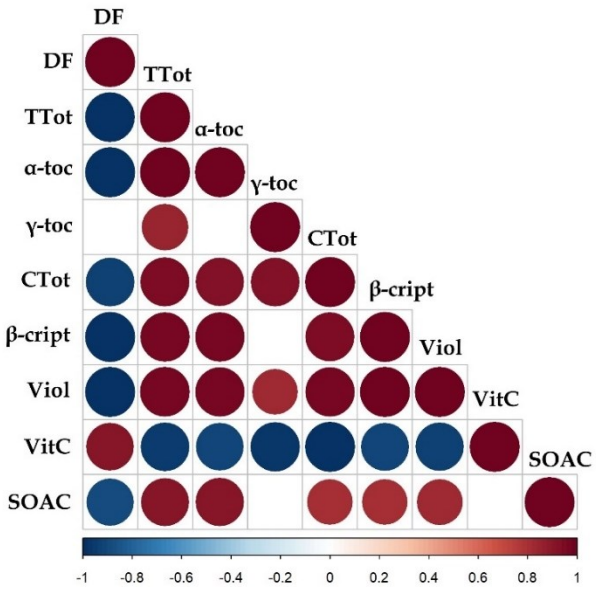

Figura 6. Matriz de correlación integrando la relación el índice de daño por frío (DF) al final de la conservación (8 semanas) y los contenidos de tocoferoles totales (TTot), α-tocoferol (α-toc), γ-tocoferol (γ-toc), carotenoides totales (CTot), β-criptoxantina (β-cript), violaxantina (Viol), vitamina C (VitC) y la capacidad antioxidante de secuestrar oxígeno singlete (SOAC). Círculos de color rojo indican correlaciones positivas, mientras que los círculos de color azul indican correlaciones negativas. El tamaño del círculo e intensidad de coloración es proporcional al valor absoluto del coeficiente de correlación de Pearson (r^2). Solo se presentan las correlaciones significativas ($p \leq 0.05$). Para la construcción de la matriz solo se han tenido en cuenta los datos para los frutos no tapados (control) utilizados en el capítulo 1 y 2, y por ello algunos resultados varían de aquellos presentados en la Tabla 4 (Capítulo 1).

Con la finalidad de analizar los cambios transcripcionales de los principales genes implicados en la síntesis de tocoferoles en los cítricos, tanto durante la conservación postcosecha como durante la maduración del fruto, se realizó un análisis bioinformático utilizando las secuencias de los genes previamente identificados en Arabidopsis y tomate frente a las bases de datos disponible del genoma de *Citrus* (Tabla 1, Capitulo 2). En base a la comparación de secuencias se identificaron 14 genes en *Citrus* implicados en la ruta específica de la síntesis de tocoferoles, así como de la síntesis de sus precursores PPP y HGA, ya que la disponibilidad de los mismos influye de forma importante en el contenido final de tocoferoles (Fritsche et al., 2017; Pellaud & Mène-Saffrané, 2017).

El análisis transcripcional de estos genes en el flavedo de mandarinas conservadas a 2 °C reveló que la regulación diferencial de algunos de estos genes parece determinar las diferencias en los contenidos detectadas entre genotipos (Figuras 4-8, Capítulo 2). En este sentido, la expresión de la mayoría de los genes de la ruta MEP en el momento de la cosecha, principalmente *DXS1*, *DXS2* y *GGDR*, fue menor en la variedad con menores contenidos, Fortune, y marcadamente mayor en Nova y Nadorcott, con mayores contenidos. Mientras que el gen *DXS* regula la entrada a la ruta MEP (Rodríguez-Concepción & Boronat, 2015), el gen *GGDR* controla la reducción directa de GGPP al precursor PPP (Pellaud & Mène-Saffrané, 2017). En tejidos vegetativos, la represión de *DXS* se ha asociado a una reducción en la acumulación de isoprenoides (Estévez et al., 2001), incluidos los tocoferoles, y en genotipos de tomate con elevadas concentraciones de tocoferoles se han detectado mayor expresiones de *DXS* y *GGDR* (Quadrana et al., 2013). Por otro lado, de los genes de la ruta específica de la síntesis de tocoferol, la expresión de las isoformas de *VTE3* tendieron a ser superiores en Nova y Nadorcott (Figura 6, Capítulo 2). En los genotipos de tomate que acumulan altos contenidos de tocoferoles también se han detectado mayores niveles de expresión de este gen (Quadrana et al., 2013), sugiriendo la importancia de este paso en el contenido de tocoferoles en los frutos. En conjunto, estos resultados parecerían indicar que la mayor expresión de *DXS* y *GGDR* podría traducirse en una mayor disponibilidad de PPP para la entrada a la ruta específica de tocoferoles, que posteriormente sería canalizado de forma preferencial hacia la rama γ/α, por la mayor expresión de las isoformas *VTE3*. Sin embargo, es importante destacar que las diferencias en la expresión de estos genes entre Nova y Nadorcott, a excepción de *VTE3b*, no parecerían explicar las diferencias en los contenidos entre ellas, indicando la actuación de otros mecanismos de regulación. En cuanto a los otros genes involucrados en la síntesis del precursor PPP (*VTE5* y *VTE6*) y HGA (*TAT1* y *HPPD*), como en la ruta específica de tocoferoles (*VTE2*, *VTE1* y *VTE4*), no se encontraron diferencias importantes entre las variedades y, por

tanto, no parecerían limitar la acumulación de tocoferoles en el flavedo de frutos maduros de mandarina. Aunque en tejidos vegetativos o semillas el gen *VTE2* limita la acumulación de tocoferoles (Collakova & DellaPenna, 2003a), en el flavedo de frutos de mandarinas su expresión no parece limitar la síntesis de tocoferoles, de acuerdo a lo observado en otros frutos como el tomate (Quadrana et al., 2013) y oliva (Georgiadou et al., 2019).

El efecto de las bajas temperaturas sobre el perfil transcripcional de los genes fue variable y dependió del gen y variedad. En general, la expresión de los genes involucrados en la síntesis de los precursores, a excepción de *GGDR* y *TAT1*, se estimuló por las bajas temperaturas, mientras que los genes de la ruta específica de tocoferoles tendieron a reprimirse (Figura 4-7, Capítulo 2). La expresión de *TAT1* disminuyó de forma temprana, pero hacia el final de la conservación en frío (5-8 semanas) incrementó su expresión, y este mismo patrón se observó en ciertos genes de la ruta específica de tocoferoles, sobre todo en la variedad Nova (Figura 6 y 7, Capítulo 2). El único gen que presentó una respuesta diferencial por el frío entre las variedades fue *GGDR*, que se indujo en la variedad con menores contenidos (Fortune) y se reprimió en Nova y Nadorcott. La inducción en la variedad muy sensible al DF podría sugerir que es una respuesta al DF, aunque Nova también fue sensible y no se observó el mismo patrón. La inducción detectada en los otros genes de la ruta MEP y en *HPPD*, y los cambios detectados en los genes específicos de la ruta, fueron similares en las tres variedades, indicando que parecerían ser respuestas directas al frío y no relacionadas con el grado de sensibilidad de las variedades al DF o sus contenidos de tocoferoles. A su vez, este incremento no se tradujo en incrementos en los contenidos de tocoferoles, sugiriendo una canalización hacia la síntesis de otros antioxidantes, como plastocromanol o carotenoides, o también la existencia de otros mecanismos de regulación, como por ejemplo a nivel postranscripcional. Por otra parte, la marcada disminución en la expresión de *VTE4* en Nova y Nadorcott durante la conservación podría significar una menor conversión de γ- a α-tocoferol y, por ende, el aumento en los contenidos de γ-tocoferol detectados en estas variedades, que no se observó en Fortune.

En una segunda aproximación se estudió el papel de los tocoferoles en la tolerancia al DF en frutos cítricos utilizando frutos de pomelo Star Ruby, variedad muy sensible al DF, en la que previamente se había demostrado que el tapado de los frutos inducía la tolerancia al DF (Lado, Rodrigo, Cronje, et al., 2015; Lado, Rodrigo, et al., 2016). De acuerdo a lo descrito previamente, los frutos tapados (T) presentaron una coloración roja uniforme y fueron tolerantes al DF durante la conservación en frío, mientras que los frutos no tapados (NT)

presentaron una coloración amarilla (con algunas zonas coloreadas) y fueron altamente susceptibles al desarrollo de DF (Figura 1, Capítulo 3).

El análisis de tocoferoles reveló que, al igual que ocurre en frutos de mandarina (Capítulo 2), los tocoferoles se acumularon principalmente como α- y γ-tocoferol en el flavedo de pomelo, y α-tocoferol fue la forma mayoritaria en ambas condiciones (Figura 2, Capítulo 3). Sin embargo, la ausencia de luz durante la maduración del fruto tuvo un efecto diferencial sobre la acumulación de γ-tocoferol. Los contenidos totales de tocoferoles incrementaron en ambas condiciones durante la maduración, pero el tapado de frutos disminuyó la acumulación de γ-tocoferol y, por tanto, redujo los contenidos totales. Por otro lado, los contenidos de α-tocoferol fueron similares en ambas condiciones y no sé vieron afectados por la ausencia de luz. Por tanto, las concentraciones de tocoferoles totales en el momento de la cosecha fueron mayores en los frutos sensibles al frío (NT) que en los frutos tolerantes (T). Durante la conservación en frío los contenidos totales y de α-tocoferol aumentaron en ambas condiciones, mientras que los contenidos de γ-tocoferol se mantuvieron constantes en los frutos NT y disminuyeron en los T. El hecho de que el incremento en los contenidos totales y de α-tocoferol fue similar en frutos sensibles y tolerantes indicaría que es una respuesta al frío independiente del desarrollo de DF. A pesar de los cambios durante la conservación, las diferencias entre condiciones se mantuvieron y los contenidos totales y de γ-tocoferol fueron superiores en los frutos sensibles. Estos resultados indican que la tolerancia adquirida mediante el tapado de frutos de pomelo parece ser independiente de los niveles de tocoferoles en el momento de la cosecha o durante su conservación en frío. Los resultados en pomelo no coinciden con los observados en frutos de mandarina, en donde se encontró una relación inversa entre los niveles de tocoferoles en el flavedo y el desarrollo de DF (Capítulo 2). Estas diferencias podrían deberse a diferentes funciones de los tocoferoles en la respuesta al DF entre especies, o a que los tocoferoles están relacionados con la tolerancia natural entre variedades, pero no a la inducida ambientalmente por la ausencia de luz. Es importante recordar que la ausencia de luz durante la maduración induce la acumulación de licopeno en el flavedo, que se detecta en trazas en los frutos NT (Lado, Cronje, Alquézar, Page, et al., 2015; Lado, Rodrigo, Cronje, et al., 2015).

Por otro lado, el análisis de la expresión de los principales genes de la biosíntesis de tocoferoles permitió identificar puntos claves en la regulación de su acumulación en el flavedo de pomelo, como también estudiar los cambios durante la maduración y el efecto de la luz y el frío sobre la transcripción de estos genes. El incremento detectado en ambas condiciones

durante la maduración estuvo acompañado de una inducción del gen *TAT1,* involucrado en la síntesis de HGA, y aquellos genes específicos de la síntesis de tocoferoles (Figura 3A y 4, Capítulo 3). Por el contrario, los genes involucrados en la síntesis del precursor PPP a través del reciclaje de fitol o de la ruta MEP, a excepción de *GGDR*, disminuyeron su expresión durante la maduración (Figura 3B y C, Capítulo 3). No obstante, la inducción en *TAT1* y la represión de los genes que regulan el PPP fue similar en ambas condiciones, sugiriendo que es una respuesta generalizada durante la maduración, independiente de la exposición a la luz. Es importante destacar que la expresión de los genes *VTE5*, *VTE6*, *DXS1* y *GGPPS1* fue superior en los frutos T en la cosecha, sugiriendo que la luz tiene un efecto negativo sobre su expresión. Este resultado fue inesperado, ya que la luz suele tener un efecto positivo sobre la transcripción de los genes de la ruta MEP (Rodríguez-Concepción & Boronat, 2015). Sin embargo, esta mayor expresión en los frutos T no se tradujo en mayores contenidos de tocoferoles, sugiriendo que su inducción puede estar relacionada con el metabolismo de otros isoprenoides (Rodríguez-Concepción & Boronat, 2015) o que está sujeta a mecanismos de regulación postranscripcional (Hemmerlin, 2013). Por el contrario, la ausencia de luz tuvo un efecto negativo y marcado sobre la expresión de G*GDR* y *VTE1,* y en menor medida *VTE2*, *VTE3a* y *VTE4*, que podrían explicar la disminución en los contenidos detectada en los frutos T (Figura 3C y 4, Capítulo 3). Mientras que el gen *GGDR* controla la reducción final de GGPP en PPP (Tanaka, Oster, Kruse, Rüdiger, & Grimm, 1999), el gen *VTE1* codifica a la enzima tocoferol ciclasa responsable de la conversión de DMPBQ en γ-tocoferol (Porfirova et al., 2002). De esta forma, la represión de *GGDR* disminuiría la disponibilidad del precursor PPP y el flujo hacia la ruta específica de tocoferoles, mientras que la reducción marcada en la expresión de *VTE1* significaría una menor formación de γ-tocoferol, que posteriormente sería convertido en su mayoría a α-tocoferol. Estos resultados son similares a los detectados por Gramegna et al. (2019) en frutos de tomate cultivados en oscuridad, donde la reducción de tocoferoles en ausencia de luz se asoció a una represión en la expresión de *GGDR* y *VTE2*, *VTE1* y *VTE4*. Por tanto, los resultados alcanzados parecerían indicar que, por un lado, el incremento en los contenidos durante la maduración en frutos NT y T está asociado a la inducción de *TAT1* y los genes específicos de la ruta de tocoferoles, mientras que la reducción en los contenidos en los frutos T parece deberse a la represión de los genes *GGDR* y *VTE1*, y en menor medida *VTE2*, *VTE3a* y *VTE4*.

El efecto del frío sobre la expresión de los genes involucrados en la síntesis de los precursores HGA y PPP fue similar para frutos NT y T, sugiriendo que los cambios fueron una respuesta al frío independiente del desarrollo de DF. A excepción del gen *GGDR*, la mayoría

de estos genes se indujeron por el frío y, en particular, el gen *HPPD* presentó una inducción marcada por el frío tan solo a 1 semana de conservación a 2 °C. Inducciones similares en la expresión de este gen se han detectado en respuesta a otros estreses abióticos, sugirieron un papel de este gen en la respuesta de las plantas al estrés (J. Ma et al., 2020). La sobreexpresión de este gen se ha asociado a incrementos en los contenidos de tocoferoles (Tsegaye et al., 2002), por lo que podría explicar el aumento en tocoferoles detectado en ambas condiciones durante la conservación. La inducción en los otros genes involucrados en la síntesis de precursores fue más variable, con algunos genes incrementando su expresión gradualmente (ej. *VTE5* y *GGPPS6*) y otros solo al final de la conservación (ej. *TAT1* y *VTE6*). La expresión del gen *GGDR* se mantuvo relativamente constante en ambas condiciones, por lo que las diferencias entre frutos NT y T fueron principalmente en la expresión de este gen y se mantuvieron durante toda la conservación. A diferencia de los genes involucrados en la síntesis de precursores, la expresión de los genes específicos de la ruta tendió a disminuir durante la conservación, aunque con diferencias entre frutos NT y T. En los frutos NT la mayoría de los genes presentaron una represión en las primeras semanas de conservación con una inducción hacia el final del periodo, mientras que en los frutos T los genes *VTE3a* y *VTE3b* disminuyeron su expresión y *VTE2*, *VTE1* y *VTE4* se mantuvieron relativamente constante. No obstante, en el gen *VTE4* se detectó una inducción al final de la conservación, lo que podría explicar, junto a la expresión constante de *VTE1*, la disminución en los niveles de γ-tocoferol detectados durante el almacenamiento.

Con el objetivo de integrar los resultados de mandarina (Capítulo 2) y pomelo (Capítulo 3) e identificar procesos comunes o diferenciales entre los mismos en respuesta a las bajas temperaturas, en la Figura 7 se resumen los cambios en los contenidos y expresión de los genes analizados con respecto al inicio de la conservación (cosecha). En esta figura se puede observar que el efecto del frío sobre la acumulación de tocoferoles fue distinto en el flavedo de pomelo y el de mandarina, indicando una respuesta diferencial entre especies. Poniendo atención sobre el efecto del frío en los niveles de expresión de los genes analizados se puede observar que, en general, los genes involucrados en la síntesis de precursores tendieron a inducirse con el frío mientras que aquellos específicos de la ruta de tocoferoles tendieron a reprimirse. La inducción más clara en respuesta al frío en las cuatro variedades fue en el gen *HPPD*, de acuerdo a lo descrito previamente en otras especies vegetales en respuesta a distintos tipos de estreses abióticos (Ma et al., 2020). Es importante destacar que el gen *HPPD* está involucrado en la síntesis de HGA que es el precursor común para todos los tococromanoles. Por tanto, la inducción marcada de este gen sin un efecto en los contenidos en los frutos de mandarina,

podría estar relacionado con la síntesis de otros tococromanoles, como plastocromanol, que también parece tener un papel en la defensa frente al frío (Liu & Lu, 2016). Los genes *VTE6*, *VTE5* y *DXS2* también tendieron a inducirse en las cuatro variedades, aunque no tan claramente como *HPPD*. Otros genes también parecieron tener un patrón dependiente de la especie, como por ejemplo el gen *TAT1*, que se reprimió durante las primeras semanas de conservación en frío en los frutos de mandarina, mientras que se indujo en pomelo. A su vez, la represión por el frío de los genes específicos de la ruta de tocoferoles fue más marcada en los frutos de Nova y Nadorcott que en Fortune, y en los frutos NT de pomelo que en los T. Por último, la expresión de los genes *DXS1* y *GGPPS6* tiende a inducirse en los frutos muy sensibles al DF (Fortune, Nova y Star Ruby NT), coincidiendo con la aparición de síntomas, y no en los frutos tolerantes (Nadorcott y Star Ruby T). Esto sugiere que la modulación en la expresión de estos genes en frutos sensibles sea una respuesta al DF, y que podría deberse a la mayor demanda de antioxidantes como carotenoides y tocoferoles, o para incrementar la síntesis de otros compuestos isoprenoides involucrados en la respuesta de las plantas a condiciones de estrés como el ABA, giberelinas o plastocromanoles.

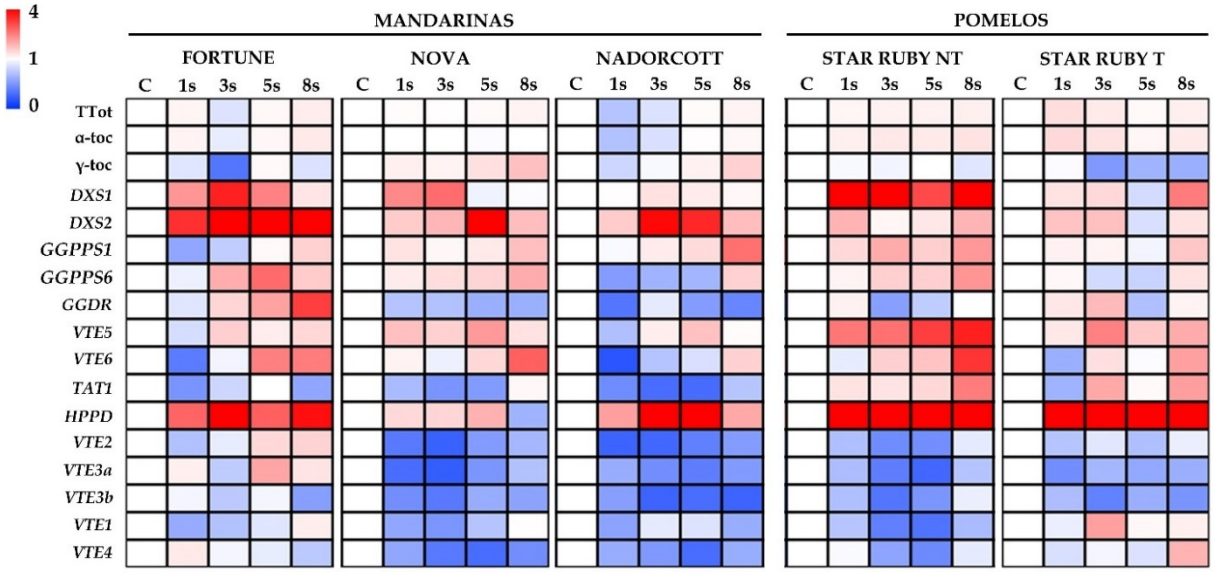

Figura 7. Representación de los cambios en el contenido de tocoferoles y en la expresión de genes en frutos de mandarina y de pomelo durante la conservación en frío respecto a la cosecha (C). Para cada variedad se utilizó el momento de cosecha como referencia (valor 1). Las tonalidades rojas indican un incremento durante la conservación, mientras que las tonalidades azules indican una disminución. Abreviaturas: frutos de Star Ruby no tapados (NT), frutos de Star Ruby tapados (T), tocoferoles totales (TTot), α-tocoferol (α-toc) y γ-tocoferol (γ-toc), y los genes involucrados en la síntesis del precursor PPP a través de la ruta MEP (*DXS1*, *DXS2*, *GGPPS1 GGPPS6* y *GGDR*) o del reciclaje de fitol (*VTE5* y *VTE6*), en la síntesis del precursor HGA (*TAT1* y *HPPD*), y de los genes específicos de la síntesis de tocoferoles (*VTE2*, *VTE3a*, *VTE3b*, *VTE1* y *VTE4*).

4.3. Biosíntesis y acumulación de tocoferoles durante la maduración de frutos cítricos

Debido a que el metabolismo de tocoferoles en el género *Citrus* ha sido muy poco estudiado, se planteó, como último objetivo de esta Tesis (Capítulo 4), realizar una caracterización bioquímica y molecular de la acumulación de tocoferoles en la piel y la pulpa durante la maduración de frutos de las principales especies cultivadas de cítricos. Para ello, y aprovechando la variabilidad genética y fenotípica del género *Citrus*, se seleccionaron 4 especies pertenecientes a los principales grupos (pomelo, limón, naranja y mandarina) y se analizaron los contenidos de tocoferoles y la expresión de los principales genes biosintéticos en el flavedo y la pulpa en cuatro estados de maduración que abarcaron desde el fruto verde inmaduro hasta la madurez comercial. El análisis de tocoferoles reveló que, al igual que lo expuesto en el Capítulo 2 y 3, y descrito previamente en estas especies (Assefa et al., 2017; Chun et al., 2006), los tocoferoles se acumulan principalmente como α- y γ-tocoferol, siendo el α-tocoferol la forma predominante en el flavedo y la pulpa durante toda la maduración (Figura 2 y 3, Capítulo 4). El conjunto de estos resultados sugiere que la rama δ-/β-tocoferol no tiene una contribución importante al total de tocoferoles en los frutos cítricos.

A su vez, los tocoferoles fueron detectados en todas las especies y estados de maduración analizados, y los contenidos variaron entre los tejidos, los estados de maduración y las especies. Al igual que sucede con otros compuestos, como los carotenoides (Alquézar et al., 2013), la vitamina C (Alós et al., 2014) o los flavonoides (Assefa et al., 2017), la concentración de tocoferoles fue mayor en el flavedo que en la pulpa en todas las especies y estados de maduración. Se ha sugerido que estas diferencias podrían estar relacionadas con la mayor exposición del flavedo a condiciones de estrés abiótico, como altas temperaturas o intensidad de luz, que pueden provocar una mayor demanda por antioxidantes para contrarrestar estas condiciones. En este sentido, los tocoferoles tienen un papel en la foto-protección (J. Ma et al., 2020), e incrementos de estos compuestos se han detectado en respuesta a condiciones de estrés por alta intensidad de luz (Collakova & DellaPenna, 2003b; Havaux et al., 2005). A su vez, la luz puede afectar la acumulación de tocoferoles debido a su efecto directo sobre la transcripción de los genes biosintéticos. En frutos de tomate se ha reportado un efecto positivo de la luz sobre la expresión de ciertos genes de la ruta de biosíntesis, lo que también concuerda con los resultados mostrados en el Capítulo 3, donde en frutos de pomelo desarrollados en ausencia de luz disminuyeron los contenidos y la expresión del gen *GGDR* y otros genes específicos de la ruta de tocoferoles. Por otro lado, otro factor que puede influir sobre las diferencias detectadas entre los tejidos es el contenido de clorofilas, ya que una fuente

importante del precursor PPP, sobre todo en tejidos inmaduros, es a partir del reciclaje de fitol liberado durante la degradación de clorofilas (Almeida et al., 2016; Valentin et al., 2006; vom Dorp et al., 2015). Así, la mayor concentración de clorofilas en el flavedo que en la pulpa (Alquézar et al., 2008b; Lado, Alós, et al., 2019) y la degradación de las mismas durante el cambio de color, puede suponer una mayor disponibilidad de PPP para la entrada a la ruta específica de tocoferoles. En este sentido, la acumulación de tocoferoles ha sido asociada a la degradación de clorofilas en distintos tipos de frutos como pimiento (Osuna-García et al., 1998) y oliva (Georgiadou et al., 2016), y también se ha descrito una reducción en los contenidos de tocoferoles en frutos de tomate que carecen clorofilas (Almeida et al., 2015). Es interesante destacar que las diferencias entre los tejidos no fueron solo cuantitativas sino también en el patrón de acumulación durante la maduración. Así, mientras que los contenidos aumentaron gradualmente en el flavedo de las cuatro especies, en la pulpa disminuyeron después del estado verde-inmaduro en pomelo, naranja y mandarina, y se mantuvieron constantes en limón. Estos resultados sugieren que la síntesis de tocoferoles parece estar regulada por distintos mecanismos en cada tejido, lo que no sería extraño ya que el flavedo y la pulpa son tejidos anatómica y morfológicamente diferentes, en los que diferentes procesos fisiológicos que ocurren durante la maduración se regulan independientemente (Tadeo et al., 2020).

El análisis transcripcional de los genes implicados en la biosíntesis de tocoferoles también reforzó la hipótesis anterior, ya que los mayores contenidos en el flavedo se asociaron a una mayor expresión de los genes analizados, y sugieren que genes distintos limitan la acumulación de tocoferoles en cada tejido (Figura 4-8, Capítulo 4). En el flavedo, el aumento en los contenidos estuvo acompañado de una inducción de los genes *TAT1* y *VTE4*, cuya expresión se correlacionó positivamente con los contenidos de tocoferoles (Figura 4, 5 y 8B, Capítulo 4). El gen *TAT1* regula la disponibilidad del precursor HGA, y una inducción en su expresión ha sido observada durante la senescencia de hojas, asociado a un incremento en los contenidos de tocoferoles (Holländer-Czytko, Grabowski, Sandorf, Weckermann, & Weiler, 2005). Por otro lado, el gen *VTE4* regula la conversión de γ- a α-tocoferol, y cambios en su expresión han sido asociados a un aumento de α-tocoferol, pero en detrimento de γ-tocoferol y sin afectar los contenidos totales (Bergmüller et al., 2003; Maeda et al., 2006). Por tanto, en el flavedo de los frutos cítricos la inducción simultanea de *TAT1* y *VTE4* proporcionaría una mayor disponibilidad de HGA y entrada a la ruta, que luego sería convertido mayormente en α-tocoferol. Sin embargo, es importante recordar que varios pasos de la ruta de tocoferoles también están involucrados en la síntesis de otros compuestos y, por tanto, es posible que muchos substratos se canalizasen hacia la síntesis de otros metabolitos (Burgos et al., 2021;

Kruk, Szymańska, Cela, & Munne-Bosch, 2014). Otros genes también se indujeron durante la maduración y pueden estar implicados en el aumento de tocoferoles detectados en cada especie, como *HPPD* en pomelo, naranja y mandarina, y *GGPPS11* en limón (Figura 4, Capítulo 4). Por el contrario, ciertos genes estuvieron claramente reprimidos durante la maduración, como *DXS2*, *VTE6* y *GGPPS6*, pero no parecen limitar el suministro de PPP hacia la ruta de tocoferoles.

A diferencia de los resultados en el flavedo, los contenidos en la pulpa se correlacionaron positivamente con la expresión de los genes *VTE6*, *DXS2* y *GGDR* (Figura 8C y D, Capítulo 4), todos involucrados en la biosíntesis del precursor PPP. Por tanto, la disponibilidad de PPP en la pulpa podría estar determinando la disminución en tocoferoles en la pulpa durante la maduración. La expresión de ciertos genes, como *TAT1*, *HPPD* y *GGPPS1,* aumento durante la maduración en las cuatro especies, pero sin un efecto aparente sobre los contenidos de tocoferoles. Es interesante destacar que durante la maduración genes específicos mostraron un patrón de expresión similar en el flavedo y la pulpa. Por ejemplo, el gen *TAT1* se indujo marcadamente en las cuatro especies en ambos tejidos, contrariamente a la evolución de los tocoferoles en la pulpa. De manera similar, el gen *HPPD* también se indujo en ambos tejidos en pomelo, naranja y mandarina, de forma opuesta a los contenidos en la pulpa. Esto sugiere que ambos genes, involucrados en la síntesis de HGA, están modulados durante el desarrollo. Inducciones en estos genes durante la maduración se han detectado en frutos de tomate y oliva (Georgiadou et al., 2015; Quadrana et al., 2013) aunque, al igual que en la pulpa de *Citrus*, sin asociarse a aumentos en los contenidos de tocoferoles. Esto podría indicar que su inducción está relacionada con la síntesis de otros compuestos, como por ejemplo los plastocromanoles (Burgos et al., 2021; Kruk et al., 2014). A su vez, ciertos genes parecieron compartir un patrón de expresión en ambos tejidos, y estuvieron correlacionados entre sí, como *VTE6* con *DXS2* y *GGDR*, involucrados en la síntesis de PPP, como *VTE4* con *VTE1*, regulando los últimos pasos de la síntesis, o *VTE4* con *TAT1* y *VTE1* con *HPPD*.

Por otro lado, los resultados del Capítulo 4 corroboraron los cambios durante la maduración observados en el Capítulo 3 en el flavedo de pomelo, y reveló ciertos patrones que parecen cumplirse entre las diferentes especies cítricas. Por ejemplo, el gen *TAT1* se indujo marcadamente en el flavedo de todas las especies analizadas. En cambio, los genes involucrados en la síntesis de PPP, tanto a través de la ruta MEP como el reciclaje de fitol parecieron reprimirse durante la maduración, y esto fue claro para los genes *DXS2* y *VTE6*. La reducción en estos genes podría estar asociada a la degradación de clorofilas que ocurre

naturalmente durante el cambio de color en los frutos cítricos (Rodrigo, Alquézar, Alós, Lado, et al., 2013). En los genes específicos de la ruta de tocoferoles se detectó una respuesta diferencial entre especies que tendieron a inducirse en el flavedo de pomelo y limón, y reprimirse en naranja y mandarina (a excepción de *VTE4* que se indujo). El hecho de que la expresión de estos genes no se estimule en el flavedo de las especies que acumulan más carotenoides podría ser indicativo de la interconexión que existe entre las rutas de ambos isoprenoides en los tejidos vegetales (Almeida et al., 2015; Estévez et al., 2001; Rodríguez-Concepción & Boronat, 2015), indicando una predominancia de la ruta de carotenoides en el flavedo de naranjas y mandarinas.

1. La tolerancia de los frutos de mandarina a desarrollar daños por frío (DF) durante la conservación a 2 °C fue en el siguiente orden: Nadorcott > Nova > Fortune. Se ha encontrado una relación positiva entre el contenido de carotenoides totales en el flavedo al inicio de la conservación (cosecha), en particular de las β,β-xantofilas β-criptoxantina y violaxantina, con la tolerancia a los DF. Asimismo, estos parámetros se correlacionaron positivamente con la capacidad antioxidante SOAC, lo que sugiere un posible papel protector de los carotenoides como eficientes secuestradores de oxígeno singlete en la protección frente al DF.

2. La susceptibilidad de los frutos de mandarina al DF se correlacionó positivamente con los contenidos de vitamina C en el flavedo en el momento de la cosecha y durante la conservación refrigerada, indicando que no parece estar implicada en la tolerancia natural al DF en frutos de mandarina.

3. La capacidad antioxidante evaluada mediante los métodos DPPH• y FRAP, reflejó la contribución de la vitamina C al sistema antioxidante, pero no así la de los carotenoides. Además, se encontró una relación positiva entre la susceptibilidad al DF y la capacidad antioxidante DPPH• y FRAP, lo que sugiere que la capacidad antioxidante total no parece estar implicada en la tolerancia natural al DF.

4. Los tocoferoles en los frutos cítricos se acumulan principalmente como α- y γ-tocoferol, siendo el primero la forma mayoritaria. La rama δ/β de la ruta no parece tener una contribución importante en la síntesis de tocoferoles en cítricos.

5. La tolerancia natural a los DF en los frutos de mandarina estudiados (Nadorcott, Nova y Fortune) también se correlacionó positivamente con el contenido de tocoferoles en el flavedo en el momento de la cosecha. Sin embargo, en los frutos de pomelo Star Ruby no se observó una relación directa entre estos antioxidantes y la tolerancia a los DF inducida por el tapado de los frutos (ausencia de luz).

6. Las diferencias en los contenidos de tocoferoles en el flavedo de los frutos de las distintas variedades de mandarina se asociaron a una mayor expresión de los genes *DXS1*, *DXS2* y *GGDR*, que regulan la disponibilidad del precursor PPP, y *VTE3b*, que redirige la ruta hacia la síntesis de γ/α-tocoferol.

7. La ausencia de luz durante las últimas etapas del desarrollo y maduración en frutos de pomelo Star Ruby redujo la acumulación de γ-tocoferol, y esta reducción estuvo asociada a una disminución en la expresión de los genes *GGDR* y *VTE1* y, en menor medida *VTE2*, *VTE3a* y *VTE4*.

8. La conservación a bajas temperaturas estimuló la expresión de la mayoría de genes involucrados en la síntesis de los precursores PPP y HGA, mientras que los genes específicos de la ruta de tocoferoles tendieron a reprimirse. Estos cambios transcripcionales fueron similares en los frutos de mandarina y pomelo tolerantes y sensibles a los DF, lo que indica que son una respuesta a las bajas temperaturas no relacionadas con el desarrollo del DF.

9. El análisis del contenido de tocoferoles en flavedo y la pulpa durante la maduración de los frutos de las cuatro principales especies de cítricos cultivadas (pomelo, limón, naranja

y mandarina) mostró que la concentración de tocoferoles fue superior en el flavedo que en la pulpa. En frutos maduros, los niveles de tocoferoles fueron superiores en el flavedo y pulpa de los frutos de limón en comparación con el resto de especies.

10. El patrón de acumulación de tocoferoles durante la maduración de los frutos de las cuatro especies de cítricos estudiadas fue diferente en el flavedo y la pulpa, lo que indica que existen diferentes mecanismos de regulación en la acumulación de tocoferoles en ambos tejidos. Así, mientras que en el flavedo los contenidos aumentan durante la maduración acompañados principalmente de la inducción de los genes *TAT1* y *VTE4*, que regulan la disponibilidad de HGA y conversión de γ- a α-tocoferol, respectivamente; los contenidos en la pulpa disminuyen o se mantienen constantes, y reflejan los cambios en la expresión de los genes *VTE6*, *DXS2* y *GGDR,* que regulan la disponibilidad de PPP.

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7.1. Anexo 1: publicación correspondiente al capítulo 1

antioxidants

MDPI

Article

Carotenoids, Vitamin C, and Antioxidant Capacity in the Peel of Mandarin Fruit in Relation to the Susceptibility to Chilling Injury during Postharvest Cold Storage

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Abstract: Chilling injury (CI) is a postharvest disorder occurring in the fruit of cold-sensitive *Citrus* species during storage at low temperatures. This study investigated the involvement of carotenoids and vitamin C, two major antioxidants of citrus peel, and the antioxidant capacity in the CI susceptibility of mandarin fruit. To that end, the fruit of three commercial varieties, Fortune, Nova, and Nadorcott, with significant differences in CI susceptibility, were selected. By on-tree fruit bagging, carotenoids and vitamin C contents were modified, and a differential effect of each cultivar on CI was observed. Carotenoid analysis in the peel revealed a strong negative correlation between total carotenoid concentration (TCC) at harvest, and specifically of β-cryptoxanthin and violaxanthin, and CI index at the end of storage. In contrast, vitamin C content was significantly and positively correlated with CI susceptibility. The antioxidant activity assessed by the DPPH• and FRAP reflected the contribution of vitamin C to the antioxidant system, while the SOAC assay correlated positively with TTC, β-cryptoxanthin, and violaxanthin. Collectively, the antioxidant capacity of carotenoids at harvest, as efficient singlet oxygen quenchers, suggests a protective role against the development of CI in mandarin fruit, while vitamin C is not likely playing a critical role.

Keywords: antioxidant capacity; chilling injury; citrus fruit; carotenoids; mandarin; vitamin C

1. Introduction

Storage at low temperatures is the technology most widely used to maintain fruit quality and extend postharvest life. However, prolonged storage at low temperature may cause physiological disorders in many species of tropical and subtropical origin, such as citrus fruit, that are prone to develop external injuries during cold storage, depreciating their quality and increasing postharvest losses. Furthermore, some countries enforce quarantine measures on citrus fruit imports, requiring storage during transport at 1–2 °C. Damage developed during storage at low non-freezing temperatures is commonly known as chilling injury (CI) and, even though macroscopic symptoms in *Citrus* fruit vary between species and varieties, they are generally manifested as brown pit-like depressions in the flavedo (outer layer of fruit peel) that expand progressively over the peel surface and become darker under prolonged exposure to low temperatures [1–3].

Many pre- and postharvest factors have been described as influencing the susceptibility of citrus fruit to CI during postharvest storage. The genotype of the species/variety is an intrinsic genetic factor determining CI sensitivity or tolerance. Among *Citrus* species, lemon and grapefruit are more sensitive to CI than orange and mandarin, which are generally more tolerant [3]. Nonetheless, within mandarins and their hybrids, there is great variability in susceptibility to CI, with cultivars ranging from

7.2. Anexo 2: publicación correspondiente al capítulo 2

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Accumulation of tocopherols and transcriptional regulation of their biosynthesis during cold storage of mandarin fruit

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ABSTRACT

Tocopherols are plant-derived isoprenoids with potent antioxidant activity, which have been implicated in the tolerance of plants to different stresses. However, tocopherol accumulation and biosynthesis in fruit, and their potential implication in postharvest chilling injury (CI), has been scarcely studied. Therefore, in this work, we have investigated tocopherol accumulation and biosynthesis in the peel of mandarin fruit of three cultivars with contrasting susceptibility to CI during storage at 2 °C ('Fortune'>'Nova'> 'Nadorcott'). α- and γ-tocopherol were the isoforms detected in the flavedo of the fruit, and a direct relationship between tocopherols content and CI-tolerance was found, since CI-tolerant fruit accumulated the highest tocopherol content whereas CI-sensitive fruit the lowest. Moreover, the transcriptional profiling of 14 genes related to the specific steps of tocopherol biosynthesis, and to their precursor's synthesis, were analyzed. Upstream genes *DXS1* and *DXS2* (1-deoxy-D-xylulose-5-phosphate synthase) and *GGDR* (geranylgeranyl diphosphate reductase), involved in the supply of phytyl pyrophosphate, and the *VTE3* (2-methyl-6-phytyl-1,4-benzoquinol methyltransferase) isoforms appear to be key for the differences in total tocopherol content among the cultivars at harvest. During cold storage, most genes involved in the precursors supply were up-regulated, whereas genes of the tocopherol-core pathway were in general repressed. Changes in *VTE4* during cold storage may account for the differences in γ-tocopherol among cultivars. Collectively, results suggest that the concentration of tocopherols at harvest may play a function in the natural tolerance of mandarin fruit to CI, and that changes in the expression of genes during storage appear to be cold-regulated responses, rather than involved in CI tolerance.

1. Introduction

A recurring problem during the commercialization of citrus fruit is the appearance of peel damage due to the exposure to low temperatures during postharvest transport and storage, which depreciates their commercial value. These damages and blemishes caused by low non-freezing temperatures are referred to as chilling injury (CI), which is usually manifested as small depressions in the flavedo that expand, sink and darken with the continuous exposure to cold (Lado et al., 2019a; Zacarias et al., 2020). Although mandarin fruit is considered more resistant to CI than fruit of other *Citrus* species, contrasting predisposition to CI can be observed among different cultivars (Sala, 1998; Rey et al., 2020).

Exposure to cold temperatures has a direct impact on the integrity of cell membranes (Lyons, 1973), and accelerates changes in membranes composition and conformation which negatively affects their functionality (Sevillano et al., 2009; Lafuente et al., 2017). Furthermore, the stability and functionality of membranes is affected by oxidative processes caused by a cold-induced overproduction and accumulation of ROS (Suzuki et al., 2012). Experimental evidence indicates that CI in *Citrus* fruit is associated with a boost in oxidative stress processes, exemplified by an enhancement of the expression of genes and enzymatic activities of the antioxidant system, and a higher singlet oxygen quenching capacity (Lado et al., 2016; Lafuente et al., 2017).

Accumulation of bioactive compounds with antioxidant properties, might play an important role in the tolerance of fruit to CI. The peel of citrus fruit contains compounds such as carotenoids, vitamin C and polyphenols which are part of the non-enzymatic defense mechanism against oxidative stress in plant tissues (Zou et al., 2016). Carotenoids are potent lipid-soluble antioxidants and have been associated with the tolerance of grapefruit (Lado et al., 2015, 2016) and mandarin to CI (Rey et al., 2020). On the other hand, changes in the concentration of vitamin

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7.3. Anexo 3: publicación correspondiente al capítulo 3

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Effect of fruit shading and cold storage on tocopherol biosynthesis and its involvement in the susceptibility of Star Ruby grapefruit to chilling injury

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ARTICLE INFO

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Fruit shading
Postharvest storage
Citrus

ABSTRACT

The aim of this study was to investigate the role of tocopherols in the susceptibility of Star Ruby grapefruit to postharvest chilling injury (CI). Fruit exposed to normal sunlight (NC, non-covered) and deprived of light (C, covered) in the last stages of development were used. Tocopherol contents increased in the flavedo of both NC and C fruit during development, concomitantly with the up-regulation of *TAT1* and most genes of the tocopherol-core pathway. Fruit shading reduced total contents by repressing γ-tocopherol accumulation, associated to a down-regulation of *GGDR* and *VTE1* and, to a lesser extent, of *VTE2*, *VTE3a* and *VTE4*. During cold storage, total and α-tocopherol contents increased in NC and C fruit, and no direct relationship between tocopherol accumulation and CI tolerance was found. Cold stress up-regulated most genes involved in the synthesis of tocopherol precursors and down-regulated those of the tocopherol-core pathway, but changes seemed to be cold-mediated and not related to CI development.

1. Introduction

Chilling injury (CI) is an economically important postharvest disorder that reduces external fruit quality, marketability and consumer acceptance, culminating in important economic losses. Because of their subtropical origin, fruit of many species and cultivars of *Citrus* are prone to develop peel injuries during storage at low temperatures (Lado, Cronje, Rodrigo, & Zacarías, 2019; Zacarias, Cronje, & Palou, 2020). Fruit of grapefruit cultivars are highly susceptible to CI when stored at temperatures below 10 °C (Lado, Rodrigo, Cronje, & Zacarías, 2015; Schirra, 1993). CI in the peel of grapefruit is manifested as a series of symptoms, referred to as peel pitting, which initiates as small brown pit-like depressions that expand forming large necrotic and depressed areas after prolonged cold storage (Lado et al., 2019; Lado, Rodrigo et al., 2015). Susceptibility to CI is influenced by many factors, including the *Citrus* genotype and environmental and agronomical conditions, such as the harvest season, maturity, growing conditions, rootstock, fruit position on the tree canopy and peel pigmentation, among others, that markedly define the initiation and development of CI during postharvest cold storage (Lado et al., 2019; Zacarias et al., 2020).

Oxidative stress is a primary mechanism in the response of *Citrus* fruit against stress caused by low temperature, and/or by the damage induced under these conditions (Toivonen, 2004). The capability of certain species and cultivars to counteract these processes appears to be essential to determine fruit tolerance or susceptibility to CI under cold stress conditions (Lado et al., 2019; Zacarias et al., 2020). Antioxidant defense mechanisms in plants include detoxifying enzymes and low molecular weight compounds such as ascorbic acid, glutathione, carotenoids and tocopherols (Decros, Baldet, Beauvoit, Stevens, Flandin, Colombié, & Pétriacq, 2019; Toivonen, 2004). Enhanced activity of enzymes of the antioxidant system has been associated with the natural tolerance to CI of several mandarin cultivars (Sala, 1998) and also with the tolerance induced by heat-conditioning treatments (Lafuente, Establés-Ortíz, & González-Candelas, 2017). Among them, a protective role of catalase against chilling has been proposed, as higher catalase activity and increased catalase transcripts have been detected in the peel of tolerant fruit of mandarin and grapefruit (Lado, Rodrigo, López-Climent, Gómez-Cadenas, & Zacarías, 2016; Maul, McCollum, Guy, & Porat, 2011; Sala, 1998). Ascorbic acid (vitamin C) and glutathione are potent water-soluble antioxidants (Decros et al., 2019) but their relation to CI in *Citrus* fruit is controversial, as contents in grapefruit and mandarin fruit did not support a direct relationship with CI tolerance (Lado et al., 2016; Rey, Zacarías, & Rodrigo, 2020). Carotenoids, on the other hand, are considered to be the first line of defense against singlet oxygen

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7.4. Anexo 4: publicación correspondiente al capítulo 4

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ORIGINAL RESEARCH
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Regulation of Tocopherol Biosynthesis During Fruit Maturation of Different *Citrus* Species

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Tocopherols are plant-derived isoprenoids with vitamin E activity, which are involved in diverse physiological processes in plants. Although their biosynthesis has been extensively investigated in model plants, their synthesis in important fruit crops as *Citrus* has scarcely been studied. Therefore, the aim of this work was to initiate a physiological and molecular characterization of tocopherol synthesis and accumulation in *Citrus* fruits during maturation. For that purpose, we selected fruit of the four main commercial species: grapefruit (*Citrus paradisi*), lemon (*Citrus limon*), sweet orange (*Citrus sinensis*), and mandarin (*Citrus clementina*), and analyzed tocopherol content and the expression profile of 14 genes involved in tocopherol synthesis during fruit maturation in both the flavedo and pulp. The selected genes covered the pathways supplying the tocopherol precursors homogentisate (HGA) (*TAT1* and *HPPD*) and phytyl pyrophosphate (PPP) (*VTE5*, *VTE6*, *DXS1* and *2*, *GGPPS1* and *6*, and *GGDR*) and the tocopherol-core pathway (*VTE2*, *VTE3a*, *VTE3b*, *VTE1*, and *VTE4*). Tocopherols accumulated mainly as α- and γ-tocopherol, and α-tocopherol was the predominant form in both tissues. Moreover, differences were detected between tissues, among maturation stages and genotypes. Contents were higher in the flavedo than in the pulp during maturation, and while they increased in the flavedo they decreased or were maintained in the pulp. Among genotypes, mature fruit of lemon accumulated the highest tocopherol content in both the flavedo and the pulp, whereas mandarin fruit accumulated the lowest concentrations, and grapefruit and orange had intermediate levels. Higher concentrations in the flavedo were associated with a higher expression of all the genes evaluated, and different genes are suitable candidates to explain the temporal changes in each tissue: (1) in the flavedo, the increase in tocopherols was concomitant with the up-regulation of *TAT1* and *VTE4*, involved in the supply of HGA and the shift of γ- into α-tocopherol, respectively; and (2) in the pulp, changes paralleled the expression of *VTE6*, *DXS2*, and *GGDR*, which regulate PPP availability. Also, certain genes (i.e., *VTE6*, *DXS2*, and *GGDR*) were co-regulated and shared a similar pattern during maturation in both tissues, suggesting they are developmentally modulated.

Keywords: tocopherol, vitamin E, *Citrus*, fruit, ripening, tocopherol gene expression

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