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ARTICLE

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Boosting carotenoid content in *Malus domestica* var. Fuji by expressing *AtDXR* through an *Agrobacterium*-mediated transformation method

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Abstract

Apple (*Malus domestica*) fruits accumulate negligible levels of carotenoids, antioxidant pigments that are precursors for vitamin A in humans. As vitamin A deficiency is an important public health issue, we aimed at increasing carotenoids in apple by constitutively expressing the *Arabidopsis thaliana DXR* gene, one of the key regulatory steps in the plastidial isoprenoid pathway. For this purpose, we optimized an *Agrobacterium*-mediated transformation method in the commercial Fuji Raku Raku variety. This resulted in a shoot establishment efficiency of 0.75% at 20 weeks after infection. Molecular and microscopical analyses revealed that 80% of the hygromycin resistant shoots contained and expressed *AtDXR:eGFP* and that the AtDXR:eGFP fusion protein located in plastids. Transgenic seedlings displayed up to 3-fold increase in total carotenoids and in individual carotenoids compared to the WT, correlating with an increased transcript abundance of endogenous carotenogenic genes such as *MdDXS*, *MdPSY1*, *MdPSY2*, *MdPSY3*, *MdLCYB1*, and *MdLCYB2*. In addition, buds of 2-year-old transgenic dormant trees showed an increment up to 3-fold in lutein, and transient transformation of fruits revealed that *AtDXR* induced a 2-fold increment in total carotenoids. Thus, these results suggest that *DXR* may be a good candidate for increasing carotenoid levels in apple fruits through metabolic engineering.

KEYWORDS

Agrobacterium apple transformation, AtDXR, carotenoids, fuji variety, *Malus domestica*

1 | INTRODUCTION

Apple (*Malus domestica*) is one of the principal fruit tree species in cold and temperate countries. Its worldwide production was 68.6 million tons for the 2018/2019 season (https://apps.fas.usda.gov/psdonline/circulars/fruit.pdf), which was led by China and followed by EU, United States, Chile, and Russia. In 2018, Chile exported 720 ton of fresh weight (FW) apple, mainly of the Royal Gala, Fuji, and Granny Smith varieties (https://www.odepa.gob.cl/), positioning it as the 5th biggest apple exporter worldwide. Apple fruits present high nutritional value reflected in their high antioxidant levels (such as vitamin C) and phenolic compounds (Patel, Kaswala, Chakraborty, & Kamath, 2012). However, commercial apple varieties accumulate very low levels of carotenoid pigments, reflected by a pale flesh, with concentrations of 3–10 $\mu g\, g^{-1}$ FW in the skin and 0.8-9 $\mu g\, g^{-1}$ FW in the flesh (Ampomah-Dwamena et al., 2012; Delgado-Pelayo, Gallardo-Guerrero, & Hornero-Méndez, 2014; Lado, Zacarías, & Rodrigo, 2016). Carotenoids are isoprenoid pigments that play

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crucial roles in plants such as photosynthesis and photooxidative protection and are also responsible for the coloration of different plant tissues in the range of light yellow to deep red colors (Rosas-Saavedra & Stange, 2016). Mammals must include carotenoids in their diet because these pigments are precursors for vitamin A synthesis. Since vitamin A deficiency affects more than 250 million people worldwide, genetically modified crops with higher carotenoid levels have been generated during the last years aiming to alleviate this problem (Al-Babili & Beyer, 2005; Alós, Rodrigo & Zacarias, 2016; Aluru et al., 2008; Burkhardt et al., 1997; Römer et al., 2000; Ye et al., 2000).

Additionally, carotenoids exhibit powerful antioxidant properties, enhance the immune response, and may delay the onset of certain types of cancer and chronic diseases (Fraser & Bramley, 2004; Odorisi & Perez-Gálvez, 2016; Giovannucci, 2002; Mares-Perlman, Millen, Ficek, & Hankinson, 2002).

Carotenoids are synthesized in plastids from isopentenyl pyrophosphate (IPP), which is a common precursor to all isoprenoids produced through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Botella-Pavía et al., 2004; Estévez, Cantero, Reindl, Reichler & León, 2001). 1-deoxy-D-xylulose 5-phosphate synthase (DXS) is a key enzyme that catalyzes the first step of the MEP pathway, and produces 1-deoxy-D-xylulose 5-phosphate (DXP) from pyruvate and D-glyceraldehyde 3-phosphate. Then, DXP is transformed into MEP by 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), another important regulatory step in the pathway (Figure 1).

Both, transient and stable expression of DXS:eGFP and DXR:eGFP proteins colocalized with chloroplast membrane structures in *A. thaliana* (Perello et al., 2016). In particular, AtDXS was found soluble in the stroma, but in a misfolded (i.e. inactive) state, while AtDXR appeared to be highly stable (Perello et al., 2016). It has also been established that AtDXR is more resistant to degradation by proteases than AtDXS and it remains active in the soluble (stromal) fraction even under stress conditions that promote general protein aggregation (Perello et al., 2016).

Overexpression of the *DXR* gene increased the concentration of monoterpene essential oil in *Mentha piperita* (Mahmoud & Croteau, 2001), and also resulted in a higher carotenoid content in transgenic *Arabidopsis* (Carretero-Paulet et al., 2006). Similarly, abietane diterpenes in *Salvia sclarea* (Vaccaro, Malafronte, Alfieri, De Tommasi, & Leone, 2014), and artemisinins in *Artemisia annua* (Xiang et al., 2012) presented higher levels after *DXR* overexpression. In addition, *Arabidopsis Atdxr* mutant exhibits abnormal chloroplast and trichome development, which are phenotypes related to carotenoid deficiency (Xing et al., 2010).

DXR catalyzes the enzymatic reaction yielding MEP, the first committed precursor for plastid isoprenoids (Figure 1). Afterwards, IPP condenses with dimethylallyl pyrophosphate (DMAPP) to produce geranylgeranyl pyrophosphate (GGPP), which is the substrate of phytoene synthase (PSY), the key enzyme for carotenoid synthesis. GGPP is also the precursor of other metabolites, such as chlorophylls (Figure 1). Phytoene is then converted into several colored carotenoids including the reddish lycopene given by the consecutive desaturations carried out by phytoene desaturase (PDS), z-carotene desaturase (ZDS), z-carotene isomerase (Z-ISO) and carotenoid isomerase (CRTISO). Then, lycopene is converted into β-carotene by the lycopene β-cyclase (LCYB) and into α-carotene by LCYB and lycopene ε-cyclase (LCYE). The α-carotene is hydroxylated by the β-carotene hydroxylase (CHYB) and ε-carotene hydroxylase (CHYE), resulting in the production of lutein (Figure 1, Rosas-Saavedra & Stange, 2016).

Given that apple fruits are commercialized worldwide and already exhibit important nutritional properties, generating apple varieties with higher carotenoid content would result in a highly

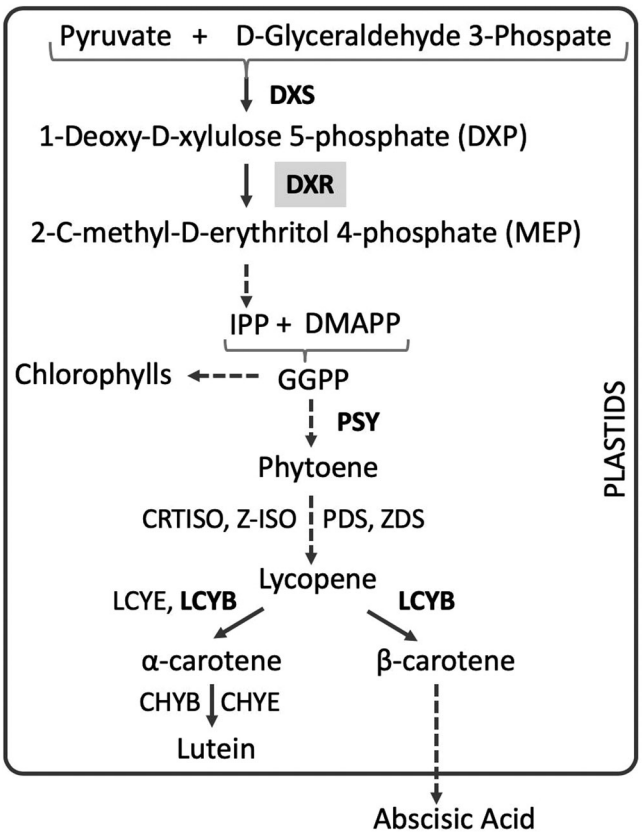

FIGURE 1 Isoprenoid and carotenoid biosynthesis pathway. Isoprenoids produced by the MEP pathway are carotenoid precursors. Pyruvate and D-glyceraldehyde 3-phosphate is taken by DXS to produce DXP which is substrate of DXR for the synthesis of MEP. After several reactions, isopentenyl pyrophosphate (IPP) is generated. IPP and DMAPP are necessary to produce GGPP, a key metabolite for chlorophylls and carotenoids biosynthesis. Enzymes in bold are analyzed in this study. DXR is featured. Continuous arrow represents one direct chemical reaction. Dotted arrow represents several chemical reactions. CHYB, β-carotene hydroxylase; CHYE, ε-carotene hydroxylase; CRTISO, Carotenoid isomerase; DMAPP, dimethylallyl pyrophosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GGPP, geranylgeranyl pyrophosphate; IPP, isopentenyl pyrophosphate; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; MEP, C-methyl-D-erythritol 4-phosphate; PDS, phytoene desaturase; PSY, Phytoene synthase; ZDS, z-carotene desaturase; Z-ISO, z-carotene isomerase

valuable product from both functional and nutritional perspectives. To achieve this, we aimed to increase the carotenoid content in the commercial Fuji variety by expressing *AtDXR:eGFP*, and to achieve this, we set out to an improved *Agrobacterium*-transformation method. We found that the transformation method had an efficiency of 0.75% and that 80% of the shoots generated, expressed the transgene in their leaves. Four transgenic lines showed AtDXR:eGFP plastidial localization and a significant increase in the transcript abundance of several genes related to the isoprenoid and carotenogenic pathways, together with a 3- to 4-fold increase in total and individual carotenoids in leaves of the transgenic plants. As a preliminary approach to assess the potential effect of the expression of *AtDXR* in fruits, we transiently expressed *AtDXR:eGFP* in Fuji apples, which also resulted in a significant increase in total carotenoids. Thus, we conclude that DXR is a suitable candidate for increasing carotenoid levels in apple fruits through metabolic engineering.

2 | MATERIALS AND METHODS

2.1 | Plant material

Malus domestica cv. Fuji Raku Raku was selected for this study because it is one of the four most important commercial varieties in Chile (https://www.odepa.gob.cl/). Sterile buds of Fuji Raku Raku apples were raised from 3-year-old trees growing in the field. Shoots were sprayed with a fungicide solution (Captan 10 mg L^{-1}) 3 days before being cut. Cuttings of 7 cm were sprayed with ethanol and stirred 30 min in a 1% Captan solution and rinsed with sterile distilled water under sterile conditions. Then, the shoots were stirred for 8 min in a commercial chloride solution 50% vol/vol with a drop of Tween 20. Finally, shoots were thoroughly washed with sterile water. The cuttings were then dried and cut into 3 cm long pieces leaving 1 or 2 buds per explant (adapted from Puite & Schaart, 1996) and placed in a shoot proliferation medium (Bolar, 1999) containing 6-benzylaminopurine (BAP) 1 mg L^{-1} and gibberellin (GA_3) 0.2 mg L^{-1} to promote differentiation of the buds. Ascorbic acid 20 mg L^{-1} was also added to prevent oxidation of the medium and of the explants (Table 1). PPM™ (Plant Preservative Mixture, Plant Cell Technology) was added to avoid microbial infections. Explants were cultivated in growth chambers at 25°C, with a 16/8 hr photoperiod using white fluorescent lamps at 50–90 μmol m^{-2} s^{-1}, with subculture every 4–6 weeks depending on the size of the buds. New shoots from in vitro cuttings appeared on Week 3. Young leaves from 3-week-old in vitro plants were used in the transformation protocol.

2.2 | Stable transformation of *Malus domestica* var. Fuji through *Agrobacterium tumefaciens*

A. tumefaciens EHA105 (provided by Dr. Elizabeth Hood, Arkansas State University) was transformed with the binary vector pB7FWG2-AtDXR (Perello et al., 2016, gently provided by Dr. Manuel Rodriguez-Concepción, CRAG-CSIC, Spain). The plasmid carries the *bar* gene, which confers resistance to the herbicide BASTA (BASF™) and the *Arabidopsis thaliana DXR* gene in tandem with eGFP, under the transcriptional control of the CaMV35S promoter. A positive clone of *Agrobacterium* containing the binary plasmid pB7FWG2 was cultured overnight in agitation at 28°C and resuspended in 20 ml of MS medium until an optical density (OD_{600}) of 0.2. Apple leaves were cut from in vitro shoots and incubated for 15 min with the *A. tumefaciens* culture at room temperature. Subsequently, each explant was dried with sterile absorbent paper and placed in coculture medium (M1 without cefotaxime) in the dark for 3 days at 25°C (Malnoy et al., 2007). Explants were then transferred to calli induction media (M1, Table SI), supplemented with Thidiazuron (TDZ) 2.2 mg L^{-1}, Naphthalene acetic acid (NAA) 1 mg L^{-1} (modified from Chen et al., 2012), cefotaxime 300 mg L^{-1}, timentin 150 mg L^{-1} (Bolar, 1999), and the herbicide BASTA, for selection. A tolerance curve was performed to determine the optimum BASTA concentration that impairs calli regeneration and shoot survival of nontransformed explants (Figure S1). Explants were transferred every 3 weeks to fresh medium supplemented with the same herbicide conditions until analysis. At the end of the experiment, alive calli, as well as the number of living shoots, were quantified.

2.3 | Calli and transformed shoots regeneration

Explants in M1 were cultured in the dark until the first calli appeared. Afterward, they were gradually transferred to light, starting with 50 μmol m^{-2} s^{-1} for 1 week and then to 90 μmol m^{-2} s^{-1} for the rest of the in vitro culture. During this period, explants were transferred to new media every 3–4 weeks. When calli reached a greater size and their characteristic green color, they were transferred to proliferation media (M2) containing TDZ 2.2 mg L^{-1}, BAP 3 mg L^{-1}, and NAA 0.1 mg L^{-1}. From Week 12 onwards, calli were transferred to shoot induction media (M3) supplemented with BAP 0.7 mg L^{-1}, GA_3 1 mg L^{-1}, and NAA 0.1 mg L^{-1} (adapted from Malnoy et al., 2007). Shoots appeared at Week 20 of culture. To evaluate the influence of GA_3 and indole-3-butyric acid (IBA) in elongation, 1 cm-high transformed shoots (24 weeks) were cultured in M4 supplemented or not with GA_3 0.1 mg L^{-1} and IBA 0.2 mg L^{-1} for 6 weeks (Figure S2). Shoots 2-cm high (30 weeks) were cultured in rooting media (M5, Table SI) for 3 weeks, then transferred to MS medium in the absence of hormones until rooting. Subsequently, 3-cm-high seedlings were moved to pots containing a 1:1:1 ratio of peat:vermiculite:leaf soil and grown in the greenhouse under a 16/8 hr photoperiod at 25°C until 5 months when they reached a height of 6 cm, for further analysis.

2.4 | DNA extraction and PCR analysis

Five-month-old (6 cm high) transformed and rooted seedlings grown in the greenhouse were analyzed by PCR to verify the insertion of

the 35S:*AtDXR:eGFP* cassette. Genomic DNA was extracted from leaf tissue using FavorPrepTM Mini Plant Genomic DNA Extraction Kit (Omega) according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry in a NanoDrop® (ND-1000 UV–Vis), and integrity was evaluated visually through agarose 1% gel electrophoresis. For PCR analysis, OneTaq HotStart DNA polymerase (New England BioLabs) was used to amplify a 115 bp of the *eGFP* gene using the qeGFPF and qeGFPR primers (Table SI). As control of DNA integrity, a 196 bp fragment of the apple 18S ribosomal gene (*MdRNA18S*) was amplified using Md18SF and Md18SR primers (Table SI).

2.5 | RNA extraction and transcript abundance analysis

Leaves were collected from 1-year-old plants (third to fifth node from the main shoot), snap-frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted from 200 mg of frozen leaves using the CTAB-Spermidine method (Reid, Olsson, Schlosser, Peng, & Lund, 2006) and RNA integrity was evaluated by gel electrophoresis. DNaseI (Thermo Fisher Scientific) treatments were achieved following manufacturer's instructions. For complementary DNA synthesis, 5 µg of DNase-treated RNA was mixed with random hexamers in the presence of Improm II reverse transcriptase (Promega). Fluorescence-based quantitative real-time polymerase chain reaction (qRT-PCR) experiments were performed in a LightCycler system (MX3000P; Stratagene), using SYBR Green double stranded DNA binding dye, as described in (Fuentes et al., 2012). To evaluate the effect of *AtDXR* expression in the isoprenoid pathway, transcript abundance of key carotenogenic genes was measured. Specific primers for *AtDXR*, *MdDXR*, *MdDXS*, *MdPSY1*, *MdPSY2*, *MdPSY3*, *MdPSY5*, *MdPDS*, *MdLCYB1*, and *MdLCYB2* were used (Table S1), based in sequences described in Ampomah-Dwamena et al. (2012; except for *MdDXR* and *MdDXS* which were identified in this study). *MdACTIN* (Ampomah-Dwamena et al., 2012) was selected as the reference gene after analysis with Normfinder (Andersen, Jensen, & Orntoft, 2004) between *MdACTIN* and *Md18SRNA*. Relative transcript abundance was calculated using the CP (crossing point) values and according to Pfaffl (2001). Each qRT-PCR reaction was performed with three biological and two technical replicates ($n = 3$). In all cases, the reaction specificities were tested with melting gradient dissociation curves, nontemplate control (NTC) and gel electrophoresis. One-way analysis of variance (ANOVA) was performed to assess differential transcript abundance between WT and transgenic lines.

2.6 | Subcellular localization

eGFP fluorescence was visualized in leaves of transgenic apple shoots carrying the pB7FWG2-*AtDXR:eGFP* vector on a confocal microscope (Zeiss LSM 710). FITC Fluorescence filter was used to visualize eGFP that excited in the blue range (478–495 nm) and emitted in the green one (510–555 nm). For chlorophyll, the Cy3 filter was selected, which excited in the green range (530–560 nm) and emitted in the red one (573–648 nm). Nontransgenic shoots were used as negative controls. The experiment was performed in triplicate.

2.7 | Transient transformation of apple fruits

Agrobacterium containing the binary plasmid pB7FWG2 (35S:*AtDXR:eGFP*) was cultured overnight at 28°C in 50 ml of liquid LB supplemented with Rifampicin (50 mg L^{-1}) and Spectinomycin (50 mg L^{-1}) under constant agitation. The culture was then centrifuged at 5,000 rpm for 5 min and resuspended in infiltration medium ($MgSO_4$ 10 mM, MES 10 mM, $MgCl_2$ 10 mM). Commercially acquired Fuji apples were cut at the base using a number 10 scalpel and submerged in the infiltration medium. The infiltration was performed for 5 min at 600 mmHg using a vacuum system. Infiltration experiments were carried out in triplicate, using the 35S:*DcALFIN2* vector carrying a non-carotenogenic gene *ALFIN2* from *Daucus carota* (Dc), which encodes for a transcription factor involved in abiotic stress response (Non-carot), and the PG:PSY-PG:CrtI (2GC) construct composed of the carotenogenic genes PSY and CrtI under the control of the polygalacturonase fruit promoter (PG). Fruits were maintained at 20–22°C, 30–50% humidity and in darkness for 7 days before total carotenoids were quantified.

2.8 | Carotenoids and chlorophyll quantification

Carotenoids and chlorophylls from extended leaves of 1-year-old plants (third to fifth node from the main shoot) and buds of 2-year-old wild-type and transgenic plants, as well as from transiently pB7FWG2-*AtDXR* transformed Fuji fruits, were extracted from 100 mg of leaves or fruit flesh, respectively, with 1 ml of hexane:acetone:ethanol = 2 : 1 : 1 (vol/vol), as described in Fuentes et al. (2012). The extract was dried with gaseous nitrogen and resuspended in 2 ml of acetone. The concentration of chlorophyll *a*, chlorophyll *b*, and total carotenoids was quantified in a spectrophotometer at 750, 662, 645, and 474 nm, at which chlorophyll *a*, chlorophyll *b*, and total carotenoids are detected, respectively. Pigment concentration was determined according to Lichtenthaler and Buschmann (2001). Individual carotenoids were quantified by HPLC using a RP-18 Lichrocart125-4 reverse phase column (Merck®), using an acetonitrile:methanol: isopropanol = 85 : 10:5 (vol/vol) mix as a mobile phase with a 1 ml/min flow rate at room temperature in isocratic conditions. The elution profile was obtained using a diode array detector. Carotenoids were identified according to their absorption spectra, retention time, and comparison with specific pigment standards, which was corroborated by comparison with the Carotenoids Handbook (Britton, 1995; Britton, Liaaen-Jensen, & Pfander, 2004). All extractions were carried out in triplicate, on ice, and under dark conditions to avoid

photodegradation, isomerization, and structural changes of carotenoids.

The Graphpad Prism (version 7.04) software package was used for statistical analysis. One-way ANOVA was performed between WT and transgenic lines.

3 | RESULTS

3.1 | Transformation and regeneration of apple var. Fuji shoots

We established and optimized a transformation protocol for Fuji apple Raku Raku based on previously reported methods for different apple varieties (Bolar, Brown, Norelli, & Aldwinckle, 1999; Chen et al., 2012; Krens et al., 2015; Malnoy et al., 2010; Szankowski et al., 2003) because none of these were specific for Fuji Raku Raku. In our improved protocol, we modified different aspects of the explant and media conditions. Specifically, we selected leaf segments from 4 weeks old in vitro cultured seedlings that were excised and transformed immediately, different to Chen et al. (2012). In addition, we used 2.2 mg L^{-1} TDZ to induce calli regeneration, instead of higher concentrations reported by others, because explants oxidized at higher TDZ concentrations. The optimized protocol for Fuji Raku Raku is summarized in Table 1.

The pB7FWG2-*AtDXR* vector used in this study contains the *bar* gene, which confers resistance to BASTA herbicide. Before transformation, a BASTA tolerance curve was performed to estimate the optimal LD50 for calli regeneration and shoot survival. Figure S1A shows the percentage of alive calli induced per explant after 6 weeks of culture on BASTA ranging from 0 to 1 mg L^{-1}. All explants placed on medium without BASTA generated calli (100%), while 40% (n/N) of those placed on 0.3 mg L^{-1} produced calli, and only 7% produced calli in 0.5 mg L^{-1} of BASTA. At a concentration of 1 mg L^{-1} no leaf explants remained alive. A representative phenotype of the calli is shown in Figure S1b. In the case of shoots, 59.5% of the explants survived in 0.3 mg L^{-1} BASTA after 6 weeks, while the survival rate decreased to 33.3% in 0.5 mg L^{-1} of BASTA (Figure S1C); none of the shoots survived at 1 mg $L^{-1}$of BASTA. Based on these results, we selected 0.3 mg L^{-1} of BASTA as the LD50 for the initial stages in apple transformation (M1 and M2) and 0.5 mg L^{-1} to select transgenic shoots (M3 and M4, Table SI).

Using this transformation protocol, the first transformed shoots were obtained 20 weeks after *Agrobacterium* infection. From a total of 9,049 transformed explants (Figure 2a), 5,826 survived and remained green during the first month. From these, 3,115 (53%) developed calli (Figure 2b), which represents 34% of the explants initially transformed. Twenty-three shoots grown in M4 (0.7% of the calli-producing explants) were finally obtained at 20 weeks of culture (Figure 2c), which represents 0.25% of the total amount of transformed explants. After 10 weeks, shoots reached 3 cm high (Figure 2d) and were transferred to M5 for

TABLE 1 Culture media for *Malus domestica* Fuji Raku Raku in vitro culture and transformant shoots regeneration

	M1 Calli induction N6 Medium[a]	M2 Calli proliferation N6 Medium[a]	M3 Shoots induction N6 Medium[a]	M4GI Shoots proliferation MS medium[b]	M5 Rooting medium MS medium[b]
Sucrose (g L^{-1})	30	30	30	15	25
Myo Inositol (mg L^{-1})	100	100	100	100	100
BAP (mg L^{-1})	–	3	0.7	2	–
TDZ (mg L^{-1})	2.2	2.2	–	–	–
GA_3 (mg L^{-1})	–	–	1	0.1	–
NAA (mg L^{-1})	0.2	0.1	0.1	–	–
IBA (mg L^{-1})	–	–	–	0.2	1.5
Calcium pantothenate (mg L^{-1})	0.5	0.5	0.5	0.5	0.5
Ascorbic acid (mg L^{-1})	20	20	20	20	20
PPM (ml)	3	3	3	3	3
Agar-agar (g L^{-1})	5.2	5.2	5.2	7	7
Phytagel (g L^{-1})	0.6	0.6	0.6	–	–
Cefotaxime (mg L^{-1})	300	300	300	300	300
Timentin (mg L^{-1})	150	150	150	–	–
BASTA® (mg L^{-1})	0.3	0.3	0.5	0.5	0.5

Abbreviations: BAP, 6-benzylaminopurine; GA3, gibberellic acid; IBA, indole-3-butyric acid; NAA, 1-naphthaleneacetic acid; TD Z, thidiazuron; PPM, plant preservation medium.

[a]Fasolo, Zimmerman, and Fordham (1989).

[b]Murashige and Skoog (1962).

rooting. Rooted plantlets (Figure 2e) were obtained after 1 month and then transferred to the greenhouse for acclimation (Figure 2f) under a 16/8 hr light/dark photoperiod. Five-month-old seedlings of 6 cm (Figure 2f) were used for molecular and biochemical analysis. To obtain a higher number of transformed shoots, we evaluated the effect of IBA and GA_3 on the elongation and proliferation of transformed shoots in M4 stage. We observed that when M4 was supplemented with 0.2 mg L^{-1} of IBA (M4 + IBA I, Figure S2), 1.5 times more shoots were induced when compared to the control M4 medium, and that the supplementation with 0.1 mg L^{-1} of GA_3 and 0.2 mg L^{-1} of IBA (M4 + GA3 + IBA, Figure S2) generated three times more shoots compared to the control M4. Thus, when M4 was further supplemented with IBA and GA_3 (Table I), the transformation efficiency increased to 0.75% of the initially transformed explants, or 2.2% of the ones that developed calli.

3.2 | Molecular analysis and subcellular localization of AtDXR:eGFP

The presence of the *AtDXR:eGFP* transgene in the transformed shoots was confirmed by PCR. Five shoots grown and maintained in selection medium were analyzed and from these, the expected segment of 115 pb of the *eGFP* gene was detected in lines DXR-L1 to DXR-L4 and absent in DXR-L5 (Figure S3). Transcript abundance levels of *AtDXR* were analyzed in these four transgenic lines, revealing that DXR-L1 and DXR-L4 had the highest transcript abundance, which correlated with higher transcript abundance levels of the endogenous *MdDXS* gene. Interestingly, the relative expression level of the paralog *MdDXR* was similar between WT and transgenic lines (Figure 3).

All transgenic lines showed a significant increase in the transcript abundance of the endogenous *MdPSY1*, *MdPSY2*, *MdPSY3*, *MdLCYB1*, and *MdLCYB2* genes, key genes for carotenoid synthesis. Additionally, the transcript abundance of *MdPSY5* increased in lines DXR-L3 and DXR-L4, whereas *MdPDS* transcript levels were higher in DXR-L4 only, when compared to the WT (Figure 3). The subcellular localization of the AtDXR:eGFP fusion protein was also monitored by confocal microscopy in the transgenic lines. As shown in Figure 4, the AtDXR:eGFP fusion protein colocalized with chlorophyll fluorescence (Figure 4a–c) and displayed the speckle pattern characteristic of plastidial subcellular localization (Figure 4d–f). Wild type Fuji apple leaves were used as a negative control and showed no fluorescence (Figure 4g–i).

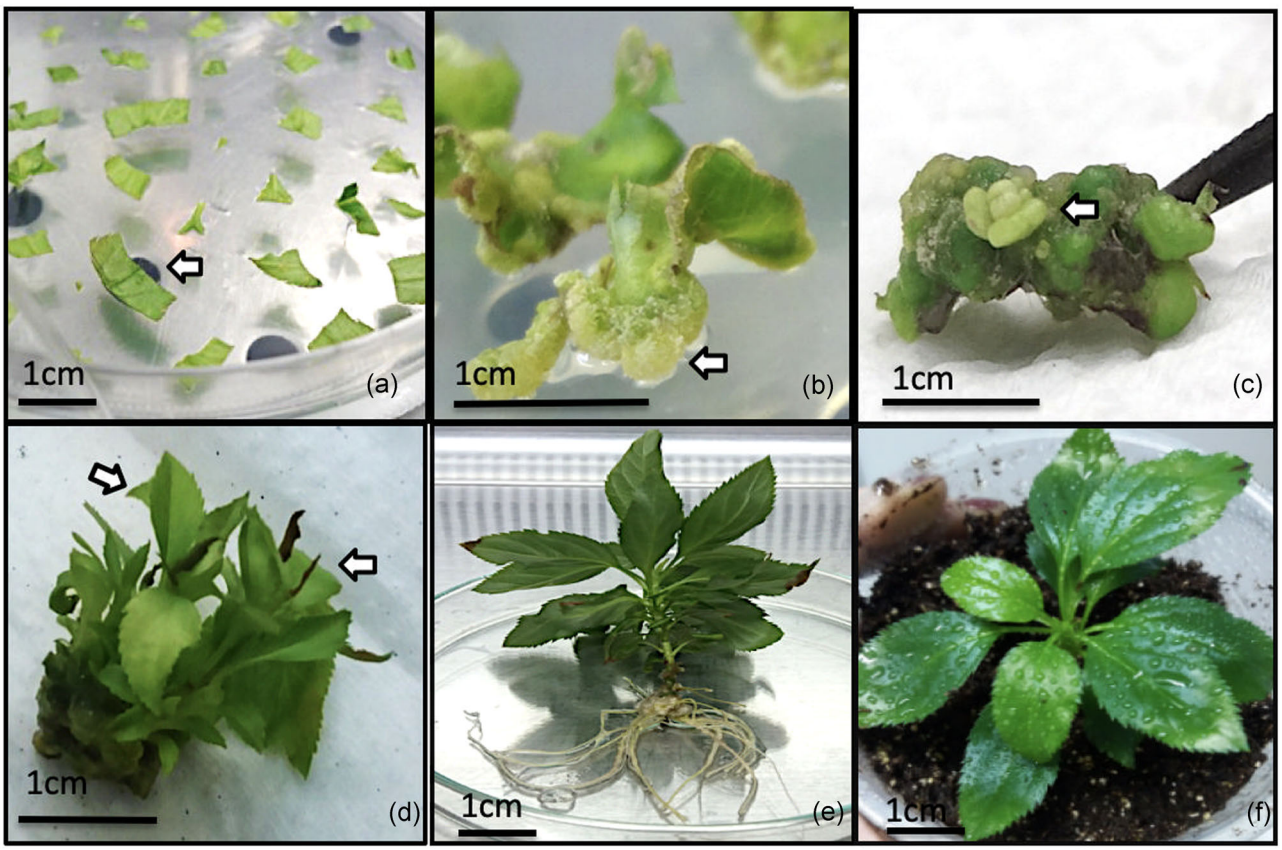

FIGURE 2 Stable transformation of *Malus domestica* Fuji Raku Raku and in vitro shoot regeneration. A) Leaves of 4 weeks old in vitro shoots were incubated with *Agrobacterium tumefaciens* carrying the AtDXR:eGFP construction They were maintained in cocultivation for 3 days and then left in darkness for 4–6 weeks in induction medium. Arrow points a representative piece of leaf used as explant. (b) Calli (shown with the arrow) appeared after 8 weeks on M1 culture. (c) Shoots (shown with the arrow) started to develop after 12 weeks on M4 media. (d) Transformed shoots (shown with arrows) of 3 cm high were obtained after 10 weeks on M4GI. (e) Rooting was induced in M5 supplemented with 1.5 mg L^{-1} IBA for two weeks. (f) A 2–3 months old seedling during acclimation in the greenhouse [Color figure can be viewed at wileyonlinelibrary.com]

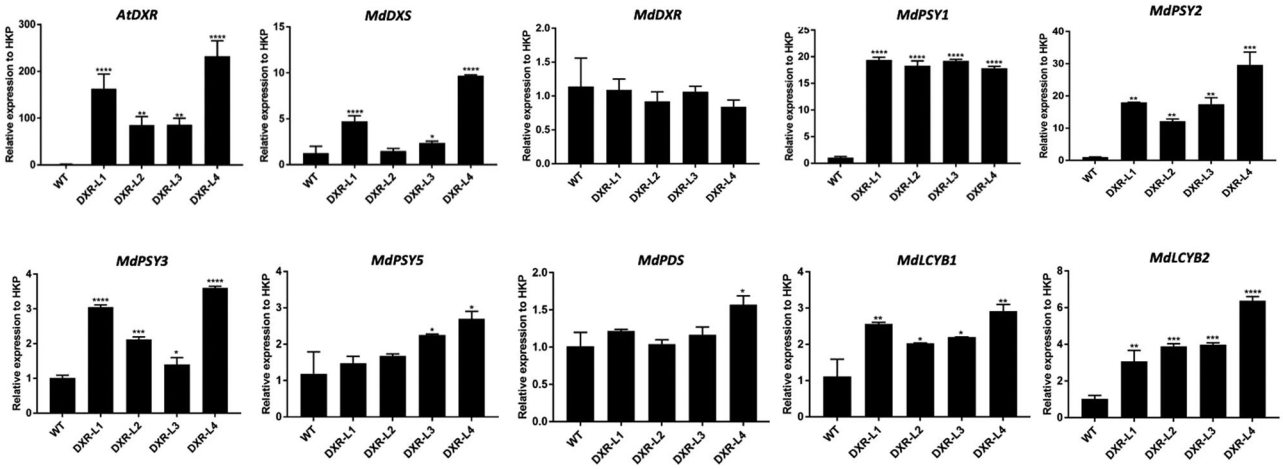

FIGURE 3 Molecular analysis of AtDXR:eGFP transformed Fuji Raku Raku seedlings. Relative expression of *AtDXR*, *Md DXR*, *MdDXS*, *MdPSY1*, *MdPSY2*, *MdPSY3*, *MdPSY4*, *MdPDS*, *MdLCYB1*, and *MdLCYB2* in wild-type (WT) and transgenic seedlings (DXR-L1 to DXR-L4). The carotenogenic genes were named accordingly to Ampomah-Dwamena et al. (2012) and Cerda et al. (2020). Transcript abundance was normalized to the housekeeping (HKP) *MdACTIN* gene and WT was taken as calibrator. Columns represent the mean and SE considering three biological and two technical replicas ($n = 3$). Asterisks indicate statistically significant differences using a one-way analysis of variance (ANOVA). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{***}p < .0001$

These results strongly supports the fact that in these transgenic shoots, the AtDXR:eGFP fusion protein is expressed with a proper and expected subcellular localization.

3.3 | Carotenoid and chlorophyll levels in AtDXR:eGFP transgenic plants

Total carotenoid content and individual carotenoid composition profile were assessed in leaves of 1-year-old transgenic lines (DXR-L1 to L4) using wild type plants as control. A 3-fold increment in total carotenoids was observed in the transgenic lines transformed with the *AtDXR-eGFP* construct when compared to WT plants, reaching up to 508 $\mu g\, g^{-1}$ FW (Figure 5a).

Regarding individual carotenoids, all transgenic lines displayed up to 4-fold increase in lutein (250–280 $\mu g\, g^{-1}$ FW), 3-fold increase in β-carotene (50–65 $\mu g\, g^{-1}$ FW), and a 3-fold increase in α-carotene (40–60 $\mu g\, g^{-1}$ FW) when compared to wild type plants, which accumulated 65 $\mu g\, g^{-1}$ FW of lutein, 18 $\mu g\, g^{-1}$ FW of β-carotene and 19 $\mu g\, g^{-1}$ FW α-carotene (Figure 5a). There were no statistically significant differences in phytoene levels between transgenic and wild type plants (data not shown). Levels of chloropyll *a* and *b* were also measured in transgenic lines (Figure 5b), since carotenoids and chlorphylls share GGPP as common precursor. Chlorophyll *a* was reduced in line DXR-L4, and increased in line DXR-L1. In the case of chlorophyll *b* levels, only DXR-L1 showed a significant albeit mild increase compared to the WT. DXR-L2 and DXR-L3 were undistinguishable from the WT with regard to chlorophyll *a* and *b* levels. These results suggest that *AtDXR* expression can affect chlorophyll content which may depend on the specific *AtDXR* transcript abundance levels presents in each transgenic line. For a deeper analysis, we determined the carotenoid content in buds of dormant 2-year-old transgenic trees growing in greenhouse conditions. As shown in Figure 6a, all transgenic lines presented between 3 to 6-fold increment in lutein levels (15–28 $\mu g\, g^{-1}$ FW) when compared to wild type buds.

3.4 | Carotenoid increment in fruits by transient expression of AtDXR:eGFP

Transient expression assay via agroinfiltration in apple fruits was performed to assess the effect of *AtDXR* expression on carotenoid biosynthesis in fruit flesh. Transient *AtDXR* expression resulted in a 2-fold increment in total carotenoids in the flesh, reaching up to 4 $\mu g\, g^{-1}$ FW, consistent with the results obtained for the positive control (2GC) composed of the carotenogenic genes *PSY* and *CrtI* (Figure 6b). As expected, the *DcALFIN2* gene (non-carot) presented similar carotenoid levels than the control treatment, as it encodes for a carrot transcription factor that is not involved in carotenoid synthesis.

4 | DISCUSSION

Currently, there is an increasing interest in the generation of new vegetable and fruit varieties with higher carotenoid levels to supplement the human diet (Alós, Rodrigo & Zacarias, 2016). Commercial apple fruits are rich in vitamins, but poor in carotenoids (< 2.5 $\mu g\, g^{-1}$ FW) when compared to other commercial fruits (Ampomah-Dwamena et al., 2012; Lado et al., 2016) such as citrus (25 $\mu g\, g^{-1}$ FW), papaya (60 $\mu g\, g^{-1}$ FW), and caquis (15 $\mu g\, g^{-1}$ FW) (Ampomah-Dwamena et al., 2012; Kato et al., 2004; Schweiggert et al., 2011; Zhou, Zhao, Sheng, Tao, & Yang, 2011). Because apples are widely consumed, they are an ideal candidate to strongly increase their carotenoid content and

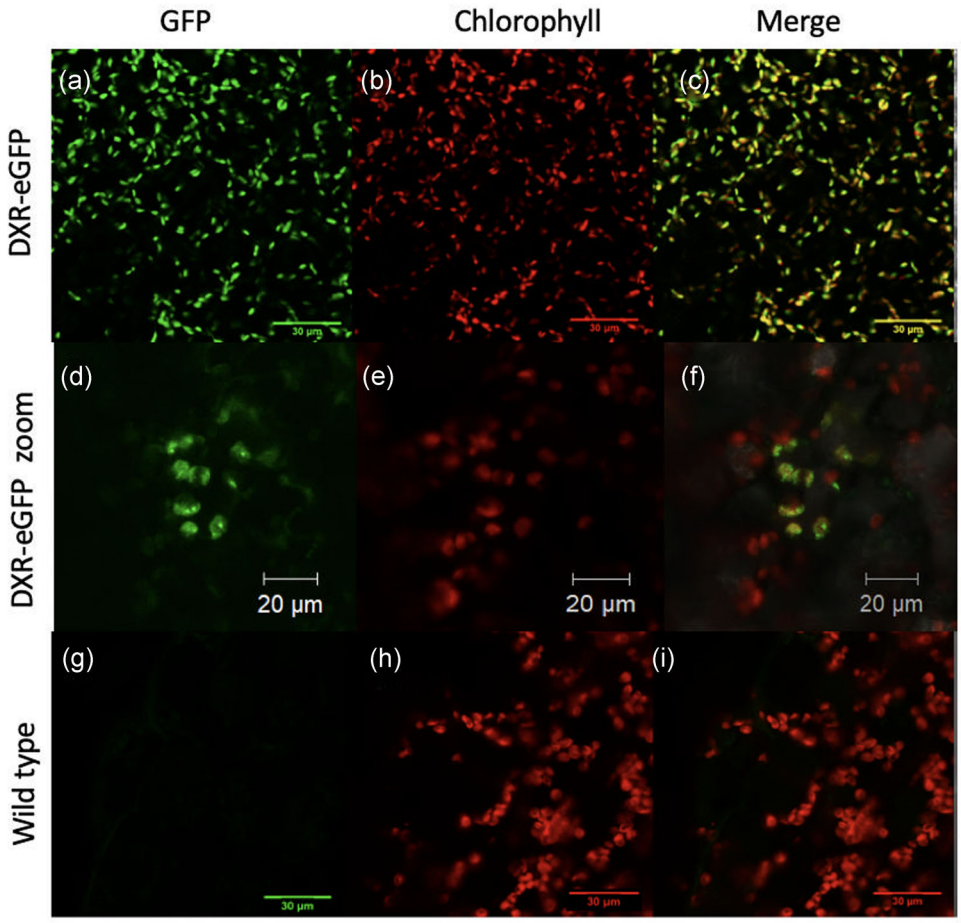

FIGURE 4 AtDXR:eGFP localization in transgenic shoots. (a–c) Visualization of AtDXR:eGFP fluorescence in 2 months old leaves of the DXR-L1 transgenic line. (d–f) Zoom of the fluorescence of AtDXR:eGFP in DXR-L1 transgenic line showing the localization in speckles inside the chloroplasts. (g–i) Wild type seedlings of nontransformed Fuji apple leaves. Scale bar: 30 µm except for (d–f) where it is 20 µm. eGFP excitation wavelength: 488-510 nm, Chlorophyll excitation wavelength: 543–618 nm [Color figure can be viewed at wileyonlinelibrary.com]

health-promoting attributes. Specifically, Granny Smith fruit flesh contains 0.17 $\mu g\,g^{-1}$ FW of β-carotene and 0.17 $\mu g\,g^{-1}$ FW of lutein, while Royal Gala accumulates between 0.2-0.5 $\mu g\,g^{-1}$ FW of several xanthophyll's but has reduced levels of lutein and β-carotene (Ampomah-Dwamena et al., 2012; Delgado-Pelayo et al., 2014). Fuji (French and Italian origin) fruit flesh contains between 0.04–0.22 $\mu g\,g^{-1}$ DW of lutein and 0.79–1.14 $\mu g\,g^{-1}$ DW of β-carotene (Delgado-Pelayo et al., 2014).

In this study, we set out to obtain apple fruits with improved nutritional value, using a herein optimized *Agrobacterium*- mediated apple transformation protocol for Fuji apples. *Malus domestica* cv. Fuji Raku Raku was used in this study as it is one of the commercially most important apple varieties that are cultivated and exported as fresh fruit in Chile (http://www.odepa.cl/boletin/boletin-de-fruta-fresca-septiembre-de-2017/). For *Malus domestica* var. Fuji transformation, we used previous protocols standardized for rootstock M26 (Malnoy et al., 2010), Elstar and Holsteiner Cox (Szankowski et al., 2003), Marshall Mcintosh (Bolar et al., 1999), Fuji (Chen et al., 2012) and Gala varieties (Krens et al., 2015). Fuji apple stable transformation was previously established for the Naga-fu variety no. 6 by Chen et al. (2012), who achieved a 20% transformation efficiency. In our improved protocol, we modified different aspects of the explant and media conditions to significantly increase the transformation efficiency up to 80%. Specifically, we selected leaf segments from 4 weeks old in vitro cultured seedlings, while Chen et al. (2012) used leaf segments of 3 weeks rooted in vitro shoots. The leaves were excised and transformed immediately, while Chen et al. (2012) maintained the leaves in calli induction medium for 4 weeks before they were transformed, so they had greater vigor when transformed and would explain the higher *Agrobacterium* concentration used (OD_{600} 0.6 vs. 0.2 in our research). In addition, the shoot regeneration medium used by them was supplemented with 8 $mg\,L^{-1}$ TDZ for the Naga-fu variety no. 6, whereas we used 2.2 $mg\,L^{-1}$ TDZ for the Raku Raku variety to induce calli regeneration, because explants oxidized at higher TDZ concentrations. This indicates that although both procedures were established for Fuji, culture protocols are specific for the variety used, and determining the optimal regeneration conditions is crucial for success.

To improve the production of transgenic plants during the in vitro culture, GA_3 and IBA was added to the proliferation media (M4). After 6 weeks the number of induced shoots per cultivated

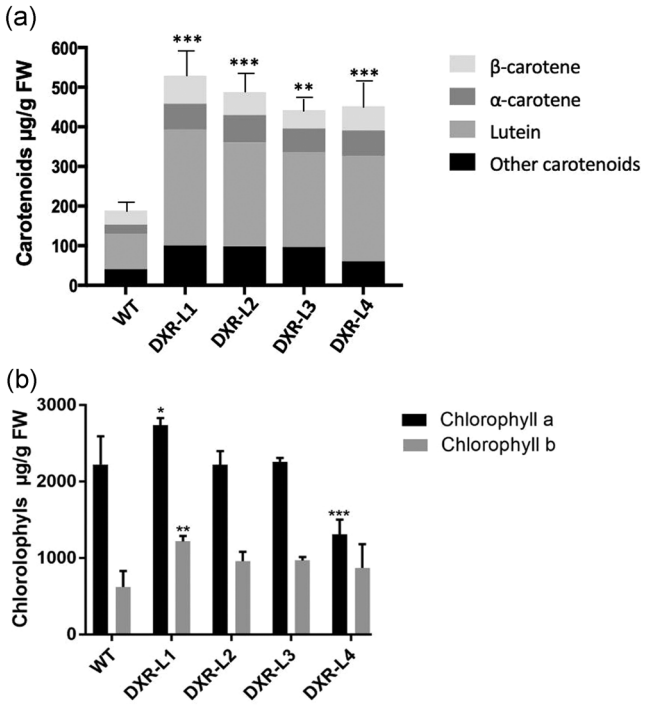

FIGURE 5 Total and individual carotenoids and chlorophyll content in leaves of AtDXR-eGFP transgenic lines. (a) Total and individual carotenoids and (b) Chlorophyll *a* and *b* in leaves of wild type (WT), transgenic DXR-L1 to DXR-L4 seedlings. Pigments were measured in three independent samples of each transgenic seedling. Asterisks indicate significant differences of carotenoid levels between transgenic lines and WT determined by one-way analysis of variance (ANOVA). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{***}p < .0001$

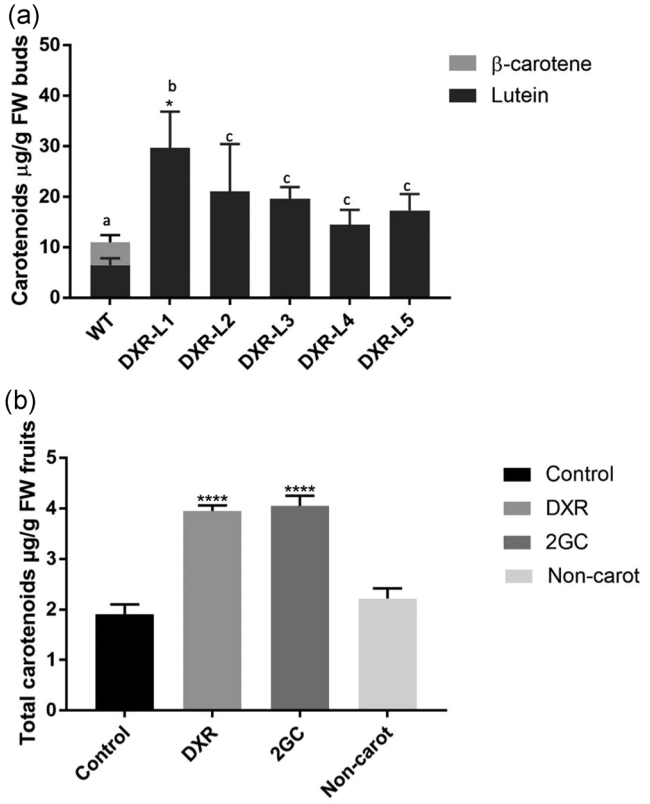

FIGURE 6 Total and individual carotenoid content in buds of AtDXR-eGFP transgenic lines and in transient agroinfiltrated fruits. (a) Total and individual carotenoids in buds of 1-year-old 35S:AtDXR:eGFP (DXR) transgenic trees and in wild type tree (WT). (b) Total carotenoid content in ripe Fuji Raku Raku fruits after 7 days of agroinfiltration with 35S:AtDXR:eGFP (DXR), PG:PSY-PG:CrtI (2GC), and the 35S:DcALFIN2 (non-carot) vectors. Control corresponds to nontransformed *Agrobacterium* infiltration media. Total and individual carotenoids were measured in three independent samples. Asterisks indicate significant differences of total carotenoid levels between transgenic lines and WT determined by one-way analysis of variance (ANOVA). $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{***}p < .0001$. Letters represent significant difference in lutein levels

shoot increased by 3-fold, in agreement with Bolar et al. (1999), where the proliferation medium was supplemented with GA_3. The use of M4 supplemented with GA_3 and IBA (M4 + GA3 + IBA) increased the total amount of shoots up to 2.2% of efficiency when total induced calli is considered, which is similar to that reported for apple rootstock (Sharma, Modgil, & Sharma, 2017). In the case of other apple varieties such as Gala, Junami, Mitchgla, and Wellant, 0.55%, 0.57%, 0.12%, and 0% transformation efficiency were obtained, respectively, when the selection method was based only in the visual production of red anthocyanins (Krens et al., 2015). A difficulty specific to the Fuji variety was its sensitivity to the *Agrobacterium* concentration. Most of the *Agrobacterium*-mediated apple transformation used an optical density (OD_{600}) between 0.8 and 1.0 (J. Li et al., 2011; Seong & Song, 2008; Szankowski et al., 2003; Vanblaere et al., 2011; Wu et al., 2011; Xu et al., 2009) or as low as 0.3–0.5 (Schaart, Puite, Kolova, & Pogrebnyak, 1995). In Fuji Raku Raku, the optimum transformation was obtained with an OD_{600} of 0.2, as higher *Agrobacterium* concentrations were lethal to the shoots, an effect that was not observed in other apple varieties assayed (data not shown).

Using this specific transformation protocol for the Fuji Raku Raku variety, transgenic lines were obtained, as shown by molecular analysis and the subcellular localization of eGFP (Figures 3 and 4). The AtDXR:eGFP fusion protein has the expected plastidial subcellular localization that was reported previously in *Arabidopsis* (Carretero-Paulet et al., 2002) with a speckled pattern distribution denoting intraplastidial localization (Perello et al., 2016).

Genes belonging to the MEP pathway have been used to increase the carotenoid levels in several plant varieties. Overexpression of DXS-encoding genes typically produce a boost of isoprenoids such as carotenoids and chlorophylls (Carretero-Paulet et al., 2006; Enfissi et al., 2005; Estévez, Cantero, Reindl, Reichler & León, 2001; Henriquez et al., 2016; Morris, Ducreux, Hedden, Millam, & Taylor, 2006; Munoz-Bertomeu, Arrillaga, Ros, & Segura, 2006; M. Zhang, Li, Zhang, Gai, & Yu, 2009). Moreover, the overexpression of *DXR* genes also leads to increased levels of MEP-derived

isoprenoids, as has been described by several authors (Carretero-Paulet et al., 2006; Chang et al., 2014; Hasunuma et al., 2008; Mahmoud & Croteau, 2001; Yang et al., 2012; H. Zhang et al., 2015), although others studies have found no effect (Mendoza-Poudereux, Munoz-Bertomeu, Arrillaga, & Segura, 2014; Rodríguez-Concepción et al., 2001; Simpson, Quiroz, Rodriguez-Concepción, & Stange, 2016). In other plant species such as *Mentha piperita* (peppermint), *Salvia sclarea* (clary sage), and *Artemisia annua* (artemisia), the overexpression of *DXR* increases the production of MEP pathway derivatives (Mahmoud & Croteau, 2001; Vaccaro et al., 2014; Xiang et al., 2012), suggesting that the effect of DXR may vary from one plant to another and therefore would be species-specific.

In our study, the higher transcript abundance of *AtDXR* in the transgenic lines correlated with an induced transcript abundance of key genes of the carotenogenic pathway such as *MdPSY1*, *MdPSY2*, *MdPSY4*, *MdLCYB1*, and *MdLCYB2*, resulting in a 3-fold increase in total carotenoids in leaves of transgenic plants. Lines DXR-L1 and DXR-L4, which presented the highest level of transcript abundance of the transgene, showed also the larger increment in *MdDXS*, although the four transgenic lines exhibited a similar carotenoid increment in leaves, indicating additional regulatory mechanisms involved in this pathway. On the other hand, transgenic lines do not exhibit changes in the transcript abundance of the endogenous *MdDXR*, which suggest that the increase in *AtDXR* transcript abundance (and the corresponding protein) produces by itself a significant increase in carotenoids, possibly through the upregulation of endogenous carotenogenic genes. In *Arabidopsis*, *AtDXR* overexpressing plants exhibit higher carotenoid content (Carretero-Paulet et al., 2006) without changes in *DXS* gene expression, suggesting that the observed increase in carotenoid levels was a direct effect of *DXR* overexpression. On the other hand, in carrot, the expression of *AtDXS* but not *AtDXR* resulted in an upregulation of endogenous *DcPSY1* and *DcPSY2* and a significant increment in carotenoids (Simpson et al., 2016). Rodríguez-Villalon, Gas, and Rodríguez-Concepción (2009) reported that *Arabidopsis* etiolated seedling with an enhanced PSY activity leads to an upregulation of *AtDXS* levels. This provides evidence of a crosstalk regulatory mechanisms between the MEP and carotenoid pathways, which ensures an optimal supply of MEP-derived precursors for the carotenoid biosynthetic pathway. Thus, the data indicate that an efficient production of carotenoids requires a balance between the amount of molecules and precursors revealing an intricate regulatory system.

In *Arabidopsis*, *AtDXR* overexpression results in up to 1.3-fold increase in carotenoid levels when compared to the wild type (Carretero-Paulet et al., 2006), while in carrot, *AtDXR* expression only has a very mild effect, increasing total carotenoids 1.1-fold in leaves exclusively (Simpson et al., 2016). Here, *AtDXR* Fuji transgenic lines exhibited a significant increase of 3-fold in total carotenoids (508 $\mu g\, g^{-1}$ FW), suggesting that *DXR* role in carotenoid synthesis is relevant in apple. Moreover, a 4-fold increment in lutein (250–280 $\mu g\, g^{-1}$ FW), a 3-fold increase in β-carotene (50–65 $\mu g\, g^{-1}$ FW) and a 3-fold increase in α-carotene (40–60 $\mu g\, g^{-1}$ FW) was obtained in all transgenic lines when compared to wild type plants. Moreover, transgenic lines, similar to wild type plants, did not accumulate phytoene. These results suggest that *DXR* expression affects downstream carotenoid synthesis indicating that endogenous apple carotenogenic enzymes codified by *PSYs*, *PDS*, *ZDS*, and *LCYBs* are functional in leaves; this is consistent with the transcript abundance analysis performed in the transgenic lines (Figure 3). Additionally, carotenoid content analysis in buds of dormant 2-year-old transgenic trees showed a higher level of lutein, which confirmed the stable transformation and the general and constitutively effect of the transgene in apple. Buds are composed of dormant embryonic cells that contain developing chloroplasts. In woody plants, buds contain over 75% of lutein and lutein-5,6 epoxide (Lx), but β-carotene is not present (García-Plazaola et al., 2004). Therefore, it was unexpected to see that β-carotene was accumulated in Fuji Raku Raku wild type buds. It is possible that in transgenic buds, the absence of β-carotene may be due to a redirection of the metabolic flux to the ε, β branch, leading to an increment in lutein. Under light conditions, Lx undergoes de-epoxidation into lutein, which constitutes the Lx cycle involved in photoprotection by modulating the rate of nonradiative dissipation and non-photochemical quenching (García-Plazaola et al., 2004). The pattern of carotenoids in buds is different from leaves (García-Plazaola et al., 2004, Ampomah-Dwamena et al., 2012), but both of them present high level of lutein, which is required for both photoprotection and photosynthesis. Therefore, the higher level of lutein in transgenic buds suggest that they might be more protected under light stress. Hence, it would be interesting for future approaches to test the survival of transgenic lines under different stress conditions such as salinity or light stress to determine if carotenoids promote abiotic stress tolerance in apple trees, as has been reported in other plant models (Simpson et al., 2018; Ruiz-Sola, Arbona, Gomez-Cadenas, Rodriguez-Concepcion, & Rodriguez-Villalon, 2014; Shi et al., 2015).

Transgenic lines also exhibited different chlorophylls levels, and we observed a correlation with *AtDXR* transcript abundance. Lines DXR-L2 and DXR-L3, which exhibit the lowest *AtDXR* transcript abundance (Figure 3), produce a significant increment in carotenoids but not in chlorophylls (Figure 5). One possible explanation is that the amount of AtDXR enzyme produced is not enough to fulfill the metabolic precursors required for both routes, and the carotenoid pathway is preferred. This led us to hypothesize that *AtDXR* expression in apple leads to an increment in GGPP common precursor that is used primarily for carotenoid synthesis and not for the synthesis of other molecules such as chlorophylls. Line DXR-L1 exhibits higher *AtDXR* transcript abundance than DXR-L2 and DXR-L3 but lower than DXR-L4, and shows an increment in carotenoids as well as in chlorophyll *a* and *b*, suggesting that this line may produce higher amount of AtDXR enzyme which could, in turn, produce the necessary amount of the common GGPP precursor, and thereof allowing the synthesis of chlorophylls as well as carotenoids. In line DXR-L4, the prominently high transcript abundance of *AtDXR* might

be triggering an inhibition in the chlorophyll *a* synthesis. This suggests a reverse effect on the metabolic regulation possibly by due to a saturation given by an overproduction of substrate.

Carotenoid accumulation is the result of regulation at multiple levels, including synthesis, degradation, and stabilization levels representing a dynamic flux which determine the amount of pigments. Interestingly, this dynamic process varies between plants (Sun et al., 2018). Carotenoid accumulation depends on the regulation of key enzymes like phytoene synthase (PSY) for synthesis, chaperones like OR that stabilizes PSY improving carotenoid storage, and carotenoid cleavage dioxygenase (CCDs) involved in carotenoid degradation (L. Li, Yuan, Zeng, & Xu, 2016; Sun et al., 2018). Thus, modifying the plant carotenogenic pathway results in different carotenoid content and stability depending on the plastidial accumulating capability (Li et al., 2012; Schaub et al., 2017). Therefore, plants with lower carotenoid content in sink organs may produce higher increases when the isoprenoid pathway is modified by overexpression of carotenogenic genes. For instance, after the expression of *crtB*, potato exhibited a 7-fold (35 $\mu g\, g^{-1}$ DW) increase in total carotenoids (Ducreux et al., 2005), similar to rice, which produced 36 $\mu g\, g^{-1}$ DW of total carotenoids (Al-Babili et al., 2005). Examples of transgenic fruits with higher carotenoid content are rare. Kiwi fruits expressing either *GGPPS* or *PSY* displayed an increase of about 1.2- to 1.3-fold in lutein or β-carotene compared to nontransgenic plants which accumulated 2 $\mu g\, g^{-1}$ FW of total carotenoids (Montefiori, McGhie, Hallett, & Costa, 2009). Similarly, transgenic banana fruits expressing *PSY1* or *PSY2* directed by a constitutive promoter displayed a 2.3-fold increase in β-carotene in the pulp (7.4 $\mu g\, g^{-1}$ DW) when compared to the wild type (Paul et al., 2017).

So far, the mechanism underlying natural low carotenoid accumulation in apple fruits is unknown. Here, we show that transient expression of the MEP gene *DXR* in apple fruits resulted in a 2-fold increment in total carotenoids, similar to the increase obtained after the transient transformation of the carotenogenic genes *PSY* and *CRTI*. This suggests that isoprenoid as well as carotenoid genes are key steps for improving carotenoid content in apple flesh (Ampomah-Dwamena et al., 2015) and that carotenogenic enzymes are functionally active in apple leaves and flesh.

It is important to mention that the transient expression assay represents just a preliminary approximation, particularly in assessing the role of *DRX* at a functional level. Because during this analysis, just some cells are transformed, the result provides a valuable tendency but not a quantitative measure. On the contrary, stable nuclear transformation should result in all the cells expressing the transgene. Therefore, we expect that fruits derived from the stable transgenic lines will accumulate even higher carotenoid levels in the flesh considering the transgene constitutive expression in the entire organ. In our experience, stable expression of carotenogenic genes using a constitutive promoter produces a relatively homogeneous increment of carotenoids in source and sink tissues in plants, as exemplified when we overexpressed *DcLCYB1* and *AtDXS* in carrot (Moreno et al., 2013; Simpson et al., 2016). In banana, authors have reported a similar accumulation of metabolic products between source (leaves) and sink (pulp) organs, at least when a constitutive promoter is used (Paul et al., 2017).

Wild type Fuji fruits accumulate around 1.8 $\mu g\, g^{-1}$ FW in total carotenoids (Figure 6b), which is similar to the amount reported in other apple varieties, such as Royal Gala (Ampomah-Dwamena et al., 2012) that corresponds to 10-14 $\mu g\, g^{-1}$ DW of total carotenoids (Delgado-Pelayo et al., 2014). However, Fuji and Royal Gala accumulate different proportion of individual carotenoids. Specifically, Royal Gala accumulates between 0.2 and 0.5 $\mu g\, g^{-1}$ FW of several xanthophylls but not lutein or β-carotene (Ampomah-Dwamena et al., 2012) and Fuji (French and Italian origin) contain between 0.04-0.22 $\mu g\, g^{-1}$ DW (0.08-0.4 $\mu g\, g^{-1}$ FW) of lutein and 0.79-1.14 $\mu g\, g^{-1}$ DW (0.16-0.27 $\mu g\, g^{-1}$ FW) of β-carotene (Delgado-Pelayo et al., 2014). In the case of Granny Smith, it accumulates 0.81 $\mu g\, g^{-1}$ FW of total carotenoids, 0.17 $\mu g\, g^{-1}$ FW of β-carotene and 0.17 $\mu g\, g^{-1}$ FW of lutein in the fruit flesh. When we expressed *AtDXR* in apple, we observed that all transgenic lines displayed up to a 4-fold increase in lutein, 3-fold increase in β-carotene and a 3-fold increase in α-carotene in leaves when compared to wild type plants (Figure 5a), showing that, just as lutein, α- and β-carotene are also more abundant in transgenic leaves. Therefore, we speculate that transgenic AtDXR:eGFP apple fruits could exhibit at least a 3-fold increase in total and individual carotenoids, corresponding to 6 $\mu g\, g^{-}1$ FW in total carotenoids, 0.24-1.2 $\mu g\, g^{-1}$ FW of lutein, and 0.48-0.81 $\mu g\, g^{-1}$ FW of β-carotene.

In summary, we successfully express *AtDXR:eGFP* in Fuji Raku Raku which resulted in an increment in the transcript abundance of three *MdPSYs* members and *MdLCYBs*, a 3-fold increase in total carotenoids, 4-fold increase in lutein, 3-fold increase in β-carotene and a 3-fold increase in α-carotene in apple leaves, revealing a relevant role of DXR in the carotenoid biosynthesis in apple. Thus, our results let us to propose that *DXR* is a good candidate for increasing carotenoid levels in apple fruits through metabolic engineering.

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CONFLICTS OF INTERESTS

The authors declare that the research was conducted in the absence of commercial or financial relationships that could be construed as potential conflict of interest.

AUTHORS CONTRIBUTIONS

A. A., F. G., and C. S. conceived and designed the experiments. Y. A., F. G., C. F., and A. A. performed the experiments. Y. A., F. G., and C. S. wrote the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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