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RESEARCH ARTICLE

Carotenoid fortification of zucchini fruits using a viral RNA vector

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Abstract

Background: Carotenoids are health-promoting metabolites in livestock and human diets. Some important crops have been genetically modified to increase their content. Although the usefulness of transgenic plants to alleviate nutritional deficiencies is obvious, their social acceptance has been controversial.

Results: Here, we demonstrate an alternative biotechnological strategy for carotenoid fortification of edible fruits in which no transgenic DNA is involved. A viral RNA vector derived from zucchini yellow mosaic virus (ZYMV) was modified to express a bacterial phytoene synthase (crtB), and inoculated to zucchini (*Cucurbita pepo* L.) leaves nurturing pollinated flowers. After the viral vector moved to the developing fruit and expressed *crtB*, the rind and flesh of the fruits developed yellow-orange rather than green color. Metabolite analyses showed a substantial enrichment in health-promoting carotenoids, such as α- and β-carotene (provitamin A), lutein and phytoene, in both rind and flesh.

Conclusion: Although this strategy is perhaps not free from controversy due to the use of genetically modified viral RNA, our work does demonstrate the possibility of metabolically fortifying edible fruits using an approach in which no transgenes are involved.

KEYWORDS
carotenoids, cucurbits, fruit fortification, phytoene synthase, RNA virus vector, zucchini yellow mosaic virus

1 | INTRODUCTION

Plants produce a vast number of secondary metabolites, many of them of great interest in food, pharmaceutical and industrial applications. However, valuable plant metabolites are not infrequently produced in limited amounts or without the desired chemical properties. The ability to manipulate endogenous metabolic pathways or to deploy novel pathways in plants via biotechnological metabolic engineering approaches should contribute to overcoming these issues and successfully establishing these autotrophic organisms as "green factories" able to sustainably provide most of the molecules required by humankind.

Abbreviations: CP, coat protein; crtB, phytoene synthase; dpi, days post-inoculation; NIa, nuclear inclusion a; NIb, nuclear inclusion b; PSY-A, phytoene synthase A; ZYMV, zucchini yellow mosaic virus

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In contrast to other systems, production in plants is cheap, easily scalable, mainly fueled by sunlight, and, if properly managed, free of human pathogens.

In fact, plants are currently being used as platforms to produce recombinant proteins and peptides in both stable genetic transformation or transient expression approaches.[1,2] Stable genetic transformation of plants is a labor-intensive and time-consuming process that frequently leads to results with undesired variability. By contrast, transient expression systems, such as those that employ *Agrobacterium tumefaciens*, viral vectors, or a combination of both, offer a rapid alternative for reaching some biotechnological goals. We recently showed that plant virus-derived vectors can be used not only to produce recombinant proteins, but also to engineer plant metabolism in cases in which the expressed proteins are regulatory factors or biosynthetic enzymes that interact with the natural host plant metabolism. Virus-based expression of transcription factors Delila and, particularly, Rosea1 from *Antirrhinum majus* has been shown to lead to massive accumulation of anthocyanins in plant tissues.[3,4] Similarly, virus-based expression of *Pantoea ananatis* phytoene synthase (crtB), the enzyme catalyzing the first step of carotenoid biosynthesis in this bacterium, has been demonstrated to lead to a substantial accumulation of phytoene and other carotenoids in plant tissues.[5,6] Virus-based coexpression of crtB and a *Crocus sativus* carotenoid cleavage dioxygenase (CCD2L) induced large accumulation in *Nicotiana benthamiana* leaves of the apocarotenoid crocins and picrocrocin, which naturally accumulate in saffron stigma and are main constituents of the valued spice.[7] However, these achievements were mainly produced in model plants, such as *N. tabacum*, *N. benthamiana* or *Arabidopsis thaliana*. Our goal here was to translate this ability to produce health-promoting metabolites such as carotenoids to edible tissues, particularly edible fruits. Generally, animals do not biosynthesize carotenoids, which are essential nutrients that must be acquired from diet.[8] Consequently, highly relevant projects aimed to the carotenoid fortification of important crops have been undertaken in last two decades.[9–12] To this end, we investigated a strategy to induce accumulation of carotenoids in edible zucchini (*Cucurbita pepo* L.) fruits using an RNA-based viral vector derived from *Zucchini yellow mosaic virus* (ZYMV; genus *Potyvirus*, family *Potyviridae*) that expresses the bacterial biosynthetic enzyme crtB.

2 | MATERIAL AND METHODS

2.1 | Virus inoculum

One-month old zucchini plants (MU-CU-16, COMAV-Universitat Politècnica de València gene bank accession BGV004370) were infiltrated,[13] with *A. tumefaciens* C58C1 cotransformed with the helper plasmid pCLEAN-S48[14] and pGZYMV or pGZYMV-crtB.[5] pGZYMV contains a wild type infectious ZYMV cDNA (GenBank accession number KX499498). pGZYMV-crtB is a derivative of pGZYMV in which the cDNA of *P. ananatis* crtB[15] was inserted between the nuclear inclusion b (NIb) and coat protein (CP) cistrons, flanked by sequences that complement the viral nuclear inclusion a (NIa) protease (NIaPro) cleavage sites (Figure 1A).[5] In pGZYMV and pGZYMV-crtB, the expression of viral infectious clones are under control of cauliflower mosaic virus (CaMV) 35S promoter and terminator. Plants were kept in a greenhouse at 25°C with a 16/8 h day/night cycle. Ten days postinoculation (dpi), symptomatic tissue was harvested, cut into pieces of approximately 50 mg, and stored at -80°C. For mechanical inoculation of zucchini plants, one tissue piece (50 mg) was ground using a 4 mm steel ball in a 2 mL Eppendorf tube in a bead beater (VWR) for 1 min at 30 revolutions s^{-1}. Powder was brought to the bottom of the tube by a brief centrifugation, after which 1 mL of inoculation buffer (50 mM potassium phosphate, pH 8.0, 1% polyvinylpyrrolidone-10, 1% polyethylene glycol-6000, 10 mM 2-mercaptoethanol) was added and the mixture thoroughly vortexed.

2.2 | Plant inoculation

A 10 μl drop of 10% carborundum in inoculation buffer was deposited on the adaxial side of a fully expanded leaf of a zucchini plant. A cotton swab soaked in virus inoculum was smoothly rubbed on the leaf surface. Plants were kept in a greenhouse at 25°C with a 16/8 h day/night cycle.

2.3 | Virus diagnosis

RNA was purified using silica gel columns (Zymo Research) from pieces of zucchini leaves and fruits. Aliquots of the RNA preparations were subjected to reverse transcription (RT) with primer PI (5′-AGGCTTGCAAACGGAGTCTAA-3′) and RevertAid reverse transcriptase (Thermo Scientific). RT products were amplified by the polymerase chain reaction (PCR) with *Thermus thermophilus* DNA polymerase (Biotools) using primers PII (5′-TCAGGCACTCAGCCAACTGTGG-3′) and PIII (5′- CTGCATTGTATTCACACCTAGT-3′). PCR products were separated by electrophoresis in a 1% agarose gel in TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.2), and the gel was stained in a solution of 0.5 μg mL^{-1} ethidium bromide.

2.4 | Carotenoid, tocopherol, and chlorophyll analyses

Fruit pieces (0.3 g) were homogenized and subjected to extraction with 10 mL hexane:acetone:methanol (50:25:25) for 30 min at 4°C with continuous gentle shaking. Then, 1 mL of water was added and samples were centrifuged for 5 min. The organic layer was recovered and dried in a speedvac. The solid residue was then resuspended in ethyl acetate:acetone:methanol (20:60:20), filtered, and frozen (-80°C) until analysis. Carotenoids were quantified in an Agilent 1200 series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Waldbronn) using a Kinetex-XB C18 fused core column (150 mm length × 4.6 mm internal diameter, and 2.6 μm particle size) from

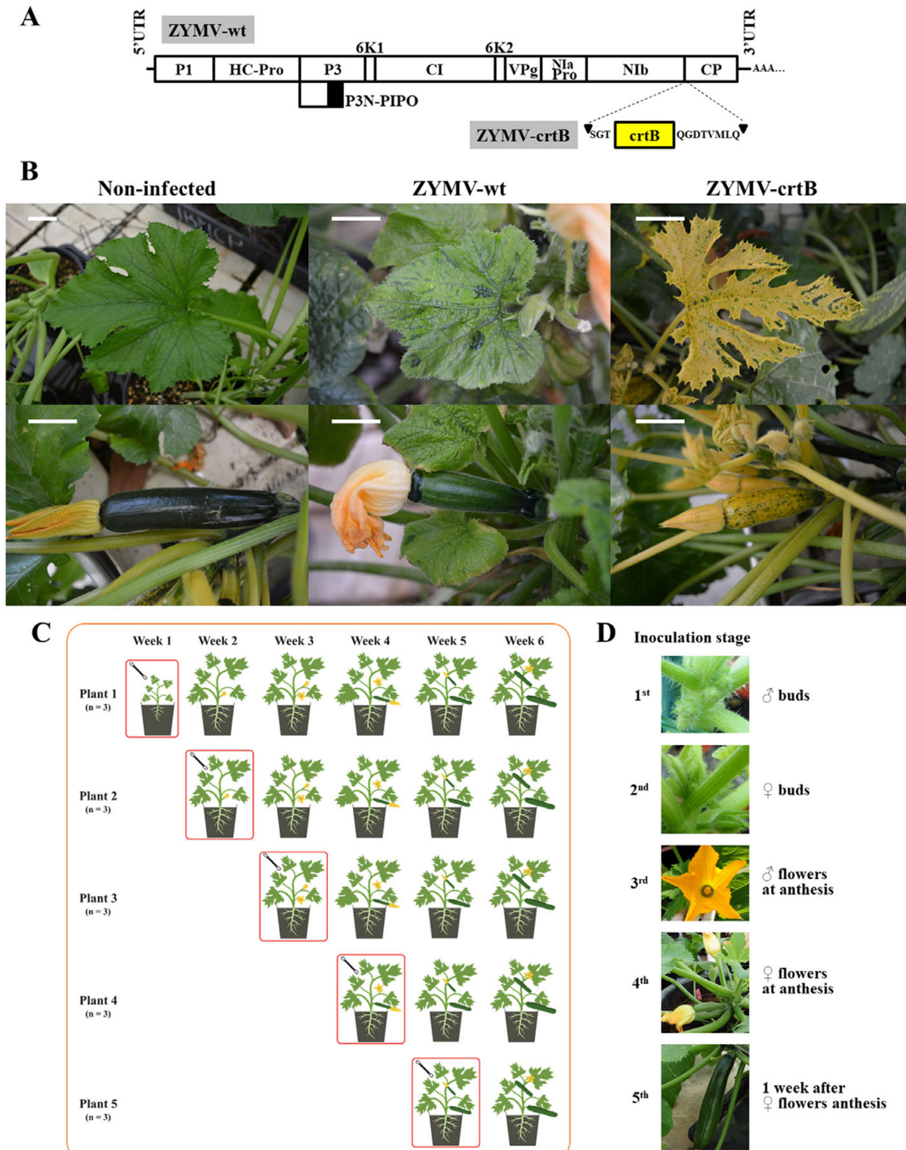

FIGURE 1 Inoculation of zucchini plants with ZYMV-wt and ZYMV-crtB. (A) Schematic representation of ZYMV-wt and ZYMV-crtB. Lines represent the virus 5′ and 3′ untranslated regions (UTR) and boxes the different viral cistrons (P1, HC-Pro, P3, P3N-PIPO, 6K1, CI, 6K2, VPg, NIaPro, NIb, and CP), as indicated. crtB is indicated by a yellow rectangle. Inserted amino acid sequences that flanked crtB and complement the split NIb/CP proteolytic site in ZYMV-crtB are indicated. (B) Representative pictures of leaves and fruits from zucchini plants noninfected or infected with ZYMV-wt or ZYMV-crtB, as indicated. Pictures were taken at approximately 1 month after inoculation. Scale bars represent 5 cm. (C) Plants, in groups of three, were mechanically inoculated (red rounded rectangle) at 1 week intervals, such that each group became infected at a different developmental stage. (D) Pictures of flower and fruit developmental stages at which plants were inoculated

Phenomenex (Torrance) following a published procedure,[16] with minor modifications. Briefly, 10 μl aliquots of the samples were injected and separated using a mobile phase with two components, solvent A (acetonitrile:methanol:water, 84:9:7 v:v:v) and solvent B (methanol:ethyl acetate, 68:32 v:v). Elution consisted of a linear gradient from 100% solvent A to 100% solvent B for 12 min; an isocratic elution of 100% B was then maintained for 7 min. Afterwards, a linear gradient to 100% solvent A was applied for 1 min, followed by an isocratic elution for 4 min to allow the column to re-equilibrate. Each sample was analyzed in duplicate. α-carotene, β-carotene, and lutein absorbance were measured at 445 nm, and phytoene absorbance at 295 nm. Chlorophyll a and b were determined at 445 nm following the same procedure. Tocopherols were determined in the same extract obtained for carotenoid analysis and using the same separation methodology, but in this case, detection was performed using a fluorimetric detector at an excitation $\lambda = 290$ nm and emission $\lambda = 330$ nm.[17]

2.5 | RNA quantification

RT-quantitative PCR (RT-qPCR) was performed to quantify the relative expression of endogenous zucchini *Phytoene synthase A*

(*PSY-A*; Cp4.1LG13g05570, http://cucurbitgenomics.org/) and exogenous *crtB* mRNAs in leaves and fruit tissues from plants noninfected and infected with ZYMV-wt or ZYMV-crtB. Total RNA was isolated from four biological replicates per treatment using the Extrazol reagent (BLIRT) and quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). After DNase I (Thermo Scientific) treatment, aliquots of 1.5 μg RNA were subjected to RT as explained above but using an oligo(dT) primer. A LightCycler 480 (Roche Diagnostics) was used to conduct qPCR in duplicate using aliquots of 1.5 μl of cDNA in a final volume containing 7.5 μl of 2 × FastStart Essential DNA Green Master (Roche), 1.5 μl 100 nM each primer and 1.5 μl of H_2O. *Actin* (Cp4.1LG08g10630) was selected as *C. pepo* control reference gene to normalize expression levels. Primer pairs previously described[18,19] were used to quantify *PSY-A* and *Actin* mRNAs. For *crtB* RNA amplification, primers PIV (5′-CTACGGCGAAGCAGGTTTAC-3′) and PV (5′-AGTAAGGGCCTGACCAGAGG-3′) were designed using the Primer3Plus software.[20] Reaction conditions consisted of 5 min at 95°C followed by 40 cycles of 5 s at 95°C, 30 s at 60°C, and 15 s at 72°C. Relative mRNA levels were obtained using the $2^{-\Delta Ct}$ method (ΔCt = Ct target gene – Ct reference gene).[21]

2.6 | Statistical analysis

Quantitative data were subjected to analysis of variance (ANOVA) using the StatGraphics statistical software (Centurion version XVIII, Statpoint Technologies Inc.). Fisher's least significant difference (LSD) post hoc test was used to evaluate differences among means.

3 | RESULTS

3.1 | Inoculation of zucchini plants with ZYMV-crtB at the right developmental stage induces yellow-orange fruits

We previously observed that inoculation of zucchini plants with a ZYMV-based vector that expresses *P. ananatis* crtB (ZYMV-crtB) caused infected leaves to turn bright yellow.[5] Figure 1B shows representative pictures of zucchini plants noninfected and infected with wild-type ZYMV (hereafter ZYMV-wt) or ZYMV-crtB. We have, more recently, understood that this phenotype is a consequence of the heterologous crtB enzyme inducing phytoene accumulation beyond a threshold that triggers transformation of leaf chloroplasts into chromoplasts, which is accompanied by the accumulation of high levels of downstream carotenoids.[6] To test whether we could trigger the chloroplast-to-chromoplast transformation in fruits of commercial size, with concomitant carotenoid overaccumulation, we grew seedlings of a zucchini cultivar (MU-CU-16),[22,23] which produces marketable dark green uniformly cylindrical fruits, and mechanically inoculated the plants (n = 3 or n = 4) with ZYMV-crtB at 1 week intervals (Figure 1C). These intervals corresponded to five different developmental stages (Figure 1D): plants with (1st) male buds, (2nd) female buds, (3rd) male flowers at anthesis, (4th) female flowers at anthesis, and (5th) female flowers 1 week after the anthesis, with fruits of approximately 20 cm.

All inoculated plants showed the first symptoms of infection at approximately 7 dpi and symptomatic tissue turned yellow during the following days (Figure 2A), as previously reported.[5] Interestingly, the development of flowers and fruits of infected plants was very different depending of plant developmental stage at inoculation time. Plants inoculated at the male bud stage (1st) did not develop female flowers. In contrast, those inoculated at the female bud stage (2nd) resulted in completely yellow female buds (Figure 2B), although flowers stopped growing before reaching anthesis. Zucchini plants inoculated at the male flower anthesis (3rd stage) completed flower development and female flowers reached anthesis (Figure 2C). However, fruits did not continue growing after pollination (Figure 2D). Despite the fruits remaining immature, these plants produced different types of fruits. Those below the inoculated leaves showed less yellow pigmentation than those above the inoculated leaf (Figure 2E). Interestingly, plants inoculated having female flowers at anthesis (4th stage) developed fruits after pollination, which displayed different degrees of green to yellow-orange color in their rinds (Figure 2F), having a uniformly orange flesh (Figure 2G). Plants inoculated 1 week after female flower anthesis (5th stage) developed fruits of commercial size. These fruits remain with dark green rinds or developed orange speckles (Figure 2H), but remarkably they exhibited a uniformly orange flesh (Figure 2I). Same results were obtained when plants at the different stages were inoculated with ZYMV-wt, although in this case no yellow-orange pigmentation was observed in any plant organ (Figure 1B). Taken together, these results suggest that a viral RNA vector can be used to specifically express a phytoene synthase, such as the bacterial crtB, in the fruits of adult zucchini plants to transform their color from green to yellow-orange. Importantly, although pigment accumulation is achieved at different developmental stages, plants with female flowers at or after the anthesis must be inoculated to produce fruit of commercial size with orange flesh.

3.2 | Pigmented tissues from inoculated plants contain the viral vector and exhibit high accumulation of *crtB* RNA

If pigmentation of zucchini fruits from inoculated plants results from viral vector-based expression of the bacterial crtB, these fruits, in contrast to green zucchinis from noninoculated controls, must contain the recombinant virus ZYMV-crtB. To confirm this prediction, we purified RNA from fruit samples of three predominantly yellow and three speckled zucchinis, each of them harvested from a different inoculated plant. As a control, we also purified RNA from three green zucchinis from three independent noninoculated plants. RT-PCR amplification of a cDNA corresponding to ZYMV CP cistron using specific primers demonstrated the presence of the virus in both the speckled and yellow zucchinis, but not in the green noninoculated controls (Figure 3A).

FIGURE 2 Photographs of zucchini plants inoculated with ZYMV-crtB. (A) Plants inoculated at 1st stage (male buds) that exhibit the first symptoms of infection and a bright yellow pigmentation at 7 and 14 dpi, respectively. (B) Yellow and green female buds from plants inoculated (14 dpi) at 2nd stage (female buds) and a noninoculated control, respectively. (C) Female flowers with yellow speckles at preanthesis (left) and anthesis (right) from plants inoculated at 3rd stage (male flowers at anthesis). (D) Preanthesis female flower from a plant inoculated at 3rd stage (left), compared to a noninoculated control (right). Longitudinal sections of the same flowers are also shown. (E) Female flowers from a plant inoculated at 3rd stage, developed over the inoculated leaf, exhibiting intense yellow pigmentation. (F) Fruit (upper) from a plant inoculated at 4th stage (female flowers at anthesis), compared to a noninoculated control (lower) at 14 dpi. (G) Details of the uniformly orange flesh of a fruit from an inoculated plant (left) compared to a control (right), at 14 dpi. (H) Fruits of commercial size from plants inoculated at 5th stage (1 week after anthesis of female flowers). From left to right, noninoculated control and fruits exhibiting different degrees of yellow-orange speckles at 5, 10, and 15 dpi. (I) Longitudinal sections of fruits from inoculated plants (left and center) that exhibited orange flesh compared to a control (right)

mRNA levels of virus-expressed *crtB* and the zucchini endogenous *PSY-A* were analyzed by RT-qPCR in leaves and fruits from plants non-infected and infected with ZYMV-wt or ZYMV-crtB. Results indicated that, while *PSY-A* mRNA levels were slightly reduced in tissues, particularly in fruit, infected with ZYMV-wt and ZYMV-crtB (Figure 3B), *crtB* mRNA strongly accumulated in both leaves and fruits from plants infected with ZYMV-crtB (Figure 3C). *crtB* RNA was, naturally, absent in tissues from plants noninfected or infected with ZYMV-wt, since this gene of bacterial origin is exogenous to zucchini (Figure 3C).

3.3 | Pigmented fruits from inoculated plants exhibit increased carotenoid accumulation

Another prediction was that the pigmented fruits produced by inoculation with ZYMV-crtB exhibited an increase in carotenoid accumulation. To confirm this, we extracted carotenoids from green and pigmented fruits harvested, respectively, from noninoculated and ZYMV-crtB-inoculated plants and quantified them after separation by HPLC. Analysis was performed at the fruit commercial ripening stage,

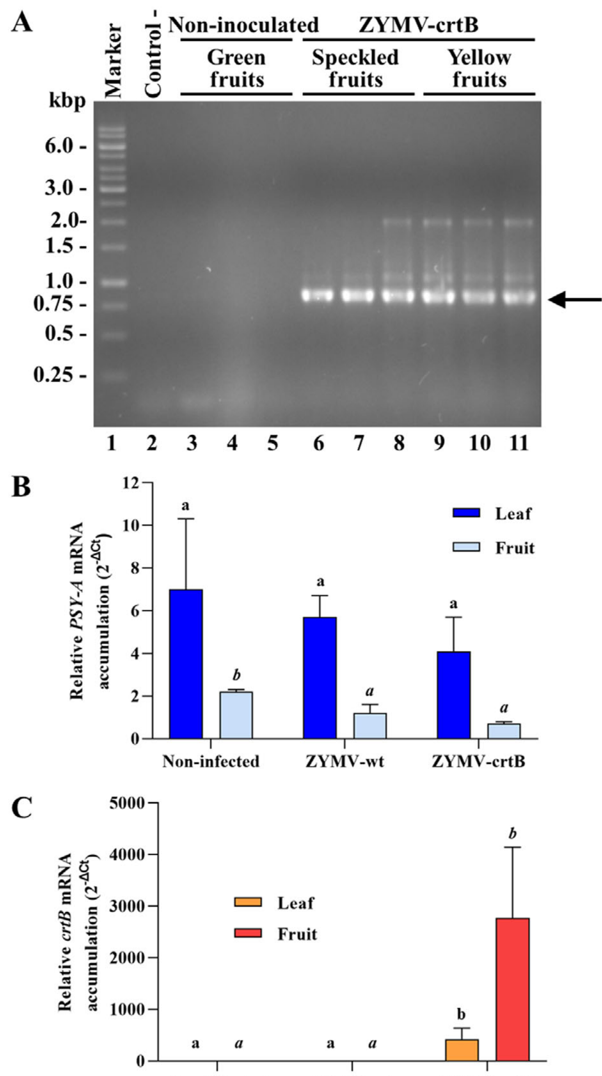

FIGURE 3 Diagnosis of ZYMV-crtB in zucchini fruits and RT-qPCR analyses. (A) RNA was purified and the ZYMV CP cistron amplified by RT-PCR. Products were separated by electrophoresis in a 1% agarose gel that was stained with ethidium bromide. Lane 1, DNA marker with some sizes (in kbp) on the left; lane 2, RT-PCR control with no RNA added; lanes 3–5, amplification products from green fruits harvested from noninoculated plants; lanes 6–8 and 9–11, amplification products from speckled (lanes 6 to 8) and fully yellow fruits (lanes 9 to 11) harvested from ZYMV-crtB-inoculated plants. Band corresponding to ZYMV CP cistron is indicated by an arrow on the right. RT-qPCR analysis of (B) zucchini endogenous *PSY-A* and (C) ZYMV-crtB-expressed *crtB* mRNAs. Columns represent mean relative mRNA accumulation in leaf and fruit, as indicated, of four independent plants noninfected and infected with ZYMV-wt or ZYMV-crtB, as indicated. Error bars represent the SD. Different letters for each tissue (regular, leaves; italics, fruits) indicate significant differences ($p < 0.01$) between the different treatments

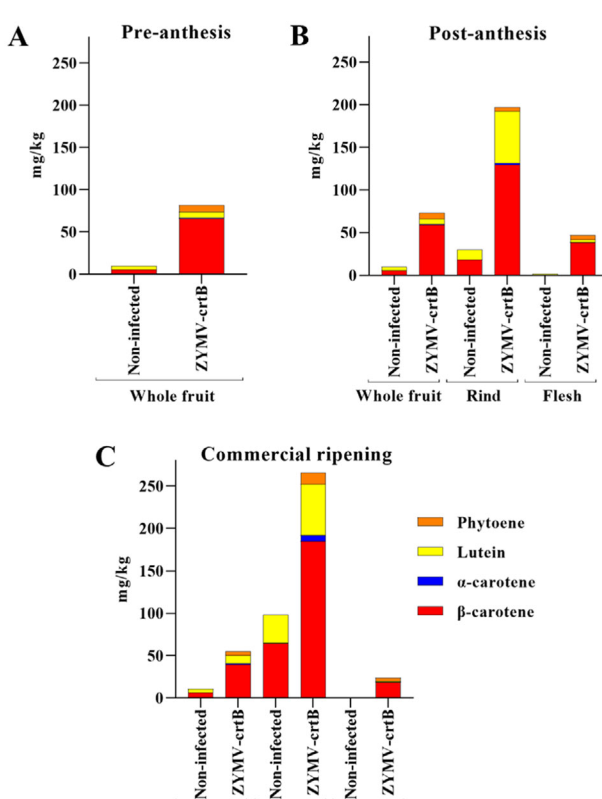

FIGURE 4 Accumulation of some representative carotenoids in whole fruit, rind, and flesh (as indicated) of green and yellow-orange zucchini fruits harvested from plants noninfected and infected with ZYMV-crtB, as indicated, at three developmental stages. (A) Preanthesis, (B) postanthesis, and (C) commercial ripening. Columns represent average accumulation in three or four fruits harvested from different plants. Numerical quantifications with SDs are in Table 1 and detailed quantitative data in Table S1

but also at two earlier developmental stages: fruit at postanthesis (medium size) and fruit at preanthesis (small size), sampled respectively from plants inoculated at 5th, 4th, and 3rd stages. Four representative carotenoids were quantified, namely phytoene, α- and β-carotene, and lutein. Remarkably, compared to the green controls from noninoculated plants, pigmented fruits from ZYMV-crtB-inoculated plants, at all three developmental stages, exhibited a dramatically increased accumulation of the four analyzed carotenoids in whole fruits, as well as in rind and flesh tissues (Figure 4, Table 1 and Table S1).

Fruits from ZYMV-crtB-inoculated plants exhibited up to 12-fold increase in β-carotene content relative to control fruits. At the commercial ripening stage, pigmented whole fruits contained 6-fold higher levels of this provitamin A carotenoid compared to controls. Although β-carotene amounts were higher in rinds, increases were more important in flesh, as that of control fruits accumulate insignificant amounts of this pigment. Conversely, lutein amounts were nearly doubled in whole fruits from inoculated plants at commercial ripening stage, mostly caused by a similar increase in the edible fruit rind (Figure 4, Table 1 and Table S1). In addition to these two main carotenoids, accumulation of phytoene and α-carotene in fruits of inoculated plants was particularly relevant, since content of these two carotenoids in control fruits was negligible (Figure 4, Table 1 and Table S1). Taken together, these results indicate that zucchini fruits from ZYMV-crtB-inoculated plants not only develop an attractive yellow-orange color, but are highly enriched in healthy carotenoids.

TABLE 1 Carotenoid content (mg kg^{-1} fresh weight; mean ± SD, n = 3 or 4) of zucchini fruits at different stages of development in noninoculated controls and ZYMV-crtB-inoculated plants. For the commercial ripening and postanthesis fruit sizes, rind and flesh were also separately analyzed. Different letters for each developmental stage and tissue indicate significant differences ($p < 0.05$) between treatments. Detailed quantitative data in Table S1.

Fruit size			**Pre-anthesis (small size)**			
Tissue			**Whole fruit**			
Treatment			**Non-inoculated**			**ZYMV-crtB**
β-carotene			5.41 ± 0.42^{a}			65.75 ± 3.37^{b}
α-carotene			0.00 ± 0.00^{a}			0.99 ± 0.10^{b}
Lutein			4.27 ± 0.37^{a}			7.03 ± 0.66^{b}
Phytoene			0.02 ± 0.01^{a}			7.73 ± 0.29^{b}
Fruit size	**Post-anthesis (medium size)**					
Tissue	**Whole fruit**		**Rind**		**Flesh**	
Treatment	**Non-inoculated**	**ZYMV-crtB**	**Non-inoculated**	**ZYMV-crtB**	**Non-inoculated**	**ZYMV-crtB**
β-carotene	5.67 ± 0.61^{a}	58.97 ± 3.60^{b}	18.23 ± 1.98^{a}	129.63 ± 0.50^{b}	0.13 ± 0.08^{a}	38.67 ± 6.15^{b}
α-carotene	0.00 ± 0.00^{a}	0.82 ± 0.11^{b}	0.00 ± 0.00^{a}	1.83 ± 0.02^{b}	0.00 ± 0.00^{a}	0.32 ± 0.08^{b}
Lutein	4.30 ± 0.60^{a}	6.26 ± 1.28^{b}	11.82 ± 0.93^{a}	60.64 ± 0.20^{b}	1.44 ± 0.20^{a}	2.69 ± 0.39^{b}
Phytoene	0.07 ± 0.01^{a}	7.08 ± 0.56^{b}	0.00 ± 0.00^{a}	5.05 ± 0.00^{b}	0.15 ± 0.04^{a}	5.29 ± 1.53^{b}
Fruit size	**Commercial ripening**					
Tissue	**Whole fruit**		**Rind**		**Flesh**	
Treatment	**Non-inoculated**	**ZYMV-crtB**	**Non-inoculated**	**ZYMV-crtB**	**Non-inoculated**	**ZYMV-crtB**
β-carotene	6.35 ± 2.11^{a}	39.78 ± 5.17^{b}	64.54 ± 34.92^{a}	184.93 ± 59.03^{b}	0.05 ± 0.09^{a}	18.83 ± 1.35^{b}
α-carotene	0.00 ± 0.00^{a}	1.26 ± 0.15^{b}	0.32 ± 0.37^{a}	6.89 ± 2.59^{b}	0.00 ± 0.00^{a}	0.21 ± 0.03^{b}
Lutein	4.54 ± 1.00^{a}	9.51 ± 1.41^{b}	33.56 ± 16.94^{a}	60.35 ± 31.04^{b}	0.99 ± 0.08^{a}	1.22 ± 0.05^{b}
Phytoene	0.00 ± 0.00^{a}	4.44 ± 0.15^{b}	0.04 ± 0.05^{a}	13.37 ± 4.14^{b}	0.00 ± 0.00^{a}	3.71 ± 0.96^{b}

To discard that increased carotenoid accumulation was the simple consequence of viral infection instead of the virus-based expression of crtB, zucchini plants (n = 4) were again inoculated at the female anthesis stage with ZYMV-crtB, but also with the empty virus (ZYMV-wt), as a control. Fruits were harvested at the commercial ripening stage. Metabolic analysis showed significant increments of phytoene and β-carotene in whole fruit and rind tissues from plants infected with ZYMV-crtB when compared to those harvested from plants non-infected or infected with ZYMV-wt (Figure S1 and Table S2). Significant increments in α-carotene and lutein were also observed in this experiment at the whole fruit level (Table S2). However, it is worth noting that these two experiments were performed at two different growing seasons, the optimal spring-summer for the first and a suboptimal summer-autumn for the second.

3.4 | Tocopherol and chlorophyll accumulation in pigmented fruits from inoculated plants

Carotenoids are synthesized from geranylgeranyl diphosphate (GGPP), a precursor that is also used for the production of phytol for chlorophylls, but also for other nutritionally relevant plastidial isoprenoids such as tocopherols (vitamin E).[24–26] The crtB enzyme is known to convert GGPP into phytoene, the first committed step of the carotenoid pathway. To test whether increased diversion of GGPP toward the carotenoid pathway might negatively impact tocopherol accumulation, the levels of these isoprenoids were also determined in the rind and whole fruit at the commercial ripening stage. ZYMV-crtB-inoculated plants exhibited not a drop but an increase in α- and γ-tocopherol contents, especially in the rind of the fruits (Figure S2A, and Table S3). Again, to analyze the potential contribution of viral infection on tocopherol accumulation, plants (n = 4) at the female anthesis stage were noninoculated or inoculated with ZYMV-wt or ZYMV-crtB. Fruits were harvested at the commercial ripening stage and tocopherols analyzed in whole fruit and rind. In this experiment performed in the suboptimal summer-autumn growing season, some differences were only observed in α-tocopherol among tissues from noninfected plants and plants infected with either ZYMV-wt or ZYMV-crtB (Figure S2B and Table S4). Finally, chlorophyll analysis in these last tissues showed a significant decrease of both chlorophylls a and b in whole fruit and rind from plants infected with ZYMV-crtB with respect to those non-infected or infected with ZYMV-wt (Figure S3 and Table S5).

4 | DISCUSSION

The goal of this work was to explore whether RNA virus-based vectors could be used to metabolically fortify edible fruits of cultivated

plants in a process that could be considered free of genetically modified DNA. Although RNA virus vectors have been instrumental in some recent plant metabolic engineering advances, these achievements mainly focused on production of metabolites in leaf tissues and in model plants.[4–7] To this aim, we analyzed the content of health promoting carotenoids in the flesh and rind of zucchini fruits harvested from plants inoculated with a ZYMV vector that expresses a bacterial phytoene synthase (ZYMV-crtB). Interestingly, our inoculation analysis indicated that yellow-orange zucchinis of commercial size can be harvested from these plants, provided they contain female flowers at or after the anthesis at the moment of inoculation (Figure 1 and 2).

Yellow-orange zucchinis harvested from ZYMV-crtB-inoculated plants exhibited a substantial accumulation of highly valuable dietary isoprenoids, such as the carotenoids phytoene, lutein, and others with provitamin A activity (β-carotene and α-carotene) (Figure 4 and Table 1). Analysis of fruit tissues from plants infected with an empty virus control (ZYMV-wt) indicated that the increased carotenoid accumulation is not a simple consequence of viral infection (Figure S1). However, this experiment also indicated an effect of growing season on accumulation of specific carotenoids, as previously reported for grapevine.[27]

Phytoene synthesis is the first committed step in carotenoid biosynthesis. Carotenoids provide some of the most important nutritional benefits of consuming squash. More specifically, β-carotene and lutein are the most abundant carotenoids in the fruits of *C. pepo*, although these carotenoids are only abundant in those fruits with yellow-orange flesh that are consumed when fully mature.[28,29] Zucchini fruits are consumed immature and naturally contain low amounts of these two carotenoids. Besides being the main precursor of vitamin A, β-carotene displays antioxidant activity that inhibits DNA damage and enhances immune system.[24] Lutein is a nonprovitamin A carotenoid involved in the macular pigment of the human retina and its intake decreases the risk of macular degeneration and other ocular diseases.[30] Phytoene is a health-promoting carotenoid normally absent from green fruits and vegetables, and α-carotene has provitamin A activity (although only half that of β-carotene).[24]

Since crtB converts GGPP into phytoene and this metabolite is also the precursor for other relevant plastidial isoprenoids such as tocopherols and chlorophylls,[24–26] we investigated a possible interference with accumulation of these metabolites in zucchini fruits of inoculated plants. Surprisingly, in our spring-summer experiment flesh and rind of zucchini fruits from ZYMV-crtB-inoculated plants accumulated higher amounts of α- and γ-tocopherol (vitamin E) compared to noninoculated controls. This differential accumulation was not repeated in the summer-autumn experiment (Figure S2 and Tables S3 and S4). In the summer-autumn growing cycle, lower accumulation of carotenoids, tocopherols and chlorophylls were in general detected, probably related to the lower radiation and temperature of the growing season. Note that although we grew zucchini plants in a glasshouse with supporting illumination and controlled temperature, plant growth is still influenced by external environmental conditions. The change in tocopherol profile, with an increase in the relative content of α-tocopherol (compare Figure S2A and B) suggests an environmental effect, as these changes have been associated with lower temperatures and shading in other crops.[31] No significant increments in tocopherols, lutein and α-carotene contents were detected in the summer-autumn suboptimal growing season when compared to the optimal spring-summer season, which might be related to a more limited supply of substrates to the pathway and preferential biosynthesis of β-carotene. In contrast to tocopherol accumulation, flesh and rind of zucchini fruits from ZYMV-crtB-infected plants exhibited decreased chlorophyll content (Figure S3 and Table S5). Chlorophyll degradation is normally associated to viral infection and it has also been observed upon virus-based expression of crtB in tobacco leaves.[5] Transition to chromoplasts also entails chlorophyll degradation.[32] It is possible that the phytol released upon chlorophyll breakage might be recycled to produce tocopherols in the zucchini fruits of virus-inoculated plants. As a result, in our spring-summer experiment, vitamin E levels were enriched 5-fold in whole fruits and 6-fold in the rind. Some zucchini yellow cultivars have been obtained introgressing the *B* gene (precocious yellow fruit color), which affects plastogenesis, bypassing the proplastid to chloroplast transition and preventing the accumulation of chlorophylls. However, these cultivars exhibit a compromised tocopherol accumulation in fruit flesh.[33] In addition to their nutritional interest, tocopherols are plant antioxidants involved in physiological processes such as abiotic stress tolerance. *B* gene expression reduced total tocopherol content in fruit rinds, which was associated with postharvest chilling sensitivity.[34]

In summary, metabolically fortified zucchini fruits of a visually attractive yellow-orange color can be produced by using a viral RNA vector to trigger carotenoid overaccumulation, with no manipulation of the plant genome. This strategy is easier and faster than both classic breeding and conventional genetic engineering. The use of engineered RNA viruses in metabolic fortification of edible plant organs, subjected to appropriate regulation,[35] may contribute to bring new products to the market.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in article supplementary material and from the corresponding author upon reasonable request.

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