The esterification of xanthophylls in tomato chromoplasts; the role of a non-specific acyltransferase?

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Abbreviations. pale yellow petal (*pyp*), *Crt*Z and *Crt*W (ZW), high β-carotene background (RI), High Resolution Melt (HRM), polymerase chain reaction (PCR), quantitive real time PCR (qRTPCR).

Abstract

The esterification of carotenoids has been associated with high-level accumulation, greater stability and potentially improved dietary bioavailability. Engineering the formation of ketocarotenoids into tomato fruit has resulted in the esterification of these non-endogenous metabolites. A genotype of tomato was created that contains; (i) the mutant pale yellow petal *(pyp)*1-1 allele, which is responsible for the absence of carotenoid esters in tomato flowers and (ii) the heterologous enzymes for ketocarotenoid formation. Analysis of the resulting progeny showed altered quantitative and qualitative differences in esterified carotenoids. For example, in ripe fruit tissues, in the presence of the *pyp* mutant allele, non-endogenous ketocarotenoid esters were absent while their free forms accumulated. These data demonstrate the involvement of the *pyp* gene product in the esterification of diverse xanthophylls.

Introduction

Replacing petrochemical derived chemical synthesis is a key component of the bioeconomy. This goal has led to intense efforts to create new renewable sources of valuable speciality chemicals, presently generated by chemical synthesis using approaches that have intrinsic poor environmental credentials. Carotenoids are an example of valuable chemicals that are used in a multi-sectorial commercial manner; as feed colourants, feed supplements and health promoting bioactives. They are also essential components of the human diet e.g. β–carotene (provitamin A). Present annual sales for carotenoids are in the region of 1.5 billion USD [1]. The production method of choice remains chemical synthesis, using petrochemical derived precursors and uses rare metals as catalysts. Metabolic engineering of the carotenoid pathway has generated numerous successful examples in various microbial and plant based chassis. In plant hosts high levels appear to accumulate in sink tissues such as tomato fruit. In the latter case economic, technical and production feasibility has been demonstrated for ketocarotenoids [2]. The hydroxyl/ketolated carotenoids, astaxanthin, phoenicoxanthin and canthaxanthin are of particular value to the aquaculture industry, where their use as feed additives is essential for intensive production and the aesthetic colour of the salmon flesh, required to attract an economic premium. It is estimated that 20% of the total cost of aquaculture is due to the cost of the carotenoid based feed additive alone.

In nature xanthophylls, which are oxygenated carotenoids, can exist in the free form or as an esterified derivative. Typically, the free form occurs in vegetative, chloroplast-containing tissues and esterified forms in chromoplast containing sink tissues. Flower tissues and *Capsicum* ripe fruits are good examples of where carotenoid esterification occurs and has been proposed to facilitate high level carotenoid accumulation. Recently, the identification of a tomato mutant with reduced flower pigmentation has illustrated the need for esterification to facilitate high level carotenoid accumulation in flower tissues and presumably xanthophyll containing fruits [3]. The gene conferring this phenotype has been termed pale yellow petal and the gene product an acyltransferase type enzyme. Interestingly, in tomato fruit which typically accumulate high levels of the acyclic red coloured carotene lycopene when the pathway is extended to produce non-endogenous ketolated and hydroxylated carotenoids, the resulting hydroxylated products are also esterified with fatty acids. This raises the questions; what enzyme(s) are responsible for this esterification of non-endogenous carotenoids in tomato fruit and is the esterification process essential for high level accumulation?

In the present study a new tomato genotype has been generated with the *pyp*1-1 mutant allele and ketocarotenoid biosynthetic pathway. This resource has elucidated the gene responsible for carotenoid esterification in tomato fruit and quantitative characterisation of carotenoid levels provides an insight into the role of esterification in carotenoid sequestration and turnover, as a complementary aspect to traditional pathway engineering.

Materials and Methods

Plant materials and cultivation.

Tomato seed for the *pyp* mutant in the microtom background (TOMJPE5508-1) was provided by University of Tsukuba, Gene Research Centre, through the National Bio-Resource Project (NBRP) of the AMED, Japan [4]. The ZW(Ø)RI(Ø) tomato line(s) were developed in-house [2]. Seeds were sown on F2+sand compost (Scotts Levington) then transferred into M3 growing media (Scotts Levington) once established. All tomato varieties were grown under glasshouse conditions with supplementary lighting, with day temperatures of approximately 25°C for 16 hours and night temperatures of 15°C for eight hours.

Molecular analyses.

Extraction of DNA and RNA

Genomic DNA was extracted from leaf material using Qiagen DNeasy plant mini kit (Qiagen Ltd. Crawley UK) using the manufacturer’s standard protocol.

Total RNA was extracted from fruit pericarp for use in quantitative real time reverse transcriptase PCR (qRT-PCR) using Qiagen RNeasy plant mini kit (Qiagen Ltd. Crawley UK) using the manufacturer’s standard protocol including on-column DNaseI digestion. cDNA was generated from the RNA pool using the Quantitect RT kit (Qiagen Ltd. Crawley UK).

Detection of the Solanum galapagnese lycopene β-cyclase allele (RI).

PuReTaq Ready-to-go PCR beads in a BioRad T100 thermocycler were used to detect the presence of the *S. galapagense* β-cyclase allele as per manufacturer's recommendations. Primer sequences are provided in supplementary table 1.

Screening for the presence of the pyp mutation.

A High Resolution Melt (HRM) approach using the Rotor-Gene Q (Qiagen Ltd. Crawley UK) and the Type-it HRM PCR kit (Qiagen Ltd. Crawley UK) was used to detect the presence of the *pyp* mutation. The programme conditions used were cycling and melt conditions of 95°C five minutes, 40 cycles of 95°C 10 seconds, 55°C 30 seconds, 72°C 10 seconds followed by an HRM from 65°C to 95°C increased in 0.2°C increments. The primer sequences used are provided in supplementary table 1.

Determination of pyp transcript levels.

The Rotor-Gene SYBR green® PCR kit (Qiagen Ltd. Crawley UK) was used to determine the expression level of the *pyp* gene. Determinations were made and normalised to *actin*. A minimum of three biological replicates were used and reactions were run on a Rotor-Gene Q with cycling conditions of 95°C five minutes, 40 cycles of 95°C five seconds, 60°C 10 seconds, then melt analysis from 50°C to 99°C in 1°C increments. For quantification calibration curves were run simultaneously with the samples and Ct calculations were made using the Rotor-Gene software. The primers used for these assays are provided in supplementary table 1.

Biochemical analysis

The analysis of carotenoid pigments.

Carotenoids were extracted as previously published [5] using 10 mg of freeze-dried fruit, leaf, petals or stamen material (10 mg). The separation, detection and identification of carotenoids present in the fruit, leaf and flower tissues were screened using ultra-high pressure liquid chromatography (UPLC) as described in [5] with a modified gradient of 50% A [methanol:water 50:50 (v:v)], 50% B [acetonitrile:ethyl acetate 75:25 (v:v)] for 30 seconds, 30% A, 70% B for four minutes 30 seconds, 0%

A, 100% B for two minutes, 30% A, 70% B for one minute before finishing on 50% A, 50% B for two minutes. High pressure liquid chromatography (HPLC) was also used to analyse carotenoid esters from flower tissue as described in [6]. Chlorophyll amounts were estimated by measuring the absorbance at 410 nm, 430 nm and 450 nm for pheophytin, chlorophyll a and chlorophyll b respectively.

The identification of carotenoid esters was performed using an Agilent infinity II (1290) LC-HRMS Q-TOF (Agilent 6560). Metabolites were separated on a C30 reverse phase column (3 µm, 150 mm x 2.1 mm). The mobile phase consisted of methanol with 0.1% formic acid (A) and *tert*-methyl butyl ether with 0.1% formic acid (B). The gradient ran from 100% A / 0% B for two minutes, changing to 80% A / 20% B over one minute, holding for three minutes, then a linear gradient to 30% A / 70% B over four minutes, holding for 10 minutes, then a linear gradient to 100% A / 0% B for two minutes with a final hold for five minutes. A flow rate of 0.2 ml/minute was used. Ionisation was provided by APCI in positive mode. Capillary temperature was 250 °C and vaporisation temperature was 450 °C. The mass was scanned from 100 – 1700 m/z with MS/MS between 700 and 1700 m/z.

Analysis of semi-volatile components.

Fresh whole fruit for semi-volatile analysis was homogenised in a blender and the juice kept frozen until analysis. 2 g ± 0.2 g was weighed into a glass vial and an internal standard of 10 ppm acetophenone-β, β, β-D3 (Sigma-Aldrich, USA; 10 µl) added. A blank of air was also included in each run.

Analysis was performed using a GC/MS (Agilent 7890B and 5977B MSD). Sample application was performed using a Gerstel multipurpose sampler (MPS) (Gerstel, Germany). Samples were incubated in the MPS heated to 60°C with shaking at 300 rpm for 30 minutes, after 10 minutes the fibre was introduced to a depth of 25 mm. A StableFlex solid-phase microextraction (SPME) fibre assembly of divinylbenzene, carboxen, polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm fibre with 23 gauge needle (Supelco, USA) was used for sampling. After head space sampling the fibre was introduced 54 mm into the injection port lined with a 0.75 mm straight/SPME inlet liner (Restek Corp, USA). The injection port was maintained at 250°C and the fibre was kept here for five minutes for complete desorption. A splitless mode was used with a solvent delay of 3.8 minutes and septum purge flow of 3 ml/minute. Volatiles were separated using a J&W HP-5ms GC Column, 30 m, 0.25 mm, 0.25 μm (Agilent) with helium as the carrier gas at 1.0 ml/min linear velocity. The oven gradient was 40°C two minutes, then increased by 5°C per minute to 120°C maintained for two minutes, then increased 5°C per minute to 250°C maintained for two minutes, then increased 6°C per minute to 300°C maintained for five minutes. After separation of the metabolites through the GC they entered the MS via a 250°C transfer line. The MS source was maintained at 230°C, the MS quadrupole was held at 150°C. Normal scan mode was used ranging from 30 m/z to 550 m/z using 70 eV positive electron impact ionisation (EI+).

Raw data was processed through automated mass spectral deconvolution and identification system (AMDIS). Compounds were identified using an in-house library which was constructed from standards and the NIST08 library with relative quantification to the internal standard. Full identification parameters of compounds are in supplementary table 4 and 6.

Statistical Analysis

Data has been presented as the mean of the biological replicates ± standard deviation. Statistical analysis was performed using XLSTAT premium 2019.4.2 (Addinsoft). Analysis of variance (ANOVA) or T-test were used as stated. Graphs were produced using Graphpad Prism 8 or MetaboAnalyst 4.0 [7].

Results

The generation of *pyp*/ketocarotenoid producing genotypes.

Previous studies have generated a stable ketocarotenoid producing line [2, 8]. This transgenic line was generated by constitutively expressing a β-carotene 4, 4' oxygenase (*Crt*W) and β-carotene 3, 3' hydroxylase (*Crt*Z) from the marine bacteria (*Brevundimonas Sp*.) in the MoneyMaker background of tomato. This ZW line, which is only viable when hemizygous for the *Crt*W and *Crt*Z transgenes, was crossed with a UC204B background containing the *Solanum galapagnese* fruit lycopene β-cyclase (B-*CYC*) allele (designated RI) responsible for high levels of β-carotene production. The resulting stable lines, designated ZWRI, produced optimal phoenicoxanthin esters. Pollen from the *pyp* mutant, in the

Microtom background of tomato was transposed onto the stamen of a ZWRI line, to create an F_1 seed population. The *pyp* mutation has been reported to be recessive in nature. Therefore, selection of genetic components was carried out in the F_2 generation, created from self-fertilisation of the F_1 population. Genotyping for the ZW (ketocarotenoid) and RI (β-carotene) components among the F_2 lines was performed on 6 week old seedlings. The presence of the ZW component was screened visually for brown leaf colouration. The presence of the RI component was detected by PCR.

The mutation in the *pyp* allele used is a single base change [3]. To detect the absence or presence of the *pyp* allele and its zygosity, High Resolution Melt (HRM) was carried out. From over 200 F_2 seedlings screened for ZW in the hemizygous or azygous state, and RI in the homozygous or azygous state, approximately 60 plants remained for *pyp* screening by HRM. The genotypes of interest recovered included: three lines with *pyp* in wild type state (P^{WT}) plus a ketocarotenoid phenotype (ZW) and the β–carotene allele (RI) present (P^{WT}ZWRI); five lines with the *pyp* mutation (P^{MT}) plus ZW and RI present (P^{MT}ZWRI), and one line with the *pyp* mutation (P^{MT}) in the absence of both ZW and RI (P^{MT}ZWØRIØ). A triple azygous (P^{WT}ZWØRIØ) control was generated from a cross of Microtom and ZWØRIØ. Figure 1 illustrates the screening workflow.

The most striking observation was the pale petals in all lines containing the *pyp* mutant allele, especially in the presence of the ZW ketocarotenoid producing component. However, the stamen of the flowers displayed no visual difference when the *pyp* mutant allele was present (Figure 1). The effect of *pyp* on the fruit phenotypes were negligible. The overall vigour of the plants did not appear compromised in the presence of the *pyp* mutation. However, phenotypic changes relating to the nature of the backgrounds used were observed. For example, the Microtom like background was observable in some lines, while in others the indeterminate, MoneyMaker like phenotypes were present. Despite these differences in plant architecture, fruit or flower colour appeared unaffected by the different backgrounds.

The effect of the *pyp* mutation on the flower and fruit carotenoid contents.

The *pyp* mutant was originally identified by the presence of pale petal coloration. Under the environmental conditions used in this present study, the azygous genotypes for ketocarotenoid production (ZWØRIØ), displayed paler petal coloration in the presence of the *pyp* allele, compared to the wild type *pyp* allele. Predominantly, this was due to reduced total carotenoid content [3] in the *pyp* flowers. Analysis of individual carotenoid components in petals showed the complete absence of esterified carotenoids and increased free violaxanthin and neoxanthin compared to the wild type allele (Table 1). Although the stamen showed a similar trend the reduction in carotenoids was not as pronounced and the visual colour reduction in the stamen not as dramatic (Supplementary table 2). Thus, these data independently corroborate previous findings. When esterification was prevented by the mutation in the *pyp* allele, the free forms of the carotenoids increase, for example 4-ketoantherxanthin showed a seven fold increase and phoenicoxanthin a four-fold increase. This is comparable to endogenous carotenoids such as violaxanthin where the content increased six times in the *pyp* mutant lines. Interestingly, those ketocarotenoids such as canthaxanthin that have no hydroxyl moieties were reduced (two fold). Overall, the total carotenoid content in the petal was also dramatically reduced.

Tomato fruit contain predominantly lycopene and β-carotene, neither of these carotenoids can be esterified due to the absence of hydroxyl moieties within their chemical structure. Lutein can potentially be esterified but in ripe fruit, it is never found to be present in the esterified form. This tradition profile of tomato carotenoid pigments is reflected in the UPLC-PDA traces displayed in Figure **2** where the presence of the wild type and mutant *pyp* allele has had no effect on the azygous (wild type) profile of carotenoids present in the ripe fruit. The only significant differences determined were decreased levels ζ-carotene (0.6-fold) with the presence of the mutant *pyp (*Table 2). A feature of the tomato fruit engineered to produce ketocarotenoids is the presence of the non-endogenous pigments in their esterified forms. LC-MS confirmed that the esterified ketocarotenoids were phoenicoxanthin (C14 and C16) and astaxanthin. Among lines (P^{WT}ZWRI) containing the wild type allele of *pyp*, over 1.5 mg/gDW of ketocarotenoid esters were formed. Subsequently in lines (P^{MT}ZWRI) where the mutant was present no esterified forms of ketocarotenoids in the fruit occurred.

Effect of carotenoid esterification on the profile of volatile metabolites?

The esterification of carotenoids is believed to provide greater stability both to enzymatic catabolism and non-enzymatic degradation [3, 9, 10], when compared to free carotenoids. In the case of carotenoids catabolism and degradation, some carotenoid derived have the potential to impact on fruit taste and aroma. Determining carotenoid derived products could potentially address the question of carotenoid stability and the role of esterification in this process. GCMS based headspace analysis of the azygous material with either the wild type or mutant revealed only two significantly different compounds; acetic acid, which was reduced three fold and *trans*, *trans*-2-6-nonadienal which was not detected in *pyp* mutant line (P^{MT}ZWØRIØ). These compounds are both associated with fatty acid catabolism.

Comparison of P^{MT}ZWRI and P^{WT}ZWRI revealed eight significantly different compounds, linked to isoprenoids. Phellandrene (0.4-fold), δ-carene (0.5-fold), cymene (0.4-fold) and β-pinene (0.2-fold) all showed a decrease in P^{MT}ZWRI. The levels of other volatiles derived from carotenoids such as β-cyclocitral and β-ionone were not altered (supplementary table 5). The fatty acid associated volatiles *n*-dodecane (0.2-fold) and acetic acid (1.7-fold) did not show a general trend,nor did the other two significant metabolites, 4-methylbenzaldehyde (2.1-fold) and propanoic acid, propanediyl diester (not detected in P^{MT}ZWRI).

Expression of *pyp* suggests other roles in tomato fruit

With the *pyp* mutation introducing an early stop codon into the Solyc01g098110 gene it was expected that the gene expression would be reduced. In order to investigate this, qPCR was used to assess the expression of *pyp* in fruit across all genotypes (Supplementary figure 2). The expression of *pyp* fruit carrying the mutation was reduced (10-fold). The presence of the ketocarotenoids, and therefore the presence of substrates suitable for esterification did not impact significantly on the expression of *pyp*. However, a trend of increased *pyp* expression was observed in fruit from the P^{WT}ZWRI line compared to the azygous comparator, further determinations with a greater pool of biological replication will help confirm the true significance of this trend. Despite an endogenous absence of carotenoid esters in tomato fruit, there was expression of *pyp* in azygous (P^{WT}ZWØRIØ) fruit. This strongly suggests there is an alternative role for *pyp* in tomato fruit.

Discussion

Previous studies have attributed the Pale Yellow Petal (*pyp*) gene product to the esterification of xanthophyll pigments in tomato flowers [3]. Introgression of the tomato *pyp* mutant allele has demonstrated that the *pyp* gene is transferable by genetic crossing and functions on endogenous flower xanthophylls in different tomato backgrounds. In the present case the studies have gone beyond the Microtom model genotype, and demonstrated functionally in established commercial genotypes, e.g. Moneymaker. The use of host genotypes engineered to produce valuable hydroxyl/keto carotenoids that are non-endogenous to tomato fruits, has also indicated that the *pyp* gene product is involved in the esterification of a broad range of hydroxyl/ketocarotenoids that are not endogenous to tomato. These data suggest we can attribute the involvement of PYP to multiple enzymatic steps in the heterologous pathway of ketocarotenoid formation. For example, the esterification of phoenicoxanthin, and astaxanthin (Figure 3). Given the esterification of these mono and bihydroxylated ketocarotenoids it is surprising that the zeaxanthin recently produced in tomato fruit is not esterified [11].

Although the present studies can attribute an involvement of PYP in the esterification of carotenoids, it is important to highlight that PYP may not be able to exert the necessary enzymatic activity directly. Potentially other proteins or macromolecular structures could be involved. This further analysis of the activity *in vitro* or as a homogenous protein will be necessary.

At the amino acid level the PYP shares 58% identity (73% similarity) with the phytol ester synthases (PES-1 and 2) identified in Arabidopsis [12]. These genes contain two domains, a lysophospholipid acyltransferase and hydrolase domain. These features are typical of enzymes that catalyse the incorporation of an acyl group from either acyl-CoAs or acyl-acyl carrier proteins (acyl –AcPs) onto acceptors such as glycerol 3-phosphate [3]. The PES enzymes have previously been shown not to act on carotenoids in leaf tissue but instead on phytol derived from degraded chlorophylls. Given that the present study indicates that the tomato PYP can act on a range of hydroxylated carotenoids including those not endogenous to tomato, the question arises; what are the other pathways/precursors that PYP can act on? For example, does the PYP enzyme work on other isoprenoids or lipids? As our data shows the *pyp* gene is expressed regardless of carotenoids present. Now the genetic resources have been created, untargeted metabolomics and lipidomics could address these questions. Recently, work has attributed the esterification of xanthophylls (lutein) in wheat to an enzyme (XAT acyltransferase) that belongs to the Gly-Asp-er-Leu (GDSL) esterase/lipase class of enzymes [13]. Thus, it would appear that plants have multiple enzymatic approaches to xanthophyll esterification. In wheat the process appears to occur in an extra-plastidial manner, with the esterification process proposed to improve carotenoid stability. The present study suggests that the tomato enzyme is a classical acyltransferase with a broad substrate specificity. It would appear that the formation of carotenoid esters in flower petals does contribute to carotenoid stability; as in the flower tissues analysis, the loss of esterified pigments and the corresponding appearance of free carotenoids, a maximum loss of 87.8% arises. In contrast in ripe fruit tissues the net loss in carotenoid from esters to the appearance of free carotenoid is 11.7%. Thus, it would appear that stability conferred by esterification is tissue-specific and in tomato fruit esterification does not enhance carotenoid stability. Presumably, in ripe tomato fruit esterification aids deposition/sequestration of carotenoids formed at high levels. Recently hydroxyl/ketocarotenoids have been shown to be preferentially stored in the plastoglobule as esterified carotenoids [8].

In conclusion, the data and resources generated demonstrate the involvement of PYP in the esterification of non-endogenous carotenoids in tomato chromoplasts, as indicated in Figure 3.

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Figure Legends.

Figure 1. Workflow for the generation, screening and phenotypic observations of the four genotypes created.

Figure 2. Example chromatograms showing the effect of the *pyp* mutation on azygous and ketocarotenoid fruit. 1) Astaxanthin, 2) Lutein, 3) Phoenicoxanthin, 4) Canthaxanthin, 5) 3'OH echinenone, 6) 3OH echinenone, 7) Echinenone, 8 and 8') Lycopene, 9) Esterified carotenoid, 10) γ-carotene, 11) esterified carotenoid, 12) β-carotene

Figure 3. Biosynthetic pathway of ketocarotenoids and ketocarotenoids esters.

*Crt*Z and *Crt*W are the bacterial carotenoid hydroxylase and oxygenase genes respectively. The *pyp* gene facilitates the esterification with fatty acids to generate ketocarotenoid esters. Phoenicoxanthin and astaxanthin accumulate within the genotypes assessed and esterified forms of these have been observed in this work.

Supplementary data

Supplementary table 1. Primer sequences used throughout this work.
Supplementary table 2. Effect of the *pyp* mutation on the carotenoid content of stamen.
Supplementary table 3. The determination of carotenoid and chlorophyll content of leaves from the P^{WT}ZWØRIØ P^{MT}ZWØRIØ.
Supplementary table 4. Chromatographic and spectral properties used in the identification of carotenoids and chlorophylls.
Supplementary table 5. Relative amounts of volatiles produced by different $P^{WT/MT}$ZW(Ø)RI(Ø) genotypes.
Supplementary table 6. Parameters used to identify semi-volatile metabolites from SPME GC/MS analysis.
Supplementary figure 1. Chromatographic profiles recorded at 450nm of flower extracts derived from P^{WT}ZWØRIØ, P^{MT}ZWØRIØ, P^{WT}ZWRI and P^{MT}ZWRI.
Supplementary figure 2. Relative expression level of *pyp* transcripts in tomato fruit from genotypes harbouring the *pyp*-mutant allele and ketocarotenoid formation.

Availability of data and materials: Processed data is available in the manuscript and appendices. Unprocessed data can be accessed after embargo at Mendeley data http://dx.doi.org/10.17632/bpgtgt65wz.1.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions: Experimental work, data analysis and presentation, and drafting the manuscript was carried out by ERL. EMAE and MN provided materials, supervision, PDF funding and supervision. All authors contributed to the writing –review, and conceptualisation,

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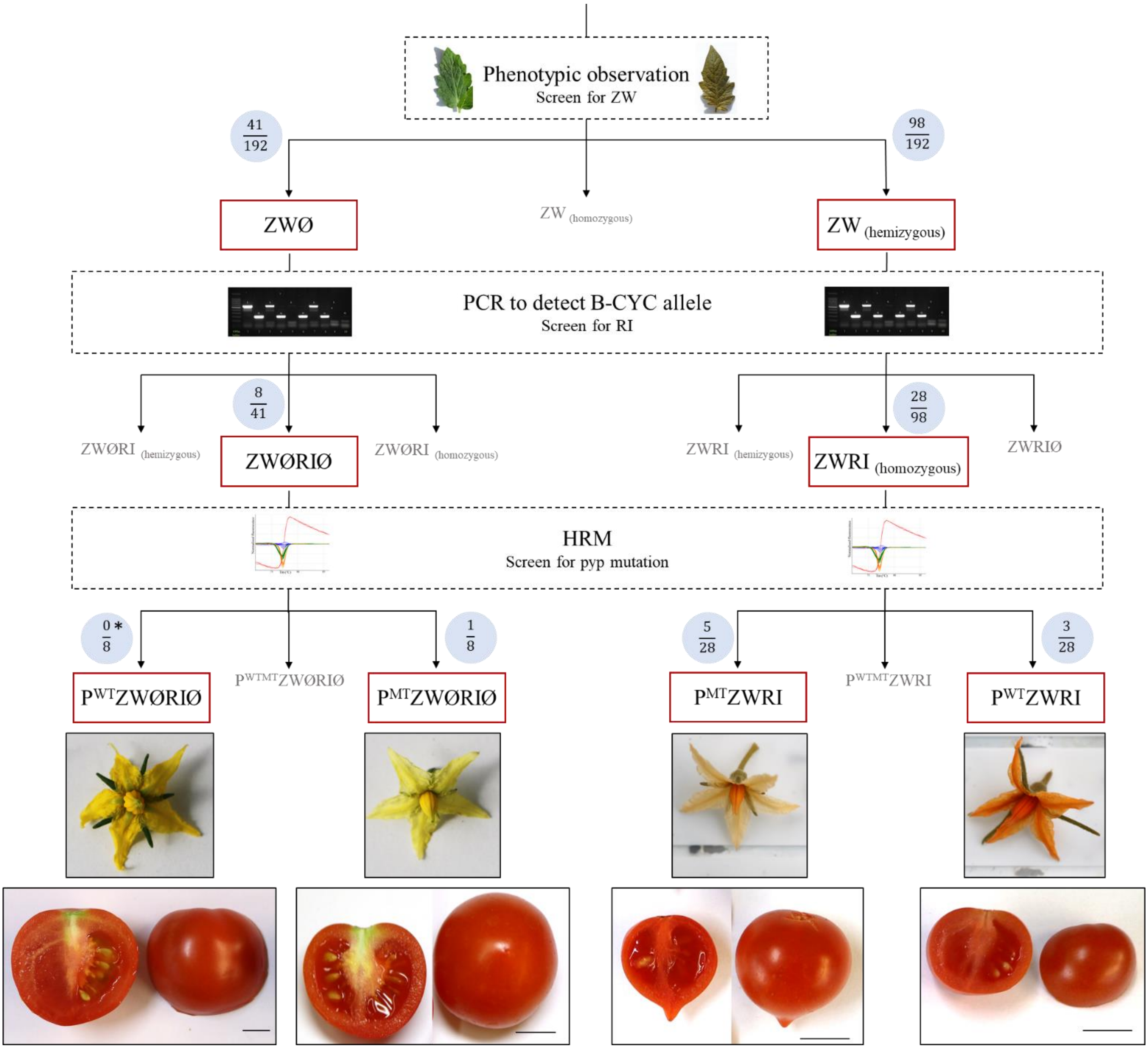

Figure 4. Workflow for the generation, screening and phenotypic observations of the four genotypes created.

The initial screen was phenotypic, the ZW genes produce ketocarotenoids in the leaf tissue, these colour the leaves brown. A PCR was then used to detect the *S. galapagense* and/or *S. lycopersicum* forms of the *B-CYC* promoter. Finally a HRM was used to detect the single base change which comprises the mutation in *pyp*1-1 plants. Scale bars represent 1 cm.

Table 1. Effect of the *pyp* mutation on the carotenoid content of ketocarotenoid containing petals.
Amounts in µg/g dry weight with ± standard deviation. Totals in mg/g. All values are calculated from three biological replicates, of 15 flowers per plant. Each biological replicate is an average of two technical replicates. Significant values from a Student's T test are in bold, * 0.05>p≥0.01, ** 0.01>p≥0.001, *** 0.001>p

Carotenoid (µg/g DW)	P^{WT}ZWØRIØ		P^{MT}ZWØRIØ		P^{WT}ZWRI		P^{MT}ZWRI	
Violaxanthin	**421.13**	**±70.37**	**1551.37**	**±209.45***	**99.68**	**±48.96**	**665.92**	**±179.32****
Neoxanthin	**84.71**	**±17.74**	**268.14**	**±64.69****	11.86	±6.69	50.90	±48.60
Lutein	53.36	±28.31	46.77	±5.36	0.00	±0.00	0.00	±0.00
Chlorophyll B	284.58	±119.63	101.46	±35.66	**156.98**	**±25.82**	**85.34**	**±29.25***
β-carotene	31.22	±10.75	18.25	±3.05	0.00	±0.00	0.00	±0.00
4-ketoantherxanthin	0.00	±0.00	0.00	±0.00	**213.48**	**±47.20**	**1446.46**	**±245.05****
Astaxanthin	0.00	±0.00	0.00	±0.00	**77.59**	**±50.45**	**403.79**	**±51.07****
Phoenicoxanthin	0.00	±0.00	0.00	±0.00	**55.10**	**±8.33**	**218.74**	**±28.05****
Canthaxanthin	0.00	±0.00	0.00	±0.00	**31.88**	**±5.07**	**15.46**	**±2.46****
3'OH echinenone	0.00	±0.00	0.00	±0.00	**3.37**	**±0.67**	**1.47**	**±0.26****
Echinenone	0.00	±0.00	0.00	±0.00	10.82	±6.40	1.42	±0.41
Total free (mg/g)	**0.88**	**±0.25**	**1.99**	**±0.27****	**0.67**	**±0.13**	**2.93**	**±0.57****
Violaxanthin monoester	**3753.80**	**±413.11**	**0.00**	**±0.00*****	1181.87	±1668.06	0.00	±0.00
Neoxanthin monoester	**1312.60**	**±202.49**	**0.00**	**±0.00*****	296.02	±512.72	0.00	±0.00
Ketocarotenoid monoester	0.00	±0.00	0.00	±0.00	**475.81**	**±130.90**	**0.00**	**±0.00****
Violaxanthin diester	**9886.49**	**±276.18**	**0.00**	**±0.00*****	1803.49	±1547.25	0.00	±0.00
Neoxanthin diester	**485.69**	**±63.79**	**0.00**	**±0.00*****	0.00	±0.00	0.00	±0.00

Ketocarotenoid diester	0.00	±0.00	0.00	±0.00	**2257.28**	**±480.37**	**0.00**	**±0.00****
Other diester	**56.03**	**±12.77**	**0.00**	**±0.00****	211.59	±155.17	0.00	±0.00
Total esterified (mg/g)	**15.49**	**±0.81**	**0.00**	**±0.00*****	**6.23**	**±3.11**	**0.00**	**±0.00***
Total (mg/g)	**16.37**	**±1.05**	**1.99**	**±0.27*****	6.90	±3.06	2.93	±0.57

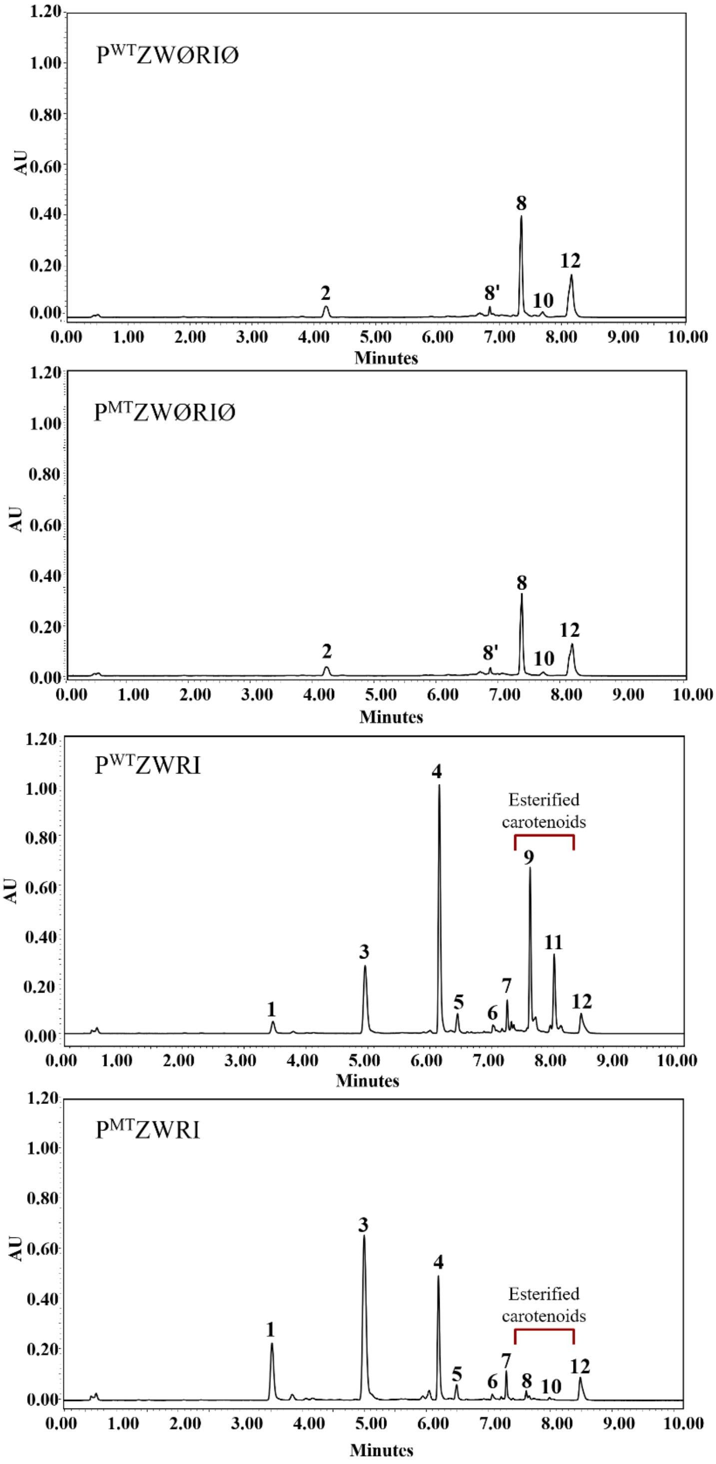

Figure 5. Example chromatograms showing the effect of the *pyp* mutation on azygous and ketocarotenoid fruit 1) Astaxanthin, 2) Lutein, 3) Phoenicoxanthin, 4) Canthaxanthin, 5) 3'OH echinenone, 6) 3OH echinenone, 7) Echinenone, 8 and 8') Lycopene, 9) Esterified carotenoid, 10) γ-carotene, 11) esterified carotenoid, 12) β-carotene

Table 2. Effect of the *pyp* mutation on the carotenoid content of fruit. Amounts in µg/g dry weight with ± standard deviation. Totals in mg/g. All values are calculated from at least three biological replicates, of eight pooled fruit per plant. Each biological replicate is an average of three technical replicates. γ-carotene and lycopene were not quantified (NQ) in fruit with esters due to co-elution. Significant values from a Student's T test are in bold, * 0.05>p≥0.01, ** 0.01>p≥0.001, *** 0.001>p

Carotenoid (µg/g DW)	P^{WT}ZWØRIØ		P^{MT}ZWØRIØ		P^{WT}ZWRI		P^{MT}ZWRI	
Astaxanthin	0.00	±0.00	0.00	0.00	**59.09**	**±46.87**	**206.57**	**±68.61****
Lutein	21.48	±6.44	23.84	±1.67	0.00	±0.00	0.00	±0.00
Phoenicoxanthin	0.00	±0.00	0.00	0.00	**750.12**	**±387.10**	**1590.76**	**±495.91***
Canthaxanthin	0.00	±0.00	0.00	±0.00	575.56	±112.98	439.65	±208.04
3'OH echinenone	0.00	±0.00	0.00	±0.00	**27.29**	**±2.90**	**19.13**	**±3.48****
Lycopene isomer	15.68	±3.06	12.27	±1.41	0.00	±0.00	0.00	±0.00
3OH echinenone	0.00	±0.00	0.00	±0.00	6.46	±4.65	11.29	±6.52
Echinenone	0.00	±0.00	0.00	±0.00	24.48	±11.62	50.24	±38.74
Lycopene	301.81	±31.28	322.73	±25.31	NQ		107.45	±120.49
Phytoene	98.00	±20.48	58.77	±17.54	14.24	±12.66	19.17	±12.88
γ-carotene	13.34	±4.76	10.97	±3.55	NQ		27.69	±20.65
ζ-carotene	**12.07**	**±2.03**	**7.93**	**±1.48***	0.00	±0.00	0.00	±0.00
Pheophytin A	635.69	±479.58	306.66	±71.40	254.46	±114.80	247.68	±185.49
β-carotene	101.78	±45.56	110.99	±14.54	68.85	±52.93	220.94	±243.87
Total free (mg/g)	1.55	±0.55	1.11	±0.03	1.78	±0.56	2.94	±0.81
Total ester (mg/g)	0.00	±0.00	0.00	±0.00	**1.55**	**±0.27**	**0.00**	**±0.00*****
Total (mg/g)	1.55	±0.55	1.11	±0.03	3.33	±0.82	2.94	±0.81

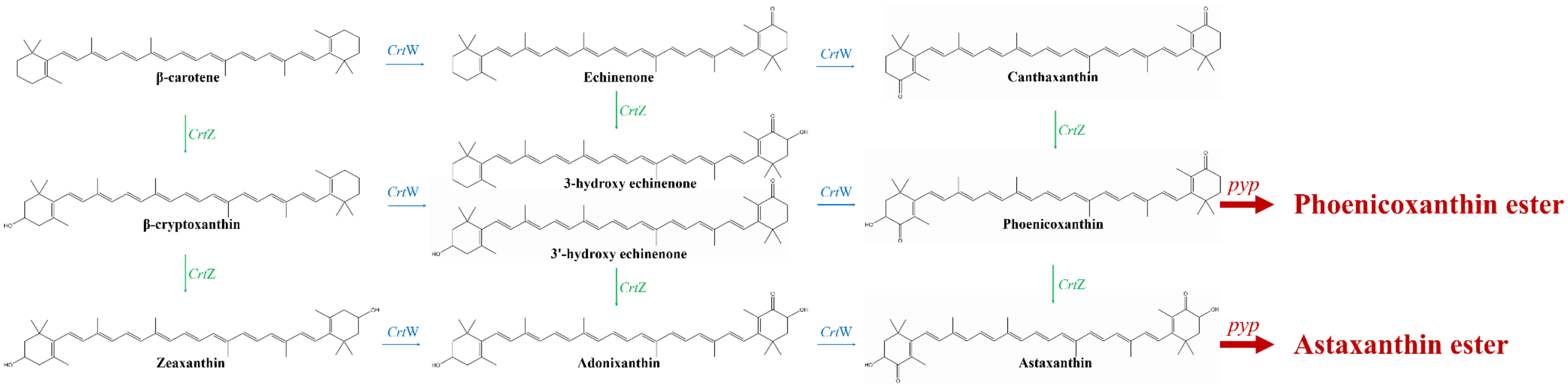

Figure 6. Biosynthetic pathway of ketocarotenoids and ketocarotenoids esters.
*Crt*Z and *Crt*W are the bacterial carotenoid hydroxylase and oxygenase genes respectively. The *pyp* gene facilitates the esterification with fatty acids to generate ketocarotenoid esters. Phoenicoxanthin and astaxanthin accumulate within the genotypes assessed and esterified forms of these have been observed in this work.