A kiwifruit (Actinidia deliciosa) R2R3-MYB transcription factor modulates chlorophyll and carotenoid accumulation

Charles Ampomah-Dwamena1, Amali H. Thrimawithana1, Supinya Dejnoprat1, David Lewis2, Richard V. Espley1 and Andrew C. Allan1,3

1The New Zealand Institute for Plant & Food Research Limited (PFR), Private Bag 92 169, Auckland, New Zealand; 2The New Zealand Institute for Plant & Food Research Limited (PFR), Private Bag 11600, Palmerston North 4442, New Zealand; 3School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

Author for correspondence:
Charles Ampomah-Dwamena
Tel: +64 99257273
Email: Charles.dwamena@plantandfood.co.nz
Received: 23 April 2018
Accepted: 11 June 2018

New Phytologist (2019) 221: 309–325
doi: 10.1111/nph.15362

Key words: carotenoid, chlorophyll, kiwifruit, overexpression, transcription factor, transcriptomics.

Summary

- MYB transcription factors (TFs) regulate diverse plant developmental processes and understanding their roles in controlling pigment accumulation in fruit is important for developing new cultivars. In this study, we characterised kiwifruit TF MYB7, which was found to activate the promoter of the kiwifruit lycopene beta-cyclase (AdLCY-β) gene that plays a key role in the carotenoid biosynthetic pathway.
- To determine the role of MYB7, we analysed gene expression and metabolite profiles in Actinidia fruit which show different pigment profiles. The impact of MYB7 on metabolic biosynthetic pathways was then evaluated by overexpression in Nicotiana benthamiana followed by metabolite and gene expression analysis of the transformants.
- MYB7 was expressed in fruit that accumulated carotenoid and Chl pigments with high transcript levels associated with both pigments. Constitutive over-expression of MYB7, through transient or stable transformation of N. benthamiana, altered Chl and carotenoid pigment levels. MYB7 overexpression was associated with transcriptional activation of certain key genes involved in carotenoid biosynthesis, Chl biosynthesis, and other processes such as chloroplast and thylakoid membrane organization.
- Our results suggest that MYB7 plays a role in modulating carotenoid and Chl pigment accumulation in tissues through transcriptional activation of metabolic pathway genes.

Introduction

The carotenoid biosynthetic pathway (Fig. 1) has been well characterised in various plant species and it is known to be controlled by factors such as channelling pathway flux, limiting enzyme steps or the availability of storage structures (Cunningham, 2002; Cazzonelli & Pogson, 2010; Yuan et al., 2015). The control of the pathway by either metabolic flux or a limiting biosynthetic step is related to enzyme activity, which is partly regulated by the modulation of gene expression. Transcriptional regulation of the carotenoid pathway has been implicated in various studies showing quantitative association between steady-state transcript levels and pigment accumulation in tissues. In tomato, the accumulation of lycopene has been partly attributed to the downregulation of lycopene cyclase genes, while the expression of upstream genes such as phytoene synthase (PSY) increases carotenoid concentration (Fraser et al., 1994, 2007). PSY plays a significant role in controlling metabolic flux down the pathway because it is the first committed step in the carotenoid pathway (Rodriguez-Villalon et al., 2009). This is essential as the carotenoid pathway shares the same substrate precursor, geranylgeranyl pyrophosphate, with other pathways such as the Chl, tocopherol and GA3 biosynthesis (Lu et al., 2013). In Arabidopsis, a single PSY is present and therefore the regulation of transcript abundance is essential to this step (Rodriguez-Villalon et al., 2009; Cazzonelli & Pogson, 2010). In other species such as maize, rice, apple and loquat, some redundancy is provided by multiple PSY genes (Li et al., 2008; Welsch et al., 2008; Fu et al., 2014; Ampomah-Dwamena et al., 2015). The tissue-specific expression observed between different PSYs in these species suggests a tightly regulated transcription of this step. The PSY step is also post-transcriptionally regulated by OR/OR-like proteins affecting PSY protein levels and carotenoid content in Arabidopsis and sweet potato without affecting PSY transcript levels (Zhou et al., 2015; Park et al., 2016). Recently, it has been reported that the OR protein, with the golden SNP mutation, may increase carotenoid content by negatively regulating the downstream beta-carotene hydroxylase activity in melon (Chayut et al., 2017).

Different kiwifruit cultivars and species display a wide range of fruit coloration due to the accumulation of pigments such as Chl, anthocyanin and carotenoids (Montefiori et al., 2005; Crowhurst et al., 2008). Developing new varieties is challenging due to the dioecism and extensive variation in ploidy levels present in the genus Actinidia. Understanding the genetic factors controlling carotenoid accumulation is valuable to plant breeding efforts to generate novel fruit phenotypes. The transcriptional control of
Isopentenyl pyrophosphate
↓ GGPPS
Geranylgeranyl pyrophosphate
↓ PSY
Phytoene
↓ PDS, ZISO
Zeta-carotene
↓ ZDS
Neurosporene
↓ ZDS, CRTISO
LCY-ε, LCY-β
Lycopene

Alpha-carotene
CYP97A CYP97C
↓ BCH1/2
Zeaxanthin
Lutein
ZEP ▲ VDE
Anthaxanthin
ZEP ▲ VDE
Violaxanthin

Fig. 1 A schematic of the carotenoid biosynthetic pathway in plants. Enzymes involved in the various conversion steps are indicated with arrows. GGPPS, geranylgeranyl pyrophosphatase; PSY, phytoene synthase; PDS, phytoene desaturase; ZISO, zeta-carotene isomerase; ZDS, zeta-carotene desaturase; CRTISO, carotenoid isomerase; LCY-β, lycopene beta-cyclase; LCY-ε, lycopene epsilon-cyclase; BCH, beta-carotene hydroxylase; CYP97A, cytochrome P450 beta-ring carotenoid hydroxylase; CYP97C, cytochrome P450 epsilon-ring carotenoid hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase.

carotenoid accumulation has been studied in kiwifruit with different fruit pigmentation, and the expression level of the lycopene beta-cyclase (LCY-β) gene was consistent with the accumulation of beta-carotene and lutein as the dominant carotenoid compounds in fruit (Ampomah-Dwamena et al., 2009). The coordinated transcriptional control of carotenoid metabolism in kiwifruit suggests that transcription factors (TFs) may play a role in regulation of this pathway. However, the mechanisms involved are not fully understood.

TFs regulate many developmental and physiological processes in plants via their ability to bind to promoters of target genes to control gene expression. A number of TFs have been implicated in the carotenoid pathway (Ye et al., 2015). MADS-box genes AGAMOUS-like 1 and FRUITFULL have been found to regulate carotenoid accumulation during ripening in tomato (Vrebalov et al., 2009), and tomato ripening-inhibitor (RIN), through its interaction with the PSY1 promoter, has been shown to regulate fruit carotenoid concentrations (Martel et al., 2011). TFs from the AP2/ERF subgroup have been reported as playing significant roles in carotenoid accumulation (Welsch et al., 2007; Chung et al., 2010; Lee et al., 2012). In tomato, the NAC TFs SINAC1 and SINAC4 have been shown to modulate carotenoid accumulation during fruit ripening (Ma et al., 2014; Zhu et al., 2014).

Two recent publications have now described a role for MYB TFs in carotenoid regulation. Plant MYBs comprise a large superfamily and are implicated in diverse roles from flower and seed development, to hormone signalling, metabolite biosynthesis and tissue pigmentation as reviewed by Jin & Martin (1999). The first of these papers reveals an R2R3 MYB, Reduced Carotenoid Pigmentation 1 (RCP1), from Erythranthe (Mimulus) lewisii (Sagawa et al., 2016). This was identified through bulk segregant analysis of E. lewisii as a positive regulator of carotenoid levels in flowers. In a mutant of E. lewisii with reduced petal colour, it was shown that RCP1 controls the entire carotenoid biosynthetic pathway. RCP1 belongs to the MYB subgroup 21 (according to Stracke et al., 2001) which, to date, has no known role in plant pigment regulation. The second recent paper to describe a role for MYBs in carotenoid accumulation presents a very different mode of action. From studies using a Citrus reticulata stay-green mutant, Green Ougan (MT), and the revertant, de-greening wild-type-like Green Ougan (MT-WT), the authors were able to identify an MYB TF, CrMYB68, which appeared to be negatively correlated with the activity of carotenoid pathway genes β-carotene hydroxylase (CrBCH2) and 9-cis-epoxycarotenoid dioxygenase 5 (CrNCED5) (Zhu et al., 2017). These two papers, while offering very different roles for MYBs, show that MYB TFs are involved in carotenoid regulation.

In fruit, carotenoid accumulation coincides with the fruit ripening process and the associated complex regulatory environment makes ascertaining the direct role of such TFs challenging. TFs involved in plant development are commonly identified through analysis of their causative phenotypes. However, identification of TFs controlling carotenoid biosynthetic pathway from this approach has been limited, possibly due to lethality of such mutations in plants. Therefore, promoter traps, deep sequencing methods or the binding of TF candidates to gene promoters have become important in understanding the regulation of the carotenoid pathway (Welsch et al., 2007; Vrebalov et al., 2009; Martel et al., 2011; Ye et al., 2015; Bond et al., 2016). Here we have used the AdLCY-β gene promoter of kiwifruit in an in planta screen assay to identify a kiwifruit R2R3 MYB (AdMYB7) that binds to and activates the expression of LCY-β. We characterised the MYB7 gene in kiwifruit and analysed its overexpression in model plants. The results suggest that MYB7 has a role in both Chl and carotenoid accumulation.

Materials and Methods

Plant material and growth conditions

Kiwifruit cultivars were selected from PFR orchards in Riwaka, New Zealand. Fruit from Actinidia macroasperma C.F. Liang and Actinidia arguta (Siebold & Zucc.) Planch. ex Miq. were picked at various stages of development from the vines. Fruit at mature green stage were stored at room temperature and sampled during ripening as described previously (Ampomah-Dwamena et al., 2009). The coordi-
Genetic distances were calculated using the Jukes–Cantor method as described previously for Nicotiana tabacum (Kalantidis et al., 2002). Transgenic plants were transferred to soil to grow under containment glasshouse conditions. Seeds from T1 plants were sterilised and grown on kanamycin (50 mg l\(^{-1}\)) selection plates to produce T2 plants used for analysis.

**Plant transformation vectors**

Kiwifruit MYB TF genes were identified from expressed sequence tag libraries (Crowhurst et al., 2008). cDNAs were amplified and cloned into pHEX2 vector using the Gateway cloning strategy as previously described (Hellens et al., 2005). Kiwifruit lycopene beta cyclase promoter fragment including 5’-untranslated region (1.1 kb) was amplified from kiwifruit genomic DNA using primers LCYBpro F (ACTGCTGTAGCTGTACTGTC) and LCYBpro R (CCTGAGAAGAGTTCCCTACAAGA). For promoter deletion constructs, primers Pro500 F (CAGCA CTATGGTTATTTGA) and Pro200 F (TGTTGAATCTC GTGCAGCATTG) were used in combination with LCYBpro R (CCTGAGAAGAGTTCCCTACAAGA) to amplify 500 bp and 200 bp promoter fragments, respectively. PCR fragments were initially cloned into pGEM-T Easy vector (Promega) for sequence confirmation and cloned into the Ndel site in pGreen II 0800-LUC upstream of the Luciferase reporter gene (Hellens et al., 2005).

**Transient assay of promoter activation**

AdLCY-β promoter sequences were analysed with PlantPan 2.0 software (Chang et al., 2008) to identify binding motifs. Transient assays were performed as previously described (Ampomah-Dwamena et al., 2009). Agrobacterium tumefaciens strain GV3101 carrying the pSOUP helper plasmid with the LCY-β promoter construct or MYB construct were resuspended in infiltration buffer (10 mM MgCl\(_2\), 0.5 μM acetosyringone) and infiltrated into the abaxial side of N. benthamiana leaves. The plants were left to grow for 2 d before 2 mm leaf discs were taken from infiltrated leaves and assayed with a Victor Multi-label Microplate Reader (Perkin Elmer, Waltham, MA, USA). Luminescence from Firefly Luciferase (LUC) expression driven by the LCY-β promoter relative to Renilla (REN) luciferase signal activity under control of the Cauliflower mosaic virus 35S promoter was measured and expressed as a ratio (LUC/REN).

**Phylogenetic analysis and tree construction**

A phylogenetic tree was constructed using Geneious 8.1.2 (Kearse et al., 2012) and MEGA7 (Kumar et al., 2016) software with Arabidopsis and kiwifruit MYB sequences retrieved from the GenBank database (Supporting Information Table S1). Genetic distances were calculated using the Jukes–Cantor distance matrix and evolutionary relationships were inferred using the maximum likelihood method with 1000 bootstrap resampling.

**Expression and purification of recombinant protein**

AdMYB7 cDNA encoding the open reading frame was amplified with MYB281F (ATGGAGTTGAGCCAGAGT) and MYB281R (CATGTTGAATTTGTTTAG) primers, modified to contain the Ndel restriction site. The amplified fragment was cloned into the Ndel site of pET30b vector to generate a C-terminal histidine-fused protein. The cloned vector was sequenced, confirmed and transformed into BL21-CodonPlus-RIL competent cells (Stratagene, San Diego, CA, USA). Recombinant protein purification followed an earlier method developed by Green et al. (2007). A 500 ml culture containing ZYM-5052 auto-induction media (Studier, 2005) was grown at 16°C, 300 rpm for 3 d to obtain an OD\(_{600}\) of 12–15. Cells were harvested by centrifugation and frozen at –80°C overnight. The cells were thawed at 4°C in 50 ml 1× His Trap Binding buffer (50 mM NaH\(_2\)PO\(_4\), 300 mM NaCl, pH 8) plus an EDTA-free protease inhibitor cocktail tablet (Roche, Penzberg, Germany) and disrupted by passing twice through an EmulsiFlex-C15 high-pressure homogeniser (Avestin, Mannheim, Germany) with a pressure setting of 15 000 psi. The sample was centrifuged 32 000 g for 30 min (4°C) to remove cell debris and filtered through a 0.45 μm filter (Merck Millipore). The supernatant was loaded onto a precharged and equilibrated 5 ml His Trap column (GE Healthcare, Piscataway, NJ, USA) and washed with 30 ml 1× His Trap binding buffer (50 mM NaH\(_2\)PO\(_4\), 300 mM NaCl, 15–35 mM imidazole, pH 8). Bound proteins were eluted at 2 ml min\(^{-1}\) using 0–500 mM imidazole gradient, pH 8, and analysed by SDS-PAGE.

**Protein mobility shift assay**

Radioactively labelled DNA probe from a AdLCY-β promoter fragment (c. 200 bp) was incubated with recombinant His-tagged kiwifruit AdMYB7 protein at room temperature for 30 min, in a binding buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 100 mM KCl, 0.1 mM dithiothreitol, 5% glycerol and 0.01 mg ml\(^{-1}\) BSA) with poly dI.dC (1 μg ml\(^{-1}\)) as nonspecific competitor. For specific competition, unlabelled DNA probe was added to the reaction. Samples were then loaded onto 4% polyacrylamide gel in TBE buffer (pH 9.2) and run at 180 V for 40 min. The electrophoresed gel was detected using an X-ray film (Kodak).

**HPLC pigment analysis**

High-performance liquid chromatography (HPLC) analysis was performed on a Dionex Ultimate 3000 solvent delivery system (Thermo Scientific, Waltham, MA, USA) fitted with a YMC RP C30 column (5 μm, 250 × 4.6 mm), coupled to a 20 × 4.6 C30 guard column (YMC Inc., NC, USA) and a Dionex 3000 PDA detector as previously reported (Ampomah-Dwamena et al., 2015). Phloem was monitored at 280 nm and coloured carotenoids and Chl\(_b\) were detected at 450 nm, while Chl\(_a\) and
its derivatives were monitored at 400 nm. Carotenoid concentrations were determined as \( \beta \)-carotene equivalents per gram dry weight (DW) of tissue. All trans-\( \beta \)-carotene, lutein and Chl \( a \) standards were purchased from Sigma Chemicals. Other carotenoids were putatively identified by comparison with reported retention times and spectral data. Total carotenoid and Chl content of the fruit tissue was also estimated using methods as previously described (Wellburn, 1994).

RNA extraction and cDNA synthesis
Total RNA was extracted by tissue homogenisation in CTAB buffer using a modified method from one previously described (Chang et al., 1993). Complementary DNA (cDNA) was synthesised from total RNA (c. 1 \( \mu \)g) using a Quantitect reverse transcription kit (Qiagen) following the manufacturer’s protocol. RNA samples were treated with the genomic DNA wipeout buffer followed by reverse transcription reaction. Reaction components included RT primer mix, Quantiscript reverse transcriptase and 

Quantitative real-time PCR analysis
Primers used (Table S2) were designed using PRIMER3 software (Rozen & Skaletsky, 2000) to a stringent set of criteria. Real-time quantitative PCR (RT-qPCR) was performed under conditions described previously (Lin-Wang et al., 2010). First-strand cDNA products were diluted 1 : 25 and used as templates for the PCR. PCR analysis was performed using the LightCycler 1.5 system and the SYBR Green master mix (Roche), following the manufacturer’s protocol. Each reaction sample was analysed from biological replicates, with each replicate being a pool of leaves of \( N. \) benthamiana. Most of the MYB constructs activated the promoter to some extent, measured by increases in the ratio of luminescence signals between the AdLCY-\( \beta \) promoter and \( A. \) deliciosa genomic DNA and the sequence was analysed for the presence of gene regulatory motifs using PlantPAN 2.0 (Chang et al., 2008). Motifs identified included those implicated in abscisic acid, light and stress response as well as MYB binding sites (Fig. S1). Due to the importance of MYBs in controlling a variety of plant-specific processes (Ambawat et al., 2013), we tested a number of MYBs for their activation of the AdLCY-\( \beta \) promoter. The promoter fragment was cloned upstream of the luciferase gene in the pGreen 0800 LUC vector (Hellens et al., 2005) and co-infiltrated with Agrobacterium transformation vectors carrying kiwifruit MYB TFs into young leaves of \( N. \) benthamiana. Most of the MYB constructs activated the promoter; to some extent, measured by increases in the ratio of luminescence signals between the AdLCY-\( \beta \) and Cauliflower mosaic virus 35S promoters (Fig. 2). This screen relies on the assumption that high translation of each construct results in high protein concentration and structure, which can be partially addressed by replication of each assay and support of other techniques. AdMYB7 (best BLAST match to AtMYB112), MYBR2 and MYBR3 (both best BLAST matches to AtMYB21), and MYB3 (match, AtMYB89) showed strong activation of the promoter, suggesting binding specificity of certain MYBs to the promoter. Among these, AdMYB7 was selected for further analysis based on its high promoter activation and gene expression pattern in fruit. AdMYB7 belongs to the phylogenetic clade including AtMYB112, AtMYB108 and AtMYB78 (Fig. 3; Stracke et al., 2001) and shows 53%, 49% and 48% amino acid sequence identity, respectively, to these Arabidopsis proteins. The AdMYB7 sequence was analysed with WolfPSORT (Horton et al., 2007), an advanced protein subcellular localisation prediction tool, which predicted AdMYB7 as a nuclear localised protein, consistent with R2R3 MYB proteins.

Transcriptome analysis by RNASeq
\( Agrobacterium \)-infiltrated \( N. \) benthamiana leaves were harvested after 24 h (T1) or 72 h (T2) and frozen in liquid nitrogen. Total RNA was extracted as described above and sequencing libraries were prepared using the TruSeq mRNA library preparation kit (Illumina) and sequenced on a HiSeq2000 (Illumina) using 2 \( \times \) 100 bp paired-end sequencing. Each treatment had three biological replicates, with each replicate being a pool of leaves from individual plants. The libraries were multiplexed and run on three lanes generating 48–54 million reads per sample. Resulting reads were then quality trimmed where FASTQ-MCF (ea-utils.1.1.2-806) was used for adapter removal with a quality threshold cut-off of 20, followed by the use of an in-house perl script to trim 15 bases of the 5’-end and remove any reads with N’s or mononucleotides. Thereafter, reads were aligned to the Solgenomics \( N. \) benthamiana genome v.1.0.1 (https://solgenomics.s.net/organism/Nicotiana_benthamiana/genome) using Bowtie2 (v.2.2.5). The number of reads aligning to the annotated genes was counted using HTSeq (v.0.6.1p1). Differentially expressed genes were identified using DESeq2 analysis with a cut-off probability of \( P < 0.05 \). Gene ontology (GO) enrichment analysis was performed using DAVID (Huang et al., 2008) and co-infiltrated with \( Agrobacterium \) transformation vectors carrying kiwifruit MYB TFs into young leaves of \( N. \) benthamiana. Most of the MYB constructs activated the promoter to some extent, measured by increases in the ratio of luminescence signals between the AdLCY-\( \beta \) and Cauliflower mosaic virus 35S promoters (Fig. 2). This screen relies on the assumption that high translation of each construct results in high protein concentration and structure, which can be partially addressed by replication of each assay and support of other techniques. AdMYB7 (best BLAST match to AtMYB112), MYBR2 and MYBR3 (both best BLAST matches to AtMYB21), and MYB3 (match, AtMYB89) showed strong activation of the promoter, suggesting binding specificity of certain MYBs to the promoter. Among these, AdMYB7 was selected for further analysis based on its high promoter activation and gene expression pattern in fruit. AdMYB7 belongs to the phylogenetic clade including AtMYB112, AtMYB108 and AtMYB78 (Fig. 3; Stracke et al., 2001) and shows 53%, 49% and 48% amino acid sequence identity, respectively, to these Arabidopsis proteins. The AdMYB7 sequence was analysed with WolfPSORT (Horton et al., 2007), an advanced protein subcellular localisation prediction tool, which predicted AdMYB7 as a nuclear localised protein, consistent with R2R3 MYB proteins.

AdMYB7 binds the AdLCY-\( \beta \) promoter
To further confirm the interaction seen in the luciferase reported assays, the physical binding of MYB7 recombinant protein to the AdLCY-\( \beta \) promoter fragment was tested using an electrophoretic
mobility shift assay (EMSA). The nucleotide coding region of AdMYB7 was amplified from fruit cDNA, cloned into the NdeI site of pET30 vector (Novagen) and expressed as a 38.7 kDa recombinant protein (339 amino acids, pI = 6.04) with a C-terminal His-tag. A specific DNA–protein complex was formed between AdMYB7 recombinant protein and the promoter fragment, and its migration was slower when compared with the labelled probe only (Fig. 4a). The formation of this complex was reduced when an increasing amount of the unlabelled promoter fragment, as a competitor, was added. By contrast, no competition was observed when an amplified fragment from kiwifruit actin cDNA was used as competitor, suggesting specificity in DNA–protein complex formation. Analysis of the promoter deletion constructs of the AdLCY-β promoter by the dual luciferase assay showed that activation of the 0.5 kb fragment by AdMYB7 was reduced compared with the longer 1.1 kb fragment while the 0.2 kb fragment was least activated (Fig. 4b).

MYB7 gene expression in kiwifruit

To understand the role of MYB7 in fruit, we examined expression levels in two kiwifruit species, A. arguta, which has green fruit throughout its development, and A. macrosperma, which turns bright orange when ripe (Fig. 5a). In A. arguta fruit, total Chl concentration increased gradually from 20 d after full bloom (DAFB) to 145 DAFB, although a decrease in concentration was observed at 90 DAFB. Total carotenoid concentration remained relatively low throughout fruit development (Fig. 5b). In A. macrosperma fruit, Chl concentration was high at the early
fruit stages and the level remained stable until fruit reached 90 DAFB. Chl concentration decreased sharply until it was barely detectable in the 150 DAFB ripe fruit. Total carotenoid concentration in this fruit, by contrast, was lower in the young fruit until after the 135 DAFB stage, when carotenoid concentration increased significantly in the ripening fruit at 142 and 150 DAFB. The dominant carotenoid compounds in both A. arguta and A. macrosperma were lutein and beta-carotene, while zeaxanthin accumulated to higher concentration in A. arguta than in A. macrosperma fruit. Alpha-carotene concentration were fairly stable throughout fruit development of A. arguta, but increased 10-fold from 90 DAFB to the ripe fruit stages in A. macrosperma (Fig. S2).

MYB7 transcript level in A. arguta was very high at 20 DAFB, reduced at 50 DAFB and then an increase in expression was observed at 70 DAFB. Expression was reduced after this fruit stage until 145 DAFB (ripe fruit stage) when a final increase in expression was seen (Fig. 6a). A similar pattern for MYB7 was seen in A. macrosperma fruit, with very high expression at 20 DAFB, decreasing from 50 DAFB until 142 DAFB (mature green), and increasing again at 150 DAFB (ripe fruit) (Fig. 6b). We then compared expression of the LCY-β gene in these fruit. Its expression pattern in A. arguta fruit showed that the transcript level was high at 20 DAFB, was significantly reduced at 50 and 70 DAFB, and showed an increase at 90 DAFB. Expression was then reduced after this stage (Fig. 6c). By contrast, AmLCY-β transcript levels in A. macrosperma fruit increased consistently from 20 to 150 DAFB (Fig. 6d).

We also examined the gene expression in fruit of the other MYB TF candidates, which activated the AdLCY-β promoter (Fig. S3). Transcript levels of MYB8 and MYBR2 were not detected in fruit. The transcript levels of MYB3, MYB13 and MYB3 were high at 20 DAFB in both A. arguta and A. macrosperma and then were reduced as the fruit developed to maturity. By contrast, MYBR1 expression in A. arguta was undetectable in the 20 DAFB fruit but increased in 50 and 70 DAFB fruit, after which expression decreased. In A. macrosperma, MYBR1 expression was low at the 20 and 50 DAFB fruit stages and transcript levels then increased in the 70, 90 and 135 DAFB fruit stages before decreasing in the later stages. Transcript levels of key carotenoid pathway genes were also assessed in the fruit of both kiwifruit species. The expression pattern for all the genes in A. arguta showed high transcript levels of the genes at 20 DAFB but reduced thereafter, except for the BCH gene for which expression appeared to increase at 90 DAFB (Fig. S4A). In A. macrosperma, the expression pattern for PSY, PDS and ZDS was similar with high transcript levels at 20 DAFB but reduced at 50 and 70 DAFB before increasing again from 90 to 150 DAFB. By contrast, CRTISO, LCY-ε and BCH gene expression in A. macrosperma fruit showed high transcript levels at 20 DAFB before reducing thereafter to 150 DAFB (Fig. S4B).

**Overexpression of AdMYB7 alters pigment accumulation in Nicotiana benthamiana**

To analyse the effects of AdMYB7 expression on carotenoid levels, N. benthamiana leaves were infiltrated with Agrobacterium carrying the AdMYB7 cDNA cloned under the control of the Cauliflower mosaic virus 35S promoter (35S:MYB7). The leaves were left to grow on the plant for 5 d and changes in leaf colour and pigment profiles were analysed. There was a gradual yellowing of the AdMYB7 infiltrated leaf patch, while the part of the leaf that was infiltrated with empty vector remained green (Fig. S5A). HPLC analysis of the leaves infiltrated with the AdMYB7 construct showed a more than two-fold increase in total carotenoid concentration (Fig. S5B).
To further analyse AdMYB7 function, we generated transgenic *N. benthamiana* plants using *Agrobacterium* transformation of leaf discs with a 35S: AdMYB7 construct. Five independently transformed T1 plants were grown in soil under glasshouse conditions to obtain seeds. T2 plants from two of these transgenic lines were selected in tissue culture, after which they were grown in the glasshouse under long-day conditions alongside empty vector control plants (Fig. 7a,b). The T2 transgenic plants showed varying levels of AdMYB7 gene expression as measured by RT-qPCR and could be grouped into high (T5-9, T3-8, T5-4, T5-7, T5-6, T5-1) and low (T5-2, T5-8, T5-3, T5-10) expression plants (Fig. 7c). In contrast to the transiently infiltrated *N. benthamiana* plants, the AdMYB7 stable transgenic plants displayed darker green leaves compared with the control.

![Fig. 5](image1.png)  
(a) Ripe fruit of *Actinidia arguta* (green) and *Actinidia macrosperma* (orange). (b) Total Chl (left) and total carotenoid concentration (right) in fruit of *A. arguta* (top panel) and *A. macrosperma* (bottom panel) during fruit development. Error bars indicate ± SE from three biological replicates.

![Fig. 6](image2.png)  
Fig. 6 Gene expression profile of MYB7 and lycopene beta-cyclase (LCY-β) in kiwifruit genotypes during fruit development. Relative gene expression of MYB7 and LCY-β in fruit of *Actinidia arguta* (a, c) and *Actinidia macrosperma* (b, d). Data were analysed using target : reference ratios (measured with LIGHTCYCLER 480 software) using actin as reference gene and presented as means ± SE from three biological replicates.
plants, which suggested increased accumulation of Chl in these tissues (Fig. 7a,b).

Chlorophyll and carotenoid concentrations in the leaves were measured by HPLC to ascertain the effect of AdMYB7 on pigment accumulation. In the seven high MYB7 expression plants, total Chl concentration was reduced in four plants (T5-9, 3-8, 5-4, 5-1), was increased in two (T5-6 and T3-5), with one (T5-7) showing a similar concentration to control plants (Fig. 7d). Pheophytin a/b compound concentrations, as a proportion of total Chl, were higher in these four plants than in the control or other transgenic plants, although artefactual conversion of Chl compounds can be caused by the extraction process (Hu et al., 2013). By contrast, the plants with lowest MYB7 expression showed somewhat increased Chl concentrations compared with the control. The main carotenoid compounds detected in the transgenic plants were lutein and beta-carotene. The pattern of total carotenoid accumulation observed between the transgenic lines was similar to the total Chl accumulation described previously. Five high MYB7 expression plants (T5-9, 3-8, 5-4, 5-7 and 5-1) showed reduced carotenoid concentrations, while T5-6, T3-5, T5-3 and T5-10 showed increased concentrations compared with the control. These results suggest that heterologous overexpression of MYB7 in transgenic N. benthamiana plants can increase Chl and carotenoid concentrations, when expression of the transgene is at a moderate level. However, transgene expression above a certain threshold significantly reduced concentrations of these pigments. In addition to the changes in pigment levels, we observed stunted plant growth in the lines with high MYB7 expression. T3-8, T5-4 and T5-9, for instance, reached only a height of c. 10 cm compared with the control plants that grew to an average height of 24 cm. There was a negative correlation ($r = -0.89, P<0.05$) between transgene expression level and plant height, suggesting that MYB7 overexpression affected plant growth. We examined the relative expression of carotenoid and Chl biosynthetic pathway genes in the transgenic N. benthamiana plants and found carotenoid genes such as NbPSY, NbPDS, NbZDS, NbLCY-$\beta$ and NbLCY-$\epsilon$ were upregulated in the high AdMYB7-expressing plants (Fig. 8a). A similar expression pattern was also observed with the Chl pathway genes such as delta-aminolevulinic acid dehydratase, magnesium protoporphyrin ester cyclase, porphobilinogen deaminase and magnesium chelatase showing increased expression in these AdMYB7 transgenic plants (Fig. 8b).

Global transcriptional changes induced by AdMYB7 in Nicotiana benthamiana

To examine the mechanism involved in AdMYB7 regulation of pigment accumulation, we analysed the transcriptomes of N. benthamiana leaves agro-infiltrated with a T-DNA construct of MYB7 compared with those carrying the empty vector (EV) or mock infiltrated with buffer. Transient
expression of genes using Agrobacterium infiltration into *N. benthamiana* as a heterologous host has been used recently to validate gene function or determine protein localisation (Leckie & Stewart, 2011; Pillay *et al.*, 2016). In this study, infiltrated leaves were left for either 24 h (T1) or 72 h (T2) on the plant before tissue was isolated for RNA extraction and analysis by transcriptome sequencing. By using RNAseq of transiently transfected 35S:MYB7 leaf patches, lists of differentially expressed tobacco genes resulting from the primary response to this TF are more likely to be revealed, rather than 4 or 5 wk of growth of a plant that has been affected by stable transformation.
The transcriptome of leaves agro-infiltrated with empty vector or mock treatments (buffer only) were first compared. A significant number of genes were differentially expressed, suggesting a background response to the Agrobacterium with empty T-DNA vector. At T1, 2659 and 2405 differentially expressed genes (DEGs) were found to be upregulated and downregulated, respectively, in empty vector vs buffer only, and at T2, 704 genes were upregulated and 35 genes downregulated (P < 0.05). There were 549 upregulated and 12 genes downregulated common to both time points. With a log2 fold cut-off (+/-2) there were 414 DEGs responsive to Agrobacterium infection (384 at T1, 108 at T2 and 78 at both time points) (Table S3). The upregulated genes included those involved in defence response and disease resistance, such as receptor kinases, systemic resistance proteins, leucine-rich repeat family proteins and WRKY TFs, indicating the plant’s response to a perceived pathogen attack (Bond et al., 2016). The downregulated genes included some chloroplast- and photosynthesis-related proteins.

The DEGs that were induced or repressed by transient expression of MYB7 compared with EV were then analysed. There were 874 upregulated and 92 downregulated genes in MYB7-treated leaves at T1 (24 h post-infiltration). By contrast, a significantly higher number (>5-fold) of differentially expressed genes were present at T2 (72 h): 2160 upregulated and 3042 downregulated genes, with 289 DEGs common to both time points (P < 0.05). With a log2 fold cut-off (+/-2), there were 290 DEGs due to AdMYB7 expression (Table S4). Some of the DEGs could be due to indirect response to AdMYB7 expression and should be interpreted with caution. However, DEGs at T1 and those common to both time points are probably targets of AdMYB7. Arabidopsis homologues of the N. benthamiana DEGs were generated and analysed at P < 0.05, for GO enrichment using the DAVID tool (Fig. 9; Huang et al., 2009). GO annotation analysis of both T1 and T2 DEGs was separated into the three main categories: biological process (BP), cellular component (CC) and molecular function (MF). For BP, the T1 DEGs were enriched for fatty acid biosynthesis (GO:0006633), carotenoid biosynthesis (GO:0016117) as well as oxidation-reduction processes (GO:0055114), all with positive z-scores that indicate these pathways were upregulated (Fig. 9a). By contrast, T2 DEGs were enriched for photosynthesis (GO:0015979), chloroplast organisation (GO:0009658), response to cytokinin (GO:0009735), Chl biosynthetic process (GO:0015995) and thylakoid membrane organisation (GO:0010027). However, these GO terms had negative z-scores, indicating downregulation of these biological processes (Fig. 9b).

For CC annotations, T1 DEGs annotated as membrane-associated genes (GO:0005886, GO:0016020, GO:0016021) made up the largest proportion and most of them were associated with the plasma membrane (GO:0005886), while in T2 DEGs plastid-associated genes were enriched (Fig. S6A,B). In the MF category, amino acid transmembrane transporter activity (GO:0015171), transferase activity (GO:0016757) and oxidoreductase activity (GO:0016491) genes were highly represented in T1 DEGs, while T2 DEGs were enriched for Chl and pigment binding (GO:0016168, GO:0031409) (Fig. S6C,D). To increase our understanding of the biological functions of the DEGs, pathway enrichment analysis was done by mapping the DEGs to the KEGG database categories. Thirteen KEGG pathways were significantly enriched at T1 (adjusted P < 0.005) with secondary metabolic pathways, such as carotenoid biosynthesis (fold enrichment 6.0) and flavonoid biosynthesis (fold enrichment 5.5) particularly indicated (Table 1). Pathway enrichment of T2 DEGs also identified metabolic pathways, including biosynthesis of secondary metabolites as well as photosynthesis-related pathways (Table 1). In general, secondary metabolic genes were highly represented in T1 DEGs, while Chl- and photosynthesis-related genes were enriched in T2 DEGs. Among the genes upregulated by AdMYB7 expression in N. benthamiana was geranylgeranyl pyrophosphate synthase (GGPPS), which catalyses the formation of geranylgeranyl pyrophosphate, a 20-carbon substrate for both the carotenoid and the Chl biosynthetic pathways. The carotenoid biosynthesis genes NsPSY and lycopene cyclase were upregulated by AdMYB7 at both T1 and T2, while phytoene desaturase (NsPDS) and carotenoid isomerase were differentially expressed at T1, but not T2 (Table 2). Chlorophyll metabolic genes, such as geranylgeranyl reductase (NbsGGR), coding for a key enzyme in the Chl biosynthetic pathway, and non-yellowing 1 (Stay-green 1 (NsSGRI)), implicated in Chl breakdown, were upregulated by AdMYB7 at both T1 and T2. We found putative MYB binding motifs in the promoter sequence of NsSGRI suggesting a direct activation by MYB7. By contrast, genes such as FAD/NADP oxidoreductase family proteins and the light harvesting Chl/a/b binding proteins were generally downregulated at T2. The transcriptomic analysis of AdMYB7 overexpression in N. benthamiana suggests it may directly regulate the expression of key genes involved in the carotenoid and Chl biosynthetic pathways.

**Discussion**

**MYB7** is a transcription factor that regulates the carotenoid pathway

Although the biosynthetic pathways responsible for Chl and carotenoid accumulation have been well described, the mechanisms involved in how they are regulated, such as during fruit development, remains to be fully understood. TFs are one such group of regulators that play crucial roles in many biological and developmental processes in plants by their regulation of spatiotemporal gene expression through recognition of specific DNA sequences in promoters (Mitsuwa & Ohme-Takagi, 2009). The identification of such regulatory genes controlling the accumulation of these pigments in fruit, for instance, provides the knowledge to develop novel cultivars for consumers. In this study, we describe a kiwifruit MYB TF, MYB7, that modulates Chl and carotenoid accumulation, via regulation of key metabolic genes. AdMYB7 was identified as an activator of the AdLCY-b gene promoter, which is a key step in the kiwifruit carotenoid pathway.

The **AdMYB7** regulation of a secondary metabolic pathway is consistent with the function of some of its closely related
homologues in other species. However, no homologue has been shown to be involved in the carotenoid biosynthetic pathway. Phylogenetic analysis placed MYB7 in a clade with AtMYB112, AtMYB108 and AtMYB78. AtMYB112 is implicated in the promotion of anthocyanin accumulation under stress treatment by inducing expression of key TFs, such as PRODUCTION OF ANTHOCYANIN PIGMENT 1 (Lotkowska et al., 2015) (Fig. 3). MYB108 has been implicated in jasmonate-mediated stamen maturation in Arabidopsis (Mandaokar & Browse, 2009), abscisic acid-induced cell death (Cui et al., 2013) and in pathogen defence in cotton (Cheng et al., 2016). Homologues of AtMYB78 have been described as playing a role during response to heat and drought stress in soybean and sorghum (Pereira et al., 2011; Johnson et al., 2014). In the phylogeny we also included the two recently published MYBs implicated in carotenoid pathway regulation, ElRCP1 and CrMYB68 (Sagawa et al., 2016; Zhu et al., 2017). These fall into separate clades and do not cluster either together or near AdMYB7. This suggests that there may be a variety of MYBs from unrelated clades involved in carotenoid regulation.

The presence of MYB binding motifs in the LCY-β promoter and the physical binding of AdMYB7 recombinant protein to a promoter fragment is further evidence of AdMYB7’s role as a transcriptional regulator in the carotenoid pathway (Fig. 4a). The promoter deletion constructs, which corresponded to the removal of MYB binding motifs, caused reduced promoter activity suggesting the removal of the binding sites affected activation by AdMYB7 (Fig 3b). Finally, it has been reported that the closest homologue to MYB7, AtMYB112, specifically binds to an 8 bp DNA fragment which contains the core sequence (A/T/G)(A/C)(A/T)(A/C)(T/C) (Lotkowska et al., 2015). Such a sequence exists in the AdLCY-β gene promoter, upstream of the ATG start site at position +31, AACCATCC. Further analysis is required to characterise this putative binding site, and the other MYB elements in the AdLCY-β promoter, to determine those that are functionally active. For example, more detailed promoter deletions or target site mutagenesis would help confirm the specificity of activation by MYB7 and the other MYBs that trans-activated AdLCY-β.

**MYB7 expression modulates Chl and carotenoid accumulation**

Expression of the MYB7 gene in *A. arguta* and *A. macrosperma* fruit, which was high in the early fruit stages and again later during fruit ripening, suggested it may have a functional role during fruit...
development. The changes in carotenoid and Chl pigment profiles in transiently infiltrated as well as stably transformed N. benthamiana plants gave an indication that MYB7 may modulate the accumulation of both pigments, at least in N. benthamiana. Some of the stably transformed plants displayed a darker green phenotype, which was observed in tissue culture and

| Treatment | Pathway term                                      | Number of genes | P value | Fold enrichment | Benjamini–Hochberg P value |
|-----------|--------------------------------------------------|-----------------|---------|----------------|----------------------------|
| T1        | ath01110:Biosynthesis of secondary metabolites    | 67              | 8.8E-08 | 1.83           | 0.000                      |
|           | ath01100:Metabolic pathways                       | 90              | 3.7E-05 | 1.40           | 0.002                      |
|           | ath01130:Biosynthesis of antibiotics               | 29              | 7.7E-04 | 1.92           | 0.023                      |
|           | ath01230:Biosynthesis of amino acids               | 19              | 2.2E-03 | 2.19           | 0.050                      |
|           | ath00906:Carotenoid biosynthesis                  | 6               | 2.6E-03 | 6.07           | 0.046                      |
|           | ath00061:Fatty acid biosynthesis                  | 6               | 1.1E-02 | 4.40           | 0.150                      |
|           | ath00040:Phenylalanine, tyrosine and tryptophan    | 6               | 3.4E-02 | 6.07           | 0.046                      |
|           | ath00945:Stilbene, diaryleptanoid and giberotin    | 6               | 5.4E-02 | 2.89           | 0.308                      |
|           | ath00280:Valine, leucine and isoleucine degradation| 5               | 6.4E-02 | 3.26           | 0.336                      |
|           | ath01212:Fatty acid metabolism                    | 7               | 3.0E-02 | 2.93           | 0.269                      |
| T2        | ath01110:Metabolic pathways                       | 457             | 1.8E-17 | 1.32           | 0.000                      |
|           | ath01130:Biosynthesis of secondary metabolites    | 289             | 1.7E-15 | 1.47           | 0.000                      |
|           | ath01230:Biosynthesis of antibiotics               | 138             | 8.2E-12 | 1.70           | 0.000                      |
|           | ath01200:Carbon metabolism                        | 89              | 7.5E-10 | 1.86           | 0.000                      |
|           | ath000195:Photosynthesis                          | 35              | 1.2E-07 | 2.48           | 0.000                      |
|           | ath00710:Carbon fixation in photosynthetic organisms| 32              | 2.7E-07 | 2.53           | 0.000                      |
|           | ath00860:Porphyrin and Chl metabolism             | 25              | 5.4E-07 | 2.84           | 0.000                      |
|           | ath00196:Photosynthesis – antenna proteins         | 15              | 3.2E-06 | 3.72           | 0.000                      |
|           | ath00630:Glyoxylate and dicarboxylate metabolism  | 30              | 1.8E-05 | 2.21           | 0.000                      |
|           | ath00620:Pyruvate metabolism                      | 31              | 1.0E-04 | 2.02           | 0.001                      |
|           | ath00260:Glycine, serine and threonine metabolism | 26              | 6.2E-04 | 1.97           | 0.006                      |
|           | ath00220:Arginine biosynthesis                    | 16              | 6.4E-04 | 2.50           | 0.006                      |
|           | ath00010:Glycolysis/glucogenogenesis              | 36              | 6.7E-04 | 1.74           | 0.006                      |
|           | ath00906:Carotenoid biosynthesis                  | 14              | 8.8E-04 | 2.64           | 0.007                      |
|           | ath01210:2-Oxocarboxylic acid metabolism          | 26              | 9.7E-04 | 1.92           | 0.007                      |
|           | ath00250:Alanine, aspartate and glutamate metabolism| 19              | 1.3E-03 | 2.16           | 0.009                      |
|           | ath00330:Arginine and proline metabolism          | 20              | 1.8E-03 | 2.06           | 0.012                      |
|           | ath00340:Histidine metabolism                     | 10              | 2.2E-03 | 3.03           | 0.014                      |
|           | ath00300:Lysine biosynthesis                      | 9               | 3.9E-03 | 3.07           | 0.024                      |
|           | ath00290:Valine, leucine and isoleucine biosynthesis| 11              | 4.5E-03 | 2.61           | 0.026                      |
|           | ath00650:Butanolate metabolism                    | 9               | 6.2E-03 | 2.89           | 0.034                      |
|           | ath00061:Fatty acid biosynthesis                  | 15              | 8.8E-03 | 2.05           | 0.046                      |
|           | ath04146:Peroxisome                              | 26              | 1.1E-02 | 1.63           | 0.054                      |
|           | ath00030:Pentose phosphate pathway                | 18              | 1.3E-02 | 1.82           | 0.063                      |
|           | ath01300:Ubinovine and other terpenoid-quinone    | 13              | 1.8E-02 | 2.03           | 0.080                      |
|           | ath01212:Fatty acid metabolism                    | 21              | 2.3E-02 | 1.64           | 0.098                      |
|           | ath00280:Valine, leucine and isoleucine degradation| 15              | 2.6E-02 | 1.82           | 0.108                      |
|           | ath00053:Ascorbate and aldarate metabolism        | 14              | 2.7E-02 | 1.86           | 0.108                      |
|           | ath00020:Citrate cycle (TCA cycle)                | 19              | 3.0E-02 | 1.65           | 0.115                      |
|           | ath00450:Selenocompound metabolism                | 8               | 3.3E-02 | 2.43           | 0.123                      |
|           | ath00640:Propanoate metabolism                    | 9               | 3.5E-02 | 2.23           | 0.126                      |
|           | ath00670:One carbon pool by folate                | 8               | 5.7E-02 | 2.18           | 0.196                      |
|           | ath00920:Sulphur metabolism                       | 13              | 5.8E-02 | 1.73           | 0.193                      |
|           | ath03010:Histamine                               | 79              | 6.0E-02 | 1.19           | 0.195                      |
|           | ath00561:Glycerolipid metabolism                  | 15              | 8.0E-02 | 1.58           | 0.245                      |
|           | ath00400:Phenylalanine, tyrosine and tryptophan    | 16              | 8.4E-02 | 1.53           | 0.252                      |
|           | ath00660:C5-Branchd dibasic acid metabolism       | 5               | 9.3E-02 | 2.73           | 0.268                      |
|           | ath00970:Aminoacyl-tRNA biosynthesis              | 28              | 9.4E-02 | 1.33           | 0.265                      |
|           | ath00261:Monobactam biosynthesis                  | 6               | 9.6E-02 | 2.34           | 0.264                      |
Table 2 List of differentially expressed genes (DEGs) associated with carotenoid and Chl metabolism in AdMYB7-infiltrated Nicotiana benthamiana leaves at T1 and T2

| Gene ID        | Description                     | Arabidopsis homologue   | Function                  | Log2 fold change |
|----------------|---------------------------------|-------------------------|---------------------------|------------------|
| NbS00043137g0007.1 | Phytolene synthase              | AT5G17230.3              | Carotenoid biosynthesis   | 2.35 0.00 0.29 NA |
| NbS00015854g0001.1 | Lycopene cyclase                 | AT3G10230.2              | Carotenoid biosynthesis   | 1.84 0.00 1.42 0.01 |
| NbS00021970g0004.1 | Lycopene cyclase                 | AT3G10230.1              | Carotenoid biosynthesis   | 1.63 0.01 1.23 0.03 |
| NbS00034641g0019.1 | Carotenoid isomerase             | AT1G06820.1              | Carotenoid biosynthesis   | 0.79 0.00 −0.56 0.01 |
| NbS00012713g0001.1 | Phytoene desaturase              | AT4G14210.1              | Carotenoid biosynthesis   | 0.78 0.00 −0.32 0.15 |
| NbS00014185g0010.1 | Phytoene desaturase              | AT4G14210.1              | Carotenoid biosynthesis   | 0.25 0.22 −0.38 0.00 |
| NbS00037135g0012.1 | Zeta-carotene desaturase         | AT3G04870.2              | Carotenoid biosynthesis   | 0.24 0.62 −0.60 0.00 |
| NbS00023229g0002.1 | Lycopene cyclase                 | AT3G10230.1              | Carotenoid biosynthesis   | 0.00 1.00 −0.45 0.01 |
| NbS00060224g0003.1 | Lycopene b/e cyclase             | AT5G57030.1              | Carotenoid biosynthesis   | −0.06 1.00 −1.00 0.00 |
| NbS00005253g0012.1 | Phytolene synthase               | AT5G17230.2              | Carotenoid biosynthesis   | −0.08 0.99 −0.85 0.00 |
| NbS00022591g0010.1 | Zeaxanthin epoxidase             | AT5G67030.1              | Carotenoid biosynthesis   | −0.10 0.96 −0.93 0.00 |
| NbS00020996g0011.1 | B-carotene hydroxylase           | AT5G52570.1              | Carotenoid biosynthesis   | −0.13 0.97 −1.31 0.00 |
| NbS00004993g0002.1 | Zeaxanthin epoxidase             | AT5G67030.1              | Carotenoid biosynthesis   | −0.17 0.92 −0.88 0.00 |
| NbS00025535g0004.1 | Lycopene b/e cyclase             | AT5G57030.1              | Carotenoid biosynthesis   | −0.19 0.92 −1.02 0.00 |
| NbS00039798g0009.1 | Violaxanthin epoxidase           | AT2G21860.1              | Carotenoid biosynthesis   | −0.28 0.67 −0.69 0.00 |
| NbS00008360g0001.1 | Geranylgeranyl reductase         | AT4G38460.1              | Chl metabolism            | 1.65 0.02 1.72 0.01 |
| NbS00026057g0001.1 | Geranylgeranyl reductase         | AT4G38460.1              | Chl metabolism            | 0.95 0.22 1.55 0.00 |
| NbS00001236g0040.1 | Non-yellowing 1 (GR1)            | AT4G22920.1              | Chl metabolism            | 1.56 0.01 1.21 0.02 |
| NbS00023456g0015.1 | Non-yellowing 1 (GR1)            | AT4G22920.1              | Chl metabolism            | 0.87 0.22 1.04 0.00 |
| NbS00001353g0003.1 | Pheophytinase                    | AT5G13800.1              | Chl metabolism            | 0.42 0.69 −0.80 0.00 |
| NbS00006039g0020.1 | Chlorophyll A-B binding family protein | AT4G17600.1 | Chl metabolism            | 0.13 0.97 −0.77 0.00 |
| NbS00002360g0024.1 | Chlorophyll A-B binding family protein | AT4G17600.1 | Chl metabolism            | 0.10 0.98 −0.70 0.00 |
| NbS00002079g0006.1 | Chlorophyll A-B binding family protein | AT1G45751.1 | Chl metabolism            | −0.04 1.00 −0.75 0.00 |
| NbS00003568g0007.1 | Chlorophyll A-B binding family protein | AT1G45751.1 | Chl metabolism            | −0.04 1.00 −0.93 0.00 |
| NbS00003416g0004.1 | FAD/NAD(P)-binding oxidoreductase protein | AT1G57770.1 | Chl metabolism            | −0.08 0.99 −0.55 0.01 |
| NbS00003626g0012.1 | Pheophytinase                    | AT5G13800.1              | Chl metabolism            | −0.28 0.75 −0.64 0.00 |
| NbS00002931g0013.1 | Chlorophyll A-B binding family protein | AT3G22840.1 | Chl metabolism            | −0.48 0.81 1.68 0.00 |

in soil-grown plants due to increased accumulation of Chl pigments. However, others with very high transgene expression showed a significant reduction in Chl and carotenoid concentrations, which correlated with changes in gene expression of key genes (Fig. 8), suggesting that transgene expression above a certain threshold may induce a different phenotype. Although this was unexpected, a similar observation was made when expression of an R2R3 MYB, RCP1, over a certain threshold in a transgenic M. lewissii line resulted in a phenotype contrary to the expected overexpression phenotype (Sagawa et al., 2016). Furthermore, in Arabidopsis Stay Green (sgr) mutants two opposing phenotypes are observed (Li et al., 2017). This means that while overexpression of TFs is an effective way to reveal their function, there are certain limitations with such an approach, particularly in a heterologous host. Because TFs may be involved in multiple pathways, their ectopic expression may result in pleiotropic phenotypes or generate a new phenotype (Zhang, 2003). The phenotype of the AdMYB7 transgenic plants combined with the gene expression observed in fruit suggested MYB7 may control accumulation of these pigments.

MYB7 induced expression of key Chl and carotenoid metabolic genes

AdMYB7 expression in transgenic N. benthamiana also increased the expression of NbLCY-β as well as other carotenoid pathway genes, such as NbPSY, NbPDS and NbZDS. The upregulation of NbLCY-β in these AdMYB7 expression lines is consistent with its interaction with the kiwifruit LCY-β promoter and suggests a regulatory role for MYB7 in the carotenoid pathway is conserved between species. The increased expression of the other pathway genes may indicate a direct activation of their promoters by AdMYB7 or be due to an indirect feedback effect of NbLCY-β expression. It is not uncommon for genes involved in the same metabolic pathway to be coordinately regulated by common TFs (Espley et al., 2007). LCY-β catalyses the conversion of trans-lycopene to beta-carotene and together with LCY-e catalyses the formation of alpha-carotene. However, this is dependent on the flux controlled by PSY and PDS genes that encode the first two committed enzymes in the carotenoid pathway. Inhibition of PSY or PDS activity affects carotenoid content and results in a bleached phenotype due to the virtual absence of carotenoid compounds (Fray & Grierson, 1993; McCarthy et al., 2004; Qin et al., 2007). It has also been shown that overexpression of LCY-β in transgenic carrot lines results in the increased expression of PSY genes, suggesting a positive feedback effect controlling the carotenoid pathway (Moreno et al., 2013). Consistent with the increased expression of the carotenoid genes in the transgenic Nicotiana benthamiana plants, the transcriptomic analysis of AdMYB7-infiltrated N. benthamiana leaves showed the upregulation of NbGGPPS, NbPSY, NbPDS, NbCRTISO and NbLCY-β.
The increased expression of Chl biosynthesis genes in AdMYB7-transgenic *N. benthamiana* plants and the upregulation of *NbGGR* and *NbSGR1* in the transcriptome of AdMYB7-infiltrated leaves suggested MYB7 may play an important role in Chl metabolism. GGR catalyses the reduction of geranylgeranyl pyrophosphate to form phytyl pyrophosphate, which is required to form the phytyl tail in the Chl molecule (Keller et al., 1998; Tanaka et al., 1999). It has been shown that the phytyl tail affects the properties of the Chl pigment, such as contributing to its conformational stability and is required for binding of the pigment by apoproteins (Fiedor et al., 2008). A *Synechocystis* sp. mutant, which lacked phytlylation ability, exhibited photooxidative stress and rapid degradation of photosystems under high light conditions (Shpilyov et al., 2013). Downregulation of GGR transcripts in transgenic tobacco using antisense RNA resulted in plants with less Chl and accumulating geranylgeranylated Chl, an intermediate compound suggesting GGR is required to increase Chl levels in plants (Tanaka et al., 1999). The rice yellow green leaf mutant, which has a single base mutation in the rice GGR (*OcCHL P*), exhibited reduced Chl levels and arrested chloroplast development (Zhou et al., 2013; Wang et al., 2014). By contrast, the significant decrease in pigment concentration observed in some of the lines showing high expression of the AdMYB7 transgene was intriguing. This phenotype suggested MYB7 may cause Chl degradation and/or interfere with plastid development, which is consistent with the upregulation of *NbSGR1* and the consequent downregulation of genes associated with chloroplast and thylakoid membrane organization. *SGR1* promotes Chl degradation by interacting with, and probably recruiting, Chl catabolic enzymes during senescence (Sakuraba et al., 2012, 2015; Oda-Yamamizo et al., 2016). SGR proteins have magnesium-de-chelatase activity, responsible for the extraction of magnesium from Chl and converting it to phophyitin a, during Chl degradation (Shimoda et al., 2016). More recently, an Arabidopsis *sgr1* (nyel) and *sgr2* (nye2) double mutant was reported to have a stay green senescent leaf and seed phenotype due to Chl accumulation. However, the seed production produced two distinct types of seeds: green seeds with accumulation of Chl and seeds that were completely devoid of Chl but contained Chl catabolic intermediates. Because the nongreen seed phenotype increased with prolonged siilque exposure to light, it was suggested that the phenotype may be due to a photodynamic effect on the accumulating Chl compounds (Li et al., 2017). AdMYB7 activation of *SGR1* to promote Chl degradation would be consistent with the transgenic phenotypes that showed significantly reduced Chl concentrations and would be similar to TFs, such as NAC016 and ANAC046, which activate the promotors of Chl catabolic enzymes (Sakuraba et al., 2012; Oda-Yamamizo et al., 2016).

Involvement of AdMYB7 in Chl and carotenoid accumulation in fruit

The gene expression pattern of MYB7 displayed a bimodal pattern with peaks associated with early fruit and fruit ripening stages. This expression pattern together with this TF's ability to modulate Chl and carotenoid accumulation, as well as its induction of key metabolic pathway genes, is consistent with MYB7 having a role in the transition between unripe and ripe fruit (Guyet et al., 2014; Fig. S7). There are examples of common regulators between these two metabolic pathways (Park et al., 2007; Luo et al., 2013). Phytochrome interaction factor 1 (PIF1) has been implicated in regulating Chl and chloroplast development as well as in carotenogenesis, as a post-transcriptional regulator of PSY (Toledo-Ortiz et al., 2010; Cheminant et al., 2011; Bout-Torrent et al., 2015). PIF1 is induced by low light conditions created by high Chl levels in the fruit, which subsequently represses PSY expression. The removal of this inhibition by reduced Chl content therefore results in increased carotenoid accumulation (Llorente et al., 2016).

The data presented here suggest a role for MYB7 in both Chl and carotenoid metabolism, possibly through transcriptional activation of biosynthetic pathway genes. This further supports recent progress in elucidating a role for MYB TFs in carotenoid regulation. Further analysis of MYB7 using transgenic kiwifruit with elevated or knockdown expression will increase our understanding of its role.

Acknowledgements

We are grateful to Trish Harris-Virgin and Tiffany Robertson for help with fruit samples, Monica Dragulescu for tending the plants in the glasshouse, Tim Holmes, Darren Snaith and Adam Sheffield for images, and Andrew Gleave and the Plant Transformation team for the cloning of *Agrobacterium* vectors. Thanks to our internal reviewers, Cyril Hamiaux, Niels Nieuwenhuizen and the PFR Science Editors, for helpful comments on the manuscript.

Author contributions

C.A-D., A.C.A. and R.V.E. designed the experiment and reviewed all the data; S.D. and C.A-D. analysed the promoter transactivation and transgenic plants; A.H.T. provided analysis of the bioinformatic and transcriptome data; C.A-D. and D.L. performed the HPLC analysis. C.A-D. wrote the body of the paper and all authors reviewed and edited the manuscript.

References

Ambawat S, Sharma P, Yadav NR, Yadav RC. 2013. MYB transcription factor genes as regulators for plant responses: an overview. *Physiology and Molecular Biology of Plants* 19: 307–321.

Ampomah-Dwamena C, Driedonks N, Lewis D, Shumskaya M, Chen X, Wurtzel ET, Espley RV, Allan AC. 2015. The *Phytoene synthase* gene family of apple (*Malus* *domestica*) and its role in controlling fruit carotenoid content. *BMC Plant Biology* 15: 185.

Ampomah-Dwamena C, McGhie T, Wikisono R, Montefiori M, Hellens RP, Allan AC. 2009. The *kiwifruit* *lycopene beta-cyclase* plays a significant role in carotenoid accumulation in fruit. *Journal of Experimental Botany* 60: 3765–3779.

Bond DM, Albert NW, Lee RH, Gillard GB, Brown CM, Hellens RP, Macknight RC. 2016. Infiltration-RNAseq: transcriptome profiling of *Agrobacterium*-mediated infiltration of transcription factors to discover gene function and expression networks in plants. *Plant Methods* 12: 41.
Regulation of carotenoid biosynthesis by shade relies on specific subsets of antagonistic transcription factors and cofactors. Plant Physiology 169: 1584–1594.

Cazenelli CI, Pogson BJ. 2010. Source to sink: regulation of carotenoid biosynthesis in plants. Trends in Plant Science 15: 266–274.

Chang WC, Lee TY, Huang HD, Huang HY, Pan RL. 2008. PlantPAN: plant promoter analysis navigator, for identifying combinatorial cis-regulatory elements with distance constraint in plant gene groups. BMC Genomics 9: 561.

Chang S, Puryear J, Cairney J. 1993. Carotenoid biosynthesis is regulated by shade in tomato. Plant Physiology 102: 745–750.

Chayut N, Yuan H, Ohali S, Meir A, Sa’ar U, Tzuri G, Zheng Y, Fraser PD, Truesdale MR, Bird CR, Schuch W, Bramley PM. 1994. The cotton MYB108 forms a positive feedback regulation loop with CML11 and participates in the defense response against Verticillium dahliae infection. Journal of Experimental Botany 67: 1935–1950.

Chung MY, Vrebalov J, Alba R, Lee J, McQuinn R, Duran C. 2012. Geneous Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28: 1647–1649.

Keller Y, Bouvier F, F. Harlingue A, Camara B. 1998. Metabolic compartmentation of plastid prenyl lipid biosynthesis – evidence for the involvement of a multifunctional geranylgeranyl reductase. European Journal of Biochemistry 251: 413–417.

Kumar S, Stecger G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution 33: 1870–1874.

Leckie BM, Stewart CN. 2011. Agroinfiltration as a technique for rapid assays for evaluating candidate insect resistance transgenes in plants. Plant Cell Reports 30: 325–334.

Lee JM, Joung J-G, McQuinn R, Chung M-Y, Fei Z, Tieman D, Klee H, Giovannoni J. 2012. Combined transcriptome, genetic diversity and metabolite profiling in tomato fruit reveals that the ethylene response factor SIER6 plays an important role in ripening and carotenoid accumulation. Plant Journal 70: 191–204.

Li F, Villalbanenzi R, Yu J, Rocheftord T, Wurtzel ET. 2008. The maize phytoene synthase gene family: overlapping roles for carotenogenesis in endosperm, photomorphogenesis, and thermal stress tolerance. Plant Physiology 147: 1334–1346.

Li Z, Wu S, Chen J, Wang X, Gao J, Ren G, Kua B. 2017. NYSERG-mediated chlorophyll degradation is critical for detoxification during seed maturation in Arabidopsis. Plant Journal 92: 650–661.

Lin-Wang K, Bolitho K, Grafton K, Korstte A, Karnarinetnem S, Mccghe T, Espley RV, Hellens RP, Allan AC. 2010. An R2R3 MYB transcription factor associated with regulation of the anthocyanin biosynthetic pathway in Rosaceae. BMC Plant Biology 10: 50.

Llorente B, D’Andrea L, Ruiz-Sola MA, Botterweg E, Pulido P, Andilla J, Loza-Alvarez P, Rodriguez-Concepcion M. 2016. Tomato fruit carotenoid biosynthesis is adjusted to actual ripening progression by a light-dependent mechanism. Plant Journal 85: 319–3211.

Luo Z, Zhang J, Li J, Yang C, Wang T, Ouyang B, Li H, Giovannoni J, Ye Z. 2013. A STAY-GREEN protein ISISGR1 regulates lycopene and beta-carotene accumulation by interacting directly with SPPSY1 during ripening processes in tomato. Plant Physiology 168: 1862–1880.

Liu YH, Rijzaani H, Karcher D, Rou S, Bock R. 2013. Efficient metabolic pathway engineering in transgenic tobacco and tomato plastids with synthetic multigene operons. Proceedings of the National Academy of Sciences, USA 110: E623–E632.

Luo Z, Zhang J, Li J, Yang C, Wang T, Ouyang B, Li H, Giovannoni J, Ye Z. 2013. A STAY-GREEN protein ISISGR1 regulates lycopene and beta-carotene accumulation by interacting directly with SPPSY1 during ripening processes in tomato. Plant Physiology 168: 442–452.

Ma NN, Feng HL, Meng X, Li D, Yang DY, Wu CG, Meng QW. 2014. Overexpression of tomato SNC1 transcription factor alters fruit pigmentation and softening. BMC Plant Biology 14: 351.
Mandaokar A, Browse J. 2009. MYB108 acts together with MYB24 to regulate jasmonate-mediated stamen maturation in Arabidopsis. Plant Physiology 149: 851–862.

Martel C, Vrebalov J, Tafelmeyer P, Giovannini JJ. 2011. The tomato MADS-box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIpening-dependent manner. Plant Physiology 157: 1568–1579.

McCarthy SS, Kobayashi MC, Niyogi KK. 2004. White mutants of Chlamydomonas reinhardtii are defective in phytoene synthase. Genetics 168: 1249–1257.

Mitsuda N, Ohme-Takagi M. 2009. Functional analysis of transcription factors in Arabidopsis. Plant and Cell Physiology 50: 1232–1248.

Montefiori M, McGhie TK, Costa G, Ferguson AR. 2005. Pigments in the fruit of red-fleshed kiwifruit (Actinidia chinensis and Actinidia delicosa). Journal of Agricultural and Food Chemistry 53: 9526–9530.

Moreno JC, Pizarro L, Fuentes P, Handford M, Cifuentes V, Stange C. 2013. Levels of lycopene β-cyclase 1 modulate carotenoid gene expression and accumulation in Daucus carota. PLoS ONE 8: e81444.

Nieuwenhuizen NJ, Green SA, Xiyin C, Bailleul EJD, Matich AJ, Wang MY, Atkinsion RG. 2013. Functional genomics reveals that a compact terpene synthase gene family can account for terpene volatile production in apple. Plant Physiology 161: 787–804.

Oda-Yamamino C, Mitsuda N, Sakamoto S, Ogawa D, Ohme-Takagi M, Ohnma A. 2016. The NAC transcription factor ANAC046 is a positive regulator of chlorophyll degradation and senescence in Arabidopsis leaves. Scientific Reports 6: 23609.

Park S, Kim HS, Jung YJ, Sun Ha Kim SH, Ji CY, Wang Z, Jeong JC, Lee HS, Lee SY, Kwak SS. 2016. Orange protein has a role in phytoene synthase stabilization in sweetpotato. Scientific Reports 6: 35563.

Park SY, Ju JW, Park JS, Li J, Yoo SC, Lee NY, Lee JK, Seong SW, Seo HS, Koh HJ et al. 2007. The senescence-induced staygreen protein regulates chlorophyll degradation. Plant Cell 19: 1649–1664.

Pereira SS, Guimaraes FCM, Carvalho JFC, Stolf-Moreira R, Oliveira MCN, Rolla AAP, Farias JRB, Neumaier N, Nepomuceno AL. 2011. Transcription factors expressed in soybean roots under drought stress. Genetics and Molecular Research 10: 3689–3701.

Pillay P, Kunert KJ, van Wyk S, Makgopa ME, Cullis CA, Vorster BJ. 2016. Agroinfiltration contributes to VP1 recombinant protein degradation. Bioinformatics 32: 459–477.

Qin G, Gu H, Ma L, Peng Y, Deng XW, Chen Z, Qu LJ. 2007. Disruption of phytoene desaturase gene results in albino and dwarf phenotypes in Arabidopsis by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. Cell Research 17: 471–482.

Rodriguez-Villalon A, Gas E, Rodriguez-Concepcion M. 2009. Phytoene synthase activity controls the biosynthesis of carotenoids and the supply of their metabolic precursors in dark-grown Arabidopsis seedings. Plant Journal 60: 424–435.

Rozen S, Skaletsky H. 2000. Primer3 on the WWW for general users and for primer biologists. Bioinformatics Methods and Protocols 132: 365–386.

Sagawa JM, Stanley LE, LaFountain AM, Frank HA, Liu C, Yuan YW, Zhou Y, Gong ZY, Yang ZF, Yuan Y, Zhu JY, Wang M, Yuan FH, Wu SJ, Wang ZQ, Yi CD et al. 2013. Mutation of the light-induced yellow leaf I gene, which encodes a geranylgeranyl reductase, affects chlorophyll biosynthesis and light sensitivity in rice. PLoS ONE 8: e75299.

Shimoda Y, Ito H, Tanaka A. 2016. The senescence-induced staygreen protein regulates carotenoid metabolism and regulation in horticultural crops. Horticulture Research 2: 15036.

Studier FW. 2005. Protein production by auto-induction in high-density shaking cultures. Protein Expression and Purification 41: 207–234.

Tanaka R, Oster U, Kruse E, Rudiger W, Grimm B. 1999. Reduced activity of geranylgeranyl reductase leads to loss of chlorophyll and tocopherol and to partially geranylated chlorophyll in transgenic tobacco plants expressing antisense RNA for geranylgeranyl reductase. Plant Physiology 120: 695–704.

Toledo-Ortiz G, Huq E, Rodriguez-Concepcion M. 2010. Direct regulation of phytoene synthase gene expression and carotenoid biosynthesis by phytochrome-interacting factors. Proceedings of the National Academy of Sciences, USA 107: 11626–11631.

Vrebalov J, Pan IL, Arroyo AJM, McQuinn R, Chung M, Poole M, Rose JK, Seymour G, Grandillo S, Giovannini J et al. 2009. Fleshy fruit expansion and ripening are regulated by the tomato SHATTERPROOF gene TAGL1. Plant Cell 21: 3041–3062.

Walter W, Sánchez-Cabo F, Ricote M. 2015. GOplot: an R package for visually combining expression data with functional analysis. Bioinformatics 31: 2912–2914.

Zhou X, Welsch R, Yang Z, Yuan H, Zhang JY, Iizuka K, Ye JB, Zhang YJ. 2015. Transcriptome profiling of tomato fruit development reveals transcription factors associated with ascorbic acid, carotenoid and flavonoid biosynthesis. PLoS ONE 10: e0130885.

Yuan H, Zhang JX, Nageswaran D, Li L. 2015. Carotenoid metabolism and regulation in horticultural crops. Horticulture Research 2: 15036.

Zhang JZ. 2003. Overexpression analysis of plant transcription factors. Current Opinion in Plant Biology 6: 430–440.

Zhou Y, Gong ZY, Yang ZF, Yuan Y, Zhu JY, Wang M, Yuan FH, Wu SJ, Wang ZQ, Yi CD et al. 2013. Mutation of the light-induced yellow leaf I gene, which encodes a geranylgeranyl reductase, affects chlorophyll biosynthesis and light sensitivity in rice. PLoS ONE 8: e75299.

Zhou X, Welsch R, Yang Z, Álvarez D, Riediger M, Yuan H, Fish T, Liu J, Thannhauser TW, Li L. 2015. Arabidopsis OR proteins are the major posttranscriptional regulators of phytoene synthase in controlling carotenoid biosynthesis. Proceedings of the National Academy of Sciences, USA 112: 3558–3563.

Zhu MK, Chen GP, Zhou S, Su Y, Wang Y, Dong TT, Hu ZL. 2014. A new tomato NAC (NAM/ATAF1/CUC2) transcription factor, SINAC4, functions as a positive regulator of fruit ripening and carotenoid accumulation. Plant and Cell Physiology 55: 119–135.

Zhu F, Luo T, Liu C, Wang Y, Yang H, Yang W, Zheng L, Xiao X, Zhang M, Xu R et al. 2017. An R2R3-MYB transcription factor represses the transformation of α- and β-branch carotenoids by negatively regulating expression of CrBCH2 and CrNCED5 in flavedo of Citrus reticulate. New Phytologist 216: 178–192.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Sequence of Actinidia delicosa lycopene beta-cyclase promoter.
Fig. S2 Concentrations of Chl and carotenoids in fruit.

Fig. S3 Gene expression of MYB genes in fruit.

Fig. S4 Relative expression of carotenoid genes in A. arguta and A. macrosperma fruit.

Fig. S5 Nicotiana benthamiana leaves infiltrated with AdMYB7.

Fig. S6 GO plot of MYB7 DEGs.

Fig. S7 A cartoon of MYB7 gene expression and pigment accumulation in fruit.

Table S1 Sequence accession numbers

Table S2 List of primers used

Table S3 List of differentially expressed genes induced by agro-transfection with empty vector, compared with mock treatment, at 24 and 72 h post-infiltration

Table S4 List of differentially expressed genes induced by MYB7, compared with empty vector treatment, at 24 and 72 h post-infiltration

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the New Phytologist Central Office.