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Comprehensive analysis of plastid gene expression during fruit development and ripening of kiwifruit

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Abstract

Key message Global survey of plastid transcriptional activities during fruit ripening in kiwifruit showed great changes in gene expression and provided *cis*-elements for engineering the plastid genome of kiwifruit in future.

Abstract A serious limitation in the application of plastid biotechnology is the low-level expression of transgene in non-green plastids like chromoplasts compared with photosynthetically active chloroplasts. Unlike other fruits, not all chloroplasts are transformed into chromoplast during ripening of red-fleshed kiwifruit (*Actinidia chinensis* vs Hongyang) fruits, which may make kiwifruit as an ideal horticultural plant for oral vaccine production by plastid engineering. To identify *cis*-elements potentially triggering high-level transgene expression in edible tissues of the ‘Hongyang’ kiwifruit, here we report a comprehensive analysis of kiwifruit plastid gene transcription in the green leaves and fruits at three different developing stages. While transcripts of a few photosynthesis-related genes and most genetic system genes were substantially upregulated in green fruits compared with leaves, nearly all plastid genes were significantly downregulated at the RNA level during fruit development. Expression of a few genes remained unchanged, including *psbA*, the gene encoding the D1 polypeptide of photosystem II. However, PsbA protein accumulation decreased continuously during chloroplast-to-chromoplast differentiation. Analysis of post-transcriptional steps in mRNA maturation, including intron splicing and RNA editing, revealed that splicing and editing may contribute to regulating plastid gene expression. Altogether, 40 RNA editing sites were verified, and five of them were newly discovered. Taken together, this study has generated a valuable resource for the analysis of plastid gene expression, and provides *cis*-elements for future efforts to engineer the plastid genome of kiwifruit.

Key words: kiwifruit; plastid; gene expression; intron splicing; RNA editing

Introduction

In recent years, expression of transgenes from the plastid genome (plastome) has received significant attention because of its capacity to serve as a cellular bioreactor. In addition to the transgene containment owing to maternal inheritance of plastid genome in most angiosperm species therefore reducing dispersion of transgene by pollen transmission (Greiner et al. 2015), the plastid's high capacity for mass production of foreign proteins has attracted particular interest as a chassis for biotechnology applications. Extremely high levels of foreign protein accumulation were obtained in green leaves (Ahmad et al. 2016), with levels exceeding 70% of the total soluble protein (TSP) in tobacco (Oey et al. 2009). In contrast, transgene expression levels in non-green plastids are conspicuously lower than that in green plastids (chloroplasts). For instance, expressing the HIV capsid protein p24 fused with the negative regulatory protein Nef (p24-Nef) reached up to 40% of TSP in leaves and 2.5% of TSP in green fruits in transplastomic tomato, whereas it was not possible to detect the fusion protein p24-Nef in red ripe (chromoplast-containing) fruits (Zhou et al. 2008).

Recent global analyses of plastid transcriptome in tomato fruits and potato tubers have revealed that nearly all genes in non-photosynthetic plastids display a marked down-regulation of expression with two exceptions, *accD*, which encodes the β -subunit of acetyl CoA carboxylase associated with lipid biosynthesis, and *clpP*, encoding a catalytic subunit of the ATP-dependent caseinolytic protease (Kahlau and Bock 2008; Moreno et al. 2017; Valkov et al. 2009). This finding has facilitated the design of chimeric expression elements (combinations of promoters and 5'-untranslated regions (5'-UTR)) to overcome the inefficient expression of transgene in non-green plastids (Caroca et al. 2013; Valkov et al. 2011; Zhang et al. 2012). While the regulation of plastid gene expression in non-green tissues of tomato has been well studied (Caroca et al. 2013; Kahlau and Bock 2008), this regulation in other economically important fruits such as kiwifruit remain largely unknown.

Kiwifruit belongs to the genus *Actinidia* that consists of approximately 54 species. China represents the center of origin of kiwifruit (Ferguson and Huang 2007). The kiwifruit has long been called "the king of fruits" due to its exceptionally high amounts of vitamin C, and the high contents of dietary fiber, minerals (e.g. potassium) and various health-promoting metabolites (Huang et al. 2013). The kiwifruit of international commerce is mainly selected from two species, *A. chinensis* and *A. deliciosa* (Zhang et al. 2010). Recently, the red-fleshed cultivar *A. chinensis* cv 'Hongyang' was developed for commercial production in China, and has become popular due to its unique anthocyanin accumulation, high sugar and vitamin C contents (Montefiori et al. 2005). Unlike tomato, partial chloroplasts maintain in ripe fruit of 'Hongyang' kiwifruit (Nishiyama et al. 2005), suggesting kiwifruit fruit is likely to a potent chassis for high-level foreign protein production.

The 'Hongyang' kiwifruit nuclear genome has a total size of 653 Mb and harbors 40,464 annotated protein-coding genes (Wu et al. 2019). In addition to the nucleus, mitochondria and plastids also contain their own genomes. Among the three distinct genomes in plant cells, plastome is the most compact one, which harbors approximately 120 genes in a 107–218 kb sequence (Daniell et al. 2016). The plastid genes can be divided into three major groups (Scharff and Bock 2014): (i) genes encoding subunits of photosynthetic complexes (ribulose 1,5-bisphosphate carboxylase/oxygenase, photosystem I (PSI), PSII, cytochrome *b₆* complex, NAD(P)H dehydrogenase (NDH), and ATP synthase) (ii) genes specifying components of the genetic system (ribosomal proteins, RNA polymerase subunits, tRNAs, and rRNAs), and (iii) genes encoding proteins related to other functions (e.g., *AccD*, *ClpP*, *Ycf1*, and *Ycf2*). With the development of high throughput sequencing technologies, several complete plastomes of *Actinidia* have

been sequenced (Kim et al. 2018; Lan et al. 2018; Wang et al. 2016; Wu et al. 2019; Yang et al. 2019; Yao et al. 2015). All of them show the typical tetrapartite genome organization found in most vascular plants, with a large and a small single-copy region separating a pair of inverted repeat regions (Bock 2015). Notably, the *clpP* gene is completely lost from *Actinidia* plastid genomes (Kim et al. 2018; Lan et al. 2018; Wang et al. 2016; Yang et al. 2019; Yao et al. 2015).

Being derived from a cyanobacterial ancestor (Palmer 2003), the plastid combines both prokaryotic and eukaryotic features of gene expression. Transcription of the plastid genes of seed plants requires at least two distinct RNA polymerases, namely the plastid-encoded multimeric RNA polymerase (PEP) and nucleus-encoded bacteriophage-type RNA polymerase (NEP) (Börner et al. 2015). It is generally assumed that PEP dominates in transcription of genes involved in photosynthetic function, while NEP mainly mediates transcription of housekeeping genes (Hajdukiewicz et al. 1997). Nascent transcripts synthesized by both PEP and NEP are usually polycistronic and undergo a series of post-transcriptional processing steps, including trimming of the 5' and 3' termini, intercistronic cleavage, intron excision by splicing, and RNA editing by cytidine to uridine (C-to-U) substitution (Barkan 2011; Bock 2000; Stern et al. 2010). These post-transcriptional maturation processes potentially activate an mRNA, affect its translatability, or alter its stability (Yagi and Shiina 2014).

Although the transcript profiles of kiwifruit were analyzed in a number of previous studies (Li et al. 2015; Wu et al. 2020; Zhang et al. 2018), information on plastid gene expression of kiwifruit has remained scarce. Typically, chloroplasts exist in the young fruit, and then differentiate into carotenoid-accumulating chromoplasts during fruit ripening process (Sadali et al. 2019). In current study, we performed a systematic analysis of kiwifruit plastid gene expression, and examined patterns of gene regulation in different tissues and plastid types. Moreover, developmental changes in two crucial post-transcriptional processes, intron splicing and RNA editing, were also determined.

Material and methods

Plant materials

Leaves and fruits at three different stages of development (60, 110 and 145 days after pollination; DAP) were collected from 'Hongyang' kiwifruit from the Center of Kiwifruit Breeding, Xianning, Hubei Province, China. The harvested tissues were frozen rapidly in liquid nitrogen and stored at -80 °C until used for nucleic acid or protein extraction.

RNA isolation and northern blot analyses

Total RNA was extracted from fresh leaves and fruits using the Quick RNA Isolation Kit (Huayueyang Biotechnology Co., Ltd., Beijing, China) following the protocol of the manufacturer. For northern blot analysis, three micrograms of RNA samples were separated in denaturing formaldehyde-containing agarose gels (1%) and transferred electrophoretically onto positively charged nylon membranes (GE Healthcare, USA) using standard blotting protocols. Gene-specific hybridization probes were amplified by PCR from cDNA or genomic DNA (intron probe preparation) using PCR primer pairs listed in Supplementary Table S4. Hybridization probes were labeled with digoxigenin (DIG High Prime DNA Labeling and Detection Starter Kit II; Roche, USA) following the manufacturer's instruction. RNA blots were hybridized at 52 °C (for intron splicing analysis) or 68 °C using standard protocols.

Quantitative reverse transcription-PCR and DNA sequencing

Reverse transcription and cDNA synthesis were done using the Hifair® II 1st Strand cDNA Synthesis Kit (gDNA digester plus, Yeasen, China). Quantitative RT-PCR amplification was carried out in a Bio-Rad CFX Connect Real-Time System (Bio-Rad, USA). Reactions were performed in 10-μL volume and contained 2 × SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; Takara, China), 2 μL of cDNA and 0.2 μL of each primer (10 μM; Supplementary Table S4). The amplification conditions include a 2-min initial denaturation at 95 °C, followed by 40 cycles of 5 sec denaturation at 95 °C, and annealing/extension at 60 °C for 30 sec. Relative expression levels of different plastid genes was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) with *actin* (GI: 149938963) gene as an internal control. Three independent biological replicates were prepared for each experiment. The RNA editing status of plastid transcripts was evaluated by directly sequencing the amplified cDNA population (Genecreate, Wuhan, China).

Protein extraction and western blot analyses

Total protein from plant samples was isolated using a phenol-based extraction method (Cahoon et al. 1992). Protein quality was determined using the Easy II Protein Quantitative Kit (BCA, TransGen Biotech, China). Samples of 25 μg of total fruit protein and a dilution series of total leaf protein were electrophoretically separated in 12% SDS-polyacrylamide gels. The protein in the gel was either stained with Coomassie Brilliant Blue R-250 stain (Biyuntian Biotechnology Co., Ltd., China) or blotted onto PVDF (polyvinylidene difluoride) membranes (GE Healthcare) using wet transfer for 1.5–2 h. The membranes were blocked in in TBS-T solution (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% (v/v) Tween-20) supplemented with 5% (w/v) fat-free milk for 1 h at room temperature (RT), and subsequently incubated with primary antibodies against PsbA and PsbD (1:1000 dilution, PhytoAB) at 4 °C overnight. Then the membranes were washed with TBS-T (10 min at RT, three times), stained with HRP conjugated anti-rabbit secondary antibody at 1/10000 dilution for 1 h at RT, and again washed with TBS-T (10 min at RT, three or more times). Detection was performed with the enhanced chemiluminescence (ECL, Biosharp, China) kit and the AI600 imager (GE Healthcare).

Results

Plastid genome of ‘Hongyang’ kiwifruit

In 2015, Yao et al. reported the first complete plastome sequences in *Actinidia*, including that of the diploid variety ‘Hongyang’ ($2n = 2 \times = 58$) (Yao et al. 2015). The plastid DNA of *Actinidia* maps as a circular genome and comprises 113 genes, including four rRNA genes, 30 tRNA genes and 79 protein-coding genes (Yao et al. 2015). However, the published chloroplast genome map (Yao et al. 2015) contained partially incorrect gene annotations (GenBank accession number NC_026690.1), and missing or incorrectly annotated introns and intergenic spacers. Here, we corrected the gene annotation of the plastid genome, and drew a new physical map of the ‘Hongyang’ plastome using OrganellarGenomeDRAW (OGDRAW) version 1.3.1 (Greiner et al. 2019) (Fig. 1).

Analysis of the plastid transcription profiles of kiwifruit

To examine the changes in transcription profiles of kiwifruit plastome upon fruit development, we monitored the RNA accumulation for all plastid-encoded genes (except the tRNA genes) using quantitative reverse transcription-PCR (qRT-PCR). Green leaves and three different developmental stages of fruits (green, turning and mature fruits; Supplementary Fig. S1) were analyzed.

Developmental changes in plastid transcriptional activities were analyzed in two manners. Firstly, by using the transcript levels in leaves as a reference, we determined the alternations in the transcript abundances of plastid genes in fruits compared with those in leaves (Fig. 2A,B). This analysis allowed us to identify alterations triggered by the fruit developmental program. Secondly, we used the transcript levels in immature green fruits as a reference (Fig. 2C,D), thus enabling dissection of the relative contributions of fruit developmental program and chloroplast-to-chromoplast differentiation.

Compared with leaves, the mRNA accumulation of most photosynthesis-related genes (ranging from *psbA* to *rbcL*) were significantly downregulated in fruits (Fig. 2 A,B). Exceptions included the transcripts of *psaI*, *ycf3*, *ndhH* and *petG*, which tended to be upregulated in the green, chloroplast-containing fruits. By contrast, most genetic system genes and all genes associated with other functions were upregulated in early-stage fruits, and several of these genes even maintained this upregulation in the later stages of fruit development (Fig. 2A,B). Moreover, chloroplast-to-chromoplast differentiation was accompanied by a remarkable general downregulation of plastid mRNA levels (Fig. 2C,D). However, the *psbA* transcript remained nearly constant during chromoplast conversions, thus representing a notable exception (Fig. 2C,D).

To test whether the qRT-PCR data accurately represented the developmental alterations in transcript levels, six genes were selected for in-depth analysis by northern blot analysis, including two photosynthetic genes (*psbA* and *psbD*), three genetic system-related genes (*rpoB*, *rrn16* and *matK*), and a fatty acid biosynthesis gene (*accD*, Fig. 2E). All six transcript patterns observed in the northern blot analyses were consistent with the qRT-PCR results (Fig. 2E).

Having seen the complexity of alterations in transcript abundance in fruits compared with leaves but largely unaltered *psbA* mRNA accumulation (Fig. 2), we were interested in investigating whether PsbA protein accumulation remained constant during fruit ripening. To this end, western blot assay using antibodies against the PsbA protein was performed. A continuous decrease in PsbA protein abundance was seen during chloroplast-to-chromoplast conversion (Fig. 3), suggesting that *psbA* is largely regulated at the level of translation and/or protein stability. PsbD, the D2 protein of PSII, was also strongly downregulated at the protein level during fruit ripening. Notably, PsbD was marginally detectable in immature green fruits, but undetectable in later ripening stages (Fig. 3).

Analysis of intron splicing in kiwifruit plastids

The expression of a subset of plastid genes relies on the excision of introns. The removal of introns from the nascent transcript by splicing is required for producing functional mRNA that can be translated into the correct protein (Barkan 2011). All plastid introns in vascular plants are members of the group II introns except *trnL*-UAA gene which contains a group I intron (Stern et al. 2010). Group II introns are usually spliced in two consecutive transesterification steps that yield ligated exons and an excised intron lariat (Stern et al. 2010). In the plastomes of vascular plants, most group II introns are not able to self-splice but rely on the assistance of a variety of splicing factors (Petersen et al. 2011). While a large

fraction of plastid group II intron splicing factors are nuclear-encoded and targeted into plastids post-translationally (Petersen et al. 2011), the maturase MatK, which is encoded by a reading frame within the *trnK* intron, is associated with splicing of seven plastid introns (Zoschke et al. 2010). Since the removal of introns from precursor mRNA is a crucial step in gene expression and is required for producing mature mRNAs, splicing can contribute to regulating plastid gene expression (Hertel et al. 2013; Karcher and Bock 2002; Petersen et al. 2011). Therefore, we analyzed plastid intron splicing to ascertain whether it had an impact on the observed differences in transcript abundance during the fruit developmental program and chloroplast-to-chromoplast transition in kiwifruit.

In ‘Hongyang’ kiwifruit, six tRNA genes and 11 protein-coding genes contain introns (Yao et al. 2015). The splicing efficiency in transcripts of *petB/D* (each containing one intron) (Stoppel and Meurer 2013) and *ndhB* were investigated by northern blotting. Although *petB/D* belong to the pentacistronic *psbB* operon (*psbB-psbT-psbH-petB-petD*) (Stoppel and Meurer 2013), only a small proportion of *petB/petD* is cleaved into monocistronic mRNAs, leaving most of the transcripts dicistronic (Zhou et al. 2007). As fruit development proceeded, the splicing efficiency of the *petB/D* introns did not change drastically in that the majority of the transcript population was intron-free also in the ripe fruits (Fig. 4A). On the contrary, the intron in the transcript of *ndhB*, encoding a subunit of the NDH complex, exhibited a gradual loss of splicing during fruit developmental stages, and the *ndhB* mRNA abundance remained mostly unspliced in mature fruits (Fig. 4B). The splicing deficiency of the *ndhB* intron was also investigated by northern blotting using an *ndhB* intron-specific probe. As shown in Fig. 4C, the unspliced transcript and the lariat intermediate released from the first transesterification reaction of the group II intron splicing accumulated throughout fruit development, while the excised intron was undetectable in mature fruits. Along with the almost total absence of the ligated exons, these observations suggest that a splicing factor participating in the second-step transesterification of *ndhB* intron removal is missing in ripe fruit chromoplasts.

Identification of mRNA editing sites in plastid transcripts of kiwifruit

A distinguishing feature of plastid gene expression in vascular plant is the requirement for a post-transcriptional modification referred as RNA editing (Small et al. 2020). Plastid genomes of flowering plants typically contain 20–60 RNA editing sites that undergo C-to-U transition at the mRNA level (Ichinose and Sugita 2017), generally resulting in restoration of evolutionarily conserved amino acid sequences (Bock 2000). Reverse change (U-to-C substitution) has been reported to be present in the plastids of hornworts (Kugita et al. 2003; Villarreal et al. 2018). To predict potential RNA editing sites in kiwifruit plastids, we compared all plastid genes with homologs from other species, including angiosperms (Kahlau et al. 2006; Maier et al. 1995; Ruwe et al. 2013), gymnosperms (Wakasugi et al. 1996) (Supplementary Table S1), and most importantly, the liverwort *Marchantia polymorpha* that presumably lost RNA editing during evolution (Oda et al. 1992). As a result, 38 candidate sites of RNA editing were predicted and 35 of them were verified experimentally in kiwifruit plastids. In addition to many conserved sites, we found five new editing sites, including *matK*-152, *matK*-386, *rps3-54*, *psbF* (5' UTR, -2), and *rps12* (*cis*-spliced intron, position 186; Supplementary Table S2).

To investigate whether RNA editing in kiwifruit plastids is regulated in a tissue- and development-specific manner and/or may even take part in regulating plastid transcriptional activities, we systematically analyzed plastid RNA editing by comparing the editing efficiency in plastids of green leaves with those of fruits at different ripening stages. Editing event in each transcript was determined

experimentally by comparing the genomic sequence with the corresponding cDNA sequence. For about two-thirds of the sites analyzed, the editing efficiencies were higher in fruits than that in leaves (Supplementary Table S3). Notably, two sites in the *matK* transcript (*matK*-386, and *matK*-415) were only edited in fruits (Fig. 5). By contrast, for editing sites *ndhB*-196, *ndhD*-1 and *petL*-2, the editing efficiency turned out to be higher in leaves than in fruits (Fig. 5). Intriguingly, editing was completely absent in green fruits at sites *ndhB*-196 and *ndhD*-1, but then reoccurred at a low level (up to ~15%) at the two later stages of fruit development (Fig. 5). Six sites (*atpA*-264, *atpF*-31, *petB*-205, *psbE*-72, *psbF*-26 and *rps12*-74) were completely edited in all tissues and fruit developmental stages (Supplementary Table S3). The editing events at *matK*-386 and *rps3*-54 are silent (Fig. 5), in that the C-to-U transition does not lead to amino acid change. For example, editing at *matK*-386 takes place in the third codon position, and both the unedited codon CCC and the edited codon CCU specify the amino acid proline.

RNA editing occurs predominantly in protein-coding regions where it typically changes the coding properties of mRNA, and occasionally, also in the untranslated regions, introns and structural RNAs (Ichinose and Sugita 2017). In kiwifruit, editing sites were detected in the 5' UTR (nucleotide position -2) of the *psbF* mRNA, and in the *cis*-spliced intron of the *rps12* transcript (Supplementary Table S3).

Discussion

In this work, we have investigated the global gene expression of the plastome in kiwifruit leaves and during fruit development. Since the vast majority of protein-coding genes in the plastome is associated with photosynthetic function, it is generally considered that plastid transcriptional activities are dramatically declined in non-photosynthetic tissues (Kahlau and Bock 2008; Valkov et al. 2009). Nevertheless, we found that compared with leaves of kiwifruits, the transcripts of a small number of photosynthetic genes and many genetic system genes to be upregulated at the RNA level in early-stage fruits and maintained at high level in late-stage fruit. This confirmed our hypothesis the kiwifruit would be an ideal chassis for high transgene expression due to its chloroplast-containing fruits. In contrast to sustained expression during the conversion of chloroplast to chromoplast in tomato fruits (Kahlau and Bock 2008), almost all plastid genes were markedly declined in developing fruits of kiwifruit (Fig. 2C,D), implying that the downregulation is triggered by chloroplast-to-chromoplast conversion. In addition to transcriptional regulation, mRNA turnover may also affect transcript levels in plastids (Barkan 2011). Whether the strong global reduction in RNA accumulation during chloroplast-to-chromoplast conversion (Fig. 2C,D) is mainly the result of decreased plastid transcriptional activities or is, at least partially, also attributed to an increment in RNA turnover remains to be elucidated. Although *psbA* transcript levels remained steady, a gradual decrease was observed in the PsbA protein level (Fig. 3), suggesting that translational regulation of *psbA* and/or regulation of protein stability override the observed pattern of mRNA accumulation (Eberhard et al. 2002).

Besides the regulation of transcript accumulation, posttranscriptional RNA processing potentially contributes to regulating plastid gene expression (Yagi and Shiina 2014). The developmentally downregulated splicing of the *ndhB* intron are in good agreement with the mRNA accumulation patterns during fruit development (Fig. 2, 4), and possibly suggest that splicing may contribute to the regulation of plastid transcription. For most genes analyzed previously in tomato plastids, the ratios of intron-containing primary transcripts to mature spliced RNAs did not altered considerably (Kahlau and Bock 2008). One of the exceptions was the *ndhB* transcript (Kahlau and Bock 2008), which had a similar splicing pattern as in kiwifruit, including release of the lariat intermediate. It would be interesting to

investigate which splicing factor(s) mediate the different splicing reactions involved in *ndhB* intron excision and how they are regulated during kiwifruit and tomato fruit development. It is noteworthy in this context that *ndhB* intron splicing has been shown to also be sensitive to temperature (Karcher and Bock 2002).

Our comprehensive analysis had identified 40 sites of RNA editing, five of which were unique to kiwifruit and had not been found in other species before (Supplementary Table S2). Several differences in tissue- and development-specific editing patterns between fruits of kiwifruit and tomato were evident. For example, in kiwifruit, the majority of editing sites were found to be changed in fruits compared with leaves, whereas only three transcripts (*ndhB*, *ndhD* and *ndhF*) underwent developmental alterations in their RNA editing patterns in tomato (Kahlau and Bock 2008). A lower level of RNA editing at sites of *ndhB*-196 and *ndhD*-1 was observed in fruits, with the unedited form being present in green fruits (Fig. 5; Supplementary Table S3). As the editing event at *ndhB*-196 changes the coding features of the mRNA, leaves and green fruits should harbor distinct NdhB protein populations, as noted previously for etiolated maize seedlings (Karcher and Bock 2002). It is apparent that loss of editing activity at the *ndhD*-1 site would affect the formation of AUG initiation codon from ACG triplet. Nevertheless, the complete lack of editing within the initiation codon does not always make the *ndhD* transcript untranslatable, since previous study has revealed that editing of an ACG codon to the AUG start codon is not strictly required for *ndhD* translation in tobacco (Zanduetta-Criado and Bock 2004). The physiological significance of the development-specific RNA editing patterns at sites of *ndhB*-196 and *ndhD*-1 on protein biosynthesis and/or the assembly and function of the plastid NDH in fruits remain to be elucidated.

We also found that plastid RNA editing in kiwifruit was not restricted to protein-coding sequences and also occurred in an intron and an untranslated region (Supplementary Table S2). RNA editing outside of coding regions was also detected in the 5' UTRs of ginkgo *psbJ* mRNAs (Kudla and Bock 1999), and of maize and rice *ndhG* transcripts (Corneille et al. 2000), but the possible functional significance of these editing events has remained unclear.

Finally, our work reported here has identified a subset of plastid genes that are expressed at relatively high levels in fruits. For example, approximately equal amounts of *rrn16* mRNA were detected in leaves and fruits (Fig. 2E). These genes may provide a precious source of chimeric expression elements that may help to overcome some of the barriers involved in inefficient transgene expression in fruit plastids (Caroca et al. 2013; Zhang et al. 2012). Thus, our work also paves the way to the design of expression cassettes for the efficient production of edible vaccines, biopharmaceuticals and antibodies in kiwifruit (Daniell et al. 2021), although protocols for kiwifruit plastid transformation remains to be established.

Author contributions

SL and JZ: project design; QC and PS: performing the experiments; SL and JZ: analysis of the data; SL and JZ: writing; RB: review and editing. All the authors read and approved the final manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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Figure legend

Fig. 1. Physical map of the kiwifruit (*Actinidia chinensis*) plastid genome. The map was drawn using the complete genome sequence (NC_026690.1) as input by OrganellarGenomeDRAW (Greiner et al. 2019). Genes on the inner circle are transcribed in clockwise direction, those on the outer circle are transcribed in counterclockwise direction. Genes belonging to different functional groups are represented by different colors. The thick lines of the circle mark the extent of the inverted repeats (IR_A and IR_B) that separate the genome into a small single-copy (SSC) region and a large single-copy (LSC) region. Note that the *clpP* gene (normally residing between *psbB* and *rps12* 5' at approximately 10 o'clock) is missing from the genome, and presumably, has been transferred to the nuclear genome by endosymbiotic gene transfer (Bock 2017; Stegemann and Bock 2006).

Fig. 2. Genome-wide analysis of kiwifruit plastid gene expression. (A, B) Alteration of RNA accumulation in three different stages of fruit ripening (green, turning and mature) relative to leaves; (C, D) Alterations in transcript levels during chromoplast development (turning and mature stages) relative to green, chloroplast-containing fruits. Downregulated (blue) or upregulated (red) transcripts are presented on a log₂ scale from -8 to +8. (A) and (C) display all data, while (B) and (D) only show statistically significant changes. (E) Changes in transcript abundance of selected genes were confirmed by northern blot analyses. Below each blot, the ethidium bromide-stained RNA gel prior to blotting is displayed as a control for equal loading. The plant samples used for RNA extractions for northern blotting was generated independently from the material utilized for the qRT-PCR experiments.

Fig. 3. Analysis of PsbA expression at the protein level in fruits. Protein accumulation of PsbA and another subunit of PSII (PsbD) were analyzed by immunoblotting. For each protein, a dilution series of total leaf protein was compared with total proteins isolated from the three fruit ripening stages. Correct loading and protein quantitation were monitored by Coomassie blue staining (upper panel).

Fig. 4. Developmental analysis of intron splicing in *ndhB* and *petB/D* by northern blotting. The hybridization probes used are indicated above each blot. (A) *petD*-specific probe. (B) *ndhB* exon probe. (C) *ndhB* intron probe. Major transcript species are indicated schematically at the right. Introns are represented as white boxes, intron-free genes in polycistronic mRNAs as dark gray boxes, and exons of intron-containing genes as light gray boxes. Asterisks denote mature transcripts. Lariats are indicated by circles. Exons are indicated with roman numerals above the shaded boxes. The ethidium bromide-stained agarose gels are shown as control for equal loading below each blot.

Fig. 5. Analysis of plastid RNA editing efficiency in kiwifruit during development. Editing at selected sites of the *matK*, *ndhB*, *ndhD*, *petL* and *rps3* transcripts is displayed below the corresponding genomic DNA sequences for green leaves and three different fruit ripening stages. Editing sites are indicated by vertical arrows that point to the corresponding peak in the sequencing chromatogram. The relative sizes of the letters C and T above each editing position in the sequence chromatograms of PCR-amplified cDNA represent the editing efficiency.

Supplementary Fig. 1. Kiwifruit samples representing different ripening stages. Cross-sections are shown for a young fruit (A), a turning fruit (B), and a mature fruit (C).

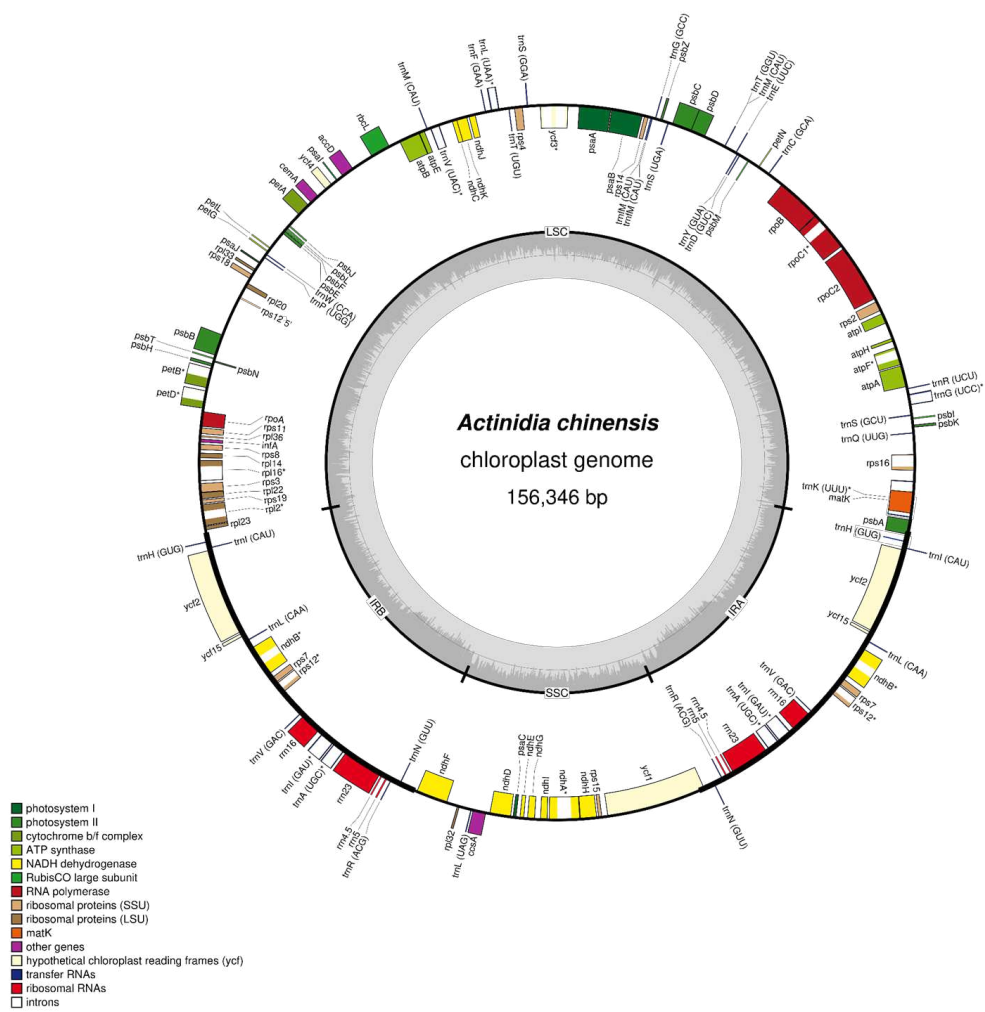


Fig. 1

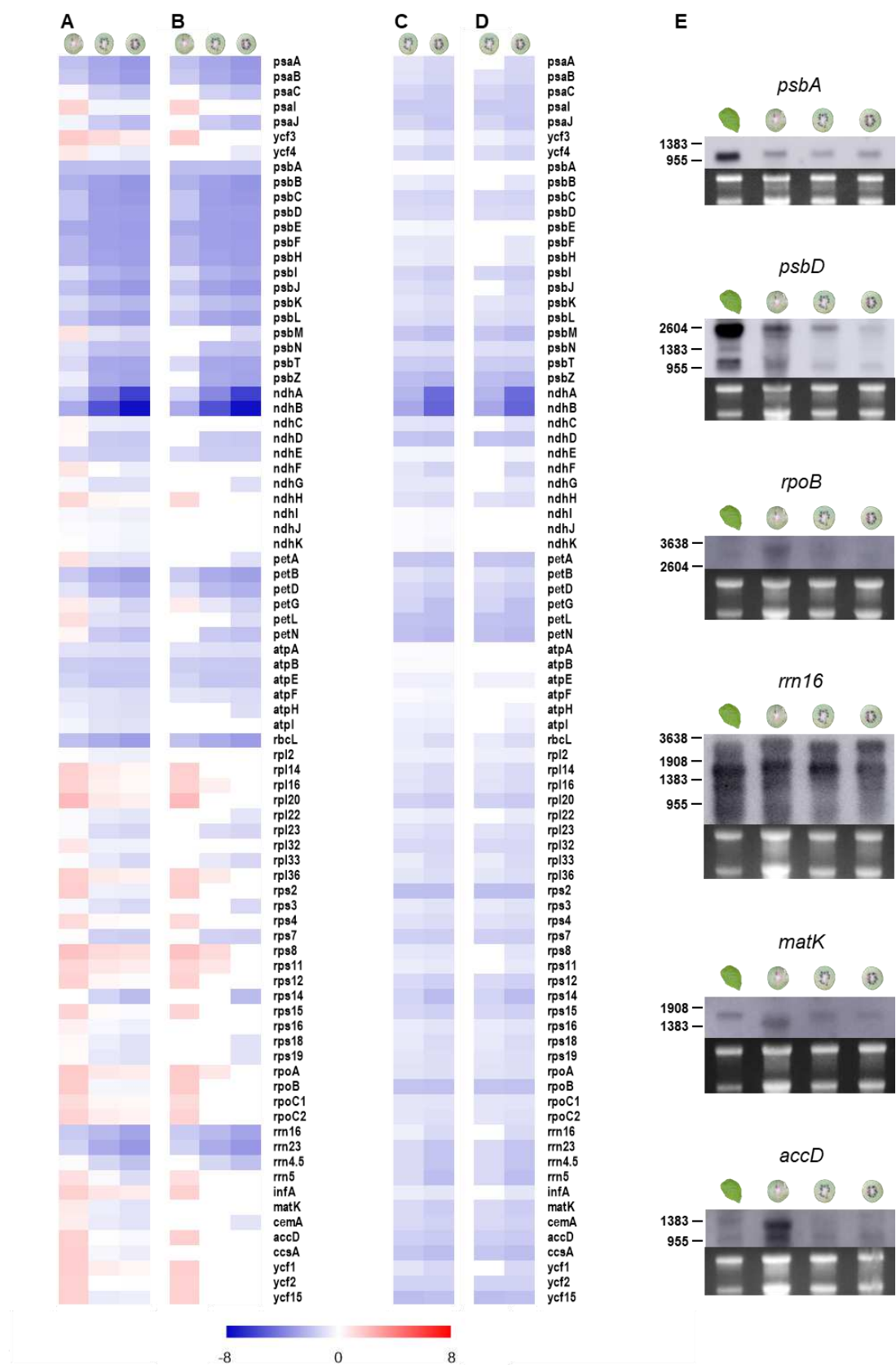


Fig. 2

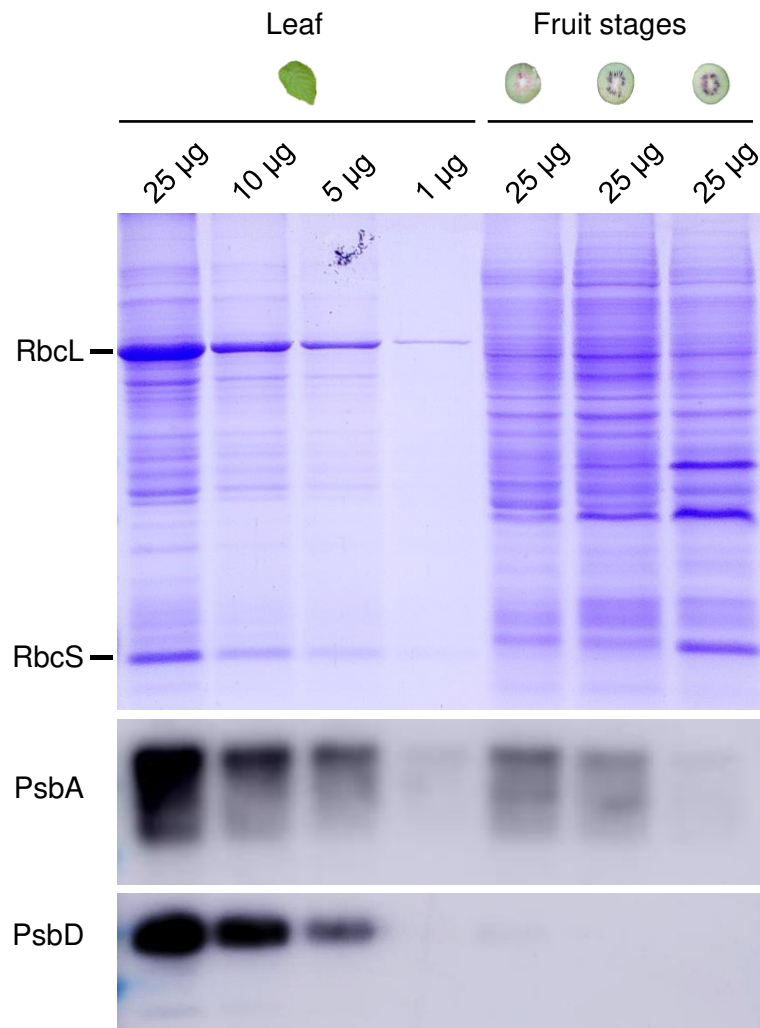


Fig. 3

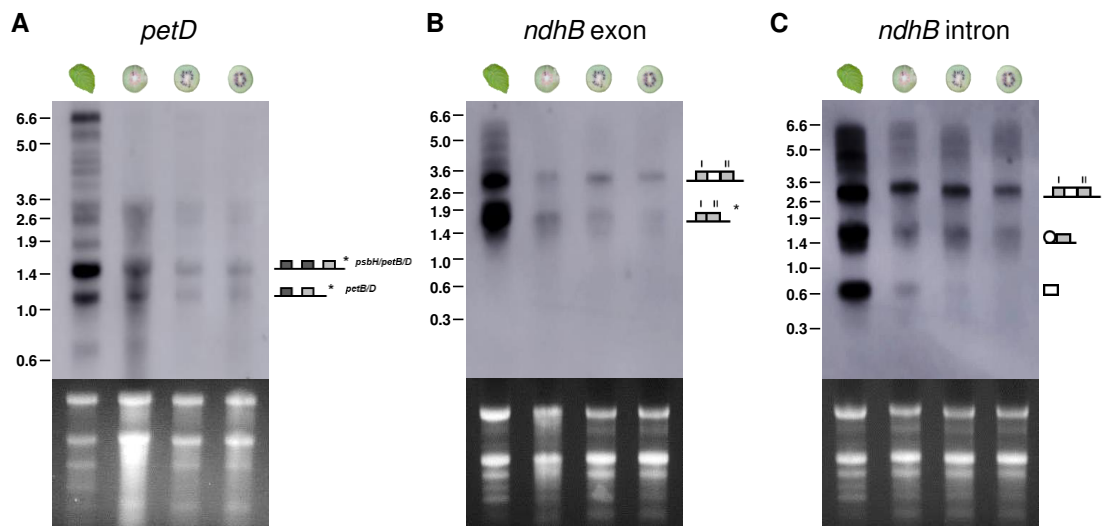


Fig. 4

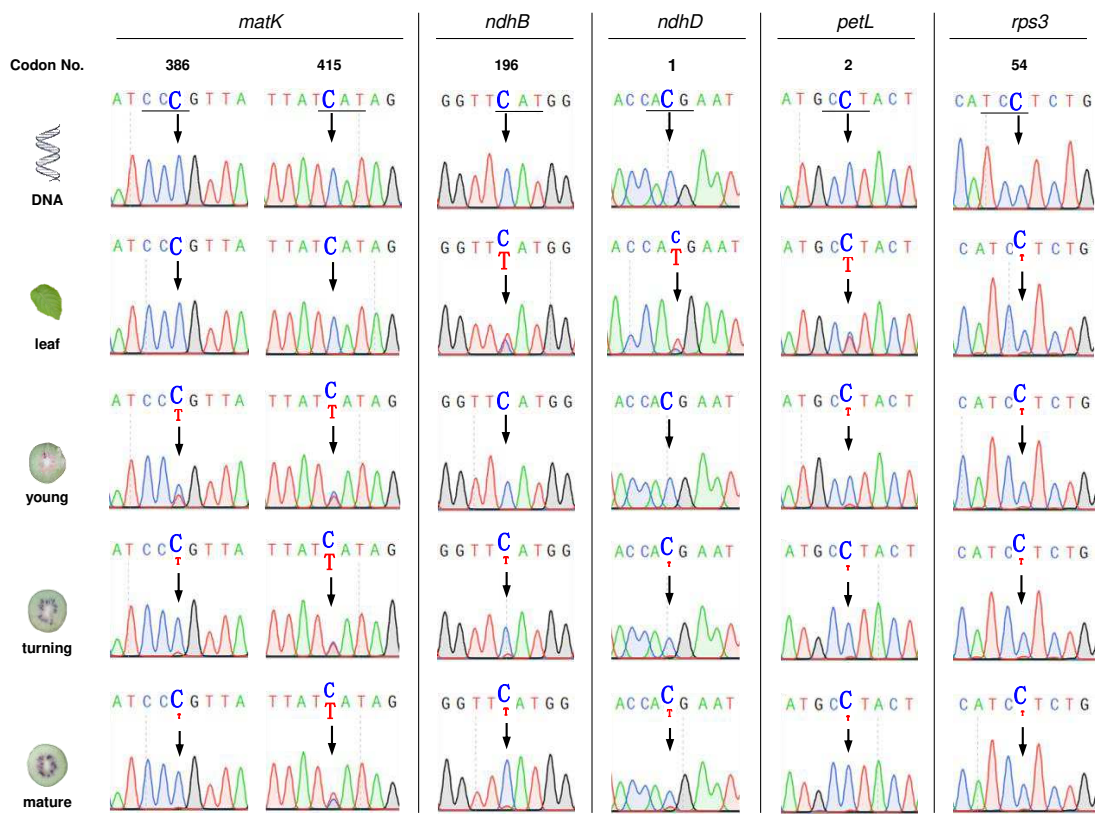


Fig. 5

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