Metabolic Engineering Communications 12 (2021) e00166

Contents lists available at ScienceDirect

Metabolic Engineering Communications

journal homepage: www.elsevier.com/locate/mec

2A-linked bi-, tri-, and quad-cistrons for the stepwise biosynthesis of β-carotene, zeaxanthin, and ketocarotenoids in rice endosperm

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ARTICLE INFO

Keywords:
2A peptide
Carotenoid
Metabolic engineering
Polycistron
Rice

ABSTRACT

Foot-and-mouth disease virus (FMDV) 2A constructs have been successfully used for the production of "Golden Rice", a β-carotene producing rice strain. However, to allay public fears and opposition to plants carrying a mammalian pathogenic viral sequence, 2A-like synthetic sequences from *Thosea asigna* virus and Infectious myonecrosis virus were used to coordinate the coexpression of carotenoid biosynthetic genes. Here, up to four carotenogenic genes encoding PSY, CRTI, BCH and BKT were concatenated and produced β-carotene, zeaxanthin, and ketocarotenoids (astaxanthin and adonixanthin) in transgenic rice seeds displaying color variation due to the difference in carotenoid content and composition.

1. Introduction

Crop biotechnology requires the simultaneous expression of multiple genes, such as in the context of the introduction of new biochemical pathway metabolons, the formation of regulons as multimeric protein complexes, and the stacking of multiple genes to increase yield or induce biotic and abiotic stress tolerances. Viral 2A/2A-like peptides, 18–30 amino acid (aa)-long oligopeptides, have been implemented to mediate self-cleavage of polypeptides at the core sequence of C-terminus of 2A, which is strongly conserved and contains the canonical motif D^{12}(x)E(x)$NPG_{2A}\downarrow P^{19}{}_{2B}$ (x is any amino acid, where P^{19} refers to the completely conserved first residue of 2B), during translation via a ribosomal pausing/shipping/jamming mechanism (Doronina et al., 2008; Luke et al., 2008, 2015). The pausing efficiencies of the 2A peptides are affected by types of host, the length of the 2A peptides or the C-terminal sequence of upstream genes (Donnelly et al., 2001; Luke et al., 2009). Of the many 2A peptides identified to date, foot-and-mouth disease virus 2A (hereafter referred to as "F_{2A}") has been the most widely used for mediating polycistronic expression in eukaryotic systems, including numerous crop plants. Examples include *CrtZ* (3,3′-β-hydroxylase, BCH) and *CrtW* (4,4′-β-oxygenase) for ketocarotenoid in tobacco and tomato (Ralley et al., 2004); the β- and δ-zein proteins in tobacco (Randall et al., 2004); *CaPsy* (*Capsicum* phytoene synthase, PSY) and *PaCrtI* (*Pantoea* carotene desaturase, CRTI) for β-carotene in rice (Ha et al., 2010); *CaBch* (*Capsicum* BCH) and *HpBkt* (*Haematococcus* β-carotene ketolase, BKT) for astaxanthin in rice (Ha et al., 2019); and *mpi* (maize proteinase inhibitor) and *pci* (potato carboxypeptidase inhibitor) for insect and pathogen resistance in rice (Quilis et al., 2014) as bicistrons, and *ZmBz1*, *ZmBz2*, *ZmC1* and *ZmR2* for anthocyanin in maize as a quadcistron (Liu et al., 2018). Another 2A peptide, 2A from porcine teschovirus-1 ("P_{2A}"), has been used in rice for two *Bacillus thuringiensis* crystal proteins, Cry1Ab and Cry2Ab, and exhibited lower cleavage efficiency than F_{2A} (Zhao et al., 2014).

Although F_{2A} has been widely used as an effective tool for multigene expression, resistance from the public to the use of a pathogenic virus may seriously inhibit the introduction of potentially beneficial crops. To appease this concern, we selected three highly active 2Avariants not found in mammalian viruses for the coordinated expression of multiple transgenes (Donnelly et al., 2001; Luke et al., 2008; Supplementary Table S1). Here, we adopted the three-steps of the carotenoid metabolic

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https://doi.org/10.1016/j.mec.2021.e00166
Received 20 November 2020; Received in revised form 17 January 2021; Accepted 29 January 2021

pathway to generate β-carotene, zeaxanthin and ketocarotenoids in transgenic rice seeds as the feasible system to prove the expression of individual polycistron using the color phenotypes (Fig. 1a). The efficiency to express bi-, tri-, and quad-cistrons was examined on the levels of transgenes and proteins and the contents and composition of carotenoids among single recombinant transcripts.

2. Materials and methods

2.1. Gene synthesis and polycistronic vector construction

Native nucleotide sequences encoding three 2A peptides, "T_{2A}", a 20 aa (+)-sense single-stranded RNA insect virus of *Thosea asigna* virus (TaV), and "I_{2A1}" (30 aa) and "I_{2A2}" (30 aa) from a double-stranded RNA shrimp virus of *Infectious myonecrosis* virus (IMNV) (Donnelly et al., 2001; Luke et al., 2008), were codon-optimized for rice (http://www.kazusa.or.jp/codon/) with the Leto software. The synthetic sequence with transit peptides (Tp, 240 bp) from rice phytoene synthase 2 for plastid targeting (Os12g43130; You et al., 2016) and four restriction endonuclease sites that generate blunt-ends were inserted into *pCR4-TOPO* vector (Entelechon, Regensberg, Germany). Four synthetic versions of carotenoid biosynthetic genes (*stPsy*, *stCrtI*, *stBch*, and *stBkt*) encoding *CaPSY*, *PaCRTI*, *CaBCH*, and *HpBKT* respectively, involved in rice carotenoid biosynthesis (Jeong et al., 2017; Ha et al., 2019), were used to construct recombinant polycistronic genes by PCRs using gene-specific primers listed in Supplementary Table S2. The recombinant genes were individually placed under the control of the rice globulin promoter to drive endosperm-specific expression as a single T-DNA into *pMJ103* vector (Ha et al., 2010) by Gateway cloning (Invitrogen, Waltham, MA).

2.2. Rice transformation, growth condition, and seed-color inspection

Five final vectors (*pstPTAC, pstPIAC, pstPTAC-IABc, pstPTAC-IABk, pstPTAC-IABc-IABk*) were individually prepared as *Agrobacteria* strains LBA4404 via tri-parental mating method as previously mentioned (Jeong et al., 2017) and co-cultivated with embryogenic calli from mature seeds of *japonica*-type Korean rice (*Oryza sativa*, cv. Ilmi). The regenerated shoots on MS medium containing phosphinothricin (3 mg/L) and cefotaxime (500 mg/L) were excised from the callus and transferred to MS medium without antibiotics for root regeneration. Putative transgenic plants were acclimatized and grown in a greenhouse under conditions of 16 h light/8 h dark cycle at 28 °C (T_0 and T_1 generation) and the field during the summer season (beyond T_2 generation). The rice seeds were harvested at full maturity of 60 days after flowering (DAF) and used for further experiments after drying for two weeks. Their color was visually inspected after dehusking (TR-200 Electromotion rice husker; Kett, Tokyo, Japan) and polishing (Pearlest polisher; Kett).

2.3. Molecular analyses for DNAs, RNAs, and proteins

Genomic DNA was extracted from leaf tissues of the of T_0 rice plants using a modified cetyltriammonium bromide method. Genomic PCR was performed to verify the integrity of the four transgenes with the EmeraldAmp® PCR Master Mix (TakaRa Bio, Shiga, Japan) using the four forward/reverse primers sets listed in Supplementary Table S2. Taqman PCR analysis was carried out as described previously (Jeong et al., 2017) and data were calculated as the T-DNA copy number in the heterozygous transgenic rice lines comparing with the homozygous transgenic rice line, *stPAC 25*, which was assigned a value of 1 as a single-copy reference (Jeong et al., 2017).

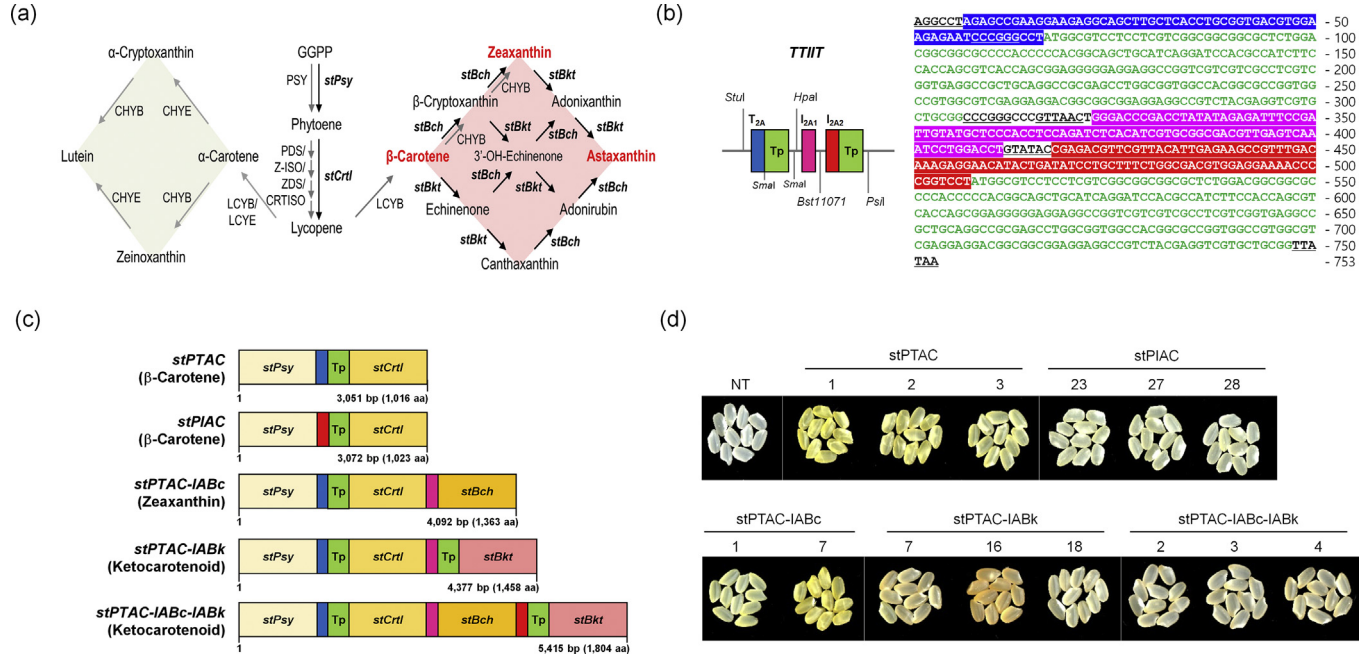

Fig. 1. Generation of transgenic color rice by introducing five polycistronic recombinant genes for stepwise production of diverse carotenoids, respectively. (a) Schematic overview of carotenoid biosynthetic pathway; solid arrows of grey for endogenous and black ones for introduced enzyme steps, with transgenes in italic. Target metabolites are in bolded red. Abbreviations are as follows: GGPP, geranylgeranyl pyrophosphate; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, ζ-carotene isomerase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; CRTI, bacterial carotene desaturase; LCYB, β-carotene cyclase; LCYE, ε-carotene cyclase; CHYB, β-carotene hydroxylase; CHYE, ε-carotene hydroxylase; *stPsy*, synthetic phytoene synthase gene; *stCrtI*, synthetic carotene desaturase gene; *stBch*, synthetic β-carotene hydroxylase gene; *stBkt*, synthetic β-carotene ketolase gene. (b) A 2A-based platform gene structure designed for convenient construction of polycistrons and its synthetic DNA sequences optimized for rice. (c) Five polycistronic recombinant genes, including two bi-, two tri-, and a quad-cistron for stepwise production of diverse carotenoids. (d) Photographs of polished seeds displaying color variation, implying the difference in carotenoid content and composition of five transgenic and a non-transgenic (NT) rice plants. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Total RNAs were extracted from the T_3 mature seeds at 60 DAF after removal of the hull using the PureLink® Plant RNA Reagent (Invitrogen). The cDNAs were synthesized and amplified from 1 μg of total RNAs using AccuPower® RT Premix (Bioneer, Daejeon, Korea). Quantitative real-time RT PCR (qRT-PCR) was carried out with CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Richmond, CA, USA) using SYBR Green Real-time PCR master mix (Bio-rad) and specific primer pairs for the four target gene. The rice ubiquitin 5 gene (*OsUbi5*, Os01g22490) was used as an internal control for normalizing the amount of seed RNAs by amplification with the primer pair *OsUbi5* QF/QR. Data were presented as formula $2^{-\Delta Ct}$ with ± standard errors from three independent experiments. Information of all primers and a probe for DNA and RNA analyses is provided in the Supplementary Table S2.

Proteins were prepared from the T_3 mature seeds at 60 DAF according to previously described methods (Jeong et al., 2017). Western blot analyses were performed using the primary antibodies of anti-CRTI (1:10,000) and anti-2A (1:10,000) (Millipore, Temecula, CA). Antibody-bound signals were developed with the anti-rabbit IgG (Fc) alkaline phosphatase-conjugated secondary antibody (1:200,000) and a BCIP/NBT color development system (Promega, Madison, WI). The images were taken using the LAS 4000 software (Fujifilm, Kanagawa, Japan) and Image QuantTL software (GE Healthcare, Piscataway, NJ) for densitometry.

2.4. Rice seed carotenoid quantitation

Carotenoids were extracted from the T_3 mature seeds at 60 DAF and prepared for high-performance liquid chromatography (HPLC) analysis as described previously (Ha et al., 2019; Ku et al., 2019). They were identified through co-elution with fifteen authentic standards: adonirubin, adonixanthin, β-apo-8′-carotenal, astaxanthin, canthaxanthin, α-carotene, (all-E)-β-carotene, 9Z-β-carotene, 13Z-β-carotene, α-cryptoxanthin, β-cryptoxanthin, echinenone, lutein, lycopene, and zeaxanthin (CaroteNature, Lupsingen, Switzerland). Experiments were carried out with three biological replicates and the results were expressed as the mean ± standard error.

3. Results and discussion

3.1. Design of polycistrons using a 2A-based platform gene and generation of transgenic color rice seeds

Previously, astaxanthin was produced in transgenic rice by the introduction of four steps (PSY, CRTI, BCH and BKT) using a single T-DNA with two cassettes for two bicistronic genes (Ha et al., 2019, Fig. 1a). In this study, we tested bi-, tri-, and quad-cistronic 2A systems for coexpression of these four genes in transgenic rice endosperm. In order to conveniently construct multicistrons, a 2A-based platform gene was first synthesized as an in-frame tandem array of *T_{2A}-Tp-I_{2A1}-I_{2A2}-Tp* with four cloning sites (*TTIIT*, Fig. 1b). The four carotenoid biosynthetic genes were concatenated into five polycistrons including two bicistrons of stPTAC and stPIAC for β-carotene, a tricistron of stPTAC-IABc for zeaxanthin, and a tricistron of stPTAC-IABk and a quadcistron of stPTAC-IABc-IABk for astaxanthin, in all cases linked *via* 2A to form a single open-reading frame (ORF; Fig. 1c). After stable transformation of the T-DNA vectors in rice plants, transgenic candidate lines were obtained on the basis of their endosperm color due to the difference in carotenoid content and composition: yellow for β-carotene and zeaxanthin and red for ketocarotenoids including astaxanthin (Fig. 1d).

3.2. Introduction and expression of bi-, tri-, and quad-cistrons in transgenic rice plants

Introduction of multiple transgenes was confirmed by genomic PCRs in all transgenic rice plants as amplified fragments of four carotenoid genes, *stPsy, stCrtI, stBch*, and *stBkt*: two in *stPTAC* and *stPIAC*, three in *stPTAC-IABc* and *stPTAC-IABk*, and four in *stPTAC-IABc-IABk*, suggesting bi-, tri-, and quad-cistrons, respectively (Fig. 2a, Top). The copy numbers of T-DNA in the rice genomes were determined by Taqman-PCR in the heterozygotic T_0 generation with a homozygous *stPAC 25* rice as a one-copy control (Fig. 2a, bottom). Results showed single T-DNA copy in all of *stPTAC*, *stPIAC*, and *stPTAC-IABc-IABk* and two of *stPTAC-IABk* lines, and multi-copies in all of *stPTAC-IABc* and one of *stPTAC-IABk* lines.

The transcript levels for the *stPsy* and *stCrtI* regions were first compared for all constructs as common genes (Fig. 2b). Results showed similar patterns of expression between these two 2A-linked genes within each polycistron. Then, the transcript levels for the *stBch* and *stBkt* regions were also shown in similar patterns of expression for the *stBch* in *stPTAC-IABc* and *stBch* and *stBkt* in *stPTAC-IABc-IABk* except *stBkt* in *stPTAC-IABk* with those for the *stPsy* and *stCrtI* within the same 2A-linked polyciston. It indicated that four recombinant genes except a *stPTAC-IABk* were well transcribed as a single transcript regardless of the polycistron length.

Protein levels were first examined to compare the cleavage efficiency among T_{2A}, I_{2A}, and F_{2A} using all bicistronic lines of *stPTAC* and *stPIAC* with a representative line of previous *stPAC* including a F_{2A} (Fig. 2c, top). In an immune-blot with seed proteins (40 μg), T_{2A} exhibited a 1.2- and 2.4-fold higher "cleavage" efficiency compared with I_{2A2} and F_{2A} respectively. Moreover, T_{2A} and I_{2A2} did not generate the any un-cleaved product, unlike F_{2A}, which produced PSY:F_{2A}:TP-CRTI – this suggests improved "cleavage" activity with T_{2A} and I_{2A2}. Further, PSY:2A forms between the *stPTAC* and *stPIAC* lines were similar using 2A antibody (Fig. 2c, bottom; Fig. 2d, left). It supported the better function of T_{2A} than I_{2A2} contributed to the translation of the second CRTI ORF (Fig. 2c, top).

The 2A-linked protein levels produced by bi-, tri-, and quad-cistrons were further evaluated among all transgenic lines (Fig. 2d). The first immune-blot using 2A antibody with seed proteins (40 μg) showed the common PSY:2As signals in all lines of *stPTAC*, *stPIAC*, and *stPTAC-IABc* except *stPTAC-IABk* and *stPTAC-IABc-IABk* (data not shown), suggesting the highest expression of PSY:2A in the two bicistrons and perhaps dependency on copy number in a tricistron. To detect the 2A-linked protein signals in another tricistronic *stPTAC-IABk* and a quadcistronic *stPTAC-IABc-IABk*, amounts of seed proteins were increased to four-times (160 μg) (Fig. 2d). Overall, results showed multiple 2A signals in all transgenic color rice seeds as the expected number: one for bicistrons *stPTAC* and *stPIAC* (PSY:2A), two for tricistrons *stPTAC-IABc* and *stPTAC-IABk* (PSY:2A and CRTI:I_{2A1}), and three for the quadcistron *stPTAC-IABc-IABk* (PSY:2A, CRTI:I_{2A1} and BCH:I_{2A2}), suggesting these 2A-linked polycistrons worked well in rice systems. However, the last two proteins, BCH and BKT, in the *stPTAC-IABc-IABk* rice lines were only detectable at the size corresponding to the uncleaved product (BCH:I_{2A2}:Tp-BKT). This suggests that the third 2A (I_{2A2}) at the 4.1 kb-position of quadcistron exhibited incomplete "cleavage" activity.

3.3. Stepwise production of β-carotene, zeaxanthin, and ketocarotenoids by bi-, tri-, and quad-cistronic expression in the transgenic rice seeds

HPLC analysis indicated an increase in the total amount of carotenoids in all transgenic seeds (Fig. 3 and Supplementary Table S3). A two-fold higher level of carotenoids in the *stPTAC* than *stPIAC* seeds was correlated with a two-fold increase in the efficacy of T_{2A}, compared to I_{2A2}, in driving CRTI translation (Fig. 2c, top). With the integration and expression of all transgenes in mature seeds, individual polycistron drove carotenoid production as intended: β-carotene by *stPTAC* and *stPIAC*, zeaxanthin by *stPTAC-IABc*, and ketocarotenoids (astaxanthin and adonixanthin) by *stPTAC-IABk* and *stPTAC-IABc-IABk*, proving that polycistronic expression of carotenoid biosynthetic genes successfully directed the metabolic flow in rice seeds. The highest levels for β-carotene, zeaxanthin, and ketocarotenoids were resulted in lines with the higher total carotenoid contents over 1 μg/g, which were reflected as more intense color traits in *stPTAC1, 2, 3* (yellow), *stPTAC-IABc 7*

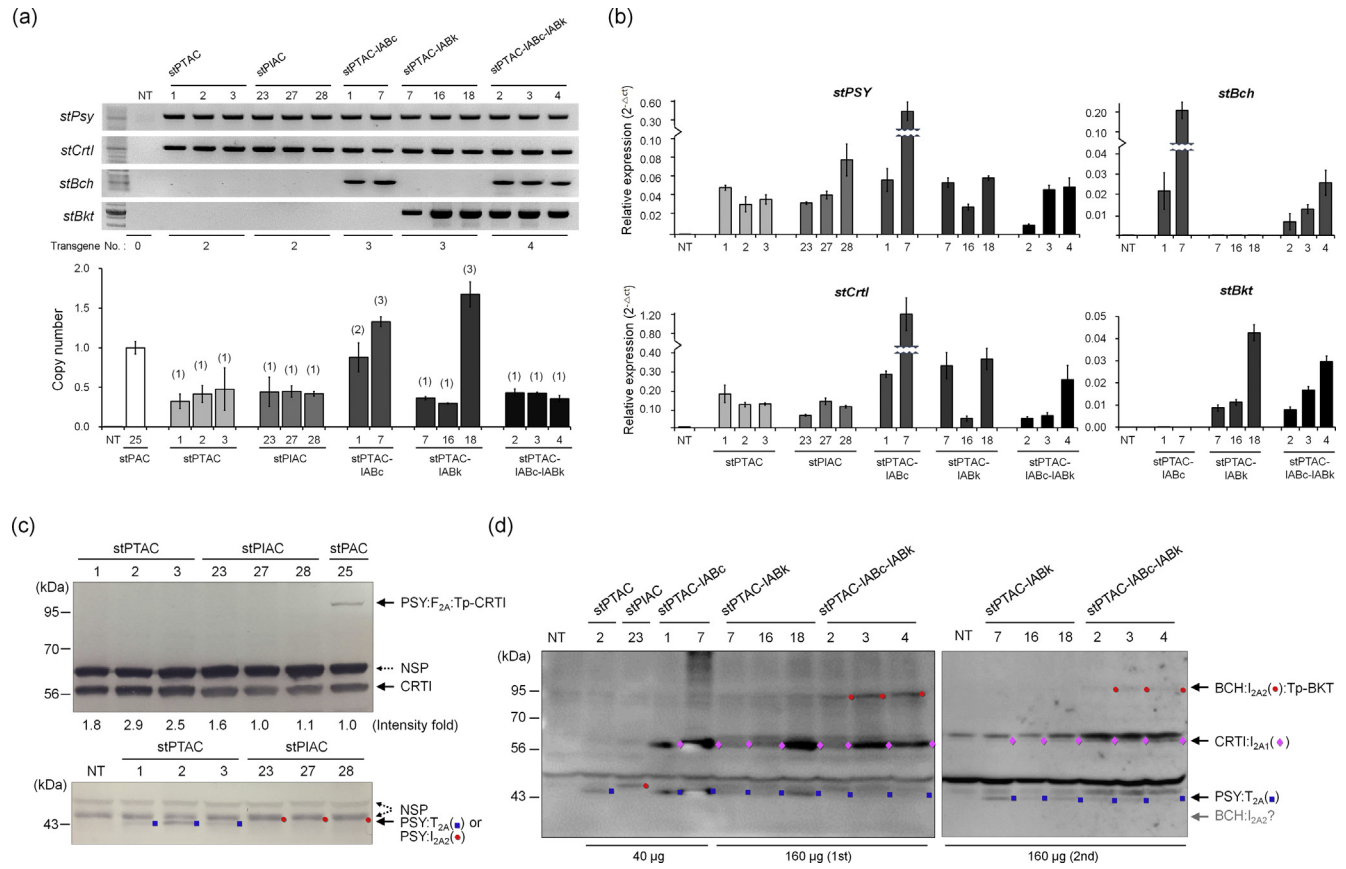

Fig. 2. The integration and expression of bi-, tri-, and quad-cistronic genes in transgenic rice. (a) Genomic PCRs (top) and Taqman PCRs (bottom) to verify transgene integration and copy number of T-DNAs in the transgenic rice genome. (b) qRT-PCRs to quantify the relative expression levels of the *stPsy, stCrtI, stBch*, and *stBkt* transcripts in T_3 transgenic rice seeds. The same amounts of seed RNA were normalized with the *OsUbi 5* gene. (c–d) Immunoblots to analyze the protein levels in T_3 transgenic seeds with anti-CRTI and anti-2A antibodies. The target proteins and nonspecific product (NSP) are indicated by solid and dotted arrows, respectively. Target proteins linked with T_{2A}, I_{2A1} and I_{2A2} are indicated as blue rectangle, pink diamond and red circle, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

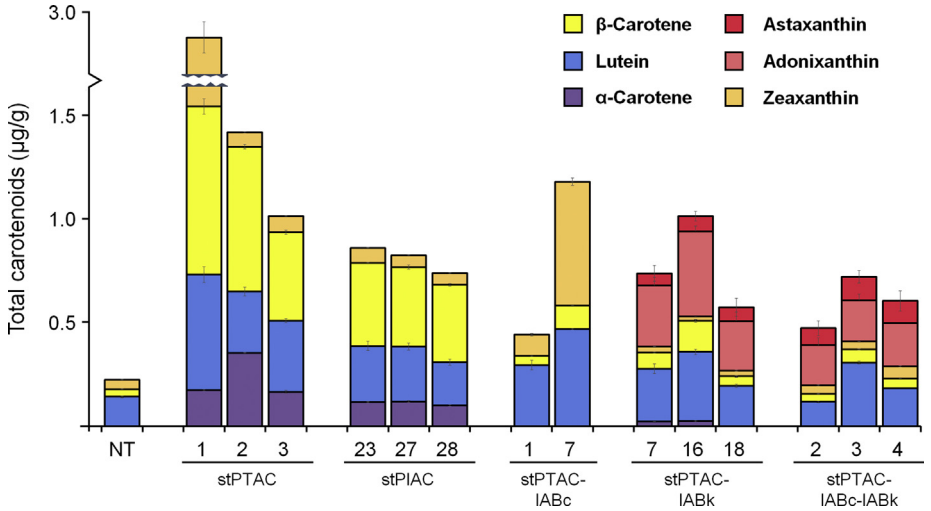

Fig. 3. Bar graph to compare carotenoid contents and composition among the transgenic rice seeds. Unpolished color seeds were used for HPLC analysis at T_3 generation. Data are expressed as mean (μg/g dry weight) ± SE from three independent experiments. Numerical results of this figure refer to Supplementary Table S3 in detail. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(yellow), and *stPTAC-IABk 16* (pinkish red) seeds (Fig. 1d). Interestingly, a *stPTAC-IABk* produced higher ketocarotenoids but less astaxanthin than a *stPTAC-IABc-IABk*. It suggested that a total capacity for carotenogenesis was improved by the tricistron than the quadcistron, while exogenous BCH aided in extending the pathway toward astaxanthin in rice seeds (Fig. 3 and Supplementary Table S3).

4. Conclusion

This study demonstrated the successfully coordinated expression of two, three, or four carotenoid biosynthetic genes linked by 2A sequences, resulting in diverse colored rice plants. Our results provide new applications for three 2A peptides, T_{2A}, I_{2A1} and I_{2A2}, of non-mammalian pathogenic viral origin, and suggest that T_{2A} is a good alternative to F_{2A}. Further, if multiple gene expression is required, different members of the 2A peptide family can be selected to disrupt sequence homology. This work indicates that polycistrons for multi-step metabolic engineering may be useful for developing new biofortified crops, although 2A-driven cleavage in quadcistron might be limited beyond 4.1-kb-long transcripts.

Author statement

S-H Ha conceived the experiments and supervised manuscript writing. YS Jeong and Y-J Jung constructed vectors and performed molecular experiments. H-K Ku statistically analyzed the data. JK Kim and KB Lee performed carotenoid analysis. S-H Lim generated transgenic rice plants. J-K Kim and D Lee conceptualized and curated data. YS Jeong and H-K Ku wrote the original manuscript.

Declaration of competing interest

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

Acknowledgements

This work was supported by grants from the Next-Generation BioGreen 21 Program (PJ01368801) and the Next Generation New Plant Breed Technology Program (PJ01477202) funded by the Rural Development Administration.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2021.e00166.

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