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

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A B S T R A C T

Foot-and-mouth disease virus (FMDV) 2A constructs have been successfully used for the production of “Golden Rice”, a \( \beta \)-carotene producing rice strain. However, to allay public fears and opposition to plants carrying a mammalian pathogenic viral sequence, 2A-like synthetic sequences from \( \text{Thosaea asigna} \) 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 \( \beta \)-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 D12(x)E(x)NPG2A↓P19 (x is any amino acid, where P19 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 “F2A”) has been the most widely used for mediating polycistronic expression in eukaryotic systems, including numerous crop plants. Examples include CrtZ (3,3’-\( \beta \)-hydroxylase, BCH) and CrtW (4,4’-\( \beta \)-oxygenase) for ketocarotenoid in tobacco and tomato (Balley et al., 2004); the \( \beta \)- and \( \delta \)-zein proteins in tobacco (Randall et al., 2004); CaPsy (\textit{Capsicum} phytoene synthase, PSY) and PaCrtI (\textit{Pantoea} carotene desaturase, CRTI) for \( \beta \)-carotene in rice (Ha et al., 2010); CaBch (\textit{Capsicum} BCH) and HpBkt (\textit{Haematococcus} \( \beta \)-carotene ketolase, BKT) for astaxanthin in rice (Ha et al., 2019); and mpi (maize proteinase inhibitor) andpci (potato carboxypeptidase inhibitor) for insect and pathogen resistance in rice (Quilis et al., 2014) as bicistrons, and ZmBz1, ZmBz2, ZmCl and ZmR2 for anthocyanin in maize as a quadcistron (Liu et al., 2018). Another 2A peptide, 2A from porcine teschovirus-1 (“P2A”), has been used in rice for two \textit{Bacillus thuringiensis} crystal proteins, Cry1Ab and Cry2Ab, and exhibited lower cleavage efficiency than F2A (Zhao et al., 2014).

Although F2A 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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pathway to generate \( \beta \)-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 *Tosea 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 four restriction endonuclease sites that generate blunt-ends were inserted into pCR4-TOPO vector (Entelechon, Regensburg, Germany). Four synthetic versions of carotenoid biosynthetic genes (\( \text{stPsy}, \text{stCrI}, \text{stBch}, \) and \( \text{stBkt} \)) encoding \( \text{CaPSY}, \text{PaCRTI}, \text{CaBCH}, \) and \( \text{HpBKT} \) 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 vectors 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 (\( \text{pstPTAC}, \text{pstPIAC}, \text{pstPTAC-IAbc}, \text{pstPTAC-IAbK} \)) 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 phosphonothricin (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 (Ti and T1 generation) and the field during the summer season (beyond T2 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 (Pearlesee polisher; Kett).

2.3. Molecular analyses for DNAs, RNAs, and proteins

Genomic DNA was extracted from leaf tissues of the of T0 rice plants using a modified cetyltrimmonium 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, sPAC 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, \( \zeta \)-carotene isomerase; ZDS, \( \zeta \)-carotene desaturase; CRTISO, carotenoid isomerase; CRTI, bacterial carotene desaturase; LCYB, \( \beta \)-carotene cyclase; LCYE, \( \epsilon \)-carotene cyclase; ChYB, \( \beta \)-carotene hydroxylase; CHYE, \( \epsilon \)-carotene hydroxylase; stPsy, synthetic phytoene synthase gene; stCrI, synthetic carotene desaturase gene; stBch, synthetic \( \beta \)-carotene hydroxylase gene; stBkt, synthetic \( \beta \)-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 genes. 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$^{-∆CT}$ 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 lyases were performed using the primary antibodies of anti-CRTI (1:10,000) and anti-2A (1:10,000) (Millipore, Temecula, CA). Antibody-bound bands were detected using the LAS 4000 software (Fuji 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 prepared for high-performance liquid chromatography (HPLC) analysis with the primer pair OsUBI5 QF/QR. Data were presented as formula 2$^{-∆CT}$ 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.

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$_2$A-Tp-I$_{2A1}$-I$_{2A2}$-Tp with four cloning sites (TTTIF, 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 stPTAC 25 rice as a one-copy control (Fig. 2a, bottom). Results showed single T-DNA copy in all of stPTAC, stPIAC, and stPTAC-IABc 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 polycistron. It indicated that four recombinant genes except a stPTAC-IABc were well transcribed as a single transcript regardless of the polycistron length.

Protein levels were first examined to compare the cleavage efficiency among T$_2$A, I$_2$A, and F$_2$A using all bicistron lines of stPTAC and stPIAC with a representative line of previous stPTAC including a F$_2$A (Fig. 2c, top). In an immune-blot with seed proteins (40 µg) showed the 2A antibody (1:10,000) and anti-2A (1:10,000) (Millipore, Temecula, CA). Antibody-bound bands were detected using the LAS 4000 software (Fuji Japan) and Image QuantTL software (GE Healthcare, Piscataway, NJ) for densitometry.

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$_2$A, compared to I$_2$A, 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 highest total carotenoid contents over 1 µg/g, which were reflected as more intense color traits in stPTAC, 2, 3 (yellow), stPTAC-IABc 7
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, T2A, I2A1, and I2A2, of non-mammalian pathogenic viral origin, and suggest that T2A is a good alternative to F2A. 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 D-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.

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Appendix A. Supplementary data

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

References

Donnelly, M.L.L., Hughes, L.E., Luke, G., Mendes, H., Ten Dam, E., Gani, D., Ryan, M.D., 2001. The ‘cleavage’ activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring 2A-like sequences. J. Gen. Virol. 82, 1027–1041.

Doronina, V.A., Wu, C., de Felipe, P., Sachs, M.S., Ryan, M.D., Brown, J.D., 2008. Site-specific release of nascent chains from ribosomes at a sense codon. Mol. Cell Biol. 28, 4227–4235.

Ha, S.-H., Kim, J.-K., Jeong, Y.S., You, M.-K., Lim, S.-H., Kim, J.-K., 2019. Stepwise pathway engineering to the biosynthesis of zeaxanthin, astaxanthin and capsanthin in rice endosperm. Metab. Eng. 52, 178–189.

Ha, S.-H., Liang, Y.S., Jung, H., Ahn, M.-J., Suh, S.-C., Kweon, S.J., Kim, D.-H., Kim, Y.-M., Kim, J.-K., 2010. Application of two bicistronic systems involving 2A and IRIS sequences to the biosynthesis of carotenoids in rice endosperm. Plant Biotechnol. J. 8, 928–938.

Jeong, Y.S., Ha, H.-K., Kim, J.-K., You, M.-K., Lim, S.-H., Kim, J.-K., Ha, S.-H., 2017. Effect of codon optimization on the enhancement of the β-carotene contents in rice endosperm. Plant Biotechnol. Rep. 11, 171–179.

Ku, H.-K., Jeong, Y.S., You, M.-K., Jung, Y.J., Kim, T.J., Lim, S.-H., Kim, J.K., Ha, S.-H., 2019. Alteration of carotenoid metabolic machinery by β-carotene biofortification in rice grains. J. Plant Biol. 62, 451–462.

Liu, X., Yang, W., Mu, B., Li, S., Li, Y., Zhou, X., Zhang, C., Fan, Y., Chen, R., 2018. Engineering of ‘Purple Embryo Maize’ with a multigene expression system derived from a bidirectional promoter and self-cleaving 2A peptides. Plant Biotechnol. J. 16, 1107–1109.

Luke, G.A., de Felipe, P., Lukashev, A., Kallioinen, S.E., Bruno, E.A., Ryan, M.D., 2008. Occurrence, function and evolutionary origins of 2A-like sequences in virus genomes. J. Gen. Virol. 89, 1036–1042.

Luke, G.A., Escuin, H., Felipe, P.D., Ryan, M.D., 2009. 2A to the fore - research, technology and applications. Biotechnol. Genet. Rev. 26, 223–260.

Luke, G.A., Roulston, C., Tihner, J., Ryan, M.D., 2015. Growing uses of 2A in plant biotechnology. In: Ekinicì, D. (Ed.), Biotechnology. InTech, Rijek, pp. 165–193. https://doi.org/10.5772/59878.

Quillis, J., Lopez-Garcia, B., Meynard, D., Guiderdoni, E., San Segundo, B., 2014. Inducible expression of a fusion gene encoding two protease inhibitors leads to insect and pathogen resistance in transgenic rice. Plant Biotechnol. J. 12, 367–377.

Ralley, L., Enfissi, E.M.A., Misawa, N., Schuch, W., Brambley, P.M., Fraser, P.D., 2004. Metabolic engineering of ketocarotenoid formation in higher plants. Plant J. 39, 477–486.

Randall, J., Sutton, D., Ghoshroy, S., Bagga, S., Kemp, J.D., 2004. Co-ordinate expression of β- and δ-zeins in transgenic tobacco. Plant Sci. 167, 367–372.

You, M.-K., Kim, J.H., Lee, Y.J., Jeong, Y.S., Ha, S.-H., 2016. Plastoglobule-targeting competence of a putative transit peptide sequence from rice phytoene synthase 2 in plastids. Int. J. Mol. Sci. 18, 18.

Zhao, Q., Liu, M., Tan, M., Gao, J., Shen, Z., 2014. Expression of Cry1Ab and Cry2Ab by a bicistronic transgene with a self-cleavage peptide in rice. PLoS One 9, e110066.