Pantoea ananatis carotenoid production confers toxoflavin tolerance and is regulated by Hfq-controlled quorum sensing

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Abstract

Carotenoids are widely used in functional foods, cosmetics, and health supplements, and their importance and scope of use are continuously expanding. Here, we characterised carotenoid biosynthetic genes of the plant-pathogenic bacterium Pantoea ananatis, which carries a carotenoid biosynthetic gene cluster (including crtE, X, Y, I, B, and Z) on a plasmid. Reverse transcription–polymerase chain reaction (RT-PCR) analysis revealed that the crtEXYIB gene cluster is transcribed as a single transcript and crtZ is independently transcribed in the opposite direction. Using splicing by overlap extension with polymerase chain reaction (SOE by PCR) based on asymmetric amplification, we reassembled crtE–B, crtE–B–I, and crtE–B–I–Y. High-performance liquid chromatography confirmed that Escherichia coli expressing the reassembled crtE–B, crtE–B–I, and crtE–B–I–Y operons produced phytoene, lycopene, and β-carotene, respectively. We found that the carotenoids conferred tolerance to UV radiation and toxoflavin. Pantoea ananatis shares rice environments with the toxoflavin producer Burkholderia glumae and is considered to be the first reported example of producing and using carotenoids to withstand toxoflavin. We confirmed that the carotenoid production of P. ananatis is dependent on RpoS, which is positively regulated by Hfq/ArcZ and negatively regulated by ClpP, similar to an important regulatory network of E. coli (HfqArcZ - RpoS ClpXP). We also demonstrated that Hfq-controlled quorum signalling de-represses EanR to activate RpoS, thereby initiating carotenoid production. Survival genes such as those responsible for the production of carotenoids of the plant-pathogenic P. ananatis must be expressed in a timely manner to overcome stressful environments and compete with other microorganisms. This mechanism is likely maintained by a brake with excellent performance, such as EanR.

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SUMMARY

Carotenoids are widely used in functional foods, cosmetics, and health supplements, and their importance and scope of use are continuously expanding. Here, we characterised carotenoid biosynthetic genes of the plant-

pathogenic bacterium *Pantoea ananatis* , which carries a carotenoid biosynthetic gene cluster (including *crtE* , *X* , *Y* , *I* , *B* , and *Z*) on a plasmid. Reverse transcription–polymerase chain reaction (RT-PCR) analysis revealed that the *crtEXYIB* gene cluster is transcribed as a single transcript and *crtZ* is independently transcribed in the opposite direction. Using splicing by overlap extension with polymerase chain reaction (SOE by PCR) based on asymmetric amplification, we reassembled*crtE –B* , *crtE –B –I* , and*crtE –B –I –Y* . High-performance liquid chromatography confirmed that *Escherichia coli* expressing the reassembled *crtE –B* , *crtE –B –I* , and*crtE –B –I –Y* operons produced phytoene, lycopene, and β-carotene, respectively. We found that the carotenoids conferred tolerance to UV radiation and toxoflavin. *Pantoea ananatis* shares rice environments with the toxoflavin producer*Burkholderia glumae* and is considered to be the first reported example of producing and using carotenoids to withstand toxoflavin. We confirmed that the carotenoid production of *P . ananatis* is dependent on RpoS, which is positively regulated by Hfq/ArcZ and negatively regulated by ClpP, similar to an important regulatory network of *E . coli* (Hfq^{ArcZ} - RpoS ClpXP). We also demonstrated that Hfq-controlled quorum signalling de-represses EanR to activate RpoS, thereby initiating carotenoid production. Survival genes such as those responsible for the production of carotenoids of the plant-pathogenic *P . ananatis* must be expressed in a timely manner to overcome stressful environments and compete with other microorganisms. This mechanism is likely maintained by a brake with excellent performance, such as EanR.

INTRODUCTION

Carotenoids are widely used in functional foods, cosmetics, and health supplements, and their importance and scope of use are continuously expanding (Song *et al .*, 2013; Ram *et al .*, 2020). Carotenoids are produced by plants and microorganisms including algae, fungi, yeast, and bacteria, but animals must obtain carotenoids from dietary sources. Interestingly, aphids, which are capable of synthesising carotenoids, are reported by later gene transfer from fungi (Moran and Jarvik, 2010).

A number of carotenoid-producing bacteria have been identified (Lorquin*et al .*, 1997; Dufossé *et al .*, 2005; Sodkova *et al .*, 2005; Sajilata *et al .*, 2008; Fasano *et al .*, 2014; Virtamo*et al .*, 2014; Lu *et al .*, 2017; Fidan and Zhan, 2019; Ram*et al .*, 2020). Carotenoids are highly hydrophobic, restricted to essential parts of the complex membrane and cell wall in bacteria, and mainly responsible for enhancing various functions related to the cell membrane and walls (Kirti *et al .*, 2014, Lutnaes *et al .*, 2004, Vila *et al .*, 2019). Carotenoids enhance various membrane functions, including physical strength, fluidity, cell wall rigidity, and lipid peroxidation. Several functions are closely related to the habitats of bacteria; in particular, the carotenoids of bacterial species living in low- or high-temperature environments are used to control the membrane fluid, while those of bacteria continuously exposed to UV radiation increase tolerance to UV (Kunisawa and Stanier, 1958; Mathews and Sistrom, 1959; Stanier, 1959; Mathew and Sistrom, 1960; Dundas and Larsen, 1963; Mostofian *et al .*, 2020). In addition, carotenoids aid bacteria in combating stress related to oxidation, salt, and desiccation (Oren, 2009; Tian and Hua, 2010). When bacteria are placed in a stressful environment, carotenoid production increases to protect against particular stressors, such as temperature, salt, light, and acidity (Paliwal *et al .*, 2017; Ram *et al .*, 2019). This is consistent with the fact that bacterial carotenoid production is closely related to habitat characteristics.

Pantoea ananatis is considered as an emerging pathogen based on the increasing number of reports of diseases occurring in a wide range of economically important agricultural crops worldwide. This pathogen can also infect humans and numerous insects (Coutinho and Venter, 2009; Dutta *et al .*, 2016; Weller-Stuart *et al .*, 2017) and cause bacteremia infection (De Baere *et al .*, 2004). *P. ananatis*PA13 causes plant diseases such as rice grain rot, sheath rot, and onion center rot disease in Korea (Choi *et al .*, 2012a; Choi *et al .*, 2012b; Kim and Choi, 2012). This pathogen is a potential threat to stable rice production, in particular during the growing season, when the weather is hot and humid.

Quorum sensing (QS) is bacterial cell-to-cell communication with extracellular signalling molecules called autoinducers that are present in the environment in proportion to cell density (Platt and Fuqua, 2010). This system facilitates community coordination of gene expression and benefits group behaviours. QS of *P . ananatis,* which uses EanRI homologous to *P . stewartii* subsp.*stewartii* EsaRI, has revealed that

EanR negatively regulates self-expression and EPS production, but not *eanI* expression (von Bodman and Farrand, 1995; Minogue *et al* ., 2005; Morohoshi*et al* ., 2007; Lee, 2015). In *P* . *ananatis* , 3-oxo-hexanoyl homoserine lactone (3-oxo-C6AHL) and hexanoyl homoserine lactone (C6AHL) signals are generated by EanI and secreted extracellularly. AHL signals bind EanR, an AHL receptor; this interaction de-represses the EanR negative regulator (Morohoshi *et al* ., 2007).

The RNA chaperone Hfq and sRNAs are important regulators of virulence in*P. ananatis* (Kang, 2017; Shin *et al* ., 2019). Hfq, a ring-shaped hexameric RNA binding protein, has many important physiological roles that are mediated by interaction with Hfq-dependent small RNAs (sRNAs) in bacteria (Brennan and Link, 2007). Hfq was first reported in *Escherichia coli* as a host factor important in the replication of bacteriophage Qβ (Muffler *et al.* , 1996). Hfq regulates the stress response protein RpoS, which controls many stress response genes (Brown and Elliott, 1996; Mandin and Gottesman, 2010; Hwang *et al* ., 2011); it also regulates virulence in several pathogenic bacteria (Sittka *et al* ., 2007; Chao and Vogel, 2010; Zeng *et al* ., 2013). In addition, it modulates a wide range of physiological responses in bacteria. The *hfq* deletion mutant exhibits several different phenotypes (Figueroa-Bossi *et al* ., 2006). The Hfq protein interacts with A/U-rich regions of untranslated sRNAs of 50–250 nucleotides with tree stem-loop sequence motifs (Lorenz*et al* ., 2010) and assists with sRNA base pairing with target mRNA (Beisel and Storz, 2010) and the regulation of gene expression (Vogel and Wagner, 2007; Fröhlich and Vogel, 2009; Bardill and Hammer, 2012). Hfq is required for the functioning of several regulatory sRNAs, including OxyS and RyhB (Storz *et al* ., 2004; Majdalani *et al* ., 2005; Aiba, 2007; Gaida *et al* ., 2013). sRNAs act as activators or repressors of protein translation through complementary base pairing with mRNA in response to change in environmental conditions (Gottesman *et al* ., 2006; Waters and Storz, 2009; Beisel and Storz, 2010). Several sRNAs regulate RpoS, including ArcZ. ArcZ (also called RyhA and SraH) binds Hfq and positively regulates regulatory RNA, which controls the translation of RpoS (Repila *et al* ., 2003). ArcZ also regulates virulence, exopolysaccharide (EPS) production, motility, and the hypersensitive response (HR) in bacterial plant pathogens (Papenfort *et al* ., 2009; Soper *et al* ., 2010; Bak*et al* ., 2014; Zeng and Sundin, 2014; Schachterle and Sundin, 2019).

Bacteria are surprisingly rich producers of carotenoids. However, bacteria with a low carotenoid content are unsuitable for commercial use. Production of plant-based carotenoids in bacteria is easier than in eukaryotic organisms such as yeasts, fungi, and plants (Ram *et al* ., 2020). Previously, biosynthesis of carotenoids has relied on bacterial carotenoid genes and DNA recombination techniques. Because these methods depend on restriction sites, generating recombinant DNA fragments and rearranging multiple carotenoid genes is problematic. The technique of splicing by overlap extension by polymerase chain reaction (SOE by PCR) using asymmetric amplification was first developed for introducing mutations into the centre of a PCR fragment (llis *et al* ., 1986; Higuchi *et al* ., 1988; Ho *et al* ., 1989), making site-directed mutagenesis more flexible. Horton *et al* . (1989) modified SOE by PCR to allow DNA segments from two different genes to be spliced together by overlap extension. SOE has been applied to enhance site-directed mutagenesis (Xiao *et al* ., 2007; Duan *et al* ., 2013; Hussain and Chong, 2016), generation of nonpolar, markerless deletions in bacteria (Merritt *et al* ., 2007; Kim *et al* ., 2013; Xu *et al* ., 2013), multiple-site fragment deletion (Zeng*et al* ., 2017), and generation of hybrid proteins of immunological interest (Warrens *et al* ., 1997).

We reassembled carotenoid genes (*crtE* , *crtB* , *crtI* , and *crtY*) of *P. ananatis* using splicing by overlap extension (SOE) to enable production of phytoene, lycopene, and β-carotene in *Escherichia coli* . We found that carotenoids were responsible for toxoflavin tolerance in *P* . *ananatis* . We confirmed that carotenoid production in *P* . *ananatis* is dependent on RpoS, which is regulated positively by Hfq/ArcZ and negatively by ClpP, similar to an important regulatory network of*E* . *coli* (Hfq^{ArcZ} - RpoS ClpXP). We also showed that Hfq-controlled quorum signalling de-represses EanR to activate RpoS, thereby initiating carotenoid production.

RESULTS

*Identification of the carotenoid biosynthetic gene cluster in*Pantoea ananatis *PA13*

We previously reported the whole genome sequence of *P .ananatis* PA13 (Choi *et al* ., 2012b), which revealed

a carotenoid gene cluster on a plasmid (PAGR_p; CP003086). Figure 1 shows the genetic map and putative pathway responsible for carotenoid biosynthesis in *P . ananatis* PA13. The open reading frames (*orfs*) in the carotenoid biosynthetic gene cluster were analysed and annotated as *crtE* , *crtX* , *crtY* , *crtI* ,*crtB* , and *crtZ* in sequence. When comparing the *crt*gene clusters between *P . ananatis* and the genetically close species *P . agglomerans* , there is a significant difference in the position of *idi* , which is located in the chromosome in the former strain (PAGR_g2908) and between *crtE*and *crtX* in the latter. The structure of the other genes is identical in the two strains (Fig. 1A).

The putative carotenoid biosynthetic pathway of *P .ananatis* was inferred from the pathways of *Pantoea* species (Misawa *et al* ., 1995; Hundle *et al* ., 1994) and plants (Guerinot, 2000). Carotenoid biosynthesis begins with isomerisation of isopenthyl diphosphate (IPP) from the mevalonate pathway to produce dimethylallyl diphosphate (DMAPP) in a reaction catalysed by IPP isomerase encoded by *idi* . Carotenoids are produced from the common precursor farnesyl diphosphate (FPP). Addition of a further IPP molecule yields geranylgeranyl diphosphate (GGPP) in a reaction catalysed by GGPP synthetase (encoded by *crtE*). The next step in the carotenoid pathway is the head-to-head condensation of two molecules of GGPP to produce phytoene in a reaction catalysed by phytoene synthase (encoded by *crtB*). Sequentially, the involved enzymes include phytoene desaturase (encoded by *crtI)* , lycopene β-cyclase (*crtY*), β-carotene hydroxylase (*crtZ*), and zeaxanthin glucosyl transferase (*crtX*) (Fig. 1B).

Carotenoid biosynthetic cluster genes crtEXYIB *of* P. ananatis *are polycistronic*

We performed reverse transcription-polymerase chain reaction (RT-PCR) to determine if the wild-type *P . ananatis* carotenoid biosynthetic cluster genes are polycistronic. We used five sets of primers to amplify *crtE –X, X –Y, Y –I, I –B* , and *B –Z* . RT-PCR followed by Southern hybridisation indicated that the *P . ananatis* carotenoid biosynthetic cluster genes *crtEXYIB* are transcribed as a single transcript, and *crtZ* is transcribed as an independent single transcript in the opposite direction (Fig. 2).

Cloning of SOE fragments and RT-PCR analysis

Each SOE product was TA-cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced to confirm the presence of the DNA sequences (Macorgen Inc., Daejeon, South Korea). Next, clones containing*crtEB* , *crtEBI* , or *crtEBIY* were digested with Xhol and Sacl and ligated into the corresponding positions of pBBR1MCS5 (Kovach *et al* ., 1995), generating pYS71, pYS69, or pYS76, respectively (Supplementary Fig. S1).

We used RT-PCR to determine if the reassembled *crtEB* ,*crtEBI* , and *crtEBIY* clones on the plasmids pYS71, pYS69, and pYS76 are transcribed as a single transcript. We used three sets of primers to amplify *crtE –B* , *B –I* , and*I –Y* . RT-PCR followed by Southern hybridisation indicated that the reassembled *crtE –B* ,*crtE –B –I* , and*crtE –B –I –Y* clones on the plasmids pYS71, pYS69, and pYS76 are transcribed as single transcripts (Supplementary Fig. S2).

Carotenoid production in E. coli

To determine if *E . coli* DH5α transformed with pYS71/pSRKGm::*crtE –B* , pYS69/pSRKGm::*crtE –B –I* , or pYS76/pSRKGm::*crtE –B –I –Y* produces phytoene, lycopene, or β-carotene, respectively, we performed high-performance liquid chromatography (HPLC). The results revealed that*E . coli* DH5α/pYS71/pSRKGm::*crtE –B* produced colourless phytoene, as confirmed by the standard peak at the same retention time (Fig. 3A and D); *E . coli*DH5α/pYS69/pSRKGm::*crtE –B –I* produced magenta lycopene, as confirmed by the standard peak at the same retention time (Fig. 3B, D); and *E . coli*DH5α/pYS76/pSRKGm::*crtE –B –I –Y* produced orange β-carotene, as confirmed by the standard peak at the same retention time (Fig. 3C, D). SOE enabled reassembly of multiple carotenoid synthetic genes and the production of carotenoids in*E . coli* .

Carotenoid confers P. ananatis *with tolerance to toxoflavin and UV radiation*

Toxoflavin is a phytotoxin produced by *B . glumae* , a rice-grain pathogen that shares rice environments

with *P . ananatis* and has antibacterial properties. To determine if carotenoid production in *P . ananatis* is responsible for tolerance to toxoflavin and UV radiation, we generated a polarised *crtE* ::pCOK184 mutant by Campbell insertion (Fig. 4A). Complementation plasmid pCOK218 was also generated by cloning the carotenoid biosynthetic genes *crtE –Z* into pBBR1MCS5 (Fig. 4A), which recovered the carotenoid deficiency in the *crtE* ::pCOK184 mutant (Fig. 4B). The wild-type is sensitive to toxoflavin concentrations $>$ 20 μg mL^{-1}(Fig. 4B). The *crtE* ::pCOK184 mutant exhibited lower tolerance than the wild-type to 20 μg mL^{-1} toxoflavin; however, the wild-type and complementation strain (+) showed greater tolerance than the *crtE* mutant (Fig. 4B). These results were consistent with those for UV radiation tolerance, but the survival of the *crtE* ::pCOK184 mutant was approximately 100 times lower than that of the wild-type (Supplementary Fig. S3).

Carotenoid production is dependent on RpoS, which is regulated positively by Hfq/ArcZ and negatively by ClpXP in P. ananatis

Figure 5A shows the Hfq^{ArcZ} - RpoS ClpXP regulatory networks of *E . coli* . In *E . coli* , the stationary sigma factor RpoS is regulated positively by Hfq and its cognate sRNA ArcZ. RpoS levels are kept low by constitutive degradation of the ClpXP protease until stationary phase (Raju *et al* ., 2012). RpoS-dependent carotenoid production has been previously reported in *P . agglomerans* (Becker-Hapaka *et al* ., 1997). To determine whether this regulation also occurs in *P .ananatis* , we constructed non-polar mutants of the *rpoS* ,*hfq* , *arcZ* , and *clpP* genes and generated complementation strains for the corresponding gene mutants. The colonies of [?]*rpoS* and [?]*hfq* mutants were white, and neither produced carotenoids (Fig. 5B); however, colonies of complementation strains (+) carrying pCOK312 and pCOK335, respectively, were orange and produced carotenoids. Colonies of the [?]*arcZ* mutant were faint orange and exhibited a slight reduction in carotenoid production (Fig. 5B), indicating involvement in carotenoid production. Colonies of the [?]*clpP* mutant were dark orange and exhibited an approximately two-fold increase in carotenoid production, indicating negative carotenoid regulation via RpoS inhibition (Fig. 5B). Complementation strains (+) of [?]*arcZ* and [?]*clpP* mutants carrying pBS28 and pOR78, respectively, produced amounts of carotenoids similar to that of the wild-type. These results suggest that carotenoid production of *P . ananatis* is dependent on RpoS, which is regulated positively by Hfq/ArcZ and negatively by ClpP, similar to an important regulatory network of *E . coli* (Hfq^{ArcZ} - RpoS ClpXP).

EanR negatively regulates carotenoid production in P. ananatis

A previous report examined EanR de-repression in the QS system of *P . ananatis* , which causes centre rot disease in onion (Morohoshi *et al* ., 2007). QS of *P . ananatis* PA13 is also similar to that of *P . stewartii* (Minogue *et al* ., 2005). Supplementary Fig. S4 shows the QS system of *P .ananatis* PA13. The *eanR* and *eanI* genes are transcribed in the opposite direction, and the *lux* box is at the *eanR* gene promoter region (Supplementary Fig. S4A). To determine if *eanI* expression is under the control of EanR, we constructed a *lacZY* integration of *eanI* ::pCOK153 (i.e., pVIK112 carrying *eanI* truncated at both ends) in PA13L and PA13L[?]*eanR* mutant backgrounds using Campbell insertion (Supplementary Fig. S4A). QS signal production of the mutants was confirmed using thin-layer chromatography (TLC) and a *Chromobacterium* indicator strain. The *eanI* mutant did not produce QS signals, whereas the *eanR* mutant did (Supplementary Fig. S4B). The expression of *eanI* was not decreased in the [?]*eanR* mutant background or increased by the addition of 3-oxo-C6AHL or C6AHL (Supplementary Fig. S4C). These data indicate that the expression of *eanI* is not under the control of EanR.

We performed functional phenotypic de-repression of EanR using [?]*eanI* , [?]*eanR* , and [?]*eanI-R* mutants of *P .ananatis* PA13. The [?]*eanI* mutant exhibited no production of QS signals or carotenoids; however, [?]*eanR* and [?]*eanI-R* mutants produced carotenoids, indicating that EanR negatively regulates carotenoid production by the binding of AHLs to EanR (Fig. 6A-C). Carotenoid production of the [?]*eanI-R* mutant was abolished by transformation with pCOK199 (pBBR1MCS5::P*lac* -*eanR*), confirming that EanR negatively regulates carotenoid production (Fig. 6B, C).

EanR negatively regulates carotenoid production via inhibition of rpoS *in* P. ananatis

To determine if *rpoS* expression is regulated by EanR, we constructed a *lacZY* integration of *rpoS* ::pYS88 (pVIK112 carrying *rpoS* truncated at both ends) in PA13L, PA13L[?]*eanI* , PA13L[?]*eanR* , and PA13L[?]*eanI-R* mutant backgrounds using Campbell insertion. The expression of *rpoS*decreased significantly in the [?]*eanI* mutant background.*rpoS* expression increased in the [?]*eanR* and [?]*eanI-R*mutant backgrounds (Fig. 6D); *rpoS* expression decreased in the presence of EanR. These results indicate that EanR negatively regulates*rpoS* expression and QS signals de-repress EanR. Although the putative *lux* box suggests that EanR binds to the promoter region of *rpoS* (Fig. 6E), there is currently no direct evidence for this. We analysed the candidate *lux* box(s) in the *crtEXYIB*gene cluster or the promoter region of the *crtZ* gene, but did not find it.

QS is delayed in the absence of Hfq

To elucidate the relationship between Hfq and QS, we performed QS signal-production assays with wild-type, [?]*hfq* mutant, and [?]*hfq* complementation strains. QS signals were extracted in the mid/late log phase (OD_{600} = 0.9, 1.5 and 1.8) and developed on C_{18} reversed-phase TLC plates. QS signalling in the [?]*hfq* mutant (-) decreased significantly but recovered to the level of the wild-type after transforming with pCOK335 (+; pLAFR3::*hfq*) (Fig. 7A, B). These results suggest that Hfq positively regulates QS signal production. We also examined whether the reduction in QS signal in the [?]*hfq* mutant was due to bacterial growth; our results showed that growth in [?]*hfq* was not retarded compared with the wild-type strain (data not shown). Using β-galactosidase activity assays, we found that expression of *eanI*decreased significantly in the absence of Hfq (-) but recovered by transformation with pCOK335 (+; pLAFR3::*hfq*) (Fig. 7C). This is consistent with the finding that QS signal production in the [?]*hfq*mutant was significantly lower (Fig. 7A, B). These results indicate that Hfq positively regulates QS, which is delayed in the absence of Hfq in*P* . *ananatis* .

DISCUSSION

Pantoea ananatis is an emerging plant pathogen that causes severe loss of many crops and trees, such as maize, onion, rice, and Eucalyptus, worldwide (Coutinho and Venter, 2009; Weller-Stuart *et al* ., 2017). This bacterium has also been associated with insects and humans. It is considered as a pathogen that is being revised because of its virulence in a wide variety of plant hosts and saprophytic ability in diverse ecological niches (Coutinho and Venter, 2009). In this study, we focused on the carotenoid biosynthesis gene cluster of *P. ananatis* and performed gene reassembly for carotenoid production. Here, we investigated the ecological and physiological functions of regulatory mechanisms of the carotenoid production of *P. ananatis* .

Much effort has focused on the biosynthesis of carotenoids using bacterial carotenoid genes (Misawa *et al* ., 1995; Guerinot, 2000; Lee *et al* ., 2003; Mijts and Schmidt-Dannert, 2003). Techniques based on recombining DNA sequences rely on restriction sites, so the primer must contain the introduced restriction site, which should not be in the centre of the fragment (llis *et al* ., 1986; Higuchi*et al* ., 1988; Ho *et al* ., 1989; Horton *et al* .,1989). Moreover, if multiple cloning vectors are to be used, plasmid incompatibility is also a limiting factor. Here, we applied a SOE by PCR technique to recombine DNA sequences without relying on restriction sites. In this report, we describe the reassembly of the genes encoding bacterial carotenoid biosynthetic proteins as *crtE-B, crtE-B-I* , or *crtE-B-I-Y* for the synthesis of phytoene, lycopene, or β-carotene, respectively. *E* . *coli* expressing *crtE-B, crtE-B-I* , or *crtE-B-I-Y* produced phytoene, lycopene, or β-carotene, respectively. Zeaxanthin biosynthesis was enabled by the addition of *crtZ* , but gene recombination failed despite numerous attempts. It seems that the likelihood of success decreases with increasing number of genes to be recombined.

In practice, simply introducing *lacZ* ribosomal binding sequences (RBSs) at the beginning of the SOE-AB product (P*lac -crtE*) enables carotenoid biosynthesis. CrtE catalyses the synthesis of GGPP, an early intermediate of carotenoid biosynthesis. We did not test whether the absolute amount of carotenoids increases as the level of GGPP increases *in vivo* .

We used a DNA template from the rice pathogenic bacterium *P .ananatis* in SOE by PCR, which is controllable and independent of restriction sequences, for carotenoid gene reassembly. *Pantoea agglomerans* , which causes palea browning of rice, is a genetically close species to *P* . *ananatis* with which it shares a biological

niche. The organisation of the carotenoid biosynthetic gene clusters of the two strains is identical, except *idi* . Interestingly, *P* . *agglomerans* has an *idi* gene between *crtE* and *crtX* , which distinguishes it from*P* . *ananatis* , suggesting that *idi* could be used to distinguish genetically similar bacteria.

SOE is a novel PCR-mediated recombinant DNA technology that does not rely on restriction sites, so its coverage is considerably wider than standard restriction enzyme-based methods for gene recombination. This enables finer control over recombination for genetic engineering. In addition, the sequence of the overlap region is determined by primer design, allowing simultaneous non-polar mutagenesis, site-directed mutagenesis, and recombination. In this study, we applied this technically simple and rapid recombinant DNA technique to the biosynthesis of three carotenoids. The technique will likely be suitable for recombination of multiple genes.

In bacteria, carotenoids are closely related to the conditions of the surrounding environment. We found that the UV radiation tolerance of*P* . *ananatis* was due to the carotenoids they produce. These results are consistent with those regarding *P* . *stewartii*subsp. *stewartii* (Mohammadi *et al* ., 2012). Considering the plant environment (particularly rice) in which *P* . *ananatis*lives, UV radiation tolerance is advantageous for survival. Interestingly, these carotenoids are unique in that they also make*P* . *ananatis* tolerant to toxoflavin. Thus, tolerance to toxoflavin via carotenoid production can be considered a survival strategy of *P* . *ananatis* . Bacteria that produce carotenoids have advantages for overcoming environmental stresses, such as UV radiation, salt, and low temperatures. *Pantoea ananatis* and*B* . *glumae* share the same rice environment and are the first reported cases of the production and use of carotenoids to overcome toxoflavin.

We found that QS and Hfq are directly or indirectly involved in regulating carotenoid production in *P. ananatis* PA13. QS regulates an extensive range of functions, including bioluminescence, virulence, biofilm formation, DNA exchange, and sporulation in bacteria (Fuqua *et al* ., 1996, Waters and Bassler, 2005). Hfq is a global RNA chaperone that interacts with sRNAs of diverse functions; it also regulates of virulence and environmental stress in many plant and animal bacterial pathogens (Ding *et al* ., 2004; Chao and Vogel, 2010; Zeng *et al* ., 2013; Shin *et al* ., 2019). The *hfq*mutant in *Erwinia amylovora* Ea1189 reduces virulence, amylovoran EPS production, biofilm formation, motility, and positive regulation of the type III secretion system (Zeng *et al* ., 2013). In*Pectobacterium carotovorum* , *the hfq* mutant exhibits defects in motility, biofilm formation, sedimentation, and virulence (Wang *et al* ., 2018). Hfq is also an important regulator of virulence, motility, and biofilm formation in *P. ananatis* LMG2665 (Shin *et al* ., 2019). We found that Hfq regulates the expression of *eanI* encoding the QS signal synthase, which was confirmed by*eanI* expression and QS signal productivity assays. These results are consistent with the finding that Hfq regulates QS signal production directly via interactions with the AHL receptor ExpR in*Sinorhizobium meliloti* (Gao *et al* ., 2015). QS systems integrate other global regulators, including noncoding sRNAs. This network is activated through the binding of Hfq and Hfq-dependent sRNA and controls gene expression via post-transcription regulation (Storz*et al* ., 2005). There are several reports that the Hfq-dependent sRNAs Qrr1–4 and RsmY interact with Hfq to directly and indirectly control QS targets in *Vibrio cholerae* and *Pseudomonas aeruginosa* (Lenz *et al* ., 2004; Kay *et al* ., 2006). Shin*et al* . (2019) suggested that the putative Hfq-dependent sRNAs pPAR237 and pPAR238 are involved in regulating QS by activating EanI without genetic analyses. Further studies are needed to identify the sRNAs in *P. ananatis* . It was previously reported that EanR mediated QS regulation by de-repression as in *P. stewartii* (von Bodman and Farrand, 1995; Morohoshi *et al* ., 2007). In *P. ananatis* , EanR represses the *ean* box (*lux* box-like sequences) in the upstream region of *eanR* , and adding AHL promoted dose-dependent de-repression (Morohoshi *et al* ., 2007). This EanR-mediated QS regulation was similar to that of the close homolog EsaR in *P. stewartii* (Minogue *et al* ., 2005). Overall, we found that QS signal production in *P* . *ananatis*was delayed in the absence of Hfq, since EanR negatively regulates RpoS. Expression of RpoS is entirely dependent on bacterial growth. Using EanR, *P* . *ananatis* must inhibit RpoS expression before reaching stationary phase, at which point EanR is removed to initiate expression of RpoS. Hfq is responsible for determining the timing of the Hfq-mediated increase in *eanI* expression to produce full QS signals. The resulting QS signals de-repress EanR, followed by Hfq to express RpoS, which turns on carotenoid biosynthesis.

We found that RpoS regulates carotenoid biosynthesis under the control of Hfq, QS, and ClpP. The regulatory networks of Hfq^{ArcZ} - RpoS ClpXP for carotenoid production are similar to those of *E . coli* . Here, we elucidated a regulatory network of carotenoid production involving Hfq-dependent QS-RpoS in *P . ananatis* . Hfq regulates full production of QS signals, thereby de-repressing the EanR negative regulator to initiate RpoS expression (Fig. 8).

EXPERIMENTAL PROCEDURES

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1. *E . coli* strains were cultured on Luria–Bertani (LB) medium at 37°C. The *P. ananatis* PA13 was cultivated at 28°C on LB medium. Antibiotics were used at the following concentrations: ampicillin, 100 μg mL^{-1}; kanamycin, 50 μg mL^{-1}; rifampicin, 50 μg mL^{-1}; tetracycline, 10 μg mL^{-1}; gentamycin, 25 μg mL^{-1}. 5-Bromo-4-chloro-3-indoyl-b-D-galactopyranoside (X-gal) was used at 40 μg mL^{-1} when necessary.

DNA manipulation and data analyses

Manipulation of genomic DNA and plasmids and DNA cloning were performed as described by Sambrook and Russell (2001). Restriction enzymes (TaKaRa) were used for DNA digestion and modification. DNA sequencing was performed by Macrogen (Seoul). DNA sequences were analyzed with the BLAST program at the National Center for Biotechnology Information (Gish and States, 1993), MEGALIGN (DNASTAR, Madison, WI, USA), and GENETYX-WIN software (Genetyx).

Carotenoid genes

Genomic DNA of *P . ananatis* PA13, a bacterial pathogen of rice, was used as the template to amplify carotenoid biosynthetic genes. The carotenoid genes are located on a plasmid (PAGR_p; CP003086). Figure 1 shows the carotenoid gene clusters of *P . ananatis*PA13 and *P . agglomerans* Eho10 (M87280; Hundle *et al* ., 1994).

Strategy for carotenoid gene reassembly

The reassembly of the carotenoid genes responsible for synthesising phytoene, lycopene, and β-carotene was performed as described previously (Horton *et al* ., 1995). The sequences of the eight primers used for reassembly are listed in Supplementary Table S2. Primers 'a' and 'd', 'a' and 'f', and 'a' and 'h' are the flanking primers for PCR amplification of the final reassembled products. Primers 'b' and 'c', 'd' and 'e', and 'f' and 'g' are the SOE primers. Bases have been added to the 5' ends of the primers in each pair to render them complementary. All of the complementary sequences have been added between primers b-c, d-e, and f-g. During SOE, the upper strands of AB and the lower strands of CD overlap to act as primers (Supplementary Fig. S5). Fragment AB was PCR amplified from *crtE* , and fragment CD from *crtB* . Fragment EF was PCR amplified from *crtI* , and fragment GH from*crtY* . The SOE primers 'b' and 'c' were used to modify the PCR products of two sequences to have an identical sequence (Supplementary Table S2). Supplementary Fig. S5B shows the reassembly of*crtE –B* genes for phytoene biosynthesis. The upper strands of AB and the lower strands of CD overlap to act as primers when the PCR products are mixed, denatured, and reannealed during PCR. Fragments of AD are formed when this overlap is extended by polymerase. Supplementary Fig. S5C shows the *crtE –B –I* gene reassembly for lycopene biosynthesis in which the upper strands of AD and the lower strands of EF overlap to act as primers when the PCR products are mixed, denatured, and reannealed during PCR. Fragments of AF are formed when this overlap is extended by polymerase. Similarly, the upper strands of AF and the lower strands of GH overlap to act as primers when the PCR products are mixed, denatured, and reannealed during PCR. Fragments of AH are formed when this overlap is extended by polymerase (Supplementary Fig. S5D). The XhoI recognition sequence and *lacZ* RBS were introduced at the beginning of the SOE-AB products.

SOE by PCR

SOE by PCR was carried out using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) for 20 cycles, each of 1 min at 98°C, 1 min at 55°C, and 2 min at 70°C. The reaction was carried out in a 50 µL volume containing 2.5 U Phusion High-Fidelity DNA polymerase (Pfu; Thermo Fisher Scientific), 200 µM dNTPs, 1 µL of primer mix (1.5 pmol per primer), and 5 µL of 10× Pfu buffer.

Purification and cloning of SOE fragments

The SOE products for use as templates were purified by electrophoresis in agarose (0.8% agarose, Promega) in TAE buffer (40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid) with 0.5 µg mL^{-1} ethidium bromide. DNA was recovered from the gel fragment using a DNA Purification Kit (GeneAll, Seoul, South Korea). The final recombinant products were gel purified prior to cloning.

The SOE products were TA cloned into pGEM-T Easy (Promega) and sequenced by Macrogen Services (Daejeon, South Korea). Error-free clones were digested with *Xho* l and *Sac* l and ligated into the corresponding positions in pBBR1MCS5 (Kovach *et al* ., 1995).

RT-PCR analysis of wild-type P. ananatis *carotenoid cluster genes*

Wild-type *P . ananatis* PA13 was grown in LB medium to exponential growth phase (12 h after inoculation); total RNA was isolated using an RNeasy Mini Kit according to the supplier's instructions (Qiagen); and the RNA samples were treated with RQ1 DNase (Promega) to remove any contaminating DNA. RT-PCR was performed according to a previous report (Kim *et al* ., 2004) as follows. Total RNA from *P . ananatis* PA13 was reverse transcribed into cDNA using M-MLV reverse transcriptase as described by the manufacturer (Promega) at 50°C for 1 h, followed by 5 min at 75°C. Next, PCR was performed using a T100 Thermal Cycler (Bio-Rad) under the following conditions: 96°C for 2 min, followed by 40 cycles of 96°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The following primers were used for RT reactions, RT1 (*crtB*) and RT2 (*crtZ*). The following PCR primers were used: PCR1f and PCR1r; PCR2f and PCR2r; PCR3f and PCR3r; PCR4f and PCR4r; and PCR5f and PCR5r (Supplementary Table S3). Southern hybridisation and DNA sequencing were carried out to confirm the RT-PCR products. As a positive control, pCOK218 DNA was used. As a negative control, PCR reactions with the same primer sets were performed using RNA samples that had not been reverse transcribed.

RT-PCR analysis of reassembled carotenoid cluster genes

E . coli harbouring recombinant pYS71, pYS69, or pYS76 was grown in LB medium to exponential growth phase (12 h after inoculation). The following primers were used for RT reactions: RT3 (*crtZ*); RT4 (*crtI*); and RT5 (*crtY*). The following PCR primers were used: PCR6f and PCR6r; PCR7f and PCR7r; and PCR8f and PCR8r (Supplementary Table S3). Southern hybridisation and DNA sequencing were carried out to confirm the RT-PCR products. As positive controls, pYS71, pYS69, and pYS76 were used. As a negative control, PCR reactions with the same primer sets were performed using RNA samples that had not been reverse transcribed.

HPLC

For carotenoid extraction and HPLC analysis, transformed *E .coli* DH5α harbouring pYS71, pYS69, or pYS76 was cultured in 250 mL flasks containing 50 mL of LB broth with 25 µg mL^{-1} gentamycin at 37°C for 24 h. After centrifugation at 10,000 × *g* for 10 min, the cultured cells were repeatedly extracted with 3 mL of acetone for lycopene and β-carotene or ethanol for phytoene until colour was completely lost. The extracted solution was centrifuged and filtered through a GHP membrane (0.45 µm pore size). HPLC was performed using 20 µL of prepared sample, with solvent A (60% acetonitrile, 38% ethyl acetate, 2% acetic acid) and solvent B (100% methanol) as the mobile phase, on a C_{18}Shim-pack GIS-DOS column (4.6 × 250 mm, 5 µm; Shimadzu) as a fixed phase at a flow rate of 1.5 mL min^{-1}. β-Carotene was measured at 450 nm using a photodiode array detector. Lycopene was measured at 470 nm and phytoene at 280 nm. Phytoene, lycopene, and β-carotene standards were purchased from Sigma-Aldrich, Inc.

Generation of lacZY*-integrations and non-polar deletion mutants*

lacZY transcriptional integration mutagenesis (Campbell insertion) was performed as previously reported (Xu *et al* ., 2013). An internal DNA fragment of *eanI* was amplified with EanI-1E-1 and EanI-2K (Supplementary Table S3). The partial *eanI*fragment was purified, cloned into pGEM-T Easy (Promega), and confirmed by sequencing. For recombinational mutagenesis, the EcoRI/KpnI-digested*eanI* fragment was cloned into the pVIK112 suicide vector (Kalogeraki and Winans, 1997), creating pCOK153. The parent strain PA13 was conjugated with pCOK153, and kanamycin-resistant colonies were selected. The mutants were confirmed through PCR using a primer that anneals upstream of the truncated fragment and the primer LacFuse followed by sequencing. We constructed *rpoS* and *crtE* null mutants using the same method as described previously.

Non-polar deletion mutagenesis was performed as previously reported (Xu*et al* ., 2013). We amplified upstream and downstream fragments (approximately 450 bp) of the targeted gene region by PCR using the corresponding primer pairs (Supplementary Table S3). After purification, the fragments were fused by overlap PCR. The final PCR products were cloned into pGEM-T Easy and confirmed by DNA sequencing. The fragments were excised using appropriate restriction enzymes and ligated into the suicide vector pNPTS138-R6KT (Lassak *et al* ., 2010). The resulting plasmids were introduced into PA13 by conjugative mating, and mating cells were spread on LB medium containing kanamycin and rifampicin. Single-crossover integrates were selected on LB plates containing kanamycin and rifampicin. Single colonies were grown overnight in LB with rifampicin (25 μg mL^{-1}) and plated on LB containing 5% (w/v) sucrose to select for plasmid excision. We checked kanamycin-sensitive colonies for targeted deletion with colony PCR using primers bracketing the location of the deletion.

Gene complementation

To generate target gene complementary strains, we cloned each intact target gene into the broad host range plasmid vectors pBBR1MCS5 (Kovach*et al* ., 1995), pSRKGm (Khan *et al* ., 2008) or pLAFR3 (Keen*et al* ., 1988), generating pCOK218 (pBBR1MCS5::*crtEXYIBZ*), pCOK197 (pBBR1MCS5::P_{lac} -*eanI*), pCOK199 (pBBR1MCS5::P_{lac} -*eanR*), pCOK312 (pSRKGm::P_{lac} -*rpoS*), pBS28 (pLAFR3::*hfq* , pLAFR3::*arcZ*), or pOR78 (pBBR1MCS5:: P_{lac} -*clpP*) which were transferred to the corresponding mutant strains by conjugation (Supplementary Table S1).

Toxoflavin and UV radiation tolerance

Overnight cultures of the PA13 derivatives were sub-cultured and grown for an additional 12 h. A 100-μL aliquot was removed and serially diluted 10-fold, and 10-μL of each culture was spotted on LB agar plates supplemented with 20 μg mL^{-1} toxoflavin. The spotted plates were incubated at 28°C for 36 h.

For the UV radiation tolerance assays, PA13 derivatives were spotted on LB plates using the above procedure and treated as previously described (Mohammadi *et al* ., 2012).

Carotenoid production

To determine the carotenoid content of cells, *P. ananatis* strains were grown in 5 mL of LB medium at 28°C. Cells were harvested by centrifugation at 10,000 × *g* for 1 min and suspended in 1ml of methanol. The samples were vortexed for 10 min and centrifuged at 10,000 × *g* for 10 min, and the methanol supernatant containing carotenoids was transferred to a new tube. We quantified the carotenoid content of the extracts by measuring the absorbance at 450 nm using a Genesys 10S UV-VIS spectrophotometer (Thermo Fisher Scientific).

AHL signal assay

The isolation and purification of AHLs were performed as described by Kim *et al* . (2004). The culture supernatants from time course cultures of *P. ananatis* PA13 and mutants were extracted with ethyl acetate (1:1). The ethyl acetate layer was dried, and the residue was dissolved in methanol. The ethyl acetate extracts were applied to C_{18} reversed-phase TLC plates (Merck) and developed with 60% methanol. The TLC plates

were dried in a fume hood and overlaid with soft agar containing *Chromobacterium violaceum* CV026 cells cultured overnight. The plates were incubated at 28°C overnight.

β-Γαλαςτοσιδασε ασσαψ

We generated non-polar deletions of *lacZY* genes from wild-type PA13 named PA13L and confirmed that all traits were identical in the two strains. Wild-type and mutant backgrounds used in the β-galactosidase assays were PA13L. All of the test strains were grown for 20 h and sub-cultured in LB broth at 28°C. The assays were performed with exponential-phase cultures at an OD_{600} of ˜ 0.4 as described previously (Choi *et al* ., 2019).

CONCLUSION

Microbial biotechnology allows bacterial carotenoids to be used as alternatives to plant-based carotenoids because of the ease of genetic manipulation of prokaryotes compared with eukaryotes, such as yeasts, fungi, and plants. Here, we used SOE by PCR for gene reassembly to redirect carotenoid synthesis from the plant-pathogenic bacterium*Pantoea ananatis* . Using SOE by PCR, we reassembled*crtE –B* , *crtE –B –I* , and*crtE –B –I –Y* for phytoene, lycopene, and β-carotene production, respectively, using *E. coli* to express the reassembled operons. We found that carotenoids confer tolerance to the phytotoxin toxoflavin. The carotenoid production of *P .ananatis* is dependent on RpoS, which is regulated positively by Hfq/ArcZ and negatively by ClpP, similar to an important regulatory network of *E* . *coli* , Hfq^{ArcZ} - RpoS ClpXP. We also demonstrated that carotenoid production is regulated by Hfq-controlled QS, since the EanR negative regulator on RpoS must be expressed in the stationary phase.

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Conflict of interest

The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1. Genetic map and putative pathway responsible for carotenoid biosynthesis by *P. ananatis* PA13 (CP003086) and*P* . *agglomerans* Eho10 (M87280). (A) The carotenoid gene cluster of *P* . *ananatis* consisted of*crtE –X –Y –I –B* and *Z* ; and that for *P* . *agglomerans* of*crtE –idi –crtX –Y –I –B* and*Z* . Gene numbers were shown on the carotenoid gene map. (B) The putative carotenoid biosynthetic pathway of *P* . *ananatis*inferred according to the pathway of *Pantoea* species (Misawa*et al* ., 1995) and plants (Guerinot, 2000). The involved enzymes include isopenthyl diphosphate (IPP) isomerase encoded by *idi,*geranylgeranyl diphosphate (GGPP) synthetase by *crtE* , phytoene synthase by *crtB* , phytoene desaturase by *crtI* , lycopene β-cyclase by *crtY,* β-carotene hydroxylase by *crtZ* , and zeaxanthin glucosyl transferase by *crtX* .

Fig. 2. Confirmation of transcriptional units in the carotenoid gene cluster of *P* . *ananatis* by RT-PCR. RT-PCR products were confirmed by Southern hybridisation. Black arrows indicate the extension and transcription directions of the *crtEXYIB* operon and*crtZ* gene. An arrow below the open arrows represents the product of RT reactions. The short thick bars below the RT arrow indicate the PCR products from the corresponding RT reactions. The expected sizes of the PCR products are indicated below the labels. Agarose gel analysis (upper panel) and Southern analysis (lower panel) of the RT-PCR products of the *crtEXYIB* operon and *crtZ* gene. Southern hybridisation was performed using pCOK128 as a probe. Lanes 1–3, 4–6, 7–9, 10–12, and 13–15 correspond to the products of PCR1, PCR2, PCR3, PCR4, and PCR5, respectively. Lanes 1, 4, 7, 10, and 13: PCR products from the DNA template as positive controls; lanes 2, 5, 8, 11, and 14: PCR products from the RNA template as negative controls; and lanes 3, 6, 9, 12, and 15: RT-PCR products.

Fig. 3. Production of phytoene, lycopene, and β-carotene in*E. coli*. HPLC analysis of phytoene (A), lycopene (B), and β-carotene (C) production. a, *E. coli*DH5α/pYS71(pBBR1MCS5::*crtE–B*) producing phytoene (retention time 2 min, 280 nm); b, *E. coli*DH5α/pYS69(pBBR1MCS5::*crtE–B–I*) producing lycopene (retention time 11 min, 470 nm); and c, *E. coli*DH5α/pYS76(pBBR1MCS5::*crtE–B–I–Y*) producing β-carotene (retention time 14.8 min, 450 nm). HPLC analysis confirmed that the *E. coli* strains harbouring pYS71, pYS69, and pYS76 produced phytoene, lycopene, and β-carotene, respectively. (D), Colour change of harvested *E. coli* cells harbouring pYS71, pYS69, or pYS76. The harvested cells showed colourless phytoene, magenta lycopene, or orange β-carotene. PS, LS, and CS indicate the phytoene, lycopene, and β-carotene standards, respectively.**Fig. 4.** Carotenoids confer toxoflavin tolerance to *P.ananatis*. (A) Construction of the *crtE*::pCOK184 mutant and complementation plasmid pCOK218. - or + indicates negative or positive carotenoid production, respectively. (B) Toxoflavin tolerance of*P. ananatis*. The wild-type and *crtE*::pCOK184 mutant carrying pCOK218 exhibited greater toxoflavin tolerance than the*crtE* mutant; however, the *crtE*::pCOK184 mutant was more sensitive than the wild-type to toxoflavin at 20 μg mL^{-1}. *Pantoea ananatis* PA13 is sensitive to toxoflavin concentrations > 20 μg mL^{-1}.

Fig. 5. Hfq^{ArcZ} - RpoS ClpXP regulatory networks. (A) An illustration showing the Hfq^{ArcZ} - RpoS ClpXP regulatory networks based on *E . coli* (Raju*et al* ., 2012). RpoS is regulated positively by Hfq and its cognate sRNA ArcZ, and negatively by ClpXP. RpoS-dependent carotenoid production in *P . agglomerans* (formerly *Erwinia herbicola*) was reported previously (Becker-Hapaka *et al* ., 1997). (B) Carotenoid production in the wild-type (W), [?]*rpoS*,[?]*hfq*, [?]*arcZ*, [?]*clpP* , and complementation (+) strains. Values are means ± standard deviation (SD) of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 vs. wild-type.

Fig. 6. EanR negatively regulates carotenoid production*via* inhibition of *rpoS* . (A) QS signal production of the wild-type and [?]*eanI*, [?]*eanR* , and [?]*eanI-R* mutants as well as the [?]*eanI-R* mutant carrying pCOK199 on *C .violaceum* CV026 biosensor-embedded plates. (B) Carotenoid production of the wild-type and [?]*eanI*, [?]*eanR* , and [?]*eanI-R* mutants as well as the [?]*eanI-R* mutant carrying pCOK199. (C) Quantification of carotenoid production of the PA13 derivatives. Carotenoid production was identical to that shown in (B). Values are means +- standard deviation (SD) of three independent experiments. ***p < 0.001 vs. wild-type. (D) β-Galactosidase activity reporting *rpoS* expression. *rpoS* expression was induced in the absence of EanR and decreased in the absence of EanI, indicating that EanR negatively regulates *rpoS* expression and QS signals de-repress EanR. Values are means ± standard deviation (SD) of three independent experiments. ***p < 0.001 vs. PA13L. (E) Genetic map of *rpoS* locus and putative *lux* box. Inverted repeat sequences are shown in bold.

Fig. 7. Hfq regulates the expression of *eanI* QS signal synthase. (A) Characterisation and quantification of AHL signals in wild-type (W), [?]*hfq* mutant (-), and complementation (+; pCOK335) strains of *P . ananatis* PA13. The culture supernatants of the PA13 derivatives were extracted with ethyl acetate at OD_{600} values of 0.9, 1.5 and 1.8. Ethyl acetate extracts were applied to C_{18} reversed-phase thin layer chromatography (TLC) plates. AHL signals were visualised with the*C . violaceum* CV026 biosensor, and synthetic C6-HSL and 3-oxo-C6-HSL were used as AHL standards. (B) 3-oxo-C6AHL signal production of the wild-type (W), [?]*hfq* mutant (-), and complementation strain carrying pCOK335 (+; pLAFR3::*hfq*). Relative percentage to the wild-type at OD_{600} 1.8. The purple area of the 3-oxo-C6AHL signals from TLC was calculated using the ImageJ program. Values are means +- standard deviation (SD) of three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 vs. wild-type. (C) β-Galactosidase activity reporting *eanI* expression in PA13L, [?]*hfq* mutant (-), and complementation strain (+; pLAFR3::*hfq*). Values are means ± standard deviation (SD) of three independent experiments. ***p < 0.01 vs. PA13L.

Fig. 8. Proposed model of carotenoid production for the previously reported regulatory network Hfq^{ArcZ} - RpoS ClpXP and that identified here in which Hfq-controlled quorum signalling de-represses EanR to activate RpoS, thereby initiating carotenoid production. Carotenoid production confers tolerance to toxoflavin and UV radiation.

Supporting information figure legends

Supplementary Fig. S1. Recombinant plasmids for rearrangement of the carotenoid genes responsible for synthesising phytoene, lycopene, and β-carotene. The SOE by PCR products were first cloned into pGEM-T Easy, digested with Xhol and Sacl, and ligated into the corresponding position of pBBR1MCS5. Open triangles indicate the *lacZ* RBS. The SacI site, which is dotted and parenthesised, was from the pGEM-T Easy vector.

Supplementary Fig. S2. Confirmation of transcriptional units in the reassembled *crtE –B* , *crtE –B –I* , and *crtE –B –I –Y* operons by RT-PCR. RT-PCR products were confirmed by Southern hybridisation. Black arrows indicate the extension and transcription directions of the *crtE –B* ,*crtE –B –I* , and*crtE –B –I –Y* operons on plasmids pYS71 (A), pYS69 (B), and pYS76 (C), respectively. Arrows below the open arrows represent the products of RT reactions. The short thick bars below the RT arrow indicate the PCR products from the corresponding RT reactions. The expected sizes of the PCR products are indicated below the labels. Agarose gel analysis (upper panel) and Southern analysis (lower panel) of the RT-PCR products of the *crtE –B* ,*crtE –B –I* , and*crtE –B –I –Y* operons. Southern hybridisation was performed using the*crtE –B –I –Y* operon region (2.2, 3, and 5 kb XhoI–SacI fragments of pYS71, pYS69, and pYS76,

respectively) as probes. Lanes 1–3, 4–6, and 7–9 correspond to the products of PCR1, PCR2, and PCR3, respectively. Lanes 1, 4, and 7: PCR products from the DNA template as positive controls; lanes 2, 5, and 8: PCR products from the RNA template as negative controls; and lanes 3, 6, and 9: RT-PCR products.

Supplementary Fig. S3. Carotenoid production confers UV radiation tolerance to *P. ananatis*. The wild-type and*crtE*::pCOK184 mutant harbouring pCOK218 exhibited greater UV radiation tolerance than the *crtE* mutant at wavelengths of 320–400 nm for 20 s; however, the *crtE*::pCOK184 mutant showed lower UV radiation tolerance than the wild-type.

Supplementary Fig. S4. QS system of *P . ananatis*PA13. (A) Genetic map of the *eanR* and *eanI* loci of the QS system in *P . ananatis* PA13 and mutant generation. The putative *lux* box is upstream of *eanR* , for comparison, the*esaR lux* box of *P . stewartii* subsp.*stewartii* and *lux* box of *Vibrio fischeri* are presented. Campbell insertion and non-polar deletion mutants were generated to determine if *eanR* regulates the expression of*eanI* ; a) PA13L, non-polar deletion of *lacZY* genes from wild-type PA13 used in the β-galactosidase assays as the wild-type; b)*eanI* ::pCOK153 (pVIK112 carrying truncated *eanI* at both ends); c) [?]*eanI* , non-polar deletion of *eanI* ; d) [?]*eanR* , non-polar deletion of *eanR* ; and e) [?]*eanR eanI* ::pCOK153. (B) Characterisation and quantification of AHL signals of the PA13 derivatives: (a) PA13L; (b) *eanI* ::pCOK153; (c) [?]*eanI* mutant; (d) [?]*eanR* mutant; and (e) [?]*eanR eanI* ::pCOK153 mutant. Ethyl acetate extracts were applied to C_{18} reversed-phase thin layer chromatography (TLC) plates. AHL signals were visualised with the *C . violaceum*CV026 biosensor, and synthetic C6-HSL and 3-oxo-C6-HSL were used as AHL standards (s). (C) β-Galactosidase activity reporting *eanI*expression.

Supplementary Fig. S5. Strategy for generating recombinant carotenoid genes responsible for synthesising phytoene, lycopene, and β-carotene. PCR products with their overlapping regions aligned and the final rearrangement products are shown. The SOE by PCR products AD, AF, and AH are shown. In each case, the overlapping region between the primers, and the priming region in which each primer recognises its template, was designed to have the ribosome binding sequence (RBS) of each gene. The XhoI recognition sequence and *lacZ* RBS were introduced at the beginning of SOE-AB products. Dotted arrows indicate the rearrangements of carotenoid genes.

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