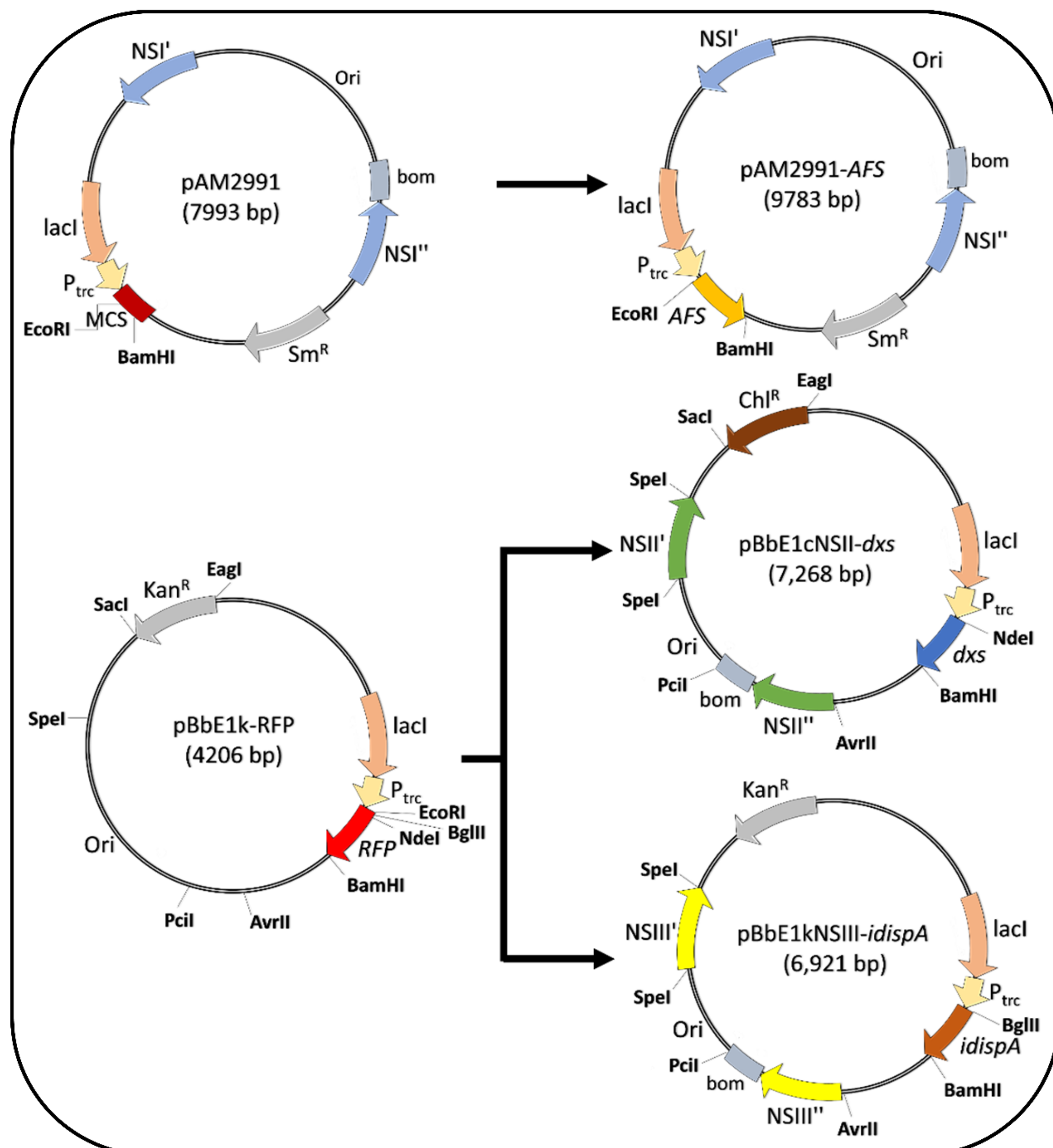
CHAPTER 3

Construction of integration vector system for *Synechococcus elongatus* UTEX 2973*



---

* Part of the work is published in **Akhil Rautela** et al. (2024) Photosynthetic production of α-farnesene by engineered *Synechococcus elongatus* UTEX 2973 from carbon dioxide. 396, 130432. Bioresource Technology.

This chapter focuses on the construction of an integration vector system which will be further used for genetically engineer *Synechococcus elongatus* UTEX 2973 (UTEX 2973, hereafter). Vectors are plasmids with properties having antibiotic resistance genes for selection, mobilization elements for transfer, and unique restriction sites for cloning. The heterologous gene can be inserted into the genome of the cyanobacteria or replicate autonomously. The former is done with the help of integrative and the latter with replicative vectors. Replicative vectors are easy to use to insert a gene of interest in cyanobacteria for bioproduction and other purposes (Heidorn et al., 2011). Autonomous expression of a gene without getting inserted in the genome gives higher expression depending on the copy number of the plasmid (Xia et al., 2019). Shuttle vectors are commonly used as replicative plasmids as they can express in two hosts. Jin et al. (2018) constructed a shuttle vector for *Synechocystis* sp. PCC 6803 utilizing its plasmid. *Synechocystis* sp. PCC 6803's plasmid pCC 5.2 consists of a replicon, and combining it with pMB1 (origin of replication of *E. coli*) leads to the formation of shuttle vector pSCB-YFP. Replicative vectors require antibiotic selection/stress to stably maintain them.

There are several neutral sites detected in the genome of cyanobacteria (Golden, 1988; Ng et al., 2015). Replacing these neutral sites with the gene of interest with the help of homologous recombination is the widely used method (Lee et al. 2017). Another strategy includes integrating the heterologous gene in place of the genes which do have a function in cyanobacteria or do not affect the robustness of the strain. In this case, the heterologous gene utilizes the promoter, RBS, and terminator sequences of the source gene. High ploidy levels in cyanobacteria prove to be the major drawback in the integration of the gene of interest as it is to be ensured that each copy has the gene (Heidorn et al. 2011). This is epitomized by Griese et al. (2011), stating *Synechococcus elongatus* PCC 7942 has 3 to 4 genomic copies per cell, and *Synechocystis* sp. PCC 6803 having 218 and 58 genomic

copies in exponential and stationary phase respectively. Earlier there was no modular cloning (MoClo) system for cyanobacteria, however, Vasudevan et al. (2019) combined plant MoClo with cyanobacteria making CyanoGate system kit (Engler et al., 2014). The kit consists of 96 parts and these can be combined from level 0 to level T to form replicative or integrative vectors. All vectors are submitted at Addgene for research purposes (Addgene Kit #1000000146).

Whether it is a replicative or integrative vector, the ultimate goal is to transfer the vector(s) inside the microorganism (cyanobacteria). Three methods of transformation are identified in cyanobacteria, namely natural transformation, electroporation, and conjugation. Model organism *Synechocystis* PCC 6803 and recently isolated *Synechococcus elongatus* PCC 11801 and PCC 11802 are naturally transformable (Pope et al., 2020; Jaiswal et al., 2020). These cyanobacteria uptake free DNA from the environment, and the process is simple and cost-effective. The only limitation is the transformation efficiency is strain-dependent. Certain species of cyanobacteria, *Spirulina platensis* and *Leptolyngbya boryana* are not naturally transformable and, therefore, require a high-voltage electric field to create transient pores in the plasma membrane through electroporation (Toyomizu et al., 2001; Tsujimoto et al., 2015). The electroporation requires specialized equipment and can cause cell damage. It sometimes also leads to reduced transformation efficiency (Wendt and Pakrasi, 2019). Conjugation is a complex method of transformation which is used to transfer large DNA fragments and has high efficiency (Satta et al., 2023). Triparental conjugation requires helper (pRL623) and conjugal plasmid (pRL443) to transfer the gene of interest into the cyanobacteria (Yu et al., 2015). The method involves direct cell-to-cell interaction, and the plasmid is transferred.

In this study, three integration vectors were constructed to direct the gene(s) to incorporate into the genomic neutral site of the host organism, i.e., UTEX 2973. For this

purpose, three neutral sites of the UTEX 2973 were identified, namely neutral site I (NSI), neutral site II (NSII) and neutral site III (NSIII). The vectors were made such that the gene of interest and other important genetic elements were flanked by the upstream and downstream regions of the neutral site. The first constructed vector pAM2991-*AFS* targets codon-optimized *M. domestica AFS* gene to the NSI of UTEX 2973. The *AFS* gene is the foremost requirement for farnesene production, as cyanobacteria lack this gene. Further, according to the host organism and the metabolic pathway of the desired product, i.e., the methylerythritol phosphate (MEP) pathway, the vectors to target bottleneck gene(s) were constructed. The vector pBbE1cNSII-*dxs* was constructed to target 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) to the NSII of UTEX 2973. Fusion of isopentenyl diphosphate isomerase and farnesyl diphosphate synthase (*idispA*) was targeted to NSIII of UTEX 2973 with the help of pBbE1kNSIII-*idispA*.

3.1. Materials and methods

3.1.1. Strains and growth conditions

All the cloning and subcloning experiments were done in the *E. coli* DH5α strain. The *E. coli* was routinely maintained at 37 °C in Luria-Bertani (LB) broth and agar with the addition of appropriate antibiotics, wherever required.

3.1.2. Contrivance of plasmid constructs

UTEX 2973 genomic DNA was extracted and purified using the GSure Algae DNA kit (GCC Biotech, India). The genomic DNA was used as a template for PCR amplification of upstream and downstream regions of neutral sites, neutral site II (NSII), and neutral site III (NSIII). List of primers used to amplify the regions during plasmid construction are listed in Table 3.1. NSI', NSII', and NSIII' are designated as upstream regions of neutral sites, while NSI", NSII", and NSIII" as downstream regions.

Table 3.1 List of primers used for vector construction and verification.

Name	Sequence	Purpose
NSI' FP	GACTAGTCAGCTTAGTCCTGCGCAATCT	NSI Upstream region amplification from UTEX 2973
NSI' RP	GACTAGTCGAAATGTTCTGGACTTGCAGC	NSI Upstream region amplification from UTEX 2973
NSI" FP	CCTAGGTGAAACAAACCACGGGCA	NSI Downstream region amplification from UTEX 2973
NSI" RP	CCTAGGGACACCAAAATCACCACG	NSI Downstream region amplification from UTEX 2973
NSII' FP	GGACTAGTCCAGCTTGTCATCTGCCGGATG	NSII Upstream region amplification from UTEX 2973
NSII' RP	GGACTAGTCCGAAAATCACCAGCTGAAAC	NSII Upstream region amplification from UTEX 2973
NSII" FP	CCTAGGACGTTGTTGCTCCAGTAAAGTC	NSII Downstream region amplification from UTEX 2973
NSII" RP	CCTAGGACCAATGCTGGGTAGTTCTC	NSII Downstream region amplification from UTEX 2973
NSIII' FP	GACTAGTCAAGAGCCAGATAGATGCG	NSIII Upstream region amplification from UTEX 2973
NSIII' RP	GACTAGTCTTCGCTACTCAAACGGC	NSIII Upstream region amplification from UTEX 2973
NSIII" FP	ATACCTAGGGACAAGCCGGGGCAG	NSIII Downstream region amplification from UTEX 2973
NSIII" RP	CCTAGG ACAGTCGGCGTCACGG	NSIII Downstream region amplification from UTEX 2973
AFS FP	ATGGAATTTCGCGTGCACCT	AFS gene amplification
AFS RP	GGATCCTTAGTGGTGGTGAT	AFS gene amplification
New NSIII" RP	ATTCTGTGGATAACCGTATTACCGCCTTTGA CAGTCGGCGTCACGG	Addition of overhangs to the 3' end of NSIII downstream region
bom FP	CAAAGGCGGTAATACGGTTATCCACAGAAT CCTGATGCGGTATTTTCTCC	Amplification of bom and adding overhangs to 5' end
bom RP	AGAACATGTCGCAGCCATGACCCAG	Amplification of bom
dxs FP	GCATGACATATGAGTTTTGATATTGCCAAA TACC	Amplification of dxs gene from *E. coli*
dxs RP	CGGGATCC TTATGCCAGCCAGGCCT	Amplification of dxs gene from *E. coli*
idi FP	TACATATGCAAACGGAACACGTC	Amplification of idi gene from *E. coli*
idi RP	TTTAAGCTGGGTAAATGCAGAT	Amplification of idi gene from *E. coli*
ispA FP	GAAGATCTTTTAAGAAGGAGATATACATAT GGACTTTCCGCAGCAA	Amplification of ispA gene from *E. coli*
ispA RP	CGGGATCCTTATTTATTACGCTGGATGATGT	Amplification of ispA gene from *E. coli*
idi-L FP	AGATCTTTTAAGAAGGAGATATACATATGC AAACGGAAC	Addition of overhangs to 5' end of idi gene
idi-L RP	TGACCCTCCGCCACCTGACCCTCCGCCACCT TTAAGCTGGGTAAATGC	Addition of linker overhangs to 3' end of idi gene
ispA-L FP	GGTGGCGGAGGGTCAGGTGGCGGAGGGTC AATGGACTTTCC	Addition of linker overhangs to 5' end of ispA gene
idispA FP	GAAGATCTTTTAAGAAGGAGATATACATAT G	Amplification of fused *idi* and *IspA*
idispA RP	CGGGATCCTTATTTATTACGCTGGATGATGT	Amplification of fused *idi* and *IspA*
chlr FP	AGCGGCCGTGATCGGCACGTAAGAGGTTC	Amplification of chloramphenicol gene from *E. coli* Rosetta gami
chlr RP	ACCGAGCTCTTACGCCCCGCCCTGCCA	Amplification of chloramphenicol gene from *E. coli* Rosetta gami
OUFP	GACGCTCAGTGGAACGAAAACTCACG	Orientation specific primers

3.1.2.1 pAM2991-*AFS*

pAM2991 (Addgene, Plasmid #40248) which already contains NSI' and NSI" regions, was used directly to target *M. domestica AFS* gene to the NSI site (Ivleva et al., 2005). The *AFS* from *Malus domestica* was codon optimized and synthesized by Gene Universal Inc., USA, such that the 5' end and 3' end have EcoRI and BamHI restriction sites, respectively (Fig. 3.1(a)). pAM2991 and *AFS* gene were both digested by EcoRI and BamHI restriction enzymes (Both New England Biolabs) and were gel purified using a QIAquick Gel Extraction kit (Qiagen, Germany). The vector (pAM2991) and insert (*AFS* gene) were both checked on a gel for their correct size and were ligated using Invitrogen, Anza™, and T4 DNA Ligase Master Mix. The ligated product was transformed into *E. coli* DH5α competent cells. The transformed mixture was spread on LB agar supplemented with 50 µg/ml streptomycin and spectinomycin plates as the pAM2991 contains the SmR gene. The next day the colonies containing ligated vector appeared on the plate, which were analyzed by colony PCR using AFS specific primers (AFS FP and AFS RP) and also by digestion. This leads to the formation of pAM2991-*AFS* vector.

3.1.2.2. pBbE1cNSII-*dxs*

pBbE1k-*RFP* (Addgene, Plasmid #35333) was used as a parent vector for the generation of the pBbE1cNSII-*dxs* vector (targeting *dxs* gene to NSII site). The sequential addition of the Chl^R gene, *dxs* gene, NSII' site, and NSII" site fused with basis of mobility sequence (bom) was done (Fig. 3.1(b)). All the amplifications were done using high-fidelity Phusion plus DNA polymerase (Thermo Scientific) with appropriate temperature programs, as mentioned in Table 3.2. At first, the kanamycin resistance gene was replaced by the chloramphenicol resistance gene (Chl^R). Chl^R gene was amplified (using chlr FP and chlr RP) from the genomic DNA of *E. coli* Rosetta gami as a template with EagI restriction site at the 5' end and SacI restriction site at the 3' end pBbE1k-*RFP* and Chl^R gene were digested

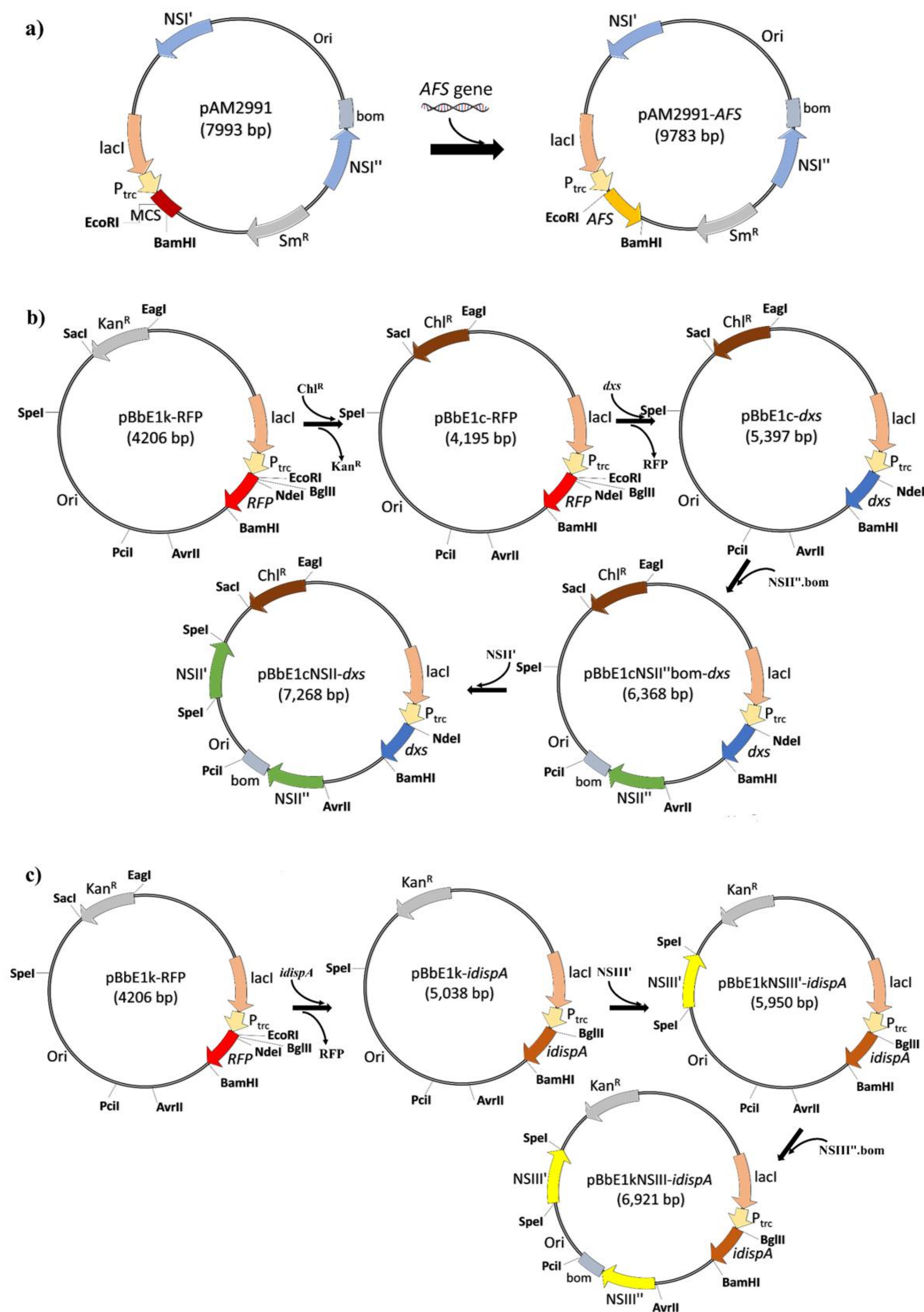


Fig. 3.1. Detailed vector construction by sequential addition of gene/inserts. **a)** Formation of pAM2991-*AFS* from pAM2991. **b)** Formation of pBbE1cNSII-*dxs* by sequential addition of chl^R, *dxs*, NSII″.bom (NSII″ fused with bom), and NSII′ in pBbE1k-RFP. **c)** Formation of pBbE1kNSIII-*idispA* by sequential addition of *idispA* (fusion of *idi* and *ispA*), NSIII′, and NSIII″.bom (NSIII″ fused with bom) in pBbE1k-RFP.

by EagI (Invitrogen, Anza™ 47 Eco52I) and SacI (Invitrogen, Anza™ 20 SacI) restriction enzymes. Both the digested products were gel-eluted and ligated. The transformed colonies were selected on LB agar chloramphenicol (10 µg/ml) plates instead of kanamycin. Further, the plasmid was modified by the addition of the *dxs* gene. The *dxs* gene was amplified (using dxs FP and dxs RP) from *E. coli* genomic DNA such that the 5' end has NdeI and the 3' end has a BamHI restriction site. The vector (pBbE1c-*RFP*) and *dxs* gene were digested with NdeI and BamHI restriction enzymes (both New England Biolabs), gel purified, and ligated. The ligated product was transformed into *E. coli* DH5α competent cells, and the positive colony was selected for further modifications. NSII' region was amplified (using NSII' FP and NSII' RP) from UTEX 2973 genome was gel purified, digested with SpeI restriction enzymes (Invitrogen, Anza™ 3 BcuI), and again gel purified. The plasmid was also digested with SpeI restriction enzyme and gel purified. Vector (pBbE1c-*dxs*) and insert (NSII') were ligated and transformed into *E. coli* DH5α competent cells and was selected on LB agar chloramphenicol plates. Finally, the bom sequence was fused with amplified, gel purified NSII" (having linker overhangs at the 3' end and AvrII restriction site at the 5' end) through overlap extension PCR (using NSII" FP and bom RP). The fused product and modified plasmid were digested with PciI (New England Biolabs) and AvrII (Invitrogen, Anza™ 15 XmaJI) restriction enzymes and were gel purified. The digested fused product and plasmid were ligated and transformed. The positive clones were selected on chloramphenicol plates. The final construct after the addition of NSII' and NSII" was named pBbE1cNSII-*dxs*.

3.1.2.3. pBbE1kNSIII-*idispA*

pBbE1kNSIII-*idispA* vector targeting fused *idi* and *IspA* gene to the NSIII region of the UTEX 2973 genome was constructed in the same manner as described above (Fig. 3.1(c)). Amplifications of the segments to be cloned were done with temperature profiles,

Table 3.2 PCR amplification conditions of genes/DNA fragments.

Gene/DNA fragments	Amplification conditions
For pBbE1cNSII-*dxs*	
Chl^R	1 cycle of 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 16 seconds, annealing at 55 °C for 18 seconds, and extension at 72 °C for a minute with final elongation at 72 °C for 10 minutes.
dxs	1 cycle of 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 59 °C for 30 seconds, and extension at 72 °C for 2 minutes with final elongation at 72 °C for 10 minutes.
NSII' and NSII"	1 cycle of 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 20 seconds, annealing at 57 °C for 20 seconds, and extension at 72 °C for 1:30 minutes with final elongation at 72 °C for 10 minutes.
bom	1 cycle of 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 20 seconds, annealing at 56 °C for 20 seconds, and extension at 72 °C for 30 seconds with final elongation at 72 °C for 10 minutes.
Fusing NSII" site and bom	1 cycle of 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 20 seconds, annealing at 56 °C for 18 seconds, and extension at 72 °C for 2 minutes with final elongation at 72 °C for 10 minutes.
For pBbE1kNSIII-*idispA*	
idi	1 cycle of 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 20 seconds, annealing at 52 °C for 20 seconds, and extension at 72 °C for 45 seconds with final elongation at 72 °C for 10 minutes.
ispA	1 cycle of 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 25 seconds, annealing at 50 °C for 25 seconds, and extension at 72 °C for a minute with final elongation at 72 °C for 10 minutes.
Fusing *idi* and *ispA* gene	1 cycle of 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 20 seconds, annealing at 56 °C for 25 seconds, and extension at 72 °C for 1:30 minutes with final elongation at 72 °C for 10 minutes.
NSIII' and NSIII"	Same as NSII' and NSII"
Fusing NSIII" site and bom	Same as fusing NSII" site and bom

as mentioned in Table 3.2. The *idi* gene (using idi FP and idi RP) and *IspA* gene (using ispA FP and ispA RP) were amplified from *E. coli* genomic DNA, such that the 5' end and the 3' end of the *idi* gene have BglII restriction site and overhangs of linker sequence (ggtggcggagggtcaggtggcggagggtca), respectively, whereas the 5' end and the 3' end of *IspA* gene have overhangs of linker sequence and BamHI restriction site, respectively (figure S5). Both the genes were gel purified separately and used as a template for overlap extension PCR, leading to the fusion of two genes. The fused *idispA* and vector (pBbE1k-*RFP*) were digested with BglII (Invitrogen, Anza™ 19 BglII) and BamHI. The digested

products were gel purified, ligated, and transformed into competent *E. coli* DH5α. The positive clones were selected, and the modified vector was designated as pBbE1k-*idispA*. Further, the NSIII' region amplified (using NSIII' FP and NSIII' RP) from UTEX 2973 genome, and pBbE1k-*idispA* was digested with SpeI (Invitrogen, Anza™ 3 BcuI) restriction enzyme. The transformed colonies were selected for positive clones. Finally, NSIII" fused with bom site and modified plasmid were digested with AvrII and PciI restriction enzymes and ligated to the modified plasmid. The final construct after the addition of NSIII' and NSIII" was named pBbE1kNSIII-*idispA*.

3.2. Results and discussion

The list of plasmids generated and the parent plasmid used in the study are listed in Table 3.3.

Table 3.3 List of plasmids used and generated in the study.

Plasmid	Genetic constitution	Resistance to antibiotic	Reference
pAM2991	pUC origin-NSI'-lacI-P_{trc}-Sm^{R}-NSI" (NSI targeting)	Streptomycin and spectinomycin	Ivleva et al., 2005
pAM2991-*AFS*	pUC origin-NSI'-lacI-P_{trc}*AFS*-Sm^{R}-NSI" (NSI targeting)	Streptomycin and spectinomycin	This study
pBbE1k-RFP	pUC origin-Kan^{R}-lacI-P_{trc}mRFP1	Kanamycin	Lee et al., 2011
pBbE1cNSII-*dxs*	pUC origin-NSII'-Chl^{R}-lacI-P_{trc}*dxs*-NSII" (NSII targeting)	Chloramphenicol	This study
pBbE1kNSIII-*idispA*	pUC origin-NSIII'-Kan^{R}-lacI-P_{trc}*idispA*-NSIII" (NSIII targeting)	Kanamycin	This study

3.2.1. pAM2991-*AFS*

M. domestica AFS, which was previously characterized as (E,E)-α-farnesene synthase (GenBank accession number AY182241), was selected based on the previous

study, which showed its superior activity compared to *C. junos* and *A. annua AFS* (Lee et al., 2022; Pechous and Whitaker, 2004; Tippmann et al., 2016b). Hence, a codon-optimized *AFS* gene from *M. domestica* was cloned in the pAM2991 vector under the influence of the trc promoter (Ivleva et al., 2005). pAM2991 vector targets the gene of interest to the NSI of PCC 7942. Since the genomic sequences of PCC 7942 and UTEX 2973 are 99.8% similar, the alignment of NSI of PCC 7942 and UTEX 2973 showed 100% similarity (Yu et al., 2015). In addition to this, pAM2991 also has a bom site, which is required for conjugation and is not orientation-specific (Finnegan and Sherratt, 1982). The successful cloning of the *AFS* gene into the pAM2991 vector was confirmed by colony PCR and digestion, as shown in Fig. 3.2.

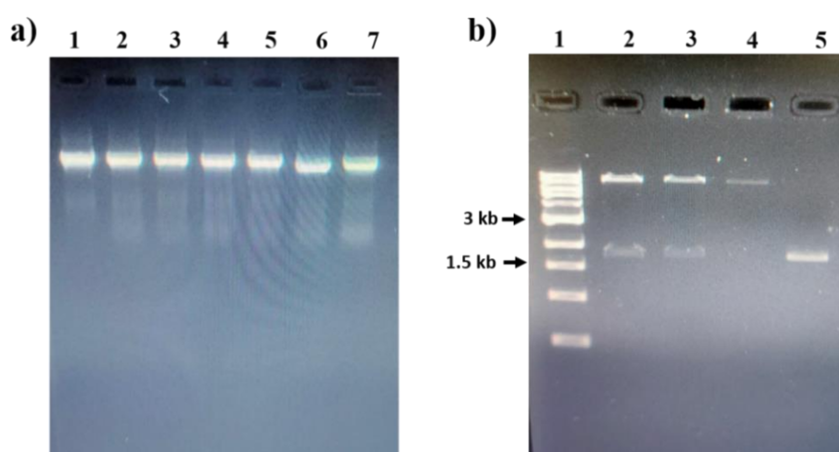


Fig. 3.2. Verification of insertion of AFS gene in pAM2991-AFS. **a)** Colony PCR using AFS FP and AFS RP (Lane 1: positive control, Lane 2-7: transformed colonies). **b)** Digestion of plasmid by EcoRI and BamHI (Lane 1: 1kb DNA ladder, 2,3: Digested pAM2991-AFS, 4: Digested pAM2991, 5: AFS insert

3.2.2. pBbE1cNSII-*dxs*

To increase the flux of DMAPP and IPP, the *dxs* gene from *E. coli was* inserted into pBbE1k-RFP under the influence of trc promoter (Satoh et al., 2012). The vector was modified such that the kanamycin resistance gene was replaced by the chloramphenicol resistance gene, and NSII′ and NSII″ were added to target the *dxs* gene to the NSII in the genome via homologous recombination. As mentioned, the importance of the bom site for

conjugation, the bom site was fused with NSII″. Each cloning step was verified by colony PCR and restriction digestion as shown in Fig. 3.2. The *dxs* gene is from *E. coli* it is not possible to do colony PCR. Therefore, the plasmids were isolated from some colonies and the PCR was done using dxs FP and dxs RP (Fig. 3.3(a)). The positive plasmids were subjected to digestion to confirm the insertion of *dxs* gene (Fig. 3.3(b)). Since the NSII' was inserted at a single restriction site, colony PCR with orientation-specific primers (OUFP and NSII' RP) was done to screen the positive colonies with the correct orientation, which was further confirmed by digestion (Fig. 3.3(c,d)). The final DNA fragment, NSII" fused bom, added to form pBbE1cNSII-*dxs* was also confirmed by colony PCR (using NSII" FP and bom RP) and digestion (Fig. 3.3(e,f)).

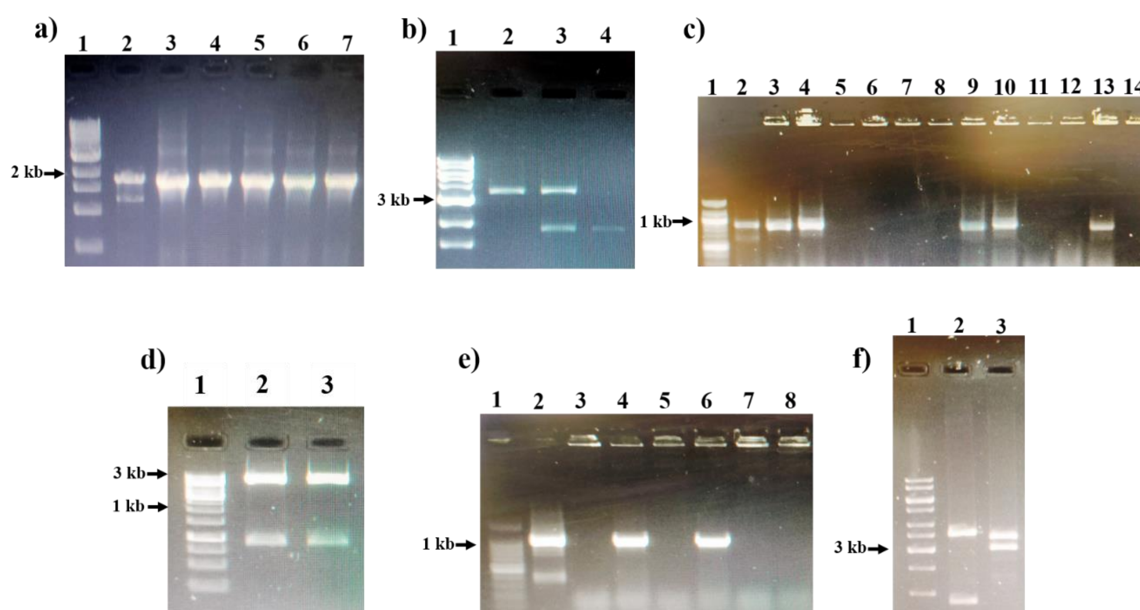


Fig. 3.3. Verification of insertion of gene/DNA fragments in pBbE1cNSII-*dxs*. **a)** PCR of isolated plasmids (Lane 1: 1kb ladder, Lane 2: +ve control, Lane 3 to 7: Isolated plasmids). **b)** Digestion of plasmid by NdeI and BamHI (Lane 1: 1kb ladder, Lane 2: Digested pBbE1k-RFP, Lane 3: Digested pBbE1c-*dxs*, Lane 4: dxs Insert). **c)** Colony PCR of NSII' with normal and orientation specific primers (Lane 1: 100 bp ladder, Lane 2: positive control, Lane 3, 5, 7, 9, 11, 13: amplification by normal primers, Lane 4, 6, 8, 10, 12, 14: amplification by orientation specific primers). **d)** Digestion of isolated plasmids by SpeI (Lane 1: 1 kb ladder, Lane 2 and 3: plasmid digestion by SpeI). **e)** Colony PCR of NSII" fused with bom (Lane 1: 100 bp ladder, Lane 2: positive control, Lane 3-11: transformed colonies). **f)** Digestion of plasmid (Lane 1: 1kb ladder, Lane 2: pBbE1c-*dxs* with NdeI and BamHI, Lane 3: plasmid digestion with NdeI)

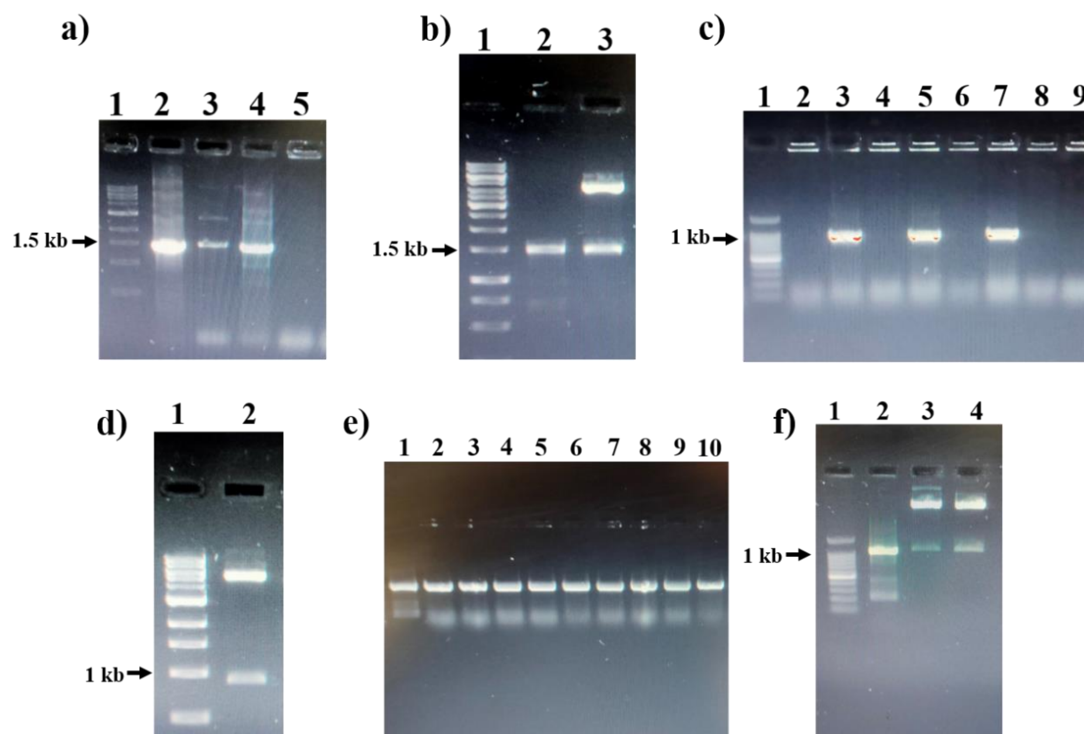


Fig. 3.4. Verification of insertion of gene/DNA fragments in pBbE1kNSIII-*idispA*. **a)** Colony PCR of transformed DH5α (Lane 1: 1kb ladder, Lane 2: +ve control, Lane 3 to 7: transformed colonies). **b)** Digestion of isolated plasmid by BglII and BamHI to detect idispA insertion (Lane 1: 25 kb ladder, Lane 2: idispA insert, Lane 3: digested vector). **c)** Colony PCR of NSIII' using orientation specific primers (Lane 1: 100 bp ladder, Lane 2 to 9: transformed colonies). **d)** Digestion of isolated plasmid by SpeI to detect NSIII′ insertion (Lane 1: 1 kb ladder, Lane 2: digested plasmid). **e)** Colony PCR of transformed DH5α having NSIII″ fused with bom (Lane 1: +ve control, Lane 2 to 10: Transformed colonies). **f)** Digestion of pBbE1kNSIII-idispA by AvrII and PciI (Lane 1: 100 bp ladder, Lane 2: NSIII″ fused bom insert, Lane 3 to 4: digested plasmids).

3.2.3. pBbE1kNSIII-*idispA*

The fusion of *idi* and *ispA* genes with the help of the linker (amino acid sequence $(GGGGS)_2$) increased the terpenoid production in PCC 7942, the genes were fused with overlap extension PCR and cloned in pBbE1k-RFP forming pBbE1k-*idispA* (Choi et al., 2017a). The vector (pBbE1k-*idispA*) was modified by inserting NSIII′ and NSIII″ fused with bom to target the *idispA* gene to NSIII in the genome. The vector generated, pBbE1kNSIII-*idispA*, was checked and verified at each cloning step. For instance, after the addition of the *idispA* gene to pBbE1k-RFP, the transformed colonies were confirmed by colony PCR (using idi FP and ispA RP) and digestion (Fig. 3.2). Further, the NSIII' addition

at a single restriction site was confirmed by colony PCR of transformed colonies with orientation-specific primers (OUFP and NSIII' RP), and restriction digestion (Fig. 3.2). Eventually the addition of NSIII" fused bom region was confirmed by colony PCR (using NSIII" FP and bom RP) and digestion (Fig. 3.2).

3.3. Conclusion

The integrative vector strategy has an advantage over the expression vector systems by generating stable engineered strains without losing the expression of genes of interest. Three integrative vectors were constructed to integrate genes into the genome of *Synechococcus elongatus* UTEX 2973. Three integrative vectors were constructed for *AFS* (pAM2991-*AFS*), *dxs* (pBbE1cNSII-*dxs*), and *idispA* (pBbE1kNSIII-*idispA*) genes to be integrated at NSI, NSII and NSIII sites of UTEX 2973 genome, respectively. All the vectors constructed were verified by PCR, restriction digestion and finally, sequencing. The vectors can be altered for any other gene of interest due to the presence of a unique restriction site.