

A. CONSTRUCTION OF PLASMIDS

1. PLASMID FOR EXPRESSION IN WHOLE PLANT

Plasmid pITB239:

The 2.7 kb *BglII-BamHI* fragment of T7 RNAP coding region along with nuclear localization signal (NLS) from pAR3283 (Dunn *et al.*, 1988, kindly provided by Studier) was cloned into *BamHI* site of plasmid pFF19 (Marja *et al.*, 1990, kindly provided by Dr. J. Messing) to yield plasmid pFF19-T7. The *BamHI* site at the carboxyl terminal end of T7 RNAP was destroyed by restriction enzyme digestion and end filling by *Klenow* enzyme. The 800bp 35S promoter from pBI221 (Clontech) was cloned into *HindIII-SmaI* sites of pFF19-T7 replacing 35S promoter and enhancer element to create pFF19-T7-35S. The 3.5 kb *HindIII-NcoI* (blunt ended) containing “CaMV 35S:T7-RNAP:35S polyA” cassette from pFF19-T7-35S was cloned into a plant transformation vector pCAMBIA1300 (kindly provided by Jefferson) at *HindIII-SmaI* sites to create pITB239 (Figure 1).

Cloning of T7 RNAP into pFF19 was confirmed by the digestion with *BamHI-SmaI*. A 2.7 kb extra band corresponding to T7 RNAP was present in the pFF19-T7 and absent in pFF19. The *BamHI* site present at the end of T7 RNAP stop codon was destroyed by restriction digestion-Klenow filling and religation. To detect whether *BamHI* site was deleted in pFF19-T7(B), plasmid DNA was digested with *BamHI-SmaI* and results are presented in Figure 2C. Presence of a linear DNA in pFF19-T7(B) and a 2.7kb band in pFF19-T7 is an indication of removal of *BamHI* site (Figure 1).

To direct the expression of T7 RNAP, CaMV 35S promoter from pBI221 was cloned in to pFF19-T7(B) as *HindIII-SmaI*. It can be seen from figure 2D that a 0.8 kb corresponding to 35S promoter was present when the pFF19-T7-35S was digested with *HindIII –BamHI* (present just before *SmaI*) (Figure 1 and 2D).

To confirm the insertion of “35S promoter:T7 RNAP:35S polyA” cassette into pCAMBIA1300, the pCAMBIA1300 and pITB239 plasmids were digested with *HindIII-Sall*. An extra 3.7kb band in pITB239 created due *HindIII* in pCAMBIA1300 and *Sall* site present at the end of T7 RNAP (Figure 2F). On the other hand pCAMBIA1300 was only linearized as both the *HindIII* and *Sall* were present in multicloning site.

Plasmid pITB250:

The PCR amplified *uidA* gene fragment from pFF19G (using primers forward GUSNCONEW and reverse GUS3-BAM) was digested with *NcoI-BamHI* and cloned into pET14b (Novagen) in the same sites to create pET14b-GUS (confirmed the correct cloning and expression of GUS gene in pET14b showed in Figure 23E). The “T7 Promoter:*uidA*:T7 Terminator” cassette was PCR amplified from pET14b-GUS (using primers forward pET14b5New and reverse pET14b3New) and cloned into *HindIII* site of pITB239 to yield pITB250.

To confirm the insertion of “T7 promoter:*uidA*:T7 terminator” cassette into pITB239, the pITB239 and pITB250 were digested with *HindIII*. Presence of an extra 2.1 kb band in pITB250 is an indication of the presence of “T7 promoter:*uidA*:T7 terminator” cassette into pITB239 (Figure 2G). In these plasmids, the T7 RNA polymerase gene was under the control of CaMV 35S promoter and the *uidA* is under the control of T7-promoter (Figure 1). To detect orientation of “T7 promoter-*uidA*-T7 terminator” cassette, with respect to T7 RNAP gene, plasmid DNA was digested with *Sall-BglIII*. It can be seen from figure 2G that the *uidA* cassette got cloned in opposite orientation.

Plasmid pITB228

The T7 RNAP from pITB250 was cloned as *BamHI-Sall* fragment into pBinHygTX, (provided by Dr. Gatz) in the same sites to yield pBin-HygTX-T7. The *HindIII-HindIII* fragment containing “T7 Proromoter:*uidA*:T7 Terminator” cassette

from pITB250 was cloned into *HindIII* site of plasmid pBin-Hyg-TX-T7 to create pITB228 (Figure 3).

Creation of pITB228 was confirmed by PCR and restriction digestion. PCR amplification using T7-1 and T7-3 primers (see materials) indicated the insertion of T7 RNAP into pBin-HygTX. Later restriction digestion also confirmed the cloning of T7 RNAP into pBin-HygTX. The *BamHI-SalI* showed a 2.7 kb T7 RNAP extra band in pBin-HygTX-T7, while it was absent in pBin-HygTX as *BamHI* and *SmaI* sites are located very close to each other in multicloning site (Figure 4F).

Similarly, PCR was used in combination of pET14bNEW5' and pET14bNEW3' primers to check the insertion of "T7 promoter:*uidA*:T7 terminator" cassette into pBin-HygTX-T7. The *HindIII* digestion also further confirmed the cloning of *uidA* cassette into pITB228 (Figure 4G) showed a 2.1 kb extra band in pITB228.

Plasmid pITB139:

The IRT1 gene was amplified from *Arabidopsis* genomic DNA using primers IRT1-5 *Xba* and IRT1-3 *Bam* (see materials) and subcloned into pGEMT-Easy to yield plasmid pGEMT-Easy-IRT1. The *XbaI-SalI* fragment of T7 RNAP gene in plasmid pITB239 was exchanged by *SalI-XbaI* IRT1 gene from pGEMT-Easy-IRT1 (Figure 5).

Cloning of IRT1 gene into pGEMT-Easy was confirmed by digestion with *XbaI* and *XbaI-SalI*. Figure 6B show that the IRT1 was inserted into pGEMT-Easy. To confirm the replacement of IRT1 with T7 RNAP in pITB239, the plasmids pITB239 and pITB139 were digested with *XbaI-SalI* (Figure 6C). Presence of 1.0 kb band in pITB139 is an indication of the IRT1 cloning, while pITB239 has 2.7 kb band corresponding to T7 RNAP.

Plasmid pITB239-GFP:

The *NcoI-BamHI* GFP gene was amplified from pCAMBIA1302 and cloned into *NcoI-BamHI* sites of pET14b. The “T7 promoter-GFP-T7 terminator” cassette was PCR amplified from pET14b-GFP and subcloned into pGEMT-Easy to create pGEMT-Easy-GFP. The *HindIII-HindIII* fragment containing “T7promoter:GFP:T7 terminator” cassette was cloned into *HindIII* site of plasmid pITB239 to yield plasmid pITB239-GFP (Figure 5).

Cloning of GFP was confirmed by the digestion of pGEMT-Easy-GFP with *HindIII* and with *BglII-BamHI* (Figure 6D). A 0.9 kb and 0.7 kb bands present in the pGEMT-Easy-GFP respectively indicated the cloning of the GFP cassette into pGEMT-Easy.

To confirm insertion of “T7 promoter:GFP:T7 terminator” cassette into pITB239, the pITB239 and pITB239-GFP plasmid DNA was digested with *HindIII*. A 0.9 kb extra band released from pITB239-GFP indicated the cloning of the “T7 promoter:GFP:T7 terminator” cassette (Figure 6E-lane 1 and 2). As expected the pITB239 was linearized. Orientation of “T7 promoter:GFP:T7 terminator” cassette in pITB239-GFP was confirmed by the digestion of pITB239 and pITB239-GFP with *BglII-SalI* (Figure 6D-lane 3 and 4). A 3.5 kb band in pITB239 as opposed to 4.5 kb band in pITB239-GFP indicates the cloning of GFP in anticlockwise direction. Result confirmed the correct cloning and expression of GFP in pITB239 (Figure 23A-B)

Plasmid pITB260

The *EcoRI-SmaI* fragment that contained TripleX promoter (a modified CaMV 35S) was subcloned into polyIII vector (gifted by Dr. Vijay Kumar) to create polyIII-Triplex (Figure 5). The 35S-promoter in pITB250 was exchanged at *BglII-SmaI* sites with that of 565 bp *BamHI-SmaI* TripleX-35S promoter from polyIII-Triplex. The pITB260 is similar with pITB228 but has a different backbone. In pITB288

backbone is pBin-HygTX (Figure 3), while in pITB260 backbone is pCAMBIA1300 (Figure 1).

To confirm cloning of modified CaMV 35S TripleX promoter into pITB260, the pITB250 and pITB260 plasmids were digested with *BglII-SmaI* (Figure 6F). Presence of a 0.8 kb extra band in pITB250, and its absence in pITB260 confirmed the cloning of the promoter. Note that due to ligation of *BamHI* end with *BglII* ends, both sites were lost. Similarly, digestion of pITB250 and pITB260 with *HindIII-SmaI* also showed loss of one *HindIII* site in pITB260 and thus only a 2.7 kb band was released, while in pITB250 two bands, 0.8 kb and 2.1 kb were present again confirming the cloning of the promoter (Figure 5 and 6F).

2. PLASMIDS FOR EXPRESSION IN PLANT TISSUE SPECIFIC

Plasmid pITB450:

The pITB450 was constructed from pITB250 by replacing CaMV 35S promoter with pea *rbcS:3A* promoter. The *rbcS:3A* promoter (Gene bank Acc. No. M21356) was PCR amplified from pea genomic DNA using primers forward *rbcS3A-5XBBg* and reverse *rbcS3A-3XS* (see materials), subcloned into pGEMT-Easy to yield pGEMT-Easy-*rbcS*. The 430 bp *BglII-SmaI* *rbcS* promoter from pGEMT-Easy-*rbcS* was cloned into pITB250 at *BglII-SmaI* sites to yield pITB450. In pITB450, T7RNA polymerase was under *rbcS* promoter and *uidA* was under T7 promoter (Figure 3).

PCR was used to check insertion of *rbcS* promoter into pGEMT-Easy. It can be seen from figure 4B that a 0.43 kb band corresponding to the size of *rbcS* promoter. Digestion of pGEMT-Easy-*rbcS* with various enzymes further confirmed the cloning of the *rbcS* promoter (Figure 4C).

To detect replacement of *rbcS* promoter with 35S promoter in pITB250, plasmids pITB250 and pITB450 were digested with *HindIII*. It can be seen from figure 4D that a 0.4 kb extra band was present in pITB450 indicating the cloning of *rbcS*

promoter, while no such band was present in pITB250. Note that in pITB250, two *HindIII* sites are expected to present one on both end of T7 promoter and T7 terminator and in pITB450 three *HindIII* sites are present (two in *rbcS* promoter and one at the end of T7 terminator).

Plasmid pITB441:

The pITB441 was created from pITB450 by removing “T7 promoter:*uidA*:T7 terminator” cassette by digesting with *BglII*-*BamHI* and religation. The “T7 RNAP:35S polyA” cassette was replaced with “*uidA*:35S polyA” cassette from pFF19G (*NcoI*-*SmaI*). In pITB441 *uidA* was under control of *rbcS* promoter directly (Figure 3).

Digestion of pITB450 and pITB440 with *BamHI*-*BglII* confirmed the removing of “T7 promoter:*uidA*:T7 terminator” cassette into pITB450. After religation both *BglII* and *BamHI* sites in pITB440 were lost.

To detect replacement of “T7 RNAP:35S polyA” by “*uidA*:35S polyA” cassette, the pITB441 and pITB440 were digested with *HindIII*-*NcoI*. As can be seen from figure 4E a 2.1 kb band (*uidA*:35S polyA) was present in pITB441, while in pITB440 has a 2.9 kb band corresponding to T7 RNAP-35S polyA.

Plasmid pITB550:

The 1.3 kb *kin1*-promoter (gene Bank Acc. No. L21929) was amplified from *Arabidopsis* genomic DNA using primers forward Kin5-HBg and reverse Kin3-BSm (see materials) and cloned into pGEMT-Easy yielding pGEMT-Easy-*Kin1*. The *SmaI*-*BglII* *rbcS*-promoter from pITB450 was replaced with *kin1*-promoter as *SmaI*-*BglII* fragment from pGEMT-Easy-*Kin1* to yield plasmid pITB550. In pITB550, T7RNA polymerase was under the control of *kin1* promoter (Figure 7).

After PCR amplification to check positive insertion of *kin1* promoter, pGEMT-Easy-*kin1* was digested with *NsiI*. Two different sizes bands (1.0 kb and 3.4 kb or 0.6 kb and 3.8 kb), depending on the orientation of *kin1* promoter in pGEMT-Easy are expected. It can be seen from figure 8B there are two *NsiI* sites in pGEMT-Easy-*kin1*, one *NsiI* site in pGEMT-Easy and the other in *kin1*.

Replacement of *kin1* promoter with *rbcS* promoter in pITB450 was confirmed by PCR amplification using Kin5-HBg and Kin3-BSm primers (Figure 8D), also digestion of pITB450 and pITB550 with *BglIII-SmaI* (Figure 8E) showed a 1.3 kb band (*kin1* promoter) in pITB550 in comparison with 0.4 kb band (*rbcS* promoter) in pITB450, and again suggesting that *kin1* is present in pITB550.

Plasmid pITB541

The *SmaI-HindIII rbcS*-promoter from pITB441 was replaced with a *kin1*- promoter as a *SmaI-HindIII* fragment to create pITB541 containing *uidA* under the control of *kin1* promoter directly (Figure 7).

PCR was used to check replacement of *rbcS* promoter with *kin1* promoter using primers Kin5-HBg and Kin3-BSm (Figure 8D). This was further confirmed by digesting pITB441 and pITB541 with *HindIII-BamHI*. (Figure 8E) Note the presence of a 1.3 kb band (*kin1* promoter) in pITB541 in comparison with 0.4 kb band (*rbcS* promoter) released in pITB441.

Plasmid pITB650

The *cor6.6*-promoter (gene Bank Acc. No. L21929) was amplified from *Arabidopsis* genomic DNA using primers forward Kin5-HBg and reverse Kin3-BSm (see materials) and subcloned into pGEMT-Easy yielding pGEMT-Easy-*cor6.6*. The *SmaI-BglIII rbcS*-promoter from pITB450 was replaced by *SmaI-BglIII cor6.6*-promoter from pGEMT-Easy-*cor6.6* to yield pITB650 that contained T7 RNAP under the control *cor6.6* promoter (Figure 7).

PCR was used to verify the insertion of *cor6.6* promoter in pGEMT-Easy using *cor6.6* specific primers (Kin5-HBg and Kin3-BSm). Further, pGEMT-Easy-Cor6.6 was digested with *ScaI* to confirm insertion and direction of *cor6.6* promoter in pGEMT-Easy (Figure 8C). There are two *ScaI* sites, one in pGEMT-Easy (at 1890 bp) and other in *cor6.6* promoter (at 352 bp). When digested with *ScaI*, two bands (1.5 kb and 2.8 kb or 2.1 kb and 2.2 kb) were noticed (showed thick band on gel) depending on the direction of *cor6.6* in pGEMT-Easy (Figure 8C).

For the replacement of *cor6.6* promoter with *rbcS* promoter in pITB450, PCR was used with *cor6.6* specific primers (Kin5-HBg and Kin3-BSm) (Figure 8D). Also digestion of pITB650 and pITB450 with *BglII-SmaI* further confirmed the replacement (Figure 8E). Note the presence of an expected 1.3 kb band (*cor6.6* promoter) in pITB650 and a 0.4 kb band (*rbcS* promoter) in pITB450.

Plasmid pITB641:

The *rbcS*-promoter in pITB441 was removed using *SmaI-HindIII* and *cor6.6* promoter was cloned in the same sites to create pITB641. In the pITB641 the *uidA* is under the control of *cor6.6* promoter directly (Figure 7).

PCR was used to confirm the cloning of *cor6.6* promoter in pITB641 (Figure 8D), Digestion of pITB641 and pITB441 with *HindIII-BamHI* further confirmed the cloning of *cor6.6* promoter (Figure 8E). Presence of 1.3 kb fragment is an indication of the cloning of the *cor6.6* promoter in pITB641.

Plasmid pITB750:

The *pal1*-promoter was amplified from *Arabidopsis* genomic DNA using primers forward (PALproAt5-2) and reverse (PALproAt3-1) and subcloned into pGEMT-Easy to create plasmid pGEMT-Easy-*pal1*. The *SmaI-BglII cor6.6*-promoter from pITB650 was replaced with a *SmaI-BglII* fragment of *pal1*-promoter from pGEMT-

Easy-*pal1* to create pITB750. In pITB750 the T7 RNAP is under the control of *pal1* promoter and *uidA* is under T7 promoter (Figure 9).

PCR was used to confirm the cloning of *pal1* using specific primers (PALproAt5-2 and PALproAt3-1). Later the pGEMT-Easy-*pal1* was digested with *BglIII-SmaI* to confirm insertion of *pal1* promoter into pGEMT-Easy (Figure 10A).

Again PCR was used to check the cloning of *pal1* into pITB650 (Figure 10B). This was further confirmed by the digestion pITB650 and pITB750 with *HindIII-SmaI* (Figure 10D). Note the presence of a 3.0 kb band (*pal1* promoter:T7 promoter:*uidA*:T7 terminator) in pITB750 and a 3.4 kb band (*cor6.6* promoter:T7 promoter:*uidA*:T7 terminator) in pITB650.

Plasmid pITB741:

The *SmaI-HindIII cor6.6*-promoter from pITB641 was removed and was replaced with *SmaI-HindIII pal1*- promoter to create pITB741 that contains *uidA* under control of *pal1* promoter directly (Figure 9).

Confirmation of insertion of *pal1* promoter into pITB641 by PCR amplification (Figure 10B) showed a 0.9 kb band; and digested pITB641 and pITB741 with *HindIII-SmaI* (Figure 10D) showed a 0.9 kb band (*pal1* promoter) in pITB741 in comparison with an 1.3 kb band (*cor6.6* promoter) in pITB641.

Plasmid pITB850:

The *pal1* Δ -promoter was amplified from *Arabidopsis* genomic DNA using forward (PALproAt5-3) and reverse (PALproAt3-1) primers and subcloned into pGEMT-Easy to create pGEMT-Easy-*pal1* Δ . The *cor6.6*-promoter from pITB650 was removed by digestion with *SmaI-BglIII* and *pal1* Δ -promoter from pGEMT-Easy-*pal1* Δ was cloned in the same sites to yield pITB850. In the pITB850, T7RNAP is regulated by *pal1* Δ promoter and *uidA* is regulated by T7 promoter (Figure 9).

Cloning of *pal1Δ* into pITB650 was verified by PCR using *pal1Δ* specific primers (PALproAt5-3 and PALproAt3-1). All positive colonies showed the presence of a 0.6 kb band (*pal1Δ* promoter) (Figure 10B). Restriction digestion of pITB650 and pITB850 with *HindIII-SmaI* (Figure 10D) further confirmed the presence of *pal1Δ* promoter in pITB850 (2.7 kb fragment) compared to a 3.4 kb band released in pITB650.

Plasmid pITB841

To create the pITB841, the *HindIII-SmaI cor6.6*-promoter from pITB641 was replaced with *HindIII-SmaI* fragment of *pal1Δ*-promoter from pGEMT-Easy-*pal1Δ* (Figure 9).

Cloning of *pal1Δ* promoter in plasmid pITB841 was confirmed by PCR amplification (Figure 10B) and restriction digestion with *HindIII-SmaI*. A 0.6 kb (*pal1Δ* promoter) was present in pITB841 as compared to 1.3 kb band (*cor6.6* promoter) in pITB641.

Plasmid pITB241

The *GluB1*-promoter was amplified from rice genomic DNA (using primers Glu B1-5H and GluB1-3BSK). Digested the *gluB1*-promoter with *HindIII-KpnI* and cloned into *HindIII-KpnI* sites of pFF19 to create plasmid pFF19-*Glu* (Figure 11). The *BglII-BamHI* T7 RNAP from pAR3283 was cloned into *BamHI* site of pFF19-*Glu* to yield pFF19-*Glu*-T7. The *BamHI* site in pFF19-*Glu*-T7 was deleted by digestion and filling by Klenow enzyme and religated the blunt ends to yield pFF19-*Glu*-T7(B) (Figure 11).

In the next step, the *HindIII-NcoI* (blunt ended *NcoI* with Klenow) fragment containing “*GluB1* promoter-T7 RNAP-35S polyA” cassette from pFF19-*Glu*-T7(B) was cloned into *HindIII-SmaI* sites of pCAMBIA1300 to yield pA4-*Glu*-T7. The ferritin gene was amplified from pea genomic DNA using gene specific primers and

cloned into pET14b at *NcoI*-*BamHI* sites yielding plasmid pET14b-Fe (confirmed the correct cloning and expression of Ferritin gene in pET14b showed in Figure 23D) . The *HindIII*-*HindIII* “T7 promoter:Ferritin:T7 terminator” cassette was amplified from pET14b-Fe using pET14b5New and pET14b3New primers (see materials) and cloned into *HindIII* site of plasmid pA4-*Glu*-T7 to yield plasmid pITB241 that contained T7RNAP under rice seed specific promoter (*GluB1*) and Ferritin gene under T7 promoter (Figure 11).

The above cloning was confirmed by restriction digestion. The insertion of *GluB1* promoter into pFF19 was verified by the digestion with *HindIII*-*KpnI* (Figure 12B). A 1.3 kb band (*GluB1* promoter) was present in pFF19-Glu, while in pFF19 a 0.8 kb band (35S promoter and enhancer) were released indicating the cloning of “T7 promoter:Ferritin:T7 terminator” cassette.

For the insertion of T7 RNAP gene into pFF19-Glu was verified by the digestion pFF19-Glu and pFF19-Glu-T7 with *KpnI* (Figure 12C). While a 2.4 kb band was released from pFF19-Glu-T7, the pFF19-Glu was just linearized. Note that one *KpnI* site came from in pFF19-Glu and the other site came from T7 RNAP that released a ~2.4 kb fragment from pFF19-Glu-T7.

Deletion of *BamHI* site in pFF19-Glu-T7 to yield pFF19-Glu-T7(B)) was confirmed by *HindIII*-*BamHI* digestion (Figure 12D). While there were two bands (3.6 kb and 4.0 kb) in pFF19-Glu-T7, the pFF19-Glu-T7(B) was linearized.

Insertion of “*GluB1* promoter:T7 RNAP:35S polyA” cassette into pCAMBIA1300 was confirmed by the digestion with *HindIII*-*NcoI* (Figure 12E). Digested pCAMBIA1300 and pA4-*Glu*-T7 showed a 4.2 kb extra band due to the cloning of T7 RNAP cassette was observed in pA4-*Glu*-T7, while there was no extra band present in pCAMBIA1300. It should be noted here that the *NcoI* site destroyed by end filling is recreated when ligated at *SmaI* site.

Insertion of Ferritin gene into pET14b was confirmed by the digestion with *NcoI*-*BamHI* (Figure 12F). There was an extra 0.7 kb band in pET14b-Fe corresponding to the size of ferritin gene. The insertion of “T7 promoter:Ferritin:T7 terminator” cassette into pA4-Glu-T7 was confirmed by the *HindIII* digestion (Figure 12G). An expected 1.0 kb extra band was present in pITB241 when compared with pA4-Glu-T7. To check orientation of ferritin cassette in pITB241, the plasmid DNA was digested with *BglII*-*Sall* and pA4-Glu-T7 was digested with *HindIII*-*Sall* (Figure 12G). Both plasmids had a 4.0 kb band, and thus the Ferritin gene was cloned in clockwise direction with respect to T7 RNAP gene (Figure 11). Note that *BglII* site is just outside T7 promoter and *Sall* is at the end T7 RNAP.

Plasmid pITB342:

The *BamHI*-*BamHI* fragment containing “T7 promoter:*uidA*:T7-terminator” cassette was amplified from pET14b-GUS and cloned into *BglII* site of plasmid pITB241 to create plasmid pITB342 (Figure 11).

To confirm insertion of the cassette into pITB241, the plasmids pITB241 and pITB342 was digested with *HindIII* (Figure 12H). Presence of a 3.1 kb bands in pITB342 and 1.0 kb band in pITB241 are an indication of insertion of “T7-promoter:Ferritin:T7 terminator” cassette. Orientation of the GUS gene in pITB241 and pITB342 was confirmed by the digestion with *BglII*-*Sall* (Figure 12H). Presences of a 4.0 kb band in both indicate that the cassette was inserted in clockwise direction.

Plasmid pITB204:

The β -lycopene gene (Lcy) was PCR amplified from tomato genomic DNA using specific gene primers. The amplified DNA was digested with *NcoI*-*BamHI* and cloned into *NcoI*-*BamHI* sites of plasmid pET14b to create pET14b-Lcy. The “T7 promoter: β -Lcy:T7 terminator” cassette was amplified from pET14b-Lcy using

pET14b5New and pET14b3New primers (see materials) and cloned into *BglII*-*BamHI* sites of plasmid pITB241 (Figure 11) to create plasmid pITB204.

Plasmid pITB245:

The *PDS* promoter was amplified from tomato genomic DNA using specific primers and clone into pGEMT-Easy to create pGEMT-Easy-PDS. The 2.1kb *SmaI*-*BglII* *PDS* promoter from pGEMT-Easy-PDS was cloned into plasmid pITB241 replacing *GluB1* promoter at *BglII*-*SmaI* sites to create plasmid pITB245. In the pITB245, the T7 RNAP is under the control of the fruit specific *PDS* promoter and the ferritin gene was under T7 promoter (Figure 13).

Insertion of *PDS* promoter into pGEMT-Easy was confirmed by the digestion of pGEMT-Easy-PDS with *BglII* (Figure 14B). Release of an expected 2.1 kb band corresponding to *PDS* promoter is a conclusive evidence for the cloning of the promoter.

To confirm the replacement of *GluB1* promoter with *PDS* promoter in pITB241, the pITB245 was digested with different enzymes (Figure 14C). Digestions with *BglII*-*SmaI* showed the presence of a 2.1 kb *PDS* band. Similarly, digestions with *BglII*-*HindIII* or *BamHI*-*HindIII* gave 1.0 kb band; and with *BglII*-*Sall* gave 4.8 kb band. These results confirmed the cloning of the *PDS* promoter in pITB245.

Plasmid pITB245 series

The 2091bp *BamHI*-*BamHI* fragment that contained “T7-promoter:*uidA*:T7 terminator” cassette was cloned into *BglII* site of plasmid pITB245 to yield plasmid pITB245G1, pITB245G2 and pITB245G3. All these plasmid varied in terms of GUS gene orientation and copy number (Figure 13).

Digestion of pITB245, pITB245G1, pITB245G2 and pITB245G3 with *HindIII*-*BamHI* (Figure 14D) showed that one copy of GUS present in pITB245G1 and

pITB245G2 (in different orientations), two copies in pITB245G3 with both the copies present in the same orientation.

To detect orientation of cassette inserted into pITB245G. The pITB245, pITB245G1, pITB245G2 and pITB245G3 plasmids were digested with *BglIII-HindIII* (Figure 14E). It can be seen from figure 14E, a 3.0 kb band was released in pITB245G1 indicating that the cassette was in clockwise direction. A 1.0 kb band was released in pITB245G2 indicating that the cassette was inserted in anticlockwise direction. Two bands of 1.0 kb and 2.1 kb were released in pITB245G3 indicating the presence of two copies of GUS cassette in anticlockwise direction.

Plasmid pITb247:

The *BamHI* site in plasmid pET-HBsAg (gifted by Dr. M.K. Reddy) was destroyed by digestion with *BamHI*, end filling with Klenow enzyme and religation to yield plasmid pET3a-HBsAg(B). The 947bp *BamHI-BamHI* fragment containing “T7 promoter:HBsAg:T7 terminator” cassette was PCR amplified from pET3a-HBsAg(B) using pET14b5New and pET14b3New primers and cloned into the *BglIII* site of plasmid pITB245 to yield pITB247. In pITB247, the T7 RNA polymerase is under fruit specific *PDS* promoter and Ferritin and HBsAg genes are under T7 promoter (Figure 15).

Cloning of the HBsAg gene cassette into pITB245 was verified using PCR amplification and restriction digestion with *HindIII-BamHI* (Figure 16B). It can be seen from the figure that a 2.0 kb band was present in pITB247. In contrast, as expected, 1.0 kb band was present in pITB245.

To detect the orientation of cassette that inserted into pITB245, digested pITB245 and pITB247 with *BglIII-HindIII* (Figure 16C), in this figure showed both pITB245 and pITB247 had a 1.0 kb band was released. It means the cassette was in anticlockwise direction.

Plasmid pITB247 series:

The 2091bp *BamHI-BamHI* fragment containing “T7 promoter:*uidA*:T7 terminator” cassette was cloned into *BglII* of plasmid pITB247 to yield plasmid pITB247G1, pITB247G2 and pITB247G3. In these vectors all the three genes *uidA*, ferritin and HBsAg are under the control of T7 promoter (Figure 15).

Digestion pITB247, pITB247G1, pITB247G2 and pITB247G3 with *HindIII-BamHI* (Figure 16D) showed one copy of the cassette was inserted in pITB247G1, pITB247G2; and two copies were inserted in pITB247G3.

To detect orientation of the cassette inserted into pITB247, the pITB247, pITB247G1, pITB247G2 and pITB247G3 plasmids were digested with *BglII-HindIII* (Figure 16E). The figure shows a 3.0 kb band released from pITB247G1 indicating that the HBsAg cassette was in clockwise direction, a 1.0 kb band released from pITB247G2 indicating the insertion of the HBsAg cassette in anticlockwise direction. Presence of two bands of 1.0 kb and 2.1 kb in pITB247G3 indicate the presence of two copies of HBsAg cassette in clockwise direction.

Plasmid pITB345 and pITB345D:

The *BamHI-NcoI* fragment containing the “*PDS* promoter:T7 RNAP” cassette from pITB245 was subcloned into *BamHI-NcoI* sites of pCAMBIA1301 to yield pB4-T7-PDS(l). The *BamHI-BglII* fragment containing the “*PDS* promoter:T7 RNAP” cassette from pB4-T7-PDS(l) was cloned into *BamHI* site of plasmid pCAMBIA2300 to yield plasmid pA8-T7-PDS(l) and pA8-T7-PDS(r). The *BamHI-BglII* fragment contained “T7 promoter:Ferritin:T7 terminator” cassette from pGEMT-Easy-Fe(B) was cloned into *BamHI* site in plasmid pA8-T7-PDS(l) to create pITB345 and pITB345D plasmids (Figure 17).

Cloning of “*PDS* promoter:T7-RNAP” into pB4-T7-PDS was confirmed by digestion with *BamHI-NcoI* (Figure 18B), an expected 5.0 kb band released from

pB4-T7-PDS as opposed to a 0.8 kb band from pCAMBIA1301 indicate the cloning of “PDS promoter-T7 RNAP”.

To confirm the cloning of “*PDS* promoter:T7 RNAP:35S polyA” cassette pA8-T7-PDS(l) and pA8-T7-PDS(r) were digested with *NcoI* (Figure 18C). Results indicate that in pA8-T7-PDS(l) the “*PDS* promoter:T7 RNAP:35S polyA” was cloned in anticlockwise direction, and in pA8-T7-PDS(r) it was cloned in clockwise direction. Digestion of pA8-T7-PDS(l), pITB345 and pITB345D with *Sall* (Figure 18D) showed that one copy of the “T7 promoter:Ferritin:T7 terminator” cassette was inserted into pITB345 and two copies into pITB345D. To detect orientation of the Ferritin cassette in pITB345 and pITB345D, pA8-T7-PDS (l), pITB345 and pITB345D, plasmids were digested with *BamHI-Sall* (Figure 18E) It can be seen that Ferritin cassette got cloned in anticlockwise direction in both pITB345 and pITB345D plasmids.

Plasmid pITB347:

The *BamHI-BglIII* fragment containing “T7-promoter:*uidA*:T7 terminator and T7 promoter:HBsAg:T7 terminator” from pITB247G2 was cloned into *BamHI* site of plasmid pA8-T7-PDS(r) to create pITB347 (Figure 19).

Digestion of pITB347 and pA8-T7-PDS(r) with *BamHI-BglIII* (Figure 20C) showed that the cassette was in clockwise direction in pITB347. A ~1.0 kb (35S promoter) from pA8-T7-PDS(r) and ~4.0 kb (35S promoter and T7 promoter:*uidA*:T7 terminator and T7 promoter:HBsAg:T7 terminator) from pITB347 were released.

Plasmid pITB348

The *BamHI-BglIII* fragment containing “T7 promoter:*uidA*:T7 terminator and T7 promoter:HBsAg:T7 terminator” from pITB247G2 was also cloned into *BamHI* site of plasmid pA8-T7-PDS(l) to create plasmid pITB348 (Figure 19).

To confirm insertion and direction of the cassette in pITB348, plasmids pA8-T7-PDS(l) and pITB348 were digested with *Sall-BamHI* (Figure 20D). While a 4.8 kb band was released in pA8-T7-PDS(l), two bands (4.8 kb and 3.0kb) were released from pITB348 indicating that the cassette was in anticlockwise direction in pITB348.

3. PLASMIDS FOR CHLOROPLAST TRANSFORMATION

Plasmid pVSR327

The *BamHI-BamHI* fragment containing “T7 promoter:*uidA*:T7 terminator” cassette was subcloned into *BamHI* site of pCAMBIA1300 to create pCAMBIA1300-GUS. In the second step, the plasmid was digested with *Sall* and *KpnI* and fragment containing the “T7 promoter:*uidA*:T7 terminator” cassette was cloned into same sites in pVSR326mod (Leelavathi *et al.*, 2002) to yield pVSR327 (Figure 21).

To confirm the insertion of “T7 promoter:*uidA*:T7 terminator” cassette into pCAMBIA1300 as well as into pVSR326mod, plasmids pCAMBIA1300, pCAMBIA1300-GUS and pVSR327 were digested with *BamHI* (Figure 22B). Presence of a 2.1 kb band can be seen in both pCAMBIA1300-GUS and pVSR327. In addition, as expected, another band of 0.13 kb was also released from pVSR327.

Digestion of pVSR327 with *XbaI* showed a 3.2 kb band, confirming the cloning of the GUS cassette (Figure 22C). Note that pVSR326mod has one *XbaI* site and the other *XbaI* site came from pCAMBIA1300-GUS. This also confirmed that the “T7 promoter:*uidA*:T7 terminator” cassette inserted into pCAMBIA1300 in anticlockwise direction. As expected, *XbaI* digestion resulted in the linearization of the plasmids pCAMBIA1300-GUS and pVSR326mod. Further restriction analysis of pCAMBIA1300-GUS, pVSR326mod and pVSR327 with *HindIII-KpnI* showed a 2.1 kb band in pCAMBIA1300-GUS, 4.0 kb band in pVSR326mod and 3.3 kb band in pVSR327 (Figure 22D), confirming the correct cloning of the GUS cassette.

Plasmid pITB255

The *Sall-NcoI* (end filled) fragment from pBin-hyg-57-T7-2 containing T7 RNAP and 57 amino acid small subunit transit peptide (SSUTP) was cloned into *Sall-SmaI* sites of pITB245 to yield pITB255 (Figure 21).

To confirm insertion, pITB245 and pITB255 were digested with *BamHI* (Figure 22E). It can be seen that a 0.8 kb band of “T7 promoter:ferritin”, 2.1 kb band of PDS promoter (see plasmid pITB245) and 2.7 kb band of T7 RNAP (along with SSUTP) were released from pITB255. As expected, only 0.8 kb band corresponding to T7 RNAP was released from pITB245.