Analysis of *cis*-acting expression determinants of the tobacco *psbA* 5'UTR *in vivo*

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For my parents and Du Heng

Contents

Abbreviations

aadA	selection marker gene encoding aminoglycoside adenine transferase
APS	ammonium persulphate
ASD	anti-SD
Bp or bp	base pair
cPABP	chloroplast homologue of poly(A) binding protein
cPDI	chloroplast homologue of protein disulfide isomerase
cpRBP, cRBP	chloroplast RNA binding protein
cpRNP	chloroplast RNA binding ribonucleoprotein
CS1	chloroplast homologue of *E.coli* ribosomal protein S1
IR	inverted repeat
IR_A, IR_B	two inverted repeats of plastid genome
kb	kilo-bases
kd	kilo-Dalton
LSC	large single copy region of plastid genome
M	molar
MCS	multiple cloning site
min	minute
mM	millimolar
NEP	nucleus encoded RNA polymerase
Nt or nt	nucleotide
OD	optic density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylenglycol
PEP	plastid encoded RNA polymerase
PNP	polynucleotide phosphorylase
pre-RNA	precursor RNA
PSI	photosystem I
PSII	photosystem II
RBP	RNA binding protein
RBS	ribosome binding site
RNase	ribonuclease
RT	room temperature
SD	Shine-Dalgarno
sec	second
SL	stem-loop
SSC	small single copy region of plastid genome
TEMED	N,N,N,N-Tetramethylethylendienamin
uidA	reporter gene encoding beta-glucuronidase
UTR	untranslated region
WT, Wt, or wt	wild-type

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1. Introduction

1.1 Overview of chloroplast and chloroplast genome

Plant cells have three compartments containing genetic information: the nuclear-cytoplastomic system, and the semiautonomous prokaryotic-like organelles of mitochondria and plastids. There are several types of plastids including chloroplasts, chromoplasts, amyloplasts, elaioplasts, and etioplasts, which are differentiated from proplastids in the meristem or arise by their inter-conversion along tissue and developmental-specific pathways (Allison, 2000; Bogorad, 2000; Heifetz, 2000). Chloroplasts are responsible for photosynthesis, in which light energy is converted into ATP and redox potential (NADPH) by thylakoid protein complexes. These are available for various stromal reactions including fixation of carbon to manufacture sugars, assimilation of nitrogen, sulfur and phosphorus, biosyntheses of amino acids, lipids, starch, and pigments (Bogorad, 2000; Zerges, 2000).

The closest free-living ancestors of chloroplasts are endosymbiotic photosynthetic eubacteria, probably of the cyanobacteria lineage (Bhattacharya and Medlin, 1998). During evolution, most ancestral genes have been abolished or shifted to the nucleus, the remaining, approximately 120 genes, as found in a variety of complete chloroplast genome sequences, are mostly conserved and can be categorised into two broad groups. The first group comprises about 40 genes encoding components for the photosynthetic machinery – photosystem I (PSI), photosystem II (PSII), the cytochrome b6/f complex and ATP synthase. The second group includes the sequences required for the genetic system of plastids, with approximate 50 genes encoding subunits of RNA polymerase, rRNAs (4.5S, 5S, 16S, 23S), tRNAs, ribosomal protein and additional factors such as elongation factor EF-Tu. These gene products resemble those in eubacteria, indicating the similarities of translation machineries between chloroplast and its progenitor (Rochaix, 1997).

The circular chloroplast genomes differ among the species of algae and higher plants in size ranging from 50 to 290kb and number of copies in each chloroplast. In the unicellular alga *Chlamydomonas* there are 80 copies of the chloroplast genome, versus up to 10000 copies in the mesophyll cells of higher plants. Most chloroplast genomes examined contain two large inverted repeats (IR_A and IR_B) harbouring the gene clusters of ribosomal RNA, with two intervening single-copy regions (SSC and LSC) (Sugiura, 1992; Sugiura et al., 1998). Chloroplast genes are mostly arranged in operon-like transcription clusters and such organisation in the chloroplast genome is generally well conserved in land plants such as

tobacco and maize, whereas extensive gene rearrangements have been observed in algae (Rochaix, 1997).

Most studies on chloroplast gene expression have been pursued in model organisms such as tobacco for land plants (Kofer et al., 1998; Maliga et al., 1993) and *Chlamydomonas* for green algae (Harris, 2001; Rochaix, 1995), particularly taking advantage of the available chloroplast transformation systems in these two species.

1.2 Regulation of chloroplast gene expression

Chloroplast gene expression involves not only the activation of a set of plastid genes for the requirements of plastid biogenesis and photosynthesis, but also the modulations of gene expression during chloroplast development and in response to environmental factors e.g. light intensity. These processes must rely on the nucleus for most of their structural proteins and regulatory factors, albeit all basic components for gene expression exist in chloroplasts. Signaling pathways have also been evolved, demonstrating the interdependence and need for coordination of gene expression between these cellular genetic compartments.

Various aspects of chloroplast gene expression including the individual roles of transcription, mRNA processing, mRNA stability and mRNA translation in chloroplasts have been reviewed in detail (Barkan and Goldschmidt-Clermont, 2000; Gillham et al., 1994; Mayfield et al., 1995; Monde et al., 2000; Rochaix, 1996; Sugita and Sugiura, 1996; Zerges, 2000). This chapter attempts to condense the current understandings of the mechanisms controlling chloroplast gene expression, with an emphasis on the post-transcriptional level during plant development.

1.2.1 Chloroplast transcription and its regulatory role

Investigation of plastid mRNA abundance in a number of species has revealed that mRNA levels fluctuate in response to both developmental and environmental signals, which are generally argued to be determined by the transcription rate and mRNA stability. However, changes of transcription rates appear to affect the RNA levels of overall chloroplast genes rather than specific individual genes.

Transcription of individual chloroplast genes is mainly directed by at least two distinct RNA polymerases PEP and NEP respectively or cooperatively. PEP resembles bacterial RNA polymerases and recognises prokaryotic-like chloroplast promoters typically containing the consensus –35 (TTGACA) and –10 (TATAAT) elements. The –35 region is shown to be

dispensable for promoter activity and unidentifiable in some chloroplast genes by both *in vitro* and *in vivo* tests (Gruissem and Zurawski, 1985; Klein et al., 1992). This implicates that chloroplast PEP promoters may require only the –10 consensus for basal transcriptional activity. In contrast, the NEP is related to certain bacteriophage polymerases (Lerbs-Mache, 1993), and the NEP promoters are found in a variety of chloroplast genes including those encoding rRNAs (e.g. rrn16), ribosomal proteins, and other 'housekeeping' genes involved in the basic metabolism of chloroplast, but are generally absent from photosynthesis-related genes (Hajdukiewicz et al., 1997; Kapoor et al., 1997). Remarkably, another distinct light-responsive promoter is found in the chloroplast gene cluster *psbD/C* of PSII. There, basal promoter elements are subject to differential transcriptional activation via their upstream light-regulated elements by distinct RNA polymerase species and additional nuclear-encoded factors (Allison and Maliga, 1995; Christopher et al., 1992). Further evidence suggests that the relative activities of the different RNA polymerases are influenced by both light and developmental signals (Allison, 2000). In general, it appears that genes for the chloroplast genetic system are transcribed by NEP polymerases during the early stage of chloroplast development, and the following transcription of those genes for photosynthesis-related components are contributed by PEP polymerases (Kapoor et al., 1997; Mullet, 1993).

Surprisingly, transcription rates of plastid genes vary largely in a single plastid. As a general rule, plastid genes transcribed at reduced rates in one specie are also expressed at low levels in other species. Transcriptional rates appear to be independent of gene position in the plastid genome and probably differentially affected by sequence elements downstream of the transcription start site, as shown by various analyses of chimeric constructs containing individual plastid promoters. The fluctuations of the transcription levels of plastid genes seem to be responsive to plastid development, plastid type, cell cycle, and environmental factors such as light-dark cycles, but the relative transcription rates of many individual plastid genes are constant compared to the dramatic change of the overall rates of plastid transcription. This indicates the regulation of transcription is not a limiting step in chloroplast gene expression, while posttranscriptional events including RNA processing (translatability), RNA stability, translation rate and protein turnover may play an important role in the differential expression of chloroplast genes (Mayfield et al., 1995). The impacts of differential transcription on chloroplast gene expression are reviewed by Stern et al. (1997). The role of a variety of nucleus-encoded sequence-specific DNA binding proteins and sigma-like factors in chloroplast transcriptional regulation have also been discussed (Allison, 2000).

1.2.2 RNA processing to control mRNA stability and translatability

Most chloroplast genes are co-transcribed in dicistronic or polycistronic forms irrespective of the types of RNA polymerase (Sugita and Sugiura, 1996). A primary gene transcript may have to undergo irreversible modifications such as RNA editing, RNA splicing, intercistronic endonucleolytic processing, or 5' and 3'-end maturation, to become translatable. These modifications are generally facilitated by numerous factors, mostly encoded by the nucleus.

1.2.2.1 RNA editing

RNA editing has been found in the chloroplasts of vascular plants as specific base changes generally from C to U. Most editing events have functional significance e.g. to create the consensus AUG start codon, making untranslatable transcripts translatable, or to subtly restore the codons for the conserved amino acids of some proteins (Bock, 2000). It is striking that RNA editing can occur very early in the pathway of RNA processing. It appears to be largely independent of RNA splicing and intercistronic cleavage of the polycistronic transcript precursors (Freyer et al., 1993), and to have no evident correlation with chloroplast translation in most cases (Zeltz et al., 1993).

The *cis*-acting determinants for RNA editing and/or their correlated trans-factors have been evaluated *in vivo* and *in vitro*. By the analyses via tobacco chloroplast transformation, the *cis*- element directing C to U editing of the plastid *psbL* mRNA is found to reside within a 22 nucleotide segment spanning the editing site (Chaudhuri and Maliga, 1996). RNA editing of chimeric *ndhB* mRNAs in tobacco chloroplast transformants differs in the size of the essential *cis*-elements or in their distance from the editing site, and the exact *cis*-acting elements that are required for editing site recognition have been defined by serial 5' and 3' deletions (Bock et al., 1996). The critical nucleotide positions for editing site recognition are further identified by scanning point mutagenesis (Bock et al., 1997). Furthermore, the *cis*-acting element required for tobacco *rpoB* editing at site II is thought to appreciably cover the sequence -20 to +6 surrounding the editing target site, in which a single nucleotide alteration at -20 surprisingly inhibits chloroplast RNA editing *in vivo* (Reed et al., 2001). Assays via an *in vitro* chloroplast RNA editing system developed recently also confirm the aforementioned *cis*-acting elements for the editing of tobacco chloroplast *psbL* and *ndhB* mRNAs (Hirose and Sugiura, 2001). Nevertheless, it must be noted that transcripts of typical dicot plant plastid genes undergo C→U RNA editing at approximately 30 locations, but there is no consensus sequence surrounding the editing sites.

Strong evidence for the involvement of nucleus-encoded *trans*-factors in the specific RNA editing of chloroplast mRNAs such as *psbL* and *rpoB* has been derived from the analysis of chloroplast transformants (Chaudhuri et al., 1995; Reed and Hanson, 1997; Reed et al., 2001). Using an elegant approach of interspecific protoplast fusion, the site-specific RNA editing deficient in tobacco *psbF* mutant can be restored by the extraplastidic *trans*-acting factor(s) originated from spinach nuclear background (Bock and Koop, 1997). Competition analysis by the system of *in vitro* chloroplast RNA editing points to the existence of site-specific *trans*-acting factors interacting with the corresponding upstream *cis*-elements, in which a 25kd chloroplast protein is found to be specifically associated with the *cis*-element involved in *psbL* mRNA editing. Furthermore, a common chloroplast RNA-binding protein, cp31, is also required for RNA editing at multiple sites, as an additional factor together with the site-specific proteins for editing recognition in chloroplasts (Hirose and Sugiura, 2001). However, no such *trans*-factors engaged in chloroplast RNA editing have so far been identified.

As RNA editing can secure the translatability of some chloroplast transcripts, differential editing activity seems to contribute directly to the multitude of chloroplast gene regulation mechanisms in response to environmental and developmental cues. In spinach *psbF-psbL*, RNA editing that creates the translatable *psbL* transcript is differentially and developmentally down-regulated in seeds and roots (Bock et al., 1993). Similarly in tobacco plastids, translatable *psbL* mRNA is generated by an editing event that converts an ACG codon to the initiator codon AUG, but this editing activity appears to be modulated by a *psbL*-specific *trans*-factor and therefore controls the translatability of *psbL* mRNA (Chaudhuri et al., 1995). Using the *in vitro* chloroplast translation system, the translation of tobacco *ndhD* mRNA begins only at the edited AUG that is created from the in-frame ACG located 25nt downstream of the canonical AUG codon, and the extent of ACG to AUG editing is partial and depends on developmental and environmental conditions (Hirose and Sugiura, 1997).

1.2.2.2 RNA splicing

A number of chloroplast genes in land plants and algae are interrupted by introns. e.g., a total of 20 *cis* introns in 18 genes of the tobacco chloroplast genome (Sugiura, 1992). These introns can be determined as either group I or group II introns by their conserved structural features. Group I introns are typically found in bacteria, also in rRNAs of both chloroplasts and nucleus, in the *psbA* gene of *C. reinhardtii* and in the *trnL*(UUA) gene in higher plant chloroplasts (Barkan and Goldschmidt-Clermont, 2000; Rochaix, 1996; Sugita and Sugiura,

1996). Group II introns are found almost exclusively in organelles. Chloroplast group II introns resemble nuclear pre-mRNA introns in terms of secondary structure, splicing mechanism and some specific base pairing interactions (Mayfield et al., 1995), and can be subclassified into group IIA and group IIB on the basis of differences in intron fine structure (Barkan and Goldschmidt-Clermont, 2000).

Efficient self-splicing of primary transcripts from chloroplast genes (*psbA* and *rrn23*) in *Chlamydomonas* containing group I introns has been demonstrated by an *in vitro* system, but only under non-physiological conditions (Rochaix, 1996). Failure to splice the group I intron of *23S rRNA* in a nuclear mutant of *Chlamydomonas, ac20*, implies the probable participation of nuclear-encoded factors in this splicing process (Holloway and Herrin, 1998). However, no other nuclear mutations influencing group I intron splicing have been reported for either *Chlamydomonas* or land plants. Among the large number of PSII mutants in *Chlamydomonas,* none are defective in *psbA* splicing. This suggests that nuclear gene products may play only a limited role in the splicing of group I introns for chloroplast gene expression (Barkan and Goldschmidt-Clermont, 2000).

The splicing and maturation of pre-RNAs of chloroplast genes harbouring group II introns probably occur by a *trans*-splicing process, as with nuclear pre-mRNAs, without any *in vivo* evidences by the self-splicing pathway (Sugita and Sugiura, 1996). The most striking feature of chloroplast group II-intron splicing is found in *psaA* in *Chlamydomonas* (Herrin et al., 1998) and *rps12* (Huebschmann et al., 1996), *rpl2* (Hess et al., 1994), *atpF* in land plants (Deshpande et al., 1995; Jenkins et al., 1997).

The mature *psaA* mRNA of *C. reinhardtii* is assembled from three separate exons by two steps of *trans*-splicing. The chloroplast encoded *tscA* RNA (430bp) is required for the *trans*-splicing of intron 1, which is composed of at least three separate RNA components. Several regions in *tscA* gene with high sequence conservation are suggested to have an important functional role probably by interacting with specific *trans*-splicing factors (Herrin et al., 1998). To date, more than 14 nuclear genes necessary for *psaA trans*-splicing have been identified genetically (Goldschmidt-Clermont et al., 1990; Rivier et al., 2001), in which at least three allelic mutations block the splicing of *psaA* intron 1 by preventing the processing of *tscA* RNA from its co-transcribed precursor *tscA-chlN* (Hahn et al., 1998; Perron et al., 1999; Rivier et al., 2001). Thus, the RNA maturation may be contributed by the co-ordinated events of both intercistronic RNA processing and RNA splicing in some cases (Hahn et al., 1998).

Genetic analysis in maize has revealed that two nuclear genes *crs*1 and *crs*2 (chloroplast RNA splicing) are required in the splicing of chloroplast group II introns (Jenkins et al., 1997). Mutations in *crs*1 lead to a specific defect in the splicing of the *atpF* intron, whereas mutations in *crs*2 disrupt the splicing of many group II introns which belong to group IIB (Jenkins et al., 1997; Vogel et al., 1999). The CRS2 protein is primarily localised in the chloroplast stroma in a complex comprising an RNA component (Jenkins and Barkan, 2001).

On the other hand, it has been shown that nuclear mutants of barley and maize lacking plastid ribosomes fail to splice such chloroplast introns of *atpF* (Jenkins et al., 1997), *rps12* (Huebschmann et al., 1996), *rpl2* (Hess et al., 1994) that fall in the group IIA, suggesting that this type of splicing process in land plants may require a factor(s) encoded by the chloroplast genome as either a protein or a small RNA transcribed specially by chloroplast-encoded RNA polymerase. This postulation is supported by the observation that an intron-encoded protein i.e. *MatK* is involved in RNA splicing of *trnK* and *rpl2* genes (Hess et al., 1994; Liere and Link, 1995), resembling a maturase that facilitates group II intron splicing in fungal mitochondria (Neuhaus and Link, 1987). Additionally, the small plastid RNA (spRNA, 218bp) encoded by tobacco plastomic *sprA* gene is probably relevant to *16S rRNA* maturation during chloroplast ribosome biogenesis (Vera and Sugiura, 1994).

It is noteworthy that the splicing of chloroplast group IIA and group IIB introns in land plants is facilitated by different factors, apparently in accordance with their distinct intron structural basis. The splicing of chloroplast introns may be species-specific or differs between algae and higher plants in distinct mechanisms. The spinach *atpF* group II intron is not spliced in transgenic *Chlamydomonas* chloroplasts, albeit the *atpF* precursor RNA is present (Deshpande et al., 1995). Furthermore, evidence indicates light regulation of group I intron splicing in *Chlamydomonas* chloroplasts (Deshpande et al., 1997) and developmental regulation of group II intron splicing in land plant chloroplasts (Barkan, 1989), which implies that RNA splicing of pre-RNAs is complex and may play a significant role in chloroplast gene expression.

1.2.2.3 Intercistronic endonucleolytic cleavage

Some discrete chloroplast transcripts may arise from primary (di-) polycistronic transcripts through an intercistronic RNA processing by endo- and/or exonuclease, to be competent for translation. In tobacco, the importance of intercistronic processing for the dicistronic *psaC-ndhD* transcript is clearly shown by an *in vitro* translation system (Hirose and Sugiura, 1997). Therein, neither *psaC* nor *ndhD* can be directly translated *in vitro* from

the dicistronic primary transcript. It has been revealed that one *cis*-element in the coding region of *psaC* mRNA is complementary to a sequence within the *ndhD* 5' UTR to form a structural barrier that inhibits translation. Thus, endonucleolytic cleavage is required to render the mRNA of both *psaC* and *ndhD* translatable (Hirose and Sugiura, 1997). In maize, monocistronic *petD* mRNA is translatable and requires a specific nuclear factor *crp1*. Mutation of *crp1* that blocks the intercistronic processing results in the deficiency of monocistronic *petD* mRNA from its polycistronic precursor and a large decrease in *petD* translation. According to structural predictions, the *petD* start codon is sequestered in a stable hairpin of its precursor but remains unpaired in the monocistronic transcript. The *crp1* function is thought to abolish the structural blockage of the translation initiation region of *petD* via RNA intercistronic cleavage and therefore affect the translatability of *petD* (Barkan et al., 1994).

Ribosomal RNAs are also co-transcribed as the polycistronic precursors that have to be processed for ribosome assembly. The maize nuclear loci *cpsl* and *hcf7* may individually function to affect the maturation of the *23S* and *16S rRNA*s (Barkan, 1993). Furthermore, in the *Chlamydomonas* mutant *ac20* which lacks ribosomes for chloroplast translation, the level of unspliced and unprocessed *23S rRNA* that can not assemble into ribosomes is unusually elevated, which is primarily caused by the defect in RNA processing, specifically by failure to process the first internal transcribed spacer (Holloway and Herrin, 1998). These data underscore the importance of rRNA processing to control the availability of functional translation machinery.

As intercistronic processing dissects single transcripts from the polycistronic precursors, this process may intertwine with their 5' or 3' end maturation upon the untranslated regions as addressed below.

1.2.2.4 5' end maturation

Maturation of chloroplast transcripts may require the 5' end processing by ribonucleolytic cleavage. Both in spinach and *Chlamydomonas*, *psbB* mRNA has two 5' ends, one corresponding to the transcription initiation site and the other to a processing site (Vaistij et al., 2000; Westhoff and Herrmann, 1988). This pattern is generally found for many chloroplast transcripts in *Chlamydomonas*, and all accumulating mRNAs appear to be the result of processing. For instance, evidence in the 5' end maturation of *Chlamydomonas petD* mRNA has implicated a combinative involvement of endonucleolytic cleavage and probable 5' to 3' exonuclease trimming (Drager et al., 1998). This is supported by similar studies on

psbB (Vaistij et al., 2000) and *psbD* (Nickelsen et al., 1999) transcripts. In case of the existence of two 5' ends for the same transcript in *Chlamydomonas* such as *psbD* (Nickelsen et al., 1999), *psbA* (Bruick and Mayfield, 1998), *psbB* (Vaistij et al., 2000), the mRNA form with shorter 5'UTR maturated by 5' end processing appears to be abundant and translatable. In addition, the ratio of the –93 (shorter) to –168 (longer) *rbcL* transcripts is found to regulate the translation efficiency in response to light changes (Shapira et al., 1997). In higher plants, the aforementioned tobacco *ndhD* and maize *petD* transcripts with fully processed 5'end are more competent for translation (Barkan et al., 1994; Hirose and Sugiura, 1997), and it is particularly remarkable that alternative processing of barley *rbcL* 5'UTR that results in inefficient *rbcL* translation is postulated to modulate the translatability of *rbcL* in response to exogenous methyl jasmonate (Reinbothe et al., 1993).

Genetic studies have shown nuclear encoded factors with or without endo/exo-ribonuclease activities may regulate the 5' end maturation and further RNA stability by direct or indirect interaction with the 5'UTR of chloroplast mRNA. In *Chlamydomonas*, the function of nuclear locus *Mbb1* is presumed to be necessary for the 5' UTR processing of *psbB* pre-mRNA and/or stabilisation of its mature transcript with shorter 5'UTR (Vaistij et al., 2000). Similarly, the abundant form with shorter 5'UTR of *psbD* mRNA in the WT fails to accumulate in the nuclear mutant *nac2*, which again underscores the importance of *nac2* in the 5'end processing and RNA stability of *psbD* mRNA (Nickelsen et al., 1999).

1.2.2.5 3' end maturation

Most chloroplast mRNAs contain inverted repeats (IRs) in their 3'UTRs that can fold into a stem-loop structure, which is the characteristic of their bacterial counterparts to promote mRNA stability and also function as *rho*-independent transcription terminators in *E.coli*. However, the chloroplast IR sequences do not effectuate transcription termination efficiently, but rather stabilise the upstream sequences and mediate correct 3'end formation (Rott et al., 1996; Stern and Gruissem, 1987; Stern et al., 1991). In general, 3' end maturation of chloroplast mRNA is thought to undergo a two step process. Endonucleolytic cleavage at a site (10~15nt) downstream of the 3' IR is followed by exonucleolytic trimming up to the stem of the 3' IR (Hayes et al., 1996; Stern and Kindle, 1993), which would also apply to those mRNAs with 3'ends generated by intercistronic processing of polycistronic precursors.

Additionally, a number of proteins mostly encoded by the nucleus have been identified to be involved in RNA 3' end processing. For example, a complex of proteins CSP55, CSP41 and CSP29 can specifically bind to the stem-loop region (IR) and a downstream AU-rich

element termed Box II of spinach *petD* pre-mRNA. The CSP41 protein has been identified as a nuclear-encoded endoribonulcease (Chen et al., 1995; Yang et al., 1996). Similarly, a 54kd protein (p54) from mustard chloroplasts, potentially binding to the 3' UTR of *rps16*, *trnK* and *trnH* mRNAs, also has endonuclease activity that can be modulated by phosphorylation and redox state (Liere and Link, 1997; Nickelsen and Link, 1993). More interestingly, a 67kd putative bacterial RNase E homologue can associate with 100kd PNP, a 33kd RNA binding protein and other several unknown proteins to form a 550kd high molecular weight (HMW) complex that is essential for 3'end processing (Hayes et al., 1996).

In the nucleus, mRNA must be processed by addition of a poly(A) tail at the 3'end to become translatable. Accordingly, 3'end maturation of chloroplast mRNA may have a possible involvement in translation, despite a dissimilarity from its nuclear counterpart in that chloroplast or bacterial mRNA with 3' poly(A) tail is susceptible to degradation (Rott et al., 1998). The evidence for this is derived from a number of experiments. In *in vivo* deletion assays of *atpB* 3'IR in *Chlamydomonas* transformant strain Δ26, only the form of discrete *atpB* mRNA with normal 3' end maturation in paucity can be associated with polysomes and thus account for the synthesis of ATPase β-subunit, whereas the majority of *atpB* transcripts with heterogeneous 3' end is untranslatable. In addition, the chloroplast *rbcL* mRNAs extending beyond the mature 3'end are found not to be polysome-associated (Rott et al., 1998). By analysing tobacco chloroplast transformants containing the *uidA* reporter gene flanked by variants of the *petD* 3'UTR, it has been revealed that the sequence and secondary structure of the 3'UTR affect 3'end maturation, RNA accumulation and further translation (Monde et al., 2000). Taken together, these results suggest that 3'end maturation also appears to modulate the translatability of chloroplast mRNA.

1.2.3 RNA stability

1.2.3.1 5' mRNA stability determinants

In chloroplasts, mRNA stability determinants have been positioned within the 5' untranslated regions (UTR). Extensive studies have been focused in the model organism *Chlamydomonas* to unravel the role of the 5'UTR by a combination of genetic, biochemical and molecular techniques. To date, several *Chlamydomonas* nuclear mutants have been obtained with defects in mRNA stability for their corresponding chloroplast genes *psbB* (Vaistij et al., 2000), *psbD* (Nickelsen et al., 1999; Nickelsen et al., 1994) and *petD* (Drager et al., 1998; Drager et al., 1999). Further investigations by using the approach of chloroplast

transformation imply that those nuclear gene products may promote mRNA stability through the interaction with the 5' UTRs.

In *Chlamydomonas*, chloroplast *psbB/T/H* mRNAs fail to accumulate in the nuclear mutant *222E* with a silenced *Mbb1* gene (Monod et al., 1992). The *psbB* 5'UTR has been mapped as the stability determinant of *psbB* mRNA and the target site for *Mbb1* function by an intensive evaluation of chimeric mRNAs in the WT or nuclear mutant background. *Mbb1* function is likely to be required in the processing and /or stabilisation of the mature *psbB* mRNA with shorter 5'UTR (-35nt relative to the translation start codon). Insertion of a poly(G) tract between transcription initiation site (-147nt) and maturation site (-35nt) can restore the mRNA stability of *psbB* in *222E* stains, suggesting the involvement of a 5'→3' exoribonuclease in *psbB* mRNA degradation (Vaistij et al., 2000). Two putative stem-loop structures upstream of the maturation site (-35nt) within the longer *psbB* 5'UTR are proposed to play a limited role in *psbB* mRNA stability, since only their simultaneous deletion can lead to the absence of all forms of *psbB* transcript in the WT strain, implying that the 5'end maturation of *psbB* mRNA requires the target sites for *Mbb1* function. Additionally, the Mbb1 protein also appears to be necessary to process and/or stabilise the *psbH* RNA independently of the *psbB* transcript (Vaistij et al., 2000). The nuclear *Mbb1* gene has been cloned, and encodes a chloroplast stromal protein as a part of a 300kd RNA-associated complex, which is most likely involved in *psbB* mRNA processing, stability, and/or translation (Vaistij et al., 2000).

In *Chlamydomonas*, another photosynthetic nuclear mutant, *nac2*, particularly fails in accumulation of chloroplast *psbD* mRNA (Kuchka et al., 1989). Its 5'UTR has been found to contain the mRNA stability determinants as the principal target sites of specific nuclear factors (Nickelsen et al., 1994). In wild-type cells, *psbD* mRNA exists in two forms with different length of 5' untranslated regions (–74nt and -47nt). Only shorter form with high abundance, likely resulting from processing of the larger RNA, can be associated with polysomes and is competent for translation. By an intensive analysis of the *psbD* 5'UTR mutants, two major *cis*-elements conferring RNA stability have been identified, one within the first 12nt at the 5' end and the second near the position of –30nt. The deduced stem-loop structures within this 5'UTR do not appear to play a role in *psbD* mRNA stabilisation. Insertion of a poly(G) tract in the longer 5'UTR can accumulate *psbD* mRNA similar to the wild-type level in the *nac2* mutant, such mRNA is however not translatable, suggesting the poly(G) tract does not convey the full protection to the downstream region and therefore *nac2* function is required for 5'end maturation, stability and/or translation of *psbD* mRNA. A

region covering the second RNA stability determinant within the shorter 5'UTR is thought to be important for the recognition and binding of the nuclear encoded protein complex (Nickelsen et al., 1999). Similar to aforementioned *Mbb1*, the nuclear *nac2* gene is cloned. The gene product Nac2 is a part of a RNA-associated complex and localises in the chloroplast stroma (Boudreau et al., 2000).

The third well-characterised *Chlamydomonas* nuclear mutant is strain F16 harbouring a mutated *mcd1-1* that contributes to the instability of *petD* mRNA and results in deficiency of the cytochrome b6/f complex. Analysis of chimeric transcripts has revealed that *petD* mRNA stability determinants are located in its 5'UTR (Drager et al., 1998). Deletion analysis of the 5' region of *petD* indicates that 5'end processing is not only required for the formation of the translatable monocistronic *petD* transcript (Sakamoto et al., 1994), but is also correlated to transcript instability in the *mcd1-1* mutant (Drager et al., 1998). The mature 5'end is postulated as the target site for *mcd1-1* function, where one RNA stability determinant has also been identified as element I spanning a maximum of the first 8 nucleotides. Mutations of this element give rise to the reduction of *petD* mRNA to less than 5% of the WT amount. This element appears to form a small stem-loop structure that may interact with *mcd1* protein to prevent 5'→3' exoribonucleolytic degradation (Higgs et al., 1999). Insertion of a poly(G) tract into the *petD* 5' UTR can allow the accumulation of chimeric transcripts in the *mcd1-1* nuclear background, indicating that the 5' →3' exoribonucleolytic activity is a normal component of chloroplast mRNA decay pathways (Drager et al., 1998; Drager et al., 1999). Recently a nucleus-encoded suppressor, *mcd2*, defining a new factor which can promote *petD* mRNA stability has also been reported (Esposito et al., 2001).

In other cases, specific *cis*-elements conferring RNA stability of *rbcL* and *atpB* transcripts in *Chlamydomonas* chloroplasts are also embedded in their 5' untranslated regions. A 10nt sequence (5'-AUUUCCGGAC-3') of *rbcL* 5'UTR (from+38 to +47 relative to the transcripts' 5' terminus) and a 12nt sequence (5'-AUAAGCGUUAGU-3') of *atpB* 5'UTR (from +31 to +42) are responsible for transcript longevity and accumulation, respectively. These 5' UTR stabilising elements are predicted to be part of RNA secondary structures, but these structural features seem insufficient to mediate the longevity of the transcripts alone (Anthonisen et al., 2001). Meanwhile, data published by another group indicates that the loop sequence (from +14 to +27) of the putative stem-loop (from +1 to +41) in the *rbcL* 5'UTR serves as another essential element for the longevity of *rbcL* transcripts (Singh et al., 2001).

In higher plants, the determinants for chloroplast RNA stability have also been demonstrated to reside in the 5' untranslated regions, especially by *in vivo* analysis via

tobacco chloroplast transformation. In tobacco chloroplasts, the abundance of *rbcL* transcripts is considerably constant, independent of light, as shown by the finding that up to 10-fold lower transcription rate in the dark can be compensated by increased mRNA stability arising from a segment at the immediate 5'end of *rbcL* 5'UTR that can form a stem-loop structure (Shiina et al., 1998). A study from our laboratory has also revealed that the *rbcL* 5'UTR confers greater mRNA stability than the *psbA* 5'UTR on chimeric *uidA* transcripts, suggesting a strong RNA stabilising element inside of the *rbcL* 5' untranslated region (Eibl et al., 1999).

The 5' terminal location of RNA stability determinants appears to be the characteristic of organellar mRNAs. In *Chlamydomonas* chloroplasts, deletions or mutations near the 5'ends can cause drastic reductions in mRNA accumulation of *psbA* (Mayfield et al., 1994), *psbD* (Nickelsen et al., 1999), *petD* (Higgs et al., 1999), and *rbcL* (Anthonisen et al., 2001; Singh et al., 2001). In yeast mitochondria, a deletion of 81nt near the 5'end of the *COX3* mRNA (Wiesenberger et al., 1995) causes an 85% decrease in its steady-state abundance, and the *COB* transcript (Mittelmeier and Dieckmann, 1995) with the removal of the first 10nt is also unstable.

1.2.3.2 5' mRNA instability determinants

In contrast to most cases, some specific elements in the 5' untranslated regions act as negative chloroplast mRNA stability determinants, in response to environmental factors. For instance, the AU-box motif (UAAAUAAA) within the 5'UTR of cyanobacterial *psbA2* transcripts has recently been found to serve as an mRNA instability determinant in darkness and to confer the signal for the light-dependent pattern of expression. The WT *psbA2* mRNA is unstable in the dark. Base changes from AT- to CG- and deletion of the AU-box can markedly increase the level of *psbA2* transcripts in the dark, and point mutations of the AU-box also affect the light dependency of the expression pattern (Agrawal et al., 2001). Another example is that the stem sequence from +21 to +41 of the stem-loop (from +1 to +41, relative to the transcripts' 5' terminus) in the 5'UTR of the *Chlamydomonas rbcL* transcript is required for the photo-accelerated degradation of *rbcL* transcripts, which may be the target sensitive to endonuclease attacks upon illumination (Salvador et al., 1993; Singh et al., 2001).

1.2.3.3 3' mRNA stability determinants

Actually, the first chloroplast RNA stability determinant comes from the earlier studies on the function of the chloroplast 3' untranslated region. Most chloroplast 3'UTRs contain inverted repeats (IRs) that can potentially fold into stem-loop structures. Instead of an efficient terminator for chloroplast transcription, the IRs of 3'UTR would rather play a major

role in RNA stability and aforementioned 3' end maturation (Rott et al., 1996; Stern and Gruissem, 1987; Stern et al., 1991).

Deletions of the 3' stem-loop (IRs) can lead to a marked reduction in the accumulation of the *atpB* (Stern et al., 1991) and *psaB* mRNA (Lee et al., 1996) in *Chlamydomonas*, suggesting that the stem-loop structure within the 3'UTR is important for impeding processive 3'→ 5' exoribonuclease. This is supported by the fact that RNA stability can be maintained, when the *atpB* 3' stem-loop is replaced by a poly(G) stretch of 18 residues (Drager et al., 1996). The *pet*A 3'UTR does not contain apparent stem-loop structures, which can account for its susceptibility to decay *in vitro* (Rott et al., 1998). Similarly, in higher plants, the spinach *petD* 3'IR has also been shown to be necessary for mRNA stability *in vitro* (Stern et al., 1989). *In vivo* analyses of chimeric *uidA* mRNA by tobacco chloroplast transformation have also indicated that the absence of a stable secondary structure (*petD* 3'IR) results in low abundance of discrete *uidA* mRNA (Monde et al., 2000), and exchanges of 3'UTRs have a moderate effect on *uidA* mRNA accumulation (Eibl et al., 1999).

On the other hand, some 3'IRs appear to have an orientation-dependent function. Synthetic RNAs of antisense *atpB* 3' UTR as well as *rbcL* 3'UTR are unstable and can not be processed to stable products *in vitro*, in contrast to those 3'UTRs in the sense orientation. *In vivo* analysis has also demonstrated that the level of discrete *atpB* transcripts in *Chlamydomonas* transformants containing heterogeneous 3'UTRs in the antisense orientation is reduced to approximately one-half of that of the sense transformants, although the accumulation of total *atpB* transcripts is almost equivalent in both cases (Rott et al., 1998). A similar phenomenon has also been observed for the *rbcL* and *psaB* 3'UTRs (Blowers et al., 1993).

Taken together, these results suggest that the secondary structure of chloroplast 3'UTRs alone may be not sufficient, and rather the correct 3'UTR sequence is required for mRNA accumulation and stability. It is proposed that the antisense 3'UTRs are less efficient at forming 3'ends or inactivate the target sites for RNA binding proteins to prevent the ribonucleases attack, although they contain the inverted repeats that can form stem-loop structures (Rott et al., 1998). Some evidence suggests the participation of 3' UTR binding proteins in regulating RNA stability. For example, 37kd and 38kd proteins have been characterised to bind specifically to the barley *psbA* 3' UTR, in a 30 nucleotide region immediately downstream of the translation termination codon and upstream of the IR. It is possible that these proteins play a regulatory role in mRNA stability during chloroplast development (Memon et al., 1996). Another hint is derived from the *Chlamydomonas crp3*

mutant, which suppresses the conditional non-photosynthetic phenotype of an *atpB* 3'IR deletion mutant. Characterisation of *crp3* demonstrates that the nuclear encoded CRP protein appears to participate both in RNA stabilisation and processing (Levy et al., 1999).

However, the 3' secondary structure (IRs) are not always required for chloroplast mRNA stabilisation. Chimeric transcripts lacking the 3'IR of *psaB* or *rbcL* mRNA are heterogeneous in size, but accumulate to normal levels in *Chlamydomonas*. Therefore, the 3' inverted repeats seem to be preferentially important for correct 3' end formation (Blowers et al., 1993). Moreover, the 3' IR does not appear to be an absolute sequence requirement to mediate RNA stability, as shown by an observation that substitution of the *atpB* 3'IR with a stable secondary structure such as the spinach *petD* 3'IR or a poly(G) tract can give rise to appreciable accumulation of discretely-sized *atpB* mRNA and normal gene expression (Drager et al., 1996; Rott et al., 1998; Stern et al., 1991). The sequence of the 3' inverted repeat of the chloroplast *rbcL* gene is also not conserved in non-flowering land plants and algae (Calie and Manhart, 1994). Additionally, compared with the regulatory importance of 5'UTR mRNA stability determinants, whose specific nuclear mutant results in a complete absence of transcript accumulation (Drager et al., 1998; Monod et al., 1992; Nickelsen et al., 1999; Nickelsen et al., 1994; Vaistij et al., 2000), the 3' UTR does not appear to play a major role in mRNA stabilisation.

1.2.3.4 mRNA stability/instability determinants in the coding region

Interestingly, an RNA stability determinant of the *psbA* transcript has also been found within its open reading frame, corresponding to the coding region for the first membrane span of the D1 protein in *Synechococcus sp.* strain PCC 7942 (Kulkarni and Golden, 1997). This stabilising element is thought to be a site for ribosome pausing, and the accumulation of ribosomes on the transcript upstream of the pause site increases transcript stability. In *Chlamydomonas*, the 5'UTR of chloroplast *rbcL* mRNA renders chimeric mRNA susceptible to rapid degradation upon illumination, but this degradation is eliminated by the addition of a segment of the *rbcL* coding region (Salvador et al., 1993). Recently, such an RNA stability determinant inside this coding segment has been finely mapped as a putative loop sequence from +329 to +334, relative to the transcripts' 5' terminus. Mutations in this loop abrogate the stabilising effect, co-operatively with another RNA stability determinant (from +14 to +27) located in the *rbcL* 5'UTR. It is postulated that the photo-accelerated mRNA degradation by endonuclease attacks on the RNA instability determinant (from +21 to +41) mentioned above is alleviated, due to the physical blockage or distortion of the target sequence by interacting

proteins that bridge the loops of both RNA stability determinants (Singh et al., 2001). This also reflects that the direct or indirect co-operation between the sequences of 5' leader and coding region may affect chloroplast mRNA stability.

By *in vitro* chloroplast mRNA decay assays, it has been revealed that spinach *psbA* mRNA degradation is initiated by endonucleolytic cleavages within the coding region, suggesting that the small structural sequences surrounding the cleavage sites are the putative mRNA instability elements (Klaff, 1995). Additionally, it is also observed that the cleavage sites can be masked by free magnesium ions leading to mRNA stabilisation (Horlitz and Klaff, 2000).

1.2.3.5 mRNA stability regulation

1.2.3.5.1 Developmental and environmental signals

Chloroplast mRNAs accumulate to different steady-state levels during plant development or in response to environmental signals. The fluctuations in transcription rates alone appear not to account for the changes in mRNA amount, deducing a concomitant contribution by mRNA stability regulation (Deng and Gruissem, 1987). Indeed, this deduction has already been substantiated by monitoring the mRNA decays *in vivo* with transcription inhibitors such as tagetitoxin (Kim et al., 1993) or actinomycin D (Klaff and Gruissem, 1991). In spinach, the two mRNAs of *psbA* and *rbcL* are examined in intact leaves after treatment with actinomycin D. Both mRNAs in young leaves decay with similar kinetics, but the relative half-life of *psbA* mRNA increases more than two-fold (~10hours) while that of *rbcL* mRNA is comparatively constant (~5hours) in mature leaves. No detectable decay is observed in the untreated control leaves. Thus, differential mRNA stability in higher plant plastids can lead to the differences in mRNA accumulation during leaf development (Klaff and Gruissem, 1991). Direct evidence has also been found for selective modulation of chloroplast RNA stability in barley shoots treated with tagetitoxin during leaf development or adjustment to illumination. The mRNAs examined differ in the estimated half-life, ranging from 6 hours for *psaA* mRNA to over 40 hours for *psbA* mRNA. Chloroplast RNA stability is regulated differentially during development in the dark, e.g. no changes in the half-life for *atpB* mRNA, two-fold decrease for *psaA* mRNA stability, but increased RNA stability for *rpoA*, *psbA* and *rbcL* transcripts and *16S rRNA*. Differential regulation of RNA stability is also found in light adaptation, in which *16S rRNA* stability can continue to increase, but the stability of *rbcL* mRNA is decreased and that of the *rpoA* and *psbA* mRNA is light-independent (Kim et al., 1993).

In *Chlamydomonas*, the RNA half-lives of examined genes vary remarkably, but are generally higher in the dark during a light/dark growth regime. For instance, the *rbcL* mRNA stability increases 6-fold in the dark relative to that in the light, and a light-accelerated RNA degradation is also observed. The enhanced RNA stabilisation during the dark phase of the cell cycle appears to compensate the concomitant lower transcriptional rate and thereby keep the steady-state mRNA levels relatively constant (Salvador et al., 1993). This is also reminiscent of *rbcL* mRNA stability regulation in tobacco (Shiina et al., 1998) and barley (Kim et al., 1993).

1.2.3.5.2 Trans-regulatory factors

In higher plants and *Chlamydomonas*, chloroplast RNA stability can be affected by magnesium ions (Klaff, 1995; Nickelsen et al., 1994). In an *in vitro* chloroplast degradation system, spinach RNAs such as *psbA* and *rbcL* mRNA, *16S rRNA*, and tRNA(His) obtain the stability at specific magnesium concentrations and each exhibits a typical magnesium concentration-dependent stabilisation profile. It is also observed that the free Mg^{2+} concentration rises during chloroplast development within a range sufficient to mediate gene-specific mRNA stabilisation *in vivo* as well as *in vitro*. More intensive investigations indicate that the magnesium ions may sequester the endonucleolytic cleavage sites of mRNA and influence the protein binding to the mRNA untranslated region. Thus, the magnesium ions are suggested as a non-proteinaceous *trans*-acting factor mediating differential mRNA stability (Horlitz and Klaff, 2000).

In addition to the involvement in aforementioned posttranscriptional RNA processing, some chloroplast RNA-binding ribonucleoproteins (cpRNPs) have been characterised as *trans*-stabilising factors of ribosome-free mRNAs. These cpRNPs are abundant in the chloroplast stroma (Nakamura et al., 1999). The majority of ribosome-free stromal *psbA* mRNAs are found in association with such cpRNPs. *In vitro* mRNA degradation assays indicate that exogenous *psbA* mRNA is more rapidly degraded in cpRNP-deficient stromal extracts but has similar decay kinetics in cpRNP-reconstituted extracts, compared with that in standard extracts (Nakamura et al., 2001). A model has been proposed for the possible role of cpRNPs in regulating mRNA stability. The cpRNPs associate with nascent RNAs or pre-RNAs immediately after transcription, and form RNA-protein complexes in the stroma which confer the RNA stability by reducing the accessibility of ribonucleases to RNAs. These complexes also serve as a scaffold for the specific catalytic machineries involved in RNA editing, splicing and maturation. When the cpRNPs dissociate from fully processed and mature mRNAs, ribosomes then attach to the mRNAs for translation. Additionally, the

association of cpRNPs with RNAs and their disassociation from RNAs may be regulated by phosphorylation and redox potential (Danon and Mayfield, 1994a; Kim and Mayfield, 1997; Liere and Link, 1997; Lisitsky and Schuster, 1995) in response to light stimuli, further suggesting that the regulation processes of mRNA stability and translation are mutually linked. A well characterised cpRNP is spinach 28kd RNA binding protein (28RNP), which appears to be a fate dominator of chloroplast mRNA after transcription and certainly an important regulator of mRNA stability. In the absence of 28RNP, precursor mRNA is left unprocessed and then degraded, while in the presence of 28RNP, the precursor mRNA is correctly processed and forms long-lived stable mRNA for translation. The RNA binding activity of this protein is also affected by light-dependent phosphorylation (Kanekatsu et al., 1995; Lisitsky et al., 1994; Lisitsky and Schuster, 1995; Schuster and Gruissem, 1991). These observations imply that the mechanisms of chloroplast gene expression have evolved to an intricate and subtle in response system to different environmental and developmental signals.

1.2.3.5.3 Ribosome association

In higher plants, some evidence also suggests that the mRNA-ribosome association affects the mRNA decay in chloroplasts. Using organelle-specific translation inhibitors such as lincomycin that affects the polysome distribution on mRNAs in an *in vivo* spinach mRNA decay assay, the results indicate that *rbcL* and *psbA* mRNAs are less stable when bound to polysomes relative to the polysome-depleted mRNAs (Klaff and Gruissem, 1991). In contrast, from the studies of maize nuclear mutants in which many chloroplast mRNAs are associated with aberrantly few ribosomes and the *rbcL* mRNA level is reduced 4-fold, the *rbcL* mRNA destabilisation is thought to be a consequence of its decreased polysome association (Barkan, 1993). Similarly, the stability of *Chlamydomonas psbA* mRNA is in parallel with ribosome association (Yohn et al., 1996; Bruick and Mayfield, 1998). An explanation for the correlation of ribosome association and mRNA stability is that the secondary structure in the 5'UTR can be affected by mRNA-ribosome association and thereby change the accessibility of internal cleavage sites to endonucleolytic ribonucleases.

1.2.3.6 Chloroplast mRNA degradation and its regulation

As described above, each chloroplast RNA has limited longevity in the range from several hours to more than 40 hours, and thereby must eventually undergo a degradation process. To date, the degradation mechanisms have been unravelled to a great extent as reviewed by Hayes et al. (1999) and Schuster et al. (1999). In brief, it is generally agreed that the chloroplast mRNA may be cleaved internally at one or more sites by endoribonucleases,

then the resultant cleavage products are polyadenylated at the 3'end followed by 3'→5' exoribonuclease degradation or occasionally directed to the decay pathway by a 5'→3' exoribonuclease activity.

As chloroplast mRNA degradation is presumably initiated by endonucleolytic cleavages, the accessibility of cleavage sites and the status of endonuclease such as amount, specificity determine the mRNA half-life. In contrast, the activities of the poly(A) polymerase for polyadenylation and exonuclease would not be rate-limiting. In this regard, some factors such as magnesium ions, cpRNPs, and/or ribosome association have already been suggested to regulate the accessibility of cleavage sites as mentioned above. On the other hand, a step following endonucleolytic cleavage could be rate-limiting. For example, the length of the poly(A) tail and the percentage of guanosine (G) residues in the poly(A) tail have been examined to influence the degradation rate *in vitro* (Monde et al., 2000).

1.2.4 Translation of chloroplast mRNAs

Translation is a key step in the control of expression of chloroplast genes. Translational regulation can provide the capacity of rapid adjustment to induce or reduce massive levels of protein synthesis in response to developmental and environmental signals. As predicted by the endosymbiotic origin of the chloroplasts, the chloroplast translational machinery features many prokaryotic characteristics such as 70S ribosomes, initial fMet-tRNA, mRNA lacking 5'-cap and 3'-poly(A) tail. However, due to the semi-autonomous genetic attribute of chloroplast, the translation mechanisms in chloroplasts considerably differ from those in prokaryotes.

After transcription and variant RNA processing, the mature chloroplast mRNA can be associated with the 30S ribosome subunit (with bound initiation factors, initiator tRNA and GTP) at the 5' leader to form the 30S initiation complex towards the initiation codon. Subsequent release of initiation factors, GTP hydrolysis, and association with the 50S ribosome subunit results in the formation of the 70S ternary ribosome complex, which is able to start the elongation phase. The polypeptide is synthesised in this period. When the ribosome reaches the termination codons by the recognition of release factors, translation is completed, the ribosome subunits are dissociated from each other and recycled. To date, more studies are focused on the step of translation initiation than on the elongation and termination phases, as the translation initiation is commonly thought to be rate-limiting in translational regulation. Therefore, the elements involved in translation initiation are particularly characterised.

1.2.4.1 *Cis*-acting translation determinants

1.2.4.1.1 Shine-Dalgarno sequence (SD)

In prokaryotes, translation initiation includes two RNA-RNA interactions that stabilise the 30S pre-initiation complex in the proper reading frame. One is the association of the small ribosome subunit with mRNA via base-paring between the 'Shine-Dalgarno'(SD) sequence located 10~30nt upstream of the translation initiation codon and a complementary anti-SD (ASD) sequence near the 3' end of the 16S ribosomal RNA in the small subunit; the other one is the codon-anticodon interaction between initiator fMet-tRNA and the initiation codon (usually AUG) (McCarthy and Brimacombe, 1994). In chloroplasts, anti-SD sequences are conserved among the 3'end of the highly conserved *16S rRNA*s from chloroplast ribosomes of higher plants, green algae, and cyanobacteria. However, the complementary SD sequences are deficient in the 5' leaders of certain chloroplast and cyanobacterial genes, and when present, are hyper-variable in position and nucleotide composition, suggesting that the SD interaction with *16S rRNA* is not an absolute requirement for translation initiation (Fargo et al., 1998; Harris et al., 1994).

By the approach of chloroplast transformation, the function of the SD sequence in the 5'UTRs of chloroplast mRNAs has been evaluated *in vivo*. In tobacco, introduction of a canonical SD sequence (GGAGG) in a short synthetic segment as the 5' leader of *uidA* and *aadA* genes which are driven by the strong constitutive *16S rRNA* promoter (Prrn) can give rise to the efficient translation of *uidA* and *aadA* mRNAs in chloroplast transformants (Eibl et al., 1999; Koop et al., 1996). In *Chlamydomonas* transformants, deletion of the SD sequence (GGAG) or sequestration of the SD by base paring with a mutation (CUCC) within the putative stem-loop in the *psbA* mRNA 5'UTR leads to dramatic reductions in *psbA* translation and RNA stability. However, a mutant leader containing a 32nt deletion immediately upstream of the SD, which eliminates the entire stem-loop region, can maintain the translation of *psbA* mRNA at a high rate (Mayfield et al., 1994). These data indicate that the SD sequence of the *psbA* 5'UTR is necessary for *psbA* translation and RNA stability. Replacement mutagenesis of the SD-like sequence in the *psbD* 5'UTR from GGAG to CCUC also results in reduced synthesis of the polypeptide to 25% of the wild-type level (Nickelsen et al., 1999). In contrast, site-directed substitution mutations of the SD-like sequences of five chloroplast mRNAs, *petD* (Sakamoto et al., 1994), *atpB, atpE, rps4* and *rps7* (Fargo et al., 1998), has little or no influence on their translation *in vivo*.

The roles of SD-like sequences in the 5' UTRs of chloroplast mRNAs in *Euglena* and barley have been addressed by *in vitro* 'toe-printing' assays. Mutations of the SD sequence of

the *Euglena* chloroplast *atpH* mRNA lead to 2~5-fold reductions in the efficiency of initiation complex formation (Betts and Spremulli, 1994). In barley, the SD-like sequence (GGAG) of the *psbA* mRNA has been mapped on its 5' UTR, unusually 33nt upstream of the bona fide initiation AUG codon. Meanwhile, a model is proposed that the chloroplast small ribosome subunit first binds to the SD-like sequence and its downstream internal non-initiator AUG within the 5' UTR, then scans in a 3' direction to the initiator AUG where it completes the initiation phase and starts the translation. The *psbA* mRNA 5'UTR is highly conserved in the region –1 to –50 relative to the initiation codon (+1), the SD-like sequence (-33) is therefore conserved in many higher plant species including tobacco (Kim and Mullet, 1994).

Concerning the tobacco *psbA* 5'UTR, an *in vitro* chloroplast translation system has been established to characterise the *cis*-elements relevant for translation. Surprisingly, mutation of the canonical SD-like element (RBS3) of the *psbA* 5'UTR has only little effect with a 12% decrease of translation (Hirose and Sugiura, 1996). In contrast, in the same system, the SD-like sequence of the tobacco chloroplast *rps14* mRNA has been examined to be required for efficient translation (Hirose et al., 1998).

Thus, it can be concluded that some SD-like sequences are required for translation while others are not. The SD-like sequences may be preferentially important for translation of highly expressed mRNAs, such as those of *psbA* and *psbD* in *Chlamydomonas* (Nickelsen et al., 1999). The absolute absence of the SD-like sequences in a certain number of chloroplast mRNAs indicates that other *cis*-acting elements might recruit ribosomes to initiation codons.

1.2.4.1.2 Initiation codon

The initiation codon for translation of most plastid mRNAs is AUG, with GUG accounting for most or all of the exceptions (Sugiura et al., 1998). For some chloroplast mRNAs such as tobacco *psbL*, the initiation codon AUG is created by RNA editing from ACG (Kudla et al., 1992). The influences of mutations of AUG on translation appear to differ among different genes. For example, changes of the initiation codon (AUG) of *Chlamydomonas psbD* mRNA into AUA or AUU lead to a defect in D1 synthesis (Nickelsen et al., 1999). In contrast, changing the initiation codon of the *Chlamydomonas* chloroplast *petA* gene from AUG to AUU, ACG, ACC, ACU, or UUC have variable effects on synthesis of cytochrome *f* and photosynthetic competence, in which all but the UUC mutant accumulate detectable levels of cytochrome *f*. The results from further mutations by introducing an in-frame stop codon UAA immediately downstream or upstream or in place of the initiation AUG codon indicate that the mutant codon is virtually used as the initiation codon. Therefore, the *petA* AUG codon appears to be unnecessary to specify the site of translation initiation in

chloroplasts, while the strength of the initiation codon-anticodon interaction may determine the efficiency of translation initiation (Chen et al., 1995).

1.2.4.1.3 Other cis-acting elements in the 5'UTR

In addition to the SD-like sequences competent for chloroplast translation initiation, other *cis*-elements within the 5'UTR have also been found either to act as the functional alternatives of the SD-like sequences, or to indirectly facilitate the translation initiation by interacting with *trans*-acting factors.

In *Chlamydomonas*, three distinct sequence elements within the *petD* 5'UTR have been defined to be necessary for *petD* translation in preliminary experiments (Sakamoto et al., 1994), and have been further finely mapped as elements I, II and III by site-directed and linker-scanning mutation assays (Higgs et al., 1999). Element I spans a maximum of 8nt at the 5' extremity of the *petD* mRNA and is required for both mRNA stability and translation. This element appears to form a small stem-loop that may interact with a nucleus-encoded factor to block 5'→3' exoribonucleolytic degradation. Both elements II and III are essential exclusively for translation. Element II is an apparently unstructured region of a maximum of 16nt centred in the 5'UTR and appears to be the binding site for *trans*-acting proteins. Element III spans a region of 14nt close to the AUG initiation codon, which has the potential to fold into a stem-loop *in vivo*. Due to no obvious complementarity to the 3'end of chloroplast *16S rRNA*, these two translation elements are proposed to interact either together or separately with gene-specific translation activators and therefore in turn facilitate the ribosomes recognition of the start codon (Higgs et al., 1999). Furthermore, a U-rich motif within the *psbD* 5' UTR and the central region of 100nt in the *psbC* 5'UTR have been shown to be required for translation (Nickelsen et al., 1999; Zerges et al., 1997). Mutations at seven positions in the 3' half of the 266nt *rps7* 5'UTR have also revealed that these residues are important for translation both in *E.coli* and *Chlamydomonas* chloroplasts (Fargo et al., 1999).

In a tobacco chloroplast transformant, a deletion of 17nt at the 5' extremity of the *psbA* 5'UTR leads to a four-fold decrease in translation efficiency and a two-fold drop in the accumulation of chimeric *uidA* mRNA, suggesting a putative element within this deletion that confers mRNA stability and enhances translation (Eibl et al., 1999). From the studies with an *in vitro* chloroplast translation system, tobacco *psbA* translation appears to be independent of a SD-like sequence. Conversely, deletion analyses have demonstrated three sequence elements (RBS1, RBS2, and AU-Box) downstream of the SD-like sequence (RBS3) in the *psbA* 5'UTR which are crucial for efficient translation. RBS1 and RBS2 may function in a co-operative manner, as the deletion of both elements has a more severe effect on translation than

either single deletion. These two elements are proposed to constitute a bipartite SD sequence responsible for the ribosome-mRNA association, based on their complementarity to co-linear sequences near the 3' end of the *16S rRNA*, and their base pairings probably expose the intervening AU-Box for the targeting of translational activator proteins (Hirose and Sugiura, 1996). It is also noted that a number of chloroplast 5'UTRs contain the consensus sequence (UGAUC), complementary to the 3' end of the *16S rRNA*, which potentially serves as the alternative ribosome binding sequence instead of the SD-like sequence (Ruf and Kössel, 1988).

In addition, the *cis*-elements that form the secondary structures in the 5'UTRs of some chloroplast mRNAs may be necessary for translation. In *Chlamydomonas*, the 5'UTR of *psbA* mRNA exhibits a putative stem-loop region immediately upstream the SD sequence. Stem-loop mutations including loop deletion and stem-base alterations greatly reduce the level of *psbA* translation. However, *psbA* translation in those stem-loop mutants is similar to wt strain during dark growth. These results suggest that the stem-loop element is required for normal *psbA* translation but appears to act as a translational attenuator that must be overcome to activate translation, and may play a certain role in light-regulated translation (Mayfield et al., 1994). In *Euglena*, the effect of secondary structure in the *rbcL* 5'UTR has been examined. A reduction of the internal weak secondary structure does not significantly affect the efficiency to form an initiation complex, while introduction of a strong secondary structure near the translational start site within approximately 10 nucleotides badly impairs the formation of an initiation complex. These results suggest that an unstructured or weakly structured region of the mRNA near the translational start site is important for ribosome recognition of the start codon (Koo and Spremulli, 1994). Actually, the aforementioned sequence regions within the 5'UTRs essential for translation of the *psbC* and *rps7* mRNAs in *Chlamydomonas* contain high order of secondary structure(s) each, in that any disturbances lead to the deficiency in translation efficacy (Fargo et al., 1999).

1.2.4.2 *Trans*-acting translation determinants

1.2.4.2.1 Chloroplast ribosomal protein or its homologue

In *E.coli*, mRNAs can associate with the 30S ribosomal subunit during the initiation process via an interaction between the 61kd S1 ribosomal protein and the U-rich sequence in the mRNA (Voorma, 1996). A smaller 43kd spinach chloroplast homologue of S1 (CS1) that is likely associated with the small ribosomal subunit has been found to specifically bind RNA sequences resembling the 5'UTR of chloroplast genes, as well as a poly(A) sequence

(Alexander et al., 1998; Franzetti et al., 1992). It has been proposed to interact with the conserved AU-Box of the *psbA* 5'UTR (Hirose and Sugiura, 1996). The 40kd RNA binding protein (RBP40), which interacts specifically with the U-rich element of the *Chlamydomonas psbD* 5'UTR, is also thought to resemble *E.coli* S1 (Ossenbuhl and Nickelsen, 2000). However, the CS1 protein has been re-examined to have a relatively high binding affinity to poly(U) stretches, and to be insufficient to direct the 30S initiation complex to the translation start codon by specific binding to the 5'UTR of chloroplast mRNAs (Shteiman-Kotler and Schuster, 2000). Alternatively, the chloroplast ribosomal protein S7 in *Chlamydomonas* has been recently characterised to dock its own coding *rps7* mRNA at least on the small ribosomal subunit by binding to the 5'UTR that lacks a putative SD sequence for translation initiation (Fargo et al., 2001).

1.2.4.2.2 Nuclear encoded trans-acting factors

It has been frequently revealed that nuclear gene products are required for the translation of specific chloroplast mRNAs by direct or indirect interactions with the 5'UTRs. The majority of these nuclear genes are defined by mutations that reduce or abolish chloroplast translation.

In *Chlamydomonas*, two nuclear mutants specifically lacking translation of the *psbA* mRNA are deficient in the 47kd protein (RB47) of the *psbA* RNA-binding complex. RB47, identified as a member of the poly(A)-binding protein family (PABP), binds to the *psbA* 5'UTR with high affinity and specificity and acts as a message-specific translational activator in the chloroplast (Yohn et al., 1998a; Yohn et al., 1998b). The nuclear mutant *F54* fails to translate *atpA* mRNA despite a 4-fold increased mRNA accumulation, but has no effect on the translation and RNA stability of the *atpB* gene (Drapier et al., 1992). The nuclear genes *Tbc1*, *Tbc2*, and *Tab1* have also been found to be required for the translation of the *psbC* and *psaB* mRNAs (Stampacchia et al., 1997; Zerges et al., 1997). For *psbD* mRNA, one nuclear gene, *Ac115*, is thought to function following the initiation of *psbD* translation and has no influence on mRNA stability (Rattanachaikunsopon et al., 1999), while another nuclear gene *Nac2* is essential for *psbD* mRNA stability, processing and/or translation (Boudreau et al., 2000).

In higher plants, only two nuclear genes, both in maize, are known to be required for chloroplast translation. A mutation in *atp1* results in a reduction in AtpB synthesis, but no changes in *atpB* mRNA structure or abundance. The *atp1* gene product may function during translation initiation or early elongation (McCormac and Barkan, 1999). Another maize nuclear gene *crp1* is found to be necessary for the translation of the chloroplast *petA* and *petD* mRNAs, and for the intercistronic processing to generate the monocistronic *petB* and *petD*

mRNAs from their co-transcribed precursor. A proposed function of *Crp1* is to coordinate the expression levels of *petA* and *petD* to produce correct stoichiometric amounts of these subunits (Barkan et al., 1994; Fisk et al., 1999).

In all, interactions between *cis*-acting elements in the 5'UTRs of chloroplast mRNAs and specific *trans*-acting nuclear gene products appear to be essential for chloroplast gene expression. This gene-for-gene relationship certainly exhibits a regulatory role of nuclear genes on the translational level. These nuclear encoded factors are proposed to function by guiding the ribosome-mRNA association and translation initiation (e.g. CS1), or by localising their target mRNAs to the thylakoid membrane for translation (e.g. Ac115), or by co-ordinating the expression of individual photosynthetic subunits that assemble into the integral membrane complex (e.g. Crp1).

1.2.4.3 Regulation of chloroplast translation

1.2.4.3.1 Light-regulated translation

The most dramatic impact on chloroplast gene expression is observed during the light-induced greening of plastids. Light can rapidly affect protein synthesis, particularly relative to photosynthesis, from a pre-existing pool of the chloroplast mRNAs. Thus, the translational control appears to be the predominant form of light-mediated gene regulation in the chloroplast.

To date, the translation of *psbA* mRNA is the most intensively characterised in higher plants and algae. It is stimulated by light to a greater extent than other mRNAs in order to compensate for photo-damaged D1 protein. In tobacco chloroplast transformants, the expression of a chimeric *uidA* gene fused to the *psbA*5'UTR is enhanced by light, suggesting that the translational regulation occurs during the initiation phase and that the *psbA* 5'UTR confers the regulatory elements in response to the light signal (Eibl et al., 1999; Staub and Maliga, 1994a). In *Chlamydomonas*, light regulation of *psbA* translation is thought to be directly mediated by the 47kd RNA binding protein (RB47) that can bind to the *psbA* 5'UTR with high affinity and specificity, within a complex containing three other proteins (RB60, RB55 and RB38) (Danon and Mayfield, 1991; Yohn et al., 1998b). The binding activity of this protein complex to the 5'UTR of the *psbA* mRNA correlates with light-enhanced translation and ribosome association of this mRNA under a variety of environmental conditions and in several mutations deficient in *psbA* mRNA translation (Danon and Mayfield, 1991; Hauser et al., 1996; Mayfield et al., 1994; Yohn et al., 1998b). This indicates that RB47 is specially required by the ribosome to initiate the translation of *psbA* mRNA, and

that the binding of RB47 to the *psbA* 5'UTR is modulated by light to regulate the ribosome association and *psbA* translation. In addition, this light-mediated RNA binding is regulated by both ADP-dependent phosphorylation (Danon and Mayfield, 1994a) and redox potential (Danon and Mayfield, 1994b), reflecting the product levels of photosynthetic activity. Both RB47 and RB60 genes have been cloned and sequenced. The RB47 protein is identified as the chloroplast homologue of poly(A)-binding protein (cPABP) (Yohn et al., 1998a), and RB60 as the chloroplast homologue of protein disulfide isomerase (cPDI) (Kim and Mayfield, 1997). The RNA binding activity of RB47 can be modulated *in vitro* by oxidation and reduction (Kim and Mayfield, 1997). The disulfide bond formation between RNA binding domains of RB47 that affects RNA binding can be regulated by redox potential via the RB60 protein (Fong et al., 2000). A model of light-regulated *psbA* translation has been established, in which RB60 acts as the switch to control the RNA binding of RB47 and *psbA* translation by responding to the ADP level in the dark and the redox potential in the light. A counterbalanced action of reducing and oxidising activities determines the status of RB60 and modulates the translation of *psbA* mRNA in parallel with the fluctuation of light intensities. In the dark, chemical reduction of the dithiol site of RB60 that can not activate the translation further indicates that RB60 is particularly silenced by ADP-dependent phosphorylation (Trebitsh et al., 2000).

In amaranth, a C4 dicotyledonous plant, the translation of plastid *rbcL* is also regulated during light-mediated development. Multiple proteins that interact with the 5'UTR of *rbcL* mRNA are found in light-grown, but not etiolated, amaranth plants, in which a 47kd protein (p47) can only bind with high specificity to the mature processed *rbcL* transcripts. The p47 RNA binding is light-associated and correlates with light-dependent *rbcL* polysome association of the fully processed transcripts in photosynthetic tissues, suggesting that the light regulation occurs in the initiation phase of *rbcL* translation (McCormac et al., 2001).

In barley chloroplasts, it has been found that light stimulates *psbA* translation at the elongation phase, which appears to be mediated through the proton (H^+) gradient that is generated across the thylakoid membrane by photosynthetic electron transport. A pH sensitive factor that affects translation elongation is proposed to regulate the rate of *psbA* translation (Edhofer et al., 1998; Muhlbauer and Eichacker, 1998).

1.2.4.3.2 Assembly-regulated translation

The assembly status of multisubunit membrane complexes in the chloroplast can also influence the translation of specific chloroplast mRNAs via variant feedback mechanisms. In *Chlamydomonas*, the translation of *petA* mRNA to synthesise cytochrome *f* is auto-regulated.

When cytochrome *f* protein is not assembled into the cytochrome b6/f complex, due to the absence of the cytochrome b6 encoded by *petB*, the translation of *petA* mRNA is repressed via an interaction between the C-terminus of cytochrome *f* and its 5'UTR (Choquet et al., 1998). Reduced synthesis of the small subunit of Rubisco in tobacco and *Chlamydomonas* causes reduced translation of the chloroplast *rbcL* mRNA encoding the large subunit, in consistence with the coordinated expression pattern of nuclear and chloroplast genes (Khrebtukova and Spreitzer, 1996; Rodermel et al., 1996). Assembly-controlled chloroplast translation is summarised in a recent review (Choquet et al., 2001).

1.3 Current biochemical and genetic approaches to unravel the mechanisms of chloroplast gene expression regulation

Up to now, a number of biochemical and genetic approaches have been developed and exploited to allow the direct demonstration of the *cis*- and *trans*- acting determinants for RNA stability and translation, and the mechanisms in RNA transcription, RNA processing, RNA degradation and translational regulation.

Among these approaches, the systems of *in vitro* transcription and *run-on* transcription in plastids are among the earliest used for the investigation of chloroplast transcription (Deng et al., 1987; Gruissem et al., 1983). After that, the systems of *in vitro* RNA processing, *in vitro* RNA decay, and *in vitro* translation have been sequentially established to unravel the mechanisms for RNA maturation (particularly at the 3' end) (Hayes et al., 1996; Stern and Kindle, 1993) and RNA degradation (Klaff, 1995; Lisitsky et al., 1996), and to determine the *cis*- or *trans*- acting factors involved in chloroplast translation (Hirose and Sugiura, 1996; Nilsson et al., 1999). Recently, an *in vitro* RNA editing system has also been developed to demonstrate that a combination of site-specific and common RNA-binding proteins recognises the editing sites within the confirmed *cis*-acting elements in chloroplasts (Hirose and Sugiura, 2001). However, an *in vitro* RNA splicing system has not yet been reported.

A 'toeprinting' assay has been used to define the ribosome binding sites (SD-like sequence) during translation initiation (Kim and Mullet, 1994) or ribosome pausing sites during translation elongation (Kim et al., 1991). This technique is also utilised to characterise the *cis*- or *trans*- elements important for the formation of initiation complexes (Betts and Spremulli, 1994; Franzetti et al., 1992). In addition, the RNA secondary structures of 5'UTRs are also evaluated by mapping nuclease-sensitive sites *in vitro* and by *in vivo* dimethyl sulfate RNA modification (Higgs et al., 1999).

With respect to nuclear encoded *trans*-acting factors involved in the regulation of chloroplast gene expression, most of them are characterised via a variety of approaches. Gel mobility shifts, UV crosslinking, RNA affinity chromatography, and enzymatic activity-associated co-purification, etc. have been prevalently exploited to identify the proteins that bind to untranslated regions of chloroplast transcripts in *Chlamydomonas* and higher plants (Danon and Mayfield, 1991; Hauser et al., 1996; Hayes et al., 1996; Schuster and Gruissem, 1991; Yang et al., 1996). However, only a few of their encoding genes have been cloned by genomic complementation (Boudreau et al., 2000; Vaistij et al., 2000), transposon-tagging (Fisk et al., 1999), or according to the amino acids information from microsequencing or conserved RNA binding motifs (Kim and Mayfield, 1997; Mieszczak et al., 1992; Yang et al., 1996; Yohn et al., 1998a).

In contrast to the *in vitro* analyses of chloroplast gene expression, the evidence from *in vivo* investigation can be more convincible and comprehensive. Chloroplast transformation that allows the integration of foreign or modified genes into the chloroplast genome by homologous recombination provides a powerful tool to address the mechanisms that control chloroplast gene expression at each regulatory level as mentioned above. For example, the *cis*-acting determinants for chloroplast mRNA stability and translation have been revealed to localise mostly on the 5' untranslated regions by analysing various chloroplast transformants (Eibl et al., 1999; Fargo et al., 1999; Higgs et al., 1999; Mayfield et al., 1994; Nickelsen et al., 1999; Staub and Maliga, 1994a; Vaistij et al., 2000).

Chloroplast transformation has been successful in *Chlamydomonas* (Boynton et al., 1988), tobacco (Koop and Kofer, 1995; Koop et al., 1996; Svab and Maliga, 1993), potato (Sidorov et al., 1999), arabidopsis (Sikdar et al., 1998), rice (Khan and Maliga, 1999), tomato (Ruf et al., 2001), Euglena (Doetsch et al., 2001), and will be achievable in other species of algae and higher plants, via bombardment microprojectiles, PEG-treatment or other technical progresses. The bacterial *aadA* gene, which encodes aminoglycoside 3'-adenyl transferase conferring the resistance to antibiotics spectinomycin and streptinomcin, is widely used as the selection marker. The alternative antibiotic-related selection markers are also available (Bateman and Purton, 2000; Carrer et al., 1993) in case of serial transformations of the chloroplast genome. Furthermore, non-antibiotic selection markers have also been developed in response to biosafety and public concerns (Daniell et al., 2001a; Sidorov et al., 1999; Ye et al., 2001). Technical details of chloroplast transformation including integration sites, transformation efficiency, advantages and pitfalls, applications and prospects, etc. have been well described in a number of reviews (Bogorad, 2000; Heifetz, 2000; Kofer et al., 1998;

Rochaix, 1997). Up to now, complete sequences of the chloroplast genomes have been achieved in a lot of plant species including blackpine (Wakasugi et al., 1994), tobacco (Shinozaki et al., 1986), oenothera (Hupfer et al., 2000), wheat (Ikeo and Ogihara, 2000), maize (Maier et al., 1995), rice (Hiratsuka et al., 1989), spinach (Schmitz-Linneweber et al., 2001), lotus (Kato et al., 2000), and arabidopsis (Sato et al., 1999). More or less sequence information has also been available in the DNA databases, relating to the chloroplast genomes of other plant species, especially with agronomic or economic traits, such as potato, tomato, sugarbeet and rape seed. Additionally, chloroplast genes are considerably conserved in the respects of DNA sequence and physical location in the chloroplast genomes among higher plants. Taken together, these have opened up the opportunities to study the function and biogenesis of chloroplasts reverse-genetically, or to engineer the chloroplast in plant biotechnological applications through the approach of chloroplast transformation.

1.4 The aims of this work

As mentioned above, the 5' untranslated regions of chloroplast mRNAs play a major role in regulating chloroplast gene expression. The *cis*-elements are mostly defined by *in vivo* analyses via chloroplast transformation in *Chlamydomonas* (Higgs et al., 1999; Mayfield et al., 1994; Nickelsen et al., 1999; Zerges et al., 1997). However, similar studies in higher plants have scarcely been reported. Up to date, only a few results are available, concerning the role of chloroplast 5'UTRs in tobacco (Eibl et al., 1999; Hirose and Sugiura, 1996; Shiina et al., 1998; Staub and Maliga, 1994a). The entire *psbA* 5'UTR has been shown to mediate light-activated translation of *psbA* and chimeric *uidA* mRNAs (Staub and Maliga, 1994a). Further studies indicate that the *psbA* 5'UTR confers a stronger translational signal than the 5'UTRs of other chloroplast mRNAs, and its 5' end that contains a stem-loop structure is suggested to be important for mRNA stability and translation efficiency (Eibl et al., 1999). Within the efforts to characterise the core sequence of the tobacco *rbcL* promoter, a segment at the extremity of the *rbcL* 5'UTR that potentially forms a stem-loop structure is proposed to promote mRNA stability for compensating the lower transcription rate in the dark (Shiina et al., 1998). However, no *cis*-acting elements for either stability or translation of chloroplast mRNAs in higher plants have been clearly determined *in vivo* yet. Remarkably, the assays via an *in vitro* chloroplast translation system indicate that three *cis*-acting elements (RBS1, RBS2, AU-Box) of tobacco *psbA* 5'UTR are required for translation of the *psbA* mRNA, while the upstream SD-like sequence (RBS3) and stem-loop structure are apparently not of functional significance (Hirose and Sugiura, 1996). Nevertheless, such an *in vitro* system may not

correctly elucidate the *cis*-acting elements influencing mRNA stability and conferring translational signals. Therefore, in this study that attempts to accurately characterise the *cis*-acting determinants for the stability and translation of tobacco *psbA* mRNA within its 5' UTR, *in vivo* analysis via chloroplast transformation is chosen.

Considering the character of chloroplast transformation as a site-directed integration of foreign genes into the plastome, the insertion site is thought to be important for a successful and stable chloroplast transformation. Hence, a plastid transformation vector with a suitable insertion site is considerably essential, not only for the characterisation of tobacco *psbA* 5'UTR in this work, but also for future studies in the fields of plastid function and plastid engineering. With respect to the *psbA* 5'UTR, a number of *cis*-elements is assumed to be functionally essential, including the poly(A) sequence, stem-loop region, canonical SD-like sequence, internal initiation AUG codon, and AU-box. The poly(A) sequence is postulated as a binding site of a putative chloroplast poly(A) binding protein in tobacco resembling *Chlamydomonas* RB47. The regulatory roles of the stem-loop region and SD-like sequence are mostly likely underestimated as mentioned above (Eibl et al., 1999; Hirose and Sugiura, 1996). The *in vitro* evaluation of AU-box appears to be convincible (Hirose and Sugiura, 1996), but still requires confirmation by *in vivo* studies. The internal 'AUG' codon together with the SD-like sequence has been suggested to participate in early ribosome-mRNA association (Kim and Mullet, 1994), however its exact function is still unknown. In addition, it is intriguing that the putative mRNA stabilising element as the 5' end stem-loop of *rbcL* 5'UTR (Shiina et al., 1998) might convey mRNA stability signals to the *psbA* 5'UTR. In order to analyse these open questions, the aims of this work are to define a suitable insertion site, preferably between two tRNA genes in the inverted repeat region, and generate a versatile plastid transformation vector. Based on this vector as a 'backbone', a series of mutant *psbA* 5'UTRs, in combination with identical promoter, reporter gene coding region and trailer are to be designed for fine analysis of *psbA* 5'UTR *cis*-elements through expression studies in transplastomic plants.

2. Materials and Methods

2.1 Biochemical, enzymes and other consumed materials

Substance	Supplier
α-^{32}P dCTP	Amersham, Braunschweig
Acrylamide solution	Bio-Rad, München
Agarose	Biometra, Göttingen
Alkaline Phosphatase (shrimp alkaline phosphatase)	Amersham, Braunschweig
Ampicillin	Serva, Heidelberg
Bacto Agar	ICN, Ohio, USA
Bacto Tryton	Serva, Heidelberg
dNTPs	MBI-Fermentas, St.Leon-Rot
Dimanin C	Bayer, Leverkusen
DNA Ligase:	
T4 DNA Ligase	MBI-Fermentas, St.Leon-Rot
Rapid DNA Ligation Kit	Boehringer Mannheim, Mannheim
DNA Polymerase:	
T4 DNA Polymerase	MBI-Fermentas, St.Leon-Rot
Klenow Large Fragment	MBI-Fermentas, St.Leon-Rot
Pfu DNA Polymerase	Promega, Mannheim
Taq DNA Polymerase	Qiagen, Hilden
Ethidiumbromide	Roth, Karlsruhe
IPTG(Isopropylthiogalactoside)	MBI-Fermentas, St.Leon-Rot
4 -MUG	Sigma, Steinheim
Nylon Membrane	Amersham, Braunschweig
Oligonucleotide	MWG-Biotech, Ebersberg
Restriction Enzymes	MBI-Fermentas, St.Leon-Rot Promega, Mannheim New England Biolabs, Schwalbach
RNase A	Amersham, Braunschweig
Spectinomycin-dihydrochlorid	Sigma, Steinheim
Trizol	Gibco BRL, Karlsruhe
X-Gal	Biometra, Göttingen
X-Gluc	Sigma, Steinheim
DNA standard Marker	MBI-Fermentas, St.Leon-Rot
RNA standard Marker	Ambion, Austin, USA

All non-listed standard chemicals were purchased from the following companies or suppliers: Baker Chemicals (Phllipsburg, USA), Biomol (Hamburg), BRL (Gaithersburg, USA), Difco (Detroit, USA), Merck (Darmstadt), Riedl de Haen (Hannover), Roth (Karlsruhe), Serva Biochemica (Heidelberg) and Sigma (Steinheim), etc.

2.2 Media, buffers and solutions

All non-specified buffers, solutions, and media for DNA cloning and molecular analysis in the methods are prepared as described by Sambrook et al. (1989).

Media for plant cell culture are listed in table 2.1.

Table 2.1: Media for plant cell culture

	(mg)	$B5_{mod}$	F-PCN	F-PIN	RMOP
Macro-Salts	NH_4NO_3				1650
	KNO_3	2500	1012	1012	1900
	$CaCl_2 \cdot 2H_2O$	150	640	640	440
	$MgSO_4 \cdot 7H_2O$	1033	370	370	370
	KH_2PO_4		170	170	170
	$NaH_2PO_4 \cdot H_2O$	150			
	$(NH_4)_2SO_4$	134			
	NH_4-Succinat*		10ml	10ml	
Micro-Salts	EDTA-Fe(III) Na^+	40	40	40	40
	KJ	0.75	0.75	0.75	0.83
	H_3BO_3	3	3	3	6.2
	$MnSO_4 \cdot H_2O$	10	10	10	22.3
	$ZnSO_4 \cdot 7H_2O$	2	2	2	8.6
	$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25
	$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025
	$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.025
Vitamin	Inosit	100	200	200	100
	Pyridoxin-HCl	1	2	2	
	Thiamin-HCl	10	1	1	1
	Biotin		0.02	0.02	
	Glycine				
	Nicotin acid	1	2	2	
Others	BAP (1 mg/ml)		1 ml	1 ml	1 ml
	NAA (1 mg/ml)		0.1 ml	0.1 ml	0.1 ml
	Kinetin				
	Polypuffer 74		10 ml	10 ml	
	Sucrose	20000		136000	30000
	Glucose		87 000		
	PH value	5.8 (KOH)	5.8 (KOH)	5.8 (KOH)	5.8 (KOH)
	Osmosis (mosm/l)		550	550	
	Agar	7000			8000
	Add H_2O to 1L	Ion-free	Ion-free and distilled	Ion-free and distilled	Ion-free
Reference		(Gamborg et al., 1968)	(Koop and Kofer, 1995)	(Koop and Kofer, 1995)	(Svab and Maliga, 1993; Murashige and Skoog, 1962)

*NH_4-Succinat (2M): Dissolve 23.6g Succinat, 10.6g NH_4CI, ~22.4g KOH in ion-free and distilled sterile water to total volume of 100ml, adjust pH to 5.8, then filter sterilise.

2.3 DNA isolation and purification

2.3.1 Plasmid DNA Isolation

2.3.1.1 'Mini' preparation of plasmid DNA

2.3.1.1.1 Alkaline lysis method for general use such as to identify recombinant clones

Fill about 1.5ml LB liquid medium containing 75μg/ml ampicillin in a 2ml Eppendorf tube, and inoculate a single bacterial clone from a solid LB plate by a 10μl short pipette tip, then incubate on a shaker at 37°C overnight. Pellet the bacteria in the 2ml tube at 15000rpm for 2 min. Decant the supernatant by a vacuum aspiration and resuspend the pellet in 50μl TE buffer thoroughly. Add 300μl TENS buffer and vortex rapidly but gently, then add in 150μl 3M KoAc (pH 5.2) and mix well by vortexing for 2~5 seconds. Centrifuge the tube at 15000rpm for 2 min, transfer the supernatant into a new 1.5ml Eppendorf tube, then mix with 900μl 100% Ethanol and store 5 min, at –20°C, for DNA precipitation. Pellet the DNA by centrifugation at 15000rpm for 5 min. Discard the supernatant, and wash the DNA pellet once with 70% Ethanol, then dry it for 5 min. Dissolve the rude plasmid DNA into 50μl TE buffer (pH 8.0) containing 10μg/ml RNase A and incubate at 65°C for 10 min to facilitate dissolving the DNA and to eliminate the RNAs. Take 5μl solution for the reaction of restriction enzyme.

TENS-Buffer (lysis buffer):

EDTA.....1mM
NaOH.....0.1 N
SDS.....0.5% (w/v)
Tris·HCI (pH7.5).....10mM

2.3.1.1.2 By 'Qiaprep Spin Miniprep Kit' for subcloning and DNA sequencing

Inoculate 100μl bacterial culture of interest into 4ml LB liquid medium in a Falcon tube and incubate on a shaker at 200 rpm, 37°C overnight. Take 2ml cell culture into a 2ml Eppendorf tube, centrifuge at 14000rpm for 2 min and discard the supernatant. Add the rest of the bacterial culture in the same tube to pellet all bacterial cells. Then, extract and purify the plasmid DNA as described in the manual of this Qiagen kit. Finally, elute the DNA from the Qiagen column into 50μl distilled H_2O and take 0.5μl for determination of its concentration by agarose gel electrophoresis (2.3.4).

2.3.1.2 'Maxi' preparation of plasmid DNA

For experiments such as plant transient expression and stable transformation, large amounts of plasmid DNA of high purity are required. To this end, the preparation of plasmids of interest were commonly carried out by 'Qiagen plasmid Maxi kit'.

The bacterial culture for maxi-preparation was cultivated by amplifying 0.5ml cell preculture for 8~12 hours into 100ml LB liquid medium on the shaker at 250rpm, 37°C overnight. The basic procedures in manipulation was carried out as described in the corresponding protocol of the kit with a supplement as follows. After the precipitation by isopropanol, the DNA pellet was resuspended in 5ml 70% ethanol thoroughly for washing, then transferred to 2ml Eppendorf tubes. The DNA was pelleted again, dissolved in H_2O completely and collected in one 2ml tube to a volume of 500µl. Then the plasmid DNA was precipitated by mixing with 1/10 volume of 3M NaAc (pH 5.2) and 2 volumes of 100% ethanol at –20°C for at least 10 min. The DNA pellet after centrifugation at 14000rpm for 10 min was washed twice with 1ml 70% ethanol and finally dissolved into 100µl distilled H_2O. The DNA concentration was measured by gel electrophoresis and comparison with appropriate standards (2.3.4).

2.3.2 Plant DNA isolation

DNA from plant tissue was extracted by 'Qiagen DNeasy plant Mini kit'.

Grind the plant tissue (~100mg leaf) under liquid nitrogen to a fine powder using a mortar and pestle, then resuspend the powder with liquid nitrogen and rapidly transfer the suspension into a 10ml Falcon tube. Place the tube at -20°C after evaporation of the nitrogen in case of many simultaneous DNA preparations.

According to the manual of the kit, add 400µl 65°C-prewarmed buffer AP1 (lysis buffer) and 4µl RNase A stock solution (100mg/ml) into the tube, mix thoroughly by vigorous vortexing and keep on ice in case of many samples. Incubate the mixture for 10 min in a 65°C shaking water-bath for cell lysis. Add 130µl buffer AP2 to the lysate, mix and place on ice for 5 min to precipitate the detergent, proteins, and polysaccharides. Centrifuge the tube at 12000rpm for 5 min optionally. Apply the rude lysate or the supernatant of the lysate to the QIAshredder spin column (lilac) attached to a 2ml collection tube and centrifuge for 2 min at maximum speed (15000rpm) for filter purification of the lysate. Transfer the flow-through fraction (typically 450µl) to a new tube without disturbing the pellet of cell-debris. Add 0.5 volumes of buffer AP3 (pre-warmed at 65°C) and 1 volume of 100% ethanol to the cleared lysate and mix by pipetting. Apply 650µl of the mixture (including the formed precipitates)

into DNeasy mini spin column inside a 2ml collection tube. Centrifuge for 1 min at 10000rpm, discard the flow-through and repeat this step with the remaining sample if possible. Transfer the column to a new 2ml collection tube, add 500µl buffer AW into the column and centrifuge for 1 min at 8000rpm for washing. Discard the flow-through, add 500µl buffer AW to the column again and centrifuge for 2 min at maximum speed to dry the column resin. Place the column to a new 1.5ml Eppendorf tube and apply 100µl preheated (65°C) AE buffer or H_2O in the centre of column resin and incubate at room temperature for 5 min, then centrifuge for 1 min at 10000rpm to achieve DNA elution. Determine the quality and concentration of the DNA preparations by gel electrophoresis (2.3.4).

2.3.3 DNA purification

2.3.3.1 Purification from the agarose gel

DNA purification from agarose gels was done by 'QIAquick gel extraction kit' as described in the respective user protocol. In the last step, the DNA was typically eluted in 30µl distilled H_2O from the column, and 0.5 or 1µl were used for determining the DNA concentration.

2.3.3.2 Purification from other sources

DNA from other applications such as restriction enzyme digestion, DNA ligation, PCR amplification and rude preparation could be purified by the QIAquick PCR purification kit, according to the user manual. To elute DNA, 30~50µl distilled H_2O was loaded in the centre of the column membrane for at least 1 min, with a following centrifugation for 1 min at 14000rpm. 0.5~1µl were used for determination of the DNA concentration if necessary.

2.3.4 Determination of DNA concentration

A certain amount of DNA mixed with 6x DNA loading buffer was loaded in a 1% mini-agarose gel with 0.5 or 1µl DNA standard (100ng/µl 1kb DNA fragment, MBI-Fermentas, St.Leon-Rot) as the reference, run at moderate voltage for a proper time and recorded under UV illumination as a '.tif' file. Then the DNA concentrations of samples could be calibrated by computer program 'Odenscan' (MWG-Biotech, Ebersberg) automatically.

2.4 DNA cloning

2.4.1 DNA ligation and purification

Almost all DNA ligations were carried out with the rapid DNA ligation kit of Boehringer Mannheim according to its user manual.

The reaction system of DNA ligation was set up as below:

Insert..... x µl

Vector .....y µl

5x DNA dilution buffer.....2 µl

Distilled H_2O.....(7-x-y) µl

2x DNA ligation buffer.....10 µl

T4 DNA ligase (5u/µl).....1 µl

---

20 µl

Mix thoroughly; the molar ratio of insert/vector was adjusted to 3:1 usually. Incubate at room temperature for 5 min. For the ligation of blunt ends or insufficient amounts of DNA, the reaction may be extended for half hour or longer.

For *E.coli* transformation by the $CaCl_2$ method, the ligation mixture can be applied directly without further purification. However, efficient transformation of *E. coli* by electroporation required purified ligation product. In this case, the ligated DNA was purified by Qiagen PCR purification kit and eluted in less than 20µl of H_2O.

2.4.2 DNA transformation of bacteria (E. coli)

2.4.2.1 Preparation of competent cells of *E. coli*

1). Competent cells for the transformation by the $CaCl_2$ method were prepared as described in 'Molecular Cloning' ^{2nd} (Sambrook et al., 1989), *E. coli* strain JM109 (Promega, Mannheim) was used.

2). Competent cells for the transformation by electroporation (*E. coli* strain: *Shure* 2 from Strategene, Heidelberg) were made by the following procedure.

Prepare a 10ml pre-culture in LB liquid medium. An overnight bacterial culture is not recommended for optimal results. Dilute 1 volume of the pre-culture (2ml) to 100 volumes of fresh LB medium (200ml) pre-warmed to 37°C, and grow the bacterial cells at 37°C on a

shaker at 250rpm. Chill the culture on ice immediately when cell growth reaches an $O.D._{600nm}$ of 0.5-1.0 (10^{10} cells/ml). Centrifuge the culture in four 50ml tubes at 4000x g for 15 min at 4°C, discard the supernatant. Resuspend the pellet in 25ml sterile cold H_2O in each tube, centrifuge at 4000x g for 15 min at 4°C and discard the supernatant with two repeats. Finally, resuspend the pellet in 400µl sterile cold H_2O in each tube, collect all suspensions together. Check the final volume of cell suspension and add 10% of cold glycerol, mix gently but thoroughly, aliquot 50~100µl in each 1.5ml sterile fresh Eppendorf tube, then quickly freeze in liquid nitrogen and keep at –70°C.

2.4.2.2 Transformation to *E. coli*

2.4.2.2.1 By heat activation on competent cells made by the $CaCl_2$ method

Thaw the competent cells (100µl) on ice for 10 min. Pipette 4µl of 0.5M β-mercaptoethanol into the cells, and optionally mix by stirring gently with the pipette tip (not by pipetting up and down). Add 2~4µl DNA ligation reaction mixture into the competent cells, mix by stirring gently with the pipette tip and keep on ice for 30 min. Apply a heat shock in a 42°C water-bath for 45~90 seconds, do not mix and shake, then put on ice immediately for 2 min. Add 900µl SOC medium (at room temperature) and incubate on a shaker at 200rpm, 37°C for 1~2 hours. Spread a proper volume of the culture on LB plates with appropriate concentration of antibiotics (e.g. 75µg/ml ampicillin). For Blue-white screening, pre-spray the X-gal/IPTG mixture (10µl 0.4M IPTG and 80µl 20mM X-gal) on the surface of LB plates. Invert the plates after the liquid has been absorbed, and place in a 37°C incubator overnight (at least 18 hours). For better colour development, plates can be shifted to 4°C for 2-4 hours.

2.4.2.2.2 By the method of electroporation

Thaw an aliquot of competent cells on ice for 10 min. Add 1~5µl of purified ligation reaction mixture into the cells and mix by pipetting gently. Load into a 2mm pre-chilled electroporation cuvette, and keep on ice for 1 min. Apply the pulse to the cuvette on the electroporation unit ‘Equibio Easyject’ (Peqlab, Erlangen) with the pre-programmed setting: 2500V (voltage), 25µF (capacitance), 201ohms (resistance), 5msec (calculated pulse time). Transfer the cuvette quickly to ice in less than 15 seconds, then add 1 ml 37°C-prewarmed SOC medium to suspend the electroporated cells by gentle pipetting. Transfer all cell suspension to a 2ml Eppendorf tube or 10ml Falcon tube and incubate at 37°C for 1~2 hours. Spread the liquid cell culture on plates as described above.

2.5 PCR amplification

Components for PCR including 10xbuffer (15mM Mg^{2+}) and *Taq* DNA polymerase were purchased from Qiagen, Hilden. *Pfu* DNA polymerase was the product of Promega, Mannheim. 25mM dNTP mixture was made from the 100mM dNTP set of MBI-Fermentas, St.Leon-Rot. Oligo primers were synthesised by MWG-Biotech, Ebersberg. PCR was carried out on the units of Hybaid (Heidelberg) or Biometra (Göttingen).

PCR amplification was applied in various experiments for this study, and differed in some respects such as the reaction setting and the programming parameter described in table 2.2 and table 2.3. After reaction, take 4µl of each sample into the test gel to see the results.

Table 2.2: The reaction settings of PCR amplification

Components \ applications	Identification of recombinant clones	DNA amplification with high fidelity	Identification of transplastomic plants
100pmol/µl Primer 1 (P1)	0.3µl	0.3µl	0.3µl
100pmol/µl Primer 2 (P2)	0.3µl	0.3µl	0.3µl
25mM dNTP mixture	0.4µl	0.4µl	0.4µl
10x *Taq* reaction buffer	5µl	-	5µl
10x *Pfu* reaction buffer	-	5µl	-
Single clone in H_2O or bacterial culture	2µl	-	-
Plant total DNA (50ng~200ng/µl) or diluted plasmid DNA (20ng/µl)	-	1µl	1µl
Taq DNA polymerase (5U/µl)	0.2µl	-	0.2µl
Pfu DNA polymerase (3U/µl)	-	1µl	-
Distilled H_2O	41.8µl	42µl	42.8µl
Total volume	50µl	50µl	50µl

Table 2.3: The program settings of PCR amplification

Parameters \ applications	Identification of recombinant clones	DNA amplification with high fidelity	Identification of transplastomic plants
95°C (Pre-denaturing)	5 min	2 min	2 min
94.5°C (Denaturing)	45~60 sec	45~60 sec	45~60 sec
Tm-5°C (Annealing)	30~60 sec	30~60 sec	30~60 sec
72°C (polymerisation)	1min/Kbp	2min/Kbp	1min/Kbp
Cycles	30~35	30~35	30~35
72 °C (extensive polymerisation)	10 min	10 min	10 min
Kept at 4°C	✓	✓	✓

2.6 RNA isolation

2.6.1 Homogenisation

Fill the glass beads (Sigma, Steinheim) into a 2ml screw tube. Put ~100mg of plant tissue (leaf) into the tube and add 1ml Trizol Reagent. Cap sample tubes securely, use a beadbeater (Biospec, California, USA) to homogenise the sample at a speed of 5000 times per minute for 1 min. For RNA isolation from 5~10 x 10^6 plant protoplasts and chloroplasts, a reduction in speed or time of the homogenisation may be an advantage. An insufficient amount of Trizol reagent may cause DNA contamination in the RNA extract.

2.6.2 Phase separation

Incubate the homogenate for 5 min at 15~30°C to permit the complete dissociation of nucleoprotein complexes. Add 0.2ml of chloroform per 1ml of Trizol reagent. Cap the sample tubes and shake vigorously by hand for 15 sec, then incubate them at 15~30°C for 2~3 min. Centrifuge the samples at no more than 12000x g for 15 min at 2~8°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, a turbid interface and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of Trizol reagent used for homogenisation.

2.6.3 RNA precipitation

Transfer the aqueous phase to a fresh 1.5ml Eppendorf tube and save the organic phase in case of the additional isolation of DNA or protein. To precipitate the RNA, mix the aqueous phase with 0.9 volumes of isopropyl alcohol thoroughly, store at 15~30°C for 10 min and centrifuge at no more than 12000x g for 10 min at 2~8°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

2.6.4 RNA wash step

Decant the supernatant. Wash the RNA pellet once by adding 1ml 75% ethanol per 1ml of Trizol reagent used for the initial homogenisation. Vortex the RNA pellet and centrifuge at no more than 7500x g for 5 min at 2~8°C. Note here that the RNA sample can be kept in the 75% ethanol at –20°C for a long time.

2.6.5 Dissolving RNA

In the end of this procedure, dry the RNA pellet in air for 5~10 min but not completely. Dissolve the RNA pellet in 20~30µl RNase-free H_2O or 30~50µl 1x RNA loading buffer by gentle pipetting and incubation at 55~60°C for 10 min. RNA can also be dissolved in 100% deionized-formamide and stored at -70°C.

2.7 Molecular hybridisation

2.7.1 Preparation of DNA probes for hybridisation

2.7.1.1 DNA labelling

For molecular hybridisation, DNA template was randomly labelled with radioactive α-^{32}P dCTP by the kit of Prime-a-Gene® labelling system of Promega, Mannheim, according to the user manual briefly described as below.

Thaw all the system components except the Klenow fragment on ice. Add xµl (~25ng) DNA template into a 1.5ml Eppendorf tube containing (33-x)µl DNase-free H_2O, and denature at 95~100°C for 2 min, then chill the tube rapidly on ice for 5 min. Afterwards, add the other components of the following reaction setting in the tube (on ice) with the DNA template.

Denatured DNA template(~25ng).....x µl (in the tube)
5x labelling buffer.....10 µl
Mixture of dNTPs:
1.5mM dATP.....0.667 µl
1.5mM dGTP.....0.667 µl
1.5mM dTTP.....0.667 µl
Nuclease-free BSA(10mg/ml).....2 µl
α-^{32}P dCTP, 50µCi, 3000Ci/mmol (red).....2 µl
DNA polymerase I, klenow large fragment(5U/µl).....1 µl
DNase-free H_2O.....33-x µl
---
to total volume of 50 µl

Mix the components gently and incubate the reaction tube at room temperature for 1~2 hours.

2.7.1.2 Purification of the labelled DNA probe

After the labelling reaction, the labelled DNA was purified by Microspin columns of Amersham, Braunschweig, to remove short-sized labelled DNA fragments and unincorporated nucleotides by the following procedure. Resuspend the resin in the column by vortexing vigorously for 1min. Loosen the cap one-fourth turn and snap off the bottom closure, place in a 1.5ml Eppendorf tube and spin the column for 1 min at 3000 rpm. Put the column in a new 1.5ml Eppendorf collection tube, open the cap of the column and slowly apply the labelling mixture to the top centre of the resin without disturbing its bed, cover the tube with the cap loosely, then spin the column at 3000rpm for 2 min. The flow-through in the collection tube is about 50µl and colourless, while the red unincorporated nucleotides and short-labelled fragments remain in the resin of the column. Before hybridisation, denature the probe in a 95~100°C water-bath for 2~3 min, then chill it on ice quickly.

2.7.2 Southern blotting

2.7.2.1 Solutions

- Hybridisation buffer (Church buffer) (Church and Gilbert, 1984):

  Na_2HPO_4/NaH_2PO_4(PH7.5).....250mM

  SDS.....7%(w/v)
- Transfer buffer: 0.4N NaOH
- Washing buffer: 0.25x SSC, 0.1% SDS
- 20x SSC
- 10% SDS

2.7.2.2 Enzyme digestion of plant DNA

Harvest the leaves from transplastomic plants grown in the culture chamber. Extract the total DNA by Qiagen DNeasy plant Mini kit. Take about 2µg total DNA of each sample for complete digestion with enzyme *BglII* at 37°C, 3~4 hours or overnight.

2.7.2.3 DNA gel electrophoresis and blotting

Prepare a large 1% agarose gel of the size 20cm x 20cm containing 0.5µg/ml ethidium bromide for southern blotting. Load the DNA digestion sample in a mixture with 6x DNA loading buffer in the gel slot and run at 30~40V overnight for optimal separation, then document the gel with a transparent ruler under UV illumination. Wash the gel with distilled

water after an optional treatment by 0.25M HCl and assemble the DNA blot unit containing the transfer buffer (0.4N NaOH) in the meantime. Apply the gel on the DNA blot unit to transfer DNA from the gel to the Hybond-N^+ nylon membrane (Amersham, Braunschweig) by capillary action. After12~18 hours or overnight, separate the DNA filter, wash in 2xSSC optionally and air-dry for half hour on 3M filter paper, then expose in the UV crossinglinker (Strategene, Heidelberg) for DNA fixation. After that, wrap the DNA filter in 3M filter paper and keep at room temperature till its use for hybridisation.

2.7.2.4 Prehybridisation and hybridisation

Roll the DNA filter and transfer to a glass tube with a thermostable plastic cap. Add proper amount of hybridisation buffer (~25ml Church buffer for a filter of 20cm x 20cm) to the tube and soak the filter gently, avoiding bubbles. Screw the cap of the tube securely and cling to the low-speed rotor of an oven at 60°C for prehybridisation. Typically perform prehybridisation for 2~4 hours, meanwhile prepare and purify the radioactive probe. Then, renew the old buffer with the same amount of Church buffer pre-warmed at 60°C and pipette the denatured probe into the buffer directly, avoiding contact with the filter. Shake the tube gently and invert for several times to evenly distribute the probe in the buffer, then return to the oven for hybridisation at 60°C overnight.

2.7.2.5 Washing and autoradiography

Discard the hybridisation solution into a 50ml plastic tube for safe disposal. Rinse the DNA filter in 50ml washing buffer, then discard. Add half tube volume of washing buffer in the tube to wash the filter at 60°C in the hybridisation oven for 0.5~1 hour, or immerse the filter in a plastic box containing 200ml washing buffer. Monitor the signal by the radioactivity meter till the optimal washing state, then wrap the filter and place it on the phosphorimager plate for a proper exposure time (1~5h). Automatically develop the hybridisation profile by the phosphorimager (Fujifilm BAS1500).

2.7.2.6 Stripping of DNA probes

In case of using the same DNA filter for more than one hybridisation, carefully keep the filter wet always after the first hybridisation. Remove the old DNA probe from the filter by the DNA stripping solution (0.2x SSC, 0.1% SDS, 0.2M Tris·HCl, pH7.5).

Incubate the DNA filter in 100~200ml 0.4N NaOH at 42°C for 5 min with gentle agitation. Rinse the filter briefly with DNA stripping solution, then wash it with the same solution afterwards. Repeat this procedure till no detectable radioactivity is left on the filter.

Place the DNA filter in the hybridisation tube and proceed with the next hybridisation experiment.

2.7.3 Northern blotting

In RNA analysis such as northern blotting, all hardware and solutions are treated to make them RNase-free.

2.7.3.1 Solutions

- RNase-free H_2O

  treat the distilled water with 0.05% (v/v) DEPC, mix well and place in the hood overnight and autoclave.

- Hybridisation buffer (Church buffer):

  Na_2HPO_4/NaH_2PO_4(PH7.5).....250mM

  SDS.....7%(w/v)

  treat with 0.1% (v/v) DEPC and autoclave.

- 10x MOPS Buffer

  MOPS.....200mM

  NaAc.....50mM

  EDTA.....10mM

  adjust pH to 7.0 with NaOH, treat with 0.1% (v/v) DEPC and autoclave.

- RNA gel running buffer (1L)

  10x MOPS buffer.....100ml

  37% formaldehyde (12.3M).....20ml

  RNase-free H_2O.....880ml

- 5x RNA sample loading buffer (1ml)

  saturated bromophenol blue solution.....1.6µl

  500mM EDTA, pH 8.0.....8µl

  37% formaldehyde (12.3M).....72µl

  100% glycerol.....200µl

  ion-free formamide.....308.4µl

  10x MOPS buffer.....400µl

  add RNase-free H_2O to 1ml

To make a saturated bromophenol blue solution, add solid bromophenol blue into distilled water and mix thoroughly until no more will dissolve. Centrifuge and carefully remove the supernatant to a new collection tube.

- 20x SSC, treat with 0.1% (v/v) DEPC and autoclave.
- 10% SDS
- Washing buffer: 0.1x SSC, 0.1% SDS

2.7.3.2 Preparation of 1.2% formaldehyde RNA gels

agarose.....1.2g

10x MOPS.....10 ml

add RNase-free H_2O to 100 ml

Heat in the microwave oven to melt the agarose, cool to 65°C, then add 1.8ml 37% formaldehyde (12.3M) and 1µl ethidium bromide (10mg/ml). Mix thoroughly and pour on the gel support. Equilibrate the gel in gel running buffer for 30 min optionally prior to use.

2.7.3.3 RNA gel electrophoresis and blotting

Load ~2 µg RNA for each sample in the slot of RNA gel, run the gel at 60V for 2~4 hours, then document under UV illumination. Wash the gel with RNase-free H_2O for 15 min twice to remove as much formaldehyde as possible. Meanwhile, assemble the RNA blot unit and fill in the transfer buffer (10x SSC). Apply the gel on the RNA blot unit and transfer RNA from gel to Hybond-N^+ nylon membrane (Amersham, Braunschweig) overnight by capillary action. Then, treat the RNA filter in a UV-crossinglinker (Strategene, Heidelberg) for RNA fixation, wrap in 3M filter paper and keep at room-temperature until the hybridisation.

2.7.3.4 Prehybridisation and hybridisation

Roll the RNA filter and transfer to a glass tube with a thermostable plastic cap. Add proper amount of hybridisation buffer (~25ml Church buffer a filter of 20cm x20cm) in the tube carefully and soak the filter gently, avoiding bubbles. Screw the cap of the tube securely and cling to the low-speed rotor of an oven at 65°C for prehybridisation. Typically perform prehybridisation for 2~4 hours, prepare and purify the radioactive probe in the meantime. Then, decant the old buffer and fill in the same amount of Church buffer pre-warmed at 65°C, pipette the denatured probe into the buffer directly, avoiding contact with the filter. Shake the tube gently and invert for several times to evenly distribute the probe in the buffer, then return to the oven for hybridisation at 65°C overnight.

2.7.3.5 Washing and autoradiography

Discard the hybridisation solution into a 50ml tube for safe disposal. Rinse the RNA filter in 50ml washing buffer, then discard. Add half tube volume of washing buffer in the tube to wash the filter at 65°C in the hybridisation oven for 1~2 hours, or immerse the filter in a plastic box containing 200ml washing buffer. Monitor the signal by the radioactivity meter to determine the optimal washing state, then wrap the filter and place it on the phosphorimager plate for a proper time (1~3h). Automatically develop the hybridisation profile by the phosphorimager (Fujifilm BAS1500).

2.7.3.6 Stripping of DNA probes

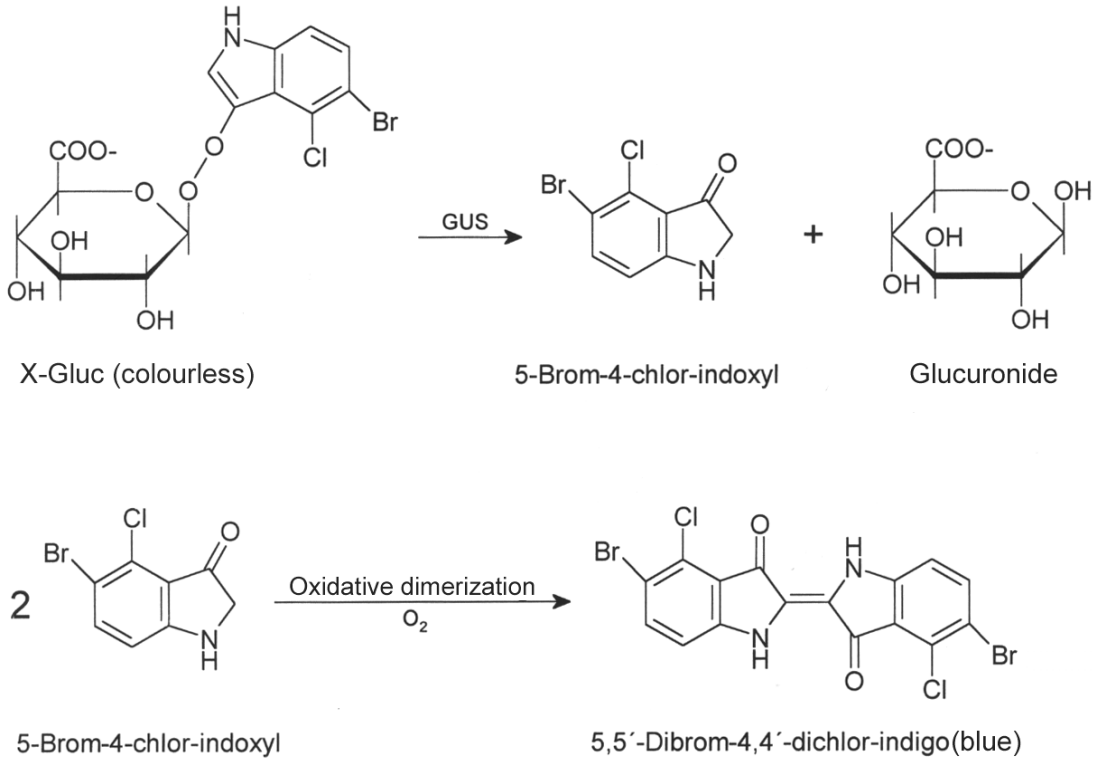
In case of hybridising the same RNA filter for more than once with different DNA probes, always be careful not to let the filter dry after the first hybridisation. Strip the old DNA probe from the RNA filter with 0.5% SDS solution.

Pre-heat 150~250ml 0.5% SDS solution to boiling (~100°C). Remove the plastic wrap from the filter and immediately immerse the filter in the boiling solution, continue to boil for 5~10 min. Remove the solution from heat and allow to cool for 10 min. Remove the filter from the solution and check the efficiency of stripping with a radioactivity counter. Repeat the stripping procedure if radioactivity can still be detected, then submit it for the next hybridisation.

2.8 Gus assay

2.8.1 Histochemical assay (qualitative assay)

As described by Jefferson et al. (1987), the colourless substrate X-Gluc (5-Brom-4-chlor-3-Indolyl-β-D-glucuronide) can be converted into 5-Brom-4-chlor-3-Indolyl and glucuronide by the catalysis of the enzyme β-Glucuronidase (Gus). Dimers of 5-Brom-4-chlor-3-Indolyl are subsequently formed as blue 5,5'-Dibrom-4,4'-Dichlor-Indolyl by oxidation. Tissues such as leaves of transgenic plants with Gus expression can be directly immersed into the Gus staining solution (X-Gluc) for in vivo reaction at 37°C. After a certain amount of time, blue spots or blocks may appear in the tissue and become more obvious after extraction of the chlorophyll.



X-Gluc Staining solution:

phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH7 .0.....100mM

EDTA.....1mM

$K_3[Fe^{III}(CN)_6]$.....1mM

$K_4[Fe^{II}(CN)_6]$.....1mM

Triton X-100.....0.3%(v/v)

X-Gluc, pre-dissolved in 10-fold (w/v) DMF.....1mM

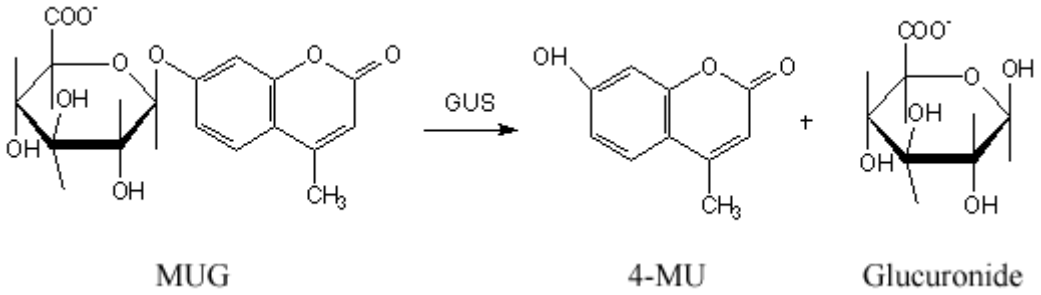
filter sterilise, aliquot and store at –20°C.

100mM phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH7.0 can be prepared by mixing 6.1% (v/v) 1M Na_2HPO_4 and 3.9%(v/v) 1M NaH_2PO_4.

Take a piece of plant tissue by forceps or scalpel and submerge into 0.1~0.5ml X-gluc staining solution. Apply vacuum infiltration and incubate at 37°C overnight. Optionally fix the tissue by removing the staining solution and adding the fixation solution (5% v/v formaldehyde, 5% v/v acetic acid, 20% v/v ethanol; store at RT) for 10 min on a shaker, then bleach the tissue by discarding the fixation buffer and incubating in 70% or 80% ethanol at RT or 60°C overnight or longer with alterations of the ethanol for several times.

2.8.2 Fluorimetric assay (quantitative assay)

Like X-Gluc, the substrate 4-MUG (4-Methyl-umbelliferyl-β-D-glucuronide) can be converted into 4-MU (4-methyl-umbelliferon or 7-hydroxy-4-methyl-cumarin) and glucuronide by the catalysis of Gus. Fluorescence of 4-MU can be obtained at a wavelength of 365nm for excitation, 445nm for emission, and measured in a fluorimeter (TKO100, Hoefer).



2.8.2.1 Solutions

- GUS extraction Buffer:

  phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH7.0.....50mM

  EDTA.....10mM

  N-Lauroylsarcosine.....0.1%(v/v)

  Triton X-100.....0.1%(v/v)

  filter sterilise and store at room temperature.

  β-Mercaptoethanol(add 70µl/100ml buffer prior to use) 10mM

- fluorescence assay buffer:

  4-MUG (4-methyl-umbelliferyl-β-D-glucuronide).....1mM

  dissolve 3.523mg 4-MUG into 10ml Gus extraction buffer to achieve a concentration of 1mM, mix thoroughly and keep on ice.

- stop buffer:

  Na_2CO_3.....0.2M, autoclaved

2.8.2.2 Preparation of Gus extracts

2.8.2.2.1 From protoplasts and chloroplasts

Prepare the protoplasts and chloroplasts of tobacco according to the corresponding protocols. Collect the suspension of protoplasts or chloroplasts into a 2ml Eppendorf tube, then centrifuge (100x g, 5 min for protoplasts; 2000x g, 5 min for chloroplasts; or full speed, 30 sec for both). Remove the supernatant carefully without disturbance to the pellet by

decanting. Add 100~200µl Gus extraction buffer and about 1g quartz sand, vortex vigorously for 2 min (or disrupt the protoplast cells or organelles by freeze-thawing in addition to strong vortexing for several times). Centrifuge at maximum speed (14000rpm) for 5 min at 4°C to pellet as much solid debris as possible. Pipette the clear supernatant into a new Eppendorf tube and place on ice.

2.8.2.2.2 From plant tissue

Fill 200µl Gus extraction buffer and a suitable amount of quartz sand into a 2ml Eppendorf tube, pre-cooled on ice. Take ~100mg plant tissue, fresh or frozen, and grind it by use of a motor-driven glass homogeniser at low speed to get a fine homogenate. Then centrifuge at 15000rpm for 3 min. Transfer the supernatant to a new Eppendorf tube and place on ice. Re-add 200µl Gus extraction buffer and vigorously for 2 min, then centrifuge again. Combine the two parts of supernatant for the same sample, centrifuge at 15000rpm for 5 min, and transfer the clean supernatant to a new tube, avoiding any sand particles. Keep the sample of protein extract on ice.

Gus extracts can be kept at –70°C for a long time, on ice or 4°C for several days. Storing at –20°C is not recommended.

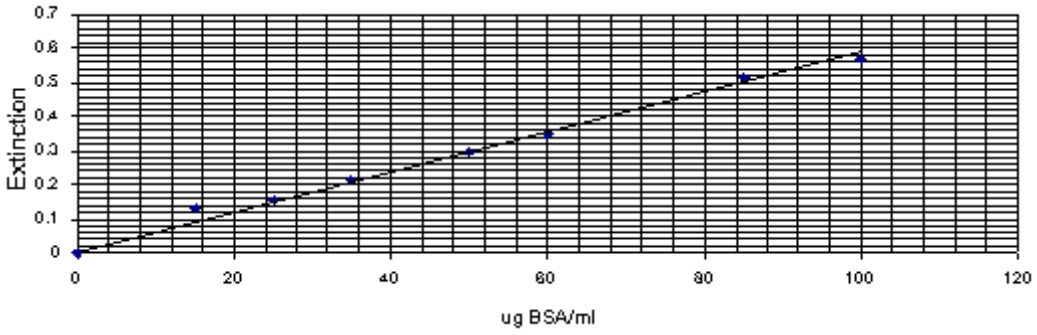
2.8.2.3 Determination of protein concentration by the 'Bradford' method

Measure the protein concentration of the Gus extract by the 'Bradford' method (Bradford, 1976).

Bradford solution:

Coomassie Brilliant Blue G (Sigma).....100mg
95-100% ethanol.....50ml
85% phosphate acid (H_3PO_4).....100ml
distilled water .....to 1000ml
Keep at 4°C in a brown glass container.

Add Xµl Gus extract and (100-X)µl H_2O into a disposable cuvette (Sarstedt), do not use the extraction buffer instead of H_2O, as it might have the negative influence on the accuracy of the protein concentration due to the interaction of Bradford solution with the detergents inside the extraction buffer. Pipette 1ml Bradford solution into the cuvette and mix thoroughly. Incubate the reaction in the dark for 15 min, then measure the absorption of the samples at a wavelength of 595nm. Use a set of BSA dilutions to make the standard curve as below for calibrating the concentration of the samples.



2.8.2.4 Gus measurement

Mix Xµl Gus extract and (450-x)µl Gus assay buffer thoroughly and incubate at 37°C, rapidly take 100µl into 900µl stop buffer at time points such as 0h, 2h, 24h, etc, mix well and measure the fluorescence value by the Fluorimeter (TKO 100, Hoefer) afterwards.

2.8.2.5 Evaluation of Gus activity

The Gus activity in pmol 4-MU per hour and µg protein can be calculated by the following equation:

$$\text{Gus activity [pmol 4-MU/(h·µg protein)]} = \frac{\text{Conc(4-MU in stopped sample)[nmol/L]} \cdot \text{dilution factor} \cdot \text{vol(assay mix)[µl]}}{\text{Vol(extract in assay mix)[µl]} \cdot \text{incubation time[h]} \cdot \text{conc(protein in extract)[µg/ml]}}$$

Notes:

Conc(4-MU): the concentration of 4-MU of the stopped sample in cuvette;

Dilution factor: the ratio of the volume (stop buffer + assay mixture) to the volume of assay mixture;

Vol (assay mix): the total volume of assay mixture;

Vol (extract in assay mix): the volume of Gus extract used for assay;

Incubation time: the time durance of the assay;

Conc (protein in extract): the concentration of total protein in the Gus extract.

2.8.3 PAGE display of Gus activity

2.8.3.1 Solutions

- Gus extraction buffer (see Gus assay)
- 1M Tris·HCI, pH 8.8
- 5x Tris-glycine buffer (for gel running)

  dissolve 15.1g Tris and 94g glycine into distilled H_2O, then add H_2O to 1 L.
- 2.5x protein gel loading buffer
- 0.01% Bromophenol Blue, 25% glycerol

  For 1.5ml, pipette 5.4µl saturated solution of Bromophenol Blue and 0.375ml glycerol in a 2ml Eppendorf tube, add distilled H_2O to 1.5ml and mix thoroughly.
- 100mM phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH7.0

  6.1%(v/v) 1M Na_2HPO_4.....6.1ml

  3.9%(v/v) 1M NaH_2PO_4.....3.9ml

  add 90 ml distilled H_2O .....to 100ml
- 0.5mM 4-MUG solution in 50mM phosphate buffer
- Gus staining buffer (X-Gluc), see Gus assay.

2.8.3.2 Polyacrylamide gel electrophoresis (PAGE)

Clean the glass plates and wash with distilled water several times, then use 100% ethanol to rinse it and air-dry them. Assemble the mini-gel apparatus of Bio-Rad. Prepare 6% native gel as described in Molecular Cloning ^{2nd} (Sambrook et al., 1989), free of SDS which can denature the proteins and may inactivate the enzymes.

6% Polyacrylamide gel (15 ml):

distilled H_2O.....8ml

30% acrylamide/Bis.....3ml

1.5M Tris·HCI, pH8.8.....3.8ml

10% APS (ammonium persulphate).....0.15ml

TEMED.....0.012ml

Add each component in a 50ml flask. Degas the mixture optionally prior to the addition of 10%APS and TEMED. Mix the solution well and let the polymerisation start for a

few of seconds, then use a 5ml glass pipette to pour the gel. Transfer the gel plate to the running chamber after solidification, remove the combs, and rinse the slots.

Load an appropriate volume of Gus extract diluted in 1x protein loading buffer in the slots of the gel. Run the gel in the cool room at 60 V for 3 hours. After electrophoresis, disassemble the gel carefully from the gel unit and put in the small plastic box. Rinse the gel with sterile distilled water and submit to the following biochemical assays.

2.8.3.3 In gel Gus fluorescence assay (4-MUG)

Equilibrate the gel with 50mM phosphate buffer for 1~2 min, then discard the buffer. Incubate the gel with 50mM 4-MUG for 10~30 min, 37°C on a gentle shaker, monitor the reaction by visualisation under UV illumination several times to obtain the optimum appearance. Remove the reaction solution, rinse with 50mM phosphate buffer and keep in the same buffer or distilled water. Place the gel on the plate of UV-cabinet carefully and photograph under UV illumination.

2.8.3.4 In gel Gus staining (X-Gluc)

Equilibrate the gel with 100mM phosphate buffer for 1~2 min, then decant the buffer. Immerse the gel into the Gus staining solution (X-Gluc) and incubate at 37°C for several hours or overnight. Remove the Gus staining solution, rinse with 100mM phosphate buffer, and keep in the same buffer or distilled water. Place the gel on a glass plate and remove as much liquid as possible. Put a layer of Whatman filter paper on the gel to enable the gel to attach the paper, turn up, then cover with a layer of Parafilm. Document by photography.

2.9 Chloroplast transformation of tobacco by microprojectile bombardment

2.9.1 Plant materials

Germinate seeds of *Nicotiana tabacum, pet. havana* on B5 or MS medium and cultivate 2~3 week-old sterile tobacco seedlings at a photoperiod of 16h light/ 8h dark in the culture chamber. Excise full expanded leaves by the sterile scalpel and put on RMOP medium (without antibiotic) with the upper side up, then pre-incubate them in the culture chamber overnight.

2.9.2 Chloroplast transformation

2.9.2.1 Preparatory work

Sterilise the workbench under UV-light and with 75% ethanol. For microprojectile bombardment, place the macrocarrier holder, macrocarrier, stopping screens and rupture discs in 100% ethanol for about 1min, then let them dry in sterile petri-dishes.

2.9.2.2 Preparation of gold-coated DNA microprojectiles

2.9.2.2.1 Wash the gold particles

Vortex the gold stock suspension (60mg ∅0.6µm gold particles in 1ml 100% ethanol) for at least 1 min, then immediately transfer 35µl to a sterile 1.5ml Eppendorf tube. Spin the gold down at 14000rpm for 10 sec by a conventional tabletop centrifuge. Discard the supernatant and resuspend the gold in 1ml sterile distilled H_2O by short vortexing. Spin down 1 min at 14000rpm and decant as much supernatant as possible.

The following steps should be executed on ice!

2.9.2.2.2 Coat the gold particles with DNA

Pipette 230µl sterile distilled H_2O, 250µl 2.5M $CaCl_2$ in the tube with gold particles (Store 2.5M $CaCl_2$ in aliquots at –20°C) and mix, then add 25µg plasmid DNA (1.0µg/µl in distilled H_2O) and suspend the gold particles well by pipetting with a 1000µl blue tip. After that, add 50µl 0.1M spermidine (stored in aliquots at -70°C) and mix thoroughly by pipetting. Incubate the suspension on ice for 10 min and vortex every 2~3 min.

2.9.2.2.3 Wash the gold particles coated with DNA

Spin down the gold particles at 10000rpm for 1 min and remove the supernatant completely. To wash the pellet, add 600µl 100% ethanol and resuspend by scraping the tube wall and pipetting up and down with a 1000µl pipette tip, then vortex gently and spin down at 10000rpm for 1 min. Repeat the washing once.

2.9.2.2.4 Suspend the gold particles coated with DNA

Spin down the gold particles at 10000rpm for 1 min, decant the supernatant and resuspend the gold carefully but thoroughly in 72µl 100% ethanol.

2.9.2.3 Bombardment of gold-coated DNA microprojectiles on plant leaves

The biolistic apparatus PDS He-1000 and all required consumables are purchased from Bio-Rad Co.

Set parameters for bombardment as below:

helium pressure:	1100psi
rupture disc:	900psi
distance rupture disc/macrocarrier:	8~10mm
distance macrocarrier/stopping screen:	10mm
distance stopping plate/leaf:	approximately 7cm
vacuum:	26~27 inches Hg

Apply 5.4µl of the DNA-coated gold suspension to the middle of each macrocarrier and air-dry completely (take gold suspension from the top of the tube). Place the macrocarrier into the macrocarrier holder. Deploy the rupture disc, the stopping screen and the macrocarrier holder correctly in the vacuum chamber, and fix them well. Put the petri-dish with plant leaf on RMOP medium at the right position inside the chamber, close the chamber door and turn on the vacuum till 26~27 inches Hg, then trigger the shot on the leaf.

2.9.2.4 Cultivation of transformed leaves

Incubate the bombarded leaves in the culture chamber for 2 days, then cut the leaves into small pieces (5mm x 5mm), place on RMOP medium containing the selective antibiotic (e.g. 500mg/l spectinomycin or streptomycin) and incubate in the culture chamber at 25°C with 16h light and 8h dark period. After 2 weeks of cultivation, partition the swelled leaf explants in small pieces and transfer all to fresh RMOP medium containing 500mg/l spectinomycin. Subsequently renew the medium every 3 weeks. About 4 weeks after bombardment, the first transformants are normally regenerated.

2.9.2.5 Cycling of shoot regeneration and cultivation of transplastomic plants

Dissect the shoots of primary resistant transformants into small pieces (2.5mm x 2.5mm), and place them on the selective RMOP medium in a small petri-dish (∅6cm) for plastid segregation. After several cycles of repeated shoot regeneration, cultivate the homoplasmic-state of shoot regenerates on B5 medium containing 500mg/l spectinomycin to grow the transplastomic plants.

3. Results

3.1 Construction of versatile plastid transformation vector

In regard to efficient transformation and high stability of discrete transcripts of foreign genes in tobacco chloroplasts, a new plastid transformation vector was developed. The space region between two plastid genes is suitable and widely used for foreign gene insertion. In addition, the transcripts of tRNA genes surrounding the insertion site are thought to promote mRNA stability of foreign genes. Foreign integration into plastomic inverted repeats can duplicate the transgene copies in the plastome in case of the requirement for highest expression of transgene. Therefore, a *MunI* site between two tRNA genes (trnR-ACG and trnN-GUU) in the inverted repeats (IR) was selected as a new insertion point (figure 3.1).

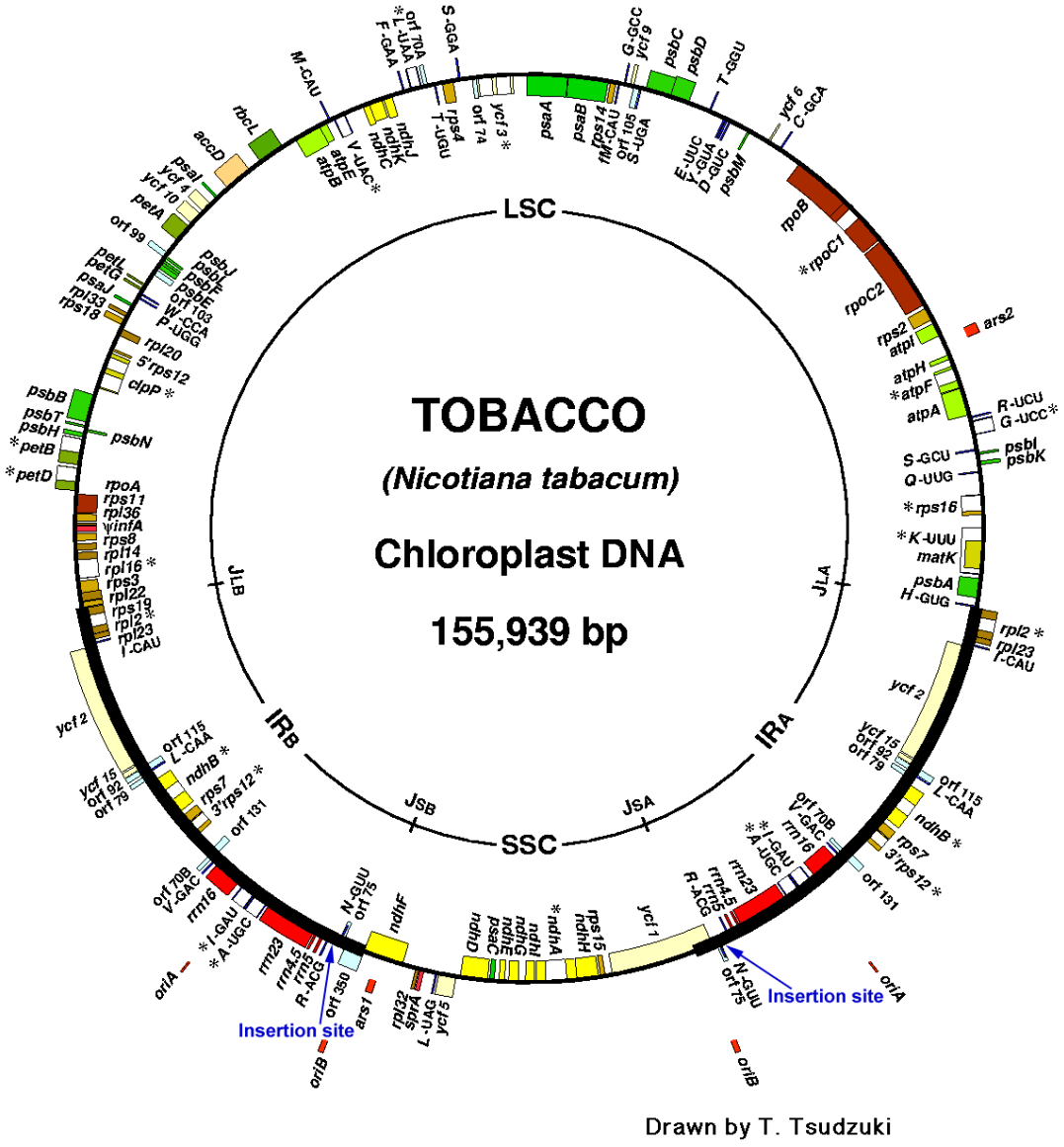


Figure 3.1: The map of tobacco plastome and the insertion site (indicated by arrow)

Considering that plastid transformation is conducted by homologous recombination, two plastid homologous fragments are required as the components of new plastid transformation vector. Those were obtained by PCR amplification as described below.

3.1.1 Amplification of tobacco plastid homologous fragments INSL and INSR

A pair of primers was synthesised to amplify the flanking fragment toward the rRNA genes and on the left side of insertion site *MunI*, termed as INSL. Forward primer INSL-Fw is identical to the sequence of the tobacco plastome, and the underlined two bases (CT) at its 5' end is derived from the recognition sequence of enzyme *BglII* in order to reconfirm the mechanism of plastid transformation via homologous recombination. The reverse primer INSL-Re contains the internal *MunI* site at the 3' end and two introduced unique enzyme sites *BglII* and *NheI* by nucleotide alterations within the part near its 5'-end.

INSL-Fw: 5'-CTTGCTGTTGCATCGAAAGAG-3'

INSL-Re: 5'-AGATCTTTGCTAGCGGCAATTGTG-3'
BglII NheI MunI

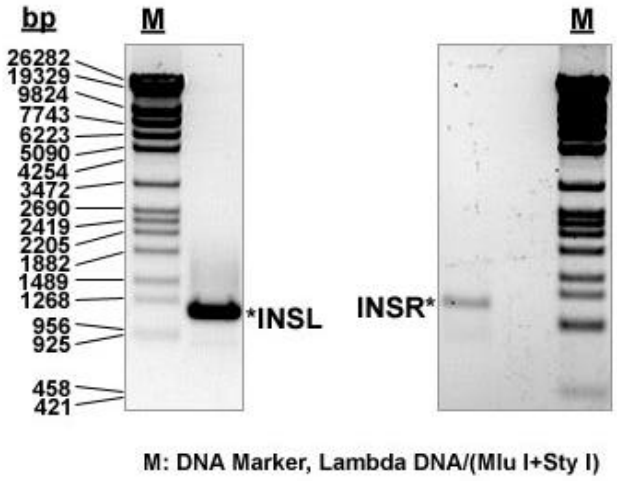


Figure 3.2: Amplification of plastid homologous fragments INSL and INSR

Similarly, another pair of primers was designed to produce the fragment close to the small single copy region (SSC) of the plastome and on the right side of insertion site *MunI*, named as INSR. Reverse primer INSR-Re is identical to the plastomic sequence with an internal *EcoRI* site at its 5'-end, while the forward primer INSR-Fw contains the internal *MunI* near the 3'-end and two additive unique enzyme sites *XhoI* and *Eco47III* created by base modifications within the part adjacent to its 5'-end.

INSR-Fw: 5'-TCTCGAGCGCTCACAATTGCCG-3'
XhoI Eco47III MunI

INSR-Re: 5'- TTGAGAATTCAAGAGAAGGTCAC-3'
EcoRI

Using tobacco total DNA as template, a ~1.2kb DNA fragment INSL and a ~1.1kb INSR were specifically amplified by their corresponding primers and *Pfu* DNA polymerase, respectively (figure 3.2, and figure 3.6, A and B).

3.1.2 PCR Cloning of INSL and INSR in pUC18

PCR products INSL and INSR were purified from 1.0% agarose gel. As *Pfu* DNA polymerase conferring high-fidelity by strong 3'→5' proofing during amplification, can only give rise to uniquely blunt DNA products. The standard vector pUC18 that contains three blunt enzymes site *SmaI*, *HincII*, and *Ecl136II* within its multiple cloning site (MCS) is applicable to clone the *Pfu*-amplified PCR products, in combination with the strategy of white-blue screening to identify the recombinant clones on LB plates containing a mixture of X-gal and IPTG.

For an overall consideration of the cloning procedures to assemble the final plastid transformation vector, the blunt enzyme *HincII* site was chosen to take in the plastid homologous fragment INSL (figure 3.6, C) as well as *SmaI* for insertion of INSR (figure 3.6, D) in plasmid pUC18. The recombinant clones with the insert of INSL or INSR in the expected orientation were nominated as pUCINSL or pUCINSR, respectively.

3.1.3 Inverting aadA cassette in pUC16SaadASma

Plasmid pUC16S*aadA*Sma contains the *aadA* gene flanked by a short synthetic ribosome-binding sequence (RBS) and the *Chlamydomonas rbcL* 3'UTR, and the *16S rRNA* promoter (Prrn). The *aadA* gene is a powerful selection marker in chloroplast transformation, conferring resistance to both spectinomycin and streptomycin. Prrn is a constitutive promoter in chloroplasts and prokaryotes. The *Chlamydomonas rbcL* 3'UTR i.e. rbcL3'(Chl) acts as a putative transcriptional terminator and mRNA stabilising element, and the 26bp synthetic RBS that comprises a canonical SD sequence (GGAGG) is sufficient to initiate *aadA* translation. Another feature of this plasmid is the presence of double *EcoRI* and *SmaI* sites, the later at both sides of the *aadA* cassette (Prrn-RBS-*aadA*-rbcL3'Chl). Thus, the *aadA* cassette was easily inverted in the same plasmid by *SmaI* cutting and self religation, which resulted in two *EcoRI* sites separated by the *aadA* cassette, suitable for the following ligation with plastid homologous fragments (figure 3.6, E).

3.1.4 Removal of the dispensable enzymes sites in pUCINSL and pUCINSR

Based on the overall design of this new plastid transformation vector with the desire that each constituent of this vector such as selection marker, promoter, etc. can be dissected by unique enzymes, duplicates of several enzymes in earlier cloning procedures must be deleted in this step. A combined digestion of plasmid pUCINSL with *PstI* and *SphI* followed by blunting with T4 DNA polymerase and religation removed both of these two enzymes sites (figure 3.6, F). Similarly, a complete cut with *EcoRI* of plasmid pUCINSR and subsequent religation led to the removal of the *KpnI* and *SacI* sites between the two *EcoRI* sites in plasmid pUCINSR (figure 3.6, G).

3.1.5 Subcloning of tobacco rbcL 3' UTR into pUC18

In order to facilitate transfer of the *aadA* cassette into another vector or to reintroduce foreign gene inserts adjacent to the selection marker, tobacco *rbcL* 3'UTR in plasmid pUC16SRBSuidArbcL3' abbreviated to '3'UTR(Tob)', as a terminator/stabiliser of *uidA*, was subcloned into plasmid pUC18. A 180bp DNA fragment of *rbcL* 3'UTR was excised by *EcoRI* and *Ecl136II* digestion and subsequently blunted with *Klenow* DNA polymerase, then ligated with *Ecl136II*-digested pUC18 DNA to create the recombinant plasmid pUCrbcL3' (Figure 3.6, H). In this plasmid, the truncated *EcoRI* site at the end of *rbcL* 3'UTR was regenerated, resulting in two *EcoRI* sites on both sides of the insert *rbcL* 3'UTR with correct orientation depicted in figure 3.3. Consequently, two enzyme sites *NotI* and *SacII* become available in pUCrbcL3', which were derived from the plasmid pUC16SRBSuidArbcL3'.

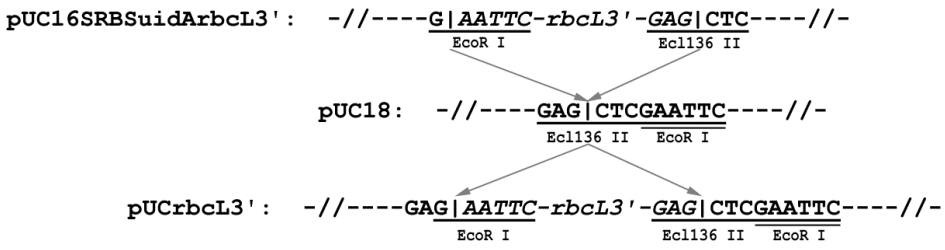


Figure 3.3: Subcloning of tobacco *rbcL* 3'UTR into plasmid pUC18

3.1.6 Combination of INSL with the aadA cassette

As enzymes *MunI* and *EcoRI* generate the same overhang ($^{\downarrow 5'}$AATT$_{\uparrow 3'}$), the vector and the insert with this overhang formed by each of these two enzymes can be ligated by nucleotide complementation. Plasmid pUCINSL was cut by *MunI* completely as the vector to accept the *aadA* cassette excised from the modified plasmid pUC16S*aadA*Sma as described

above (context 3.1.3) by *EcoRI* digestion to generate the recombinant plasmid pUCINSL*aadA* (figure3.6, I).

3.1.7 Combination of INSR with tobacco rbcL 3'UTR

In plasmid pUCINSR, the PCR insert INSR contained two *MunI* sites, only the one within the primer INSR-Fw was proper to incorporate the tobacco *rbcL* 3'UTR. Thereby, plasmid pUCINSR had to be partially digested with *MunI*, the full-length of DNA fragment with single *MunI* cut was carefully recovered and purified as the vector to take in the insert of tobacco *rbcL* 3'UTR from pUCrbcL3' by *EcoRI* digestion. A positive recombinant clone with correct insertion site and orientation was identified by restriction and termed pUCrbcL3'INSR (figure 3.6, J).

3.1.8 Removal of the dispensable enzyme site in pUCINSLaadA

Plasmid pUCINSL*aadA* was fully cut by *SacII*, blunted by T4 DNA polymerase, then purified and self-religated. A positive clone with the *SacII* site removed was identified by enzyme digestion as pUCINSL*aadA*(Δ*SacII*). Deletion of the *SacII* site in this step supplies a unique *SacII* site in the final plastid transformation vector (figure 3.6, K).

3.1.9 Assembly of the final vector

As plasmid pUCINSL*aadA* (Δ*SacII*) has two required *EcoRI* sites that could cause the loss of the *aadA* cassette by full digestion, this plasmid was partially digested with *EcoRI* under careful monitoring, after the full reaction with *XbaI*. The full size DNA fragment with a single *EcoRI* cut was gel-purified, as the vector to combine the insert of rbcL3'INSR obtained by double digestion with *XbaI* and *EcoRI* of plasmid pUCrbcL3'INSR and achieve the resultant plasmid pUCINSL*aadA*rbcL3'INSR, which was finally termed plastid transformation vector pKCZ (figure 3.6, L).

3.1.10 Confirmation of vector pKCZ by enzyme profiling and sequencing

Plasmid pKCZ DNA was prepared in large scale by Qiagen plasmid Maxi-kit. Proper amounts of DNA were used for enzyme profiling (figure 3.4). About 10μg pKCZ DNA was submitted for automatic sequencing by ABI 377 through a strategy of primer walking in *Toplab* company. The full sequence of pKCZ was achieved and identical to the computer-imitated data by program Vector NT6 suite (Informax Co.), with an insert 3934bp (INSL-

aadA cassette-rbcL3'Tob-INSR) on the backbone of pUC18. Meanwhile, the plasmid map of pKCZ was created automatically by Vector NT6 as shown in figure 3.5.

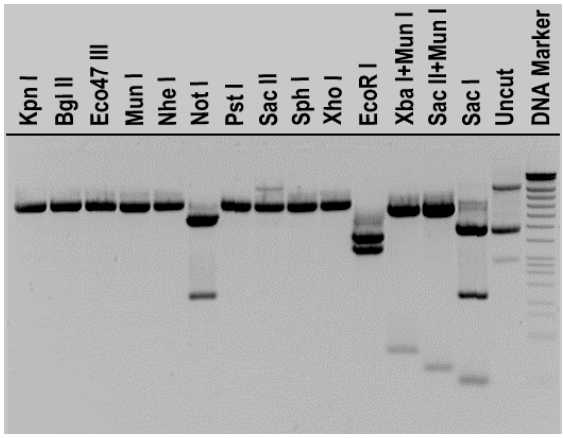


Figure 3.4: The restriction enzyme profiling of vector pKCZ

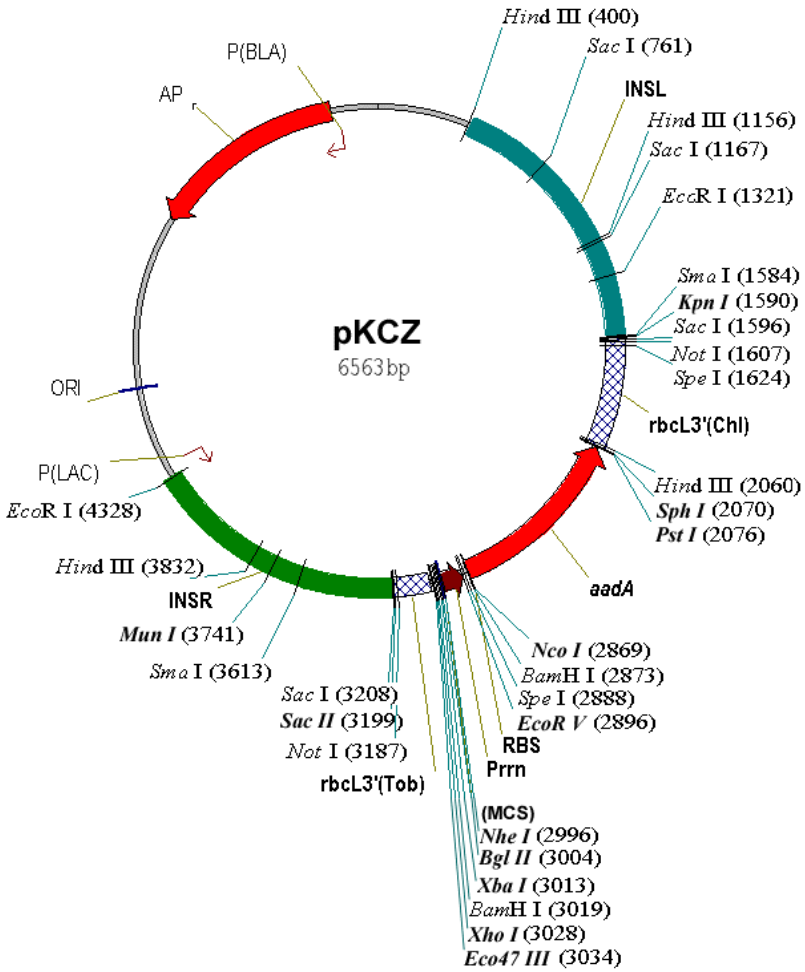
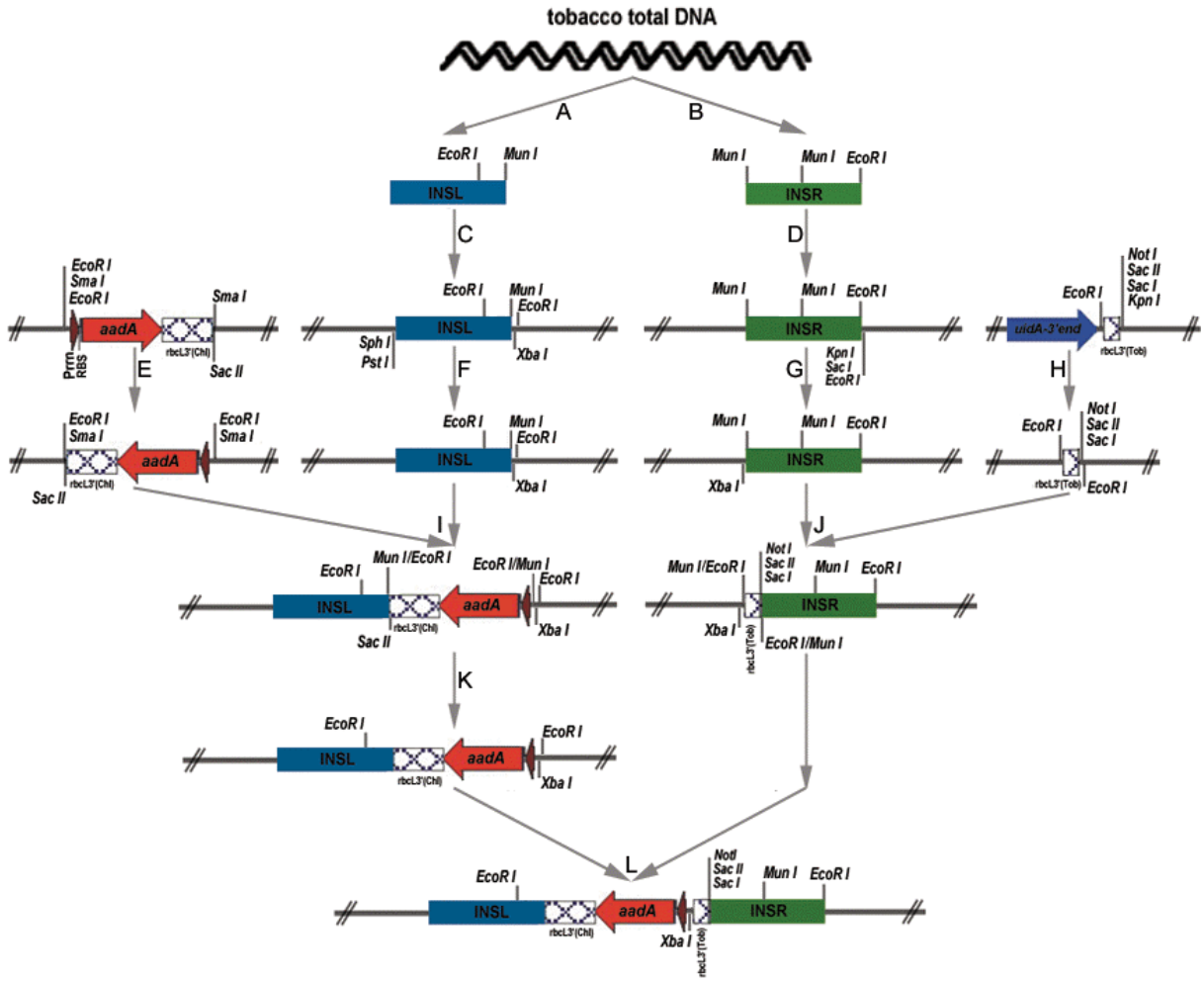


Figure 3.5: The physical map of plastid transformation vector pKCZ



The process to construct the versatile plastid transformation vector pKCZ

A: PCR amplifying plastid homologous fragment INSL(~1.2kb) from tobacco total DNA;
B: PCR amplifying plastid homologous fragment INSR(~1.1kb) from tobacco total DNA;
C: Cloning INSL into pUC18 at *Hinc II* site as pUCINSL;
D: Cloning INSR into pUC18 at *Sma I* site as pUCINSR;
E: Inverting aadA cassette in pUC16SaadASma;
F: Removing *Sph I* and *Pst I* in pUCINSL;
G: Removing *Kpn I* and *Sac I* in pUCINSR;
H: Subcloning rbcL 3'UTR from pUC16SRBSuidArbcL3' into pUC18 at *Ecl136 II* site as pUCrbcL3';
I: Combining aadA cassette and INSL at *Mun I* site as pUCINSLaadA;
J: Combining rbcL 3'UTR and INSR at *Mun I* site as pUCrbcL3'INSR;
K: Removing *Sac II* from pUCINSLaadA;
L: Combining INSLaadA and rbcL3'INSR to form the final vector pUCINSLaadArbcL3'INSR named as pKCZ.

Figure 3.6: The overall procedures to complete the construction of vector pKCZ

As seen in figure 3.5, vector pKCZ contains eight components: pUC18 as the backbone, INSL, *rbcL* 3'UTR(Chl), *aadA*, RBS, Prrn, *rbcL* 3'UTR(Tob) and INSR. Each component between INSL and INSR can be removed by a pair of unique enzymes such as *KpnI*, *SphI*, *PstI*, *NcoI*, *EcoRV*, *BglII*, *NheI*, *XbaI*, *XhoI*, *Eco47III*, and *SacII*. Furthermore, this vector also offers a *NotI* site that is exploitable to transplant the fragment between INSL and INSR into another vector featured with a specific insertion site. Tobacco *rbcL* 3'UTR can serve as a universal transcript terminator/stabiliser for another gene insert of interest.

Nucleotide modifications in synthetic primers for amplifications of INSL and INSR introduced several useful unique enzyme sites thus generating convenient multiple cloning sites (MCS). This MCS can also act as a short exogenous space region to spatially separate a future gene insert from the selection marker *aadA*, especially in case that both genes utilise the same promoter (Prrn) in the same construct.

3.2 Tobacco psbA 5' UTR modification and its related transformation construct

3.2.1 Basis and overall process of psbA 5'UTR modification

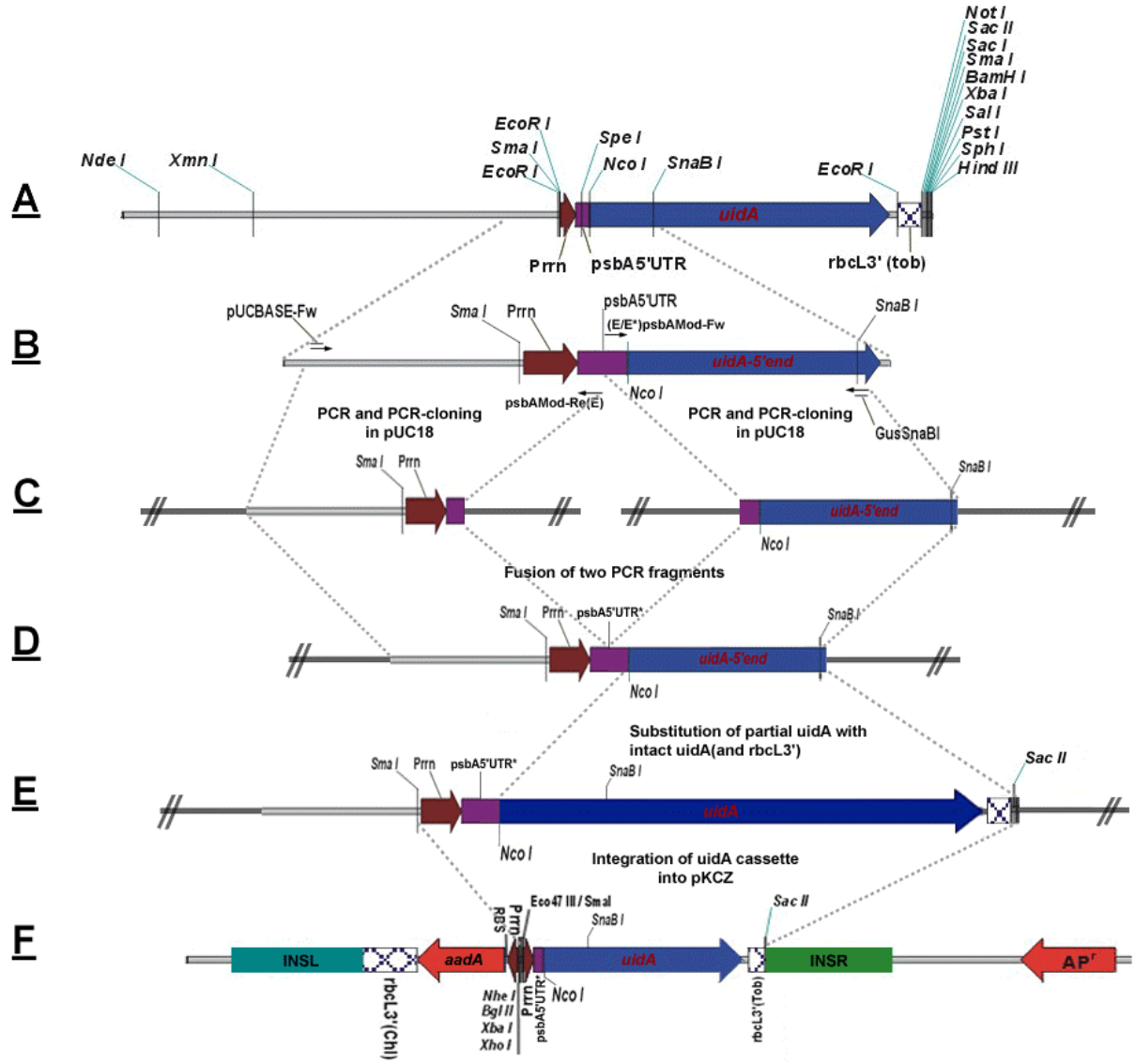
The *psbA* gene is a main component in photosynthesis. The light-regulated gene expression of *psbA* is conducted by phosphorylation and redox potential that modulate the interaction of a complex of RNA binding proteins (RBPs) and *psbA* 5'UTR. A working model of *psbA* gene regulation has been postulated (Trebitsh et al., 2000). The role of the tobacco *psbA* 5'UTR has been previously investigated by *in vivo* analyses via chloroplast transformation (Eibl et al., 1999; Staub and Maliga, 1994a). Those investigations did not supply detailed information about the *cis*-acting elements for the stability and translation of *psbA* in chimeric transcripts. By use of an *in vitro* chloroplast translation system, three *cis*-acting sequences essential for *psbA* translation have been determined (Hirose and Sugiura, 1996), whereas other elements required for mRNA stability were inevitably disregarded. Thus, the *in vivo* analysis via chloroplast transformation appears to be the most suitable approach to simultaneously define the *cis*-acting determinants for mRNA stability and translation in higher plants as well as in *Chlamydomonas* (Higgs et al., 1999; Mayfield et al., 1994; Nickelsen et al., 1999). Accordingly, it is exploited in this study that aims to characterise the *cis*-acting elements of tobacco *psbA* 5'UTR correlated to the modulations of mRNA stability and translation efficiency. For this purpose, serial modifications of the *psbA* 5'UTR were created and used as the 5' leader sequence of reporter *uidA* gene in a set of plastid transformation constructs. By comparing the Gus activities and chimeric *uidA* mRNA levels in the corresponding transplastomic plants, the *cis* elements of *psbA* 5'UTR conferring the particular regulatory function could be determined.

The full-length (85nt) tobacco *psbA* 5'UTR existing in plasmids pUC16SpsbA5'uidA-rbcL3' or pUC16SpsbA5'uidApsbA3' is shown in figure 3.16 (page 75). From the 5'end to the 3' end of this 5'UTR, some *cis*-elements might be of regulatory significance, including the

poly(A) sequence (AAAAA), stem-loop (SL), SD-like RBS (GGAG), internal AUG codon and AU-Box (UAAAUAAA). Furthermore, the 5'end structural sequences of the *rbcL* 5'UTR were presumed to convey high mRNA stability signals to the *psbA* 5'UTR. Based on these concerns, the following modifications of the *psbA* 5'UTR were introduced.

In plasmid pUC16SpsbA5'uidArbcL3', the reporter *uidA* is flanked by *psbA* 5'UTR and *rbcL* 3'UTR as the leader/trailer and driven by promoter Prrn in the framework of plasmid pUC18. This plasmid was used as the template for *Pfu* DNA amplification to modify the *psbA* 5'UTR through a strategy as indicated in figure 3.7. Two common primers were used in all PCR-based mutagenesis of *psbA*5'UTR, in which one primer pUCBASE-Fw (5'-CTGCGTTATCCCCTGATTCTGTG-3') was based on the sequence of pUC18, and another primer GusSnaBI (5'-TCACACAAACGGTGATACGTAC-3') was according to the coding region of *uidA* gene. For each *psbA* 5'UTR modification, a pair of primers was designed with introduction of specific restriction enzyme sites (termed as E or E* in all cases). One generally named as PsbAMod-Re(E) together with primer pUCBASE-Fw could be used to generate the PCR fragment PucPsbAmod (450~530bp) containing Prrn and 5' part of *psbA* 5'UTR, another as (E/E*)PsbAMod-Fw could couple with primer GusSnaBI to amplify the fragment PsbAmodGus (420~500bp) containing 3' part of *psbA* 5'UTR and partial *uidA* gene. Both PCR products with a part of *psbA* 5'UTR were cloned into plasmid pUC18 at proper sites by blunt end ligation. Then, the *psbA* 5'UTR was regenerated within the merged insert PucPsbAmodGus by digestions with enzymes (E or E*) and the related cloning operations. The partial *uidA* was further substituted with intact *uidA* from plasmid pUC16SpsbA5'uidA-rbcL3' by digestions with *NcoI* and another appropriate enzyme. The resultant plasmid resembled pUC16SpsbA5'uidArbcL3', but with a modified *psbA* 5'UTR. Finally, the fragment comprising Prrn, either form of *psbA* 5'UTR (wt or modified), *uidA* and tobacco *rbcL* 3'UTR (Prrn-psbA5'-*uidA*-rbcL3') was isolated by *SmaI* (blunt end enzyme) and *SacII*, then replaced the tobacco *rbcL* 3'UTR of vector pKCZ cut by *Eco47III* (blunt end enzyme) and *SacII* to create the plastid transformation construct containing wt or mutated *psbA* 5'UTR. The detailed process for each *psbA* 5'UTR modification and the generation of the final corresponding construct is individually described as below. In most cases, the versatile plastid transformation vector pKCZ digested by *Eco47III* and *SacII* with the removal of tobacco *rbcL* 3'UTR served as the frame in a general term of pKCZ (*Eco47III+SacII*) to take in the *uidA* cassette. However, few constructs were obtained by alternative pathways. As the controls, plastid constructs with wt *psbA* 5'UTR were also included. It is necessary to note here that the plasmid pUC16SpsbA5'uidArbcL3' applied for PCR-based mutagenesis of *psbA* 5'UTR had

a variant form of wild-type tobacco *psbA* 5'UTR termed as *ori**, which was resulted from its own PCR cloning in the previous work where the small poly(A) sequence at the 5' end was slightly changed into 'ACTAA' by two nucleotide alterations. Therefore, another control construct with original *psbA* 5'UTR termed as *ori* was specially required to examine the consequence of this small modification. For those constructs containing the *psbA* 3'UTR and wild-type or original *psbA* 5'UTR, this *psbA* 5'UTR could be distinctly marked as *Ori*-P* or *Ori-P*.



A: Plasmid pUC16SpsbA5'uidArbcL3'; B: the fragment of plasmid A containing Prrn, psbA 5'UTR and uidA 5' end, as the DNA template for PCR; C: PCR amplifications by primers pUCBASE-Fw and psbAMod-Re(E), GusSnaBI and (E/E*)psbAMod-Fw, and PCR cloning into pUC18 by blunt end ligation; D: Combination of PCR inserts to regenerate the modified psbA5'UTR; E: Replacement of uidA 5'end with intact uidA including the tailor rbcL3'UTR; F: Integration of uidA cassette into vector pKCZ.

Figure 3.7: The process to modify *psbA* 5'UTR and create plastid transformation constructs containing reporter *uidA* gene and various forms of *psbA* 5'UTR

3.2.2 Deletion of the poly(A) sequence (PolAD)

A pair of primers PolADel-Li and PolADel-Re was synthesised to delete the poly(A) sequence located at the 5'extremity of *psbA* 5'UTR. Either primer included a unique *SgfI* site at the 5'end (figure 3.8) that was used as the conjunction point for the reunion of *psbA* 5'UTR. After a multiple cloning process as shown below [1], the poly(A)-comprised sequence upstream of the stem-loop was entirely substituted with tri-bases (CGC) (figure 3.8; figure 3.16, page 75).

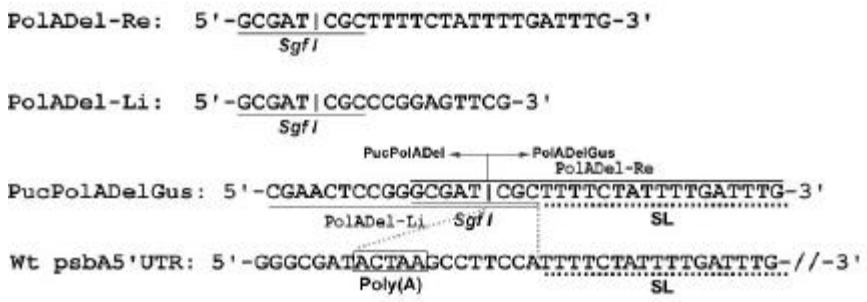


Figure 3.8: The strategy to create the poly(A) deletion of *psbA* 5'UTR

[1] Forward primer PolADel-Re together with the primer GusSnaBI was used to amplify the fragment PolADelGus (~490bp) containing the main part of the *psbA* 5'UTR and a partial *uidA* gene, while the reverse primer PolADel-Li coupled with primer pUCBASE-Fw could generate the Prrn-comprised PCR product PucPolADel (~450bp). Both PCR fragments were cloned into plasmid pUC18 at the *SmaI* site by blunt end ligation. The *psbA* 5'UTR with poly(A) deletion could be presumably regenerated by combining two above PCR inserts into PucPolADelGus at the *SgfI* site (figure 3.8). Surprisingly, all recombinant plasmids with insert PucPolADel failed in digestion by *SgfI*, due to a probable loss or blockage of this enzyme site. It was difficult to verify the PCR products by *SgfI* digestion or direct sequencing before their cloning into pUC18, as the *SgfI* site was located exactly at the 5'ends of those primers. Therefore, an alternative pathway had to be conceived to achieve the same goal.

Fortunately, the plasmids with PCR insert PolADelGus in either orientation were examined to contain *SgfI* site, which appeared to be indispensable to achieve the poly(A) deletion in this case. It was noted that the ancestral plasmid pUC16SaadASma comprised promoter Prrn and an *EcoRV* site within the synthetic RBS, from which plasmid pUC16SpsbA5'uidArbcL3' was originated by localising the *psbA* 5'UTR behind promoter Prrn exactly at the *EcoRV* site that was incidentally flawed. Interestingly, this *SgfI* cut-end with 3'-TA overhang can be blunted as 5'-CGC-//-3', and joined behind *EcoRV* cut-end 5'-Prrn-//-GGGCGAT↓-3' to recover the *SgfI* site, as indicated in figure 3.8.

The *aadA*-truncated pUC16SaadASma DNA available in the work of SL deletion (SLD, context 3.2.3) with the cut ends of *EcoRV* and *XbaI* was also selected as the cloning vector here. The plasmid with insert PolAdelGus containing *SgfI* site distal to *XhaI* was sequentially treated with *SgfI*, T4 DNA Polymerase and *XbaI* to dissect the insert PolADelGus that was further ligated to the prepared vector. In the resultant plasmid pUC16SpolADelGus, *SgfI* was restored, the entire part upstream of the stem-loop was substituted with tri-bases (CGC) and the poly(A) sequence of *psbA* 5'UTR was concurrently deleted. Afterwards, this plasmid was renewed with intact *uidA* gene from plasmid pUC16SpsbA5'uidA-rbcL3' by digestions with *NcoI* and *PstI* to create the intermediate plasmid containing the full *uidA* cassette PrrnpsbA5'(PolAD)uidArbcL3' that was subsequently excised by *SmaI* and *SacII* and subcloned into vector pKCZ (*EcoR47III +SacII*) to generate the plastid transformation construct pKCZ-psbA5'(PolAD)::rbcL3'. It was remarkable that this construct contained a unique *SgfI* site for future applications such as the replacement of plastid promoters.

3.2.3 Deletion of the stem-loop (SLD)

As the *psbA* 5'UTR offers an internal *SpeI* site adjacent to the stem-loop, it is feasible to truncate its forepart comprising the stem-loop and the 5' terminal sequence by digestions with *SpeI* and other appropriate enzyme outside of the *psbA* 5'UTR [2] (figure 3.16, page 75).

3.2.4 Local deletion of the stem-loop (NurSLD)

In deletion SLD, it was noted that both stem-loop region and its anterior part comprising the poly(A) sequence were simultaneously removed, which might have a coordinated contribution to regulation of chloroplast gene expression. Thus, it is necessary to examine local deletion of the stem-loop region in *psbA* 5'UTR independently. This *psbA* 5'UTR modification (NurSLD) was achieved as described below [3] and indicated in figure 3.16 (page 75).

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[2] Plasmid pUC16SpsbA5'uidArbcL3' DNA was linearised by *SpeI* completely, blunted by *Klenow* DNA polymerase, and further digested by *XbaI*. A ~2.0kb DNA fragment psbA5'(SLD)uidArbcL3' containing SL-truncated *psbA* 5'UTR was subsequently obtained and gel-purified. The plasmid pUC16SaadASma was fully digested by *EcoRV* and *XbaI* to remove the *aadA* cassette except promoter Prrn, then used as the vector to incorporate the insert psbA5'(SLD)uidArbcL3' to form the intermediate plasmid pUC16SpsbA5'(SLD)uidA-rbcL3'. There, the *psbA* 5'UTR as the 5'leader of *uidA* was mutated with a deletion of stem-loop region and its anterior sequence. Afterwards, the DNA fragment 16SpsbA5'(SLD)uidArbcL3' was dissected by *SmaI* and *SacII* and further subcloned into pKCZ(*Eco47III+SacII*) to generate plastid transformation construct pKCZ-psbA5'(SLD)::rbcL3'. A similar construct pKCZ-psbA5'(SLD-P)::psbA3' was created simultaneously. SLD-P differs from SLD in the respect of 3' trailer of *uidA* as *psbA* 3'UTR instead of *rbcL* 3'UTR.

[3] The intermediate plasmid with merged insert PucSLM1Gus during the process of SLM1 generation (context 3.2.5) contained the reconstituted *psbA* 5'UTR and an additional *MunI* site near the conjunction position of stem-loop and its anterior sequence. Therefore, it became feasible to locally delete the stem-loop region by digestions with *MunI* and *SpeI* internal, albeit in an indirect way due to the existence of another *MunI* site in the partial *uidA* gene.

Plasmid with the insert PucSLM1Gus was treated with *MunI*, *Klenow* DNA polymerase and *SacI* in turn to remove the *uidA*-related fragment, then served as the cloning vector with a small remainder of *psbA* 5'UTR i.e. the anterior sequence of the stem-loop region. Likewise, plasmid pUC16SpsbA5'uidArbcL3' was successively treated with *SpeI*, *Klenow* DNA polymerase and *SacI* to dissect the intact *uidA* gene flanked by *rbcL* 3'UTR and partial *psbA* 5'UTR covering the sequence exactly downstream of its stem-loop. Then the vector and *uidA* fragment were ligated to form an intermediate plasmid containing the full *uidA* cassette Prrn-psbA5'(NurSLD)-*uidA*-rbcL3' that was further excised by *SmaI* and *SacII*, then integrated into vector pKCZ (*EcoR47III+SacII*) to create plastid transformation construct pKCZ-psbA5'(NurSLD)::rbcL3'. In this construct, the stem-loop region was solely removed, with the 5' terminal sequence remained in the mutated *psbA* 5'UTR.

3.2.5 Mutation of the stem-loop (SLM1 and SLM2)

In the stem-loop region of the barley *psbA* 5'UTR, several nucleotides at the side of the stem adjacent to the 5' terminus were speculated to be crucial for the binding of a 95kd RBP (RNA binding protein) (Mullet, unpublished data). Since the *psbA* 5'UTR was found to be highly conserved in plants (Kim and Mullet, 1994), the interaction of 95kd RBP with the stem-loop sequence was proposed to occur similarly in tobacco. Thus, the putative 'core' nucleotides (asterisked in figure 3.9 and 3.10) in the stem-loop of tobacco *psbA* 5'UTR were submitted to mutagenesis, termed SLM1, with additional concern that nucleotide alterations did not change the melting temperature (Mt) of the stem-loop. Mt is known to be an important aspect to evaluate the status of stem-loop that might fluctuate among the linear, hairpin, and other intermediate structural states through its interaction with cpRBPs. Changes in Mt of the stem-loop were presumed to have an influence on chloroplast gene expression. Therefore, another stem-loop mutation was also conceived, termed as SLM2 in which the GC content of the stem-loop was increased by alternating some nucleotides excepting those putative 'core' bases (figure 3.10). The generations of SLM1 and SLM2 were conducted as shown below [4].

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[4] **First mutation of the stem-loop (SLM1)**

Two primers SLMut1-Li and SLMut1-Re were designed and synthesised (figure 3.9). Primer pair SLMut1-Li/pUCBASE-Fw was used for the generation of PCR product, Prrn-included PucSLM1, as well as another primer pair SLMut1-Re/GusSnaBI for the amplification of PCR product, SLM1Gus comprising partial *uidA* gene. Both PCR products with identical size (~470bp) were further cloned into plasmid pUC18 at *Ecl136II* and *SmaI* sites by blunt end ligation, respectively.

Because base residues 5'CTC3' (at the 5' end of primer SLMut1-Li) localised behind the cut-end (5'-//-GAG↓3') of *Ecl136II* in pUC18 could restore the *SacI* site (5'GAGCTC3'), the recombinant plasmids with insert PucSLM1 was preliminarily identified by *SacI* digestion. Likewise, primer SLMut1-Re contained an *SspI* site (5'AAT↓ATT3') at its 5' end, therefore the positive clones with insert SLM1Gus were primarily detected by *SspI* digestion, and those with introduced *SspI* site distal to *SacI* were selected. The pUC18 DNA was dually cut by *XbaI* and *SacI* to act as the cloning frame to accept the insert PucSLM1 with the cut-ends of *XbaI* and *SacI* (blunted), and another insert SLM1Gus with the cut-ends of *SspI* and *SacI*, from their related PCR clones. The resultant plasmid contained a merged insert PucSLM1Gus, in which the full-length of *psbA* 5'UTR with local nucleotide mutations in the stem was regenerated. With respect to this mutation, the stem-loop Mt was maintained, whereas four alterations of base-pairs occurred in the stem to inactivate the 'core' nucleotides, incidentally with an additive *MunI* site (5'C↓AATTG3') (figure 3.9). Then, the partial *uidA* was substituted with intact *uidA* by *NcoI* and *SacI* digestions to form the intermediate plasmid comprising the full *uidA* cassette Prrn-psbA5'(SLM1)-uidA-rbcL3' that was subsequently separated by *SmaI* and *SacII*, and introduced into vector pKCZ (*EcoR47III+SacII*) to create plastid transformation construct pKCZ-psbA5'(SLM1):: rbcL3'.

Second mutation of the stem-loop (SLM2)

Two primers SLMut2-Li and SLMut2-Re were synthesised, with internal nucleotide alterations for the mutation SLM2 (figure 3.10). Primer pairs SLMut2-Li/pUCBASE-Fw and SLMut2-Re/GusSnaBI were individually used to amplify the same size (~ 470bp) of PCR products PucSLM2 (Prrn-included) and SLM2Gus

The nucleotide substitutions for SLM1 and SLM2 were marked in distinct colours (see figure 3.16, page 75).

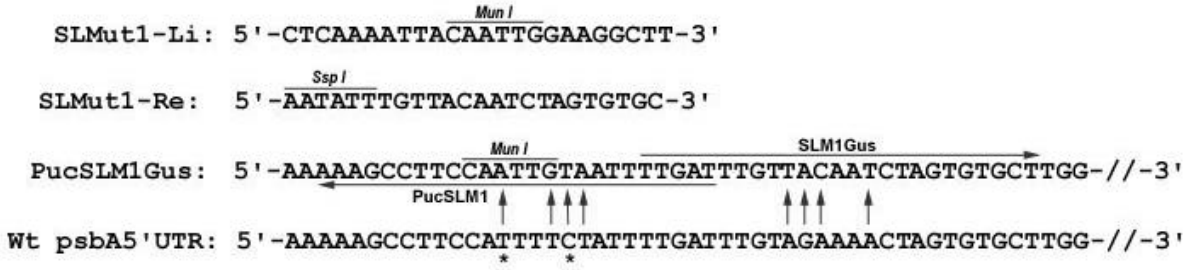

Figure 3.9: The strategy to create stem-loop mutation (SLM1) of *psbA* 5'UTR. Nucleotide substitutions are indicated by arrows and 'core' bases are asterisked.

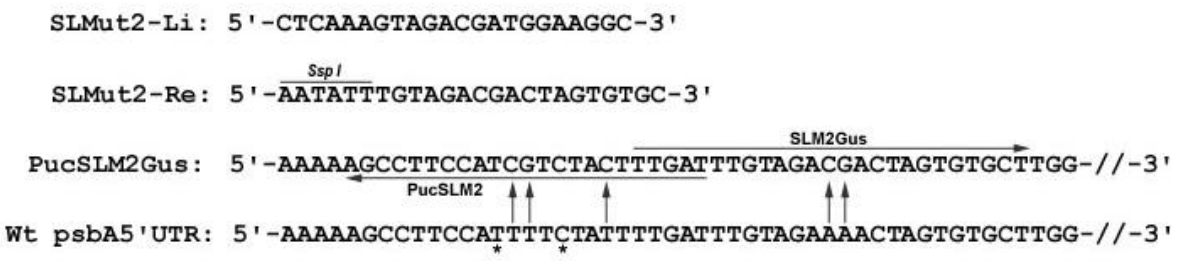

Figure 3.10: The strategy to create stem-loop mutation (SLM2) of *psbA* 5'UTR. Nucleotide substitutions are indicated by arrows and 'core' bases are asterisked.

The secondary structures of wild-type *psbA* 5'UTR and two mutants (SLM1 and SLM2) were predicted, using software programme 'RNA Structure 2.52' (Zuker, 1999). Their stem-loop structures near the 5' end are demonstrated in figure 3.11.

(*uidA*-related) that were further cloned into plasmid pUC18 at *Ecl136II* and *HincII* site by blunt end ligation, respectively.

According to the same strategy as for SLM1, the PCR clones of PucSLM2 and SLM2Gus were preliminarily identified by *SacI* and *SspI*, respectively. The recombinant plasmids with PCR inserts in desired orientation were selected. Plasmid with insert PucSLMut2 was sequentially treated with *SacI*, T4 DNA polymerase and *PstI*, then served as the vector to incorporate the insert SLMut2Gus released from its recombinant plasmid by *SspI* and *PstI*. The resultant plasmid contained a combined insert PucSLM2Gus, in which the original size of mutated *psbA* 5'UTR was formed. This mutation of *psbA* 5'UTR was attributed to several nucleotide substitutions from A/T to G/C in the stem, and therefore increased the stem-loop Mt from dG (-11.9 kcal/mol) to dG (-17.6 kcal/mol), but the putative 'core' nucleotides were unchanged (figure 3.10 and 3.11). Afterwards, the partial *uidA* was replaced with intact *uidA* by *NcoI* and *PstI* to form the recombinant plasmid containing entire *uidA* cassette Prrn-psbA5'(SLM2)-uidA-rbcL3' was excised by *SmaI* and *SacII*, then integrated into vector pKCZ (*EcoR47III+SacII*) to achieve plastid transformation construct pKCZ-psbA5'(SLM2)::rbcL3'.

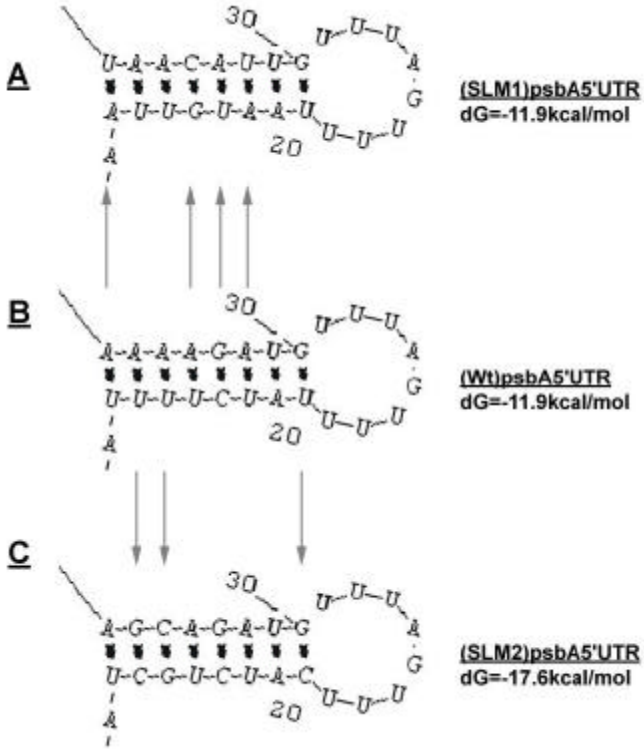

Figure 3.11: Comparison of the free energy (dG) of the stem-loop structures among the wild-type *psbA* 5'UTR (B) and its two mutants SLM1 (A) and SLM2 (C). Arrows indicate nucleotide substitutions

Comparing their potentials for structural maintenance, the SLM1 mutant of *psbA* 5'UTR had the same value of dG (-11.9 kcal/mol) (figure 3.11, A) as well as the wild-type (figure 3.11, B), indicating the nucleotide alterations of SLM1 in the stem was neutral to the structural state. However, the value(dG) of mutant SLM2 was considerably increased up to –17.6 kcal/mol, due to a higher GC content in the stem resulting from nucleotide changes (figure 3.11, C), implicating the conformation of the stem-loop structure in mutant SLM2 was comparatively stable.

3.2.6 Mutation of the RBS (RBSM)

The tobacco *psbA* 5'UTR contains a typical SD-like RBS, unusually 32nt upstream of the authenticated start codon AUG and consequently different from its prokaryotic counterpart. Thus, the *psbA* translation is thought to be initiated by ribosome recognition to the start codon via a 'scanning' mechanism. Direct evidence for this hypothesis might be derived from *in vivo* analysis of RBS mutants.

Mutation of RBS was carried out by similar procedures as mentioned above and described below[5]. Finally, the SD-like RBS was changed from 'GGAG' to 'TCCC' in the mutant RBSM of the *psbA* 5'UTR (figure 3.12; figure 3.16, page 75).

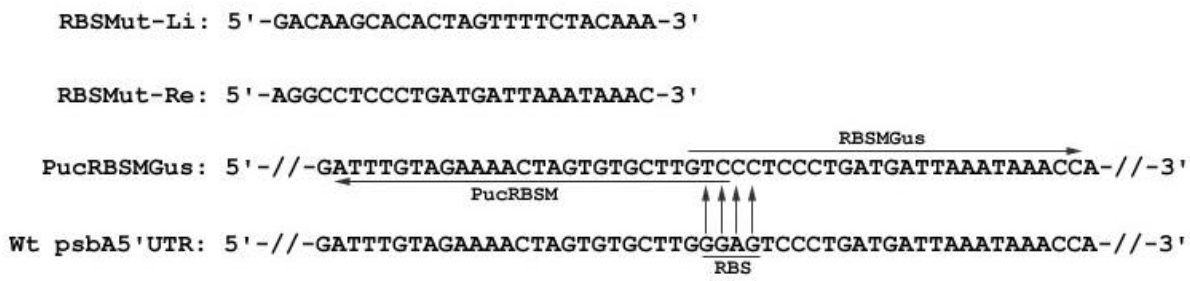

Figure 3.12: The strategy to create mutation of *psbA* 5'UTR (RBSM). Arrows indicate nucleotide substitutions.

3.2.7 Mutation of the AU-Box (AUM)

A pair of primers AUBMut-Li and AUBMut-Re was synthesised with the introduction of an *RsrII* site (CGG*A/T*↓CCG) at their 5' ends (figure 3.13). This *RsrII* site was used for the regeneration of *psbA* 5'UTR in which the AU-Box was replaced with the recognition sequence of *RsrII* (CGGACCG) (figure 3.13; figure 3.16, page 75)[6].

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[5] Synthetic primer pairs RBSMut-Li/pUCBASE-Fw and RBSMut-Re/GusSnaBI were used to amplify PCR fragments PucRBSMut and RBSMutGus, respectively. Both PCR products were subsequently cloned into plasmid pUC18 at the *HincII* site by blunt end ligation.

Because the cut-end (5'-//-GTC↓3') of *HincII* in pUC18 followed by base residues (5'GAC3' at the 5' end of primer RBSMut-Li) could regenerate the *HincII* site (5'GTC↓GAC3'), the positive clones of insert PucRBSM were primarily identified by *SalI* or *HincII* digestion. On the other hand, the primer RBSMut-Re comprised a blunt *StuI* site (5'AGG↓CCT3') at its 5' extremity, thus the recombinant plasmid with insert RBSMGus was initially examined by *StuI* digestion. Then, the plasmid containing insert PucRBSM was dually digested by *HincII* and *SacI*, and served as the vector to take in the fragment RBSMGus dissected by *StuI* and *SacI* from its recombinant plasmid. The resultant plasmid contained an integrated insert PucRBSMGus, in which the full-length of *psbA* 5'UTR with mutated RBS from 'GGAG' to 'TCCC' was reconstructed (figure 3.12). Afterwards, the partial *uidA* was substituted with intact *uidA* by *NcoI* and *SacI* digestions to create an intermediate plasmid comprising the full *uidA* cassette Prrn-psbA5'(RBSM)-uidA-rbcL3' that was subsequently isolated by *SmaI* and *SacII*, and introduced into vector pKCZ (*EcoR47III+SacII*) to generate the plastid transformation construct pKCZ-psbA5'(RBSM)::rbcL3'.

[6] Primer AUBMut-Li coupled with pUCBASE-Fw was used to generate Prrn-containing PCR product PucAUBMut (~520bp), while primer AUBMut-Re together with GusSnaBI could amplify AUBMutGus(~ 420bp) that comprised the partial *uidA* gene. Both PCR products were cloned into plasmid pUC18 at the *SmaI* site. Since only the plasmid clones with insert PucAUBMut in one orientation were obtained, in which the *RsrII* site was proximal to *XbaI*, the AUBMutGus-inserted plasmid with *RsrII* site distal to *XbaI* had to be selected for

Figure 3.13: The strategy to create AU-box mutations of *psbA* 5'UTR. Arrows indicate nucleotide substitutions.

3.2.8 New mutation of the AU-Box (AUMN)

As an internal AUG exists in the original *psbA* 5'UTR, upstream of the AU-Box but 6nt downstream of the SD-like RBS, its function is speculated, e.g. to serve as an alternative translation start codon. However, it appears unlikely to initiate *psbA* translation from this codon because of the incorrect reading frame. Thus, it is required to introduce nucleotide insertions for an accurate reading frame in order to evaluate this internal start codon.

Proposed that this internal AUG could act as the start codon for translation, its downstream AU-Box would become the 'coding' sequence and hence the above mutation of the AU-Box (AUM) might be overridden. Remarkably, the creation of AUM offered the intermediate plasmids for introducing a message translatability from the internal AUG in a simple manner. This AUM-derived mutation of the *psbA* 5'UTR was therefore termed as AUMN, in which the AU-Box was changed to the recognition sequence of *RsrII* and an additional nucleotide 'A' (CGGACCGA) (figure 3.16, page 75)[7].

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the fusion of two PCR inserts into PucAUBMutGus by dual digestions with *RsrII* and *XbaI*. In the resulting recombinant plasmid, the *psbA* 5'UTR was reconstituted by replacing the AU-Box (TAAATAA) with the recognition sequence of *RsrII* (CGGACCG) as depicted in figure 3.13. The partial *uidA* was further substituted with the intact *uidA* from plasmid pUC16SpsbA5'uidArbcL3' by *NcoI* and *PstI* digestions to form an intermediate plasmid containing the whole *uidA* cassette Prrn-psbA5'(AUM)-uidA-rbcL3' that was subsequently disassembled by *SmaI* and *SacII* and integrated into vector pKCZ (*EcoR47III+SacII*) to create plastid transformation construct pKCZ-psbA5'(AUM)::rbcL3'.

[7] For this purpose, a new reverse primer AUBMutNew-Re was synthesised which differed from primer AUBMut-Re by an additional nucleotide A just behind the *RsrII* site. Primer AUBMutNew-Re together with GusSnaBI was used to amplify the PCR fragment AUBMutNewGus (~ 420bp) that was subsequently cloned into plasmid pUC18 at the *SmaI* site by blunt end ligation. The plasmid with the PCR insert in such orientation that *RsrII* was proximal to *BamHI* was selected and renewed with an intact *uidA* by *NcoI* and *SacI*. A specific DNA

3.2.9 Chimeric 5'UTR consisting of rbcL 5'UTR and psbA 5'UTR (Rd-Pd and Rd-Pf)

The 5' end stem-loop of the *rbcL* 5'UTR (182nt length) was suggested as a putative stabilising element to maintain the steady-state mRNA level (Shiina et al., 1998), while that of the *psbA* 5'UTR was proposed to confer a strong translational signal (Eibl et al., 1999). Thus, it would be of interest to investigate whether their structural union could be paralleled with their functional integration, and the consequence of their mutual exchanges.

For this purpose, two chimeric 5'UTRs, Rd-Pd [8] and Rd-Pf [9] (figure 3.16, page 75), on the basis of the *psbA* 5'UTR were generated, by localising the stem-loop of *rbcL* 5'UTR

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fragment from the resulting recombinant plasmid was excised by *RsrII* and *SacII* to replace its counterpart in construct pKCZ-psbA5'(AUM)::rbcL3' and create the plastid transformation construct pKCZ-psbA5'(AUMN)-::rbcL3'. In this mutation of *psbA* 5'UTR, AU-Box (TAAATAAA) was changed to the sequence (CGGTCCGA) that embedded an *RsrII* site and allowed the in-frame translation of the *uidA* gene initiated from the internal AUG codon upstream of the modified AU-Box.

[8] From tobacco total DNA, primers rbcL5'-Fw and rbcL5'-Re were used to amplify a ~600bp PCR fragment rbcL5'end consisting of the entire *rbcL* 5'UTR and partial *rbcL* coding sequence. Primer rbcL5'-Re: 5'-TCGCAGATCTTCCAGACGTAG-3' was identical to the *rbcL* coding sequence, while primer rbcL5'-Fw: 5'-CAGCTGTATTTGGCAAATCAAATA-3' was synthesised according to the 5'-terminal sequence of *rbcL* 5'UTR with an introduced *PvuII* site (5'CAG↓CTG3'). Then, the PCR product rbcL5'end was cloned into plasmid pUC18 at the *SmaI* site by blunt end ligation, and positive clones were primarily identified by *PvuII* digestion.

Afterwards, the aforementioned plasmid containing inverted-*aadA* (context 3.1.3) was cut by *EcoRV* and *SacI* to remove the *aadA* cassette except promoter Prrn, then accepted the insert rbcL5'end released from its recombinant plasmid by *PvuII* and *SacI*. The resulting plasmid pUC16SrbcL5'end was sequentially treated with *NcoI*, *Klenow* DNA polymerase and *SacI*, then served as the cloning vector where the stem-loop of *rbcL* 5'UTR was kept behind promoter Prrn. Plasmid pUC16SpsbA5'uidArbcL3' was treated by *SpeI*, *Klenow* DNA polymerase and *SacI* in turn to excise the fragment comprising the curtailed *psbA* 5'UTR (SLD) and intact *uidA* gene that was subsequently inserted into the vector. The resultant recombinant plasmid contained a 74nt chimeric 5'UTR by combining the stem-loop (-182~-160) of *rbcL* 5'UTR and *psbA* 5'UTR(SLD), accordingly termed as psbA5'(Rd-Pd). Finally, the full *uidA* cassette Prrn-psbA5'(Rd-Pd)-uidA-rbcL3' was dissected by *SmaI* and *SacII*, then integrated into vector pKCZ (*EcoR47III+SacII*) to generate the plastid transformation construct pKCZ-psbA5'(Rd-Pd)::rbcL3'.

[9] To construct another chimeric 5'UTR containing wild-type *psbA* 5'UTR, the tobacco *psbA* 5'UTR had to be generated again, due to the absence of proper enzyme cleavage site at its 5'end in those former plasmids. Primer psbA5'wt-14 (5'-CCTTTAAAAAGCCTTCCATTTTCTATTT-3') based on the 5' end sequence of *psbA* 5'UTR contained several additive nucleotides (5'CCTTT3') for the introduction of an *DraI* (5'TTT↓AAA3') site, and was coupled with primer GusSnaBI to amplify a ~490bp DNA fragment psbA5'wtGus comprising the wild-type *psbA* 5'UTR and partial *uidA* gene, from the common plasmid DNA template. This PCR product was subsequently cloned into plasmid pUC18 at the *SmaI* site by blunt end ligation. The recombinant plasmid pUCpsbA5'wtGus with an additional *DraI* site distal to *SacI* was selected to release the insert psbA5'wtGus by digestions of *DraI* and *SacI*. The plasmid pUC16SrbcL5'end was fully digested by *SacI*, then partially cut by

ahead of the wild-type *psbA* 5'UTR and its mutant (SLD), respectively. In the *rbcL* 5'UTR, the putative stem-loop of interest is situated exactly at its 5' end, followed by an adjacent *NcoI* site and a distal *SspI* site (34nt downstream). These two enzyme sites were particularly useful for the construction of the chimeric 5'UTRs (Rd-Pd, Rd-Pf) that were also accounted as *psbA* 5'UTR modifcations.

The secondary structures and dG values of these two chimeric 5'UTRs i.e. psbA5'(Rd-Pd) and psbA5'(Rd-Pf) were computed, using the programme 'RNA Structure 2.52' (Zuker, 1999), as well as that of wild-type *psbA* 5'UTR (figure 3.14). Therein, the structural pattern of psbA5'(Rd-Pd) (figure 3.14, B) was identical to that of wild-type *psbA* 5'UTR (figure 3.14, A), also with the same dG values (-11.9 kcal/mol). However, the structural prediction of chimeric psbA5'(Rd-Pf) was complicated by the introduction of *rbcL* 5'UTR(SL+38) that had the potential to form the typical stem-loop at the 5'end and one additional bi-hairpin structure, therefore its dG value was increased to –22.8 kcal/mol (figure 3.14, C).

3.2.10 Constructs with psbA 5'UTR(ori)*

Intact *uidA* cassette composed by Prrn, *psbA* 5'UTR(*ori**), *uidA*, and *rbcL* 3'UTR was excised from plasmid pUC16SpsbA5'uidArbcL3' by *SmaI* and *SacII* then integrated into pKCZ(*Eco47III+SacII*) to form such a construct as pKCZ-psbA5'(*ori**)::rbcL3'. Similarly, an additional construct pKCZ-psbA5'(*ori**-*P*)::psbA3' was obtained by inserting *psbA* 3'UTR-tailed *uidA* cassette into pKCZ, for the purpose to investigate the regulatory role of plastid 3'UTR e.g. to affect the mRNA stability or translation, solely or by hypothetical interaction with the 5'leader sequence.

3.2.11 Constructs with psbA 5'UTR(ori)

Most *psbA* 5'UTR modifications as described above were derived from the plasmid pUC16SpsbA5'uidArbcL3' where the 5' end poly(A) motif ('AAAAA') of *psbA* 5'UTR was slightly changed into 'ACTAA'. By sequence alignment of *psbA* 5'UTRs from dicot and monocot plant species, the consensus sequence of this poly(A) motif is found as

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SspI to serve as the vector that contained a region (-182~-122) of *rbcL* 5'UTR, covering the stem-loop and 38nt additional sequence, therefore termed as *rbcL* 5'UTR(SL+38). The resulting recombinant plasmid was obtained, in which a 125nt chimeric 5'UTR named as psbA5'(Rd-Pf) was created by following the *rbcL* 5'UTR (SL+38) with wild-type *psbA* 5'UTR. Then, the partial *uidA* gene was renewed with intact *uidA* by *SpeI* and *SacI* to result in an intermediate plasmid that contained the complete *uidA* cassette. Afterwards, this cassette Prrn-psbA5'(Rd-Pf)-uidA-rbcL3' was dissected by *SmaI* and *SacII*, then incorporated into vector pKCZ (*EcoR47III+SacII*) to generate plastid transformation construct pKCZ-psbA5'(Rd-Pf)::rbcL3'.

'AATAACAA', in which two A-rich sequences 'AATAA' and 'AACAA' are overlapping (figure 3.15). The sequence 'AACAA' is shown as the common poly(A) motif at the 5' end, but some plants may contain the sequence 'AATAA' or full consensus sequence instead. Among these poly(A) motifs, the central nucleotide is very flexible. Thus, the slight modification at the 5' end of *psbA* 5'UTR appeared to be insignificant, but an additional control construct with the original *psbA* 5'UTR was required to confirm this.

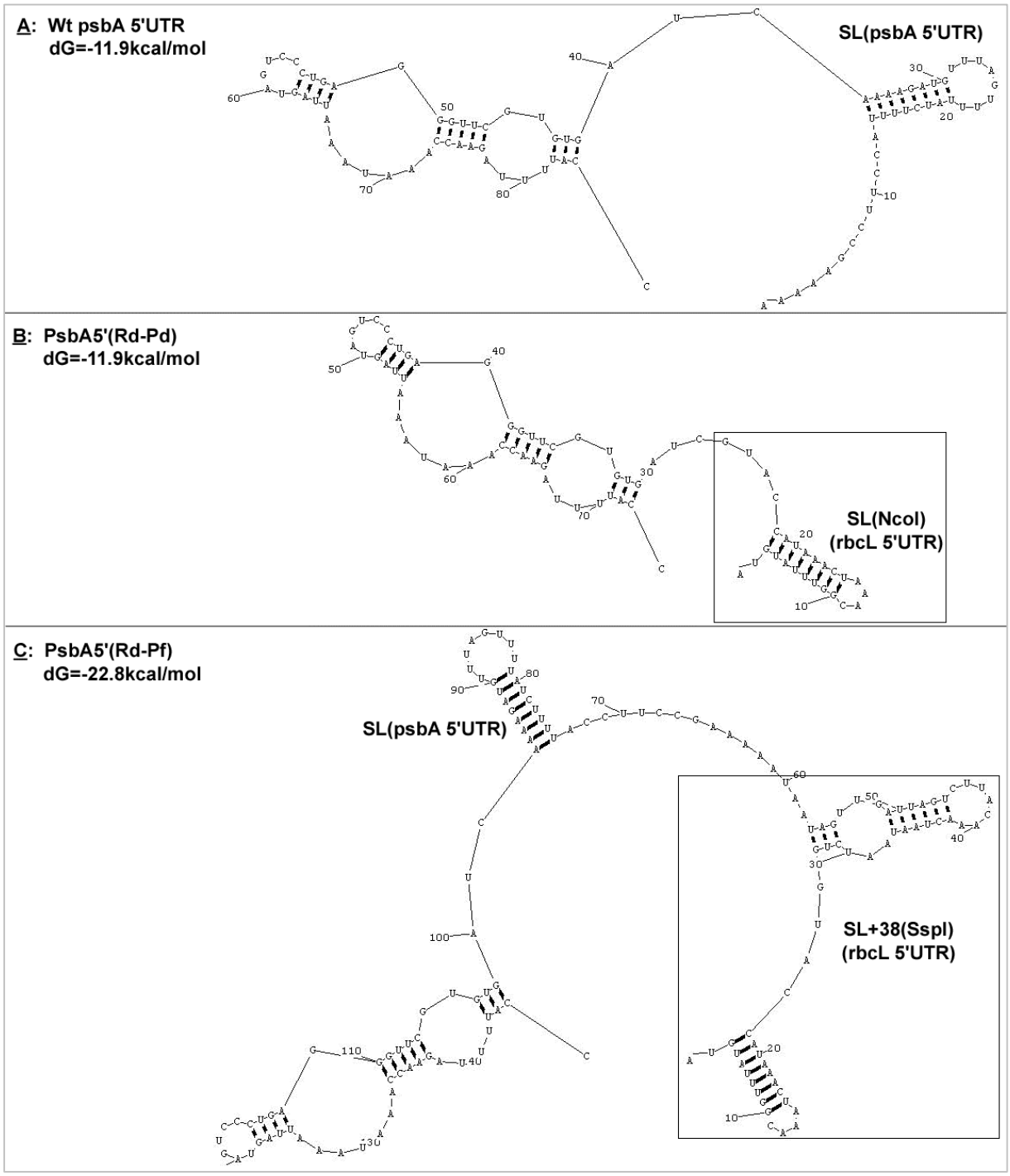


Figure 3.14: Structural predictions of wild-type *psbA* 5'UTR (A) and two chimeric *psbA* 5'UTRs, Rd-Pd (B) and Rd-Pf (C)

The full-length of original *psbA* 5'UTR(*Ori*) was singularly available in the plasmid pUC16SpsbA5'uidApsbA3', surrounding the *uidA* gene together with *psbA* 3'UTR. Then, this plasmid was cut by *SmaI* and *SacII* to release the *uidA* cassette Prrn-psbA5'(*Ori-P*)-*uidA*-psbA3' that was subsequently introduced into vector pKCZ (*EcoR47III+SacII*) to generate the plastid transformation construct pKCZ-psbA5'(*Ori-P*)::psbA3'.

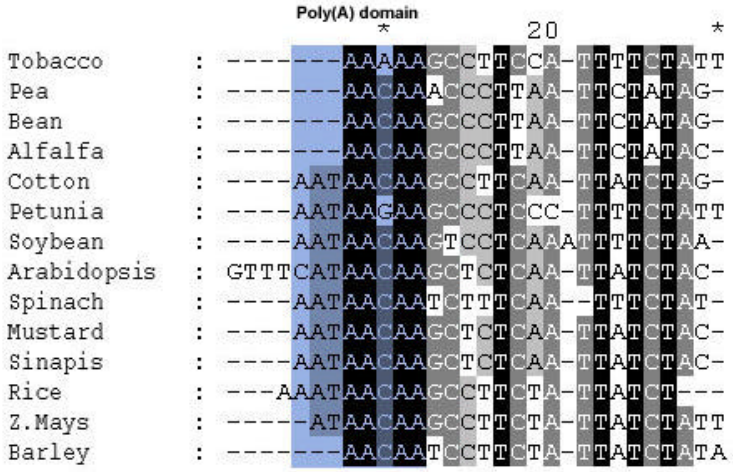
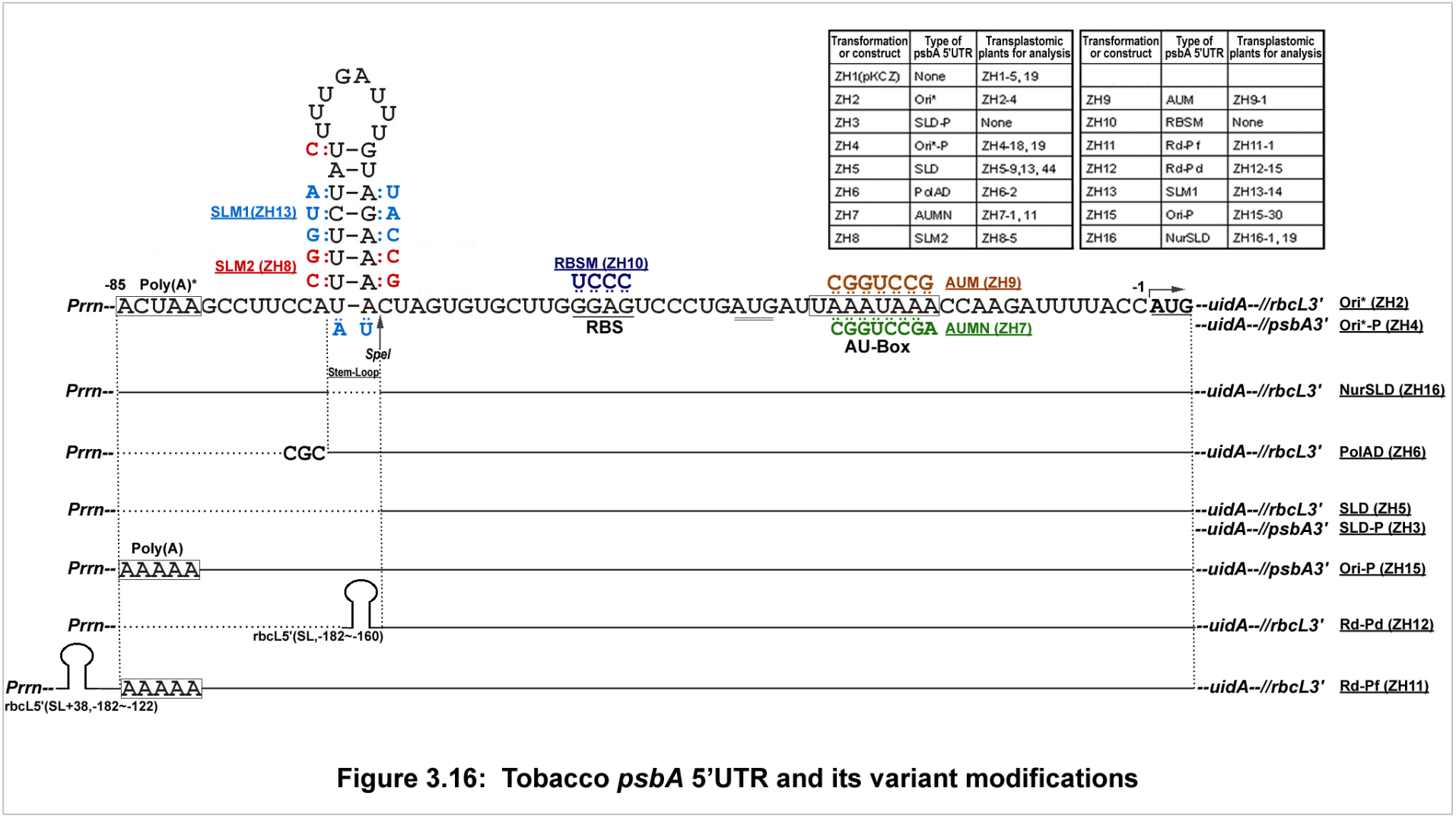


Figure 3.15: The alignment of poly(A) sequence of *psbA* 5'UTR in variant plants

3.2.12 The outlines to create all modifications of psbA 5'UTR

Finally, fourteen plastid transformation constructs containing variants of *psbA* 5'UTR have been achieved for this study. These are depicted in figure 3.16 (page 75).

In this figure, the wild type *psbA* 5'UTR(Ori*) is displayed as the complete sequence. Therein, the typical stem-loop structure near the 5' end is specially demonstrated, the putative poly(A) (AAAAA) or poly(A)* (ACUAA) sequence immediately at the 5' extremity is enclosed in the blank rectangle, the canonical SD-like RBS (GGAG) behind the stem-loop is underlined, the internal AUG and the start codon AUG are individually marked by duplicate and single underlining, the AU-Box (UAAAUAAA) near the 3' end is bordered. Furthermore, the mutations of *psbA* 5'UTR including SLM1, SLM2, RBSM, AUM, AUMN are shown in different colours, and the deleted sequence of *psbA* 5'UTR is labelled as dot line in the cases of mutations SLD, NurSLD and PolAD. In addition, the chimeric 5'UTRs (Rd-Pf or Rd-Pd) are delineated by capping a hairpin miniature of *rbcL* 5'UTR on the whole or truncated *psbA* 5'UTR, respectively. Taken together, the *uidA* cassettes differ in the form of *psbA* 5'UTR alone in most constructs, containing the same promoter Prrn and *rbcL* 3'UTR.



Transformation or construct	Type of psbA 5'UTR	Transplastomic plants for analysis
ZH1(pKCZ)	None	ZH1-5, 19
ZH2	Ori*	ZH2-4
ZH3	SLD-P	None
ZH4	Ori*-P	ZH4-18, 19
ZH5	SLD	ZH5-9,13, 44
ZH6	PolAD	ZH6-2
ZH7	AUMN	ZH7-1, 11
ZH8	SLM2	ZH8-5

Transformation or construct	Type of psbA 5'UTR	Transplastomic plants for analysis
ZH9	AUM	ZH9-1
ZH10	RBSM	None
ZH11	Rd-Pf	ZH11-1
ZH12	Rd-Pd	ZH12-15
ZH13	SLM1	ZH13-14
ZH15	Ori-P	ZH15-30
ZH16	NurSLD	ZH16-1, 19

Figure 3.16: Tobacco *psbA* 5'UTR and its variant modifications

3.2.13 Verification of plastid transformation constructs by sequencing

Before applied for tobacco plastid transformation, all above constructs were sequenced using primer GusSeq1 (5'-GCTGATACCAGACGTTGC-3') complementary to the 5' end of *uidA* gene in an ABI sequencer 377. However, it was striking that the sequencing process towards promoter Prrn for most constructs was prematurely terminated, with an output just extending the 5'UTR sequence (figure 3.17, SLD and AUM), probably due to the high percentage of G/C nucleotides proximal to the 5' end of *psbA* 5'UTR. This situation was slightly improved in some constructs by an optimised sequencing program, where the sequencing reaction was able to override the promoter Prrn maximally (figure 3.17, Rd-Pd).

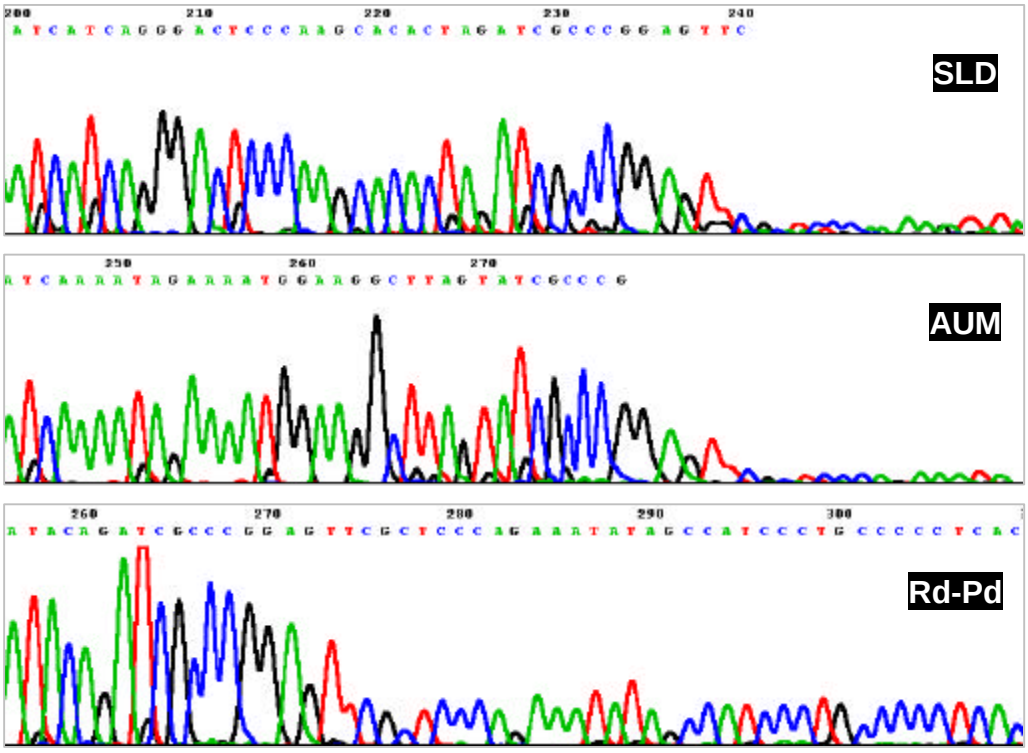


Figure 3.17: Fluorescent sequencing of plastid transformation constructs

The sequence data of all constructs were integrated into a FASTA file, then aligned by program ClustalX1.6b. The output file in '.msf' format was displayed by program 'Genedoc' as in figure 3.18, in which all modifications of *psbA* 5'UTR are consistent with the predictions. The TATA box of promoter Prrn, the stem-loop of *rbcL* 5'UTR, the poly(A) sequence, stem-loop, RBS, and AU-Box of *psbA* 5'UTR are distinguishable as indicated.

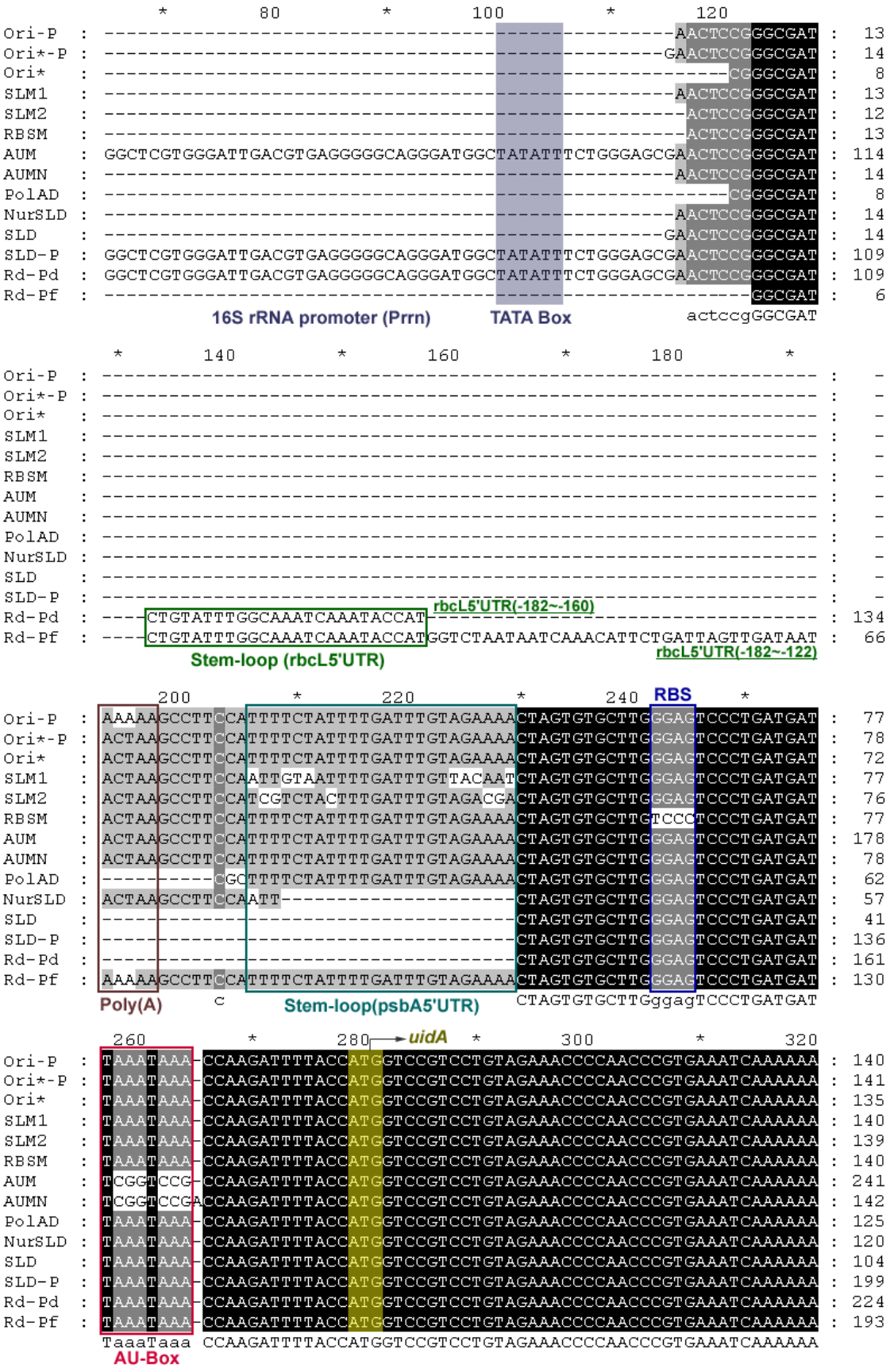


Figure 3.18: The alignment of sequencing data of all plastid transformation constructs

3.3 Plastid transformation

After verification by sequencing, all constructs described above were applied for plastid transformation by microprojectile bombardment. Each transformation event corresponds to a specific construct, thus the two terms of 'transformation' and 'construct' contain the same connotation in the following contexts. As described in Materials and Methods, DNA-coated gold particles were injected into young tobacco leaves by high helium pressure. After a pre-incubation for two days, the bombarded leaves were cut into small pieces, then placed on selective RMOP medium (containing 500 mg/l spectinomycin) for selection and regeneration of resistant transformants. After two weeks, swollen leave explants were cut again and transferred to new selective ROMP medium. In general, the medium was renewed every three weeks to keep the selection stress. Within 4~8 weeks after shooting, resistant green shoots or calli were generated from the bleached explants, then transferred into small petri-dishes (∅6cm) containing the selective RMOP medium for growth of the shoots or regeneration of new shoots from the calli after a few of days. Then leaves were dissected into small pieces and cultured on the selective RMOP medium in ∅6cm petri-dishes for segregation of wt and transgenic plastomes.

Generally, eight bombardments with microprojectiles were carried out for each transformation. A number of resistant transformants ranging from 4 to 12 were obtained and confirmed by initial identification via Gus staining or PCR amplification. Thus, an average transformation efficiency of one transformant per bombardment was achieved. Spontaneous mutations that acquired resistance to spectinomycin were found repeatedly in all transformations. In addition, it was frequently observed that aberrant recombination occurred in some constructs, causing a partial loss of transgene, especially of *uidA*. For example, in construct ZH3, all resistant transformants except the spontaneous mutations were identified as negative by Gus staining, but contained the *aadA* gene as shown by PCR amplification (context 3.4.3), suggesting that the *uidA* gene was partially or entirely removed by an unknown mechanism. Nevertheless, such kind of transformant were accounted as positive.

Since numerous transformants were obtained at different time points from the discrete transformation events, it was time-consuming and laborious to subculture all transformants and further cultivate them into plants. Thus, only a few of transplastomic plant lines per each construct with preliminary identification were selectively generated and applied for further analyses, e.g. ZH1-19, ZH2-4, ZH16-1 (see table 3.1; figure 3.16, page 75). Unfortunately, the correct transplastomic plants from transformations ZH3 and ZH10 were not achieved and hence not included in the following parallel analyses.

Table 3.1: Constructs and transformations

Transformation	Construct	Type of psbA 5'UTR	Number of transformant (by PCR or Gus staining)	Transplastomic Line for analysis
ZH1	pKCZ	None	8	ZH1-5, 19
ZH2	pKCZ-psbA5'(Ori*)::rbcL3'	Ori*	5	ZH2-4
ZH3	pKCZ-psbA5'(SLD)::psbA3'	SLD-P	7	None
ZH4	pKCZ-psbA5'(Ori*)::psbA3'	Ori*-P	9	ZH4-18, 19
ZH5	pKCZ-psbA5'(SLD)::rbcL3'	SLD	10	ZH5-9,13, 44
ZH6	pKCZ-psbA5'(PolAD)::rbcL3'	PolAD	7	ZH6-2
ZH7	pKCZ-psbA5'(AUMN)::rbcL3'	AUMN	9	ZH7-1, 11
ZH8	pKCZ-psbA5'(SLM2)::rbcL3'	SLM2	7	ZH8-5
ZH9	pKCZ-psbA5'(AUM)::rbcL3'	AUM	8	ZH9-1
ZH10	pKCZ-psbA5'(RBSM)::rbcL3'	RBSM	4	None
ZH11	pKCZ-psbA5'(Rd-Pf)::rbcL3'	Rd-Pf	7	ZH11-1
ZH12	pKCZ-psbA5'(Rd-Pd)::rbcL3'	Rd-Pd	8	ZH12-15
ZH13	pKCZ-psbA5'(SLM1)::rbcL3'	SLM1	12	ZH13-14
ZH15	pKCZ-psbA5'(Ori)::psbA3'	Ori-P	9	ZH15-30
ZH16	pKCZ-psbA5'(NurSLD)::rbcL3'	NurSLD	7	ZH16-1, 19

3.4 Preliminary identification and analysis of transplastomic plants

3.4.1 Identification of transplastomic plants by Gus staining

For those constructs comprising the reporter *uidA* gene, it is feasible and rapid to screen their positive transgenic plants by histochemical Gus staining. Using this method, the positive transformants have been obtained for most constructs in this study and differed in Gus activity significantly (table 3.2).

Table 3.2: Gus staining to identify the positive transformants

Transformation	Type of psbA 5'UTR	Annotation	Gus staining
ZH1	- - -	aadA only, without psbA 5'UTR and uidA, as the control	
ZH2	Ori*	Wt type-like psbA 5'UTR with a slight modification at the 5' end	
ZH4	Ori*-P	Wt type-like psbA 5'UTR as above, but with psbA 3'UTR as uidA trailer	
ZH5	SLD	Deletion of stem-loop and its anterior sequence	
ZH6	POlAD	Deletion of poly(A) sequence at the 5' end	
ZH7	AUMN	New mutation of AU-Box by nucleotide substitutions, but with an additive nucleotide 'A'	
ZH8	SLM2	Mutation of stem-loop with increased GC content and melting temperature(Mt)	
ZH9	AUM	Mutation of AU-Box by nucleotide substitutions,	
ZH10	RBSM	Mutation of SD-like RBS by nucleotide substitutions	
ZH11	Rd-Pf	Chimeric psbA 5'UTR consisting of rbcL 5'UTR(SL+38) and wild-type psbA 5'UTR	
ZH12	Rd-Pd	Chimeric psbA 5'UTR consisting of rbcL 5'UTR(SL) and SLD of psbA 5'UTR	
ZH13	SLM1	Mutation of stem-loop by nucleotide substitutions, and with the same Mt	
ZH15	Ori-P	Wild-type psbA 5'UTR	
ZH16	NurSLD	Local deletion of stem-loop, with the maintenance of its anterior sequence	

Moreover, the expression patterns of *uidA* in the same and other transformant for the same construct were considerably identical, some of which related to the constructs ZH2, ZH4, ZH5, ZH7 and ZH9 are demonstrated in figure 3.19 as the representatives.

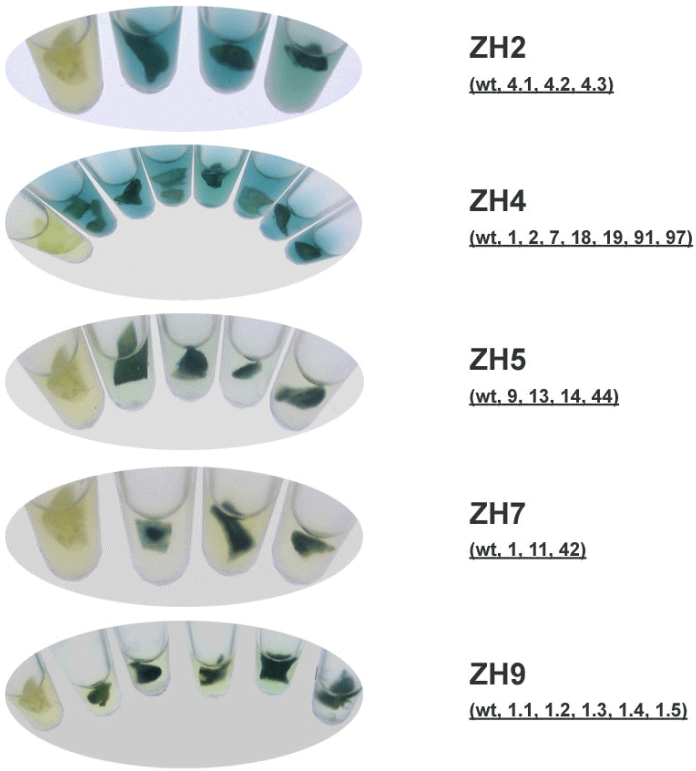


Figure 3.19: Similar Gus staining pattern in variant transformants for the same construct

The *uidA* expression in different transplastomic plant lines cultivated in the culture chamber were also compared, by staining variant leaf pieces with similar size in Gus staining solution (figure 3.20). Therein, one transplastomic plant for each construct is represented, albeit including a transformant shoot from transformation ZH10 that failed to regenerate the transplastomic plantlets.

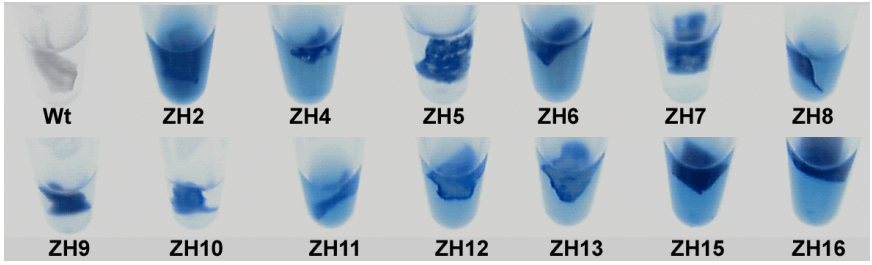


Figure 3.20: Different *uidA* expressions correlated to the different constructs by Gus staining to leave pieces of transplastomic plants

From figure 3.20, a significant difference in *uidA* expression was observed among those transplastomic plants corresponding to the different version of tobacco *psbA* 5'UTR. The expression of *uidA* was evidently inclined to become weak in those plant lines with *psbA* 5'UTR modifications, predominantly in the cases of constructs ZH5 (SLD), ZH7 (AUMN) and ZH9 (AUM), compared with that from ZH2 conferring the wild-type *psbA* 5'UTR. Low *uidA* expression in a shoot of ZH10 resulted from the RBS mutation of *psbA* 5'UTR (RBSM), indicating that this canonical SD-like RBS element appeared to be essential for the translation of its following gene. Since the approximate appearances of Gus staining were observed in the tissues of both transformant shoot and transplastomic plantlet leaf for the same construct such as ZH9, ZH2, or ZH4 (figure 3.19 and 3.20), it was postulated that this scenario might also exist in ZH10. Therefore, it was reasonable to include the transformant of ZH10 in above overall comparison of *uidA* expression in all constructs by Gus staining (figure 3.20).

3.4.2 PCR amplification to identify transplastomic plants[10]

To identify transplastomic plants, two pairs of primers specific to the *aadA* and *uidA* genes were synthesised, respectively as shown in the following list.

Primer type	Name	Sequence	The size of PCR product
aadA primers	aadANew-Fw	5'-AGCACTACATTTCGCTCATCGC-3'	430bp
	aadANew-Re	5'-CACAGTGATATTGATTTGCTGG-3'	
uidA primers	uidANew-Fw	5'-GACTTTGCAAGTGGTGAATCC-3'	~690bp
	uidANew-Re	5'-AGCACATCAAAGAGATCGCTG-3'	

Total DNA was extracted from the young leaves of transgenic plants, and finally dissolved into sterile distilled water (50µl in general) to a proper concentration. By *aadA* primers, a ~430bp specific DNA fragment within the *aadA* gene was amplified from the Gus-positive transplastomic plants (figure 3.22, B), in which a unique ~690bp PCR product

[10] The positions and orientations of all primers used for PCR analyses (context 3.4.2 and 3.4.3) are indicated in a standard plastid transformation construct of the *psbA* 5'UTR (figure 3.21).

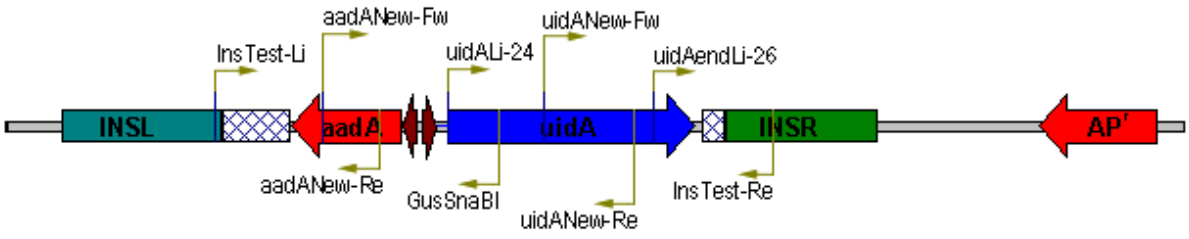


Figure 3.21: The positions and orientations of all primers used for PCR analyses

corresponding to a part of the *uidA* gene was obtained by *uidA* specific primers (figure 3.22, C), and a ~1.3kb intergenic PCR fragment was amplified by *aadA* primer aadANew-Fw together with *uidA* primer GusSnaBI (figure 3.22, A). These results indicate the selective marker *aadA* and reporter *uidA* genes associated in the transformation constructs had been concomitantly integrated into at least one of the plant genomes. Additionally, an aberrant PCR fragment with shortened size (~750bp) was found with construct ZH5 (SLD) (figure 3.22, C, arrow indications for both normal and abnormal PCR products). This scenario was also observed in other constructs as described below.

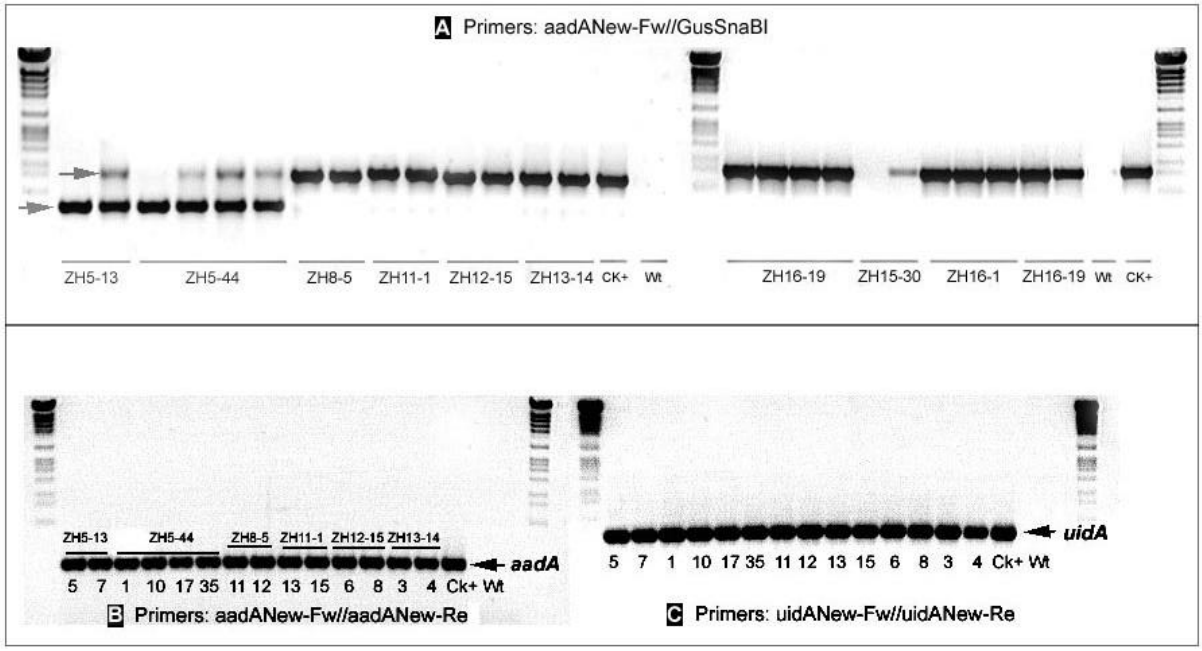


Figure 3.22: PCR identification of transplastomic plants. A: intergenic amplification of *aadA*- *uidA*; B: *aadA*-specific amplification; C: *uidA*-specific amplification

Further PCR amplifications were performed in an attempt to get more accurate evidence about the integration of foreign genes into the plastome. Thus, additional primers InsTest-Li and InsTest-Re based on the proximal end sequences (relative to the foreign genes) of plastid homologous fragments (INSL and INSR) were synthesised for this duty. The sequences and features of these primers are indicated individually in the following list.

Primer	Sequence	Feature
InsTest-Li	5'-CTCAGATATACTAGCACTGCATC-3'	Within INSL, 60bp far from the rbcL3'(Chl) of aadA cassette
InsTest-Re	5'-GTAGCTCAGAGGATTAGAGCAC-3'	Within INSR, 370bp far from the rbcL3'(Tob) of uidA cassette

Transplastomic plant line ZH1-19 for the vector pKCZ is exemplified in this case. By primers InsTest-Li and InsTest-Re, two distinct PCR fragments were obtained, the large one (~2kb) might comprise the full *aadA* cassette between INSL and INSR, while the small one (0.43 kb) is thought to correspond to the wild-type plastome sequence (figure 3.23, lane 1). Moreover, a specific PCR product with the expected size (~1.3kb) was amplified by primers aadANew-Fw and InsTest-Re (figure 3.23, lane 2). Additional test by a specific primer outside the distal end of INSL (with respect to plastomic insertion site) and primer InsTest-Re could generate a large fragment of ~3.6kb containing the putative transgene (*aadA*) and another fragment (~2.0kb) probably originating from the wild-type plastome (data not shown). Thereby, it could be concluded that the transplastomic plant ZH1-19 contained the transgene of *aadA* in the plastome by homologous recombination, but appeared to be heteroplastomic due to the presence of a paucity of wild-type plastome copies that were not completely removed by the selection stress. Since other constructs with *psbA* 5'UTR modifications were derived from the vector pKCZ, it could be predicted that similar evidence like in ZH1 might also be observed in other transformations.

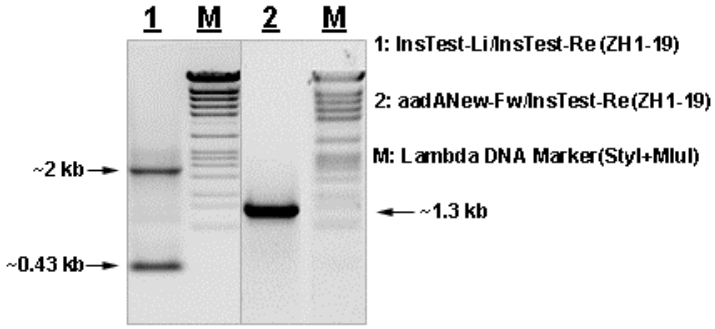


Figure 3.23: PCR analysis of transplastomic plant ZH1-19

3.4.3 The loss of transgene as shown by PCR analysis

Although the transplastomic plants were obtained for most constructs and identified by Gus staining and PCR amplification, it was observed that the number of transformants with *uidA* expression was not identical to the total number of transformants resistant to spectinomycin except the concurrent spontaneous mutants. This was strikingly severe in the constructs ZH3 and ZH5 where the stem-loop and its anterior sequence of *psbA* 5'UTR was deleted. In ZH3, astonishingly, no transformant with *uidA* expression was obtained among up to 10 cultivated resistant transformants. Therefore, it is speculated that the loss of transgene activity particularly related to the *uidA* gene might occur in some constructs.

This postulation was substantiated by an intensive PCR analysis of the resistant transformants of constructs ZH3 and ZH5. Therein, the specific PCR product by *uidA* primers uidALi-24[11] and GusSnaBI was absent in all samples of ZH3 and most of ZH5 with few exceptions only (figure 3.24, A), whereas a majority of samples of ZH3 and ZH5 could enable the generation of a specific PCR product by *aadA* primers (aadANew-Fw and aadANew-Re) (figure 3.24, B). For example, in ZH3, 6 out of 8 *uidA*-deficient transformants were *aadA*-positive and thus could be accounted as transplastomic ones, while the rest was thought to come from spontaneous mutations.

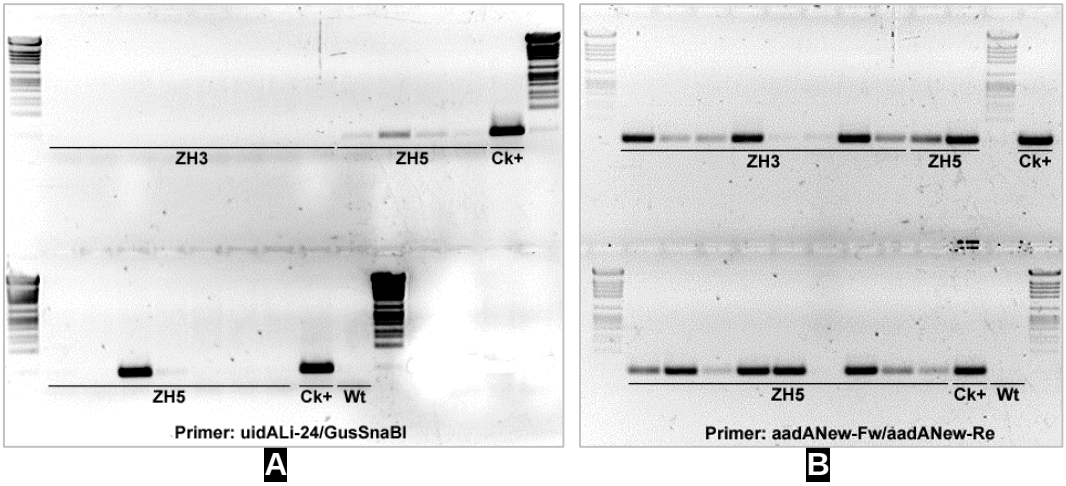


Figure 3.24: Primary screening the positive transformants of ZH3 and ZH5 by PCR amplification with primers specific to *uidA* (A) and to *aadA* (B)

As primer uidALi-24 was identical to the 5'-terminal sequence of *uidA* gene, the missing PCR product in ZH3 and ZH5 with *uidA* primers might be accounted for a loss of the 5' partial sequence of *uidA*. Further PCR analysis was carried out to test for this possibility.

In all transformation constructs for the study of the *psbA* 5'UTR, the *aadA* and *uidA* cassettes are associated between the plastid homologous fragments INSL and INSR (figure 3.21). Thus, the gene internal and external primer combinations InsTest-Li/aadANew-Re (IL-AD), aadANew-Fw/GusSnaBI (AD-UA), and uidAendLi-26[12]/InsTest-Re (UA-IR) were used for an overall characterisation of the integration of *aadA* and *uidA* into the plastome, especially in constructs ZH3 and ZH5. By primers IL-AD, a correct size (~1.2kb) of PCR fragment was amplified in most transformant samples of ZH3 and ZH5 (figure 3.25, B), while an expected amplification was obtained in most ZH5 samples but few ZH3 samples by primers UA-IR (figure 3.25, C). However, an aberrant PCR product with short size (~0.75kb)

[11] uidALi-24: 5'-ATGGTCCGTCCTGTAGAAAC-3'

[12] uidAendLi-26: 5'-TCATCACCGAATACGGCG-3'

was observed in most samples of ZH3 and ZH5 compared with the control (~1.3kb) by primers AD-UA (figure 3.25, A), in which one sample of ZH5 (ZH5-9 with Gus activity) also presents the expected PCR product (~1.3kb, indicated by arrow).

In conclusion, the results in ZH3 and ZH5 imply that the *aadA* and *uidA* genes had been correctly incorporated into the plastome at first by homologous recombination. During cell division and plastid propagation in these transformants, an intermolecular or intramolecular recombination might occur at high frequency in ZH3 and ZH5, resulting in the excision of 5' partial *uidA* gene and abolishment of *uidA* expression.

Such abnormal *uidA* gene defects as in ZH3 and ZH5 were also observed in other constructs such as ZH6 and ZH8, but not as frequently. Nevertheless, each construct except ZH3 could offer adequate transplastomic plant lines with the presence of both functional *uidA* and *aadA* genes for further molecular characterisations.

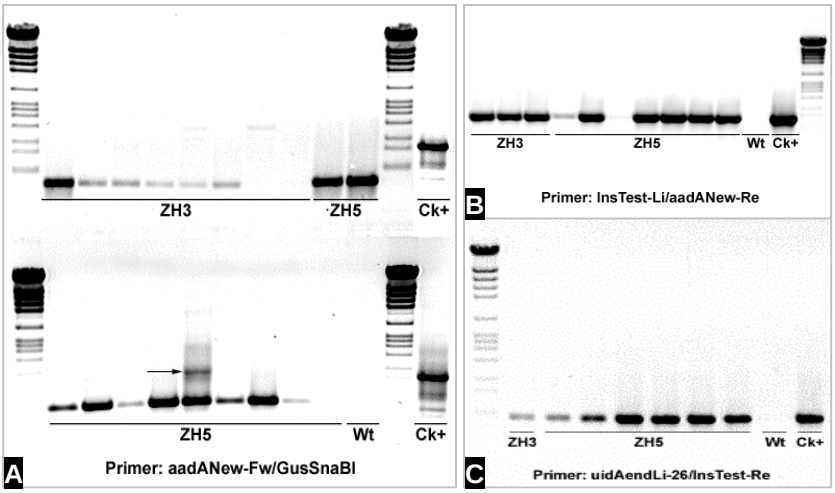


Figure 3.25: Characterisation of the transformants of ZH3 and ZH5 by PCR with different pairs of primers. A: intergenic amplification of *aadA-uidA*; B: 'intergenic' amplification of *INSL-aadA*; C: 'intergenic' amplification of *uidA-INSR*

3.5 Further analysis of transplastomic plants

After the primary identifications by Gus staining and PCR amplification, the transplastomic plants were allowed for an optimal growth in the culture chamber, then simultaneously offered the proper leaves for the following integral analyses at different molecular levels.

3.5.1 DNA analysis of transplastomic plants by Southern blotting

3.5.1.1 General southern analysis

Leaves from the transplastomic plants were sampled for DNA extraction and analysis. About 2µg total DNA for each sample was restricted with enzyme *BglII* at 37°C 3~4 hours or overnight for complete digestion, then run on a 1% agarose gel overnight at 30~40V for optimal separation. The gel was photographed, and washed with distilled water after an optional treatment by 0.25M HCI, then applied on the DNA blot unit for transferring DNA from the gel to nylon membrane. After12~18 hours, this nylon filter was exposed in the UV crossinglinker for DNA fixation and ready for hybridisation.

A fragment (~0.8kb) within the coding region of the *aadA* gene excised by *PstI* and *NcoI* from vector pKCZ was used to prepare the DNA probe for *aadA* detection, thus termed as *aadA* probe, while a *HindIII* segment (~0.76kb) within the plastid homologous fragment INSL of vector pKCZ was chosen for the probe preparation to detect wild-type plastomic sequence (*Wt* probe). Likewise, a ~0.54kb *HincII* fragment of plasmid pUC16SpsbA5'uidA-rbcL3' corresponding to the central part of the *uidA* gene was selected for the preparation of a *uidA* probe. In addition, a ~0.6kb PCR fragment amplified by specific primers of *16S rRNA* gene served as the *16S rRNA* probe. All above templates for probes (*Wt*, *aadA* and *uidA*) are indicated together in figure 3.28.

The DNA hybridisation was sequentially carried out with the probes (*uidA, 16S rRNA* and *aadA*) for the same DNA filter, and revealed by the phosphorimage detection system of Fujifilm BAS1500.

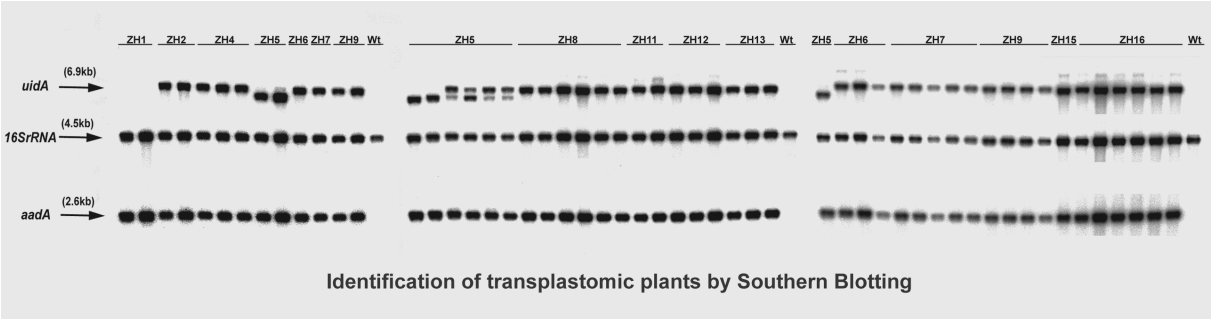


Figure 3.26: The collection of southern analyses for all transplastomic plants

From a collection of all southern blots, the plant samples examined corresponding to the vector pKCZ and most constructs exhibited a distinct hybridisation band with the expected size of 2.6kb or 4.5kb by *aadA* or *16S rRNA* probe, respectively. The *uidA* signal

with correct size of 6.9kb was observed in most constructs, except some samples of ZH5 where the *uidA* signal appeared as an aberrant band of ~6.3kb or dual bands (6.9kb and ~6.3kb). This result confirmed the finding of PCR identification as mentioned above. The hybridisation signals by *uidA, 16S rRNA* and *aadA* probes are individually indicated by arrow with the molecular size tags (figure 3.26). A sample hybridisation profile is represented in figure 3.27 and described in following context.

As the transplastomic plants for most constructs were regenerated in different periods, it was reasonable to preliminarily identify all plant lines at the DNA level in batches before their integrative analyses of RNA abundance and Gus activity. In figure 3.27, southern blotting was applied for a set of transplastomic plants related to constructs ZH5, ZH8, ZH11, ZH12 and ZH13.

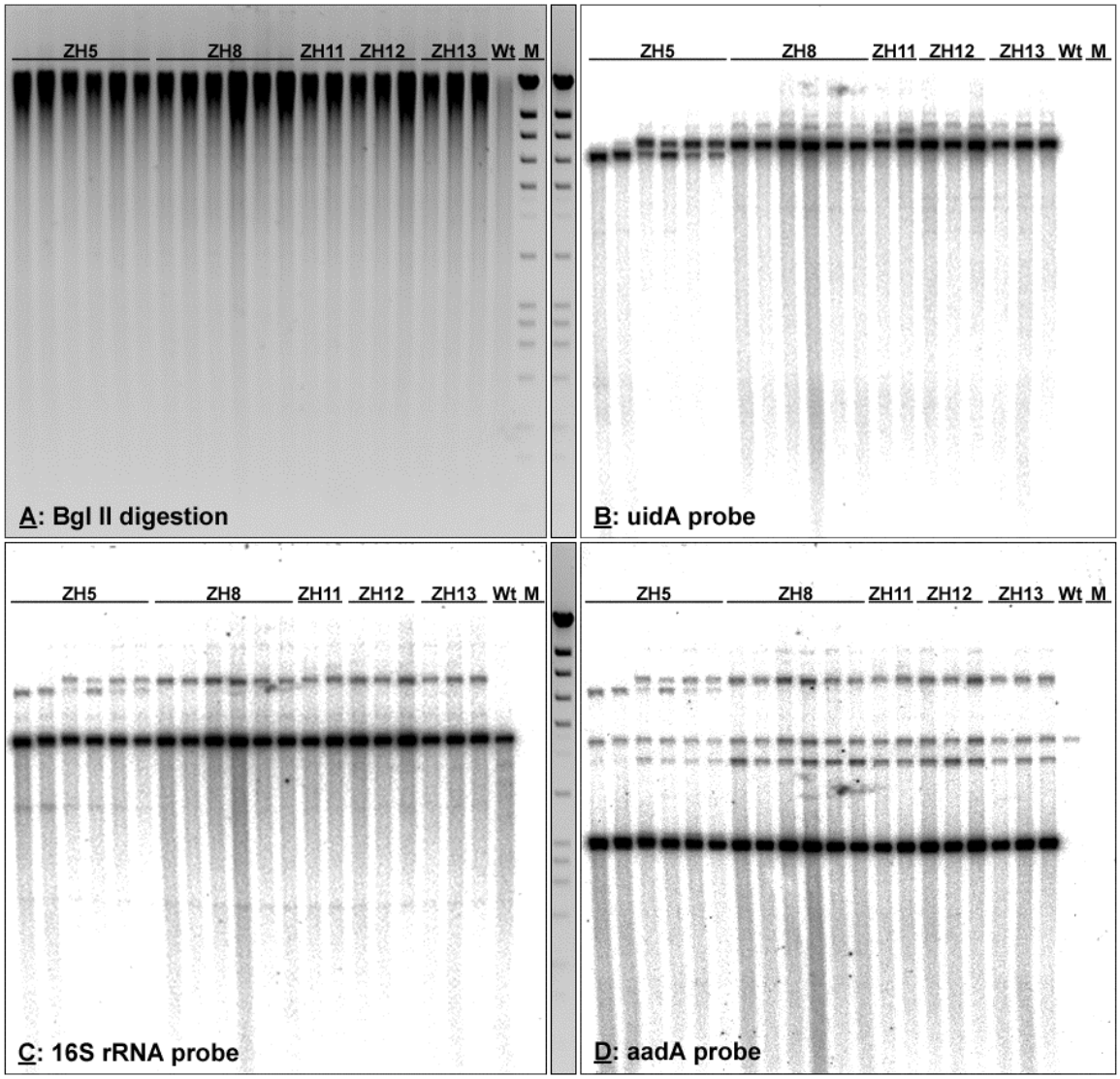


Figure 3.27: A representative of southern analyses of transplastomic plants. A: agarose gel electrophoresis of DNA samples with *BglII* digestion; B: first hybridisation with *uidA* probe; C: secondary hybridisation with *16S rRNA* probe; D: third hybridisation with *aadA* probe

Their total DNAs were cut by *BglII* and run on a 1% agarose gel (figure 3.27, A), then blotted on nylon membrane. The DNA filter was primarily reacted with the *uidA* probe, in which a distinct *uidA* hybridisation band (6.9kb) was observed in most constructs except ZH5 with the aberrant signals in discrete size (~6.3kb) or heterogeneous sizes (6.9kb and 6.3kb) (Figure 3.27, B). The weak band larger than the main *uidA* signal might come from an abnormal plastid recombination event or unspecific hybridisation, and was not found in all cases (figure 3.27 B). The same DNA filter was stripped, and re-hybridised with the *16S rRNA* probe, then an obvious signal (~4.5kb) of *16S rRNA* gene was subsequently obtained as the evidence for the examination of endogenous plastid genes (figure 3.27, C). Afterwards, the DNA filter was stripped once again for the third hybridisation with the *aadA* probe. Therein, a predominant signal of the expected size (~2.6kb) was proposed as the large percentage of normal transplastome, while a weak band (~4.0kb) was more or less found in all constructs, implying the existence of low abundance of the aberrant transplastome that comprised the intact *aadA* gene and flawed *uidA* gene (figure 3.27, D). This hybridisation pattern by *aadA* probe was also corroborated by another similar blotting with a *wt* probe for the determination of transplastomic homoplasmy (figure 3.32), because the plastid wt sequence (INSL) and *aadA* gene was associated within a *BglII* fragment in all constructs.

3.5.1.2 The essence of plastid transformation

Generally in plastid transformation, two flanking plastid fragments are required to facilitate the site-directed gene integration into the plastome by homologous recombination, thus the foreign gene insertion is predictable. To reinforce this transformation mechanism, plastid homologous fragment INSL was designed to contain two residues (CT) of the recognition sequence of *BglII* (G↓AGTCT) immediately at its 5' end distal to the insertion site (context 3.1.1). The *BglII* site was proposed to be maintained in the plastome if the plastid transformation was virtually conducted by homologous recombination.

In figure 3.28, the transformation events in the study of *psbA* 5'UTR is simulated according to the typical plastid transformation mechanism. Preliminarily, the transformation construct, e.g. pKCZ-psbA5'(Ori)::rbcL3' is associated with the wild-type plastome by the recognition and complementation of the homologous fragments (INSL and INSR) to their plastomic counterparts. Then, an intermolecular recombination occurs to result in the immigration of transgenes (*aadA* and *uidA*) from their construct into the wild-type plastome to form the transplastome in which the joint *BglII* site of INSL is remained. The *aadA* and *uidA* genes are theoretically compartmentalised by *BglII* into two discrete fragments with the

size of 2.6kb and 6.9kb respectively, and can be demonstrated by their corresponding probes in Southern analysis. The evidence in the aforementioned southern analyses substantiated the applicability of the canonical plastid transformation mechanism in this study.

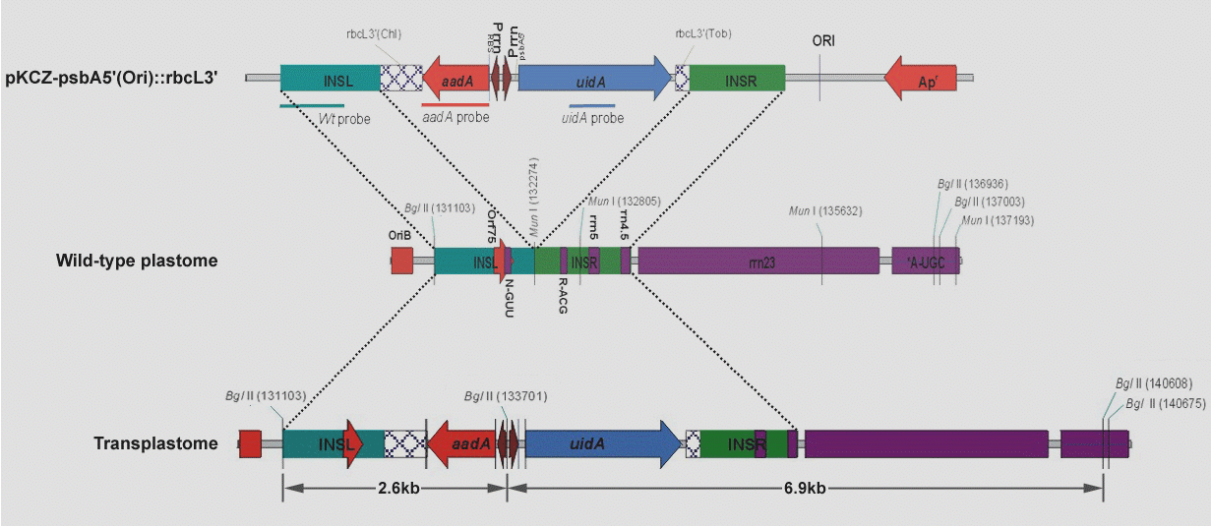


Figure 3.28: Homologous recombination between transformation construct pKCZ-psbA5'(Ori)::rbcL3' and tobacco wild-type plastome

3.5.2 RNA analysis of transplastomic plants by Northern Blotting

Total RNA was extracted from transplastomic plant leaves, and finally dissolved into the proper amount of RNase-free distilled water or 1x RNA loading buffer. The quality and concentration of RNA samples were evaluated on a test RNA gel. Then about 2µg total RNA for each plant sample undergoing the analysis of transplastomic homology was loaded into a RNA gel for Northern blotting. After running for several hours at 60~80V, the gel was applied to the RNA blot unit, by which the RNA was shifted from the gel to the nylon membrane. Then, the RNAs were fixed on the filter by UV-crosslinking, and became available for molecular hybridisations.

The RNA hybridisation was conducted with the identical probes (*uidA, 16S rRNA* and *aadA*) as in Southern analysis, but differed in that the temperature for RNA hybridisation and filter washing was increased to 65°C, all solutions were treated to eliminate RNases, and the washing was more stringent to erase more unspecific background. Finally, the hybridisation signals were identically detected by Fujifilm BAS1500.

A complete RNA analysis is represented in figure 3.29. RNA samples from the transplastomic lines (ZH2, ZH5, and etc) were run on a 1.2% agarose RNA gel for northern blotting (figure 3.29, A), where the samples visualised under UV transillumination were almost evenly loaded. The corresponding RNA filter was first reacted with the *uidA* probe,

then stripped and secondarily hybridised with the *16S rRNA* probe, followed by the third hybridisation with the *aadA* probe, in which the distinct hybridisation signals correlated to the transcripts of *uidA*, *16S rRNA* and *aadA* gene with the expected sizes (2.0kb, 1.5kb, 1.3kb) were revealed, respectively (figure 3.29, B, C, D).

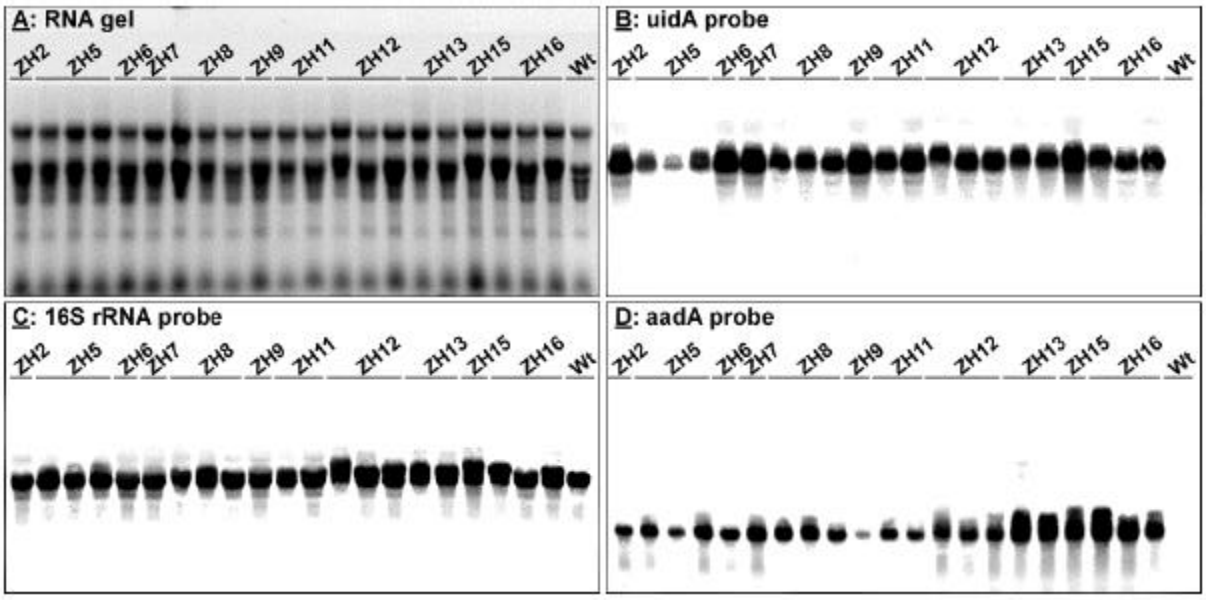


Figure 3.29: RNA analysis to transplastomic plants by Northern blotting. A: RNA gel electrophoresis; B: first hybridisation with uidA probe; C: secondary hybridisation with *16S rRNA* probe; D: third hybridisation with *aadA* probe

3.5.3 Qualitative analysis of uidA expression in transplastomic plants

While the total RNA was extracted from the transplastomic plant samples, the total protein was prepared with Gus extraction buffer from the same plant tissue, of which the concentration was subsequently determined by Bradford method. Then, a certain amount of protein extract for each sample was applied for the PAGE display of Gus activity and Gus fluorescent assay under UV illumination. The protein extracts could be stably stored at -70 °C for a few of months.

3.5.3.1 PAGE display of Gus activity

An appropriate amount of protein extract for each plant sample was loaded in the native polyacrylamide gel assembled in the mini-unit (*Bio-Rad*), and run at 60V in the cool room. After electrophoresis, the gel was rinsed with sterile distilled water, subsequently equilibrated with phosphate buffer, then incubated with the Gus staining solution (X-Gluc) or Gus fluorescent assay buffer (4-MU) at 37°C on a shaker for a proper period. Finally, the gels were rinsed with water after the removal of the reaction buffers. The *in gel* Gus fluorescence

(4-MU) was documented under UV illumination, as well as the *in gel* Gus staining was recorded by photography, in which the *uidA* gene product (Gus) with correct molecular weight was directly reflected by blue (figure 3.30, A) or fluorescent signal in the gel (figure 3.30, B).

Particular care was taken that the protein loading for the *in gel* Gus staining was evenly maximised up to the capacity of the gel slot to enable the Gus reaction product to become visible. During the *in gel* Gus fluorescence assay, the Gus reaction with the substrate (4-MUG) was drastic, and the fluorescence was intensely prone to peripheral diffusion. Thus the protein sample was loaded in gel after an appropriate dilution, and the gel incubation in the substrate solution(4-MU) was carefully monitored to reach an optimal fluorescent appearance (figure 3.30, B).

From figure 3.30, it can be seen that the *uidA* expression was decreased in all constructs with *psbA* 5'UTR modifications, but drastically in ZH5 (SLD), ZH7 (AUMN) and ZH9 (AUM), compared with the control construct ZH2 containing the wild-type *psbA* 5'UTR.

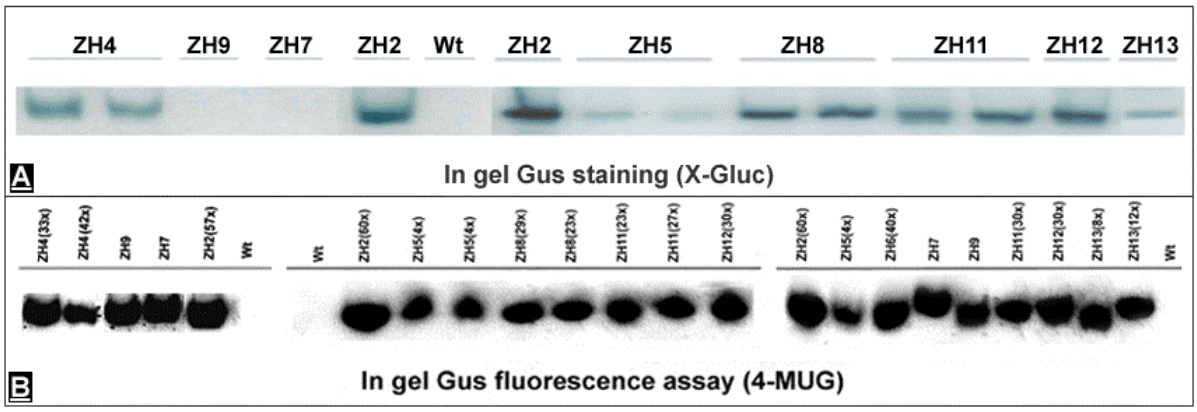


Figure 3.30: PAGE display of *uidA* expression in the transplastomic plants. A: *In gel* Gus staining (X-Gluc); B: *In gel* Gus fluorescence assay (4-MUG)

3.5.3.2 Gus fluorescent assay under UV illumination

The Gus fluorescent reaction was set up by incubating appropriate amounts of total protein extract of the transplastomic plant in Gus assay buffer containing the fluorescent reactant 4-MUG at 37°C for a certain period of time, then stopped. The samples were mainly submitted for the aftermentioned quantitative Gus fluorimetric measurement, but activities could also be demonstrated directly (figure 3.31).

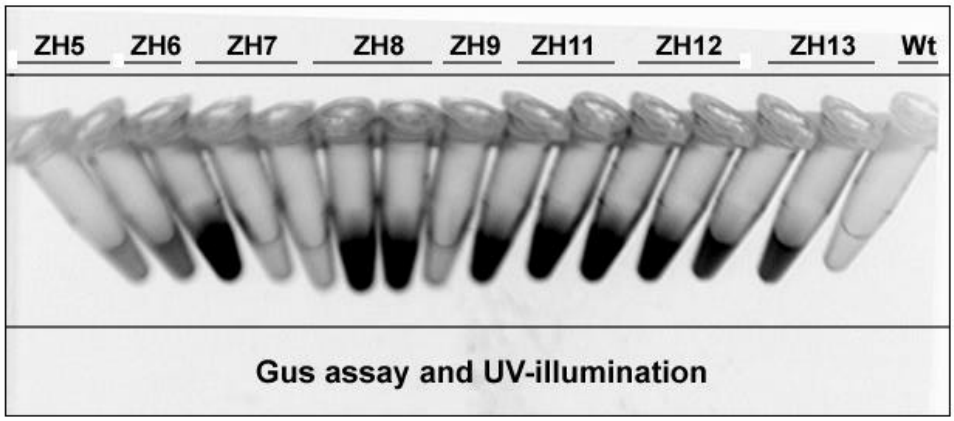


Figure 3.31: Gus fluorescent assay of transplastomic plants. Gus reaction samples were recorded in black and white under UV illumination

3.5.4 Quantitative analysis of transplastomic plants

3.5.4.1 Determination of the proportion of transplastome

For quantitative evaluations of transplastomic plants at the RNA and translational levels, it was essential to disclose their degrees of homoplastomy conferring the intact *uidA* gene, due to two particular concerns that the instability of transgene was occasionally observed and the plant samples might differ in the physiological status and subculturing cycles. Therefore, a group of plant DNA samples undergoing above DNA analyses and covering all constructs were submitted for a new round of southern blotting by *BglII* digestion and subsequent hybridisation with *wt* probe (figure 3.32).

From this blotting, the *wt* probe derived from plastid homologous fragment INSL generated three hybridisation signals (2.6kb, 4.0kb and ~5.8kb) concurrently visible (figure 3.32, left). The former two signals (2.6kb and 4.0kb) were coincident with the hybridisation profile by the *aadA* probe as the aforementioned, due to the intimate association of the *aadA* gene with the fragment INSL in all constructs. The predominant 2.6kb signal represented the normal transplastome comprising the intact *uidA* gene in majority, while the weak 4.0kb signal implied the coexistence in plastids of a paucity of aberrant transplastomes where the *uidA* gene was thought to be excised. Nevertheless, both these two signals constituted the entirety of transplastome. In addition, the large hybridisation signal (~5.8kb) was found singularly in the wild-type sample and slightly in all constructs. This implied that the transplastomic plants examined were heteroplastomic, with a low abundance of wild-type plastome in their plastids that had not been segregated out during the selection process.

All hybridisation signals derived from the *wt* probe were measured using *Tina* program as indicated in the following description. The same signals (2.6kb, 4.0kb or 5.8kb) in all constructs were preliminarily grouped in a transparent rectangular box with a central crossing line, then labelled as P1, P2 or P3 that could accordingly correspond to the *uidA*-containing normal transplastome, *uidA*-flawed aberrant transplastome, and wild-type plastome, respectively (figure 3.32, left). The signals in group P1, P2 or P3 were optimally figured as discrete peaks for all plant samples (figure 3.32, right), whose average densities were subsequently determined by arithmetical calculus and listed in the table 3.3.

In this case, the density values of above discrete hybridisation signals could be termed as different kinds of plastomy including the normal transplastomy, aberrant transplastomy, and wild-type plastomy that was also relevant to the labels P1, P2 and P3, respectively. Therewith, the sum of P1, P2 and P3 for each plant sample was equal to its full plastomy, as well as that of P1 and P2 was accounted as the total transplastomy. Thus, the percentage of normal transplastome (*uidA*%) and total transplastome (transgene%) could be determined by the formulas of P1/(P1+P2+P3) and (P1+P2)/(P1+P2+P3), respectively. Similarly, the formula of P3/(P1+P2+P3) was accorded to measure the remainder of wild-type plastome (wt%) in those transplastomic plants.

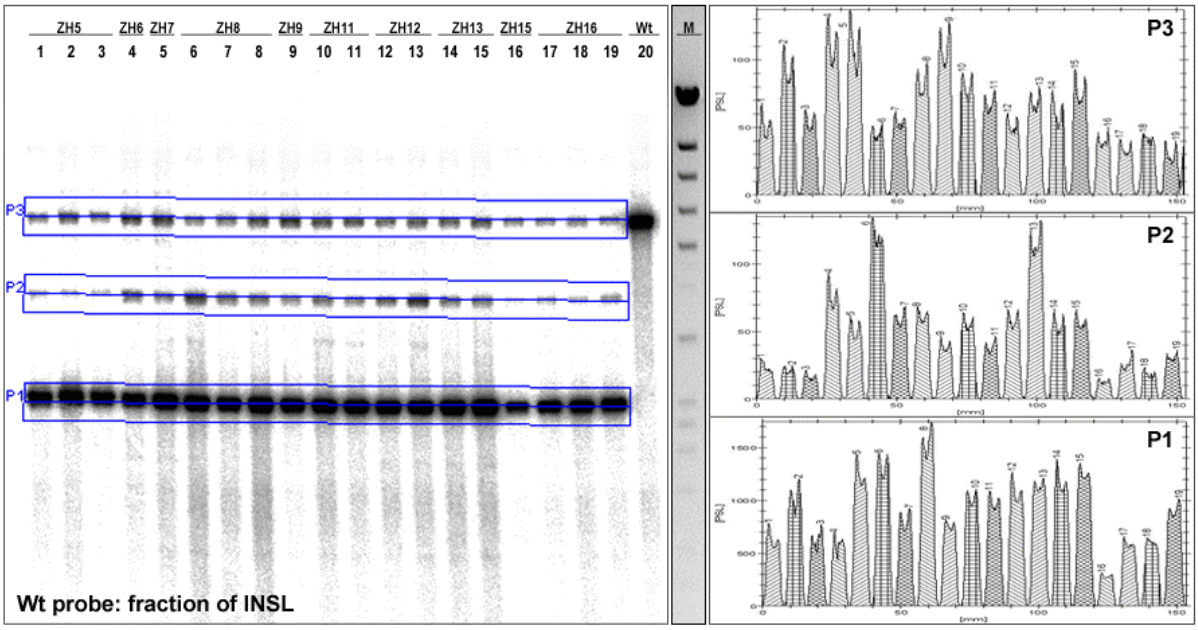


Figure 3.32: Determination of the plastomic proportion in transplastomic plants by Southern blotting with *wt* probe. P1, P2, P3: groups of 2.6kb, 4.0kb and 5.8kb hybridisation signals and their density values corresponding to normal transplastome (*uidA*-containing), aberrant transplastome (*uidA*-flawed) and wild-type plastome, respectively

Table 3.3: The density of hybridisation signals from Southern blotting

Sample Nr.	Sample Name	Cycle Nr.	P1(uidA$^+$) (PSL-Bkg)/mm	P2(uidA$^-$) (PSL-Bkg)/mm	P3(wt) (PSL-Bkg)/mm	Total (PSL-Bkg)/mm	P1/Total UidA%	(P1+P2)/Total Transgene%	P3/total Wt%
1	ZH5-13	IV	2201	82,56	181,8	2465,36	89,28 *	92,63	7,37
2	ZH5-44, a	III	3573,94	71,02	301,42	3946,38	90,56 *	92,36	7,64
3	ZH5-44, b	III	2143,45	56,13	186,9	2386,48	89,82 *	92,17	7,83
4	ZH6-2	IV	1957,72	240,33	365,98	2564,03	76,35	85,73	14,27
5	ZH7-1	IV	3910,13	171,34	347,94	4429,41	88,28	92,14	7,86
6	ZH8-5, a	III	4299,47	376,81	161,32	4837,6	88,88	96,67	3,33
7	ZH8-5, b	III	2801,5	210,41	170,38	3182,29	88,03	94,65	5,35
8	ZH8-5, c	IV	4917,9	198,91	262,91	5379,72	91,42	95,11	4,89
9	ZH9-1	I	2527,66	130,04	356,6	3014,3	83,86	88,17	11,83
10	ZH11-1, a	I	3435,83	170,63	262	3868,46	88,82	93,23	6,77
11	ZH11-1, b	I	3051,15	132,34	226,71	3410,2	89,47	93,35	6,65
12	ZH12-15, a	I	3551,65	198,31	172,56	3922,52	90,55	95,60	4,40
13	ZH12-15, b	I	3667,11	371,41	226,16	4264,68	85,99	94,70	5,30
14	ZH13-14, a	I	4019,32	174,78	205,17	4399,27	91,36	95,34	4,66
15	ZH13-14, b	I	4012,56	170,03	258,97	4441,56	90,34	94,17	5,83
16	ZH15-30	I	1019,11	47,07	125,84	1192,02	85,49	89,44	10,56
17	ZH16-19, a	I	1924,74	86,89	114,93	2126,56	90,51	94,60	5,40
18	ZH16-19, b	I	2062,14	60,58	132,37	2255,09	91,44	94,13	5,87
19	ZH16-1	I	3029,21	104,1	114,03	3247,34	93,28	96,49	3,51
Sum.			3076,55	163,11	220,49	3460,15	88,91	93,63	6,37

*: the proportion of transplastome containing intact *uidA* in ZH5 samples is adjusted and indicated in figure 3.33, corresponding to the hybridising band (6.9kb) in figure 3.27.

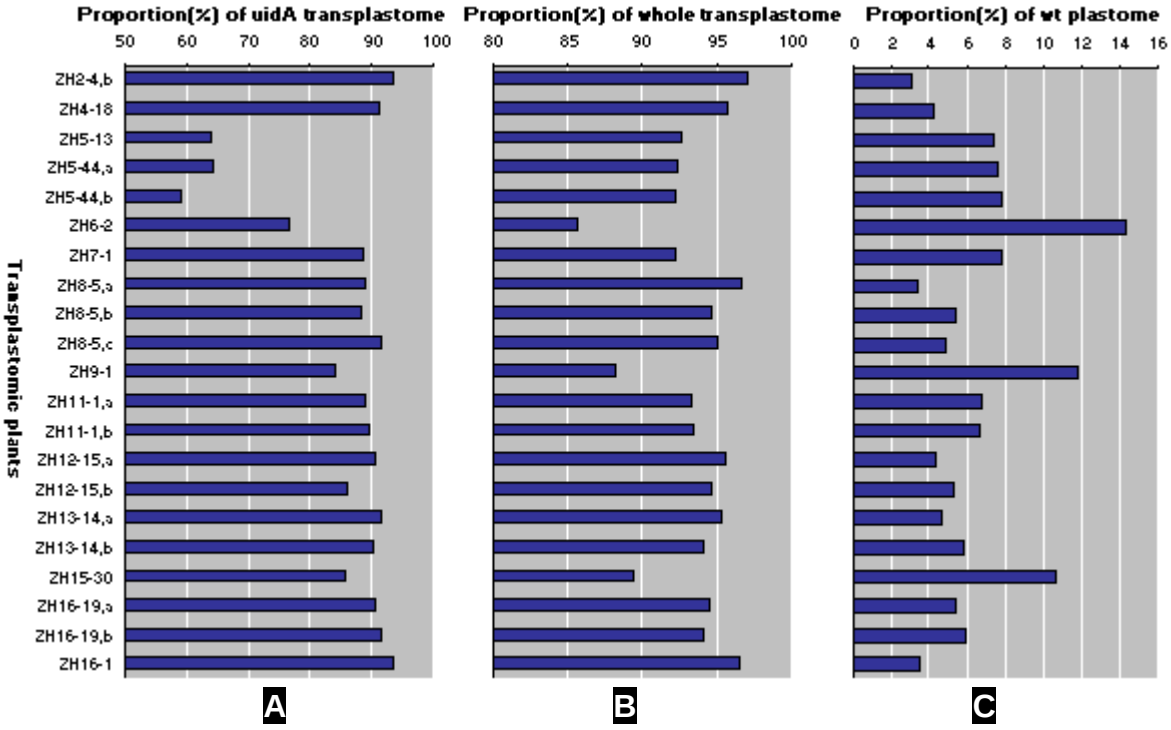


Figure 3.33: Proportions of *uidA*-containing transplastome (A), whole transplastome (B) and remained wild-type plastome (C) in transplastomic plants

From this quantitative analysis (table 3.3 and figure 3.33), the proportions of normal transplastome (*uidA*%) ranged from 58% to 95%, while the whole transplastomic proportion (transgene%) was relatively higher and varied from 85% to 97%. Moreover, in those constructs undergoing several cycles of subculturing, no apparent changes in the respective proportions were observed. These data indicate that it is unlikely to achieve the homogeneous transplastome in this study, albeit an average proportion of the whole transplastome up to 93.7% has been obtained.

Similar results in the DNA analysis using *wt* probe for some transplastomic plants obtained earlier confirmed the above finding. There, the transplastomic homoplasmy appeared to be unachievable, even in the transformation of vector pKCZ (ZH1) without detected aberrant recombination events after three cycles of tissue subculturing. In the constructs for the study of *psbA* 5'UTR, the heterogeneous transplastome was observed as well as above. Surprisingly in some constructs such as ZH4 and ZH7, the 2.6kb signal of normal transplastome was almost or fully absent, while the 4.0 kb signal of aberrant *uidA*-deficient transplastome and 5.8kb wild-type signal were consequently intensified (figure 3.34). This result implied that the *uidA* gene appeared to be more unstable than the *aadA* gene in the transplastome. The resultant *uidA*-deficient transplastome was also not very stable and inclined to the conversion into the wild-type plastome probably by a secondary deletion of the *aadA* gene.

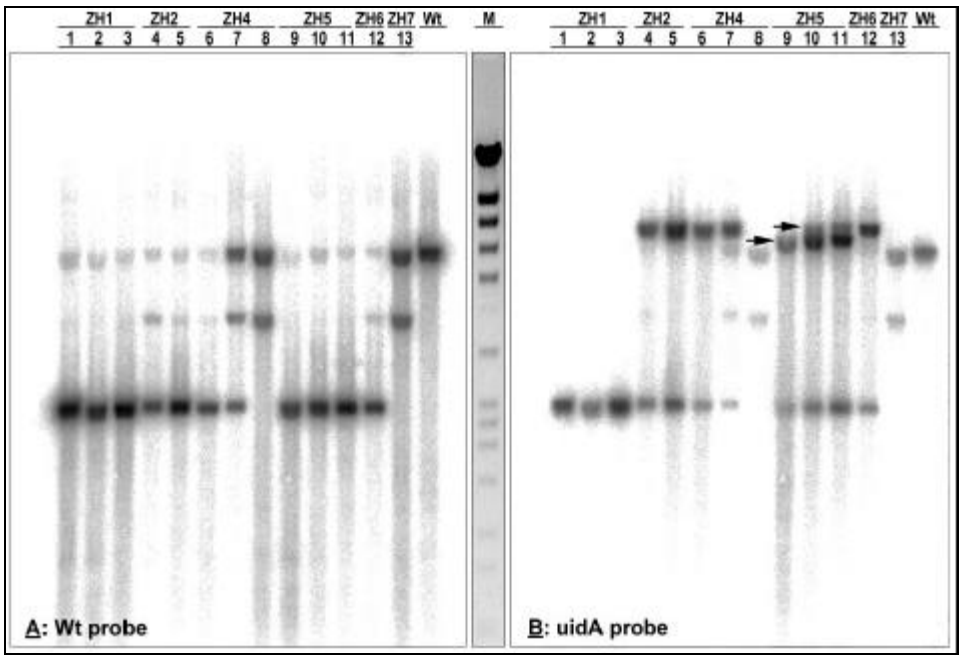


Figure 3.34: Characterisation of the transplastomic plants obtained earlier by Southern blotting with the *wt* probe(A) and the *uidA* probe(B). Arrows indicate two hybridisation signals of ZH5 samples

The proportion of transplastome was also finely determined by the same tactic as described above, and listed in the table 3.4. It was found that the proportion of normal transplastome in some samples of ZH4 and ZH7 was badly reduced even below detection level, and their whole plastome was nearly 50% aberrant transplastome and 50% wild type. Particularly, the proportions of different types of plastomes in transplastomic plants ZH2-4, b and ZH4-18 were highlighted in the table 3.4 and demonstrated in the figure 3.33.

Table 3.4: The density of hybridisation signals from DNA blotting (figure 3.34, A)

Sample Nr.	Sample name	Cycle Nr.	UidA+(%)	Transgene(%)	Wt(%)
1	ZH1-5, a	III	-	95,36	4,64
2	ZH1-5, b	III	-	95,95	4,05
3	ZH1-19	III	-	97,91	2,09
4	ZH2-4, a	III	77,64	91,19	8,81
5	ZH2-4, b	III	93,34	96,97	3,03
6	ZH4-18	III	91,18	95,72	4,28
7	ZH4-19, a	I	34,41	54,10	45,90
8	ZH4-19, b	I	6,30	40,54	59,46
9	ZH5-9, a	I	93,13	97,79	2,21
10	ZH5-9, b	I	90,83	94,73	5,27
11	ZH5-9, c	I	91,45	96,40	3,60
12	ZH6-2	II	88,56	96,87	3,13
13	ZH7-11	I	6,85	43,48	56,52

3.5.4.2 Determination of the relative *uidA* mRNA level

From northern blotting as indicated in figure 3.29 and another RNA blot with respect to ZH4 (data not shown), the transcript levels of *uidA*, *16S rRNA* and *aadA* genes examined in all transplastomic plants were evaluated as the densities of their corresponding hybridisation signals (determined by Tina program as described above, see context 3.5.4.1). The density value of the *16S rRNA* signal was used to standardise the RNA loading, then the *uidA* mRNA level could be normalised as $uidA_{16S}$ by the formula of value(uidA)/value(16S). Finally, the creditable abundance of *uidA* mRNA was calibrated by dividing the value of $uidA_{16S}$ with its related proportion of the normal transplastome (*uidA*%), and further adjusted as the relative *uidA* mRNA level for an overall comparison in respect of mRNA stability (figure 3.35, A). Therein, a similar decrease (2~3 fold) of the relative *uidA* mRNA level was apparently found in those *psbA* 5'UTR mutants related to the stem-loop region, including SLD (ZH5), SLM2 (ZH8), Rd-Pf (ZH11), Rd-Pd (ZH12), SLM1(ZH13) and NurSLD (ZH16). However, other mutants such as PolAD (ZH6), AUMN (ZH7) and AUM (ZH9) could accumulate *uidA* mRNA approximately to the level of Ori* (ZH2) conferring the wild-type *psbA* 5'UTR. Therefore, the stem-loop region of the *psbA* 5'UTR appeared to be an essential mRNA

stability determinant, the mutations of AU-Box and poly(A) sequence did not affect *uidA* mRNA accumulation. Furthermore, it was remarked that the *uidA* mRNA level was similarly reduced in mutants Rd-Pd (ZH12) and Rd-Pf (ZH11), indicating that the 5' end stem-loop structures of the *rbcL* 5'UTR did not significantly behave for the mRNA stabilisation and convey stability signals to the *psbA* 5'UTR. Additionally, it was also observed that the RNA abundance in the controls of Ori* (ZH2), Ori*-P (ZH4) and Ori-P (ZH15) was nearly equivalent, thus the slight change in the 5' end of original *psbA* 5'UTR appeared to have only an insignificant influence on mRNA stability of the *uidA* gene (figure 3.35, A).

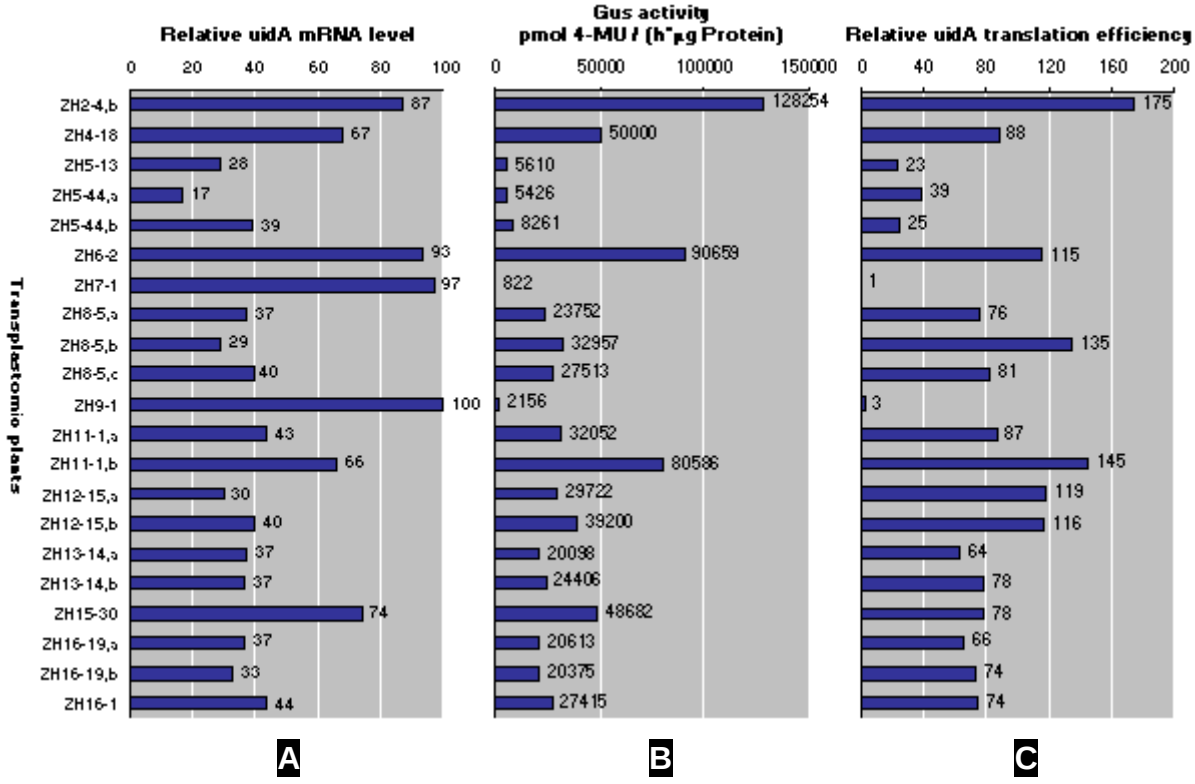


Figure 3.35: Overall quantitative analysis of transplastomic plants in the respects of relative *uidA* mRNA level (A), Gus activity (B), and relative translation efficiency (C)

3.5.4.3 Measurement of Gus activity by the fluorimetric assay

A proper amount of protein extract for each plant sample was pipetted for Gus fluorescent assay. After reaction, the fluorescence was measured in a Fluorimeter. In case of some plant samples with very high *uidA* expression, the reaction was diluted properly and measured again. The value of Gus activity for each sample was finally calibrated as described in the method, and represented in figure 3.35, B.

It was observed there that the Gus activity was extremely high (~129,000 pmol 4-MU/(h*µg Protein) in the control transplastomic plant containing the wild-type *psbA* 5'UTR, Ori* (ZH2), but reduced 2.5-fold in both Ori*-P (ZH4) and Ori-P (ZH15). A drastic decrease up to 150 fold of Gus activity was found in the *psbA* 5'UTR mutant AUMN (ZH7), as well as 50-fold or 20-fold decrease in AUM (ZH9) or SLD (ZH5) respectively. There was also a considerable (3~6 fold) decrease of Gus activity in the mutants mostly related to the stem-loop of *psbA* 5'UTR such as SLM2 (ZH8), Rd-Pf (ZH11), Rd-Pd (ZH12), SLM1 (ZH13) and NurSLD (ZH16). However, only a slight decrease of Gus activity was found in the mutant PolAD (ZH6) with the deletion of poly(A) sequence.

Since translation is correlated to the steady-state mRNA level, it is reasonable to evaluate the translation relatively. In this study, the Gus activity normalised to the *uidA* mRNA level was defined as the *uidA* translation efficiency that was compared in all constructs to disclose potential *cis*-elements in the *psbA* 5'UTR required for translation.

The *uidA* translation efficiencies specific to all constructs were calibrated as shown in figure 3.35, C. Therein, a dramatic decrease up to 175-fold of the translation efficiency was found in the *psbA* 5'UTR mutant AUMN (ZH7), as well as a 58-fold decrease in mutant AUM (ZH9). A range from 1.5 to 6-fold decrease of the translation efficiency was observed in those mutants related to the stem-loop structure of the *psbA* 5'UTR, and two-fold decrease in both Ori*-P (ZH4) and Ori-P (ZH15).

In conclusion, the AU-box of *psbA* 5'UTR was definitely required for *psbA* and its chimeric gene translations, since its mutations resulted in dramatic decreases in translation efficacy. The internal AUG within the space region of RBS and AU-box did not appear to serve as a virtual translation start codon, even though an in-frame *uidA* translatability was introduced in mutant AUMN. The stem-loop was essential for mRNA stabilisation and further translation, since its modifications led to various reductions in the mRNA level and translation efficiency of the reporter *uidA* gene. The poly(A) sequence did not play an apparent role in *psbA* gene expression, but its deletion might slightly affect the function of the stem-loop. Furthermore, the 5'end structural sequences of the *rbcL* 5'UTR did not confer distinct RNA stabilising signals in this case. From the comparison of chimeric *uidA* expression in Ori* (ZH2) with those in Ori*-P (ZH4) and Ori-P (ZH15), the slight modification at the 5'end poly(A) sequence in previous work had no significant influence on mRNA accumulation, while the *rbcL* 3'UTR appeared to moderately enhance the translation of the *uidA* gene possibly by an interaction with the 5' leader sequence.

3.6 Characterisation of transplastomic plants in greenhouse

All transplastomic plants undergoing the analyses were cultivated in the greenhouse for setting the seeds in batches, some of which were selected for characterisation again in the respects of RNA abundance and *uidA* expression. Leaves from large plants were harvested and quickly frozen by liquid nitrogen. The total DNA, RNA and protein were extracted simultaneously for each plant sample examined. PCR was carried out and revealed the maintenance of both *aadA* and *uidA* genes in the transplastomic plastids (data not shown). Quantitative Gus fluorimetric assay indicated that the Gus activity of the transplastomic plant in the greenhouse was generally lower than that in the culture chamber, but the pattern of *uidA* expression was the same in both cases, i.e. ZH2>ZH15>ZH16 (figure 3.36, A). Furthermore, the RNA blotting and *in gel* Gus staining were applied to detect the *uidA* mRNA and its gene product (Gus) that were also accumulated in the greenhouse plants to a certain of degree (figure 3.36, B).

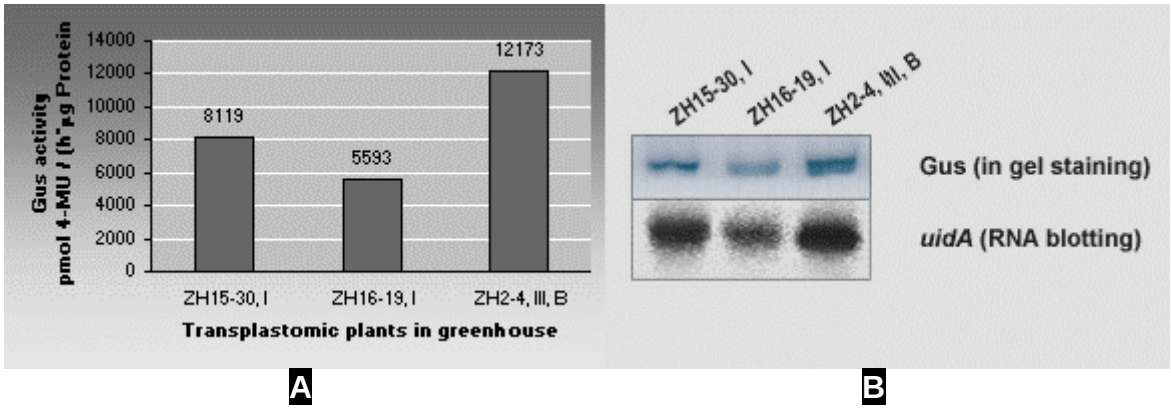


Figure 3.36: Molecular analysis of transplastomic plants in greenhouse. The expression of *uidA* was evaluated by Gus fluorimetric assay (A), in gel Gus staining (B, up) and RNA analysis (B, down)

4. Discussion

Chloroplast gene expression is mainly modulated at multiple posttranscriptional levels, such as RNA processing (editing, splicing, intercistronic cleavage and end maturation), RNA stability and mRNA translation, in which the latter two events are thought to be predominant regulatory checkpoints. Through various biochemical, genetic, and molecular approaches, the *cis*-acting determinants for mRNA stability or/and translation within the 5'UTRs of some photosynthesis-related chloroplast mRNAs have been defined, and a number of correlated *trans*-acting nuclear factors have also been characterised, particularly in the alga *Chlamydomonas* (Boudreau et al., 2000; Higgs et al., 1999; Mayfield et al., 1994; Nickelsen et al., 1999; Vaistij et al., 2000; Vaistij et al., 2000; Yohn et al., 1998a). In contrast, only limited data was obtained from similar studies in higher plants, especially for the 5'UTR of tobacco *psbA* mRNA. Therein, three *cis*- elements were revealed to be crucial for the translation *in vitro* (Hirose and Sugiura, 1996), and a rough region covering the putative stem-loop structure was suggested to be essential for both mRNA stability and translation by the *in vivo* analysis (Eibl et al., 1999). Among all analytic systems, chloroplast transformation is regarded as a potent tool to unravel the intricate regulatory mechanism of chloroplast gene expression, since it offers the potential to characterise the *cis*-acting elements required for mRNA stability and translation simultaneously under a variety of physiological and environmental circumstances. Thus, this approach was utilised in this work to characterise the tobacco *psbA* 5'UTR in detail. A series of *psbA* 5'UTR modifications were generated and up to fourteen plastid transformation constructs with either version (wt or modified) of *psbA* 5'UTR were made for chloroplast transformation. Spectinomycin-resistant transformants were obtained for all constructs, while thirteen constructs had the positive chloroplast transformants by Gus staining and twelve had the transplastomic plants regenerated. By analysis of all transplastomic plants, the following results were found: 1) the AU-box of *psbA* 5'UTR was absolutely required for the translation of *psbA* and its chimeric genes; 2) the stem-loop structure was generally responsible for mRNA stability and associatively affected the translation efficiency; 3) the internal AUG did not artificially act as a translation start codon; 4) the 5'end poly(A) sequence appeared to be not of functional significance; 5) the secondary structures at the 5' end of *rbcL* 5'UTR alone were incapable to confer mRNA stability. Furthermore, the SD-like RBS appeared to be an indispensable element for translation initiation as seen by Gus staining. Prior to this UTR's study, a versatile plastid transformation

vector pKCZ was constructed for the basis to generate all constructs involved in the functional analysis of *psbA* 5'UTR.

4.1 Insertion site and chloroplast transformation vector

Chloroplast transformation, as a site-directed transformation via homologous recombination, means that foreign genes are allowed to be integrated into the chloroplast genome at any insertion sites of interest. However, improper insertion sites might account for the instability of transgene in the transplastomes and the dysfunction of chloroplast endogenous genes. Thus, the insertion site is proposed to be a limiting rate in chloroplast transformation, and should be delicately evaluated prior to subsequent applications.

From the gene structures of a few of available chloroplast genomes in higher plants, it is noteworthy that most chloroplast genes are clustered into transcriptional operons, the tRNA genes are interspersed inside of operons or within the space region of two chloroplast transcriptional units (operon or single chloroplast gene). Therefore, it is suggested that tRNA transcripts might act as a stabilising element to promote RNA stability of primary transcripts. This is somewhat reflected by an observation in *Chlamydomonas* that a chimeric 3' UTR consisting of *petD* 3'IR and its downstream *trnR* could result in a higher level of *atpB* mRNA than the *petD* 3'IR alone, and the *trnR* appeared to singularly enhance the *atpB* mRNA stability independently of the strand orientation of the chimeric 3'UTR (sense or antisense) to flank the *atpB* gene (Rott et al., 1998).

Since the chloroplast genome (or plastome) generally contains two identical inverted repeat regions (IR_A and IR_B), a transgene inside this region is undoubtedly duplicated by copy correction in each plastome. Thus, such an integration site within the inverted repeat is thought to be contributive to highest expression of foreign valuable gene in chloroplasts.

Based on above concerns, two tobacco plastid transformation vectors (pPRV, pLD-CtV) containing the insertion site inside of the inverted repeat region had already been achieved in two laboratories (Daniell et al., 2001a; Zoubenko et al., 1994), and prevalently applied in a variety of biotechnological and fundamental biological realms (Chaudhuri and Maliga, 1996; Daniell et al., 2001a; Daniell et al., 2001b; De Cosa et al., 2001; Guda et al., 2000; Khan and Maliga, 1999; McBride et al., 1995; Serino and Maliga, 1997; Shiina et al., 1998; Staub et al., 2000; Ye et al., 2001). The insertion position in vector pPRV and its derivatives is located in the *trnV-rps7/12* intergenic region, precisely at the *ScaI* site between *trnV* gene and open reading frame *70B* (*Orf70B*) (Zoubenko et al., 1994); while in vector pLD-CtV, the insertion site is situated within the space region of two intron-containing tRNA

genes (*trnI* and *trnA*) individually flanked by two conserved rRNA genes (*16S rRNA* and *23S rRNA*) (Daniell et al., 2001a).

In our lab, a tobacco plastid transformation vector pFaadAI had also been constructed and proved to be effectual by PEG-mediated plastid transformation (Koop et al., 1996), in which the *SnaBI* site proximal to the 3' end of *rpl32* gene was chosen for the insertion of the selection marker gene *aadA* into the tobacco plastome. This vector was further modified with the removal of *SmaI/NdeI* fragment, then converted into pFaadAI ΔS/N that carried a unique *HincII* site (90bp downstream of *SnaBI*) for the plastomic integration of reporter *uidA* cassette with different UTR combinations (Eibl et al., 1999). However, both discrete insertion sites (*SnaBI* and *HincII*) were subsequently reexamined to reside in the *rpl32* 3'UTR that was certainly disrupted by the transgenes (*aadA* and *uidA*) in the chloroplast transformants, whereas the consequence of these disruptions appeared to be inapparent. Additionally, the integration of *uidA* gene at *HincII* site did also disturb the *sprA* gene that overlaps with *rpl32* 3'UTR. The exact function of *sprA* is still unknown. The 90bp plastid homologous sequence between the two insertion sites was assumed to account for the loss of the *uidA* gene with the trailer of *rpl32* 3'UTR through an aberrant intramolecular recombination (Eibl et al., 1999). Therefore, it appeared to be essential to establish a new plastid transformation vector for the future studies of chloroplast transformation.

Taken all above into consideration, a versatile vector pKCZ was generated to fulfil the requirement for the study that aimed to characterise the *cis*-acting elements of the tobacco *psbA* 5'UTR *in vivo*. In this vector, the position for foreign gene integration was chosen at the unique *MunI* site in the intergenic region of two tRNA genes (trnR-ACG, and trnN-GUU) in the inverted repeat region of the tobacco plastome. This insertion site was also close (~1.4kb) to the replication sequence (*OriB*) at the end of the IR proximal to the small single copy region (SSC). All replication sequences (*OriA* and *OriB*) in the tobacco plastome have been finely mapped and suggested to act as extrachromosomal replicons for transformation vectors whose copies in plastids can be increased by replication to facilitate homologous recombination (Kunnimalaiyaan and Nielsen, 1997). To construct vector pKCZ, two flanking plastid homologous fragments (INSL and INSR) were required and obtained by fidelity PCR amplification. The fragment INSR (~1.1kb) comprises the *trnR-ACG*, *5S* and *4.5S rRNA* genes that are located at the 3' end of the *23S rRNA* transcriptional operon, while fragment INSL (~1.2kb) covers an open reading frame (orf75) and its embedded *trnN-GUU* gene, but does not extend to the *OriB* sequence. It was important to exclude *OriB* from the plastid transformation vector because otherwise it become autonomous and would be maintained in

the chloroplasts. Its derivatives containing the *uidA* cassette would lead to an ambiguous Gus activity in chloroplast transformants that might be contributed by both transplastomic and extrachromosomal *uidA* genes. Therefore, the *OriB* sequence was deliberately excluded in vector pKCZ and its derivatives of all constructs containing variant versions of *psbA* 5'UTR in this study.

Vector pKCZ was constructed by a complicate process that included two initial PCR amplifications of homologous fragments (INSL, INSR) and the following ten steps of cloning (figure 3.6) to assemble the large insert consisting of INSL, *aadA* cassette, ISNR and additional tobacco *rbcL* 3'UTR into the framework of plasmid pUC18. The full sequence of vector pKCZ was identified, with an insert of 3934bp, and will be submitted to the DNA databases (GeneBank, EMBL, etc).

Two further versatile features of vector pKCZ include the convenience in future DNA manipulations and the introduction of two artificial enzyme markers to monitor chloroplast transformation. Unique restriction sites derived from the primary plasmids or introduced by PCR primers allow the integration of new genes or substitution of each component in the region flanked by homologous fragments (INSL and INSR) (figure 3.5). A truncated *BglII* and complete *EcoRI* site are particularly localised at the borders of fragments INSL and INSR distal to the insertion site, respectively. Through DNA analysis with these two enzyme digestions, the chloroplast transformation mechanism could be understood directly.

4.2 Plastid promoter and chloroplast transformation vector

Considering the attribute of chloroplast genetic system that resembles the prokaryotic counterpart but relies on nuclear factors to some extent, plastid endogenous promoters are generally selected to drive the target gene in plastid transformation constructs and following transplastome, except for some special applications such as bacteriophage T7 promoter required for the constitutive or inducible *trans*-activation of plastid genes by virtue of heterogeneous T7 RNA polymerase (Heifetz, 2000; McBride et al., 1994). These plastid promoters could be derived from a variety of plastid genes, e.g. tRNA promoter of *trnV* (Zoubenko et al., 1994), photosynthesis-related promoters of *psbA* (Zoubenko et al., 1994) and *rbcL* (Shiina et al., 1998), ribosomal promoters of *16S rRNA* (Prrn) (Svab and Maliga, 1993) and *rps16* (Staub and Maliga, 1994a) genes. From an overall analysis of barely chloroplast gene transcription, it was found that the transcription rate of translational RNA genes (tRNA and rRNA) was generally higher than that of messager RNA genes encoding the photosynthesis subunits, ribosomal proteins and RNA polymerase. In the latter, the

transcription of photosynthesis-related genes was predominantly strong, especially that of the *psbA* gene. Among all barley plastid genes examined, the transcription rates of *16S rRNA*, *tRNA-fMet* (*trnfM*) and *psbA* genes could be classified into the highest group despite an apparent rank: *trnfM* > *psbA* > *16S rRNA* (Rapp et al., 1992). The transcription of *psbA* was light-regulated, while the *trnfM* gene appeared to be constitutively transcribed as well as the well-known *16S rRNA* gene. Taken together, the promoter of *trnfM* gene (PtrnfM) was thought to be hyperactive and constitutive in chloroplasts, resembling the *16S rRNA* promoter (Prrn). Hence, it can be potentially used as the alternative of Prrn in case of requirement.

Promoter PtrnfM from tobacco plastome was obtained by PCR amplification. This promoter could drive the selection marker *aadA* and reporter *uidA* genes in their corresponding plasmids to acquire spectinomycin-resistance and Gus activity in *E.coli*, respectively (see appendix). This result demonstrated that the tRNA promoter (PtrnfM) did primarily activate in prokaryotes as well as the ribosomal promoter (Prrn) and photosynthesis-related promoter (PpsbA) characterised elsewhere (Brixey et al., 1997), whereas an *in vivo* functional analysis of this promoter by chloroplast transformation was required in parallel with other promoters such as Prrn.

4.3 Strategy for modifications of psbA 5'UTR

DNA sequence alterations can be effectuated by site-directed mutagenesis, PCR-based mutations (e.g. reverse PCR) and further methods. Nonetheless, with respect to the modifications of tobacco *psbA* 5'UTR in this study, a strategy taking the advantages of both fidelity PCR amplification and site-orientated restriction enzyme digestion was deliberately exploited, and hence termed as PCR-Enzyme associated DNA mutagenesis.

As described in results chapter, the wild-type *psbA* 5'UTR within the template plasmid was split by two distinct PCR events, then reconstituted by PCR-introduced enzymes during the related cloning steps. With that, the *psbA* 5'UTR was differentially modified, including base alterations, local sequence deletions and chimeric formation. PCR amplification with fidelity *Pfu* DNA polymerase is thought to prevent base-mismatches during the elongation phase, unlike in reactions with *Taq* DNA polymerase. Moreover, the enzyme-governed reformation of *psbA* 5'UTR could be more predictable and reliable. By DNA sequencing, all *psbA* 5'UTR modifications were confirmed. In addition, a universal manipulative platform was explicitly established and proven to be very favourable on the creations of *psbA* 5'UTR modifications and their related plastid transformation constructs. Conversely, other strategies such as reverse PCR (generally using *Taq* DNA polymerase) might not only take the risk of

amplification errors in UTR's region of the obligatory template plasmid, but also be incapable to accomplish all experimental tasks alone.

From sequencing of all constructs, it was unexpectedly found that template plasmid pUC16SpsbA5'uidArbcL3' carried a slight modification immediately at the 5' terminus of *psbA* 5'UTR, i.e. the small poly(A) tract (AAAAA) was changed into a sequence (ACTAA) with two nucleotide alterations. Since this study was the extension of a previous work in the lab (Eibl, 1999), this incidental modification was found to arise from the introduction of appropriate restriction enzymes for the convenience in subcloning PCR-amplified *psbA* 5'UTR. In a manner, this modification of *psbA* 5'UTR could also be regarded as one mutant. However, the 5' end poly(A) motif of *psbA* 5'UTR was not highly conserved in plants, with the consensus sequence as 'AATAACAA' seemingly overlapping two poly(A) motifs (AATAA and AACAA) (figure 3.15). Hence, this modified poly(A)* (ACTAA) was assumed unchanged with respect to its regulatory role.

4.4 Plastid transformation efficiency of vector pKCZ and its derivatives

Tobacco plastid transformation by biolistic DNA delivery was done after the completion of vector pKCZ and its derived constructs. Generally, one bona fide plastid transformant was obtained per each bombardment among all constructs, in consistence with the efficiency of plastid transformation reported in the literature (Svab and Maliga, 1993). However, it was found that this transformation frequency by the same selection marker differed with some experimental conditions and technical particulars, hence was meant to be improvable. For instance, frequent renewal of the selective RMOP medium was applied to not only avoid the false transformants under constant antibiotic selection stress, but also facilitate the regeneration of potential transformants by virtue of nutrition replenishment. The evenly small dissection of swollen leaf explants was useful to enable regeneration of many more resistant shoots. A prolonged selection process was also found to be contributive to increase of transformant numbers, despite a concurrent augmentation of spontaneous mutants. In addition, the physiological status of initial plant tissue for transformation was important. In this regard, the fully expanded, dark green leaves of about 2-month-old tobacco plants were recommended (Daniell et al., 2001a), since the chloroplasts in such kind of tissue are well-developed.

However, a remarkable escape of genuine plastid transformants accompanied the plastome segregation, due to the interference of spontaneous mutation and the phenotype masking of initial chimeric transgenic tissue where both the transplastomic and wild-type

sectors were green and indistinguishable. This bottleneck has been overcome by an advantageous fluorescent antibiotic marker FLARE-S as an in frame fusion of GFP (green fluorescent protein) marker behind the *aadA* (Khan and Maliga, 1999). The employment of this bifunctional marker in plastid transformation not only facilitates the distinction of plastid transformants and spontaneous antibiotic-resistant mutants, but also directly visualises the transplastomic sectors in the chimeric tissue, significantly simplifying the routine process to achieve homoplasmic transplastomic plants. Thus, this marker could also extend the plastid transformation to cereal crops that generally lack the pigment phenotype (Khan and Maliga, 1999). Additionally, this visual marker could enhance plastid transformation efficiency. In this respect, the influence of marker gene exploitation on tobacco plastid transformation was intensively compared, in which the bacterial *aadA* marker was much more efficient than the *16S rRNA* spontaneous mutant and improved the transformation efficiency up to 100-fold (Svab et al., 1990; Svab and Maliga, 1993), the fluorescent marker FLARE-S could duplicate the transformation efficiency of *aadA* as well (Khan and Maliga, 1999), and a non-antibiotic marker BADH (betaine aldehyde dehydrogenase) gene from spinach could increase plastid transformation efficiency 25-fold higher than the *aadA* gene with antibiotic selection of spectinomycin as declared by Daniell et al. (2001a).

4.5 Proportion of transplastome

From all constructs except ZH10, transplastomic plants were obtained. By the analyses of PCR amplification and southern blotting to those plant lines, the transgenes *aadA* and *uidA* were revealed to be integrated into the plastid genome at the directed site by homologous recombination (figure 3.22, 3.26, 3.28). Nevertheless, it was found that all transplastomic plants examined were heteroplastomic even after repeated cycles of subculturing, with the maintenance of differently low levels of wild-type plastome (figure 3.32, 3.33, 3.34; table 3.3, 3.4). Thus, the transplastomic proportion for each construct was required to be determined for the functional analysis of *psbA* 5'UTR. A similar scenario had also been reported elsewhere, and that heteroplastomy was thought to be related to inactivation of essential genes and hence to cell viability (Drescher et al., 2000; Kofer et al., 1998). However, the heteroplastomy in this study would rather be attributed to the instability of transgenes (*aadA* and *uidA*), since no plastid endogenous gene surrounding the insertion site was disrupted by foreign gene integration, but transgene loss as discussed below was observed and thought to occur in all constructs. Therefore, on one hand, the repeated cycles of antibiotic selection could accelerate plastome segregation to sort out the wild-type plastome in plastids; on the other hand, the

removal of transgenes from the transplastome adversely increased the heteroplastomy and hence compensated the elimination of wild-type plastome. These two reciprocal processes were discreetly proposed to reach a dynamic equilibrium in case of no concomitant occurrence of spontaneous antibiotic-resistant mutants. Otherwise, the *wt*-plastomic 'restoration' appeared to become predominant over its dismissal, and finally had the transplastomic plant regressed to the 'wild-type'-like plant in which the antibiotic resistance was possibly conveyed by the spontaneous *16S rRNA* mutations.

4.6 Loss of transgenes in plastid transformants

From the DNA analysis of all transplastomic plants by Southern blotting (figure 3.27, 3.32, 3.34), it was observed that the transgene loss occurred in all constructs containing the *uidA* cassette, featured by an unexpected 4.0kb hybridisation signal that is assumed to represent the proportion of transplastome with the excision of *uidA* gene and remainder of *aadA* gene. A plausible interpretation for this phenomenon might point to the inevitable employment of plastid promoter Prrn to govern both selection markers (*aadA*) and reporter (*uidA*) in this study. The promoter Prrn sequence (91bp) was present in three copies in each transplastomic inverted repeat that was separated by ~87kb LSC and ~19kb SSC, of which two specific to transplastomic *aadA* and endogenous *16S rRNA* (*rrn16*) genes were orientated as the direct repeats. It had been demonstrated that intramolecular recombination between two direct repeats could occur within the chloroplast genomes of *Chlamydomonas* (Kunstner et al., 1995) and tobacco (Svab and Maliga, 1993), resulting in the loss of the DNA fragment between the two repeats. Consequently, this 'direct repeat' strategy was used to recycle the selectable marker (e.g. *aadA*) for sequential chloroplast transformation (Fischer et al., 1996) or gain antibiotic marker-free transplastomic plants (Iamtham and Day, 2000), with a notable feature that one of the two obligatory repeats was still left in the chloroplast genome after the removal of their intervening DNA fragment. Therefore, the 4.0kb hybridisation signal herein is assumed to be the consequence of intramolecular recombination via direct repeats of Prrn. This 'direct repeat'-mediated gene excision is computer-simulated and shown in figure 4.1. Therein, the normal transplastome with the incorporation of both *aadA* and *uidA* cassettes could give rise to 2.6kb and 6.9kb hybridisation signals by DNA digestion with *BglII* and using *wt*/or *aadA* and *uidA* probes, respectively. The direct repeats of Prrn (marked with horizontal single arrows in the same orientation) have the potential to lead to the removal of their space region of ~10kb comprising the entire *uidA* cassette, all *rRNA* (5S, 4.5S, 23S and 16S), and several *tRNA* genes; meanwhile the intergenic *BglII* site between two divergent Prrn

for *aadA* and *uidA* cassettes and another two endogenous *BglII* inside of *trnA* gene are also likely to be removed. The resultant aberrant transplastome would exactly generate a 4.0kb *BglII* fragment that could be detectable by *aadA* and *wt* probes, but undetectable by *uidA* and *16S rRNA* probes. The remaining Prrn whether from *rrn16* or *aadA* itself appeared to ensure the expression of the *aadA* gene and therefore spectinomycin resistance. This hypothetical simulation is consistent with the hybridisation patterns in figures 3.27, 3.32 and 3.34. Another Southern analysis of DNA samples with *EcoRI* digestion also confirmed this simulation (data not shown).

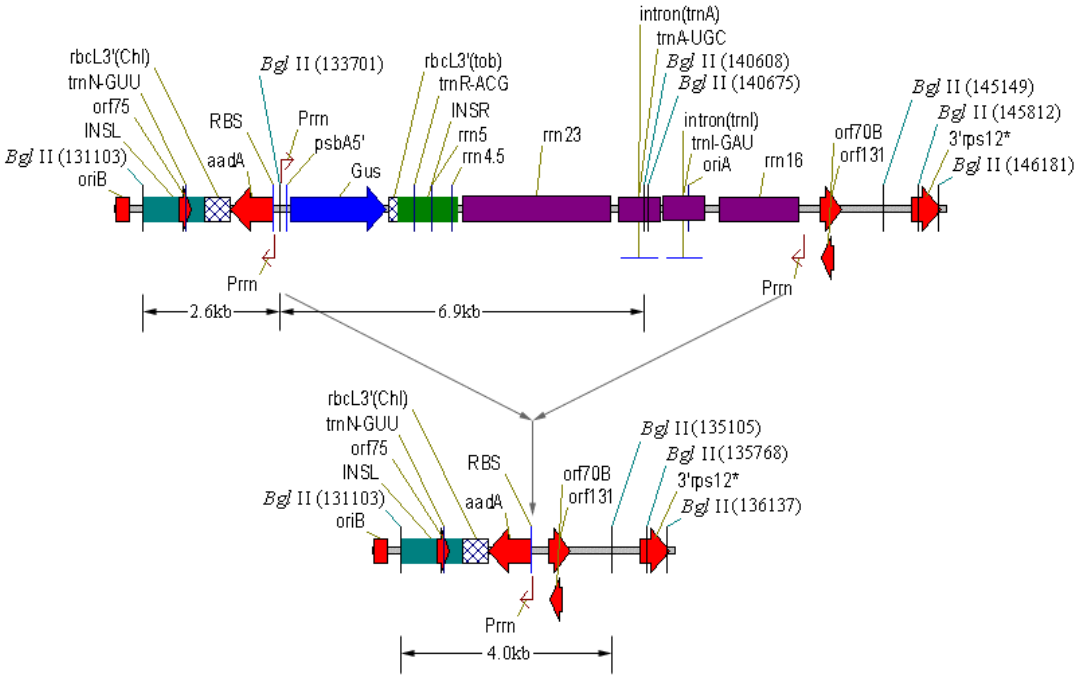


Figure 4.1: The gene loss mediated by direct repeats of Prrn (91bp) in transplastomic plants

From a variety of studies involving the application or incidental occurrence of direct repeat-mediated recombination in chloroplasts, it was found that the size of direct repeats appeared to control the frequency of gene excision (see overview in table 4.1). In *Chlamydomonas*, the 100bp and 230bp repeats did not allow detectable excision of the selection marker gene *aadA*, while 483bp and 832bp repeats could lead to efficient *aadA* loss, predominantly with respect to the latter (shaded in dark-grey) (Fischer et al., 1996). Likewise, the repeat of 418bp *psbA* 3'UTR had more potential than 174bp Prrn+rbcL5'UTR$_{RBS}$ to generate antibiotic marker-free tobacco transplastomic plants (shaded in light grey) (Iamtham and Day, 2000). However, a small direct repeat (90bp of *rpl32* 3'UTR) was also observed to result in the unexpected loss of the transgene *uidA* (~2.0kb) in tobacco chloroplast transformants (Eibl et al., 1999). In this study, the Prrn repeat (91bp) in transplastomic IR region could astoundingly direct the expurgation of a large fragment up to 10kb that

comprised the transgene *uidA* and all ribosomal RNA genes, and an average excision frequency of 5% was estimated among all constructs (table 3.3). This is in accordance with the statement that short direct repeats mediated plastid genome rearrangements during chloroplast evolution (Aldrich et al., 1988; Kanno et al., 1993).

Table 4.1: Overview of direct repeat-mediated gene excision in chloroplasts

Source of Direct repeat	Size of direct repeat (bp)	Chloroplast transformants	Possibility and relative strength of gene excision	Excised DNA size	Citation
psbA 3'UTR (tob)	392	Tob	+	26kb of LSC	(Svab and Maliga, 1993)
Plastome (Tob), imperfect, 136586-136601, 137438-137453	16	Tob	+ (?)	0.868kb (NICE1 within IR)	(Staub and Maliga, 1994b)
Not determined (Chl)	216	Chl	+	Not determined	(Cerutti et al., 1995)
atpA promoter (Chl)	100	Chl	Undetectable	None	(Fischer et al., 1996)
atpA promoter (Chl)	230	Chl	Undetectable	None	(Fischer et al., 1996)
Plasmid pACYC184 (non-plastid sequence)	483	Chl	+ (*)	~ 2.5kb (aadA)	(Fischer et al., 1996)
psbC Promoter +5'UTR (Tob)	832	Chl	+ (**)	~1.6kb (aadA)	(Fischer et al., 1996)
Prrn+rbcL5'UTR_{RBS} (Tob)	174	Tob	+ (*)	~3.8kb (uidA+aadA)	(Iamtham and Day, 2000)
psbA 3'UTR (Tob)	418	Tob	+ (**)	~2.5kb (aadA+bar)	(Iamtham and Day, 2000)
rpl32 3'UTR (Tob)	90	Tob	+	~2.0kb (uidA)	(Eibl et al., 1999)
Prrn+$16S_{18nt}$ (Tob)	91	Tob	+	~10kb within IR (uidA-included)	(This study, 2001)

Note: Tob, tobacco; Chl, *Chlamydomonas*; IR: inverted repeat; LSC: large single copy region; +, positive gene excision; *: the significance of gene excision; ?, the extrachromosomal NICE1 generated by 16bp imperfect direct repeat was only observed in particular tobacco chloroplast transformation published (Staub and Maliga, 1994b), so this gene excision by minor sequence of direct repeat appeared to be an occasional event.

Foreign gene excision by direct-repeat mediated recombination events was a continuous process in transplastomic plants during *in vitro* cultivation under selection stress (e.g. for generating transplastomic homoplasmy), and tended to be accelerated in the absence of antibiotics (e.g. while producing the progeny in the greenhouse). For example, the 90bp repeat of the *rpl32* 3'UTR led to the loss of the *uidA* transgene in an aberrant transplastome that retained the selection marker *aadA* for maintenance of spectinomycin resistance, this gene excision without any deterioration to cell viability could result in the homoplasmy of

aberrant transplastome (Eibl et al., 1999). The expression of *uidA* was further revealed to vary in the seedlings of corresponding transplastomic line (108-6) by Gus staining, some of which were completely deficient of Gus activity (data not shown). Additionally, it was also observed that when plastid recombination was activated by multiple direct repeats, recombination events did not stop until all local direct repeats had been removed (Iamtham and Day, 2000).

In this study, direct repeat-activated loss of the transgene *uidA* and all vital rRNA genes (e.g. *rrn16* and *rrn23*) was proposed to be lethal for plastid function, despite an unaffected contribution to antibiotic resistance due to the maintenance of an active *aadA* gene. Therefore, such resultant abnormal transplastome would be unstable and susceptible to be expelled as well as the wild-type plastome under selection stress. However, the plastome elimination appeared to be alleviated by the continuous gene excision, until a relatively dynamic equilibrium was obtained in most constructs with an average proportion of 4.7% for the aberrant transplastome (table 3.3).

Nevertheless, in some transplastomic plant lines (e.g. ZH4 and ZH7), this aberrant transplastome generated by direct repeats of Prrn became the uniform transplastome whose proportion was lowered, conversely with an accompanied increase in the proportion of wild-type plastome (figure 3.34). This finding was indicative of continuous gene excision, and is presumably attributed to such factors as spontaneous antibiotic resistance or decreased concentration of spectinomycin occasionally present in the RMOP medium. In the presence of either factor, the antibiotic resistance did not appear to be essentially provided by the normal transplastome, the gene excision was hence accelerated to result in the increase of aberrant transplastome, and the plastome sorting seemed to advance towards the absence of intact transplastome (i.e. Gus activity) and the increase of wild-type plastome. Particularly in case of spontaneous mutations, the antibiotic-resistant ‘wild-type’ plastome had the potential to further oust the aberrant transplastome and consequently restore the ‘wild-type’ plant lacking any transgenes that could not be detected by PCR and Gus staining. This might be a convincing interpretation for the failure in regenerating the desired transplastomic plants for construct ZH10 despite the obtainment of initial Gus-positive transformants. An overall plastomic fluctuation is generalised from above comments (figure 4.2). Meanwhile, excision of *aadA*-included DNA fragment might be generated by direct repeats of Prrn allocated in both inverted repeat regions. This gene loss is nonsensical but rather supplemented in this figure. Likewise, the slight *aadA* excision induced by direct repeats should be theoretically existent in plastid transformation by using the vector pPRV series and their derivatives, but the resultant *aadA*-deficient transplastome as well as the wild-type was extinguished sooner or

later by selection stress, and hence imperceptibly disregarded (Chaudhuri et al., 1995; Staub et al., 2000; Ye et al., 2001; Zoubenko et al., 1994).

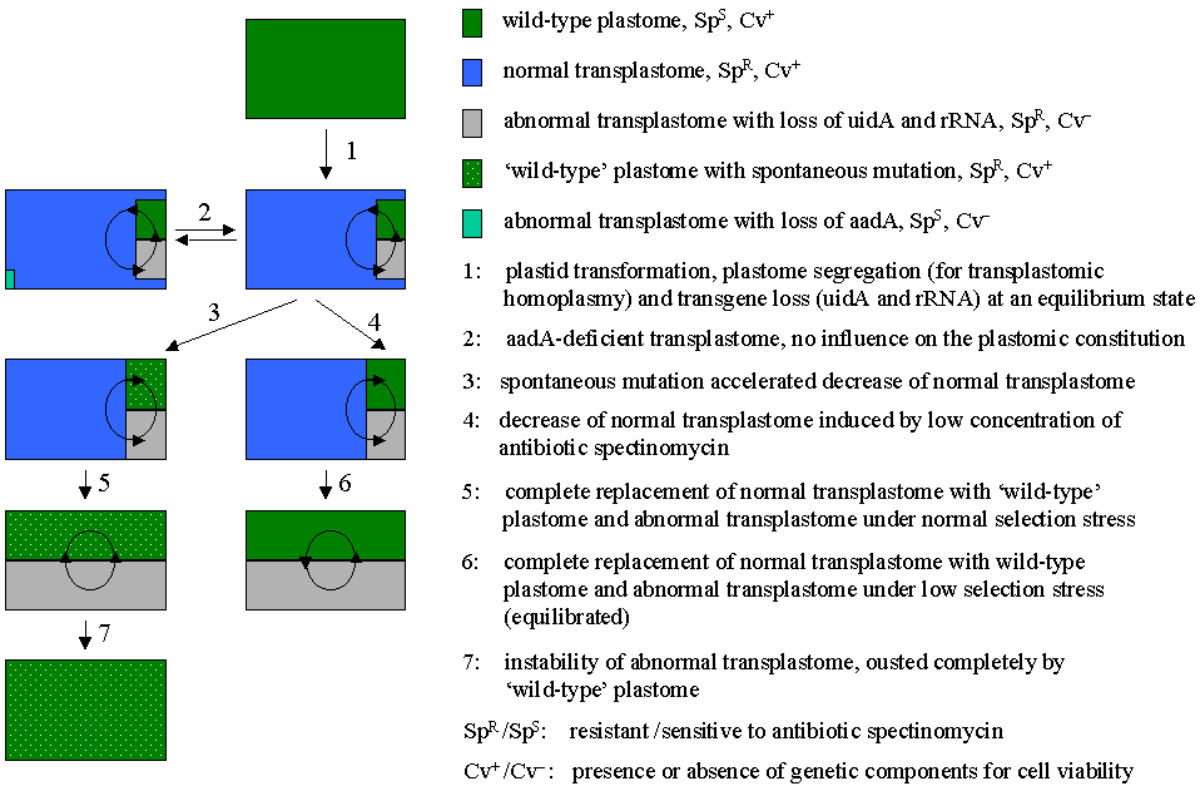


Figure 4.2: Outline of the plastomic fluctuation in transplastomic plants

Besides this common Prrn-directed gene loss in all constructs, another unexpected recombination was observed particularly in ZH5 and ZH3, which accounted for the partial loss of the *uidA* gene (figure 3.24, 3.25, 3.27, 3.34). With respect to both of these constructs that identically conferred the stem-loop deletion (SLD) of tobacco *psbA* 5'UTR, only aberrant antibiotic-resistant transplastomic plants were obtained, those of which corresponding to ZH5 were found to contain a *uidA* gene that was discretely truncated (relative to 6.3kb signal) or heterogeneous in size (relative to 6.3kb and 6.9kb signals) in figures 3.26 and 3.27, while all those to ZH3 appeared to be fully deficient of an intact *uidA* gene and hence Gus activity, but with a remained *aadA* gene (figure 3.24, 3.25). This type of gene excision also seemed to be a continuous process, and all copies of intact *uidA* genes were finally impaired to silence the Gus activity in plastid transformants. However, those transplastomic plants (ZH5-13, ZH5-44) with *uidA* expression by Gus staining were included in the integral quantitative assays for the functional characterisation of tobacco *psbA* 5'UTR. To elucidate the mechanism responsible for this particular gene loss, further analysis is required.

Furthermore, additional abnormal recombination was observed in transformants, resulting in hybridisation signals larger than 6.9kb (*uidA* probe) and at least one weak signal smaller than 4.5kb (*16S rRNA* probe). In ZH5, such recombination event was antagonistic to the process of partial *uidA* loss (figure 3.27). More detailed analysis would be required to elucidate the origin of these signals.

Taken all into consideration, the gene loss arisen from direct-repeat mediated and other unknown recombination events is assumed to account for the underestimation of transformation efficiency and inability to achieve transplastomic homoplasmy (even for vector pKCZ) in this study.

The 91bp Prrn for expression control of the selection marker *aadA* was able to activate a gene loss of up to 10kb, in combination with its direct repeat of endogenous cognate (in *16S rRNA*). Therefore, this promoter was not optimal for expression of transgenes incorporated at the chosen insertion site (intergenic *MunI* of trnR-ACG and trnN-GUU) that was regarded to be functional. To prevent the reuse of Prrn specifically with this insertion site, plastid promoters from other plant species might be a solution but need to be discreetly judged (Sriraman et al., 1998). Additional endogenous plastid promoters outside of the invert repeat region such as PtrnfM (promoter of trnfMet gene) might be advantageous, as well as the *psbA* promoter. In this study, this promoter PtrnfM functionally resembling Prrn was shown to direct the expression of *aadA* and *uidA* genes in *E.coli*. Hence, it could be used as an alternative to drive the selection marker or other genes of interest in designation of further plastid transformation constructs.

4.7 Functional architectural analysis of tobacco psbA 5'UTR

By the approach of chloroplast transformation, transplastomic tobacco plants that carried a series of *psbA* 5'UTR modifications were obtained. After a systematic analysis of those plants with respect to transplastomic proportion, mRNA accumulation and protein activity of chimeric reporter *uidA* gene, some *cis*-elements within the *psbA* 5'UTR were revealed to play an important role in modulating mRNA stability and translation efficiency.

4.7.1 The role of the AU-Box and dual RBS-mediated translation model

Among those elements, the AU-Box of *psbA* 5'UTR was identified to be a major *cis*-acting translational determinant, since its mutations led to dramatic decreases in Gus activity (up to 150-fold) and translation efficiency (up to 175-fold) of chimeric *uidA* gene, but did not

impair (rather slightly enhance) *uidA* mRNA accumulation (figure 3.35). This data confirmed the previous report by an assay of *in vitro* chloroplast translation system where the local deletion and base alterations to this AU-rich region almost abolished translation (Hirose and Sugiura, 1996). In addition, other two *cis*-elements (RBS1 and RBS2) complementary to the 3' end of *16S rRNA* were revealed to be essentially involved in translation, suggesting a regulatory model for tobacco *psbA* translation that the bipartite interactions between RBS1/RBS2 and *16S rRNA* resulted in a loop-out of the AU-box optimally for the targeting of nuclear *trans*-factors to modulate translation initiation (Hirose and Sugiura, 1996).

In plastid gene transcripts, the AU content is relatively higher than that in nuclear RNAs, especially in their 5' untranslated regions. The small A or U tract within the 5'UTR was likely favoured for the interaction with nuclear encoded RNA binding proteins (Alexander et al., 1998; Ossenbuhl and Nickelsen, 2000; Shteiman-Kotler and Schuster, 2000). However, according to the *psbA* translation model in tobacco, the function of this A/U-rich region appeared to need the coordination of its flanking sequences (RBS) to associate with 30S small ribosome subunits. These RBS elements could be accounted as any sequences that were relatively adjacent to the translation initiation codon and complementary to the 3' end of *16S rRNA*, including the canonical SD (GGAGG) which was nonetheless absent in a certain number of plastid genes and examined not to be an absolute requirement for translation (Fargo et al., 1998).

In higher plants such as barley, spinach, and tobacco, the region (-1 to -50 with respect to authentic AUG initiation codon) of the *psbA* 5'UTR is highly conserved (Kim and Mullet, 1994), including all putative three ribosome binding sites (RBS1, RBS2, RBS3) (figure 4.3). The SD-like RBS3 (GGAG) is relatively distant to the initiation codon, generally at the site –33 (Kim and Mullet, 1994). Since the translation initiation complex was found to only protect a region of mRNA from –20 to +13 in contact with ribosomes from nuclease digestion (Hartz et al., 1988), this distal SD-like RBS3 was unlikely to guide the translation initiation directly. *In vitro* toeprint assay also indicated that the barley *psbA* mRNA had a main toeprinting pattern similar to that of *rbcL* mRNA by chloroplast polysomes (Kim and Mullet, 1994). The RBS1 (AAG, -9) is fully identical, while RBS2 (generally at –22) appears to be variable in position and nucleotide composition among plant species. Both of these non-SD RBS sequences are complementary to the 3'end of *16S rRNA*, and relatively adjacent to the AUG codon with an intervening AU-rich region that has the consensus sequence of 'UAAAUAAA' (Kim and Mullet, 1994) (figure 4.3).

In contrast, the *Chlamydomonas psbA* 5'UTR lacks the RBS2 but retains a putative RBS1 derivative, i.e. RBS(?), -9, matching the immediate 3' extremity (UUU) of *16S rRNA* (figure 4.3). The SD-like RBS3, i.e. RBS(SD), -27, a little close to AUG, is remained beyond the area of ribosome protection and hence incapable to initiate translation alone. Nevertheless, this RBS(SD) was shown to be required for ribosome association that had a tight correlation with the processing, stability and translation of *psbA* 5'UTR (Bruick and Mayfield, 1998). Additionally, the AU-rich space between RBS(?) and RBS(SD) was also essential, resembling the tobacco AU-Box and thus termed as AU-Box(Chl), since its local deletion led to the deficiency of translation but accumulated wild-type level of *psbA* mRNA (Bruick and Mayfield, 1998). Therefore, the RBS(SD) appears to integrate the functions of tobacco RBS2 and RBS3, embracing the AU-box(Ch1) together with RBS(?) to modulate the translation.

A similar scenario to that of *psbA* 5'UTR was also found in the *Chlamydomonas psbD* gene encoding D2 in PSI (Nickelsen et al., 1999). The *psbD* 5'UTR contains a consensus SD ('GGAG', PRB1, -10), rather proximal to the AUG codon, thus the translation of *psbD* mRNA was proposed to be executed via the typical prokaryotic translation mechanism (Sugiura et al., 1998). However, the PRB1 activity did not appear to be absolutely sequence-dependent, because base-altered mutants PRB1A ('AAAG') and PRB1B ('GAAG') had no influence on *psbD* translation, despite a significant decrease (75%) of D2 synthesis in another mutant PRB1C ('CCTC') (Nickelsen et al., 1999). In this regard, it was noticed that the mutants PRB1A and PRB1B comprised the tobacco RBS1 sequence ('AAG') with the ability to complement 3'end of *16S rRNA* and hence associate 30S ribosome. Moreover, all these mutants accumulated the wild-type level of *psbD* mRNA and did not affect the 5' end maturation of *psbD* mRNA, suggesting a different function of SD-like PRB1 from its counterpart in *psbA* 5'UTR (Bruick and Mayfield, 1998). Interestingly, an anterior element PRB2 ('AGTTGT', -25) fully entailed the role of RBS(SD) in *psbA* 5'UTR, and was responsible for 5' end processing, stability and translation of *psbD* mRNA by an assumed ribosome association, due to its potential to complement the 3'end sequence upstream of 'CCUCC' in *16S rRNA* (Nickelsen et al., 1999). In addition, the 11mer U-tract between PRB1 and PRB2 was identified as a translation determinant. However, the function of this element appeared to be sequence-independent, and rather length-dependent with a minimal 8nt spacing between PRB1 and PRB2. The secondary RNA structure within this element was also suggested to significantly impair translation efficiency, and whatever mutations of this U-rich region only slightly affected the mRNA accumulation (Nickelsen et al., 1999; Ossenbuhl and Nickelsen, 2000). These data indicate a considerable difference from the studies related to the

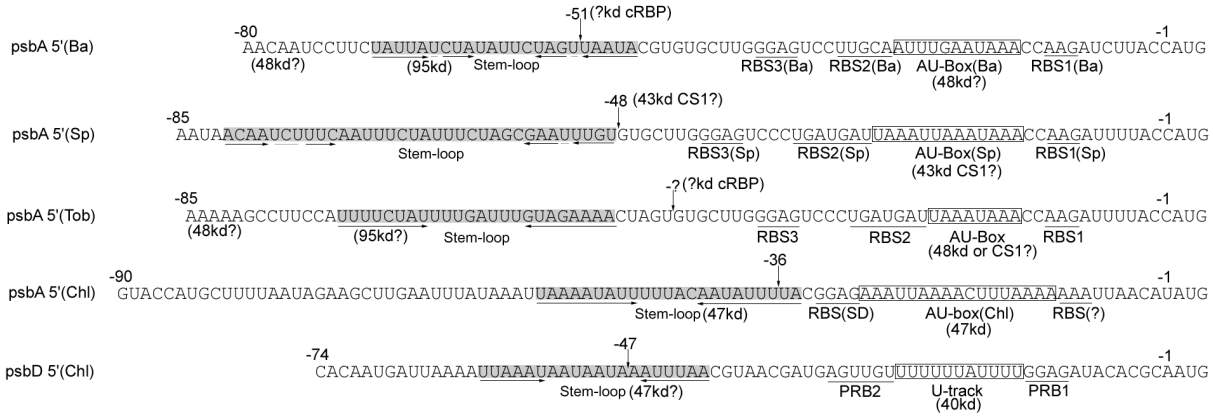
AU-Box (UAAAUAAA) of the *psbA* 5'UTR in tobacco. By an *in vitro* assay, two base-altered mutants of the AU-Box, M4 ('UCGCUAAA') and M5 ('UAAAUCGC'), significantly affected the translation efficiency (less than 15% remained) (Hirose and Sugiura, 1996). By *in vivo* analysis in this work, most A bases of the AU-box were changed to G/C bases in mutant AUM ('UCGGUCCG') and its derivative AUMN ('UCGGUCCGA'). This change almost abolished the translation of the chimeric reporter *uidA* gene. Hence, the efficacy of the AU-Box of tobacco *psbA* 5'UTR is likely to be sequence-dependent. Additionally, a more severe decrease of translation efficiency was observed in mutant AUMN (175-fold) than AUM (58-fold) (figure 3.35). This discrepancy might be attributed to the potential formation of a strong secondary structure rather than length extension (with extra base 'A') of mutated AU-Box in mutant AUMN.

Taken together, the dual RBS-mediated translation model appears to be a common mechanism underlying the regulation of some photosynthetic genes such as *psbA* and *psbD*. The PRB1/PRB2 elements of *Chlamydomonas psbD* 5'UTR resemble RBS(?)/ RBS(SD) of *psbA* 5'UTR in the same alga, and RBS1/RBS2 of *psbA* 5'UTR in tobacco and other higher plants, while its U-tract is similar to the putative AU-box of *psbA* 5'UTR in all plant species (figure 4.3). These bipartite interactions of two RBS stems with the 3' end of *16S rRNA* extrude a unstructured loop-out of A/U-rich motif for binding the *trans*-acting factors to regulate translation.

According to this model, the internal 'initiation codon' of *psbA* 5'UTR (AUG, frequently used; UUG, AUU, scarcely used) adjacent to the SD-like RBS3 was found to reside in the RBS2 or A/U-rich region between RBS1 and RBS2, and not to confer a consistent reading frame (Kim and Mullet, 1994). In mutant AUMN of this study, an in frame mRNA translatability starting from the 'AUG' upstream of the AU-Box was introduced. However, this mutation did not suppress its maternal mutant AUM to conduct a normal translation, and further impair the translation efficacy instead. Thus, this internal 'initiation codon' does not appear to direct translation.

Through molecular and biochemical efforts, some nucleus-encoded factors (cpRBP) have been identified to bind the 5'UTR to regulate plastid gene expression, in a relatively gene-specific manner. In spinach, the 43kd CS1 (chloroplast homologue of E.coli S1) with high affinity to the A/U-rich sequence within the 'central protein binding element' (from –49 to –9) of *psbA* 5'UTR was suggested to guide the translation initiation through the prokaryotic S1-mediated translation mechanism, and to participate in the 5'end endonucleolytic processing at the –48 site and subsequent degradation of *psbA* mRNA (Alexander et al.,

1998). In barley, the stromal 95kd RBP could specifically interact with the stem-loop sequence at the side adjacent to the 5' termini of *psbA* 5'UTR, while the binding region of membrane-associated 48kd RBP was not clearly defined. In addition, two other stromal RBPs (60kd and 38kd) was also observed, and the binding of 60kd RBP to *psbA* 5'UTR was reduced along with 48kd RBP by addition of ATP (Mullet, unpublished data). However, none of such proteins have been examined or referred to the *psbA* 5'UTR in tobacco. In *Chlamydomonas*, a protein complex was characterised, including 38kd, 47kd and 60kd RBPs with high affinity and specificity to either form (-90 or –36) of the *psbA* 5'UTR (Bruick and Mayfield, 1998; Danon and Mayfield, 1991). The 47kd RBP (or RB47) was identified as membrane-associated chloroplast poly(A) binding protein (Yohn et al., 1998a; Zerges and Rochaix, 1998), and 60kd RBP (or RB60) as the chloroplast homologue of protein disulfide isomerase partitioned between stroma and thylakoid (Kim and Mayfield, 1997; Trebitsh et al., 2001). Furthermore, RB47 could also bind to the longer form of *psbD* 5'UTR exclusively, suggesting its role in the 5' end processing of *psbD* mRNA (Nickelsen et al., 1994). With respect to the *psbD* 5'UTR, a 40kd RBP was revealed to interact with the U-rich motif between two RBS elements mentioned above, PRB1 and PRB2, and a clear correlation between D2 synthesis and RNA binding capacity of 40kd RBP was observed (Ossenbuhl and Nickelsen, 2000). This appears to be reminiscent of RB47 activity specific to the *psbA* 5'UTR in regulating the light-activated *psbA* translation (Yohn et al., 1998b).



Ba: Barley; Sp: Spinach; Tob: tobacco; Chl: Chlamydomonas; ?: undefined; -48, -36, -47, -51: cleavage or maturation sites of 5'UTR;
RBS1, RBS2, RBS3, PRB1, PRB2, RBS(SD), RBS(?): ribosome binding sites complementary to 3' end of 16S rRNA, dependently of plastid gene;
cRBP: chloroplast RNA binding protein; 95kd, 48kd: cRBPs for barley psbA 5'UTR; 43kd CS1: chloroplast homologue of E.coli S1 to bind spinach psbA 5'UTR;
47kd: cRBP to bind psbA 5'UTR and longer form of psbD 5'UTR in Chlamydomonas; 40kd: cRBP to bind psbD 5'UTR U-track in Chlamydomonas;
References: psbA 5'(Ba), Kim et al.,1994 and Mullet (unpublished data); psbA 5'(Sp), Alexander et al.,1998; psbA 5'(Tob), Hirose et al.,1996;
psbA 5'(Chl), Mayfield et al.,1994 and Bruick et al.,1998; psbD 5'(Chl), Nickelsen et al.,1999 and Ossenbühl et al.,2000.

Figure 4.3: Dual RBS-mediated translation model for chloroplast genes

Taken these into consideration, the *Chlamydomonas* RB47 protein might interact with the loop-out of the AU-Box(Chl) to modulate translation, and with another A/U-rich motif

upstream of the distal RBS to affect 5' end maturation and stability of *psbA*/or *psbD* mRNA. This protein might also target the mRNAs to the thylakoid membrane for translation or recruit other functional *trans*-factors such as RB60 and RB38. The 40kd protein likely entails the partial role of RB47 to modulate translation of the *psbD* mRNA, whereas the regulation of its binding activity to the U-rich region is still unknown. With respect to the *psbA* 5'UTR in higher plants, the spinach 43kd CS1 protein appears to resemble *Chlamydomonas* RB47. The set of barley RBPs (38, 48, 60kd) is proposed as the counterparts of the RNA binding complex (RB38, RB47, and RB60) in *Chlamydomonas*. Therefore, the 48kd protein as well as the 43kd CS1 might interact with the AU-box to execute the light-activated regulation of *psbA* translation in barley or tobacco (figure 4.3). Additional roles of this protein in guiding mRNA loading on the thylakoid membrane and mRNA stabilisation are also postulated.

4.7.2 The role of the SD-like RBS

Referring to above dual RBS-mediated translation model, the RBS1 as well as its upstream flanking element (AU-box or U-tract) that is more proximal to the initiation AUG codon appears to solely act as the translation determinant (Nickelsen et al., 1999; Ossenbuhl and Nickelsen, 2000; this study). The RBS2 might be simply essential for translation in higher plants (Hirose and Sugiura, 1996), but plays a multitude of roles in 5' end processing, stability and translation of chloroplast mRNA in *Chlamydomonas* (Bruick and Mayfield, 1998; Nickelsen et al., 1999). This *Chlamydomonas* RBS2 (*psbA*/*psbD* 5'UTR, RBS3-absent) might integrate the functions of RBS2 and RBS3 (*psbA* 5'UTR) in higher plants. Thus, a question is raised that either RBS2 or RBS3 of *psbA* 5'UTR might be obligatory for mRNA stability or translation, with additional concern that *psbA* mRNA in higher plants does not require 5' end processing for translatability (Kim and Mullet, 1994). *In vitro* evidence indicates that the SD-like RBS3 does not significantly affect the translation efficacy as strongly as RBS2. It was noticed that the mRNA amount of reporter gene as the template for *in vitro* chloroplast translation was relatively similar (Hirose and Sugiura, 1996). Therefore, the RBS3 of *psbA* 5UTR appears to carry the essential signal for mRNA stabilisation. Another possible role of RBS3 is that it might be involved in an early phase of translation initiation. This was considerably evidenced by the common toeprinting signals of chloroplast polysomes and *E.coli* 30S ribosomes to barley *psbA* mRNA (Kim and Mullet, 1994). In this study, RBS3 mutant RBSM ('UCCC') of *psbA* 5'UTR was obtained. Initial analytic results from Gus staining to primary shoot transformants suggested a dramatic decrease of *uidA* expression in RBSM (ZH10) as well as in mutants AUM (ZH9) and AUMN (ZH7) (figure 3.20). This

decrease could have arisen from a reduction in mRNA level or translation efficiency of chimerical *uidA* gene. Unfortunately, the transplastomic plant related to RBSM could not regenerated, likely due to the severe gene loss by abnormal plastid recombination events as described above. Therefore, the distinct role of the SD-like RBS3 of tobacco *psbA* 5'UTR still remains to be elucidated. Additionally, the accurate function of the RBS2 element appears to require an *in vivo* verification.

4.7.3 The role of the stem-loop structure

In this study, about half of the *psbA* 5'UTR modifications were correlated to the stem-loop region upstream of the SD-like RBS3 element. There, the entire stem-loop region and its anterior sequence were removed in mutant SLD, a deletion of the entire stem-loop sequence resulted in the mutant NurSLD. The 'core'-binding bases within stem-loop were modified in mutant SLM1, and a more stable stem-loop structure was created in mutant SLM2. Moreover, the stem-loop and its extension of *psbA* 5'UTR was substituted with the 5' terminal stem-loop region of *rbcL* 5'UTR in a chimeric mutant Rd-Pd, while in another chimeric mutant Rd-Pf, the stem-loop conformation of *psbA* 5'UTR was proposed to be stabilised or obstructed by adding the 5' end structural sequence of *rbcL* 5'UTR (see details in results). All these *psbA* 5'UTR mutants were revealed to have a similar decrease (2~3 fold) in *uidA* mRNA accumulation (figure 3.35), indicating that the stem-loop region is required for mRNA stabilisation.

In barley chloroplasts, 75% of total *psbA* mRNA was found in non-polysome fractions of the soluble phase, and less than 5% of total *psbA* mRNA was associated with polysomes in the soluble phase. The remaining *psbA* mRNA was associated with the thylakoid membrane, mostly in the polysome fraction (Klein et al., 1988). This majority of ribosome-free stromal *psbA* mRNA was further found in association with stromal cpRNPs (i.e. cpRBPs) (Nakamura et al., 1999). *In vitro* mRNA degradation assays indicated that cpRNPs conferred the stability of ribosome-free *psbA* mRNAs (Nakamura et al., 2001). It is proposed that the nascent RNAs or pre-RNAs are immediately protected from ribonuclease attacks by their association with cpRNPs. After a putative maturation process (not for all RNAs), RNAs dissociate from cpRNPs then associate with ribosomes for translation, and this transition is probably regulated in response to environmental and developmental signals (Nakamura et al., 2001). The barley stromal 95kda RBP appears to be one of such cpRNPs specific to *psbA* mRNA. It has also been suggested to sequester *psbA* mRNA in a non-polysomal pool in the soluble phase of chloroplasts by specific binding that blocks formation of the stem-loop structure and inhibits

both binding of 48kd RBP and ribosome association to *psbA* 5'UTR (Mullet, unpublished data). Such cpRNPs could be also accounted as Nac2 for *psbD* (Nickelsen et al., 1994), Mcd1 for *petD* (Drager et al., 1998), and Mbb1 for *psbB* (Vaistij et al., 2000) mRNAs in *Chlamydomonas*. The specific sequence of 5'UTR interacting with such *trans*-stabilising factor (cpRNPs) is generally adjacent to the 5'-termini of chloroplast RNAs or pre-RNAs and mostly within a stem-loop region (Higgs et al., 1999; Nickelsen et al., 1999; Vaistij et al., 2000; Shiina et al., 1998; this study).

With respect to the tobacco *psbA* 5'UTR, a model is favoured to address the influence of stem-loop structure on mRNA stability and translation (figure 4.4). The tobacco chloroplast has a similar set of RNA binding proteins (48kd, 60kd, and 95kd RBPs) specific to *psbA* mRNA as those in barley chloroplasts. After transcription, the newly-synthesised *psbA* mRNA immediately associates with 95kd RBP at the sequence within the stem-loop region. This association can stabilise *psbA* mRNA by masking the endonucleolytic cleavages site within its 5' UTR or spatially reduce the accessibility of endoribonucleases to mRNA. In addition, this association blocks the formation of the stem-loop structure, then prohibits the binding of tether protein (48kd RBP ?) and further ribosome association, allowing a majority of ribosome-free stable *psbA* mRNA left in the stroma of chloroplasts (figure 4.4, A). The 95kd RBP-associated *psbA* mRNAs are subsequently processed for mature 3' end formation by a different set of cpRBPs (figure 4.4, B). Another distinct feature of 95kd BRP association is its flexibility for the change of endo- or exogenous factors such as light stimuli. In case of illumination and increase of light intensity, the 95kd RBP dissociates from *psbA* mRNA to enable the formation of the stem-loop structure for binding of tether protein 48kd RBP (figure 4.4, C). This tethering can successively protect the *psbA* mRNA from ribonuclease attacks, according to the finding that the *psbA* mRNA stability is light-independent (Kim et al., 1993). Then, a large fraction of 95kd RBP-free *psbA* mRNA is allowed to target to the thylakoid membrane and to associate with polysomes by dual-RBS (RBS1 and RBS2) mediation. The membrane-bound *psbA* mRNA is then subject to translation (figure 4.4, D) facilitated by a putative light-responsive activator, probably the same tether protein 48kd RBP or another *trans*- factor.

According to this model, the association of 95kd RBP and tethering of 48kd RBP appear to be rate-limiting for mRNA stability and translation efficacy, respectively, in despite of aforementioned *cis*- and *trans*- translation determinants. These two critical processes are likely linked, due to their common correlation with the stem-loop of *psbA* 5'UTR. Here, we report that *psbA* 5'UTR mutants (SLD, NurSLD, SLM1, SLM2, and Rd-Pd) related to this

stem-loop region had a moderate and similar decrease in mRNA stability (2~3 fold) (figure 3.35), suggesting a parallel decrease in the binding activity of 95kd RBP to *psbA* 5'UTR. This could be reflected by the *in vitro* binding assays of *psbA* 5'UTR mutants in barley. Therein, the stromal 95kd RBP could specifically bind to a sequence of the stem-loop at the side proximal to the 5'-terminus of *psbA* 5'UTR. Deletions covering this sequence abolished the binding of 95kd RBP completely. In case of base alterations to this sequence that potentially masked the target sites ('core' bases) and/or disrupted the secondary structure, binding activity of 95kd RBP was also deficient. In addition, this binding was reduced when the stem-loop structure was stabilised by alternating this sequence (Mullet, unpublished data). Therefore, binding of 95kd RBP appears to considerably depend on the sequence and correct conformation of stem-loop of *psbA* 5'UTR. Direct *in vitro* evidences specific to tobacco *psbA* 5'UTR is undoubtedly desirable, and plasmids conferring all *psbA* 5' UTR modifications available in this study likely open up this opportunity.

Furthermore, these *psbA* 5'UTR mutants were found to have an additional decrease in translation efficiency (~2-fold in SLM1, SLM2 and NurSLD; ~1.5-fold in Rd-Pd and Rd-Pf; ~6-fold in SLD) (figure 3.35), suggesting a tight correlation of mRNA stability and translation efficiency mediated by the stem-loop structure. The tethering of 48kd RBP was proposed to be impaired in these mutants, which led to the decreases in both of its abilities to stabilise mRNAs and to target mRNAs onto the thylakoid membrane for translation. This tethering is thought to require the stem-loop structure of the *psbA* 5'UTR that is formed after the release of 95kd RBP. Mutants SLM2 and SLM1 contained a stabilised or 'core' bases-altered stem-loop structure, respectively; while the defect of stem-loop in mutant NurSLD was alleviated by a potential weak structure correlated to the 5' terminal sequence. Similar lowered affinity to 48kd RBP in these three mutants could account for the similar decrease in translation efficiency. Remarkably, mutant Rd-Pd employed a heterogeneous AU-rich stem-loop structure from the 5' end of *rbcL* 5'UTR, while mutant Rd-Pf might use the same structure of *rbcL* 5'UTR or obstructed inner stem-loop of *psbA* 5'UTR for mRNA targeting to membrane. Therefore, equivalent binding activity of 48kd RBP might contribute to the same reduced translation efficiency in these two mutants. Finally, mutant SLD without secondary structure and its anterior 5'end sequence rationally had lowest possibility for 48kd RBP recognition, and hence had more deleterious influence on translation. This was also suitable to interpret another independent mutant 124-1 where a deletion of 17nt at the 5'-termini of *psbA* 5'UTR had the similar consequences as those in mutant SLD (Eibl et al., 1999).

Taken together, the function of 48kd RBP to tether *psbA* mRNA onto the membrane is probably dependent on the stem-loop of the *psbA* 5'UTR with respect of its sequence and conformation. This has already been shown in detail by the functional characterisation of 5'UTRs of *psbA* (Mayfield et al., 1994), *psbC* (Rochaix et al., 1989; Zerges et al., 1997), *rps7* (Fargo et al., 1999; Fargo et al., 2001) mRNAs in *Chlamydomonas*.

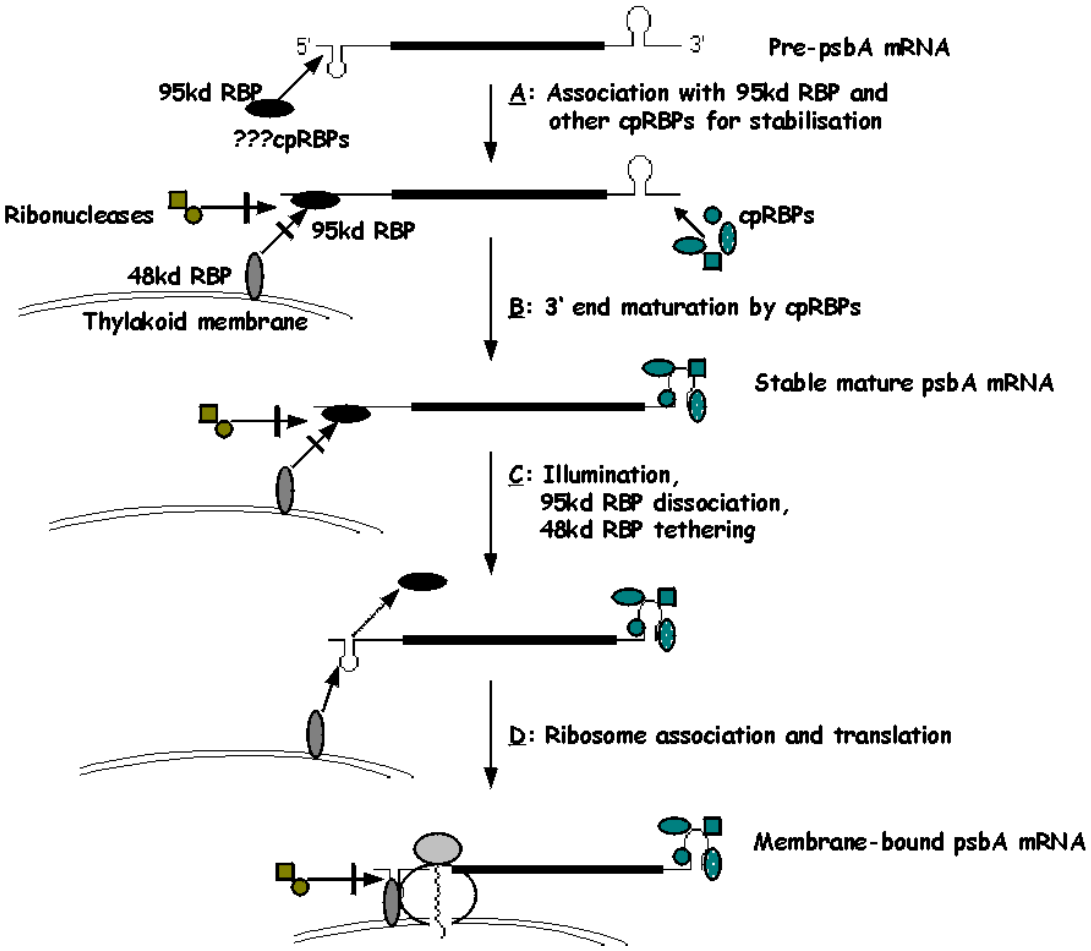


Figure 4.4: Interaction of *cis*- and *trans*- factors to regulate *psbA* mRNA stability and translation

4.7.4 The role of the 5' end poly(A) sequence

The identification of *Chlamydomonas* RB47 as a chloroplast homologue of poly(A) binding protein (cPABP) (Yohn et al., 1998a) suggests that the poly(A) sequence at the 5' extremity of *psbA* 5'UTR could be the target site for an RB47-like cpRNP in tobacco chloroplasts. To examine this, a mutation of the *psbA* 5'UTR (PolAD) was generated, in which the poly(A)-containing 5'end sequence upstream of the stem-loop was deleted. From our analytic results, this mutation did not significantly affect the mRNA level and translation of the chimerical *uidA* gene in tobacco chloroplast transformants (figure 3.35), indicating that

the 5'end poly(A) sequence does not apparently confer a regulatory signal and act as the binding site of a tobacco RB47-like protein. However, this mutation might have a slight influence on the conformation of the stem-loop structure, resulting in a slight increase in 95kd RBP binding but a slight decrease in 48kd RBP tethering according to the model in figure 4.4. Then, these two opposite changes could account for the moderate decrease in translation efficiency (~1.5 fold) in mutant PolAD.

Additionally, the form of *psbA* 5'UTR as Ori* could be accounted as a mutant of the *psbA* 5'UTR, where the poly(A) sequence was changed from 'AAAAA' to 'ACTAA' in the previous work (Eibl, 1999). However, no significant influence with respect of mRNA level, translation amount and translation efficiency resulted from this unintentional mutation (figure 3.35). It is also noted that this 5'end poly(A) motif is not strictly conserved, with a consensus sequence as 'AATAACAA' from the sequence alignment of *psbA* 5'UTRs (figure 3.15). Therefore, these data further confirm that the 5'end poly(A) sequence is not a functional *cis*-acting determinant.

4.8 The role of the 5'end secondary structure of tobacco rbcL 5'UTR

In our previous study (Eibl et al., 1999), the entire *rbcL* 5'UTR (182bp) was found to confer a stronger signal for mRNA stabilisation than the *psbA* 5'UTR. Shiina et al.(1998) also suggested that the 5' end structural sequence of the *rbcL* 5'UTR might be responsible for the high mRNA stability in the dark. Therefore, in attempts to examine whether the *rbcL* 5'UTR could convey its stabilising effect to the *psbA* 5'UTR, two chimeric 5'UTRs (Rd-Pd and Rd-Pf) were included in this work. Therein, the mutant Rd-Pd comprised a 5'end segment (-182~-160) of the *rbcL* 5'UTR embedding a putative stem-loop structure as suggested by Shiina et al. (1998), while the mutant Rd-Pf contained a longer 5'end sequence (-182~-122) of this 5'UTR conferring more complex secondary structures (see details in the results). From quantitative RNA analysis, it was found that these 5' end structural sequences of the *rbcL* 5'UTR in mutants Rd-Pd and Rd-Pf were incapable to promote mRNA stability (figure 3.35).

These results indicate that other mRNA stability determinants of the *rbcL* 5'UTR appear to reside elsewhere of the 5'end region. It is noted that the transcription of *rbcL* in many plants results in a transcript containing a 178 to 344nt 5'UTR. This primary transcript is subsequently processed to yield mRNAs with ca. 59nt 5'UTRs that are mostly associated with polysomes in the stroma of chloroplasts (Mullet et al., 1995; Hanley-Bowdoin et al., 1985; Kim and Mullet, 1994; Klein et al., 1988). Ribosome association has been suggested to stabilise the maize *rbcL* mRNA (Barkan, 1993). However, when the *rbcL* 5'UTR was reduced

to a 26bp fragment containing the canonical ribosome binding site (SD sequence), a threefold decrease of mRNA level was observed, despite of its capacity to direct translation initiation (Eibl et al., 1999). An RNA secondary structure just downstream of the 5'-terminus of barley mature *rbcL* mRNA was revealed by toeprint analysis, and proposed to aid cleavage at the processing site (-58) and modulate RNA stability or translation (Kim and Mullet, 1994). Therefore, the *cis*-acting stability determinants for the mature *rbcL* mRNA might be located within the mature 5' terminal sequence region conferring high-order secondary structures. Interactions between these *cis*-elements and their correlated *trans*- factors might regulate the stability of mature *rbcL* mRNAs, and be affected by ribosome association.

Accordingly, the *cis*- structural elements upstream of the processing site appear to stabilise the primary *rbcL* transcripts (Shiina et al., 1998). But this stabilising effect is likely eliminated upon illumination. The *rbcL* mRNA stability decreased 2-fold in barley chloroplasts during light adaptation (Kim et al., 1993), while it increased 6-fold in the dark relative to that in the light, and light-accelerated degradation of the *rbcL* mRNA was observed in *Chlamydomonas* (Salvador et al., 1993). This situation could also be explained by the function of cpRBPs as mention above. The nascent *rbcL* pre-mRNA is immediately protected by a 'cpRBP' association with the 5' end structural sequence. This association appears to be required for 5' end maturation of the *rbcL* mRNA but down-regulated by light stimuli. However, due to a strikingly increased rate of *rbcL* transcription upon illumination, the 'cpRBP' can still sequester and stabilise a similar amount of *rbcL* pre-mRNAs as that in the dark for mRNA maturation and translation. Another possibility is that the amount and binding activity of such 'cpRBP' are equivalent in both dark and light conditions, hence the amount of cpRBP-bound *rbcL* pre-mRNAs for 5' end maturation and further translation is unchanged. Since the *rbcL* mRNA does not require targeting to the thylakoid membrane, the large fraction of 'cpRBP'-free *rbcL* pre-mRNAs can allow attacks of ribonucleases for rapid degradation. Therefore it becomes understandable that the mRNA level and translation of chimeric *uidA* genes with the *rbcL* 5' leaders are relatively constant and light-independent (Shiina et al., 1998; Eibl et al., 1999)

The RNA analysis herein were carried out with transplastomic plants gown at a cycling photoperiod of 16h light /8h dark, while the mRNA turnover of *rbcL* is relatively slow (Kim et al., 1993). This could account for the indistinct roles of the 5'end structural sequences of the *rbcL* 5'UTR in this study. An investigation for these mutants (Rd-Pd, Rd-Pf) under dark adaptation would give an insight in the regulatory mechanisms underlying mRNA stabilisation in the dark.

5. Summary

Chloroplast gene expression is predominantly regulated at the posttranscriptional levels of mRNA stability and translation efficiency. The expression of *psbA*, an important photosynthesis-related chloroplast gene, has been revealed to be regulated via its 5'-untranslated region (UTR). Some *cis*-acting elements within this 5'UTR and the correlated *trans*-acting factors have been defined in *Chlamydomonas*. However, no *in vivo* evidence with respect to the *cis*-acting elements of the *psbA* 5'UTR has been so far achieved in higher plants such as tobacco. To attempt this, we generated a series of mutants of the tobacco *psbA* 5'UTR by base alterations and sequence deletions, with special regard to the stem-loop structure and the putative target sites for ribosome association and binding of nuclear regulatory factors. In addition, a versatile plastid transformation vector pKCZ with an insertion site in the inverted repeat region of the plastid genome was constructed. In all constructs, the *psbA* 5'UTR (Wt or modified) was used as the 5' leader of the reporter gene *uidA* under control of the same promoter, Prrn, the promoter of the rRNA operon. Through biolistic DNA delivery to tobacco chloroplasts, transplastomic plants were obtained. DNA and RNA analyses of these transplastomic plants demonstrated that the transgenes *aadA* and *uidA* had been correctly integrated into the plastome at the insertion site, and transcribed in discrete sizes. Quantitative assays were also done to determine the proportion of intact transplastome, the *uidA* mRNA level, Gus activity, and *uidA* translation efficiency. The main results are the following:

1) The insertion site at the unique *MunI* between two tRNA genes (trnR-ACG and trnN-GUU) is functional. Vector pKCZ has a large flexibility for further DNA manipulations and hence is useful for future applications.
2) The stem-loop of the *psbA* 5'UTR is required for mRNA stabilisation and translation. All mutants related to this region showed a 2~3 fold decrease in mRNA stability and a 1.5~6 fold reduction in translation efficiency. The function of this stem-loop depends on its correct sequence and secondary conformation.
3) the AU-box of the *psbA* 5'UTR is a crucial translation determinant. Mutations of this element almost abolished translation efficacy (up to 175-fold decrease), but did not significantly affect mRNA accumulation. The regulatory role of the AU-Box is sequence-dependent and might be affected by its inner secondary structure.

4) The internal AUG codon of the *psbA* 5'UTR is unable to initiate translation. An introduction of mRNA translatability from this codon failed to direct the translation of reporter *uidA* gene, overriding the mutation of the AU-Box.
5) The 5'end poly(A) sequence does not confer a distinct regulatory signal. The deletion of this element only insignificantly affected mRNA abundance and translation. However, this mutation might slightly disturb the conformation of the stem-loop, resulting in a moderate decrease in translation efficiency (~1.5 fold).
6) The SD(Shine-Dalgarno)-like RBS (ribosome binding site) of the *psbA* 5'UTR appears to be an indispensable element for translation initiation. Mutation of this element led to a dramatically low expression of the *uidA* gene as seen by Gus staining.
7) The 5'end structural sequence of the *rbcL* 5'UTR does not convey a high mRNA stabilising effect to the *psbA* 5'UTR in a cycling condition of the light and the dark. Their distinct roles appear to be involved in darkness adaptation.

Furthermore, with respect to the overall regulatory function of the *psbA* 5'UTR, two models are proposed, i.e. dual RBS-mediated translation initiation, and cpRBPs-mediated mRNA stability and translation. The mechanisms for mRNA stabilisation entailed by the *rbcL* 5'UTR are also discussed. Direct repeat-mediated transgene loss after chloroplast transformation and other aspects related to the choice of insertion site and plastid promoter are also analysed.

6. Appendix

6.1 New plastomic insertion site

Foreign gene integration into the plastome at improper sites might cause a deleterious effect on the function of plastid endogenous genes near the insertion site, or give rise to a consequence that the transgene is susceptible to expurgation by aberrant gene rearrangement during plastid propagation. Therefore, a new insertion site within the large single copy (LSC) of tobacco plastome was selected as an alternative in case of the probable dysfunction of the insertion site in vector pKCZ. This insertion site was appointed as the cut position of the unique blunt enzyme *SwaI* (ATTT↓AAAT) between two tRNA genes trnS (GCU) and trnG (UCC). Remarkably, it has not so far been used in the studies by the approach of chloroplast transformation.

A pair of primers InsNewSG-Fw (5'-AAGCTTTACCGTTTGAAACCTCG-3') and InsNewSG-Re1 (5'-AAGGAATCAAGCGTCTTGGATTC-3') was specially synthesised for amplification of a ~2.5kb DNA fragment, as shown in figure 6.1 simulated by software Vector NT6.

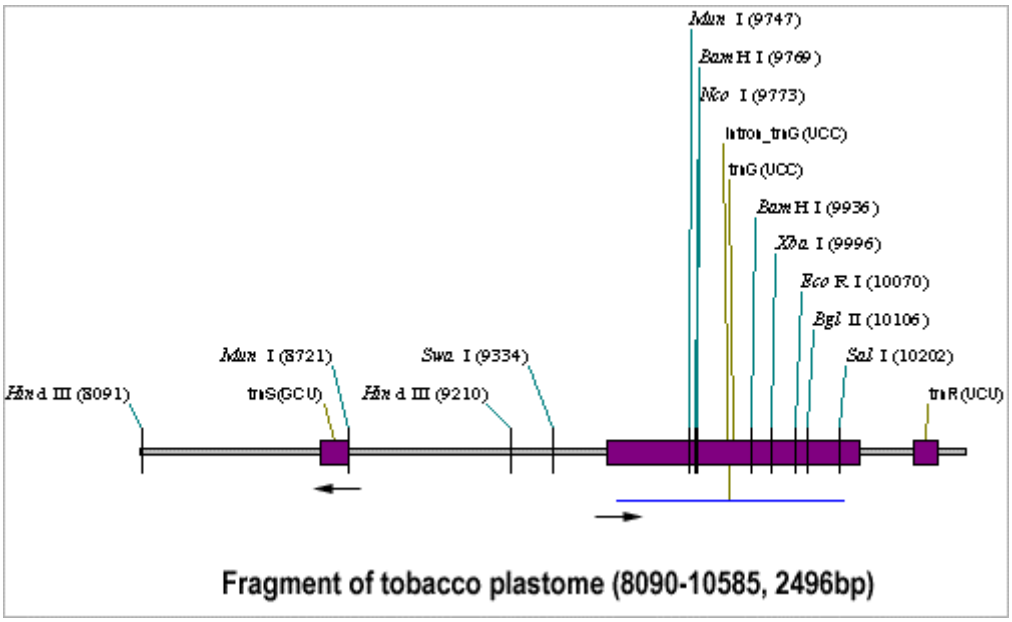


Figure 6.1: Simulation of the expected PCR fragment containing the new insert site

Within this putative PCR fragment, no coding sequence was included. The unique *SwaI* site in the centre was defined as the insertion site that resulted in two similarly sized plastid homologous fragments for constituting the new transformation vector. Notably, this *SwaI* site was situated in the space region of two tRNA genes trnS(GCU) and trnG(UCC)

containing their own promoters oriented oppositely as indicated by arrows in figure 6.1. Therefore, a transgene at this site would be transcribed in the same orientation as one of the tRNA genes to avoid potential anti-sense silencing. In the plastid genome, most tRNA genes are interspersed within or among the operons to compartmentalise the protein or rRNA genes. This is thought to confer a better transcriptional termination and RNA stability on their flanking genes. Taken together, it can be assumed that this insertion site would be advantageous and very appropriate for foreign gene integration.

PCR amplification was carried out by *Pfu* DNA polymerase via a special cycling program to generate the specific DNA fragment InsNewSG (~2.5kb). This fragment was primarily identified by digestions of *SwaI* and *HindIII* (figure 6.2, A) and further cloned into plasmid pUC19 at the *Ecl136II* site by blunt end ligation. The resultant ~5.2kb plasmid pUCInsNewSG was carefully investigated by enzyme profiling (figure 6.2, B), consistent with computer simulation as the aforementioned (figure 6.1). Moreover, the insert InsNewSG in plasmid pUCInsNewSG was fully sequenced, and no PCR errors were found (data not shown).

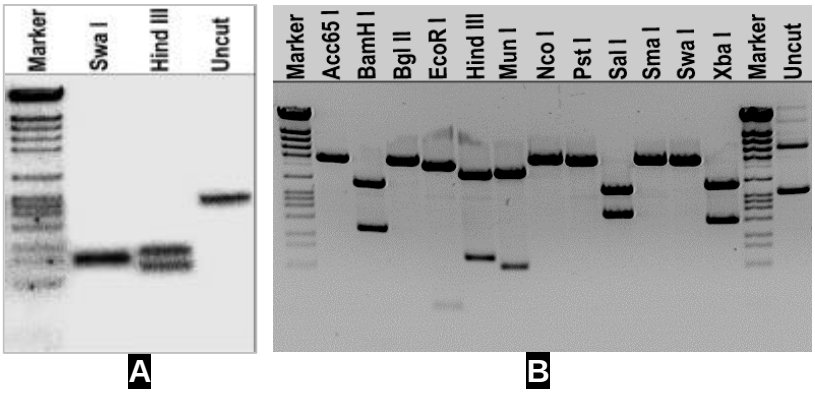


Figure 6.2: Restriction enzyme identifications of PCR fragment InsNewSG and its recombinant plasmid related to the new insertion site

6.2 New plastid promoter (PtrnfM)

In most publications related to chloroplast transformation, the constitutive promoter Prrn was used to govern transgene expression (e.g. *aadA*) in the transplastome. In this study to particularly characterise the *psbA* 5'UTR, a heterogeneous constitutive promoter for the reporter *uidA* gene was required to prevent any regulatory events at the transcriptional level. Since only the promoter Prrn was practical and available on hand, it was used to control *uidA* gene expression in all above constructs. Nevertheless, a new 'constitutive'-like promoter appeared to be essential as an alternative of Prrn for future studies.

From the quantitative analysis of transcription and RNA levels of barley plastid genes, it was found that the transcription rate of the tRNA (*trnfM*-trnG) gene cluster was about two times higher than that of the *16S rRNA* gene, but its RNA abundance was more than 20 times lower than the latter (Rapp et al., 1992). In principle, the plastid tRNA as well as rRNA are indispensable components involved in plastid gene translation, and their abundance is comparatively constant. Considering that most plastid genes accept fMet as the initial amino acid in translation, the transfer-RNA for fMet (*trnfM*) is likely to be prioritised with an optimum supply. Thus, it becomes understandable that the trnfM is transcribed so highly. However, the promoter of trnfM (PtrnfM) has not yet been discretely characterised.

To date, the plastid genome of more than 10 plant species have been fully sequenced, with at least one representative for each evolutionary plant group, e.g. Chlorella vulgaris for algae, liverwort for bryophytes; black pine for gymnosperms; arabidopsis, spinach, oenothera, tobacco, lotus for dicot angiosperms, and rice, wheat, maize for monocot angiosperms. From the plastome databases of most forenamed plants, the *trnfM* and its upstream 'promoter' sequences were collected into a FASTA file, together with their counterparts from barley and pea. Then, this sequence set was aligned as described above (context 3.2.13) in figure 6.3. Therein, the *trnfM* bordered in garnet red was highly conserved in all plant species examined, as well as the core region of its promoter PtrnfM with the green edge that contained a TATT repeat and the typical TATA box shaded in pale red. Strikingly, a SD-like RBS sequence with blue border was also observed downstream of the promoter region of *trnfM*, particularly in tobacco, pea and most monocot plant species such as rice, wheat and maize.

Before cloning the promoter PtrnfM, the capacity of the *aadA* gene to confer resistance to the antibiotic spectinomycin in *E.coli* was investigated. Plasmid pUC16SaadA-Sma was used as the host of the *aadA* gene that was controlled by promoter Prrn. The bacterial culture of this plasmid was spread on LB plates containing 75µg/ml ampicillin and various concentrations of spectinomycin, then incubated at 37°C overnight. The resistance to spectinomycin was estimated by the appearance of bacterial cell growth on the plate and listed in following table 6.1.

Table 6.1: The *aadA* resistance to spectinomycin *in E.coli*

Trial No.	Concentration (µg/ml)of		pUC18	*Sure(E.coli)*	pUC16SaadASma
	Ampicillin	Spectinomycin			
1	75	100	-	-	+++
2	75	200	-	-	++
3	75	300	-	-	+
4	75	400	-	-	-
5	75	500	-	-	-

From table 6.1 it could be revealed that the concentration of 100µg/ml of spectinomycin was the optimum selection concentration for bacteria in case of the *aadA* gene as the selection marker. Meanwhile, control experiments were included by using plasmid pUC18 in strain *Sure E.coli* (Strategene Co.).

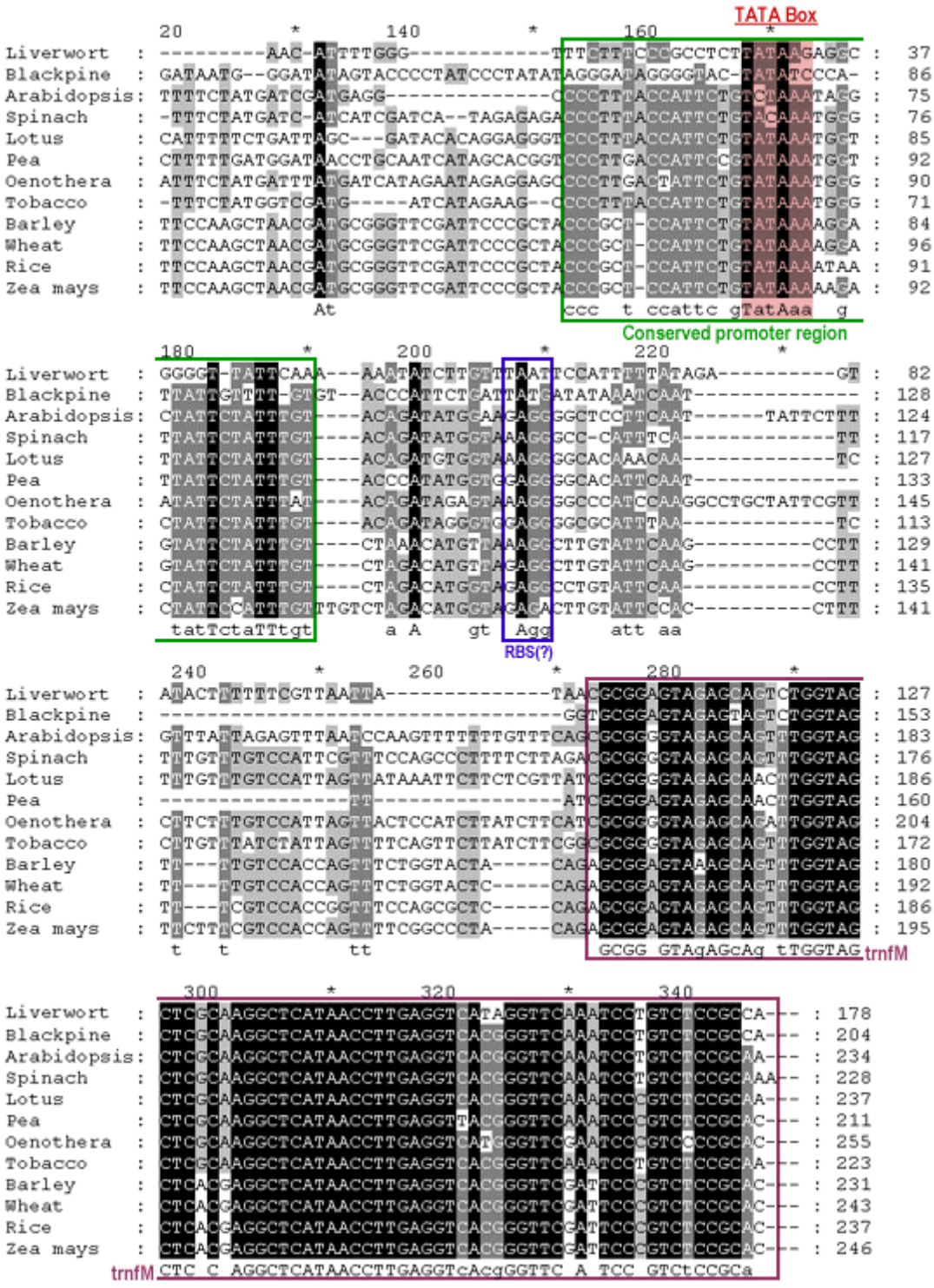


Figure 6.3: DNA sequence alignment of chloroplast trnfM gene

To clone the promoter of *trnfM* (PtrnfM), a pair of primers PtrnfMet-Re and PtrnfMet-Fw was synthesised with introduced *EcoRV* and *XhoI* sites at their 5'end respectively, and used to amplify a small fragment (~100bp) locally covering the promoter PtrnfM that was further cloned into plasmid pUC18 at the *SmaI* site by blunt end ligation. The recombinant plasmid pUCPtrnfM with additive *EcoRV* site adjacent to *SalI* was fully cut by *EcoRV* and *SalI*, then accepted an insert of *aadA* originated from plasmid pUC16SaadASma by digestions with *EcoRV* and *SalI*. The resultant plasmid pUCPtrnfMRBSaadArbcL3' was preliminarily screened under a selection stress of 75µg/ml ampicillin and 100µg/ml spectinomycin, then identified by enzyme digestion, in which the *aadA* gene was governed by promoter PtrnfM instead of the former promoter Prrn. Afterwards, the *aadA* gene and its 3' trailer in this plasmid were subsequently substituted with the *uidA* gene and its 3' UTR from plasmid pUC16SpsbA5'uidArbcL3' by digestions with *NcoI* and *PstI* to result in the recombinant clone pUCPtrnfMRBSuidArbcL3' with a blue appearance on LB plates containing appropriate amounts of X-Gluc (figure 6.4). This indicated that the promoter PtrnfM was active to promote *uidA* expression in *E.coli*. Finally, the full *uidA* cassette PfMet-RBS-uidA-rbcL3' was inserted into vector pKCZ by digestions with *XhoI* and *SacII* to create the plastid transformation construct pKCZ-PtrnfM(RBS)::rbcL3'. Subsequently, the large fragment consisting of both, *aadA* and *uidA*, cassettes was dissected from this construct by *Ecl136II* and integrated into the plasmid pUCInsNewSG at the new insertion site *SwaI* by blunt end ligation to generate a new plastid transformation construct pTrnSG-PtrnfM(RBS):: rbcL3'.

PtrnfMet-Re: 5'-GAT↓ATCTGTACAAATAGAATAGCCC-3'
EcoRV

PtrnfMet-Fw: 5'-C↓TCGAGGATTAACGCTTCATTTC-3'
XhoI

Figure 6.4: The recombinant clone pUCPtrnfMRBSuidArbcL3' containing new plastid promoter PtrnfM on LB plate (X-Gluc)

For the confirmation of promoter PtrnfM, the construct pKCZ-PtrnfM(RBS)::rbcL3' was locally sequenced by primer GusSeq1 in ABI 377 sequencer. From the reverse DNA strand of the single sequencing read, a region covering the promoter PtrnfM and synthetic RBS element are selectively demonstrated in figure 6.5, where the sequence of promoter PtrnfM is shaded in grey, with several distinguishable nucleotides in pale grey caused by the introduction of enzyme sites (*XhoI* and *EcoRV*). The TATA Box (TATAAA) is highlighted by dot underline. The synthetic SD (GGAGG, shaded in dark yellow)-like RBS sequence originated from the *rbcL* 5'UTR is situated between the promoter PtrnfM and the *uidA* gene, and applied to ensure the translation of the *uidA* gene. The internal SD-analogous RBS of *trnfM* is thought to be effectual for translation initiation, but not suitable for the singular purpose to characterise the promoter of *trnfM* (PtrnfM) in this case.

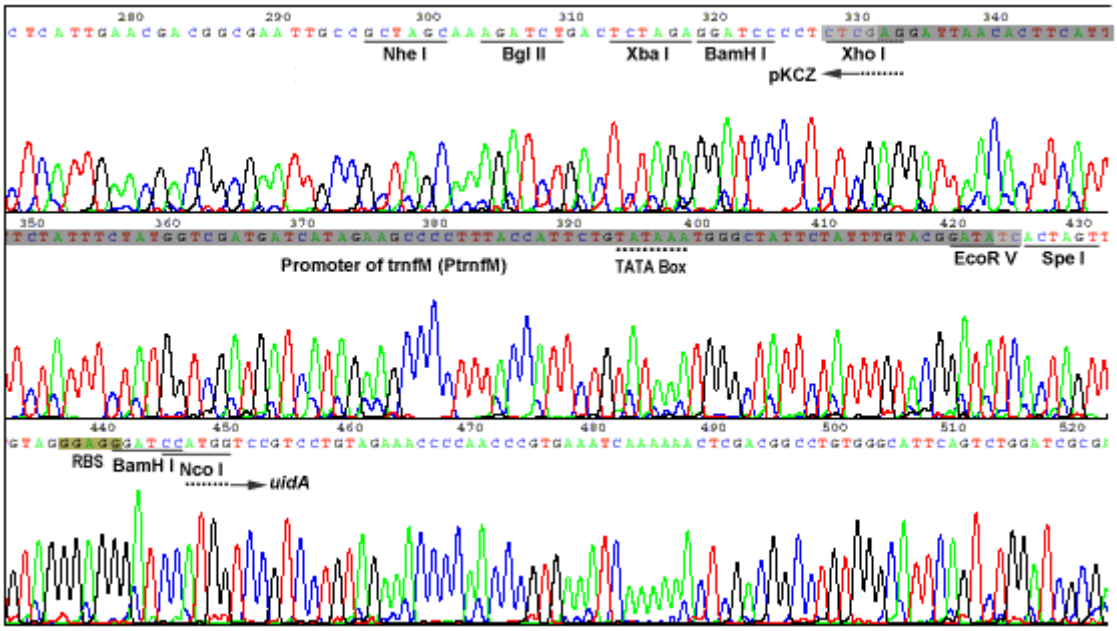


Figure 6.5: The fluorescent sequencing of plastid transformation construct pKCZ-PtrnfM (RBS)::rbcL3' containing the new plastid promoter PtrnfM

In conclusion, the promoter PtrnfM was preliminarily examined to be capable in directing the transcription of the *aadA* or *uidA* genes in bacteria and is believed to perform well in future studies related to chloroplast transformation.

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