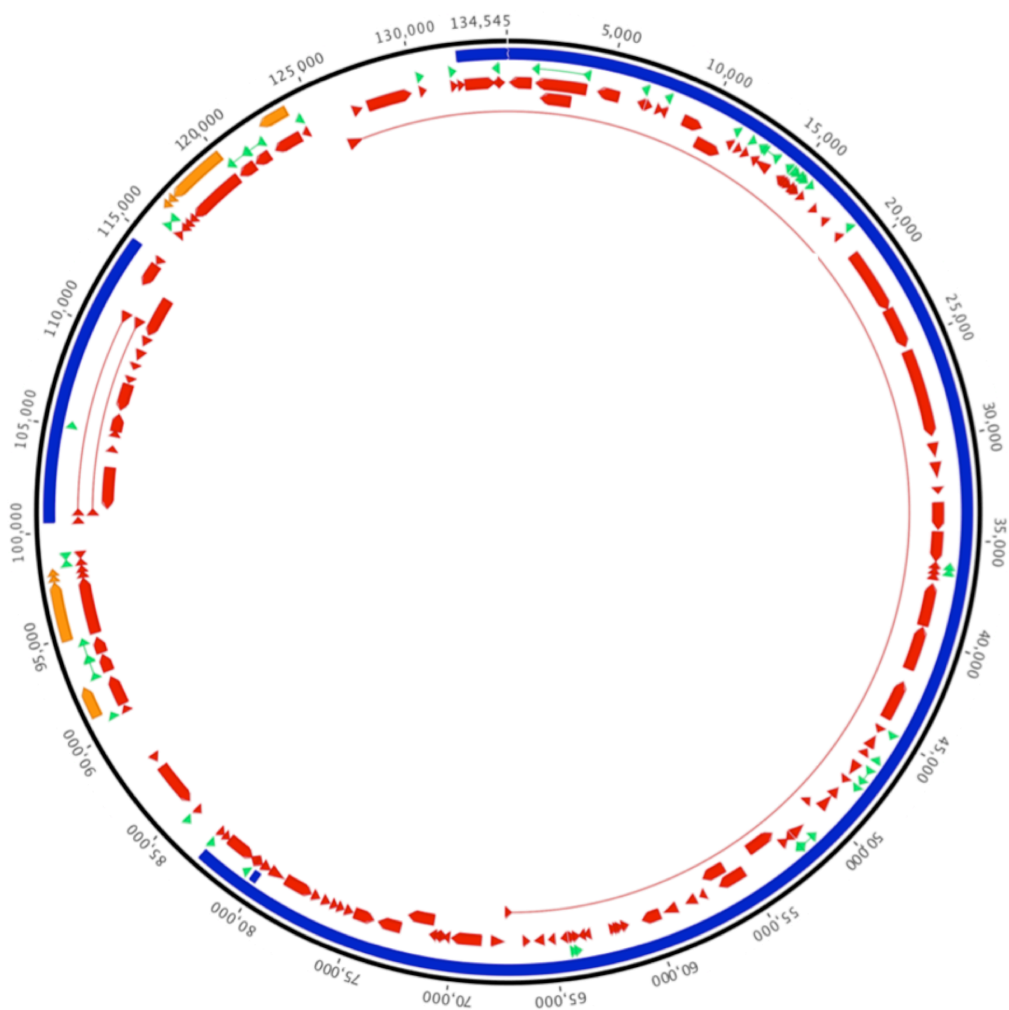# An investigation into the chloroplast transformation of wheat, and the use of a cyanobacterial CCM gene for improving photosynthesis in a C₃ plant

Parwez Samnakay PhD thesis
Lancaster University
Department of Plant Biology and Crop Sciences, Rothamsted Research
Sept 2015

Acknowledgements

Abstract

Wheat is a major component of the UK diet, and provides approximately 20% of global caloric intake. Wheat is grown on more land area than any other crop, and the continued supply of wheat is essential for global food security. Biotechnology is likely to play an important role in the sustainable increase of wheat yields, and the genetic manipulation of chloroplasts for photosynthetic improvement has many potential advantages over transformation of the nuclear genome. The genetic modification of the chloroplast genome via transformation was first demonstrated in the late 1980's, and since then, chloroplast transformation of many *Dicotyledonous* (dicot) plant species such as *Nicotiana tabaccum* has been routinely performed. In comparison, the transformation of chloroplasts in *Monocotyledons* (monocot) plant species, which includes all cereal crops, has made far less progress. To date, there has been no reproducible homoplasmic plastid transformation event in the monocots.

This study identifies a number of bottlenecks responsible for the prevention of chloroplast transformation in wheat. One such bottleneck is the lack of a suitable explant for plastid transformation, as traditional nuclear transformation targets are absent of metabolically active plastids. This study has developed a robust regeneration protocol for a previously undescribed tissue, termed the primary inflorescence leaf sheath (piLS), which is rich in active chloroplasts. Functional wheat specific chloroplast transformation vectors have been generated, and bombardment studies have been conducted with these on piLS and a second tissue, the immature embryos-derived callus. Immature embryo callus (IEC) does not contain active plastids, however contains pro-plastids and is highly embryogenic.

To uncover novel ways of increasing photosynthesis in C_3 plants, a number of transplastomic tobacco lines expressing the *Synechococcus elongatus* PCC 7942 *ictB* gene were generated. Previous studies suggest that ictB may be an inorganic carbon transporter. In a number of transplastomic lines produced in this study, the intercellular carbon concentration (Ci) is significantly increased. This increased Ci did not result in an increased photosynthetic rate, however did cause a number of phenotypic differences, such as smaller plants, wider leaves, and earlier seed pod formation.

The results, with regards to chloroplast transformation, and its implications in improving photosynthesis within C_3 plants, are discussed in this thesis.

Table of Contents

Abbreviations

ATP   Adenosine triphosphate

CA   Carbonic anhdrase

CCM   Carbon concentrating mechanism

Ci   Intercellular carbon concenration

CIM   Callus induction medium

DB   Downstream box

DNA   Deoxyribonucleic acid

EDTA   Ethylenediaminetetraacetic acid

FLARE-S   3'-adenyltransferase + GFP fusion protein

GFP   Green fluorescent protein

GM   Genetically modified

GUS   β-glucuronidase

iC   Inorganic carbon

ictB   Inorganic carbon transporter B

IEC   Immature embryo callus

IR   Inverted repeat

LS   Leaf sheath

LSC   Large single copy

MCS   Multiple cloning site

MES   2-(N-morpholino)ethanesulfonic acid

MS   Murashige and Skoog

NEP   Nuclear encoded plasmid

PCR   Polymerase chain reaction

PEG   Polyethylene glycol

PEP   Plastid encoded plasmid

PGA   Plant growth activator

piLS   Primary inflorescence leaf sheath

Prrn   16srRNA promoter

PMT   Photomultiplier

RA   RubisCO activase

RBS   Ribosomal binding site

RNA   Ribonucleic acid

RNAP RNA polymerase

siLS   Secondary inflorescence leaf sheath

SD   Shine-Dalgarno

SSC   Saline sodium citrate

TBE   Tris Borate EDTA

tiLS   Tertiary inflorescence leaf sheath

UTR   Untranslated region

WT   Wild type

YT   Yeast tryptone

Figures

Tables

1. General Introduction

Plastids are specialized organelles located within the plant cell. They contain their own genome of between 100-200kb which encodes approximately 120 genes, and have their own protein production machinery (Maliga, 2004). The plastid genome exists in a large copy number (~100/plastid), and each circular genome is attached to the organelle membrane in clusters called plastid nucleoids (Kobayashi et al., 2002, Sato et al., 1993, Kuroiwa, 1991). The term "plastid" is derived from the ancient Greek word "plastós", meaning formed or molded. This is a fitting name, as when the plastid differentiates from its pro-plastid progenitor, it can take on a number of different functions within the plant cell. Pro-plastids can develop into a range of different plastid types including chromoplasts for pigment storage, amyloplasts for starch storage and gravity detection, elaioplasts which store fats, and most famously, the chloroplasts which host photosynthesis.

1.1   The Chloroplast

Chloroplasts are organelles found in plant cells and some eukaryotic algae. The chloroplast is best known for its role in photosynthesis, where inorganic carbon from the air (CO_2) is converted into organic carbon in the form of sugars, with O_2 produced as waste. The chloroplast was not always a cytoplasmic resident of the photosynthetic eukaryotes. To understand the origin of the chloroplast, and all plastids for that matter, we must delve back 1.5 billion years to the primary endosymbiosis event. It is during this proterozoic occurrence, that it is believed a free living prokaryote-like cyanobacteria was integrated into a eukaryote by endocytosis, and a symbiotic relationship was forged (Gould et al., 2008). Over time, the cyanobacterial ancestor was reduced to a primary plastid, giving rise to the formation of three ancestral autotrophic lineages, the red algae, the glaucophytes and the green algae, who are the ancestors of all land plants, (Adl et al., 2005). These primary plastids maintained the double membrane from their gram-negative cyanobacterial heritage (Cavalier-Smith, 2000, Cavaliersmith, 1982, Cavalier-Smith, 2010), which is still evident today as

concluded by the presence of galactolipids (Jarvis et al., 2000), β-barrel proteins in both membranes (Schleiff et al., 2003), and the occurrence of peptidoglycans (not in all cases) beneath these membranes (Steiner et al., 2005). Phylogenetic analyses suggests that the red and green algae diverged later than the glaucophytes approximately 550 million years ago (mya), with plants emerging from their green algae ancestors approximately 400 to 475 mya, resulting in terrestrial take over, and hence creating the environment to allow animals to appear on land. Phycobilisomes, the light harvesting antennae of photosystem II in cyanobacteria, red algae, and glaucophytes, was replaced with chlorophyll *b* in green algal/plant lineage plastids, and a suite of accessory pigments developed to capture light and provide protection to the photosynthetic machinery from unfiltered earthbound light (Melkonian, 1990).

As a whole, primary plastids have undergone a plethora of changes and modifications during their time optimizing the relationship between their progenitor (eubacterial endosymbiont) and its eukaryotic hosts. These changes, or innovations, include (a) the consolidation of genetic information within the cyanobacterial endosymbiont genome by removal of redundant genetic information, (b) the establishment of a metabolite exchange system to facilitate the movement of photosynthetic products from the endosymbiont into its host, (c) substantial transfer of genetic information from the endosymbiont genome into the host genome (Bauer et al., 2001, Bock and Timmis, 2008), (d) the creation of a targeting system for protein products of these transferred genes from the host cytoplasm to the endosymbiont (Bauer, Hiltbrunner et al. 2001), and (e) the development of a protein import machinery for the transport of targeted protein products outside the double membrane of the endosymbiont, into its cytoplasm (Reumann et al., 2005, Andres et al., 2010, Schwenkert et al., 2011, Shi and Theg, 2013, McFadden, 1999). The majority of intracellular genetic transfer between endosymbiont and host most likely occurred early on in the optimization process, at least before the divergence of the three primary endosymbiotic lineage (glaucophyta, chlorophyta, and rhodophyta), as they all share a similar suite of common genes (Martin et al., 1998). It is also important to note that this gene transfer, from organelle to nucleus, is not limited to the period of time in the early evolution of plastids, but continues to occur in present day plant species (Martin, 2003, Kleine et al., 2009). However, given that the majority of gene transfer occurred pre-divergence, it is fairly likely that the protein targeting and import systems were developed soon after these

gene transfer events, which is supported by the fact that the protein targeting/import machinery are very similar in both rhodophyte and green algae lineages (McFadden and van Dooren, 2004).

With time, further modifications, adaptations, and innovations occurred to the endosymbiont and its relationship with its new host, until such a point where the endosymbiont became an integrated component of the host cell, an organelle that has reached a stable suite of core metabolic functions.

1.2 The chloroplast genome

1.2.1 Structure

The common and standard picture of the plastid genome consists of a circular genome, between 100-200kbp, containing approximately 120—130 genes in higher plants, and the highly conserved genome is organized into a quadripartite structure. This quadripartite structure defines two inverted repeat regions (IRs: IR_A and IR_B), separating the large single-copy (LSC) and small single-copy (SSC) regions of the genome (Fig 1.1). The IRs are normally identical in their nucleotide composition but differ in their relative orientation, and mainly include ribosomal RNA genes. In land plants, it is the variation within the inverted repeat regions, in terms of number of genes, that provides the main difference in genome sizes between different plastid species, given that protein coding genes and tRNA genes are very similar (Chumley et al., 2006, Gao et al., 2010, Ravi et al., 2008). The major differences that allow one to distinguish between plastid genomes of different lineages are the original eubacterial genes that are retained, presence or absence of introns and repeats, transcript edition, and the organisation and orientation of genes within the genomes. In land plants and green algae, these characteristics are largely conserved between species, once again indicating a common lineage.

1.2.2 Content

Gene content of the chloroplast DNA (cpDNA) between different species is largely conserved, and can be divided into three main categories. The first category contains genes responsible for the photosynthetic apparatus of the chloroplast, such as photosystem I (*psa*A, *psa*B), photosystem II (*psb*A, *psb*B), Large subunit of RubisCO (*rbc*L), ATP synthase (atp*A*, atp*B*), NAD(P)H dehydrogenase genes (*ndh*A, *ndh*B), and cytochrome 6bf (*pet*A, *pet*B). The second is composed of a suite of RNA genes and genes for the chloroplast genetic apparatus, such as ribosomal RNA (*rrn*16, *rrn*5), transfer RNA (*trn*H, *trn*K), RNA polymerase (*rpo*A, *rpo*B), and ribosomal subunit genes (*rps*2, *rps*3). The third and final category is comprised of conserved plastid-specific open reading frames (ORFs) and protein coding genes (*mat*K, *cem*A). See (Green, 2011) for a basic list of plastid genes. It is also important to note here that although the common consensus is that cpDNA are constructed into circular genomes,

there is a body of evidence to suggest linear cpDNA genomes, varying in length and branched structure, do exist in modern plant species (Bendich, 2004).
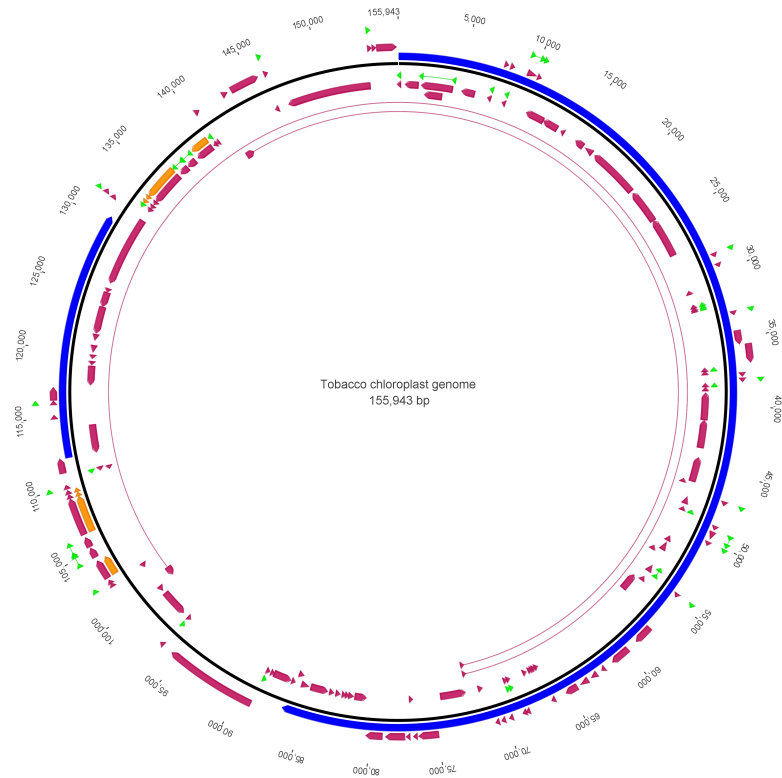
Fig 1.1 The tobacco chloroplast genome (Shinozaki et al., 1986). Large single copy, LSC, and small single copy, SSC, regions (0-86,687 and 112,030-130,601bp respectively) are highlighted in blue. Area between the LSC and SSC are the inverted repeat regions, IRa and IRb (86,687-112,030bp and 130,601-155,943bp respectively). Highlights: Red, all chloroplast genes; green, chloroplast transfer RNA genes; orange, chloroplast ribosomal RNA genes.
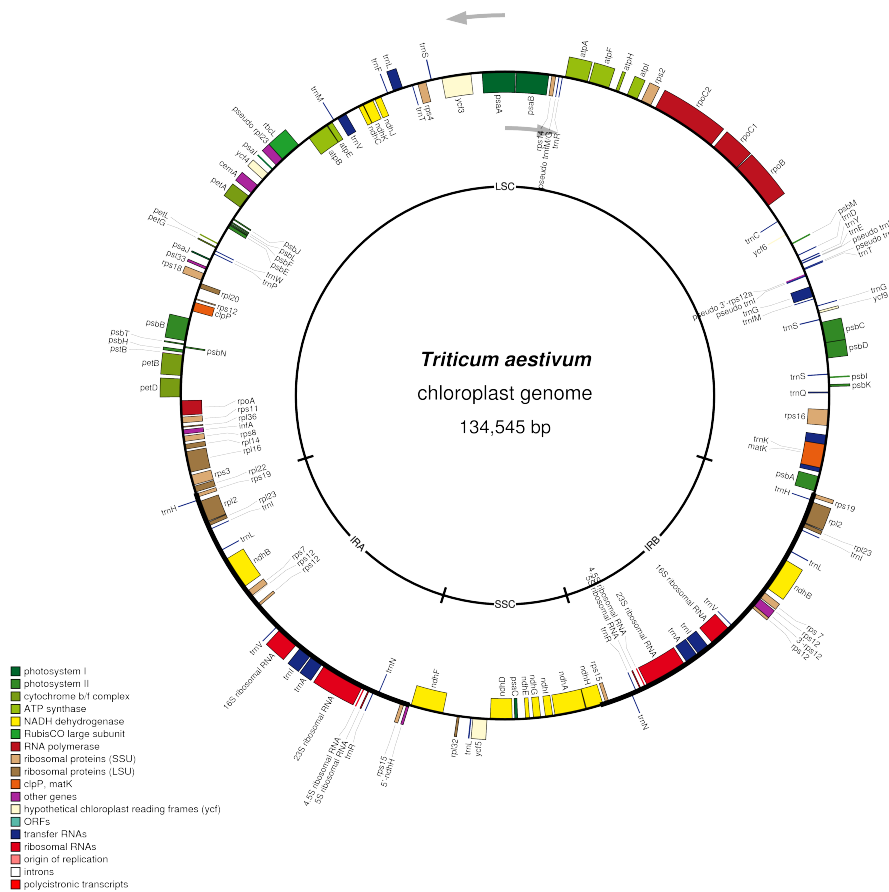
Fig 1.2 An annotated diagram of the wheat chloroplast genome, drawn using the complete genome sequence as input (GenBank accession number NC_002762.1) in version 1.1 of the OrganellarGenomeDRAW software tool (Lohse et al., 2013). The gray arrows denote the direction of transcription for the two DNA strands of the genome, and the inner circle shows its tetrapartite structure. IRA, inverted repeat A; IRB, inverted repeat B; LSC, large single-copy region; SSC, small single-copy region.

1.3 Plastid transcription and translation

1.3.1 Plastid transcription

As mentioned above, the plastome of most algae and land plants contains genes for the construction of the plastid RNA polymerase (RNAP), which is necessary for plastidial transcription. One of these plastid RNAP is a cyanobacterial-type RNAP, commonly known as the plastid-encoded plastid RNAP (PEP), whose core subunits $((\alpha)_2, \beta, \beta', \text{ and } \beta'')$ are coded for within the plastome (by *rpoA, ropB, rpoC1,* and *rpoC2* respectively). Further evidence of the plastids bacterial origins comes from the observation that PEP β and β' subunits are capable of substituting the homologous subunits of *E.coli* RNAP (Severinov et al., 1996). What is interesting is that while the core components of the PEP holoenzyme is derived from the plastid genome, the sigma (σ) factors which combine with the holoenzyme, are derived from nuclear encoded genes. Sigma factors are encoded by the small family of *sig* genes (Lysenko, 2007, Shiina et al., 2009), and are necessary for plastidial promoter recognition and transcription initiation. Other nuclear derived additions to the PEP holoenzyme are various accessory proteins (Schweer et al., 2010, Shiina et al., 2005), which are also believed to aid transcription.

Approximately 90% of plastidial transcription is believed to be conducted by PEP (Liere and Boerner, 2007), which means 10% of the remaining transcriptional activity is conducted by another RNAP. This RNAP is the single subunit nuclear-encoded plastid RNAP (NEP) (Hedtke et al., 2002). Initial studies demonstrating active transcription in plastids with impaired protein synthesis (Hess et al., 1993) suggested the existence of NEPs. Furthermore, transcription of plastid genes were shown to still occur from transplastomic tobacco plants absent in PEP (Legen et al., 2002, Krause et al., 2000, Allison et al., 1996), and nonphotosynthetic plastids of the parasitic plant *Epifagus virginiana*, which is absent of PEP genes (Ems et al., 1995, Morden et al., 1992). The nuclear encoded RNAPs that are responsible for transcription within plastids are the bacteriophage T3/T7 type, encoded by the *RpoT* gene family. In the dicotyledon plants such as *Nicotiana tabacum*, and *Arabidopsis thaliana*, there are three *RpoT* genes, two of which are targeted to the plastid for NEP function, *RpoTm* and *RpoTp* (Azevedo et al., 2008, Liere et al., 2004). In the cereals however, the *RpoTp* is the only NEP enzyme (Kusumi et al., 2004).

Although PEP are believed to be responsible for the transcription of photosynthetic genes, most genes within the plastome have promoters for both NEP and PEP, with only a few genes having promoters exclusively limited to either. Interestingly, the *rpoB* operon which codes for 3 out of the 4 core subunits of the PEP holoenzyme is solely transcribed by NEP (Silhavy and Maliga, 1998, Swiatecka-Hagenbruch et al., 2007). Thus it would seem that PEP activity is controlled by the nucleus in two ways, by supply of sigma factors and accessory proteins for PEP holoenzyme binding, and also by the transcription of PEP core subunits by NEP. Further research has demonstrated that NEP promoters are more active in early leaf development (Lysenko and Kusnetsov, 2005), while PEP activity increases during chloroplast maturation (Swiatecka-Hagenbruch et al., 2007, Zoschke et al., 2007). NEP activity is also light regulated, and appears to show light induced binding to thylakoid membrane proteins, thus reducing the transcription of PEP holoenzyme subunits (Azevedo et al., 2008). A model for the regulation of NEP and PEP proposes that nuclear *RpoTp* encodes NEP, which in turn transcribes and regulates the expression of the plastid *rpoB* operon and this PEP. PEP transcribes genes for photosynthetic complexes such as PSI and PSII, and it is the products of photosynthesis (e.g. reactive oxygen species) that signal to the nucleus to modulate NEP transcription. PEP transcription of tetrapyrrole biosynthesis (chlorophyll and haem) precursors are also believed to "signal" the nucleus to regulate NEP transcription (Liere et al., 2011). Thus it is possible that NEP and PEP machineries are used to adjust expression of genes within the nucleus and plastids depending on changes to the internal and external environment.

Most plastid promoters posses the eubacterial σ^{70}–type signals for binding the sigma factors of PEP. These signals are the -35 (TTGaca) and -10 (TATaaT) consensus sequences, and promoters that contain them are termed PEP promoters. Bacterial RNAP are able to faithfully recognize PEP promoters, thus further demonstrating the cyanobacterial origins of plastids (Gatenby et al., 1981, Liere et al., 2011). NEP promoters resemble plant mitochondrial promoters, and to date have been described as existing in three types. The detailed structure of NEP promoters is beyond the scope of my thesis, however research in this are is reviewed in Liere et al, 2011.

1.3.2 **Plastid translation**

Most of the primary transcription products of both NEP and PEP activity are polycistronic, and require significant posttranscriptional modifications such as intron removal, processing into monocistronic or oligocistronic mRNAs, RNA editing, and trimming of 5' and 3' ends (Bock, 2000, Stoppel and Meurer, 2012, Sugiura, 2008). As mentioned in the previous section, gene expression is under significant transcriptional control (Mullet and Klein, 1987, Liere and Boerner, 2007). However during the process of evolution, it is generally believed that gene expression in plastids shifted towards a more posttranscriptional level of control (Eberhard et al., 2002), specifically during RNA stabilisation and translation (Staub and Maliga, 1993, Eberhard et al., 2002, Kahlau and Bock, 2008, Stern et al., 2010). Plastid mRNAs are stabilised by RNA secondary structures as well as their 5' and 3' UTRs. Ribonucleases are employed to remove protective stem-loop structures and polyadenylation sites, which initiates degradation of plastid mRNAs (Stern et al., 2010, Stoppel and Meurer, 2012).

Plastid translation is conducted by ribosomes similar to eubacterial 70s ribosomes, with the translational machinery being close homologues to those in cyanobacteria, again pointing to the plastids bacterial past (Manuell et al., 2007, Marin-Navarro et al., 2007). Translation is initiated when the 30s ribosome combined with the initiator tRNA finds and binds to the initiator start site in the mRNA, AUG, although on rare occasions, this can be GUG or UUG (Sugiura et al., 1998). In bacteria, a Shine-Dalgarno (SD) sequence is required for the initiation of translation. This SD site, or ribosomal binding site (RBS), binds to a purine rich sequence known as the anti-SD sequence, at the 3' end of the 16s rRNA (which is part of the 30s ribosomal subunit). The plastids also share the same translation mechanism. The SD site in some plastid genes is clearly detectable (such as the rbcL RBS, GGAGG), however many plastid genes lack clearly defined SD sequences. Continuation into elongation phases of translation occurs when the 50s subunit binds with the pre-initiation complex to from an active initiation complex. Translation is terminated when the ribosome complex reaches one of the three standard stop codons, UAA, UAG, or UGA, which requires the assistance of ribosome release factors (Meurer et al., 2002, Motohashi et al., 2007), which is subsequently followed by disassembly of translation complex (Wang et al., 2010). The role of the plastid translation apparatus in plant development is reviewed in depth by Tiller and Bock, 2014.

1.4 Manipulating the chloroplast genome

The plastid genome can be engineered by genetic transformation, and cells containing modified plastid DNA can be regenerated to form plants with transgenic plastid genomes, known as transplastomic plants.

1.4.1 Plastid transformation methods in green plants and algae

The key innovation that began plastid transformation technologies was the gene gun (also known as the biolistics/bioballistics device). In 1987, driven by the limitations of current technologies for DNA delivery in to plant cells, Sanford and Klein et al. (1987) transformed the way in which genetic manipulation could take place. They demonstrated that small tungsten particles, termed micro-projectiles, could be accelerated and fired into plant cells without causing fatal injury. Furthermore, they demonstrated that DNA/RNA bound micro-projectiles could be delivered into onion tissue, and these DNA/RNA sequences be genetically expressed. Although this method was initially developed for the transformation of nuclear targets, a year later in 1988, Boynton (Boynton et al., 1988) used this same methodology to successfully transform the chloroplast in *Chlamydomonas Reinhardtii*. These *C.reinhardtii* were deletion mutants for the gene *atp*B (which codes for an ATP synthase essential for photosynthesis), and due to the mutation, were unable to photosynthesise. One μm sized gold particles coated with plasmid DNA containing a functional (wild type) *atp*B gene, were fired into *Chlamydomonas* on agar plates using gunpowder charge. The transgene was incorporated into the chloroplast genome by homologous recombination (Blowers et al., 1989), and rescued the photosynthetic phenotype. It is quite surprising that bombardment of chloroplasts with gold or tungsten particles results in viable transformed plastids, as plastids are in the same size range as the standard particles themselves (0.4-1.7 μm), depending on the target species.

Soon after the initial successes in *Chlamydomonas*, transformation of chloroplasts in tobacco (*Nicotiana tabacum*) was achieved (Svab et al., 1990). This landmark event, the first Embryophyta plastids to be transformed, proved to be the only published evidence for many years. Plastid transformation appeared to have hit a wall at *Chlamydomonas* and Tobacco, however within these two systems, the basic principles of plastid genome engineering and a toolbox were developed (Day and Goldschmidt-Clermont, 2011), with information of plastid genome content, gene function, and transcription and translation (Stern et al., 1997) also being uncovered. Over the

24

coming decades, other Embryophyta species of the dicotyledon family were transformed, including soybean, potato, and tomato (see Bock 2015).

The speed of the biolistic bombardment system, coupled with the robustness and low maintenance of the tobacco chloroplast transformation protocol, in comparison to other cell culture, glass bead (Economou et al., 2014), or polyethylene glycol (PEG) treated protoplast techniques (Oneill et al., 1993), leaves this methodology unrivaled in time and transformation efficiency at present. Unless another cheaper, more efficient system is developed, there is no reason why particle bombardment of explant tissue would not remain as the chosen tool for chloroplast transformation of a specific species of interest. Especially given the evidence that particle bombardment can be used to simultaneously alter the nuclear genome as well as the chloroplast genome (Elghabi et al., 2011).

1.4.2   Vector **design for successful integration of foreign DNA into the plastid genome**

As mentioned above, the basic and most efficient method for delivery of exogenous DNA into plastids is to use biolistic bombardment. However, to achieve stable chloroplast transformation, that is where (a) the transforming DNA integrates into a specific location within the chloroplast genome, and (b) where all untransformed copies of plastid DNA are eliminated to give a homoplasmic plastid genome content, an effective expression cassette needs to be constructed.

1.4.2.1   Homologous recombination and targeted insertion of transgene into plastome

The first step, DNA integration, is reliant on successful homologous recombination. This relies on "flanking regions" placed either side of the transformation cassette with sufficient nucleotide homology to the nucleotide sequence within the plastid genome where integration is required to take place. Although gene content and orientation within land plants are observed to be highly conserved, the use of 'universal' flanking regions is not advised unless dealing with closely related species (Ruf et al., 2001). RNA editing patterns can differ even between closely related species (Kahlau et al., 2006), and heterologous RNA editing sites have been shown to remain unprocessed when transferred into another species (Bock et al., 1994, Schmitz et al., 2009, Schmitz-Linneweber et al., 2005). It is therefore wise to carefully select flanking

regions for homologous recombination that closely match the target sequence in the germplasm to be transformed.

The genomes of most higher plant chloroplasts that have been sequenced contain two inverted repeat regions (IRs) (Ravi et al., 2008), IRa and IRb, separated by a large and small single copy region (LSC and SSC respectively, Fig 1.1, Fig 1.2). IRa and IRb are identical in their DNA sequence, and contain genes responsible for ribosomal RNA (rRNA) and transfer RNA (tRNA) production. Genes targeted and inserted into the one of the IRs is rapidly copied over into the second IR by gene conversion (Daniell et al., 1998), thus a gene targeted to the IRs is present in two copies per chloroplast genome. It must be noted that both transformed and non-transformed IRs can be templates for gene conversion, thus transgenes can also be eliminated by gene conversion (Lutz et al., 2007). The *trnI-trnA* gene region within the IRs (Fig 1.3a) is one of the most frequently targeted transformation regions (Chakrabarti et al., 2006, Cheng et al., 2010, Chiyoda et al., 2007, Davarpanah et al., 2009, Jeong et al., 2004, Lelivelt et al., 2005, Li et al., 2012, Liu et al., 2007, Lutz et al., 2007, Zhu et al., 2011, Maliga, 2004, Lee et al., 2006, Daniell et al., 1998) due to its integration success rate, and its conservation between higher plant species. The *trnI-trnA* gene region has also been used to express several proteins (Daniell et al., 2005). Insertion of foreign DNA into the plastome has also been achieved in over 14 other sites (Maliga, 2004).

Due to the chloroplasts' bacterial origin, chloroplast genes exist mostly in di- or poly-cistronic operons. It has been shown that promoter-less constructs can still generate exogenous gene transcripts and translation products, so long as they are inserted downstream of a plastid promoter, and have the necessary 3' regulatory elements (Staub and Maliga, 1995). The *trnI-trnA* gene region resides in a polycistron (Fig 1.3) driven by the strong plastidial 16s ribosomal RNA promoter (Prrn). Therefore, it is possible to insert exogenous DNA into the *trnI-trnA* gene region without a promoter, and still get transcription of the exogenous gene via read-through from the Prrn. If a promoter-containing construct is inserted into the *trnI*-trnA region, transgene transcription would occur from both the constructs promoter, and via read-through. This increases mRNA transcript levels of the exogenous gene/genes. Thus if the desire is to avoid interference from read-through transcription, for example, if studying promoter activity, then insertion into the *trnI-trnA* should be avoided. An alternative

location within the IRs that is not affected by read-through transcription is the *trnV*-3'*rps12* intergenic region (Zoubenko et al., 1994).
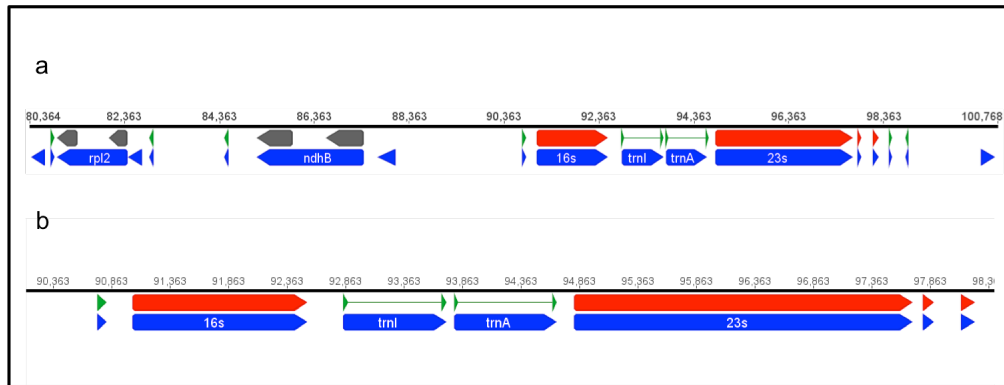
Fig 1.3 a, The inverted repeat region a (IRa), showing, all chloroplast genes (blue), transfer RNA genes (green), ribosomal RNA genes (red), and exons (grey). b, showing from left to right, trnV, 16s rRNA, trnI, trnA, 23s rRNA, 4.5s rRNA and 5s rRNA genes of the IRa.

1.4.2.2   Selection to attain homoplasmy

The second step, creating a homoplasmic transgenic chloroplast genome, requires a combination of an effective transformation cassette, and the expression of a suitable selectable marker. The basic structure of an expression cassette (see Fig 1.4, and section 3.1) for the purposes of plastid transformation consists of a promoter upstream of the coding region (gene of interest, selectable marker, etc), followed by a 3'UTR downstream. For transgene expression within the plastid, promoters of the bacterial type are used as they are recognized by the plastid-encoded plastid RNA polymerases (PEPs), as well as conferring a much higher gene expression in comparison to nuclear-encoded plastid RNA polymerases which favour phage type promoters (Hajdukiewicz et al., 1997). For successful translation, a SD sequence is required for binding to the 3' end of the 16s rRNA, and this is provided by the 5'UTR. As a note, the distance between the SD and translation initiation is of critical importance for efficient translation initiation in plastids, as it is in bacteria (Drechsel and Bock, 2011, Esposito et al., 2001, Chen et al., 1995). The 3'UTR is essential for successful transgene transcript stability and processing by forming stable stem-loop RNA structures (Stern and Gruissem, 1987, Stern et al., 1997).
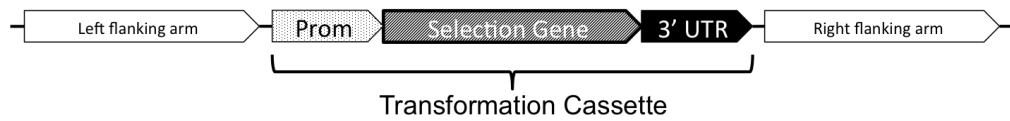
Fig 1.4 The structure of a basic transformation cassette. A promoter (Prom) is required to drive the expression of the gene of interest (in this case a selection gene), and the transcription product of the gene requires stability from a 3' untranslated region (3' UTR). The cassette is inserted into a specific location within the chloroplast genome by flanking the cassette with DNA sequences homologous to the region into which insertion is required (Left flanking arm, Right flanking arm).

Selectable makers (see Table 1.1) can provide both positive selection, and negative selection. However, for the creation of homoplasmic chloroplast transformants, positive selection markers are utilised, whereas negative selection markers are likely to be useful in genetic screens for the regulators of plastid gene expression (Serino and Maliga, 1997, Gisby et al., 2012). The most commonly used positive selectable marker by far has been the protein product of the *aad*A gene, 3"-adenylytransferase, originally used in *Chlamydomonas*, and adapted for use in the tobacco (Goldschmidtclermont, 1991, Svab and Maliga, 1993). This aminoglycoside confers resistance to spectinomycin and streptomycin, both potent inhibitors of plastid translation that bind to the chloroplast 70s ribosome. Although alternative selectable markers have been developed over the years (Day and Goldschmidt-Clermont, 2011, Bock, 2015) *aad*A remains the most utilised due to its effectiveness. There is considerable attractiveness to removing selection/marker gene following plastid transformation, especially for the biotechnological modifications of agronomic crops. Mechanisms of marker removal following plastid transformation are described in Maliga (2004), and Day and Goldschmidt-Clermont (2011).

Marker	Selection	Organism	Reference
Antibiotic Resistance			
aadA	Spectinomycin/Streptomycin	Chlamydomonas	(Goldschmidtclermont, 1991)
		Rice	(Lee et al., 2006)
		Tomato	(Ruf et al., 2001)
		Oilseed rape	(Hou et al., 2003)
		Carrot	(Kumar et al., 2004)
		Soybean	(Dufourmantel et al., 2004)
		Potato	(Sidorov et al., 1999)
		Lettuce	(Lelivelt et al., 2005)
Nptll	Kanamycin	Tobacco	(Carrer and Maliga, 1995)
		Cotton	(Kumar et al., 2004)
rrnS	Spectinomycin/Streptomycin	Chlamydomonas	(Newman et al., 1990)
		Tomato	(Nugent et al., 2005)
		Tobacco	(Svab et al., 1990)
rrnL	Erythormycin	Chlamydomonas	(Newman et al., 1990)
aphA-6	Kanamyin	Cotton	(Kumar et al., 2004)
Visual			
gfp	Green Fluorescence	Tobacco	(Khan and Maliga, 1999)
		Rice	(Lee et al., 2006)
		Potato	(Sidorov et al., 1999)
Herbicide resistance			
bar	Phosphinothricin	Tobacco	(Iamtham and Day, 2000)
psbA	DCMU, metribuzin	Chlamydomonas	(Przibilla et al., 1991)
HPPD	Diketonitrile	Tobacco	(Dufourmantel et al., 2007)
EPSP	Glyphosphate	Tobacco	(Ye et al., 2003)

Table 1.1 Selectable markers for plastid transformation, adapted from (Day and Goldschmidt-Clermont, 2011).

1.4.2.3   Promoter and 5'-UTR choice

Levels of protein expression from transgenes is dependent on promoter strength and mRNA turnover (which is dependent on the protective action of 5'- and 3'-UTR stem-loop structures (Barkan and Goldschmidt-Clermont, 2000), and will be discussed further in 1.4.2.5). Promoters are normally selected from the plastid genome of the organism that is being modified. The plastid genome contains a number of promoters, suitable for use in chloroplast transformation cassettes. The 16s rRNA promoter (Prrn) and *psbA* promoter (PpsbA) are considered to be strong plastid promoters (Hanson et al., 2013, Maliga, 2004), and are readily used in chloroplast transformation studies.

Chloroplasts originate from cyanobacteria (Green, 2011), and therefore contain the -35 (TTGaca) and -10 (TAtaaT) consensus sequence of the eubacterial sigma-70 type promoters (Reznikoff et al., 1985, Liere et al., 2011). The bacterial origins of chloroplasts are further supported by studies that demonstrate the ability of *E-coli* RNA polymerases to recognize PEP (plastid-encoded plastid RNA polymerase) promoters (Gatenby et al., 1981, Liere et al., 2011). Therefore, a suitable promoter must contain the elements necessary for RNA polymerase binding and successful transcription.

Most transformation vectors use entire or truncated strong plastid promoters, such as the *psbA, atpB,* or *rbcL* (Staub and Maliga, 1993, Kuroda and Maliga, 2001b). In more recent times, the most widely used promoter in chloroplast transformation studies is the Prrn. In the plastid genome, this promoter is responsible for the transcription of chloroplast genes downstream of it, as part of a single operon. The Prrn contains both -35 and -10 consensus sequences required for transcription. When comparing the wheat Prrn to that of other monocot and dicot Prrn (Fig 1.5), the -35 and -10 boxes are highly conserved. When comparison is made between the total Prrn sequence of wheat and tobacco, rice, and maize, percentage identity is 86.6%, 97.8%, and 97.8% respectively.
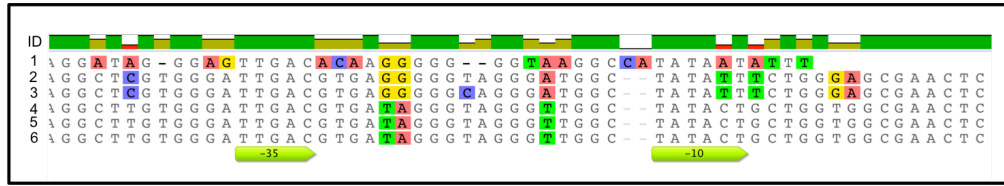
Fig 1.5 Sequence alignment of the -35 and -10 upstream region in three dicot species (1, Pea; 2, Spinach; 3, Tobacco) and three monocot species (4, Wheat; 5, Rice; 6, Maize).

The decision to use a promoter to drive transgene expression, or utilise the read-through mechanism, requires significant consideration. Promoterless *uidA* inserted downstream of the plastid *rbcL* gene resulted in 4 fold higher β-glucuronidase protein levels than constructs containing a heterologous ribosomal promoter inserted at the same site in the plastid genome (Staub and Maliga, 1995). This was despite there being a greater monocistronic *uidA* mRNA produced in the latter case. The successful use of promoterless constructs has been demonstrated in a number of chloroplast transformation studies (Herz et al., 2005, Chakrabarti et al., 2006, Gray et al., 2009, Gray et al., 2011)

While a strong promoter like the Prrn is desirable, the translatability of a transgene is more affected by the 5' UTR chosen (Maliga, 2003, Barkan, 2011). The Prrn is a promoter that controls transcription of ribosomal and transfer RNA genes, which means that there is no ribosomal binding site present upstream of any of the genes, or at the 5' end of the Prrn. Therefore, if the Prrn is chosen to drive transcription and translation of exogenous genes in a transformation vector, a ribosomal binding site, a SD sequence, needs to be inserted at the 3' end of the Prrn. The *rbcL* SD site, GGAGG, placed at the 3' end of the Prrn, is sufficient to initiate translation of a transcript (Day and Goldschmidt-Clermont, 2011). Alternatively, the use of a bacterial 5' UTR has been shown to be able to successfully initiate ribosomal binding and translation (Kuroda and Maliga, 2001a). In *E.coli*, translation is facilitated by the interaction between the SD sequence upstream of the AUG start codon, and the anti-SD sequence at the 3' end of the 16sr RNA (Sprengart et al., 1990). The promoter of gene 10 of the T7 bacteriophage (T7g10), has a well-defined downstream box (see next section) sequence (Sprengart et al., 1996), and has been shown to accumulate high levels of exogenous protein in bacteria (Studier et al., 1990). Placing the 5' UTR of T7g10 downstream of the Prrn, can successfully produce exogenous transcripts and

translation products (Herz et al., 2005, Bohmert-Tatarev et al., 2011, Kuroda and Maliga, 2001a), while also increasing protein expression (Kuroda and Maliga, 2001a, Oey et al., 2009). Other bacteriophage 5' UTRs can be used to provide sufficient marker gene expression to recover transplastomics, however expression levels are low (Yang et al., 2013). The creation and successful use of inducible promoters has also been demonstrated in plastid transformation, and should be considered for studies temporal or spatial transgene expression is required (Muhlbauer and Koop, 2005, Verhounig et al., 2010).

1.4.2.4   Downstream Boxes

The downstream box (DB), first identified in *E. coli* (Sprengart et al., 1996), is defined as the first 10-15 codons downstream from the start codon, and works synergistically with the SD sequences upstream to regulate protein accumulation. *E. coli* DB sequences have been shown to be functional in higher plants (Gray et al., 2011, Kuroda and Maliga, 2001a, Kuroda and Maliga, 2001b, Ye et al., 2001), as well as significantly changing the magnitude of protein accumulation (Gray et al., 2009). However, results have also shown that which DB to use to optimise expression has to be selected empirically, as final protein accumulation is dependent upon the coding region under DB control.

1.4.2.5   Spacers and transcript stability

Expression of each gene in a multigene transformation vector can be achieved by inserting a promoter 5' to each individual gene. However, by increasing the number of plastidial promoters in a transformation vector, could increase the probability of unwanted rearrangements within the chloroplast genome, or rearrangements within the vector. In order to avoid this, genes can be arranged into a polycistron, with each individual gene preceded by a leader or spacer, short plastidial sequences (Herz et al., 2005) that have translation capabilities. In essence, each gene has its own translational element, however all are under the same single transcriptional control. The *psbD/C* overlap region from the tobacco chloroplast genome has been used successfully in tobacco transformation vectors (Bohmert-Tatarev et al., 2011) as has the SD containing region of the *rbcL* gene (Svab and Maliga, 1993).

Although plastid expression is similar to that of prokaryotic organisms, gene expression in plastids is largely controlled at the post-transcriptional level (Stern et al., 1997, Quesada-Vargas et al., 2005, Marin-Navarro et al., 2007). Translation efficiency is primarily dependent on the 5' UTR (Barnes et al., 2005), as well as the DB (Kuroda and Maliga, 2001a) in some chloroplast genes. Similar to eubacterial mRNA, chloroplast mRNA does not have a cap structure or Poly A tail. The mRNAs also lack any conserved SD like sequences, which suggests that other *trans*-acting factors must be involved in the regulation of translation from chloroplast mRNA.

The 3' UTR is necessary for mRNA stability (Herrin and Nickelsen, 2004), which it achieves by forming stem-loop structures necessary for mRNA processing (Rott et al., 1996), and preventing degradation of the mRNA by ribonucleases (Stern et al., 2010). The translation and accumulation of *psbA*, which encodes the D1 protein of photosystem 2, has been shown to be light dependent and under the control of both the 5' and 3' UTR (Staub and Maliga, 1993). The 3' inverted repeat sequences, a common occurrences in plastid mRNA sequences, can fold in to stem and loops and appear to be important for mRNA processing and stability (Rott et al., 1998), and proteins with endonuclease activity are known to bind to these regions (Lisitsky and Schuster, 1995). Furthermore, interactions between 5' and 3'UTRs of plastid genes, such as *psbA,* has been demonstrated to be involved in mRNA stability (Eibl et al., 1999).

1.5 Chloroplast transformation for the improvement of photosynthesis

By manipulating various open reading frames (ORFs) or genes (Bock and Khan, 2004), chloroplast transformation has made possible the improved understanding of plastid genes and their roles in orchestrating photosynthesis. By better understanding how chloroplasts work, we can better understand how photosynthesis can be improved. By utilising our understanding of plastid genome function, chloroplast transformation also provides an opportunity to engineer the plastid genome to supercharge photosynthesis.

1.5.1 RubisCO

One way in which this can be achieved is through the manipulation of ribulose 1,5-bisphosphate carboxylase oxygenase (RubisCO) subunits (Andrews and Whitney, 2003). The RubisCO enzyme plays its role within the first step of converting inorganic carbon (iC) into carbohydrates in plants and algae, by catalyzing the incorporation of CO_2 into ribulose 1,5-bisphosphate. Accumulating carbohydrates is paramount for increasing biomass and growth. RubisCO is an ancient enzyme, whose evolution began at a time when the atmosphere was rich in CO_2 due to the absence of photosynthetic organisms, approximately 3.5 billion years ago. Despite its importance in the process of biomass production, RubisCO is largely inefficient (Long et al., 2006b), and has therefore been targeted as an area of improvement for more improving photosynthesis (Parry et al., 2007, Raines, 2006). All RubisCO enzymes, of which there are three forms, are comprised of at least two large 50kD subunit arranged head to tail to form a dimer (L2), with two active sites located at the interface. During evolution of photosynthetic organisms, the arrangements of subunits within RubisCO has changed and diverged into three forms, I, II, and III, while the catalytic site in all forms has remained conserved (Andersson and Backlund, 2008). Land plants, algae, and photosynthetic bacteria use the most abundant form of RubisCO, form I, which is comprised of 4 L2 dimers (L2) 4 capped by 8 small 13-17kD subunits (organised in 2 groups of 4). Form I is thus denoted L_8S_8. The small subunits are not required for catalysis, however have been found to be essential for maximal activity and RubisCO stability (Andersson and Backlund, 2008).

RubisCO shows natural variation in its catalytic activity between families (Carmo-Silva et al., 2010), although this is based on limited comprehensive catalytic data (Whitney et al., 2011). Algae and cyanobacteria, or other photosynthetic organisms that have developed in a high CO_2, although have higher carbon fixation rates, have lower affinities and specificities for CO_2 over O_2. The same is said for C_4 plants and other organisms that adopt energy expensive methods for increasing carbon concentration in close proximity to RubisCO. C_3 plants on the other hand, which include most agronomical crops and algae, possess RubisCO that have high affinities and specificities for CO_2 over O_2, however have a slower carbon fixation rate (Whitney et al., 2011). It would seem that nature has fine tuned each system accordingly, given the differences in evolutionary history and intracellular inorganic carbon concentrations (Tcherkez et al., 2006). The argument is thus, can photosynthesis be improved by making adjustments to processes developed through billions of years of evolution. The amount of RubisCO in a cell is thought to be in excess; therefore an approach to increase RubisCO levels is unlikely to improve photosynthesis (Bally et al., 2009, Bally et al., 2011). In theory however, it is possible to raise yields of a C_3 crop, by transferring RubisCO with higher affinities for CO_2 and reduced photorespiration, for example from the red alga *Griffithsia monilis* (Long et al., 2006b). However it is not as simple as that. In higher plants, the large subunit of RubisCO is coded for (*rbcL* gene), transcribed, and translated in the plastid. The gene for the small subunit (*rbcS*) on the other hand, are housed within the nucleus, translated in the cytoplasm, and unfolded small subunit protein transported to the chloroplast prior to processing into the "caps" for the RubisCO enzyme (Nishimura et al., 2008, Jarvis, 2008). N terminal modifications, along with other post-translational modifications, is conducted to L-subunits in order to prevent proteolytic degradation (Houtz et al., 2008). To ensure correct folding, L-subunits also associate with Hsp70 chaperone proteins (Nishimura et al., 2008). While the S-subunits most certainly associate with cytoplasmic chaperone proteins prior to transport into chloroplasts, their folding and protein associates prior to binding to L-dimers within the chloroplast is unclear. Any protein product of heterologous RubisCO genes that are inserted into the nucleus or chloroplast, must be able to undergo the same alterations and processing that native RubisCO genes would be able to. Indeed, the ability to create hybrid Rubsico has been demonstrated by replacing tobacco *rbcL* with the tomato *rbcL* to give pale green photoautotrophic plants with hybrid tomato L-subunit/tobacco

S-subunit RubisCO (Zhang et al., 2011). Replacing higher plant *rbcL* genes with protobacterium *rbcM* (L_2), archaeabacterium RubisCO (L_{10}) (Whitney and Andrews, 2001a, Alonso et al., 2009), and sunflower *rbcL* (Sharwood et al., 2008, Sharwood and Whitney, 2010, Kanevski et al., 1999) via chloroplast transformation has also been successfully demonstrated. Although catalytic properties of RubisCO were as expected, sunflower L subunits showed compatibility issues with folding and assembly, thus limiting the formation of L_8S_8 hybrid RubisCOs. Furthermore, efficient red algae RubisCOs were unable to be introduced into higher plants plastids due to incomplete folding and assembly (Whitney et al., 2001) as a result of significant evolutionary divergence. Most L-subunit substituted transplastomics often demonstrate a pale green phenotype or/and require elevated CO_2 for growth (Hanson et al., 2013). Attempts to express S-subunits within the chloroplast directly were met with disappointing results, as the plastids favoured assembly of Rubsicos with nuclear derived S-subunit as opposed to those transcribed and translated within the plastid (Whitney and Andrews, 2001b). Expression of a linked L and S-subunit RubisCO transcribed and translated from a single operon within the plastome, produced plants that required elevated CO_2 as juvenile plants. The requirement of supplementary CO_2 was not due to an impaired catalytic activity of the RubisCO, but an inadequacy in folding, which may have arisen due to the build up of insoluble fused RubisCO aggregates (Whitney et al., 2009). Thus it would appear that although the integration of exogenous RubisCO genes into the plastid genome is clearly possible, adequate translation, folding and assembly into hybrid RubisCO might not be so forthcoming. Even if functional hybrid RubisCO is obtained, an improvement in RubisCO activity is unlikely. Furthermore, the import of RubisCO proteins from the cytosol into the plastid to form functional RubisCO has also been demonstrated, however this approach is also unlikely to result in increased RubisCO activity (Kanevski and Maliga, 1994). The production of a tobacco "master line" (^{cm}trL) which allows the relatively rapid production of homoplasmic L-subunit replacement transplastomics, could increase the probability of uncovering RubisCO subunit modifications/combinations that improves RubisCO catalysis (Whitney and Sharwood, 2008). Time will tell whether RubisCO modification can provide supercharged photosynthesis.

1.5.2 RubisCO Activase

While work on RubisCO continues, alternative routes to improved photosynthesis is necessary. Photosynthesis is particularly sensitive to heat stress, and such inhibition can result in reduced yield (Lobell and Field, 2007), and correlates with a reduced activity of RubisCO (Salvucci and Crafts-Brandner, 2004). With global temp set to increase by up to 2.5 in the next 10 years, reducing yield losses due to heat stress will be important. One enzyme that is particularly sensitive to temperature increases is RubisCO activase (RA), which is responsible for priming RubisCO activity. RA frees the RubisCO active site off sugar phosphate inhibitors, which are used to regulate RubisCO (Parry et al., 2008). A more thermostable RA mutant did improve photosynthesis and growth in Arabidopsis (Kumar et al., 2009), however overexpression of activase in the cereal crop rice did not provide such improvements (Fukayama et al., 2012). Unfortunately, poor understanding of RAs interaction with RubisCO impedes furthering this as an option for improving photosynthesis.

1.5.3 Ribulose-1,5-bisphosphate (RuBP)

At saturating levels of CO_2, the availability of RuBP can be rate limiting for photosynthesis. Sedoheptulose-1,7-bisphosphatase (SBPase), fructose-1,6-bisphosphate aldolase, and transketolase are the enzymes responsible for the regeneration of RuBP (Zarzycki et al., 2013), and are thus an area of interest for the improvement of photosynthesis. Overexpression of SBPase in rice chloroplasts enhanced CO_2 assimilation at higher temperatures, increased photosynthetic rates under salt stress (Feng et al., 2007), and resulted in greater biomass accumulation in *Arabidopsis thaliana* and *Nicotiana tabacum* (Miyagawa et al., 2001, Lefebvre et al., 2005). SBPase is bi-functional, in that it also acts as a fructose-1,6-bisphosphataseFBPase, and both SBPase and FBPase catalytic functions are enhanced in overexpression lines, and both have a positive effect on photosynthetic rate (Tamoi et al., 2006). However, under CO_2 limiting conditions, SBPase overexpression can be toxic.

1.5.4 Increasing inorganic carbon concentrations around RubisCO

An attractive approach for improving photosynthesis in C_3 crop plants would be to introduce the carbon concentrating mechanisms employed in C_4 plants to increase

intracellular inorganic carbon, thus favouring carboxylation and reducing losses through photorespiration. Photorespiration in C_4 plants has been shown to remain slow even under limiting conditions (Carmo-Silva et al., 2008).

Encouraging results from nuclear transformed higher plants suggest that increasing concentrations of CO_2 in the vicinity of RubisCO, or by introducing multi-enzyme photorespiratory bypass pathways (Maurino and Peterhansel, 2010) targeted to the chloroplasts that utilise the glycolate substrate, can improve biomass production by reducing photorespiration (Kebeish et al., 2007, Maier et al., 2012). The bypass pathways not only removed glycolate from engaging in photorespiration, but converted glycolate into glycerate, which can be used to regenerate RuBP. Carbon dioxide is a by-product of these enzymatic pathways, thus increasing inorganic carbon concentrations to be used by RubisCO. These examples of successful bypass pathways can be constructed into polycistrons and inserted into the plastome (Khan, 2007).

While work continues on introducing the "Kranz" anatomy and the CO2 concentrating mechanisms of C4 plants into C3, a complex multi-gene approach, alternative single or double transgene insertions into the plastome or nucleus that would mimic the cyanobacterial and algae carbon concentrating mechanism (CCM) is an option (Lieman-Hurwitz et al., 2003, Price et al., 2013, Price et al., 2008). With a reduction in transgene insertions and no anatomical modifications, such an approach is likely to be less technically challenging, and may also require lower energy costs in comparison the C4 pathway. CCMs are not observed in land plants, and therefore CCM mechanisms need to be exogenously introduced within these plant cells. In cyanobacteria, RubisCO and carbonic anhydrase (CA) are housed within a compartment known as a carboxysome. Carboxysomes are semi permeable protein shells, not present in land plants (except the earliest land plants *Anthocerotophyta*), which increase the concentration of inorganic carbon around RubisCO. The organisation of RubisCO and CA within one compartment and consequential elevated CO_2 levels, reduces the possibility of photorespiration, and compensates for the low efficiency of RubisCO. Developing carboxysomes in the plastids of higher land plants is very complex, and faces many obstacles (Zarzycki et al., 2013).

A simpler option could be to insert cyanobacterial bicarbonate pumps into the chloroplast envelope, such as *Chlamydomonas* HCO_3^- transporters (Spalding, 2008). There are five import mechanisms known to transport inorganic carbon in cyanobacteria, three for HCO_3^-, and two for CO_2 (Price, 2011). Three well characterised HCO_3^- transporters are the BCT1 (Price et al., 2008, Omata et al., 1999), SbtA, and BicA (Price et al., 2004) transporters. BicA shows the lowest affinity for HCO_3^-, however has a faster transport rate, and like SbtA, is coupled to Na+ symport. In theory, insertion of these transporters into the chloroplast membrane, would provide increased flow of inorganic carbon into the stroma, where HCO_3^- will be converted to CO_2 by carbonic anhydrase, thus increasing CO_2 around RubisCO and subsequent photosynthetic carbon fixation. Expression of the inorganic transporter B (ictB), initially believed to be a HCO_3^- transporter (Bonfil et al., 1998), in the nucleus and transported to the chloroplast in *Arabidopsis thaliana and Nicotiana tabacum*, demonstrated increased photosynthetic rates and plant growth under CO_2 limiting conditions. Overexpression studies in cyanobacterial strains absent of most inorganic carbon transporters can increase our understanding and characterisation of increased bicarbonate/CO_2 uptake. Having a variety of protein channels/transporter options would be useful to test for in higher plant species.

1.6   Nuclear verses chloroplast transformation

For many plant species, nuclear transformation is most efficiently achieved using the bacterium *Agrobacterium tumefaciens* (Bevan, 1984). *A. tumefaciens* has the innate ability to transfer a copy of part of Ti plasmid into a plant cell via a pilus. Once in the plant cell, the bacterial DNA is transported to the nucleus where it integrates randomly into the nuclear DNA. By inserting a gene of interest between the left and right border sequences of the Ti plasmid, it too will be copied along with the rest of T-DNA transferred into the plant cell cytoplasm, and directed into the nucleus. The position of T-DNA insertion into the plant nucleus appears to be largely random and unpredictable, hence unless inserted into intergenic regions, would result in undesired disruption of particular genes. Furthermore, even if insertion of T-DNA has occurred in an intergenic region, and that fertile offspring have been produced from these transformants, expression of the transgene can be surprisingly varied in each tissue type and between different transgenic events. This is because the expression of the

transgene depends on the activity of its promoter and also the genomic location of the insertion and on number of transgene copies /rearrangements etc that have occurred during the initial transformation (Grevelding et al., 1993).

Insertion of exogenous genes into a chloroplast with *A. tumefaciens* has been conducted (Deblock et al., 1985), although the experiment has since never been reproduced. The chosen method for chloroplast transformation today is biolistic transformation via a particle gun (Sanford, 1990, Klein et al., 1987). Chloroplast transformation has many advantages over nuclear transformation. Firstly, because each chloroplast contains a certain number of circular chromosomes, it is easy to calculate the number of transgene insertions that is possible. This is an advantage when the dosage of transgene protein product is important to control, for example if the transgene product is of pharmaceutical importance. Protein production of chloroplast transgenes is far greater than nuclear transgenes (18% of total proteins, and 0.5-3% of total proteins respectively (Maliga, 2004)). This is another advantage if the transgene is of medical or pharmaceutical importance. The higher level of protein production is due to a several factors including: the high number of chloroplast chromosomes per cell (approximately100) as well as the lack of gene silencing, epigenetic mechanisms and pre/post transcriptional silencing. It is also possible to express multiple genes from polycistronic mRNA (Maliga, 2001, Staub and Maliga, 1995, De Cosa et al., 2001, Quesada-Vargas et al., 2005). Secondly, transcription and translation of a transgene would occur only in the chloroplast, hence any mRNA/protein product would be compartmentalised within the chloroplast. The prevention of protein product entering the cytoplasm of the cell has three advantages: avoiding adverse reactions between cellular components and the exogenous protein, no alteration of the cellular water potential, and reduced labour of exogenous protein extraction (if protein is of pharmaceutical importance). Thirdly, the chloroplast genomes do not undergo genetic alterations as does the nuclear genome does (and rather frequently), such as polyploidy, gene duplication, or recombination events. Lastly, and most importantly, plastids are inherited maternally in the vast majority of angiosperms (Birky, 1995, Mogensen, 1996, Hagemann, 2002). Maternal inheritance occurs due to the exclusion of pollen-derived chloroplasts from the male gamete during fertilization. This would mean that any alterations made to the chloroplast genome of plant tissue that is eventually grown to an adult plant primed for reproduction, cannot transmit the transgenic chloroplast to its offspring via its pollen.

This is an enormous advantage as gene flow of GM genotypes via pollen into wild relatives is a commonly-cited environmental risk hindering the development and commercialization of GM crops. Although some genetic leakage has been observed in some species (Ruf et al., 2007, Svab and Maliga, 2007), plastid transformation provides a strong level of biological containment, and is still considered a safe method of transgene containment following risk assessment (Wilkinson et al., 2003).

The advantages associated with chloroplast transformation has resulted in a rapid increase in scientific interest, especially from the biopharmaceutical sector. This has lead to a large number of different plant species chloroplasts being sequenced, with more chloroplast sequencing expected in the near future (Wani et al., 2010).

1.7 Chloroplast transformation in the monocots.

From the suggestion that chloroplasts contain DNA in 1951, the cytological identification of chloroplast DNA over a decade later, to the first reports of integration of exogenous DNA into plastid DNA, modern plastid transformation research has provided many examples of successful and stable plastid genome alterations to date. These successes have largely, if not exclusively, been limited to the dicotyledon plant species and algal species. In comparison, chloroplast transformation in the monocotyledon species has been restricted to rare events. The first event of exogenous DNA expression in monocot chloroplasts came in the form of transient GUS expression in wheat. Following his work with transient gene expression in the plastids of higher plants (Daniell et al., 1990), Henry Daniell turned his attention to transient expression in wheat using the *uidA* gene (Daniell et al., 1991). Biolistic bombardment (Klein et al., 1987) of albino wheat leaf with the *uidA* gene under the transcriptional control of a dicot plastid promoter, the catalytic activity of the uidA translational product (beta-glucuronidase, which cleaves its substrate X-gluc to produce an indigo dye) could be observed compartmentalised within the chloroplast. In comparison, bombardment of the same tissue with a nuclear *uidA* transformation vector showed the insoluble blue die evenly spread throughout the cell. It was hoped that these results could provide a positive benefit the prospects for chloroplast transformation in the monocots. However it would not be until the end of the decade when a second report of chloroplast transformation in the monocots would be presented.

Encouraged by the expression of the FLARE-S fusion gene (a 3"-adenyltransferase + GFP fusion protein) in non green plastids in tobacco, Khan and Maliga attempted to create chloroplast transformed rice cells by bombarding white embryogenic tissue culture cells formed from mature seeds (used for rice nuclear transformation) with a rice specific chloroplast transformation vector containing the FLARE-S expression cassette (Khan and Maliga, 1999). Integration of FLARE-S was confirmed using PCR and confocal microscopy, however, only a fraction of the chloroplasts in the PCR positive transformants were shown to express FLARE-S. Although plastid homoplasmy was unable to be achieved, this presented a step forward in that it clearly presented chloroplast transformation within the monocots is possible, plastid encoded promoters (PEP) are functional in non green plastids, and that streptomycin can be

used as a selection mechanism (however, the lack of homoplasmy indicates an alternative selection mechanism may be necessary).

Bombardment of rice embryogenic callus, formed from mature seed, once again proved to be the target for a chloroplast transformation study published in 2006 (Lee et al., 2006). A rice-specific plastid transformation vector, containing an expression cassette consisting of *gfp* and *aad*A under the expressional control of the *Prrn* promoter (PEP promoter), targeted to the *trn*I-*trn*A genes of the rice plastid genome, was bombarded into embryogenic calli of rice. GFP expression was confirmed using confocal microscopy and western blot analysis, however homoplasmy was once again not achieved, and due partly to the low efficiencies and labour intensive nature of the procedure (4000 bombarded calli produced just 2 independent lines), has not been repeated by other laboratories (or at least not published to date).

1.8   Bottlenecks and outlook

Successful plastid transformation is reliant on three things, (a) an adequate and robust method of DNA delivery into plastids, (b) a tissue target that contains plastids possessing active homologous recombination enzymes to facilitate the integration of exogenous DNA into the plastid genome, (c) a tissue target with a robust regeneration system, and (d) an effective selection mechanism. As described above, the biolistic method of DNA delivery is the most effective and cost efficient method of depositing DNA into plastids, and this method has universal capabilities. Plastid homologous recombination is also known to be highly efficient and active within *Chlamydomonas* and the seed plants, and therefore DNA delivery and homologous recombination are unlikely to be limiting factors in successful monocot chloroplast transformation (especially as partial transformation has been demonstrated). The factors causing the bottleneck are most likely to be the tissue culture methods and selection processes.

There is no universal method for plastid transformation across the land plant species and algae. Each new species targeted for chloroplast transformation would require the development of a suitable tissue culture system which may also show differences in response between cultivars for that particular species. This presents a great challenge that would involve laborious and time-consuming optimization work, based largely on a trial and error principal. Furthermore, a novel selection mechanism is likely to be required for the plastid transformation of the monocotyledon embryophytes.

1.9 Aims of project

- Identify novel tissues suitable for use in the chloroplast transformation of wheat, and develop a robust tissue culture process for these novel tissues.
- Produce functional wheat specific chloroplast transformation vectors.
- Develop an optimised protocol for the chloroplast transformation of wheat.
- Produce transplastomic tobacco containing the cyanobacterial carbon concentrating gene, *ictB,* for the improvement of photosynthesis in a C_3 plant.

2   General Material and Methods

2.1   Chloroplast transformation via biolistic bombardment

2.1.1   *Nicotiana tabaccum* (tobacco) plant preparation.

Seeds were sterilised in eppendorf tubes by exposure to 70% ethanol for 30 seconds, followed by 10% bleach (available chlorine 10-15% in stock) for 5 mins on a roller mixer. Under sterile conditions, seeds were then washed 5 times with 1ml of sterilised distilled H_2O. Seeds were allowed to dry for 5 mins, and then were placed (up to 100 seeds) on a 9cm petri dish containing MS (see 4.2) media at 26°C, in the dark for 4-7 days to encourage germination. Once the seedlings had germinated, the required numbers were moved to magenta boxes (1 per box) containing MS media. Magenta boxes containing tobacco seedlings were moved to the culture room at 26°C on a 16/8 hour day/night cycle at 10-50µE light intensity. After 4-6 weeks, the first five leaves below the unexpanded top leaf are suitable for transformation.

2.1.2   Tobacco leaf explant preparation

Suitable leaves were excised from tobacco plants grown *in vitro*. For each leaf, the midrib and edges were removed to leave two rectangle pieces (Fig 2.1). These were then placed in the centre of a 9cm petri dish containing RMOP (Day and Goldschmidt-Clermont, 2011) media abaxial (bottom) side up, ready for bombardment. Once prepared, plates were used immediately.
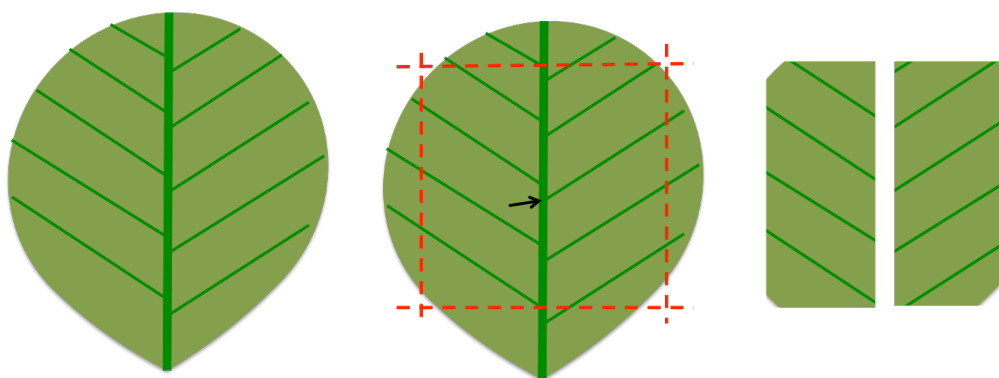
Fig 2.1 Preparation of tobacco leaf for bombardment. Leaf is placed abaxial side up (left), and using a sterile scalpel, the midrib (arrow) and edges are removed (red dashed lines) to leave two rectangular pieces (right).

2.1.3 Gold preparation

40mg of 0.6μm gold (BIO-RAD, USA) was weighed into an eppendorf, and 1mL of 100% ethanol added. The mixture was sonicated for 2 mins, then pulse centrifuged for 3 seconds. Supernatant was removed, and the ethanol wash followed by centrifugation was repeated twice. 1mL of sterile distilled H_2O was added and the mixture sonicated for 2 mins. The mixture was again pulse centrifuged for 3 seconds, supernatant removed, and repeated. Gold was then re-suspended in 1mL sterile distilled H_2O. 50mL amounts were aliquotted into eppendorfs, vortexing between each aliquot.

2.1.4 DNA coated gold particle preparation

50μL aliquot of gold mixture was allowed to thaw, and placed in a sonicator for 3 mins. 5μL of plasmid DNA (1mg/mL) was added to the gold, mixed by pipetting, vortex for 5 seconds, and then placed on ice for 60 seconds. The lid of the eppendorf containing gold and plasmid DNA mixture was opened, and 50μL of 2.5M $CaCl_2$ pipetted into the lid of the eppendorf. 20μL of 0.1M spermidine was added to $CaCl_2$, mixed by pipetting, and allowed to drop into the gold/plasmid mixture by closing the lid. The mixture was vortexed for 5 seconds and place on ice for 1 min. The DNA coated gold particles were centrifuged at 3,000 rpm in a microcentrifuge for 1 min, and the supernatant discarded. 150μL of 100% ethanol was added to the pellet, while dislodging and breaking up the pellet with the pipette tip. It was important that large clumps were broken down at this stage. The mixture was mixed well with a pipette, and vortexed for 5 seconds. A second centrifugation step was performed at 3,000 rpm for 1 min in a microcentrifuge. The supernatant was discarded, and the pellet re-suspended in 60μL of 100% ethanol (per 10 shots), once again dislodging and breaking up the pellet with the pipette. The DNA/gold preparation was vortexed for 5 seconds, and distributed onto macrocarriers (5 μL), or store on ice. Preparation was vortexed for 5 seconds for every 5 macrocarriers.

2.1.5 DNA delivery

Bio-Rad PDS 1000/He particle delivery system was set up as per manufacturer's instructions, and microcarrier launch assembly placed into the top shelf position (groove 2). A 9cm Petri dish containing target tissue on the relevant media was placed in the fourth shelf position (groove 4). This produced a 6cm distance from the stopping screen in the launch assembly to the target plate. 5μL of the DNA coated

gold particle preparation was pipetted on the centre of the macrocarrier, and ethanol allowed to evaporate in a laminar flow hood. The device was operated according to manufacturers instructions, with biolistic bombardments conducted at a vacuum of 27-28in Hg. Following bombardment, plates containing bombarded target tissue were moved to the subsequent relevant conditions.

2.1.6 Selection of tobacco plastid transformants

Bombarded leaves were cut into 0.5cm square pieces, and placed on RMOP medium containing 500mg/L spectinomycin abaxial side up under sterile conditions. Blades and forceps were sterilised between each bombarded plate. Plates were sealed with parafilm (twice) and incubated in 10-50μE light on a 16/8 hour day/night cycle at 26°C for 4-8 weeks. Green resistant shoots or cell clumps appeared between 6-8 weeks, and these were separated from the callus piece it grew from, then placed on RMOP supplemented with 500mg/L spectinomycin in a 9cm petri dish, in 10-50μE light on a 16 hour day cycle at 26°C. After 8 weeks, plates that had not produced resistant shoots were disposed of. Resistant shoots that had grown into a mass of several shoots were then subjected to second round of selection on RMOP supplemented with 500mg/L spectinomycin, in a magenta box. Alternatively, if the resistant shoots were large enough, MS was used instead of RMOP in order to encourage root formation.

2.1.7 Transferring plants to greenhouse

To transfer rooted transplastomic tobacco shoots to greenhouse, the agar was gently broken up, the roots washed in running tap water to remove as much solid media as possible, and then planted into soil. The plants were placed into a covered micro-propagator, and left to incubate in the greenhouse after watering. To collect seedpods once matured, the plants were no longer watered and seedpods allowed to dry, and removed from the plant only when they turned brown.

2.2 Molecular Characterization

2.2.1 Isolation of genomic DNA

Whole genomic DNA from both tobacco and wheat were extracted from young adult leaves using a DNeasy Plant Mini Kit (Qiagen, 27220 Turnberry Lane, Suite 200, Valencia, CA 91355), using the manufacturer's protocol.

2.2.2 Polymerase Chain Reaction (PCR)

Each reaction tube contained a total reaction mixture of 20μl. Components of each PCR reaction would include a 4μL 5x buffer, 0.4 μL dNTPs (10mM), 1μL forward primer (10μM), 1μL reverse primer (10μM), 50-250ng of genomic DNA/ 1pg-10ng plasmid DNA, 0.5 units polymerase enzyme, dH$_2$O up to make volume up to 20μL. For all PCR reactions, the Phusion®High-Fidelity DNA polymerase was used (New England Biolabs, 240 County Road, Ipswich, MA 01938-2723, USA). Once PCR reaction mixtures were ready, they were moved to a PCR machine. Thermocycling conditions were set as per Phusion® High-Fidelity DNA polymerase manufacturers instructions.

2.2.3 Agarose Gel

Electrophoresis gels were 1% molecular biology grade agarose, dissolved in 0.5x TBE buffer (5x TBE buffer: 53g Tris base, 27.5g boric acid, 10mM EDTA, made up to 1 litre with H$_2$O). Once the agarose had been dissolved by heating in a microwave oven 0.01% v/v 10mg/ml ethidium bromide was mixed into the buffer as it cooled. Gels were left to solidify in a microgel former with appropriate combs before being placed in a microgel bath and submerged in 0.5x TBE buffer. The PCR mix was mixed with 6x tri track loading buffer (Thermo Fisher Scientific Inc., 81 Wyman Street, Waltham, MA 02451, USA) and the resultant mixture loaded into wells. Hyperladder™ 1kb DNA ladder (5μl) (Bioline USA Inc., 305 Constitution Drive, Taunton, MA 02780, USA) was added to a well for each experiment. Once PCR products and ladder had been loaded, gels were run between 40-90V, then photographed under ultraviolet light.

2.2.4 DNA extraction from agarose gel

Using ultraviolet light, a DNA band was first identified in the agarose gel, and then excised with a clean sharp blade. As much of the agarose was removed at the time of

excision. The excised band was then placed in an 2ml eppendorf, and DNA extracted from the band using a Wizard® SV gel and PCR clean up kit (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA), following manufacturer's instructions.

2.2.5   Chloroplast DNA extraction

Chloroplasts from green tobacco and wheat leaves were extracted using a chloroplast extraction kit (Sigma-Aldrich, 3050 Spruce St, St. Louis, MO 63103, USA), as per manufacturer's instructions.

2.3   Gene Cloning

2.3.1   Poly A Tailing DNA fragments

PCR amplified DNA fragments that were extracted from agarose gel, needed to have a single deoxyadenosine base attached to the 3' ends of each amplified fragment for cloning into the pGEM-T Easy vector. Each PCR reaction tube contained 1µL 10x PCR buffer, 0.2µL aNTP (10mM), up to 100ng fragment DNA, Hot start Taq (New England Biolabs, 240 County Road, Ipswich, MA 01938-2723, USA), and dH$_2$O up to a total reaction volume of 10µL. The mixtures were then placed in a PCR machine at 95°C for 15 mins, then 72°C for 30 mins.

2.3.2   Ligation into pGEM-T Easy Vector

Adenine tailed DNA fragments from 2.3.1 were ligated into the pGEM-T easy vector (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA) as per manufacturer's instructions.

2.3.3   Ligation into other vectors

T4 DNA ligase (New England Biolabs, 240 County Road, Ipswich, MA 01938-2723, USA) was used for all ligations. Each reaction contained a minimum insert to vector ratio of 3:1, 200 units of T4 DNA ligase, 1µL of 10x T4 ligase reaction buffer (New England Biolabs, 240 County Road, Ipswich, MA 01938-2723, USA), and dH$_2$O up to a total reaction volume of 10µL. Reactions were left at room temperature for 2 hours, prior to transformation into *E.coli* (2.6.1).

2.3.4 Restriction Endonuclease digestions

All restriction endonuclease enzymes used were purchased from New England Biolabs (New England Biolabs, 240 County Road, Ipswich, MA 01938-2723, USA), and digestions were performed as per manufacturers instructions. If larger amounts of restriction products were required, reaction volumes were scaled up accordingly.

2.4 Microbiological techniques

2.4.1 *E.coli* transformation and culture

For all *E.*coli transformations, NEB 10-beta competent cells (New England Biolabs, 240 County Road, Ipswich, MA 01938-2723, USA) were used. Cells were thawed at room temperature on ice and 10-20µL were pipetted into 1.5mL eppendorf tubes (in an ice bucket at room temperature), and 10ng of DNA/2.5µL of ligation mix was pipetted into cells and mixed (by pipetting). The mixture was incubated on ice for 30 mins, followed by a heat shock step at 42°C for 30 seconds, then snap cooled on ice. Following this, 150µL of SOC medium was added, and the mixture incubated at 37°C for 60-120 mins. Pre-prepared 2xYT agar (Sigma-Aldrich, 3050 Spruce St, St. Louis, MO 63103, USA), mix was warmed using a microwave oven, and allowed to cool before pouring into sterile 9cm petri dishes. If selection was required, then the relevant antibiotic was added to the warmed 2x YT agar after it has cooled to a temperature that it can be held comfortably in the hand, but not too cool that the agar has begun to harden and set. For colony screening using the disruption of the LacZα gene to give blue/white colonies for failed insertion/successful insertion of DNA into the multiple cloning site, X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) was added to the agar. X-gal (Melford Laboratories, Bildeston Road, Chelsworth, Ipswich, Suffolk, IP7 7LE, UK) was added under conditions to that of selectable agent. Under sterile conditions, 75µL of incubated mixture was then pipetted onto agar plates containing 2x YT, and spread with sterile disposable spreaders. Plates containing transformed *E.coli* cells were then left to incubate at 37°C overnight. For the preparation of Miniprep volumes (5mL) of transformed *E.*coli, a single colony was "picked" off the overnight culture with a sterile toothpick, and placed in a vial containing 5mL of 2x YT. Selection was added to the 2x YT if it was required. This was then placed in an incubator at 37°C, rotating at 220 rpm, overnight. For maxiprep volumes (50-100mL) of transformed *E.*coli, 5mL of 2xYT containing a

"picked" colony was incubated for 8-10 hours at 37°C, rotating at 220 rpm. A 1/1000 aliquot of this was then diluted in 50-100mL of 2xYT (with or without selection). This was then left to incubate overnight at 37°C, rotating at 220 rpm.

2.4.2 Plasmid Miniprep

Plasmid DNA was recovered from transformed *E.coli* using Wizard® *Plus* Minipreps DNA Purification kit (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA), following manufacturer's instructions.

2.4.3 Plasmid Maxipreps

Plasmid DNA was recovered from transformed *E.*coli using a Qiagen Plasmid Maxi Kit (Qiagen, 27220 Turnberry Lane, Suite 200, Valencia, CA 91355), following manufacturers instructions.

2.5 Microscopy

2.5.1 Digital Photography

Digital photographs were taken using either a Canon EOS 350D digital SLR Compact systems camera, with zoom lens EF-S18-55mm f/2.5-5.6, or an Olympus OM-D E-M10 compact system camera, with a M.Zuiko Digital ED 60mm f2.8 Macro lens. For macro images using the Canon, the lens was disconnected, turned around, and held in place. Camera was then moved towards or away from object in order to focus. For macro images using the Olympus, the camera was connected to a SZ40 Olympus microscope using 60S T camera adapter. A JJC macro LED ring light attached to the microscope provided illumination.

2.5.2 Light Microscopy

Light microscopy was conducted using a Leica M205 FA stereomicroscope (Leica Camera AG, Am Leitz-Park 5, 35578 Wetzlar, Germany). A DsRed filter was used for the detection of DsRed proteins (Excitation wavelength 510- 560nm, Emission wavelength 590-650nm).

2.5.3 Laser Microscopy

Laser microscopy was conducted using a Zeiss LSM780 (Carl-Zeiss-Strasse 22, 73447 Oberkochen, Germany). For detection of DsRed and GFP proteins, the recommended manufacturer settings were used, with digital gain adjusted to amplify detected protein emission wavelengths accordingly. Images were processed with the accompanied image processing program (ZEN 2011).

3 Design and construction of wheat and tobacco chloroplast transformation vectors

3.1 Introduction

As mentioned in Chapter 1, chloroplast transformation is dependent upon the delivery of a transformation cassette into chloroplasts via particle bombardment, subsequent integration of the transformation cassette into the chloroplast genome, and finally the expression of transgenes thus permitting selection of positively transformed chloroplasts. Thus, the design of the transformation cassette is equally as important as the method of delivery. A basic transformation cassette is shown in Fig 3.1, however in the majority of chloroplast transformation cassettes, both a gene of interest and a selection gene are required. The cassettes can be designed and orientated as shown in Fig 3.2. It has been successfully demonstrated that up to 4 genes can be transcribed in a single polycistronic operon by using a combination of spacers and leaders between each gene (Bohmert-Tatarev et al., 2011).
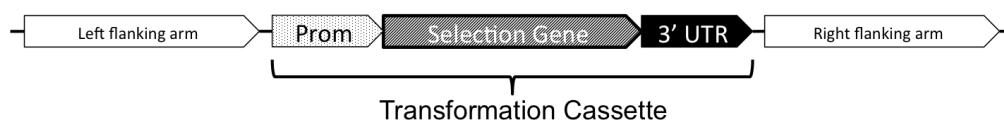
Fig 3.1 The structure of a basic transformation cassette. A promoter (Prom) is required to drive the expression of the gene of interest (in this case a selection gene), and the transcription product of the gene requires stability from a 3' untranslated region (3' UTR). The cassette is inserted into a specific location within the chloroplast genome by flanking the cassette with DNA sequences homologous to the region into which insertion is required (Left flanking arm, Right flanking arm).

The flanking arms, DNA sequences homologous to the regions within the plastid genome the transformation cassette is targeted to, are placed either side of the transformation cassette, and both arms should ideally be 1-2kb in length (Maliga, 2004). Plastid targeting sequences can be selected from any part of the plastid genome, but should be designed to avoid disrupting native functional genetic elements. For example, interruption of the *petB-petD* region with *aadA* resulted in a sucrose dependent phenotype (Zhang et al., 2001). For a list of plastid targeting sites

previously used, please see (Maliga, 2004) and (Day and Goldschmidt-Clermont, 2011). The *16srRNA/trnI - trnA/23srRNA* gene region of the chloroplast genome has been successfully targeted in a number of tobacco chloroplast transformation studies, and it is this region that I will target in the wheat chloroplast genome.

a

b

c
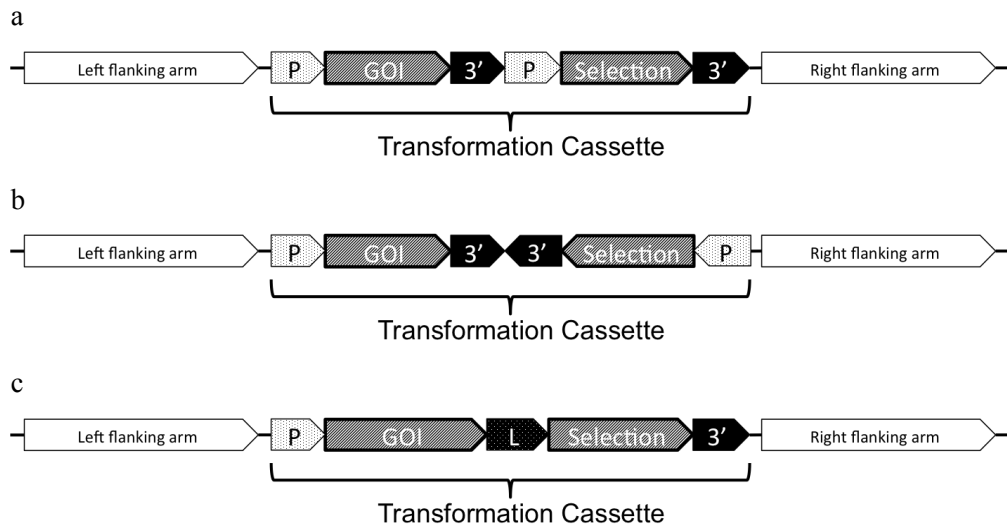
Fig 3.2 The structure of a transformation cassette for the expression of multiple genes. a, Each gene is driven by its own promoter (P) and each transcript stabilised with its own 3' UTR (3'); b, as in a, the "selection" gene is driven by its own promoter, and transcript stabilised with a 3' UTR, however the orientation is in the opposite direction. By having the promoter orientated in opposite orientation, you avoid deletions by repeated sequences (Lutz et al., 2007); c, The promoter drives the expression of both the gene of interest and selection gene, to produce a single mRNA transcript, however a leader/spacer sequence (L) is placed between the two genes to ensure separate translation. A 3' UTR is again used for stability of the single transcript. See (Bohmert-Tatarev et al., 2011) for further information.

For the purposes of my investigation, the chloroplast transformation of wheat, I decided to construct a transformation cassette that would consist of two coding genes, a visual reporter gene and a selection gene. As there was no previously published data of direct exogenous gene expression within the chloroplasts of wheat, I attempted to keep the design of the cassette as simple as possible, and based on previously-validated genetic elements to minimise the possibility that any absence of transformation was not due to failed transcription of marker or selection genes. It is for this reason that I initially chose to construct a chloroplast transformation cassette that contained the *gfp* reporter gene and *aadA* selection gene, transcribed in opposite orientations, each under the control of a promoter (as described in Fig 3.2.b). However, cloning two identical promoters in opposite orientations proved problematic, and therefore I decided to design a cassette that would express *gfp* and *aadA* in a single polycistronic transcript (see Fig 3.2.c). As described in the Chapter 1, the tobacco *Prrn* is a strong plastid promoter, and its fusion with the 5'UTR of the T7g10 bacterial promoter produces higher levels of exogenous protein accumulation (Maliga, 2002, Oey et al., 2009) when compared to the *Prrn* fused with other 5'UTRs containing ribosomal binding sites. It is for this reason that I chose to design a cassette driven by the wheat *Prrn* promoter fused with the T7g10 5'UTR. To ensure separate translation of *gfp* and *aadA* from a single polycistronic mRNA, a spacer was inserted between marker and selection gene. A number of options are available to choose from (Bohmert-Tatarev et al., 2011), and I elected to use the psbD/C spacer sequence (9,983-10,036 of *Triticum aestivum* chloroplast genome) to separate the *gfp* and *aadA* genes in the transformation cassette. The transformation cassette (TCas1 Fig 3.3) was synthesised, and provided in the pUC57 vector. Once constructed, the transformation cassette will be tested in the tobacco transformation system to confirm its function.
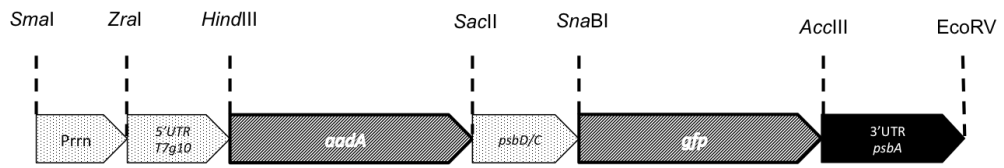
Fig 3.3 The transformation cassette TCas1. Prrn, wheat 16srRNA promoter; 5'UTR T7g10, the 5' untranslated region of gene 10 from T7 *E.coli;* aadA, aminoglycoside resistance gene; psbD/C, the wheat psbD/C intergenic region; gfp, green fluorescent protein gene codon optimised for plastids; 3' UTR psbA, the 3' untranslated region of the tobacco psbA gene. Restriction endonuclease sites are indicated with dotted lines.

3.1.1 **Aims**

- Design and construct wheat specific chloroplast transformation vectors.
- Confirm function of the chloroplast transformation cassette by testing in the tobacco chloroplast transformation system.

3.2 Material and Methods

3.2.1 Vectors

Detailed in this section are the vectors used and referenced to in Chapter 3.

3.2.1.1 pRRes14m
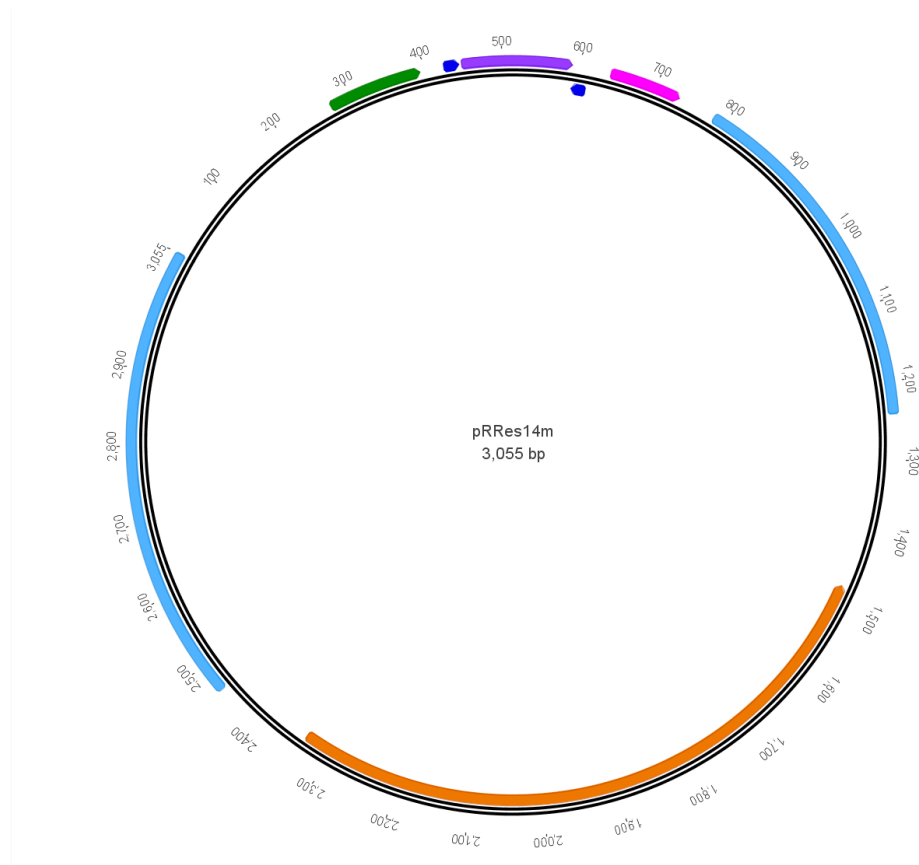
Fig 3.4 pRRes14m, a gift from Dr A. Huttly (Rothamsted Research). pRRes14m was generated from the pGEM (PROMEGA) backbone. It contains a pMB1 and an F1 ORI (origin of replication, light blue), which are located on the right and left of the Ampicillin resistance gene (orange) respectively. The lacZ promoter (green) and lacZ α-peptide gene (pink), are separated by the multiple cloning site (purple). The multiple cloning site is flanked by a T3 (dark blue, left) and a T7 (dark blue, right) primer sequence. 5'-3' orientation of genetic elements are indicated by blunt (5') and pointed (3') ends of annotations.

3.2.1.2 pRRES14m multiple cloning site
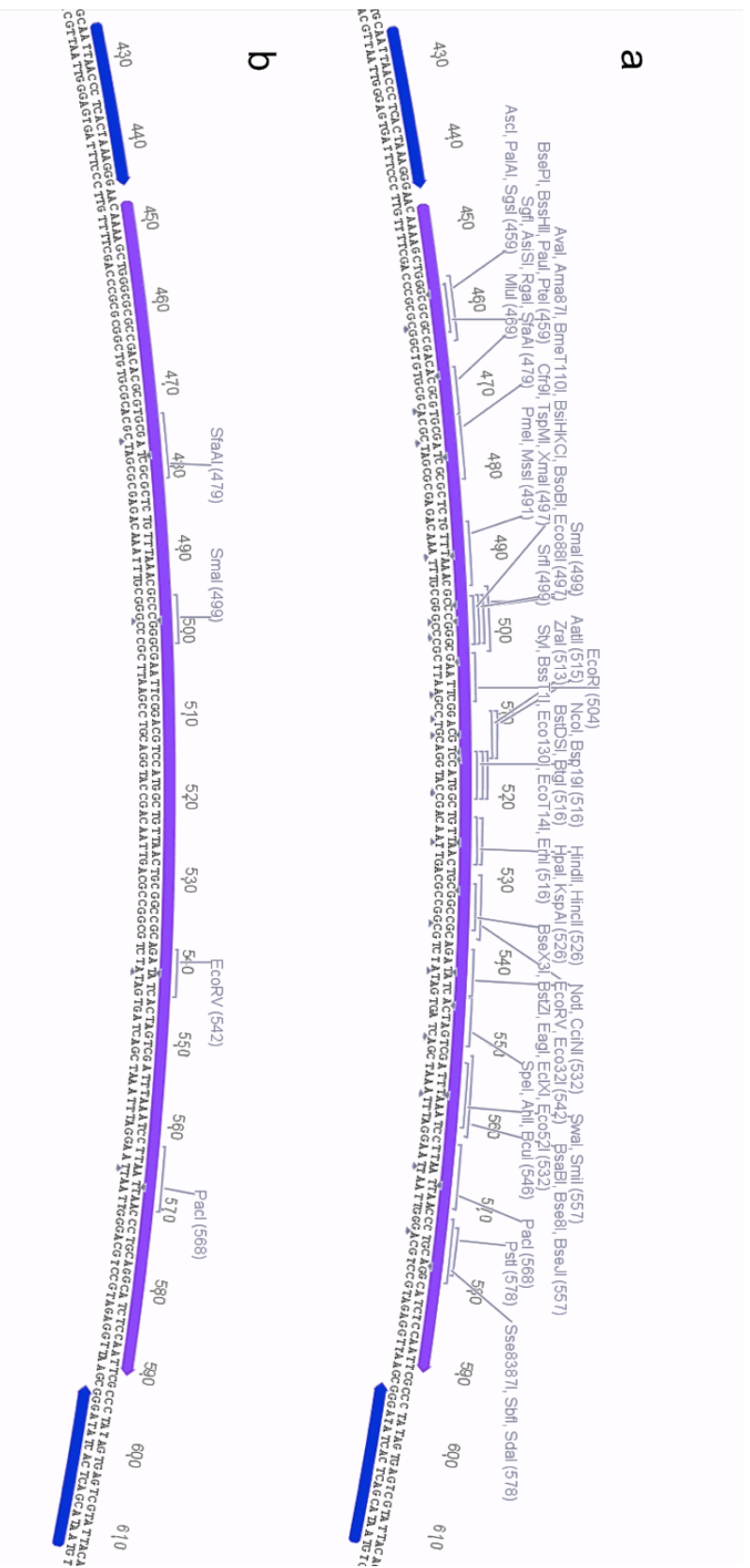
Fig 3.5 Restriction endonuclease sites in the pRRES14m multiple cloning site. a, total sites; b, sites used for cloning in this chapter. Blue annotations indicate T3 (left) and T7 (right) primers.

3.2.1.3 pPRV323CloxII
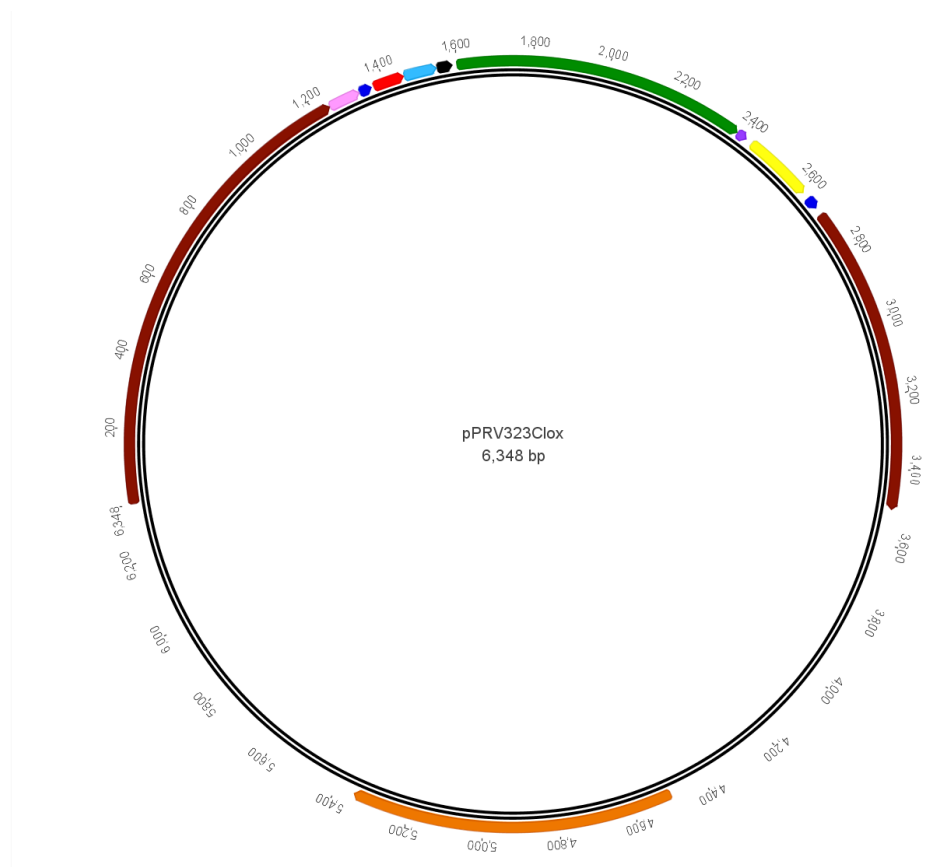
Fig 3.6 pPRV323Clox (Lutz et al., 2007), GenBank Accession No. DQ489715. Formerly known as pPRV323Clox (Chakrabarti et al., 2006). A chloroplast transformation vector targeted to the *rrn16/trnI-trnA* gene region (burgundy), containing ampicillin resistance (orange), multiple cloning site (pink), *aad*A gene (green) driven by the tobacco Prrn (TPrrn, Red). Prrn is followed by the tobacco atpB gene leader (light blue) and N terminus (black). *aad*A is tagged with a c-myc tag (purple), and transcript stabilised with the psbA 3'UTR (yellow). Transformation cassette can be post-transformationally excised by Cre due to *lox*P sites (dark blue) located either side of the transformation cassette. *Swa*1 restriction endonuclease sites (not shown) are located to the left of the multiple cloning site, and to the right of the second *lox*P site. Pointed ends indicate direction of genetic elements.

3.2.2 Isolation of genomic DNA

Whole genomic DNA wheat were extracted from young adult leaves using a DNeasy Plant Mini Kit (Qiagen, 27220 Turnberry Lane, Suite 200, Valencia, CA 91355), using the manufacturers protocol.

3.2.3 Generating flanking arms for homologous recombination

Total genomic wheat DNA (100ng) was amplified in 25µL PCR reactions (see 2.2.2) with primers (Table 3.1) deigned to only amplify the *16srRNA/trnI – trnA/23srRNA* gene region (corresponding to 92,304-93,761 and 93,762-95,494 respectively) of the wheat chloroplast DNA. PCR primers were designed to add specific restriction sites to the 5' end of amplicons.

Primer pair	Sequence (5'-3')	Tm (°C)	5' Restriction Endonuclease	Amplicon length (bp)
trnI F2	CGTCACACTATAGGAGCT	52	SfaAI	1359
trnI R1	CAGAGTGCTTCTTCTATTCTT	53	SmaI	
trnA F1	AAGAATAGAAGAAGCATCTG	53	EcoRV	1752
trnA R2	CCCTATTAAGACTCGCTT	53	PacI	

Table 3.1 primers used for the amplification of flanking arms.

3.2.4 Cloning into pGEM®-T easy vector, pRRes14m, and pPRV323Clox

All cloning into vectors was conducted as per manufacturers instructions, or as described in Chapter 2.3.

3.2.5 DNA extraction from agarose gel

Using ultraviolet light, a DNA band was first identified in the agarose gel, and then excised with a clean sharp blade. As much as possible of the agarose was removed at the time of excision. The excised band was then placed in a 2ml eppendorf, and DNA extracted from the band using a Wizard® SV gel and PCR clean up kit (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA), following manufacturers instructions.

3.2.6 Production of WPrrn and TPrrn promoters

Wheat *Prrn* (WPrrn, 90,830-90,990 in the wheat chloroplast genome) was PCR amplified with primers (Table 3.2) containing a *Sma*1 restriction endonuclease site at the 5' end, and a ribosomal binding site (RBS) + *Hind*III restriction site engineered into the 3' end. The tobacco *Prrn* (TPrrn) was PCR amplified from the pUM78 vector, with *Sma*1 restriction endonuclease site at the 5' end, and a ribosomal binding site (RBS) + *Hind*III restriction site engineered into the 3' end.

Primer	Sequence (5'-3')	Tm (°C)	5' Restriction Endonuclease
TPrrn F1	CTTAGGTTTTCTAGTTGGATTTG	58	SmaI
TPrrn R1	GATCCCTCCCTACAACTG	57	HindIII
WPrrn F1	AACCCAATGTGGTATTAGG	60	SmaI
WPrrn R1	CCCTCCCAAGGATAACTTG	61	HindIII

Table 3.2 Primers used for the amplification of WPrrn and TPrrn from wheat and tobacco chloroplast DNA respectively. Yellow highlights engineered ribosomal binding sites.

3.2.7 Chloroplast transformation of tobacco

See chapter 2.1.

3.3 Results

3.3.1 Generating flanking arms for homologous recombination

As explained in section 3.1 I chose to target my transgenes to the *16srRNA/trnI –
trnA/23srRNA* gene region. To generate the flanking arms for the transformation
cassette, I first amplified this region (corresponding to 92,304-93,761 and 93,762-
95,494 respectively) from the wheat chloroplast genome of Apogee. PCR primers
were designed to amplify the two regions and to also add a specific restriction site to
the 5' end (Table 3.1). Total genomic DNA was obtained from wheat leaves 10 days
post sowing using a protocol that also retained chloroplast DNA (see 2.2.1). Primer
pairs trnI F2/trnI R1 and trnA F1/trnA R2 (Table 3.1) were used to amplify the
corresponding regions and produced amplicons of 1,359 bp and 1,752 bp,
corresponding to the *16srRNA/trnI* and *trnA/23srRNA* gene regions respectively (Fig
3.7).
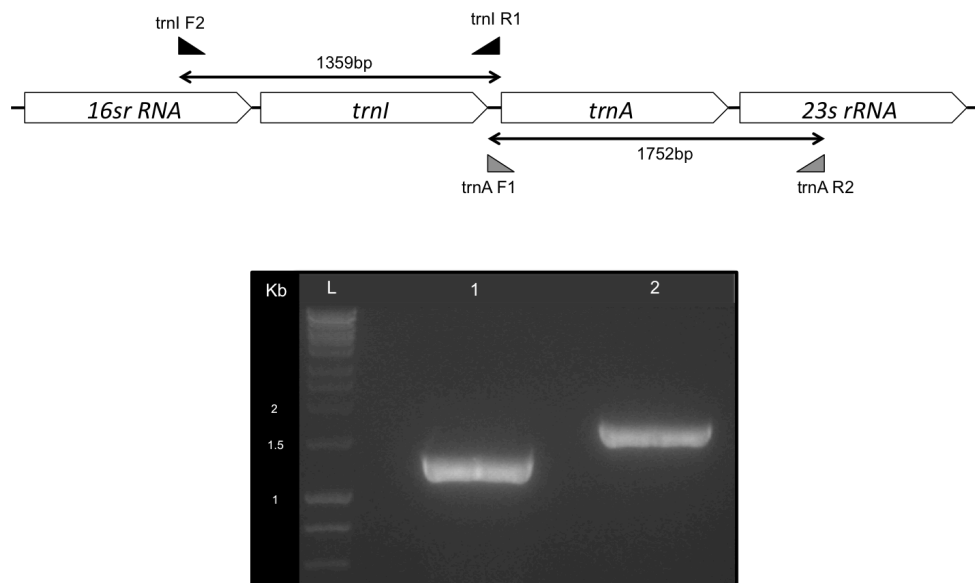
Fig 3.7 Generation of homologous recombination arms. Above, amplification of
16srRNA/trnI (trnI) and *trnA/23srRNA* (trnA) gene regions with primers(triangles,
direction of which indicates direction). Below, PCR products run on a agarose gel.
Lane 1, trnI amplicon; Lane2, trnA amplicon; L, 1kb DNA ladder; kb, Kilobase.

PCR amplicons were ligated into the pGEM®T-Easy vector to generate vectors pGEM®T-easy + trnI amplicon (pG-I) and pGEM®T-easy + trnA amplicon (pG-A), which were then transformed into competent *E.coli* cells. Diagnosis of successful amplification, ligation, and transformation was demonstrated by double digesting aliquots of pG-I and pG-A extractions, with SfaAI/SmaI and EcoRV/PacI respectively, and running on an agarose gel (Fig 3.8).
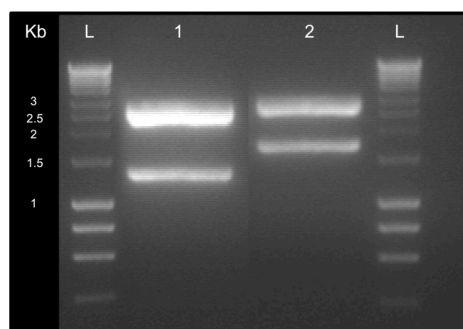
Fig 3.8 Diagnostic digestion of pG-I and pG-A. Lane 1, pG-I; Lane 2, pG-A; L, 1kb Ladder; kb, Kilobase. Gel confirm the expected sizes of the vector backbone (3015 bp), the trnI (1359 bp) and trnA (1752 bp) fragments.

pG-I and pG-A were sequenced between T3 and T7 primers (insertion site), and to confirm they contained no point mutations, were aligned with their corresponding regions in the apogee chloroplast genome that I had previously sequenced. Vectors pG-I and pG-A were double digested with SfaAI/SmaI and EcoRV/PacI respectively, to separate the trnI and trnA fragments which were extracted from the gel and purified. pRRes14m vector was linearised with a SfaAI/SmaI double digest, aliquots run on a gel, and linearised vectors extracted and purified. The two flanking arms were sequentially ligated into pRRes14m to give the plasmid pRRes14mF2 (Fig 3.9.b), which was validated by diagnostic restriction endonuclease digestion.
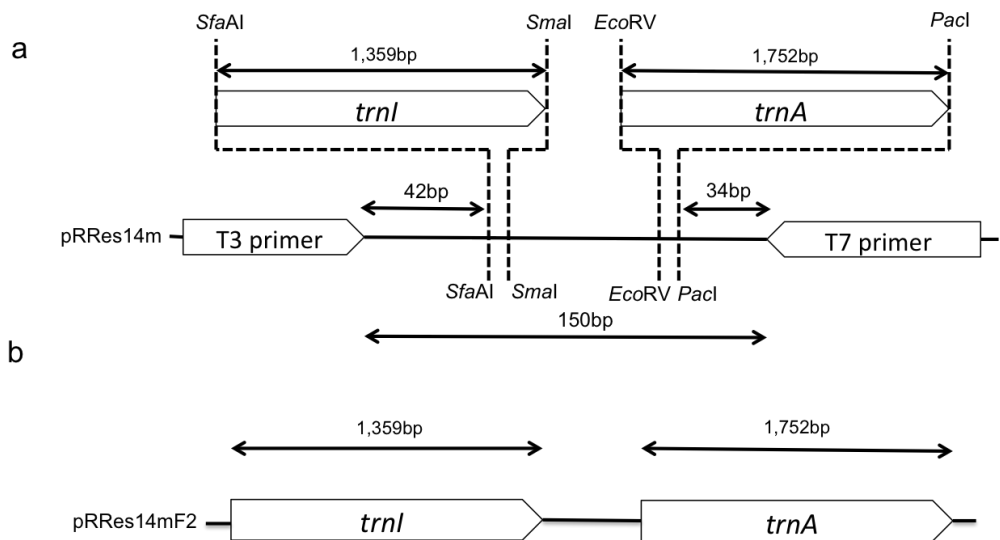
Fig 3.9 Insertion of flanking arms into pRRes14m (a) to give pRRes14mF2 (b).
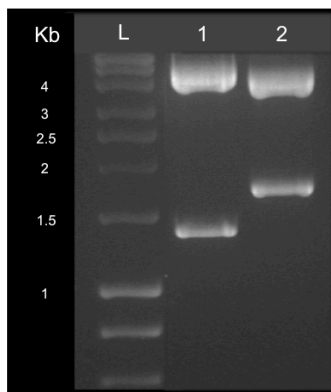
Fig 3.10 Diagnostic digestion of pRRes14mF2 Lane 1, pRRes14mF2 digested with *Sfa*A1/*Sma*1; Lane 2, pRRes14mF2 digested with *Eco*RV/*Pac*1; L, 1Kb ladder; kb, kilobase.

3.3.2 **Construction of pRRes14mF2-T7g10, pRRes14mF2-TPrrn, pRRes14mF2-WPrrn**

Once the integrity of pRRes14mF2 was confirmed by sequencing, I then ligated the synthesised transformation cassette (TCas1, Fig 3.3) between the trnI and trnA flanking arms of pRRes14mF2. The plasmid containing the TCas1 (pUC57-TCas1) was double digested with *Sma*I/*Eco*RV to release TCas1 from the vector. This was confirmed by gel electrophoresis (Fig 3.11).
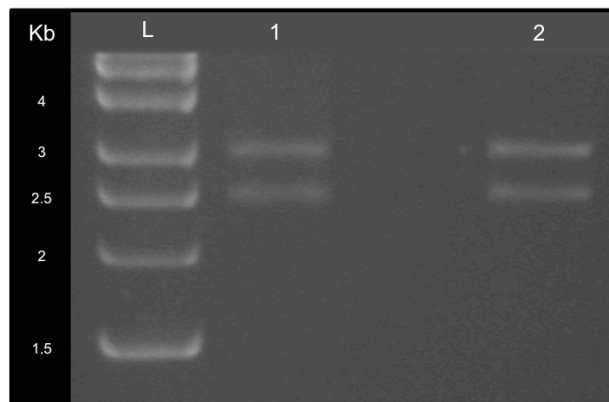
Fig 3.11 Digestion of pUC57-Tcas1 with *Sma*I and *Eco*RV. Lane 1 and 2 both contain digested pUC57-Tcas1. L, 1kb Ladder; kb, Kilobase.
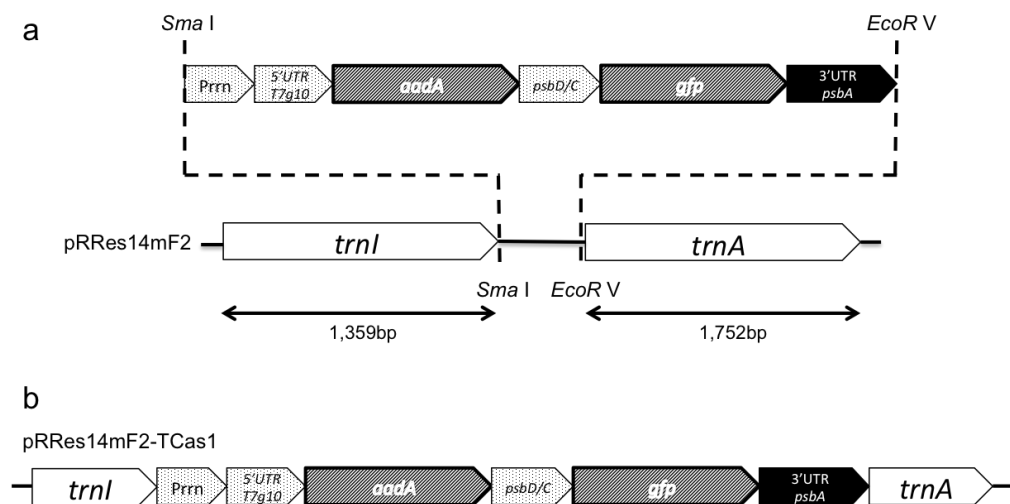
Fig 3.12 Formation of pRRes14mF2-Tcas1. a, Ligation of TCas1 into pRRes14mF2 to give b, pRRes14mF2-TCas1.

A successful ligation of TCas1 into pRRes14mF2 (Fig 3.12) would present two bands of 6734bp and 1675 bp. However, the diagnostic digest (Fig 3.13) showed that the ligation was unsuccessful. The ligation of TCas1 into pRRes14mF2 was repeated a number of times, varying the ratio of insert (TCas1) to vector (pRRes14mF2). Subsequent diagnostic digests of ligation products showed that ligations continued to be unsuccessful. Furthermore, sequencing results using T3 and T7 primers (flanking the MCS) indicated that sections of bacterial genes had been inserted into the MCS.
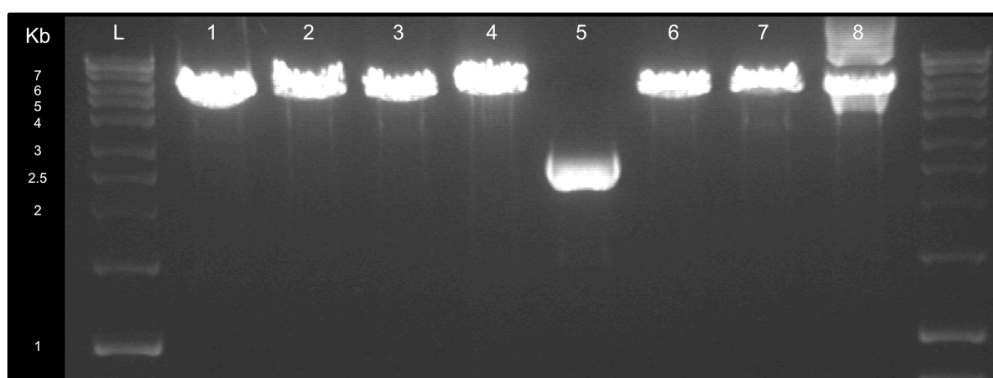
Fig 3.13 Digestion of ligation products from the ligation of Tcas1 into pRRes14mF2. Lanes 1-8 all contain ligation products from ligation reactions at varying ratios of insert:vector. L, 1kb ladder; kb, kilobase.

All the components of the transformation cassette, except the wheat Prrn, have been previously cloned into a number of transformation vectors without any reported difficulties. For this reason, I performed a series of diagnostic cloning experiments to diagnose and confirm that the wheat Prrn is the cause of cloning failure.

I digested pUC57-TCas1 with *Zra*1/*Eco*RV and *Zra*1/*Sna*B1 in two separate digestions, to give TCas1.2 and TCas1.3, and cloned both into the pRRes14mF2 linearised with *Sma*1/*Eco*RV (Fig 3.14). Both ligations were successful, indicating that the problem with cloning of the whole TCas1 sequence is occurring in sequences upstream of the *Zra*1 restriction site, which is the wheat Prrn. The successful clone of TCas1.3 into pRRes14mF2 was retained, and labelled pRRes14mF2-T7g10 (Fig 3.14c). The vector pRRes14mF2-T7g10.2 was generated by cloning TCas1.3 into pRRes14mF2 linearised with *Zra*1/*Eco*RV (Fig 3.15).
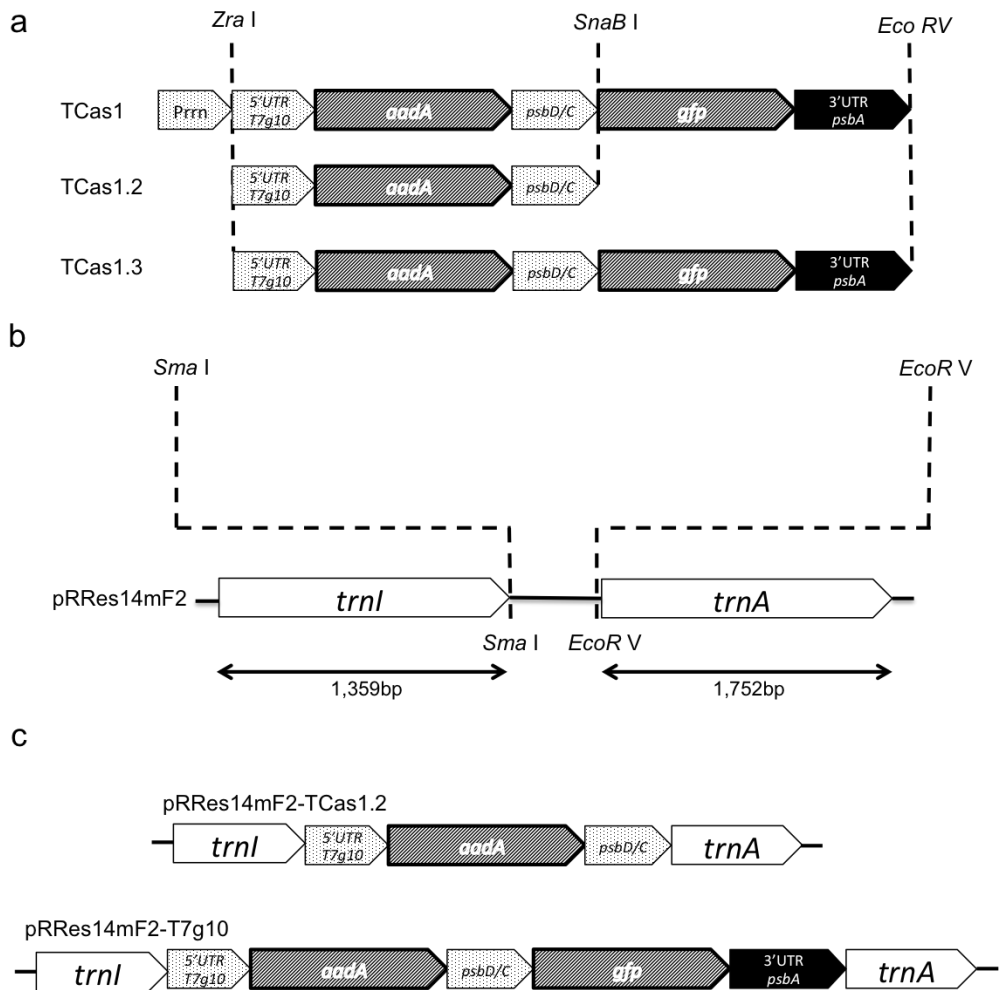
Fig 3.14 Formation of pRRes14mF2-T7g10 and pRRes14mF2-TCas1.2. a, Double digestion of Tcas1 with *Zra*1/*Eco*RV, and *Zra*1/*Sna*B1 to give Tcas1.2 and Tcas1.3; b, Double digestion of pRRes14mF2 with *Sma*1/*Eco*RV to create a linearised pRRes14mF2; c, Ligation of linearised pRRes14mF2 with TCas1.2 and Tcas1.3 to give pRRes14mF2-TCas1.2 and pRRes14mF2-TCas1.3 (renamed pRRes14mF2-T7g10).
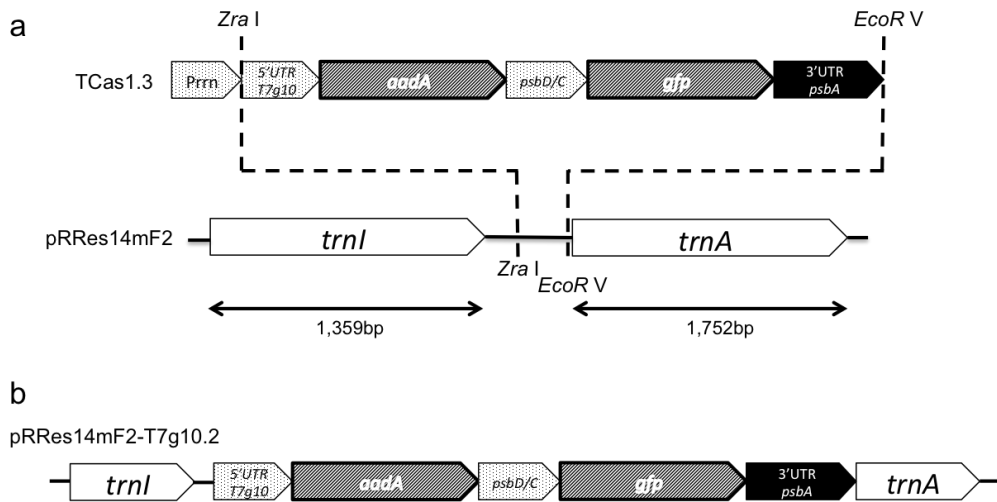
Fig 3.15 Tcas1.3 ligated into pRRes14mF2 linearised with *Zra*1/*Eco*RV (a), to give pRRes14mF2-T7g10.2.

Once I had established that the wheat Prrn was causing the problems with cloning TCas1 into pRRes14mF2, I PCR amplified a shorter portion of the wheat Prrn (WPrrn, 90,830-90,990 in the wheat chloroplast genome), with primers containing a *Sma*1 restriction endonuclease site at the 5' end, and a ribosomal binding site (RBS) + *Hind*III restriction site engineered into the 3' end. The tobacco Prrn (TPrrn) was PCR amplified from the pUM78 vector (Dr Anil Day, Manchester University, UK), with *Sma*1 restriction endonuclease site at the 5' end, and a ribosomal binding site (RBS) + *Hind*III restriction site engineered into the 3' end. This then allowed the insertion of both WPrrn and TPrrn into pRRes14mF2-T7g10.2 linearised with *Sma*1/*Hind*III, to create pRRes14mF2-TPrrn and pRRes14mF2-WPrrn (Fig 3.16)
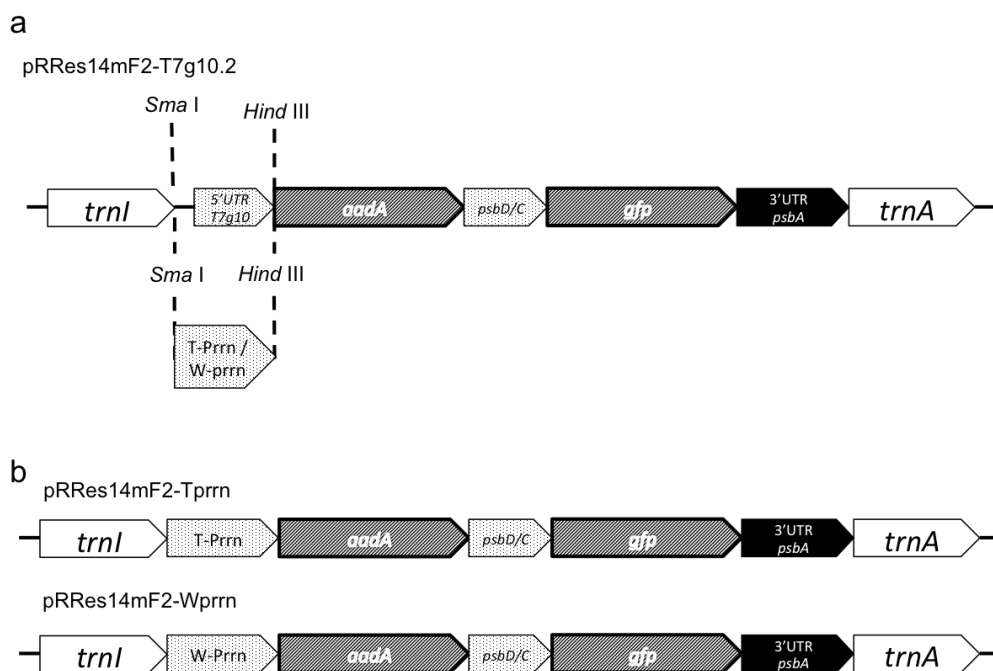
Fig 3.16 Formation of pRRes14mF2-TPrrn and pRRes14mF2-WPrrn. a, pRRes14mF2-T7g10.2 linearised with *Sma*I/*Hind*III is ligated with TPrrn and WPrrn to give b, pRRes14mF2-TPrrn and pRRes14mF2-WPrrn.

3.3.3 Construction of pPRV323Clox-T7g10, pPRV323Clox-TPrrn, and pPRV323Clox-WPrrn

To test that the chloroplast transformation cassettes were functional, they were inserted into the chloroplast transformation vector pPRV323CloxII (gift from Pal Maliga). pPRV323CloxII is a tobacco chloroplast transformation vector targeted at the tobacco *trnI-trnA* gene region. pPRC323CloxII was linearised with *Swa*I, which resulted in the release of the transformation cassette. TCas1.3 from pRRes14mF2-T7g10 was excised using a *Zra*I/*EcoR*V double digest, and cloned into the linearised pPRV323CloxII, to give pPRV323Clox-T7g10 (Fig 3.17). The transformation cassettes from pRRes14mF2-TPrrn and pRRes14mF2-WPrrn were excised with a *Sma*I/*EcoR*V double digest, and inserted into the linearised pPRV323CloxII, to give pPRV323Clox-TPrrn, and pPRV323Clox-WPrrn (Fig 3.18).

69
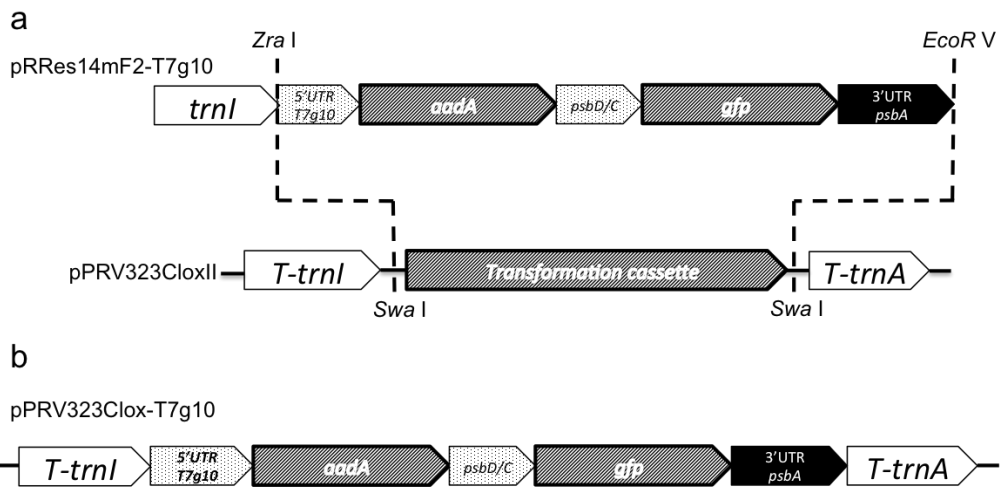
Fig 3.17 Production of pPRV323Clox-T7g10. a, TCas1.3 was digested out of pRRes14mF2-T7g10 with *Zra*I/*Eco*RV, and ligated into pPRV323CloxII linearised with *Swa*I to give b, pPRV323Clox-T7g10.
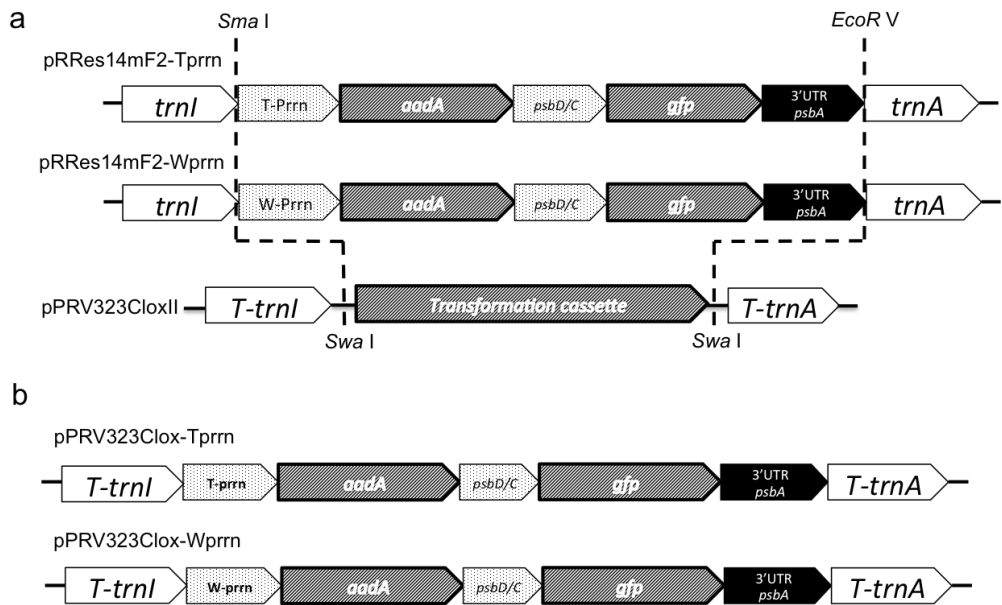
Fig 3.18 Production of pPRV123Clox-TPrrn and pPRV123Clox-WPrrn. The transformation cassettes from pRRes14F2-TPrrn and pRRes14mF2-WPrrn were excised with *Swa*1/*Eco*RV, ligated into pPRV323CloxII linearised with *Swa*I to give b, pPRV323Clox-TPrrn and pPRV323Clox-WPrrn.

70

3.3.4 **Validation of transformation vector function**

The transformation cassettes within pPRV323Clox-T7g10, pPRV323Clox-TPrrn and pPRV323Clox-WPrrn were all tested for function in the tobacco chloroplast transformation system (Svab et al., 1990). Following bombardment and subsequent selection with spectinomycin, resistant plants that regenerated from callus tissue on antibiotic selection (Fig 3.19) were examined for GFP expression using a confocal microscope (Fig 3.20, 3.21, 3.21).
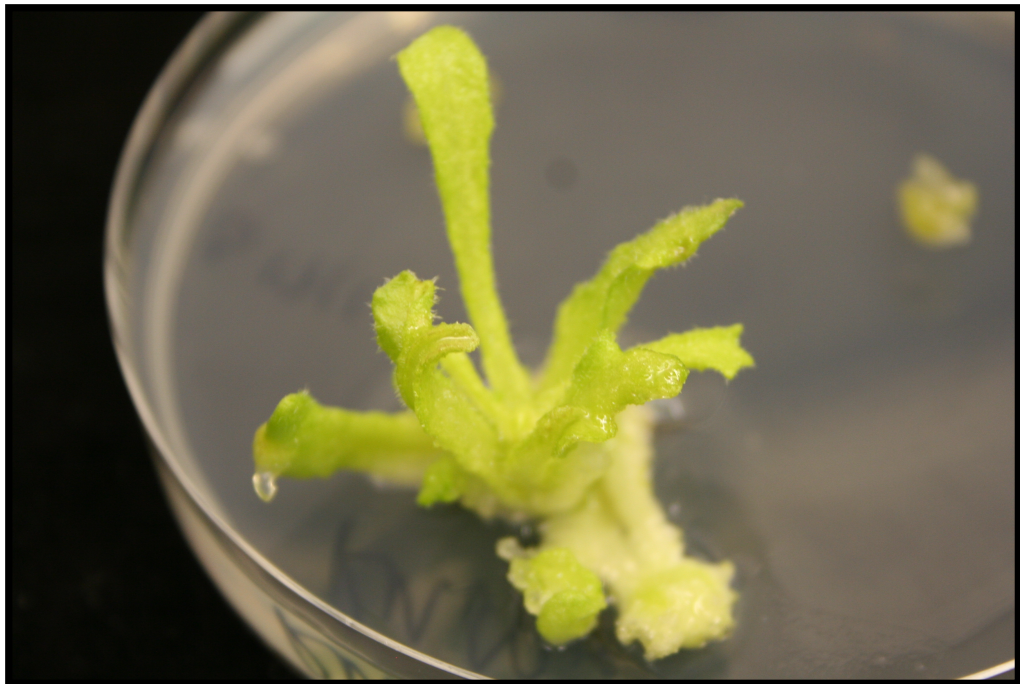
Fig 3.19 Resistant shoots developing from callus on RMOP media containing antibiotic selection.

Results show clear GFP expression of GFP in leaf cells of regenerated tobacco plants from pRRes14mF2-T7g10, pRRes14mF2-TPrrn, and pRRes14mF2-WPrrn bombardment studies. This indicates that all elements within the transformation cassettes are functioning as expected. Interestingly, it also demonstrates that the wheat Prrn is functional in a tobacco chloroplast.
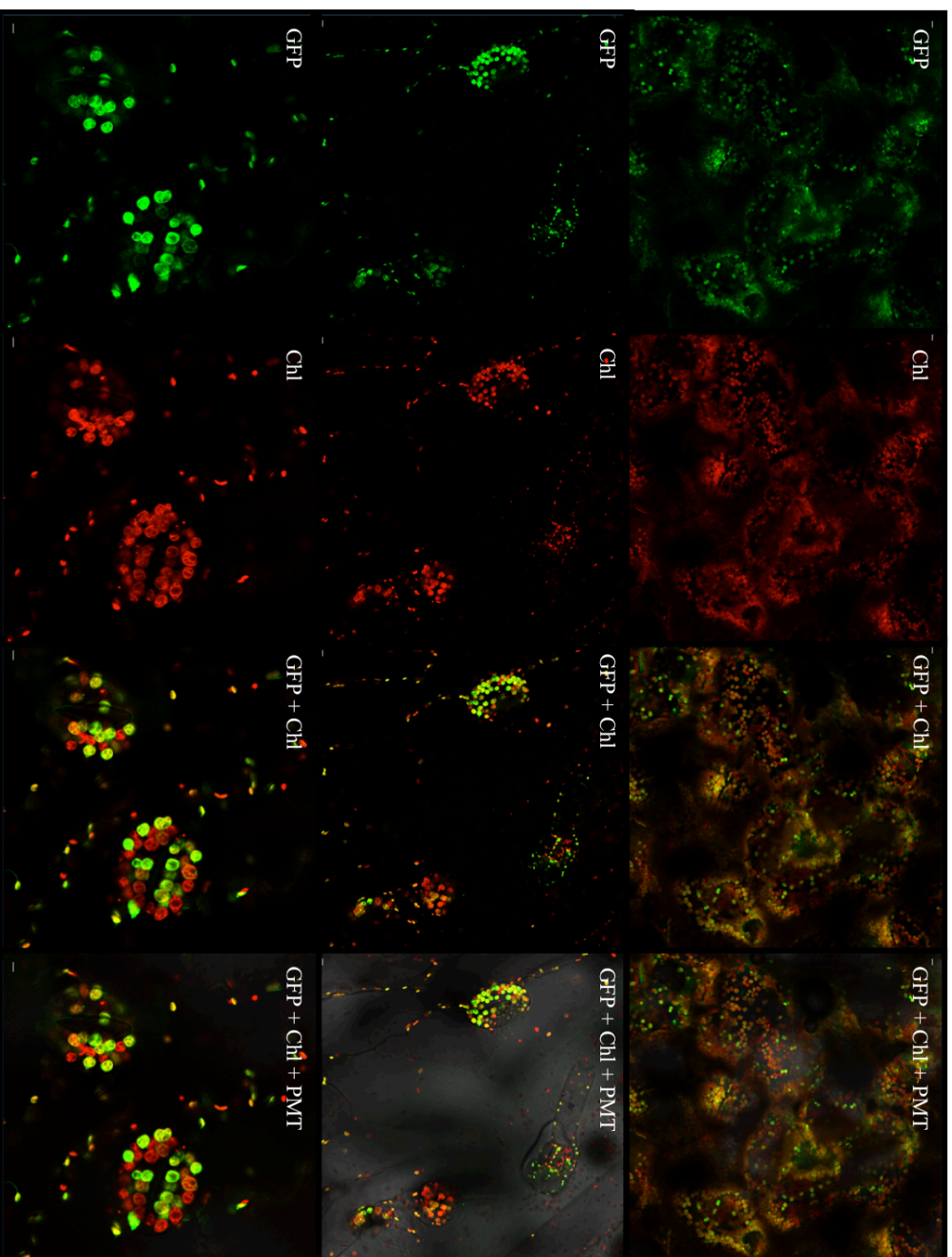
Fig 3.20 pPRV123Clox-T7g10 transplastomics. Regenerated shoots resistant to antibiotic selection were analysed using a confocal microscope. Small sections of shoot were mounted onto a glass slide, and imaged for gfp (GFP) and chlorophyll (chl) production. Each row of four images represents an independent transplastomic line. PMT (photomultiplier) indicates the background image. Scale bar = 5μm.

GFP Chl GFP + Chl GFP + Chl + PMT

GFP Chl GFP + Chl GFP + Chl + PMT

GFP Chl GFP + Chl GFP + Chl + PMT
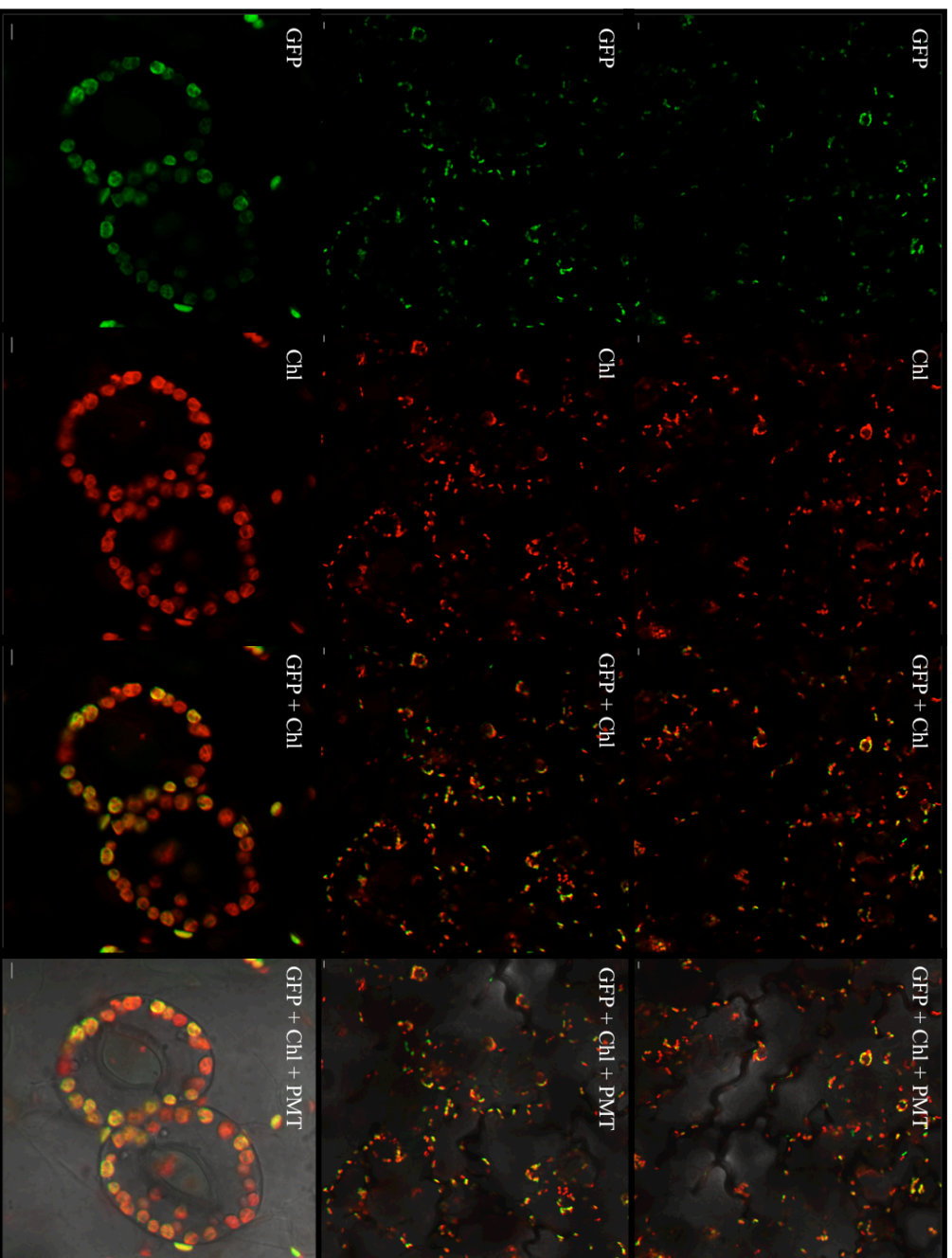
Fig 3.21 pPRV123Clox-TPrm transplastomics. Regenerated shoots resistant to antibiotic selection were analysed using a confocal microscope. Small sections of shoot were mounted onto a glass slide, and imaged for gfp (GFP) and chlorophyll (chl) production. Each row of four images represents an independent transplastomic line. PMT (photomultiplier) indicates the background image. Scale bar = 5µm.

Fig 3.22 pPRV123Clox-Wprn transplastomics. Regenerated shoots resistant to antibiotic selection were analysed using a confocal microscope. Small sections of shoot were mounted onto a glass slide, and imaged for gfp (GFP) and chlorophyll (chl) production. Each row of four images represents an independent transplastomic line. PMT (photomultiplier) indicates the background image. Scale bar = 5µm.

3.4   Discussion

The construction of a chloroplast transformation cassette requires careful consideration. Given that the current knowledge of chloroplast transformation in the monocot species is limited, the components in the wheat chloroplast transformation cassette were chosen based on their demonstrated functionality within plastids.

The *trnI-trnA* gene regions of the chloroplast genome have been the most frequently targeted in tobacco chloroplast transformation, and are the regions also used to transform rice plastids (Khan and Maliga, 1999, Lee et al., 2006). Given the frequency of successful use of this target region in transformation studies, I decided to use the wheat *trnI-trnA* gene region for the chloroplast transformation in wheat. The rational was the same for the choices of promoters driving the transgenes, spacer sequence separating the transgenes, and the 3' UTR for transcript stability. The 5'UTR of the T7g10 bacterial gene, which contains a ribosomal binding site at its 3' end, has been demonstrated to be a strong promoter in tobacco chloroplast transformation studies. The expression of transgenes under the control of this promoter have been shown to constitute high levels of total leaf protein, and in some cases, the high protein production has resulted in cytotoxicity and sterility. The 16s rRNA gene is a plastid gene, and its promoter, the *Prrn*, has also been successfully utilised as a promoter in chloroplast transformation vectors (Day and Goldschmidt-Clermont, 2011). However, because the *Prrn* lacks a ribosomal binding site (RBS), a plastid RBS such as that in the *rbcL* 5'UTR, must be engineered at the 3' end. Alternatively, the *Prrn* can be fused to the T7g10 promoter as it has a plastid functional RBS, and has been demonstrated to consistently produce relatively large amount of exogenous protein. Hence my choice of promoter was the wheat *Prrn* (WPrrn) fused with the T7g10 5'UTR. To date, the WPrrn has not been used in any published chloroplast transformation study. Choosing to have the transgenes driven by this promoter meant that both the selection (*aadA*) and reporter (*gfp*) genes in the cassette would be transcribed into a single transcript. It was therefore necessary to have a spacer gene inserted in between the *aadA* and *gfp*.   The *psbC/D* intergenic region has been demonstrated to be successful at providing separate translation of multiple genes from a single transcript (Bohmert-Tatarev et al., 2011). Due to its success in this synthetic construct, I chose the *psbC/D* gene region to provide separate translation of *aadA* and *gfp*. The final component to consider was the 3'UTR, which is necessary to ensure

transcript stability. The 3'UTR of the *psbA* tobacco chloroplast gene has been shown to provide transcript stability in tobacco and in monocot species, hence the *psbA* 3'UTR was chosen to provide mRNA stability to our wheat chloroplast transformation construct. Due to the relatively long length of the *psbA* 3' UTR (approximately 400bp) and the well validated status of the tobacco *psbA* gene, I chose to use this version as opposed to the wheat *psbA*, so as not to induce unwanted homologous recombination events.

The transformation cassette, TCas1, was synthesised by GenScript, and I initially attempted to insert TCas1 into pRRes14mF2, a chloroplast transformation vector containing the wheat *trnI-trnA* homologous recombination flanking arms. As detailed within the results of this chapter, I was unable to achieve the insertion of TCas1 into pRRes14mF2. During the troubleshooting process, I managed to insert a truncated (Fig 3.23) TCas1 (TCas1-T7g10), the cassette without the wheat Prrn (WPrrn), into pRRes14mF2 to give pRRes14mF2-T7g10. It was clear from this result, that there was a genetic component within the WPrrn that was preventing successful cloning of TCas1 into pRRes14mF2. Cloning a truncated WPrrn (90,830-90,986 in the wheat chloroplast genome) into pRRes14mF2-T7g10.2 provided further indication to the location of the problematic genetic sequence, which was within the final 69bp of the full length WPrrn sequence.
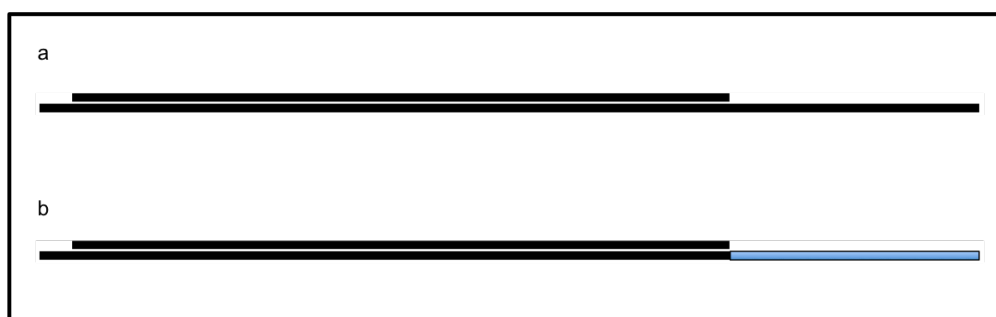
Fig 3.23 showing, a, the alignment of full length wheat Prrn (long black line, 90,830-90,986bp of wheat chloroplast genome) with the truncated wheat Prrn (short black line, 90,830-90,986 of wheat chloroplast genome); and b, the 3' excluded sequence (blue, 69bp) was analysed for secondary structures.

When the 69 bp excluded WPrrn sequence was entered into a program that predicted DNA secondary structure formation, results indicated that at least two hairpin

structures are likely to form (Fig 3.24). Given that the 3' end of the 5' UTR is important for transcriptional control, it is possible that these hairpin structures are involved in interactions with plastid encoded ribosomes during translation. These hairpin structures may explain the issues experienced with the cloning of TCas1 into pRRes14mF2. Following ligation of TCas1 into linearised pRRES14mF2, the successful clones are then amplified by incubation in E.coli cells, where the replication of plasmids occurs due to bi-directional DNA polymerase activity from the origin of replication. The hairpins that formed in the full WPrrn sequence may have inhibited the DNA polymerase during replication, and resulted in failed or reduced amplification. When the TCas1 was delivered from Genscript, they mentioned that they had difficulty amplifying the plasmid in standard E.coli cells due to its instability, and instead had to supply the plasmid in CopyCutter™ EPI400™ E. coli. CopyCutter™ EPI400™ E. coli cells significantly lower the copy number of a wide variety of common vectors so that unstable DNA sequences may be cloned more readily. DNA that is unstable at high-copy number often code for proteins that inhibit cell growth, or contains AT- and GC-rich sequences, or as appears likely in my cloning difficulties here, sequences with strong secondary structure.
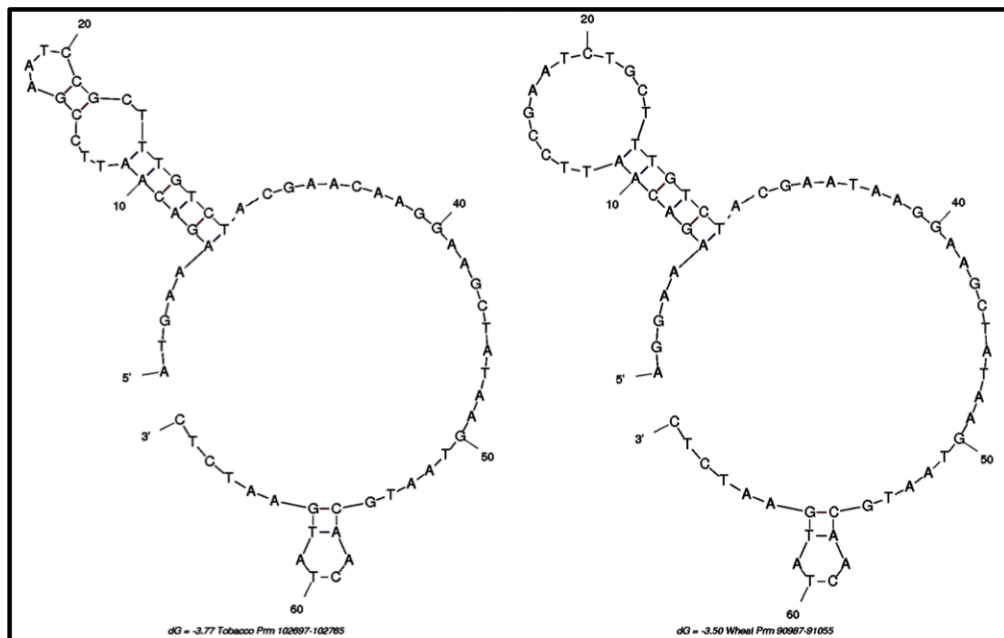
Fig 3.24 showing the secondary structure formations that occur in the 3' sequences of the tobacco *Prrn* (left), and the wheat *Prrn* (right).

77

Removal of secondary structures enabled successful cloning, and also allowed the transformation cassettes to be tested in the tobacco chloroplast transformation system. As can be seen from Fig 3.20, 3.21, 3.22, all transformation cassettes are functional, indicating complete promoter function, and successful transcription and translation of exogenous genes. Therefore, if the chloroplast transformation of wheat is not attained during the time course of this project, it would not be due to a poorly constructed transformation vector. Interestingly, the wheat *Prrn* is shown to be functional within the chloroplasts of tobacco.

4 Tissue Culture

4.1 Introduction

Plant regeneration from somatic cells is possible through either organogenesis or somatic embryogenesis, with both pathways leading to the production of fertile regenerants. It is widely accepted that the somatic embryogenesis pathway is more suitable for the production of transgenic crops, as it presents a developmental pattern resembling that of zygotic embryogenesis in plants (Ammirato, 1983). For cereals, the road leading to somatic tissues capable of embryogenic callus formation has been a long one. In 1969, the first report emerged of regeneration of whole *Triticum aestivum* plants by in vitro tissue culture of stem tissue (Shimada et al., 1969). However it was not until almost a decade later, that regeneration from immature embryo derived callus was demonstrated (Chin and Scott, 1977). Prior to the 1980s, regeneration of cereal and grass species had been conducted from shoot meristems (Thomas et al., 1979, Vasil et al., 1979, Vasil and Vasil, 1980). These meristems are multicellular in origin, and therefore any transgenic plant obtained from this source would likely be chimeric. The advantages of regeneration from single cells or somatic embryoids, for the production of fertile nuclear genetic transformation of wheat make the possibility of chimerism much lower.. It was not until the beginning of the 1980s that the creation of transgenic wheat appeared optimistic. The publishing of scutellum-derived embryoid formation resembling zygotic embryoids in Guinea grass (Lu and Vasil, 1982), were quickly followed by reports of regeneration from immature embryos (Sears and Deckard, 1982), and immature inflorescences (Oziasakins and Vasil, 1982) of *Triticum aestivum*, with the anatomy of somatic embryogenesis from embryo derived callus being well characterized (Magnusson and Bornman, 1985).

It is clear from the literature that the immature embryo is the favoured and most consistent choice of explant for the regeneration of *Triticum aestivum* (Reviewed by Jones (2005)). However other sources of tissue have been explored for the tissue culture and regeneration of *Triticum aestivum*. As mentioned above, the immature inflorescence is another explant that provides a robust method of plant regeneration, although does not show to work for all wheat varieties (Oziasakins and Vasil, 1982). A third previously used explant are segments of young leaves. Cells from a small area

close to the leaf base, which were in close proximity to the original leaf meristem, were shown to have retained a capacity for totipotency and form callus (Wernicke and Milkovits, 1984) in 6 different *Triticum* genotypes. Anther and microspores have also demonstrated to be capable of regeneration. The culture of immature anthers containing haploid microspores, under conducive conditions, diverts the normal development of the male gametophyte to a sporophytic pathway, resulting in embryogenic callus formation (Dunwell, 1985, Wei, 1982). Theoretically, plants regenerated via this method are genetically haploid, however, the chromosome complement may double spontaneously or after the application of spindle assembly inhibitors such as colchicine to produce fertile diploid plants. Therefore, anther culture provides a rapid method for the production of homozygous (double-haploid) lines, which is of interest to producers of breeding lines. The regeneration of plants from microspores separated from anthers prior to tissue culture has also been demonstrated in *Triticum aestivum* (Datta and Wenzel, 1987).

Out of all the explant tissues capable of regenerating into new plants in *Triticum aestivum*, they all share a common factor; they do not contain an abundant number of chloroplasts. This is problematic if the desired end product is a chloroplast transformed wheat plant. In the tobacco chloroplast transformation system, the most successful chloroplast transformation protocol (Svab et al., 1990, Svab and Maliga, 1993), targets young tobacco leaves. This explant is highly regenerable, and contains an abundant number of chloroplasts (approximately 100 chloroplasts per cell), of 5-8μm in size (Fig 4.1). There is simply no equivalent tissue in wheat for the purpose of chloroplast transformation.
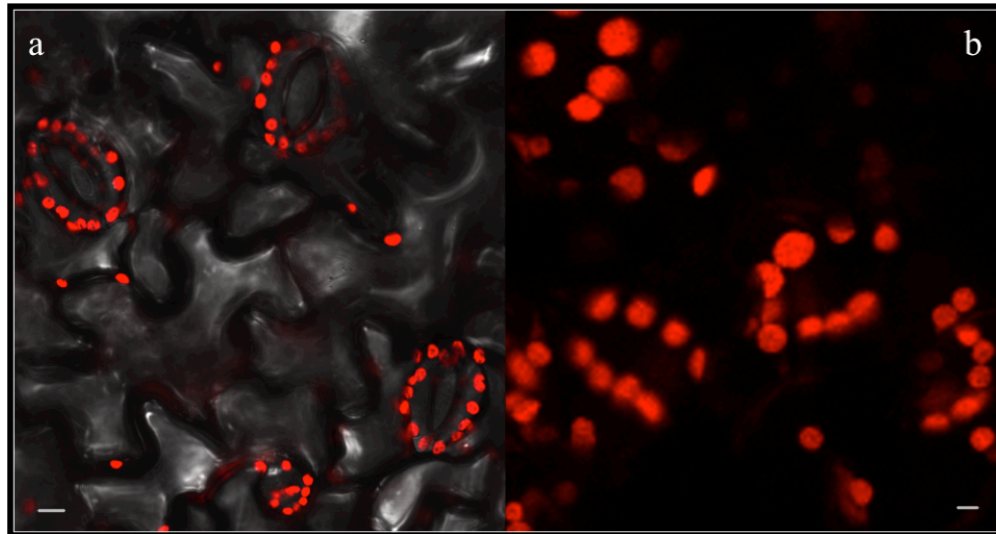
Fig 4.1 Chloroplasts in young tobacco leaves. Red indicates chlorophyll auto-fluorescence. Scale bars represent 5µm.

4.1.1 **Aims**

- Identify immature wheat tissue that contained chloroplasts.
- Develop and optimize tissue culture procedures to regenerate fertile new wheat plants via organogenesis from the identified tissues.

4.2 Material and Methods

4.2.1 Media Composition

Media	Conc	Components	pH
L7	2x	200mL Macrosalts (10x) 2mLMicrosalts (1000x) 20mL Fe Na EDTA 400mg *Myo*-Inositol 40mL 3AA (25x) 2.5gL Asparagine 60g Maltose (for L7 3%)	5.7 (KOH)
R	2x	200mL Macrosalts (10x) 2mLMicrosalts (1000x) 20mL Fe Na EDTA 10mL L Vitamins/Inositol (200x) 60g Maltose	5.7 (KOH)
MS	2x	8.8g MS Basal Medium 10mL Fe Na EDTA 0.2g *Myo*-Inositol 2mL Thiamine (1mg/mL) 1g MES 60g sucrose	5.8 (KOH)
M	2x	200mL Macrosalts (10x) 2mLMicrosalts (1000x) 20mL Fe Na EDTA 2ml MS Vitamins (1000x) 200mg *Myo*-Inositol 40mL 3AA (25x), 2.5gL Asparagine 180g sucrose (for M 9%)	5.7 (KOH)
WLS	2x	200mL Macrosalts (10x), 2mLMicrosalts (1000x), 20mL Fe Na EDTA, 2ml MS Vitamins (1000x), 200mg Ascorbic acid*, 200mg Caserin hydrolysate, 3.8g MES, 1g glutamine, 1.5g $MgCl_2$, 1mg 2,4-D*, 4.4mg picloram*, 1.2mg $AgNO_3$*, 2.5gL Asparagine, 80g maltose	5.8 (KOH)
To attain 1x, mix with equal amounts of (2x) Agargel^{TM} (10g/L)			

Table 4.1 Components of L7, R, MS, M and WLS basal media (2x). Conc, concentration; *, do not autoclave.

	Component	Amount (g/L)
Macrosalts (10x)	Ammonium nitrate (NH_4NO_3)	2.5
	Potassium nitrate (KNO_3)	15
	Potassium phosphate monobasic (KH_2PO_4)	2
	Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	3.5
	Calcium chloride dihydrate ($CaCl_2.2H_2O$)	4.5
Microsalts (1000x)	Manganese sulfate monohydrate ($MnSO_4$)	15
	Boric Acid (H_3BO_3)	5
	Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	7.5
	Potassium iodide (KI)	0.75
	Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0.25
	Copper sulfate pentahydrate ($CuSO_4.5H_2O$)	0.025
	Cobalt chloride hexahydrate ($CoCl_2.6H_2O$)	0.025
L Vitamins/ Inositol (200x)	Inositol	40
	Thiamine HCL	2
	Pyridoxine	0.2
	Nicotinic acid	0.2
	Ca-Pantoethane	0.2
	Ascorbic acid	0.2
3AA (25x)	L-Glutamine	18.75
	L-Proline	3.75
	L-Asparagine	2.5

Table 4.2 Components of macrosalts, microsalts, L Vits/inositol, and 3AA.

The tables in this section detail the composition of media used in Chapter 4. MS Basal Medium was obtained from Sigma-Aldrich (3050 Spruce St, St. Louis, MO 63103, USA).

	Component	Amount (mg/L)
MS Basal Medium	Ammonium nitrate (NH_4NO_3)	1650.00
	Boric Acid (H_3BO_3)	6.2
	Calcium chloride ($CaCl_2$)	332.2
	Cobalt chloride hexahydrate ($CoCl_2.6H_2O$)	0.025
	Cupric sulfate pentahydrate ($CuSO_4.5H_2O$)	0.025
	Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$)	27.8
	Magnesium sulfate (anhydrous) ($MgSO_4$)	180.7
	Manganese sulfate monohydrate ($MnSO_4$)	16.9
	Potassium iodide (KI)	0.83
	Potassium nitrate (KNO_3)	1900.0
	Potassium phosphate monobasic (KH_2PO_4)	170.0
	Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0.25
	Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	8.6
	Disodium EDTA dihydrate	37.26
	Glycine	2.00
	Myo-Inositol	100
	Nicotinic acid	0.5
	Pyridoxine hydrochloride	0.5
	Thiamine HCL	0.1

Table 4.3 Components of MS Basal medium

4.2.2 Tissue Culture

4.2.2.1 Immature embryo callus

Ears were harvested from wheat plants 15-21 days post anthesis, and immature caryopses were surface sterilised with 70% (v/v) ethanol for 5 min and 10% (v/v) sodium hypochlorite for 15 min followed by three washes with sterile distilled water. The immature embryos, translucent at this stage of development, were aseptically removed from early-medium milk stage caryopses, and the scutella isolated by removal of the embryonic axis. Scutella were placed cut side down in the centre of a 9cm petri dish containing immature embryo callus induction medium, with 30 scutella per plate. Explants were cultured in the dark at 26°C for 21-27 days to produce embryogenic callus.

4.2.2.2 Immature inflorescence

Apogee and Cadenza tillers were harvested 28-32 days and 40-44 days (respectively) post sowing (Fig 4.2). The top 3 cm of stem was removed (and the bottom 5 cm in cadenza), and the remainder surface sterilised with 100%(v/v) ethanol for 30 seconds, and 10% (v/v) sodium hypochlorite for 10 mins, followed by three washes with sterile distilled water. The location of the immature inflorescence in the bottom 5 cm of the stalk was identified by running the blunt side of a no.11 scalpel along the length of the stalk. The inflorescence was felt as a bump. The immature bundles were dissected out by making incisions 1 cm either side of the inflorescence. Each leaf layer covering the inflorescence was rolled away until the immature inflorescence was revealed. Each inflorescence was cut into 1-2mm sections and placed on the required media.

4.2.2.3 Peduncle Node

Apogee and cadenza stems were harvested 28-32 days and 40-44 days (respectively) post sowing. The top 3 cm of stem was removed (and the bottom 5 cm in cadenza), and the remainder surface sterilised with 100%(v/v) ethanol for 30 seconds, and 10% (v/v) sodium hypochlorite for 10 mins, followed by three washes with sterile distilled water. The location of the immature inflorescence in the bottom 5 cm of the stalk was identified by running the blunt side of a no.11 scalpel along the length of the stalk. The inflorescence was felt as a bump. The immature bundles were dissected out by making incisions 1 cm either side of the inflorescence. Each covering of the inflorescence was rolled away until the immature inflorescence was revealed, with the

node attached to the base of the inflorescence. The node was separated from the inflorescence and cut into 2-3 cross sectional pieces. Each piece was placed on L7 callus induction media.
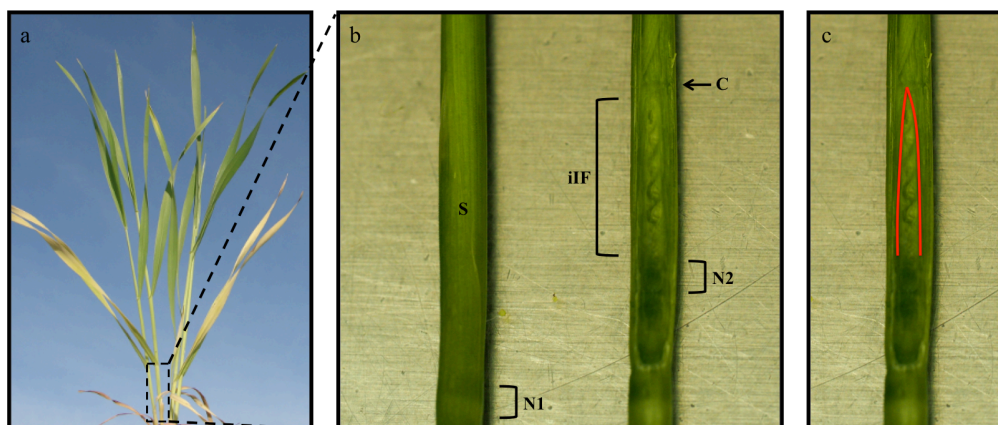
Fig 4.2 Locating the immature bundle. a, Apogee plants of 28-32 days post sowing are harvested; b, the immature inflorescence (iIF) is located as a bump in the lower 5cm of the stem (s), just above node 1 (N1). Once tiLS and siLS are removed, incisions are made above Node 2 (N2) and below the collar (C) to remove the iIF and piLS; c, location of the piLS.

4.2.2.1   Leaf Sheaths

Apogee and cadenza stalks were harvested 28-32 days and 40-44 days (respectively) post sowing. The top of each stem was removed, and the remainder surface sterilised with 70% (v/v) ethanol for 30 seconds, and 10% (v/v) sodium hypochlorite for 10 mins, followed by three washes with sterile distilled water. The location of the immature inflorescence in the bottom 5 cm of the stem was identified by running the blunt side of a no.11 scalpel along the length of the stalk. The inflorescence was felt as a bump. The immature bundles were dissected out by making incisions 1 cm either side of the inflorescence. Each covering of the inflorescence was rolled away until the primary leaf sheath remained. Removals of leaf sheath are made easier by separating from the node they are attached to. The primary leaf sheaths, (piLS, see Fig 4.3) the first sheath surrounding the immature inflorescence, were aseptically separated from the immature bundle, cut into 6-12 pieces, and placed adaxial side up (waxy side up) on callus induction media.
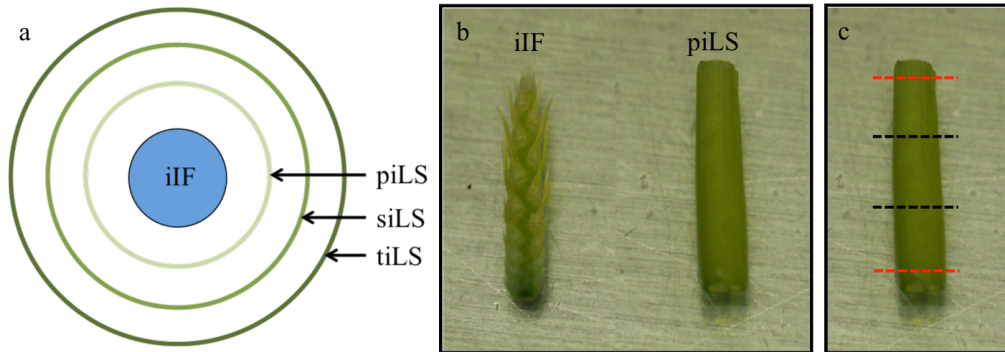
Fig 4.3 a, immature inflorescence (iIF), surrounded by primary, secondary, and tertiary leaf sheaths (piLS, siLS, tiLS respectively); b, an iIF and its piLS separated. c, tissue above the top red line and tissue below the bottom red line are disposed because they are the most recalcitrant. This also aids the ease at which a piLS can be rolled flat onto filter if they are to be biolistically bombarded (Fig 4.4). For tissue culture, LS are cut into equal sections (maximum 3 as indicated by black lines), and each section rolled out and cut into 3-4 pieces each.
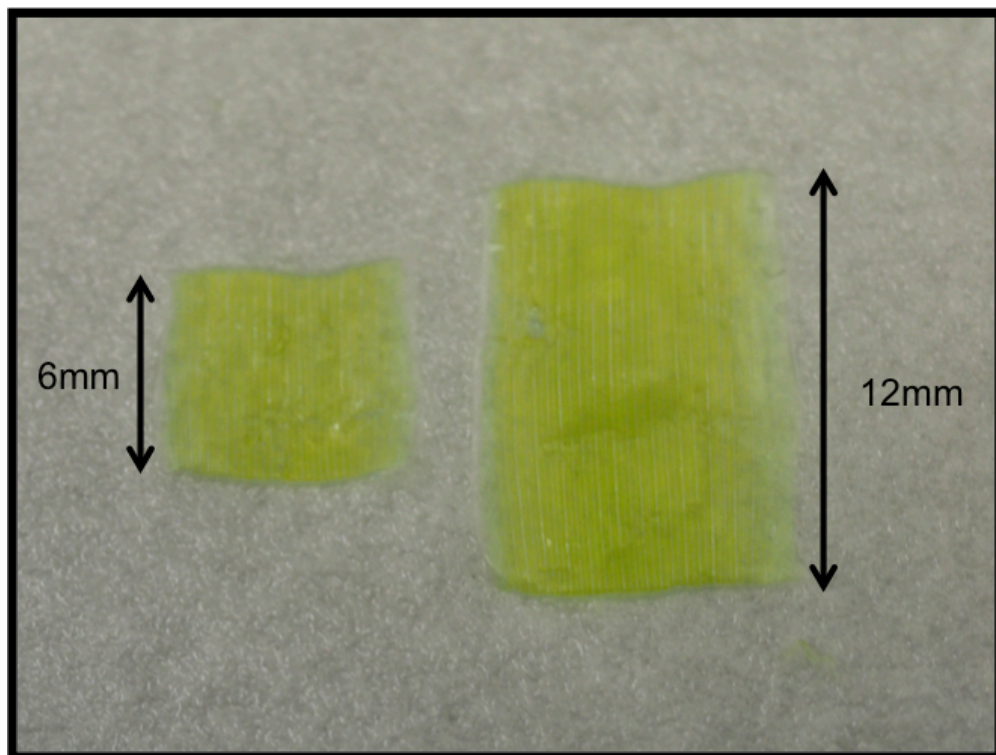
Fig 4.4 piLS of different sizes rolled flat onto filter paper, abaxial side up.

4.3   Results

I started with the assumption that the target tissue for transformation must possess metabolically active chloroplasts or pro-plastids. Unlike in tobacco and some other species, the obvious mature green tissues with chloroplasts in wheat cannot be induced to from regenerable callus. Thus I focused on immature tissues and on callus tissue.

4.3.1   Immature embryo-derived callus

The most commonly used wheat explant for nuclear transformation, the immature embryo, does not contain chloroplasts. Furthermore, any pro-plastids within the immature embryo appear to be smaller than 0.5μm in diameter (Fig 4.5) and the activity of enzymes to enable homologous recombination of the transformation cassette into the plastid genome would be very low. It is likely that the relative size of micro-projectile particles (typically 0.6μm in diameter) compared to the pro-plastid would result in irreparable damage to the pro-plastid, therefore preventing transformation. Callus derived from immature embryos are also absent of chloroplasts, however it has been reported that under certain conditions, callus cells derived from immature embryos in tissue culture can turn green. I investigated whether pro-plastids in callus derived from wheat immature embryos were larger in size than those in the immature embryos, and whether they may be more metabolically-active.

Immature embryos were callus induced, and between 21 and 30 days (when callus is most embryogenic), were moved into light (100-130μE) for incubation at 24°C. After 4 days exposure to light, chloroplasts can be observed in the embryogenic callus (Fig 4.6). These chloroplasts are approximately 1μm in size, and therefore the pro-plastid that form these chloroplasts are significantly larger and more active than those in the immature embryo (smaller than 0.5μm). Given the highly embryogenic nature of immature embryo callus, the presence of metabolically active non-green plastids of approximately 1μm exist within callus cells, and the previous use of callus as explant targets for chloroplast transformation in rice (Lee et al., 2006), I determined that targeting wheat immature embryo callus for the purpose of chloroplast transformation would be a sensible approach.
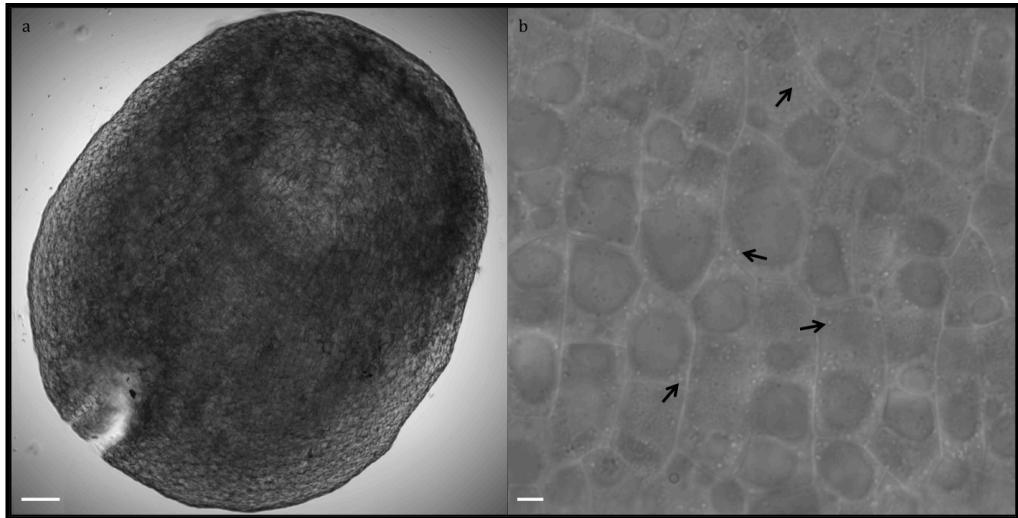
Fig 4.5 a, Immature Embryo. Scale bar represent 100µm. b, cells within the immature embryo. Black arrows point to circular structures that could be pro-plastids. Scale bar represents 5µm.
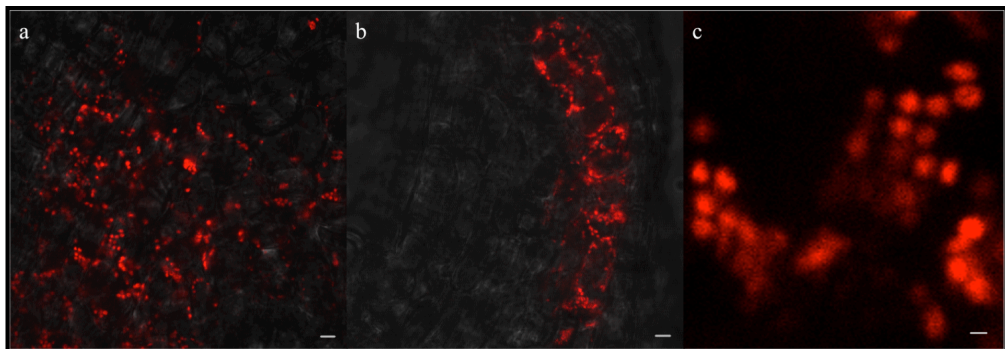
Fig 4.6 Chloroplasts in callus derived from immature embryo. Exposure of 21 day immature embryo callus tissue to light for 4 days (a) and 7 days (b) induces the activity of chloroplasts, indicated by chlorophyll auto-fluorescence (red). Scale bare represents 5µm (a, b) and 1µm (c).

4.3.2 **The immature inflorescence**

The immature inflorescence (iIF) was also considered as a suitable target because parts of this organ appears green and is an alternative explant for nuclear genetic transformation, albeit a much less commonly-used one.

To investigate the potential of iIF for chloroplast transformation, I analysed the callus induction and regeneration phases of immature inflorescence tissue culture, to investigate whether the embryogenic callus could be developed from green starting tissue.

The iIF (5-10mm in length) were isolated and cut into 1-2mm sections and cultured using standard callus induction media. The origin of the regenerable callus and the position of cells possessing chloroplasts were analyzed. It was found that the distribution of active chloroplasts was restricted to the rachis of the iIF, with no chloroplasts or chlorophyll expressing plastids being observed in the floret tissue (Fig 4.7 and Fig 4.9). However, the regenerable callus originated only from the floret regions and all regenerated plants came from this callus (Fig 4.8).
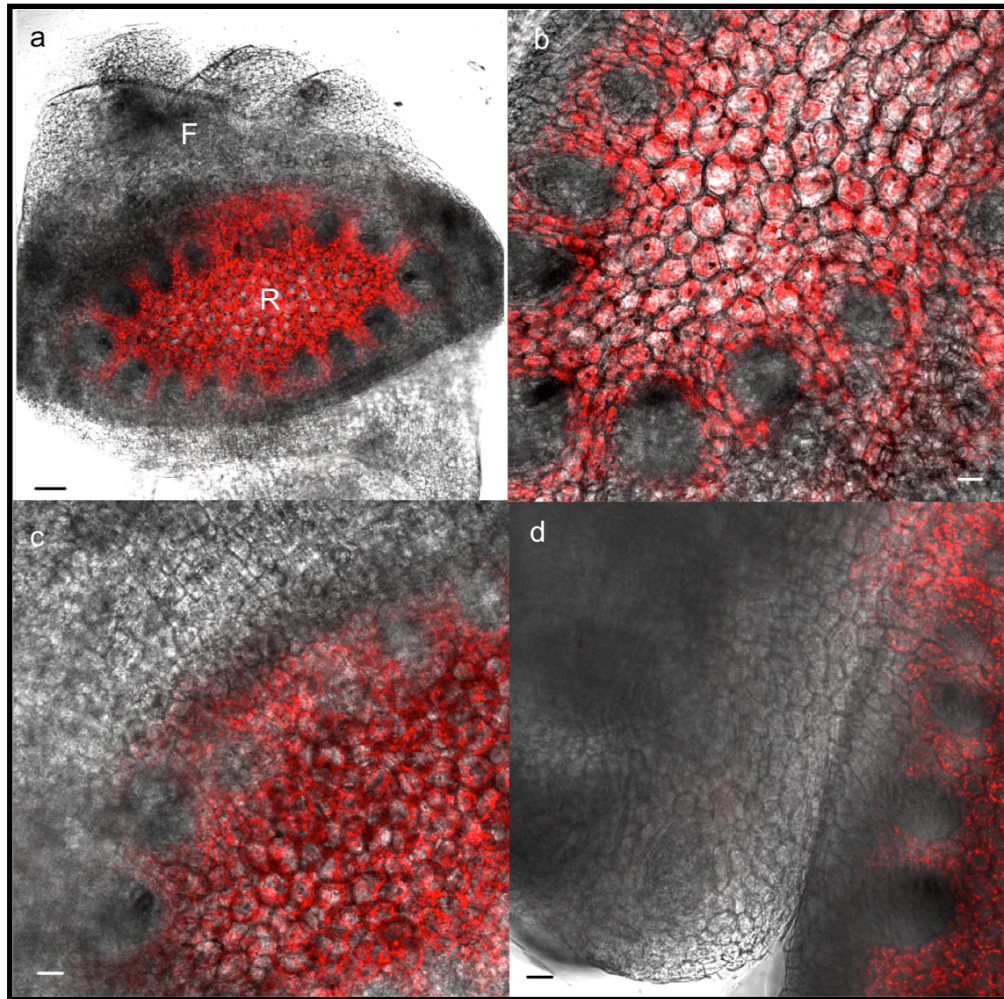
Fig 4.7 Chloroplast distribution within the immature inflorescence. a, horizontal cross section of the iIF showing floret (F) and rachis (R), with chloroplasts (red) clearly located only in the rachis regions. Close up images of the rachis (b), and both floret and rachis (c, d) further support the observation that the floret regions are absent of chloroplasts. Scale bars represent 50μm (a), 20μm (c) and 10μm (b, d).
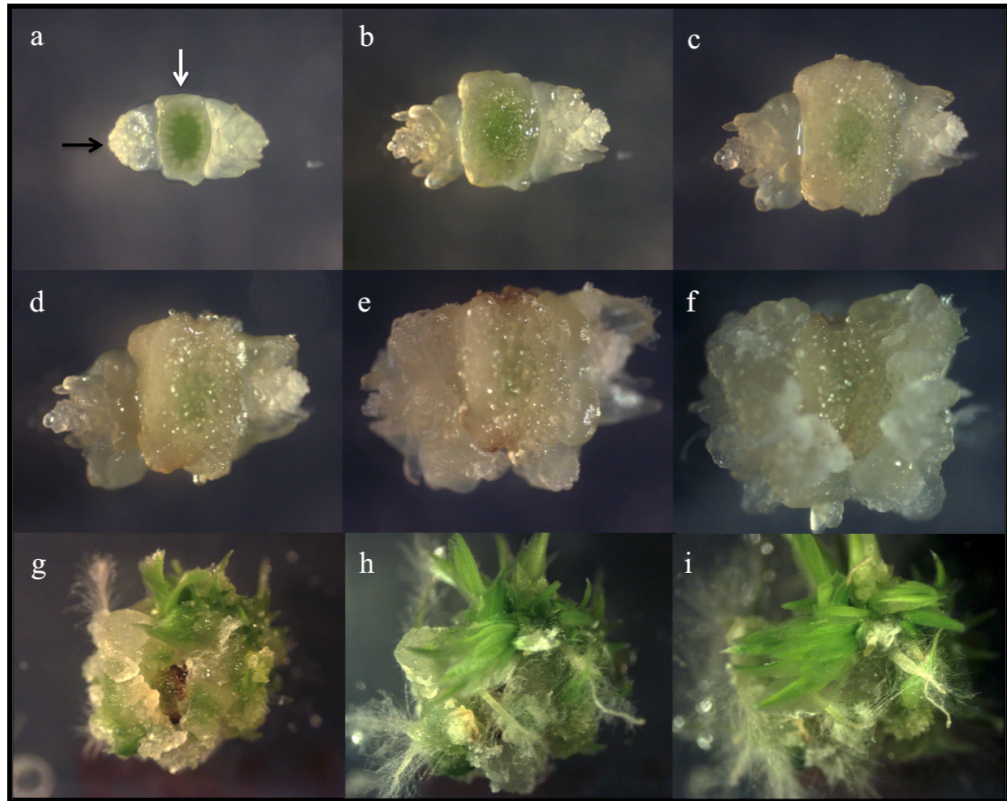
Fig 4.8 Tissue culture of an immature inflorescence (iIF) cross section (~ 4mm across in a). a-f, Callus induction of iIF piece showing callus formation occurring from floret tissue (black arrow) and rachis (white arrow). g-i, regeneration of callus tissue shows shoots forming from floret derived callus, while rachis derived callus degenerates (dark areas, see g).
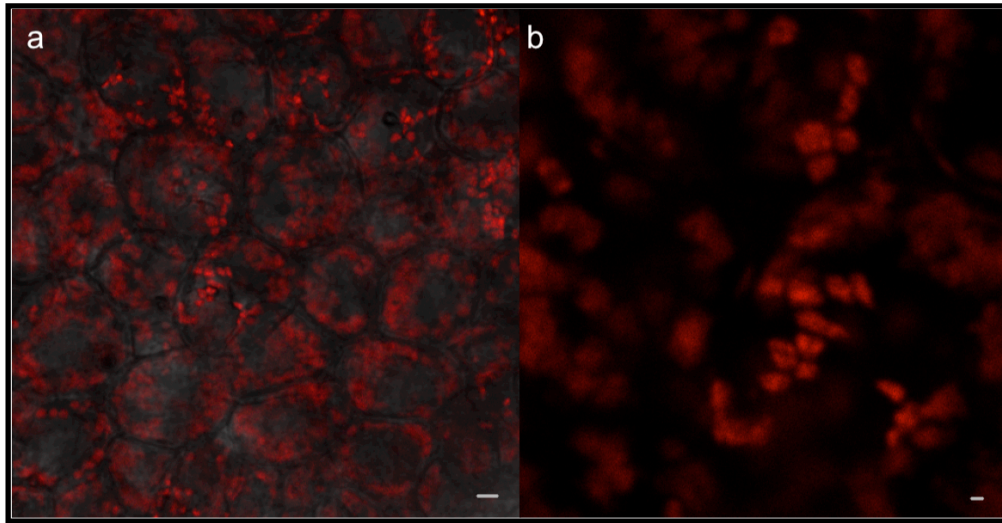
Fig 4.9 Chloroplasts within the rachis are abundant (a), and are approximately 1μm in size (b). Scale bars represent 5μm (a) and 1μm (b).

To test whether I could induce chloroplast development within these floret regions, I exposed iIFs to a period of 2 to 7 days exposure in light post extraction. After 4 days exposure, I found that chloroplasts of 1-2μm in size developed within the floret tissue, although in a very low number (Fig 4.10). Unfortunately, this period of light exposure renders the iIF recalcitrant to regeneration. Given that the regenerable tissue of the inflorescence derives from the chloroplast absent floret regions, and the immature embryo callus (IEC) is more embryogenic than the iIF, I favoured the latter as a target for chloroplast transformation. In further support of the use IEC as an explant target, the two only reports of chloroplast transformation in monocots also used embryo callus tissue.

4.3.3   A novel green explant for wheat chloroplast transformation

The primary immature leaf sheath (piLS), is the first and closest of 5 leaf sheaths that surrounds the iIF as it develops at the base of the wheat plant. The leaf sheaths provide rigidity to the developing shoot as it rapidly grows from nodal meristem, and those surrounding the iIF provide it with protection from shoot damage. These tissues are considerably green (Fig 4.4), indicating the presence of chloroplasts (Fig 4.11), with the abundance of chloroplasts and rigidity increasing from primary to tertiary iLS.
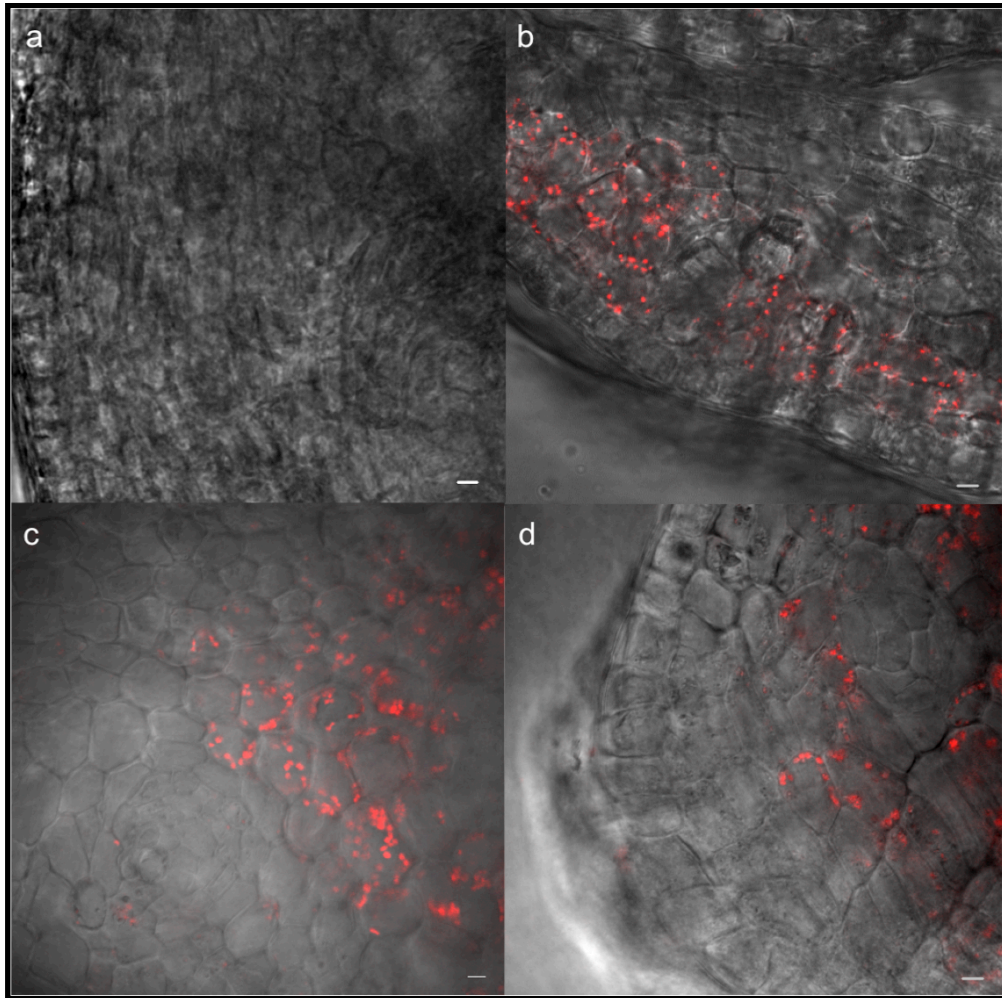
Fig 4.10 Induction of chloroplast development in floret tissue. a, floret tissue is absent of any chloroplasts, however exposure to 4 days (b), and 7 days of light (c, d), induces the formation of active chloroplasts (red). Scale bars represent 5µm.
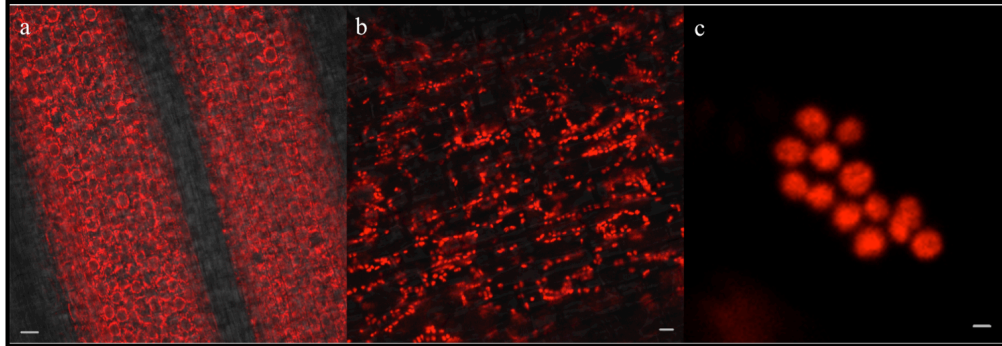
Fig 4.11 Chloroplasts (red) within the leaf sheaths of Triticum Aestivum. Scale bars represent a, 20μm, b, 5μm, and c, 1μm.

To investigate whether regenerable callus could be induced from leaf sheaths a series of experiments were performed.

4.3.3.1 Effect of 2,4-D

Once the piLS was isolated, it was cut into 6, 9 or 12 pieces, if the LS's were 6mm, 7-8mm, or 9-13 mm in length respectively. Cut pieces were placed abaxial side down onto 9mm petri dishes containing callus induction media. Callus induction media was composed of L7 (3%) media supplemented with 10mg/L $AgNO_3$, and varying concentrations of 2,4-Dinitrophenylhydrazine (2,4-D). L7 (3%) was chosen as it is used for the callus induction of the iIF, and the iIF and iLS develop in close proximity and from the same nutrition source. Leaf sheath pieces which were covered in over 75% of callus tissue, were scored as having formed callus. Pieces that had formed less than 75% of callus tissue were scored as not having formed callus.

From Fig 4.12 it is clear that L7 (3%) supplemented with 10mg/L $AgNO_3$ and 0.5mg/L 2,4-D (n=45 piLS) provides the highest and most consistent amount of callus formation in Apogee piLS. Increasing levels of 2,4-D reduced the number of piLS pieces that would form callus, as well as increasing the inconsistency of callus formation. L7 (3%) supplemented with 0.5mg/L 2,4-D was therefore used as the basis for callus induction of piLS. Callus induction media, (CIM), from here on refers to L7 (3%) supplemented with 10mg/L $AgNO_3$ and 0.5mg/L 2,4-D. CIM produces firm, dry embryogenic callus (Fig 4.13 and 4.14). I also observed that larger concentrations of 2,4-D increased the incidence of non-embryogenic callus formation (Fig 4.15).
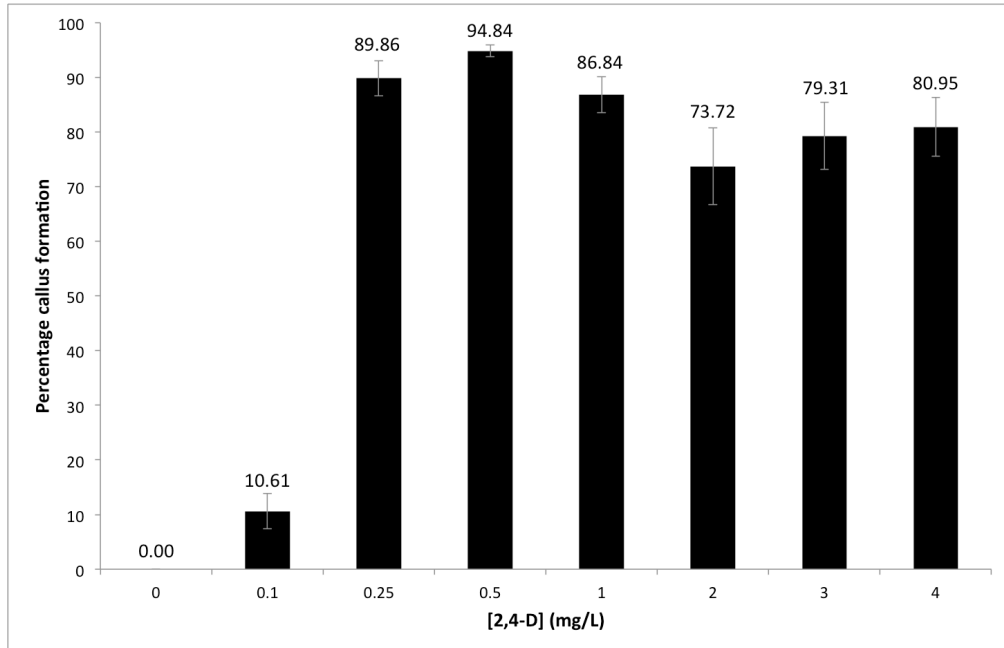
Fig 4.12 Callus formation of Apogee piLS on L7 (3%) callus induction media with varying concentrations of 2,4-Dinitrophenylhydrazine (2,4-D). For each condition, n=20.
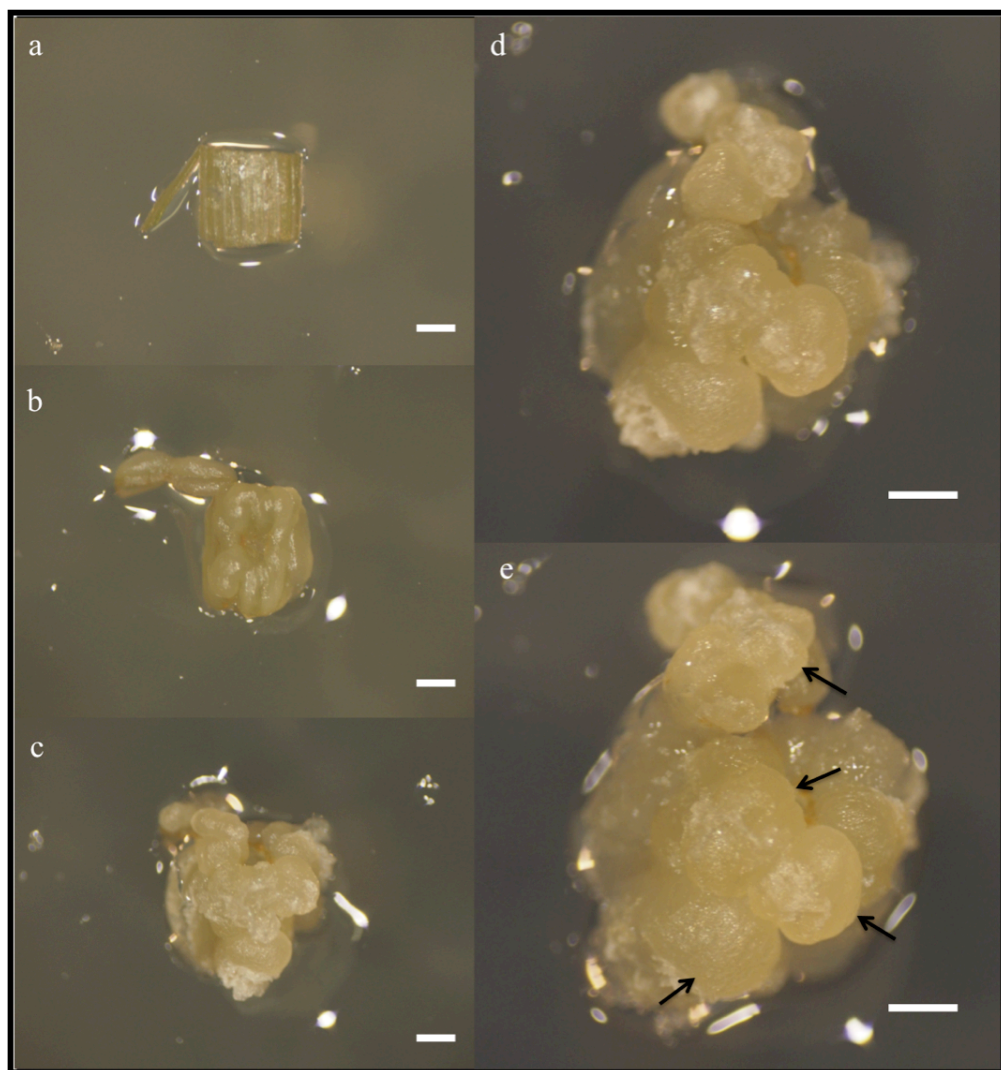
Fig 4.13 Embryogenic callus formation in Apogee piLS on CIM at time point a, 0 days, b, 7 days, c, 14 days, d, 21 days, e, 28 days. Scale bars represent 2mm. Black arrows indicate embryoids.
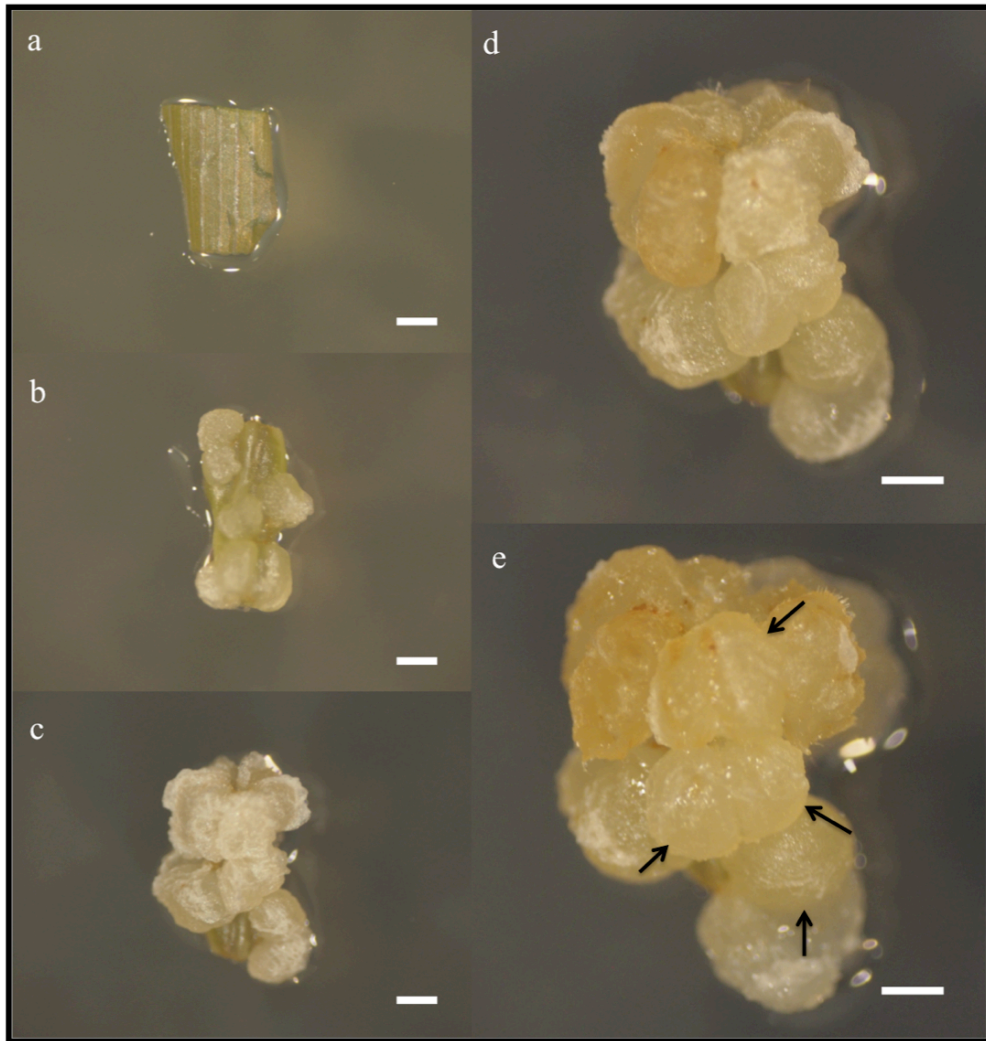
Fig 4.14 Embryogenic callus formation in Apogee piLS on CIM at time point a, 0 days, b, 7 days, c, 14 days, d, 21 days, e, 28 days. Scale bars represent 2mm. Black arrows indicate embryoids
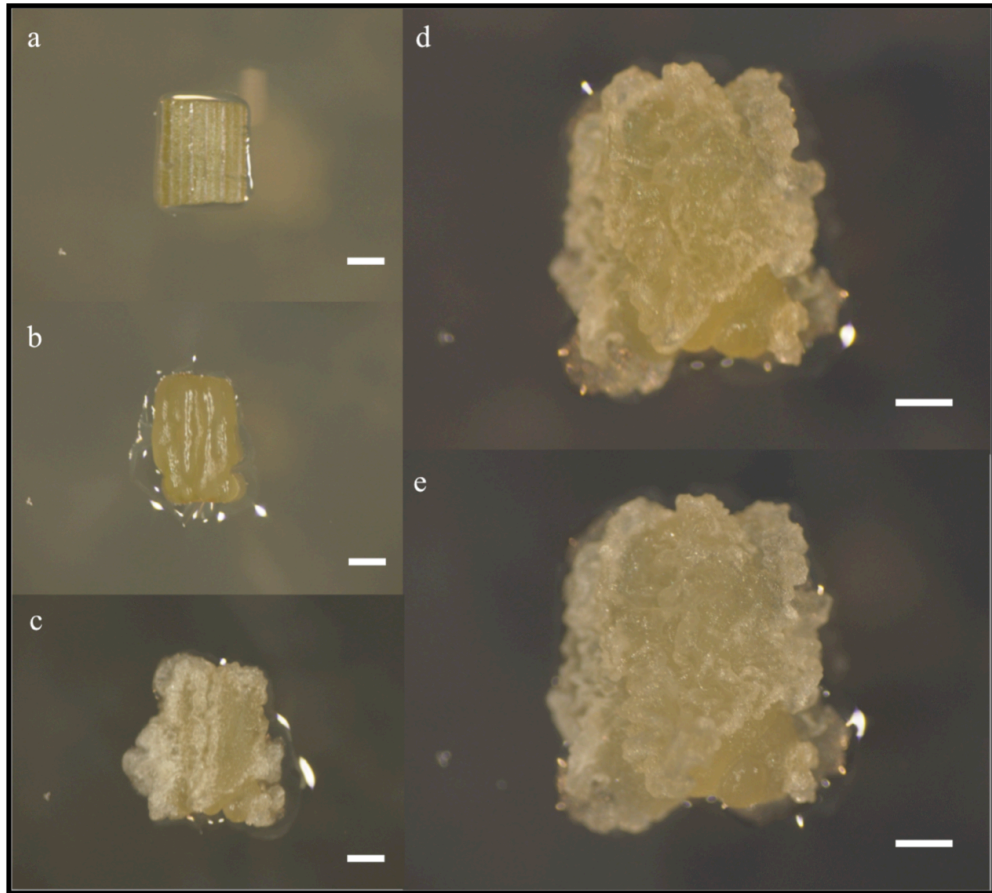
Fig 4.15 Non-embryogenic callus formation in Apogee piLS on CIM at time point a, 0 days, b, 7 days, c, 14 days, d, 21 days, e, 28 days. Scale bars represent 2mm.

Both embryogenic and non-embryogenic callus were observed for shoot formation on regeneration media (Fig 4.16 and Fig 4.17).
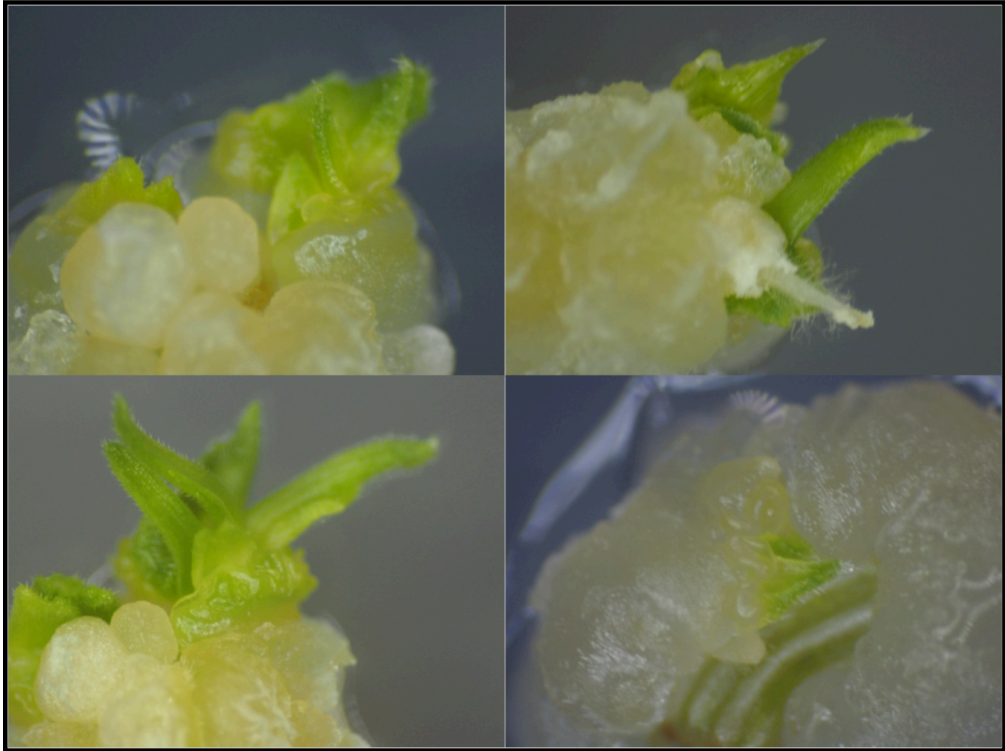
Fig 4.16 Shoots regenerating from embryogenic callus of Apogee piLS after 7 -14 days on regeneration media.
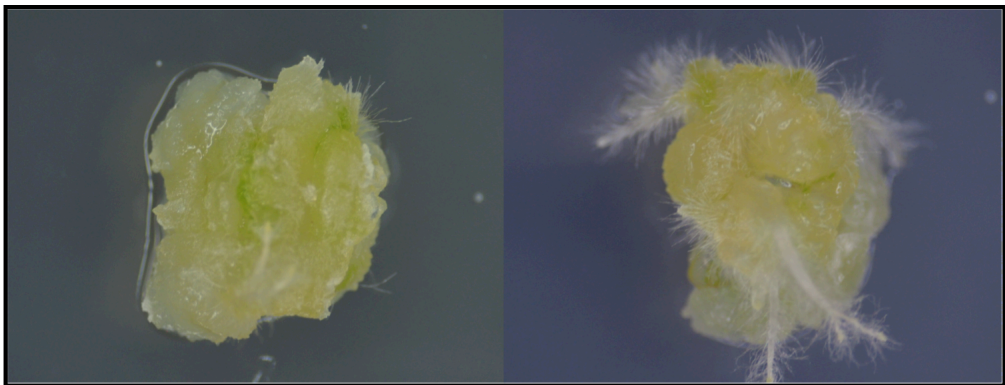
Fig 4.17 Non-embryogenic callus after 2 weeks on regeneration media. Although greening may occur, callus forms roots or root hairs rather than shoots.

4.3.3.2 Optimisation of mineral salts

Callus formation of Apogee was also tested with two other commonly used tissue culture media, MS and M (9%), supplemented with 0.5mg/L 2,4-D (Fig 4.18). Both showed callus formation at similar percentages (70.5% and 73.7% respectively) and consistencies, however CIM still provided superior callus formation (99.6%). CIM is also most effective on Apogee piLS of sizes of 8-13mm (Fig 4.19).
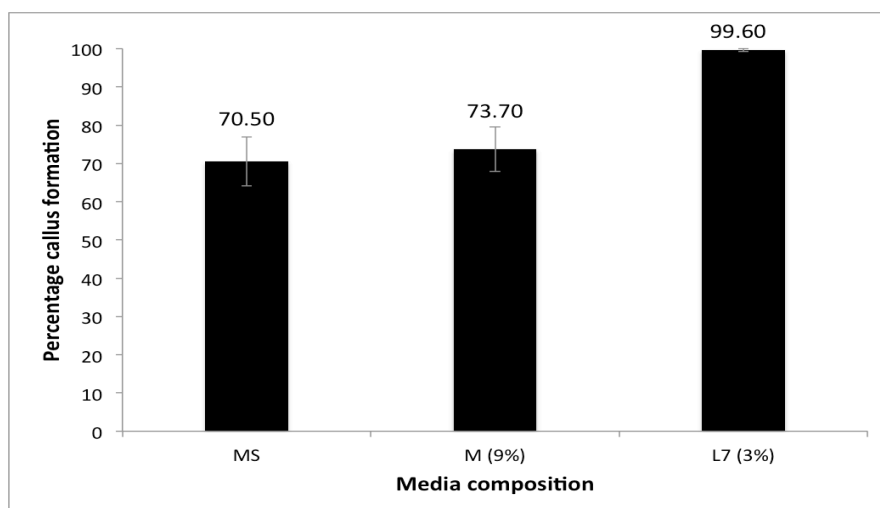
Fig 4.18 Callus formation of Apogee piLS on commonly used tissue culture media. 20 replicates per condition. For each condition, n=20.
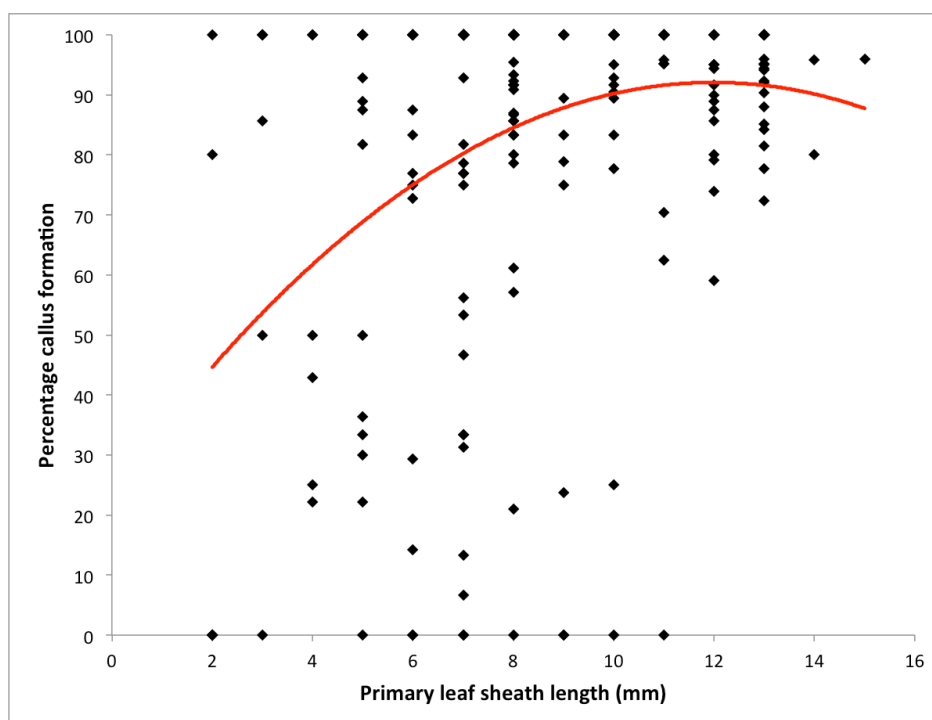
Fig 4.19 Callus induction of Apogee piLS on CIM. 240 total samples. n=243.

4.3.3.3   Genotype-dependency of leaf sheath regeneration

The Rothamsted transformation lab uses the spring wheat variety Cadenza for much of its conventional genetic engineering work. To test whether the emerging protocol for leaf sheath regeneration was specific to the Apogee genotype, I compared the response of Cadenza.

CIM was able to also induce consistent embryogenic callus formation in piLS of cadenza (Fig 4.20). Callus formation in Apogee siLS and tiLS with CIM was also attempted, however failed to produce any callus.
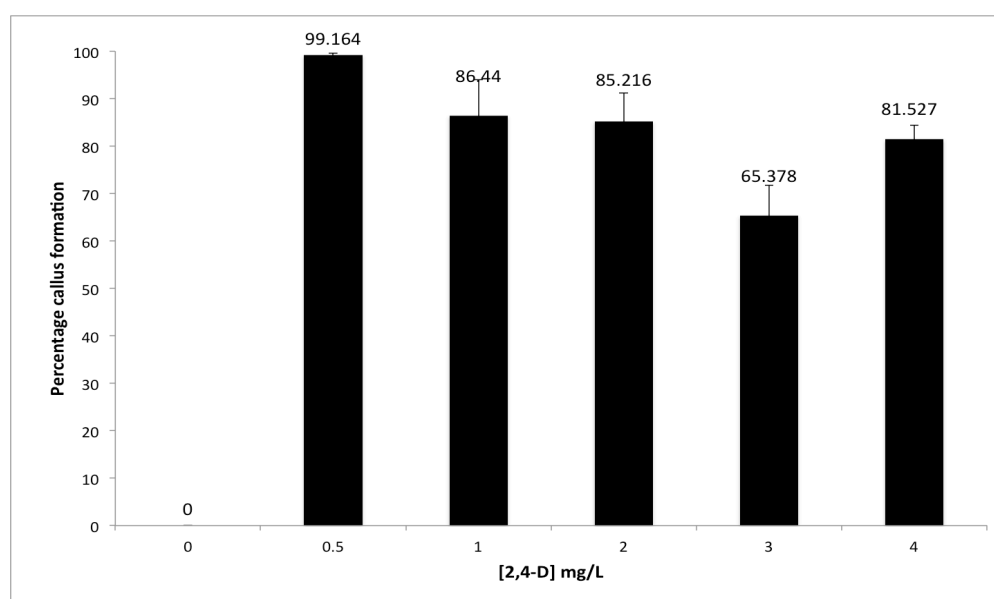
Fig 4.20 Callus formation of cadenza piLS on L7 (3%) callus induction media supplemented with 10mg/L AgNO$_3$ and varying concentrations of 2,4-Dinitrophenylhydrazine (2,4-D). For each condition, n=20.

Following 4 weeks on CIM, moving callus into light (on the same media) was shown to induce shoot (Fig 4.21) and root (Fig 4.22) formation in both Apogee and cadenza.
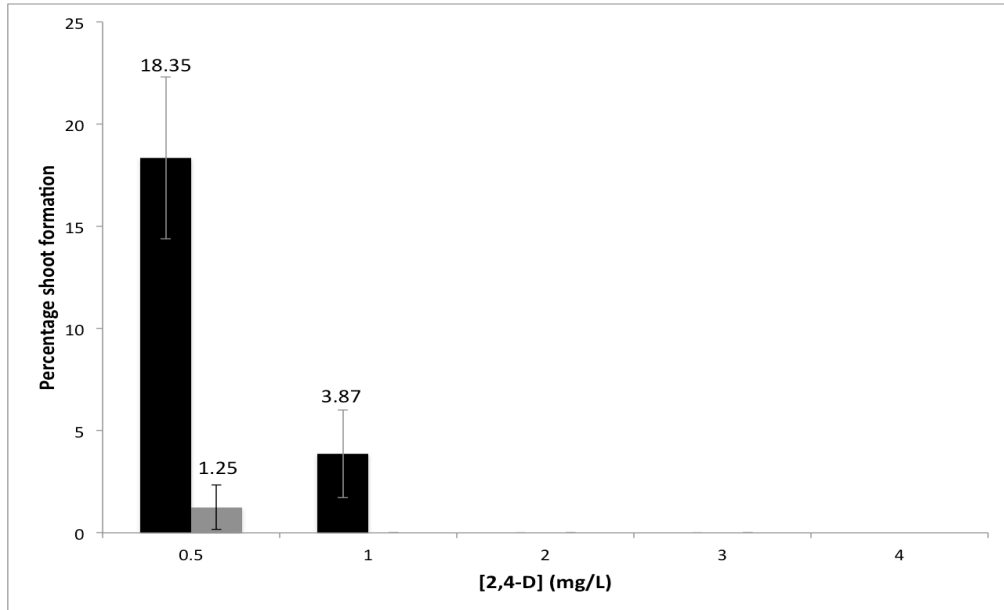
Fig 4.21 Shoot formation following light exposure. piLS callus from Apogee (black) and Cadenza (grey) show shoot formation when exposed to light while on callus induction media. For each condition, n=20.
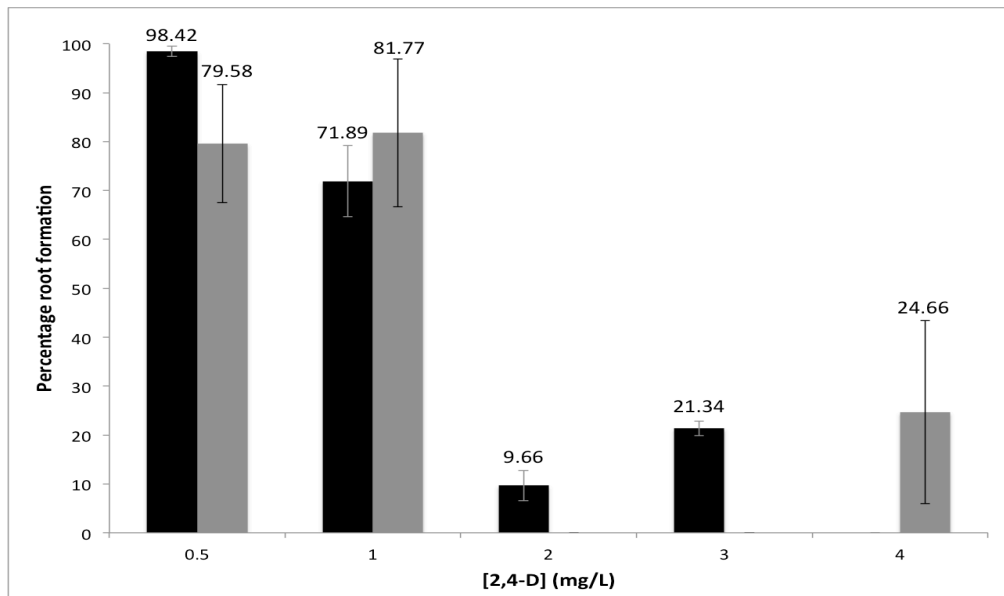
Fig 4.22 Root formation following light exposure. pILS callus from Apogee (black) and Cadenza (grey) show root formation when exposed to light while on callus induction media. For each condition, n=20.

4.3.3.4   Balance of cytokinin & auxin is significant

The regeneration steps for wheat immature embryos and immature inflorescences is conducted on R regeneration media supplemented with 5mg/L zeatin and 0.1mg/L 2,4-D (cytokinin:auxin ration of 50:1). This medium has been optimized for Cadenza over many years of research into conventional transformation of the nuclear genome. Given the observation that piLS embryogenic callus from both Cadenza and Apogee varieties are capable of forming shoots and roots when exposed to light, I investigated a range of different hormone ratios. The regeneration of piLS callus on R regeneration media supplemented with 1mg/L zeatin and 0.1mg/L 2,4-D (10:1), 2.5mg/L zeatin and 0.1mg/L 2,4-D (25:1), and 5mg/L zeatin and 0.1mg/L 2,4-D (50:1) was compared (Fig 4.23).
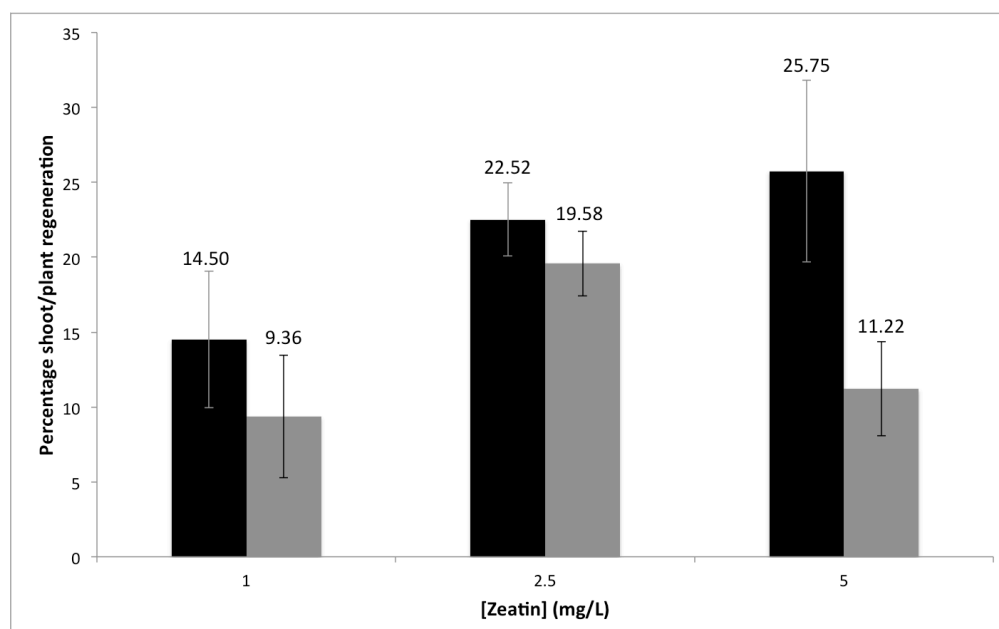
Fig 4.23 Shoot (black) and plant (grey) regeneration from Apogee piLS on R media supplemented with 0.1 mg/L 2,4-D and varying concentrations of Zeatin. For each condition, n=20.

Although the 50:1 (5mg/L zeatin) conditions encouraged greater shoot formation per piLS section (25.75%), it was the 25:1 conditions that produced the greatest number of regenerated plants (19.58%). Neither of the cytokinin:auxin ratios were suitable for regeneration in Cadenza.

Apogee piLS embryogenic callus when placed on hormone free MS media, showed to green up significantly more when compared to that on hormone free R media (Fig 4.24).
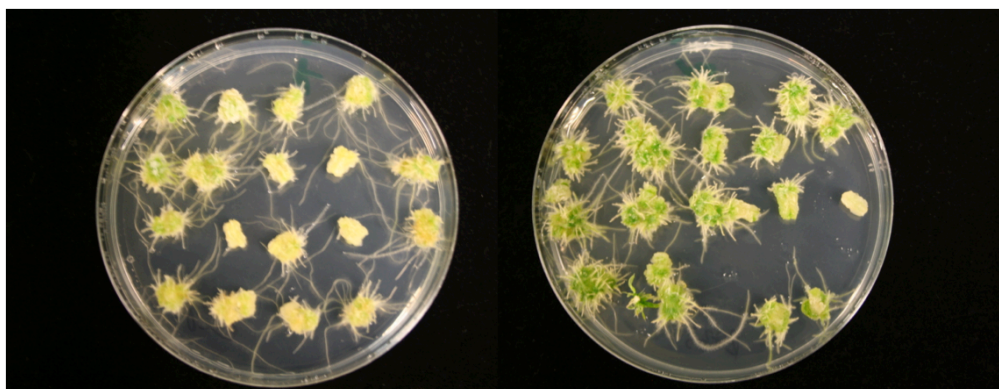
Fig 4.24 Apogee piLS callus after 4 weeks on hormone free R media (left), and hormone free MS (right) in light.

Following these observations, regeneration of Apogee piLS on MS media supplemented with varying concentrations of Zeatin was conducted (Fig 4.25).
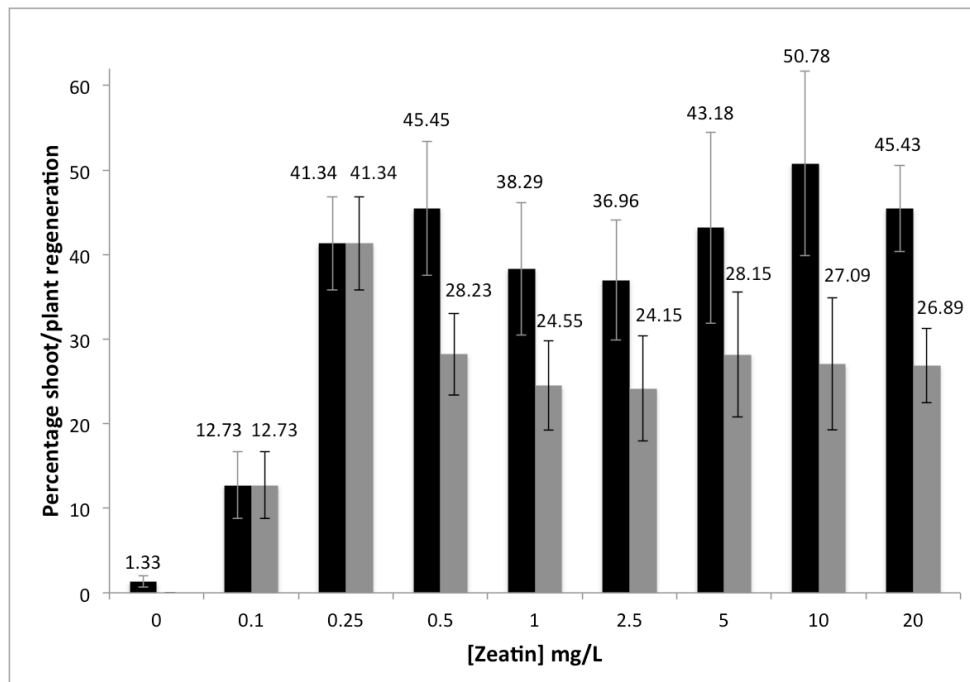
Fig 4.25 Shoot (black) and plant (grey) regeneration from Apogee piLS on MS media supplemented with 0.1 mg/L 2,4-D and varying concentrations of Zeatin. For each condition, n=20.

Between Zeatin concentrations of 0.5-2mg/L, the percentage of shoot and plant regeneration remains relatively similar, with no significant difference observed. However at 0.25mg/L Zeatin, regeneration was at its maximum (41.34 %), even though shoot regeneration was not significantly higher than any of the other concentrations. Callus formed from Apogee piLS of between 6-8mm (Fig 4.26) were also observed to be most responsive on MS media supplemented with Zeatin (concentrations varying from 0.25-10mg/L).
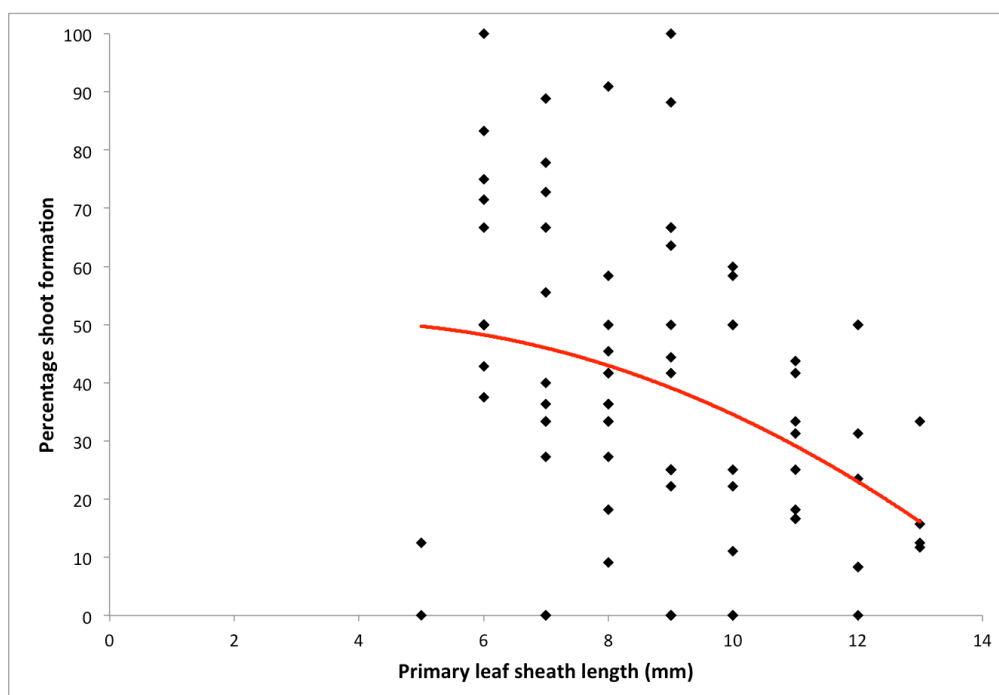
Fig 4.26 Regeneration of shoots from Apogee piLS callus on MS media supplemented with Zeatin. Percentage values were taken from regeneration experiments conducted on 0.25, 0.5, 1, 2.5, 5, and 10mg/L Zeatin. n=243.

4.3.3.5 Summary

In the tables below, I have summarized the results from the tissue culture investigations with apogee piLS. In each table, the conditions which produced the greatest callus induction or plant regeneration are highlighted with a " * ". All experiments were conducted at 24°C. Callus induction was conducted in darkness, and regeneration conducted in light (100-130μE) on a day/night cycle of 12h/12h.

Media	[2,4-D] (mg/L)	AgNO$_3$] (mg/L)	CI (%)	
L7 (3%)	0	10	0	
L7 (3%)	0.1	10	10.61	
L7 (3%)	0.25	10	89.86	
L7 (3%)	0.5	10	94.84	*
L7 (3%)	1	10	86.84	
L7 (3%)	2	10	73.72	
L7 (3%)	3	10	79.31	
L7 (3%)	4	10	80.95	

Table 4.4 Callus induction (CI) of Apogee piLS with varying concentrations of 2,4-D.

Media	[2,4-D] (mg/L)	[AgNO$_3$] (mg/L)	CI (%)	
MS	0.5	10	70.5	
M (9%)	0.5	10	73.7	
L7 (3%)	0.5	10	99.6	*

Table 4.5 Callus induction (CI) of Apogee piLS on varying mineral salts.

Media	[Zeatin] (mg/L)	[2,4-D] (mg/L)	CuSO$_4$	Reg (%)	
R	0	0.1	1M	0	
R	1	0.1	1M	9.36	
R	2.5	0.1	1M	19.58	*
R	5	0.1	1M	11.22	

Table 4.6 Plant regeneration (Reg) from Apogee piLS callus on R regeneration media.

Media	[Zeatin] (mg/L)	[2,4-D] (mg/L)	CuSO$_4$	Reg (%)	
MS	0	0.1	1M	0	
MS	0.1	0.1	1M	12.73	
MS	0.25	0.1	1M	41.34	*
MS	0.5	0.1	1M	28.23	
MS	1	0.1	1M	24.55	
MS	2.5	0.1	1M	24.15	
MS	5	0.1	1M	28.15	
MS	10	0.1	1M	27.09	
MS	20	0.1	1M	26.89	

Table 4.7 Plant regeneration (Reg) from Apogee piLS callus on MS regeneration media.

Optimum embryogenic callus induction was achieved with Apogee piLS of 8-13 mm in length, on L7 (3%) callus induction media supplemented with 0.5mg/L 2,4-D and 10mg/L AgNO$_3$ (Table 4.4 and 4.5). Optimum plant regeneration from Apogee piLS callus was first achieved on R regeneration media supplemented with 2.5mg/L zeatin, 0.1mg/L 2,4-D, and 1M CuSO$_4$ (Table 4.6). Further investigations demonstrated that plant regeneration from Apogee piLS callus could be further increased by regeneration on MS regeneration media supplemented with 0.25mg/L zeatin, 0.1mg/L 2,4-D, and 1M CuSO$_4$ (Table 4.7). Typically, the duration from piLS isolation to a reproducing adult plant is approximately 4 months, with a further 1-2 months required for seeds to mature (Fig 4.27). Seeds from regenerated Apogee wheat plants appear phenotypically normal, germinate as WT, and provide no indication of somaclonal variation.

Although the protocol developed for Apogee piLS was unable to demonstrate regeneration in Cadenza piLS, MS regeneration media supplemented with 0.1mg/L 2,4-D and 5 mg/L zeatin or 4 mg/L 6-Benzylaminopurine, benzyl adenine (BAP) did encourage regeneration of Cadenza piLS callus, although this was at a significantly lower rate (1 plants produced per piLS).
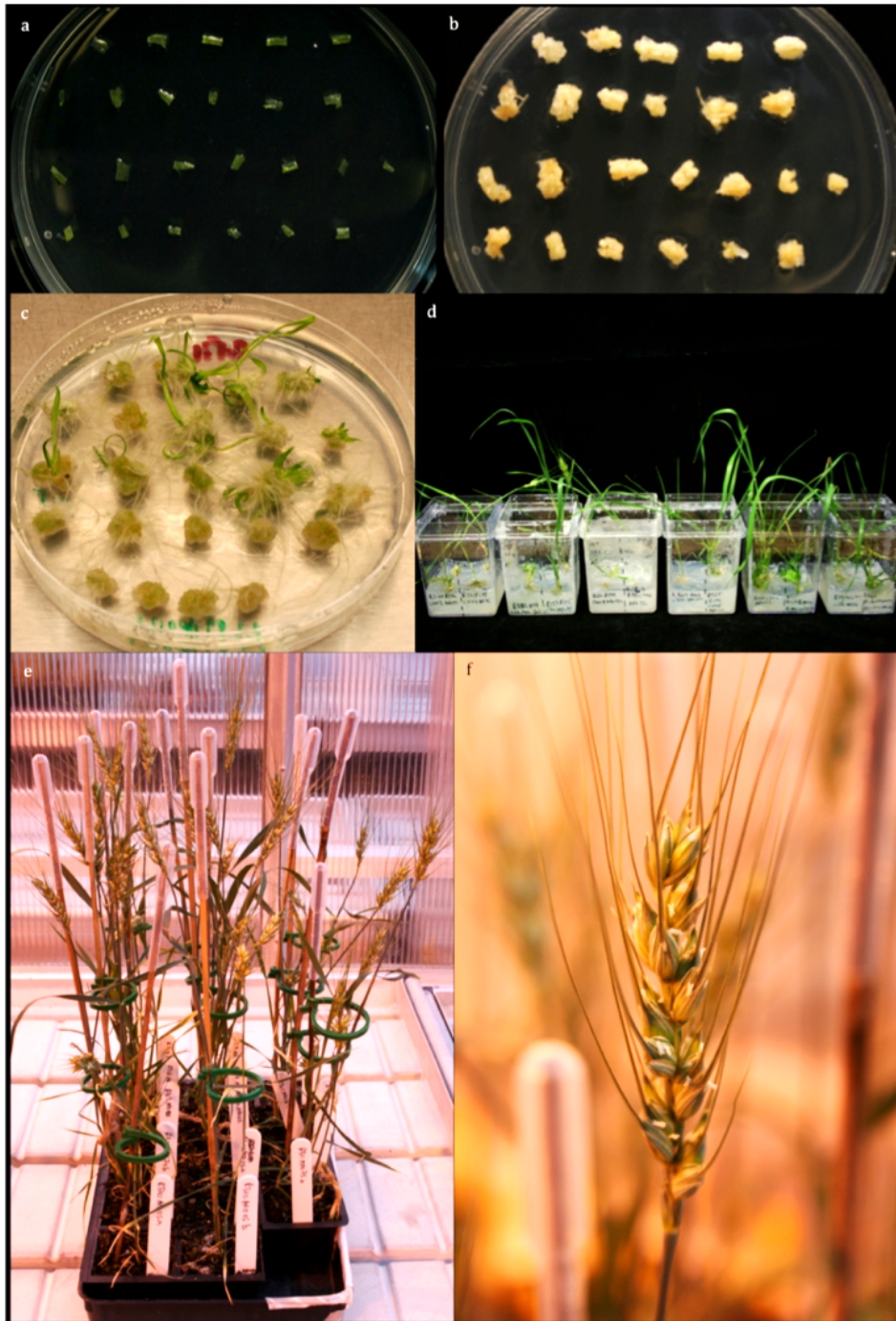
Fig 4.27 Regeneration of new wheat plants from Apogee piLS via organogenesis. piLS pieces are placed on CIM (a) and incubated in the dark for 25-30 days to produce embryogenic callus (b). These callus pieces are incubated on R regeneration media in light for 3-4 weeks (c), and then moved to magentas (d). Typically 3-4 weeks later, plantlets are potted on. Plantlets form adult apogee plants that are small (e), otherwise phenotypically normal and fertile (f).

4.4 Discussion

The absence of chloroplast-containing regenerable explant tissues, presents an obvious problem for the development of a chloroplast transformation of wheat. If there are no active chloroplasts present in a target tissue, then the biolistic bombardment of DNA coated particles will ultimately fail to transform any chloroplasts. Due to this restriction, it was necessary to identify alternative options for the chloroplast transformation of wheat.

Due to the recalcitrant nature of adult wheat tissue, regeneration of new wheat plants through tissue culture is restricted to immature tissues. The most embryogenic of these are the immature embryo and the immature inflorescence which are well-characterised as part of established protocols. One method for nuclear transformation of wheat, is to biolistically bombarded the immature embryo with a nuclear transformation DNA vector. However, I have shown that the immature embryo lacks any active chloroplasts, and that any possible pro-plastids would be too small to successfully repair following bombardment of even the smallest of bombardment particles. I have also shown that the regeneration of the immature inflorescence occurs from the floret regions, which are absent of any chloroplasts. Incubation in light for 4 to 7 days post extraction does induce the formation of active chloroplasts in floret tissue, however this incubation period also renders the inflorescences recalcitrant to further regeneration. Furthermore, activated chloroplasts are not present in every cell of the florets. This suggests that, (a) light incubated floret tissue may not be suitable even if not rendered recalcitrant due to the limited chloroplast distribution and low number, and (b) the number of pro-plastids large and active enough prior to light incubation are also of poor distribution and insufficient number for biolistic bombardment. Light incubation of 21 day old immature embryo callus also produces active chloroplasts throughout the callus tissue, indicating that immature embryo callus at 21 days, contains a large number of pro-plastids of consistent distribution throughout the tissue. For this reason, I decided to use immature embryo callus as an initial target for the biolistic chloroplast transformation of wheat while also looking at other options.

Despite immature embryo callus providing one potential starting tissue, I also set about identifying a regenerable chloroplast-containing explant To find this, I looked at other immature tissues that would contain enough cambium tissue in order to regenerate new plants. Given the immature inflorescence being highly regenerable, I

decided to investigate tissues associated with the immature inflorescence, which were (a) the immature leaf sheaths and (b), the peduncle node that the immature inflorescence develops from and is attached to (node 2).

Initial experiments where nodal pieces were cultured on CIM, showed that callus induction and regeneration was possible, however at a very low efficiency (1 plant per 10 nodes). Furthermore, the inability to generate sterile enough nodal tissue reduced the capacity for regeneration further as plate contamination was high (1 in 3 plates). For these reasons, I did not pursue the node as a viable option for the regeneration of whole wheat plants, despite it containing vast numbers of chloroplasts.

The immature leaf sheaths are abundant in chloroplasts, with the density increasing from the primary leaf sheath to the tertiary. Rigidity also increases from primary to tertiary. In this chapter, I have described a protocol for the regeneration of new wheat plants, via embryogenesis, from the primary leaf sheath (piLS) of *Triticum Aestivum* var. Apogee. I also attempted to develop a protocol for *Triticum Aestivum* var. Cadenza, however in the results, I was unable to do so. However a small follow up study showed that regeneration from Cadenza piLS is possible on MS regeneration media supplemented with 5mg/L zeatin, or 4mg/L BAP, all be it at a much lower efficiency (1 plant per 5 piLS). The reason for this may be due to the length of incubation on CIM. Due to Apogee's faster life cycle, they may reach the embryogenic stage of callus induction sooner than Cadenza. Therefore a longer period of Cadenza piLS on CIM may produce more embryogenic callus. These results with cadenza suggest that regeneration via embryogenesis in piLS from other varieties can occur, however the conditions need to be optimised.

5 Biolistic bombardment of wheat for chloroplast transformation

5.1 Introduction

Chloroplast transformation is achieved by the biolistic bombardment of an explant tissue with gold/tungsten particles coated in DNA, using a gene gun (also known as a biolistics device). First developed to demonstrate transient nuclear expression (Klein et al., 1987), it was not long until the technology was used to create transgenic chloroplast transformants in *Chlamydomonas reinhardi* (Boynton et al., 1988, Blowers et al., 1989), *Nicotiana tabacum* (Svab et al., 1990), and many other dicotyledon species (Day and Goldschmidt-Clermont, 2011).

Chloroplast transformation has many advantages over nuclear transformation. Firstly, each chloroplast contains a certain number of circular chromosomes, therefore making it easy to calculate the number of transgene insertions that is possible. It is also possible to express multiple genes from polycistronic mRNA (Maliga, 2001, Staub and Maliga, 1995, De Cosa et al., 2001, Quesada-Vargas et al., 2005). Secondly, transcription and translation of a transgene only occurs in the chloroplast, hence any mRNA/protein product would be compartmentalised within the chloroplast, thus removing cytotoxicity. Lastly, and most importantly, plastids are inherited maternally in the vast majority of angiosperms (Birky, 1995, Mogensen, 1996, Hagemann, 2002). Thus, any transgene escape via pollen is vastly reduced. This provides a strong level of biological containment as it reduces, and is still considered a safe method of transgene containment following risk assessment (Wilkinson et al., 2003).

Progress in chloroplast transformation of the monocot species has been very limited, especially when compared to the achievements attained with dicot species such as *N.tabacum* (tobacco). The two published monocot transformation reports were both in rice (Khan and Maliga, 1999, Lee et al., 2006), and neither of these produced homoplasmic plants. Both instances used the biolistic bombardment method for DNA delivery, and the target tissue was embryogenic callus derived from mature seed. Although the rate of heteroplasmic chloroplast transformation was very low (2

transformants from 4000 bombarded explants, (Lee et al., 2006)), these studies demonstrated that limitations of chloroplast transformation in the monocots are not due to the DNA delivery method, but instead the target tissue and subsequent selection. In chapter 1, I have explained the rationale behind requiring a chloroplast-containing regenerable target for chloroplast transformation. I have subsequently demonstrated the successful identification of such a tissue in wheat, the primary inflorescence leaf sheath (piLS), and developed a regeneration protocol for this as described in chapter 4. Wheat piLS will therefore serve as a tissue target in our bombardment experiments. I will also be targeting embryogenic callus derived from the immature embryo. Wheat embryogenic callus derived from the immature embryo contains pro-plastids that can be encouraged to form abundant chloroplasts after just 4 days of light exposure, indicating that the pro-plastids are abundant and active. Given the highly embryogenic nature of this callus, active and abundant pro-plastids reside in this tissue, and that previous rice chloroplast transformation studies have successfully used embryogenic callus as targets for biolistic bombardment, wheat embryogenic callus was also used as a target for the chloroplast transformation of wheat.

The transformation cassettes from the wheat-specific chloroplast transformation vectors described in chapter 3 (pRRes14mF2-T7g10, pRRes14mF2-TPrrn, pRRes14mF2-WPrrn) were all tested for function in the tobacco chloroplast transformation system (Maliga, 2002). These results provided confidence that any absence of stable chloroplast transformation in wheat would not be due to the bombardment system, or the vector DNA.

5.1.1 Aims

- Optimise the bombardment conditions for the biolistic bombardment of piLS.
- Bombard the piLS and IEC with wheat specific chloroplast transformation vector to attain chloroplast transformed wheat plants.

5.2 Materials and methods

5.2.1 Chloroplast transformation vectors pRRes14mF2-T7g10, pRRes14mF2-TPrrn, pRRes14mF2-WPrrn

The vectors pRRes14mF2-T7g10, pRRes14mF2-TPrrn, pRRes14mF2-WPrrn are described in chapter 3. They are targeted to the *trnI-trnA* gene region within the wheat plastome, and are composed of a promoter/5'UTR, selection marker, leader, and 3'UTR (Fig 5.1).
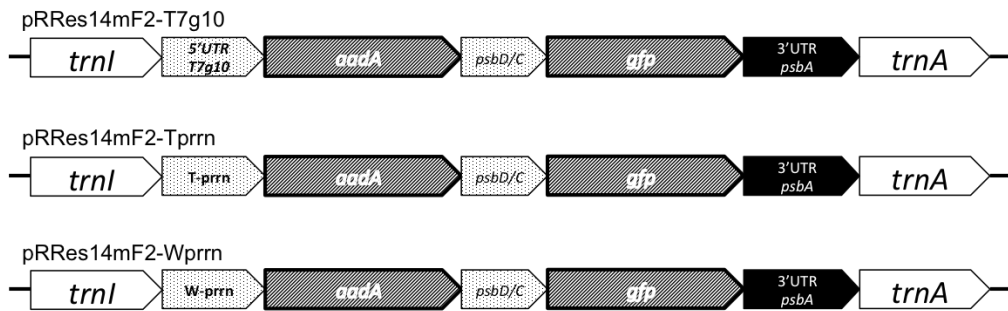
Fig 5.1 pRRes14mF2-T7g10, pRRes14mF2-TPrrn, pRRes14mF2-WPrrn. The 5'UTR T7g10, T-Prrn (tobacco *Prrn*), W-Prrn (wheat *Prrn*), serves as a promoter, *aada* as the selection marker, *psbD/C* as a leader, *gfp* (codon optimised for plastids) as a visual marker, and 3' UTR stabilises the transcribed mRNA.

5.2.2 Biolistic bombardment of wheat explants

Wheat explants (piLS and IEC) were bombarded using the same parameters as set out for tobacco leaf biolistic bombardment in chapter 2.1.

5.2.3 IEC formation and preparation for bombardment

Ears were harvested from wheat plants 15-21 days post anthesis, and immature caryopses were surface sterilised with 70% (v/v) ethanol for 5 min, 10% (v/v) sodium hypochlorite for 15 min, then three washes with sterile distilled water. The immature embryos, translucent at this stage of development, were aseptically removed from early-medium milk stage caryopses, and the scutella isolated by removal of the embryonic axis. Scutella were placed abaxial side up in the centre of a 9cm petri dish containing immature embryo callus induction medium (M 9%), with 30-40 scutella per plate. Explants were cultured in the dark at 24°C for 14-21days to produce embryogenic callus. To allow for a longer period of callus formation, immature embryos were cultured on M(9%) for 7 days, then moved to WLS for up to 6 weeks.

For bombardment, 9-10 embryogenic calli were moved to the centre of a 9cm petri dishes containing M(9%) or WLS (depending on what media callus induction was conducted on). Following bombardment, each plate was left in the dark for 48 hours at 24°C.

5.2.4 piLS preparation for bombardment

Apogee piLS were extracted as described in chapter 4.2.2.4, and four placed abaxial side down in the centre of a 9cm petri dish containing CIM. A sterile filter paper was placed on top of the CIM prior to preparation of piLS, so as piLS would be placed on the damp filter paper. Following bombardment, each sheath was spread out on the filter paper, and left in the dark for 48 hours at 24°C.

5.2.5 Selection of transformed IEC

Bombarded calli were incubated in the dark for 48 hour. Incubated bombarded IEC were then either moved to selective callus induction for a period of time, or transferred to selective R regeneration media, 10 bombarded callus pieces per plate. Bombarded IEC on selective callus induction media, after a period of time, were then transferred to selective R regeneration media. Regeneration plates were double sealed with parafilm, and incubated at 25 °C in a 12-h/12-h day/dark cycle, light intensity of 100-130μE.

5.2.6 Selection of transformed piLS

Bombarded piLS were placed in the dark for 48 hours to rest, then cut into 9-12 pieces, and transferred to selective CIM. Plates were double sealed with parafilm, and incubated at 25 °C in the dark for 21-30 days. Leaf sheath pieces which formed embryogenic callus, were transferred to selective R regeneration media, sealed with parafilm, and incubated at 25 °C in a 12-h/12-h day/dark cycle, light intensity of 100-130μE.

5.2.7 Microscopy

Images in this chapter were taken using equipment and settings described in chapter 2.5.

5.3 Results

5.3.1 Optimisation of bombardment for piLS

The adaxial epidermis of all the leaf sheaths are waxy, and therefore are thicker than the abaxial surface. Thus I chose the adaxial surface to bombard. To ensure that gold particles could enter the cells of the piLS through the adaxial epidermis, optimisation of bombardment parameters was necessary. To achieve this, piLS pieces were bombarded with both 0.4 and 0.6μm gold particles, at 900, 1100, and 1350 psi, using an easily scorable visual reporter plasmid pDsRed (gifted to us by Ann Blechl at USDA-ARS, USA and Jorge Dubcovsky at UC Davis, USA). The DsRed vector contains the *DsRed* gene under the control of a ubiquitin promoter. The DsRed protein can be easily detected at certain wavelengths (See 2.5.2). At all pressures and gold particle sizes, transient DsRed protein expression was observed in leaf sheath pieces (Fig 5.2 and 5.3), however, only at 1100 and 1350 psi were gold particles viewed to be in the same plane as chloroplasts. These results confirmed that both 0.4μm and 0.6μm gold particles can be used for the delivery of exogenous DNA into piLS tissue, and that the optimum bombardment pressures are the 1100 and 1350 psi.
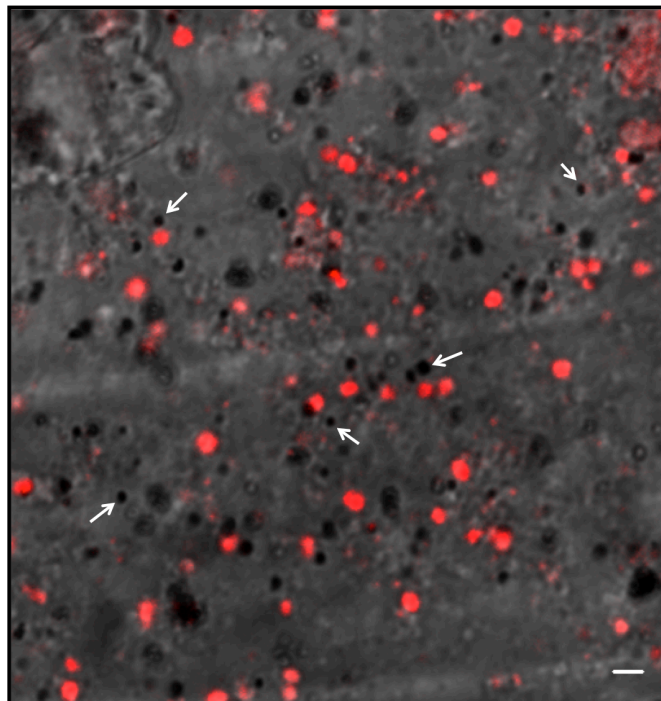
Fig 5.2 piLS bombarded with 0.6μm gold particles at 1100 psi. Gold particles/clumps (white arrows) can be seen in the same plane as chloroplasts (red, indicating chlorophyll auto fluorescence). Scale bar represents 2μm.
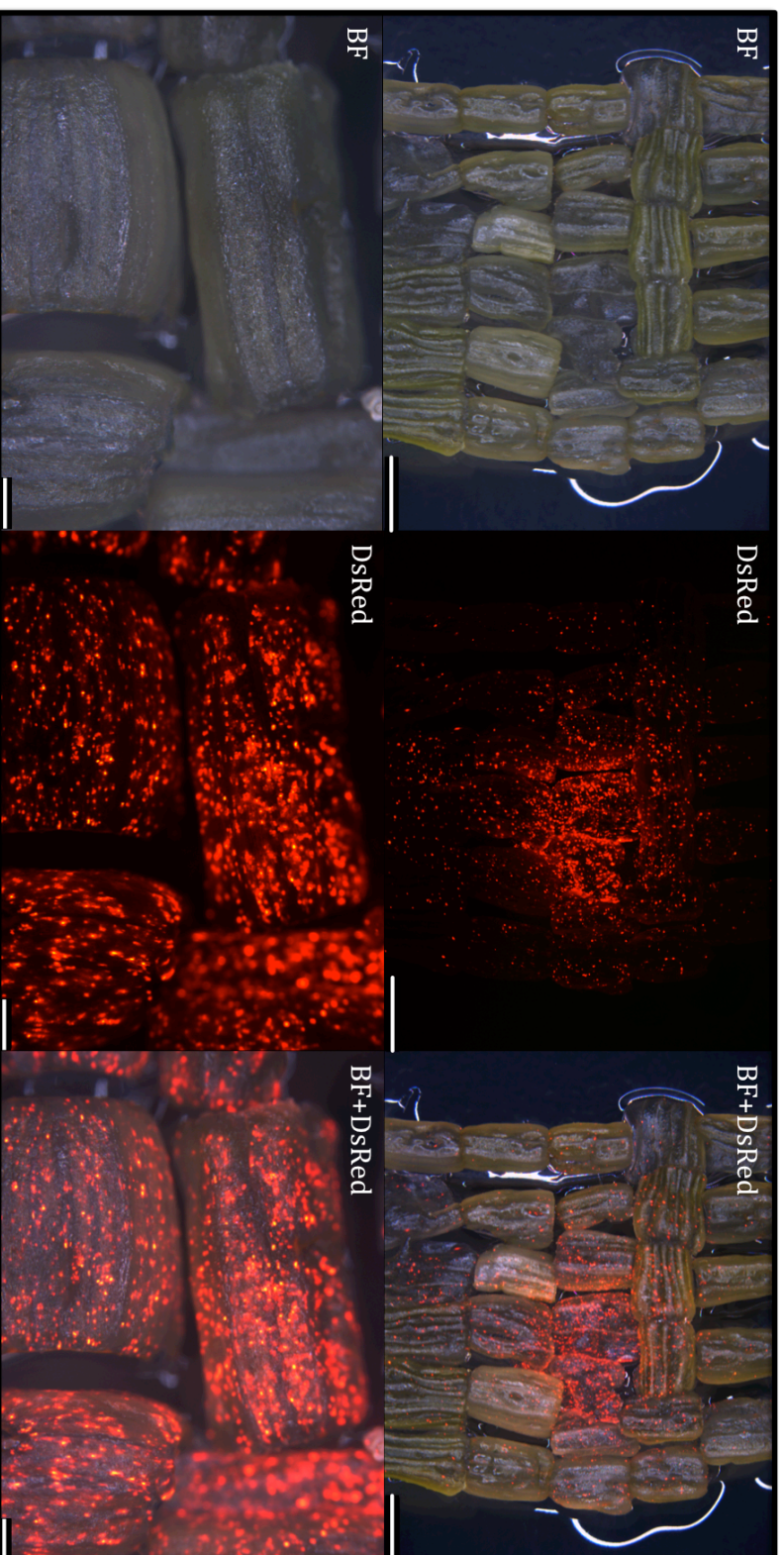
Fig 5.3 Bombardment of piLS pieces with pDsRed. Apogee piLS pieces were bombarded with 0.6µm gold carrying pDsRed at 1100 psi. 4 days post bombardment, piLS were analysed for DsRed expression using light microscopy (see 2.5.2). The DsRed filter ensures that only DsRed protein excitation is observed, and all chlorophyll auto fluorescence is excluded. BF, bright field; DsRed, DsRed filter applied, BF+DsRed, bright field and DsRed filter images combined. Scale bars represent 25mm (top row) and 500µm (bottom row).

piLS leaf sheaths bombarded at 1100 psi with 0.6µm gold particles carrying pDsRed were allowed to form callus on CIM. Callus pieces were then moved to R regeneration media for 1 week and analysed using light microscopy. I found that large portions of the callus tissue stably expressed the DsRed protein (Fig 5.4), even in the absence of any selection. This suggested that piLS tissue is not only capable of transiently expressing exogenous DNA, but can also form callus stably expressing exogenous DNA.

Fig 5.4. piLS callus expressing the DsRed protein. Callus was analysed for DsRed expression using light microscopy (see 2.5.2). BF, bright field; DsRed, with DsRed filter applied, BF+DsRed, bright field and DsRed filter images combined. Scale bars represent 500µm.

Bombardment of piLS at 1100 psi with a single shot of 0.6μm gold did not have any adverse affect on callus induction and subsequent regeneration efficiency of the tissue, however two shots reduced the percentage callus formation to approximately 50%.

5.3.2 **Optimisation of bombardment for immature embryo callus (IEC)**

Previous chloroplast transformation studies in rice have used callus tissue as the bombarded explant, and given that heteroplasmic transformation was achieved (even at low efficiencies), this indicates that bombardment of callus tissue using standard bombardment pressures and gold sizes is suitable. Furthermore, bombardment of wheat IEC at 600, 900, and 1100 psi with 0.6μm gold carrying a GUS (β-glucorinidase) expression vector produced transient β-glucorinidase expression in callus tissue, indicating that that these parameters are suitable for DNA delivery into callus tissue.

5.3.3 **Optimisation of antibiotic selection.**

5.3.3.1 Immature embryo callus (IEC)

Wheat cells, like most cereals, have a natural resistance to spectinomycin (Fromm et al., 1987). However, the *aad*A gene also confers resistance to streptomycin (Svab and Maliga, 1993), therefore can be used to select for transformed chloroplasts in wheat. Immature embryos were callus induced on M (9%), and after 21 days were transferred to M (9%) supplemented with varying concentrations of streptomycin (0-500mg/L). IEC were still capable of forming callus at 500mg/L streptomycin (the highest concentration tested), and appeared less compact when exposed to concentrations of of 250mg/L and above. However, when 21day old IEC were placed on R regeneration media supplemented varying concentrations of streptomycin for 1 month, the effect on regeneration was clear. As the concentration of streptomycin increased, the percentage of green shoot regeneration reduced (Fig 5.5 and Fig 5.6). At 500mg/L streptomycin, no green shoot formation was observed, and few bleached shoots formed from IEC pieces.
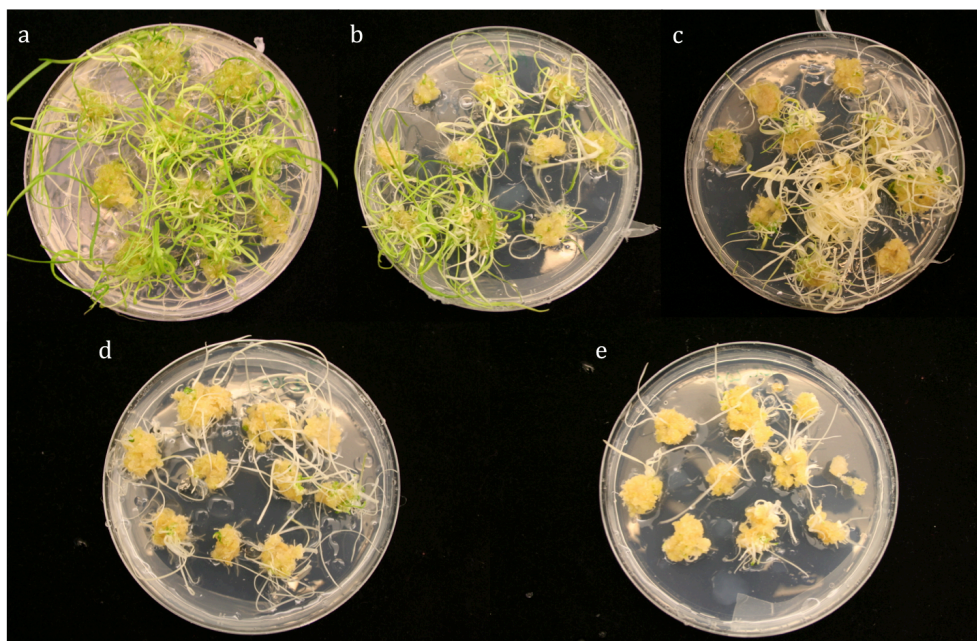
Fig 5.5 IEC (21 day old) placed on R regeneration media supplemented with varying concentrations of streptomycin for 4 weeks. a, 0; b, 50; c, 100; d, 200, e, 400 mg/L .
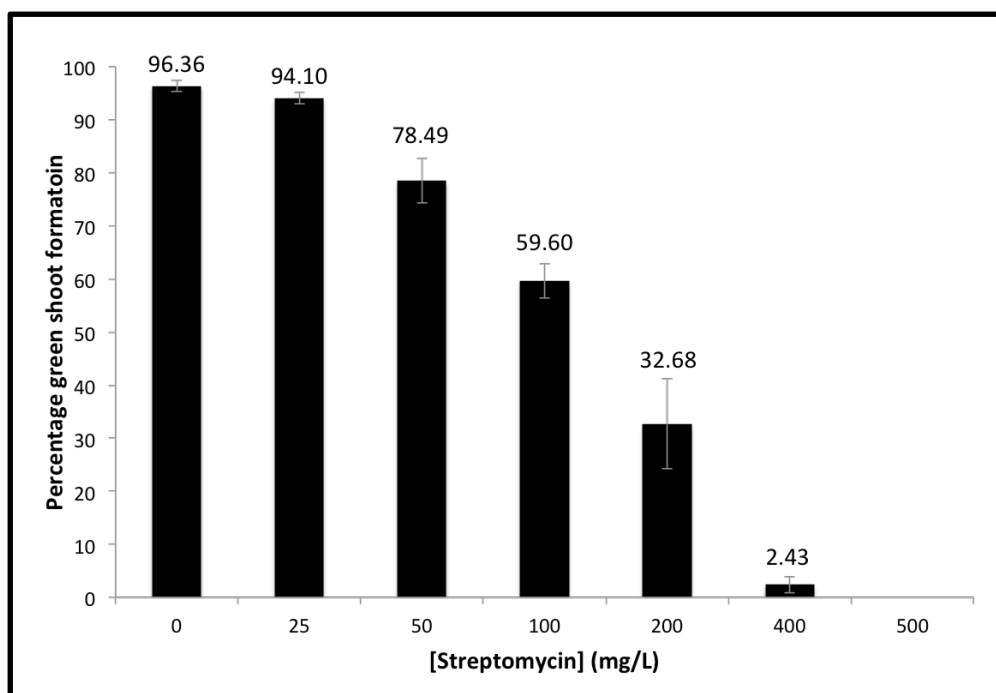
Fig 5.6 Green shoot formation from IEC on R regeneration media supplemented with varying concentrations of streptomycin. Percentage was calculated as the number of IEC that formed green shoots from total IEC

From these results, I decided that selection of bombarded IEC would be conducted on callus induction media supplemented with streptomycin concentrations between 200-500 mg/L, and on regeneration media supplemented with streptomycin concentrations between 400 and 500 mg/L.

5.3.3.2   Primary Inflorescence Leaf Sheath (piLS)

The effect streptomycin has on the callus induction of piLS was also tested. piLS were placed on CIM supplemented with varying concentrations of streptomycin and scored for % callus formation?. In the tobacco chloroplast transformation system, kanamycin has also been demonstrated as a successful selection agent (Carrer et al., 1993, Day and Goldschmidt-Clermont, 2011), and its affect on piLS callus induction was also tested (Fig 5.7).
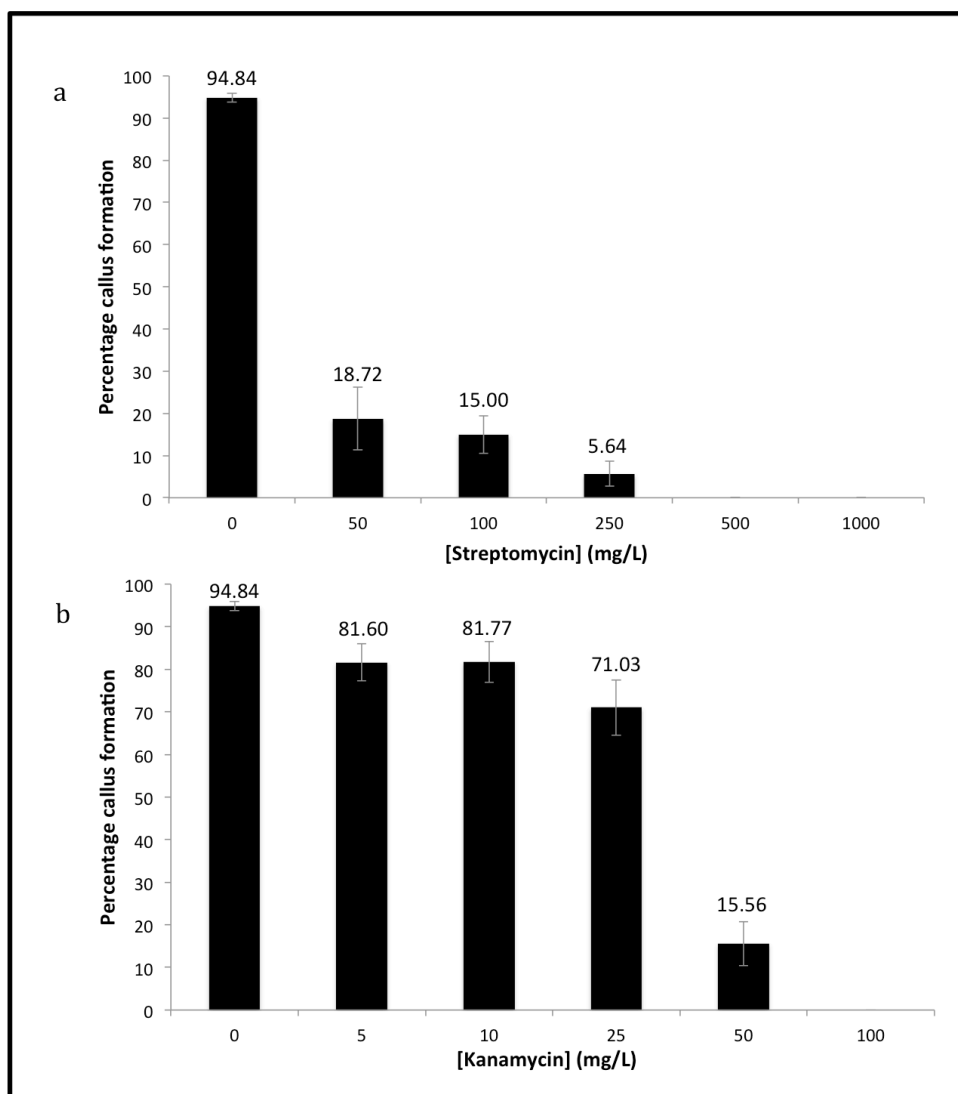
Fig 5.7 Percentage callus induction of piLS on CIM supplemented with varying concentrations of a, streptomycin; and b, kanamycin. Percentages for 0mg/L were calculated as described in chapter 4, and percentages above 0mg/L were calculated as piLS pieces forming any amount of callus out of total piLS pieces.

Both streptomycin and kanamycin inhibit embryogenic callus formation from piLS tissue on CIM, with maximum inhibition occurring at 500mg/L and 100mg/L respectively. Antibiotics bleached the tissue, killed it completely (Fig 5.8), or allowed the formation of only non-embryogenic callus. Callus pieces that formed on CIM supplemented with 250mg/L streptomycin, failed to regenerate when placed on R regeneration media. However, selection on Kanamycin appeared to be harsher than that with streptomycin, as the frequency of piLS pieces having undergone cell death was observably much greater.
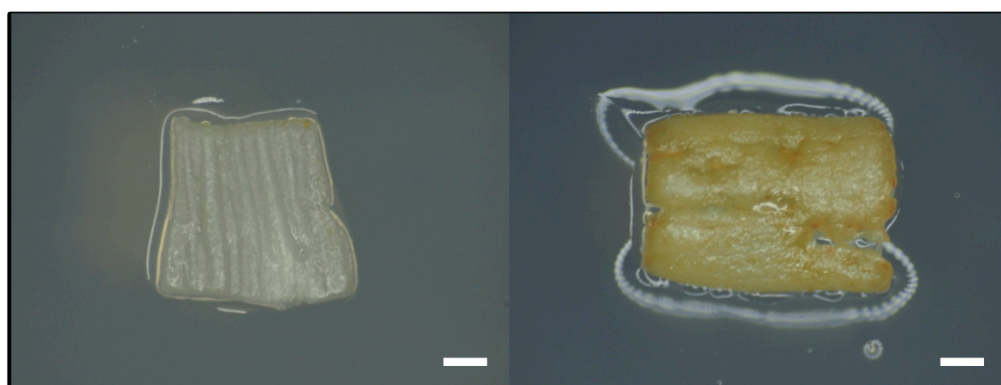
Fig 5.8 Effect of antibiotics on piLS during callus induction. piLS pieces would either bleach and remain inactive (left), or undertake cell death (right). Scale bars represent 1mm.

With these observations, I decided that selection of transformed chloroplasts from bombarded piLS pieces would be conducted on CIM supplemented with streptomycin between 250-500 mg/L, or kanamycin at 100mg/L.

5.3.4 Bombardment of piLS to observe transient GFP expression.

As an added control, I attempted to see if transient expression of GFP was possible in the chloroplast of piLS. Transient expression of fluorescent proteins in plastids following biolistic bombardment has been previously described (Hibberd et al., 1998). piLS were bombarded with 0.6μm gold carrying pRRes14mF2-TPrrn, and after 2-4 days incubation in the dark, were analysed for GFP expression using laser microscopy (see 2.5.3). In one bombardment experiment (10 piLS), clear GFP expression can be seen, although this was not localised to a chlorophyll expressing compartment (Fig 5.9

and 5.10). At this early stage of development, it is not unusual for chloroplasts or proplastids to lack chlorophyll expression, as has been confirmed by transient nuclear chloroplast targeted GFP expression (results not shown). The GFP expressing compartments appear similar in morphology to the chloroplasts that surround them, and are in similar number, therefore it would appear that these too are indeed plastids. The bombardment experiment was repeated numerous times, however similar results could not be obtained.
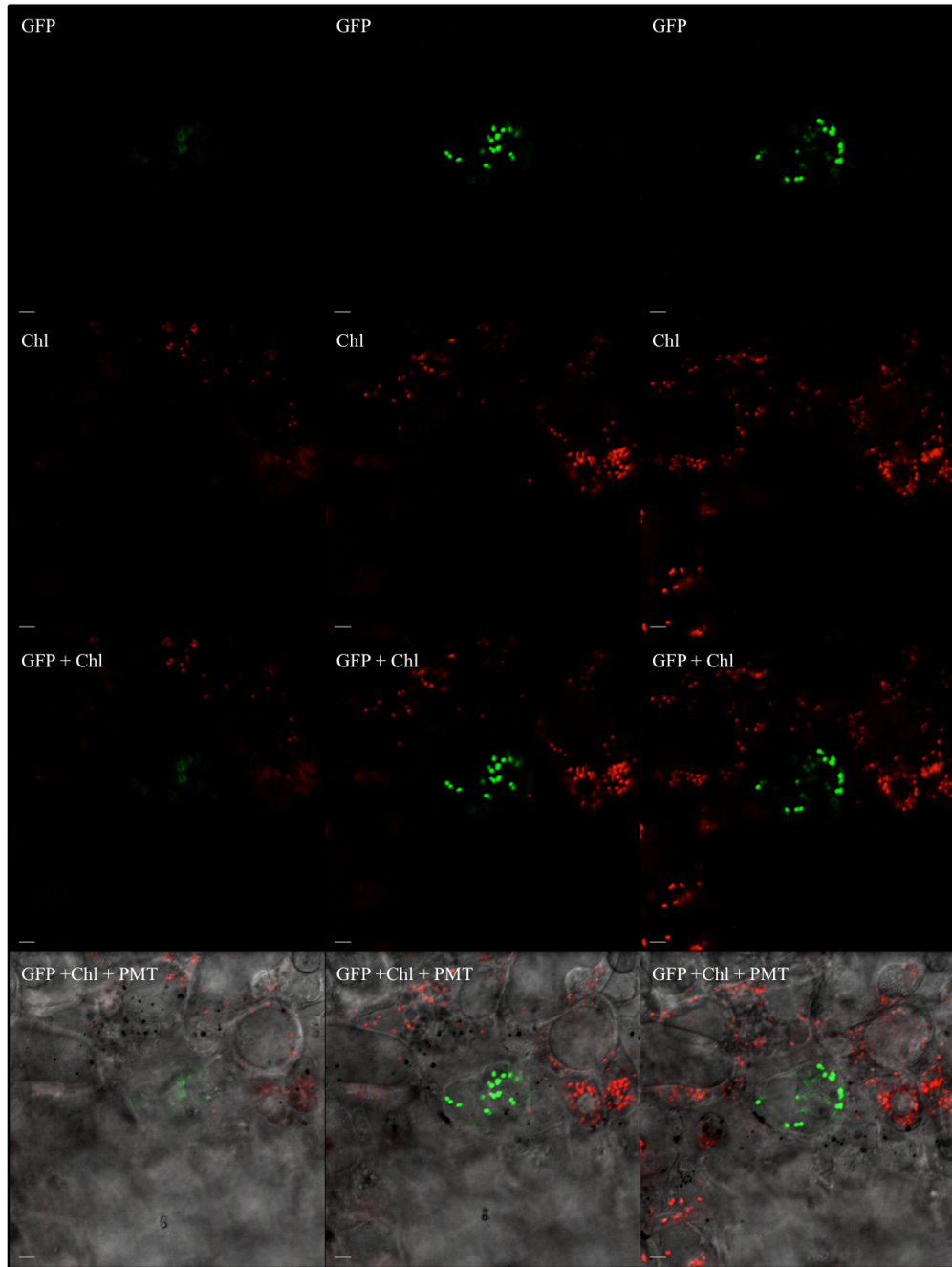
Fig 5.9 Transient GFP expression in bombarded piLS tissue. Each column of images represents a section through a specific location in a bombarded piLS piece. GFP, green fluorescent protein fluorescence; Chl, chlorophyll auto fluorescence; PMT, photomultiplier tube (background). Scale bar represents 5µm.
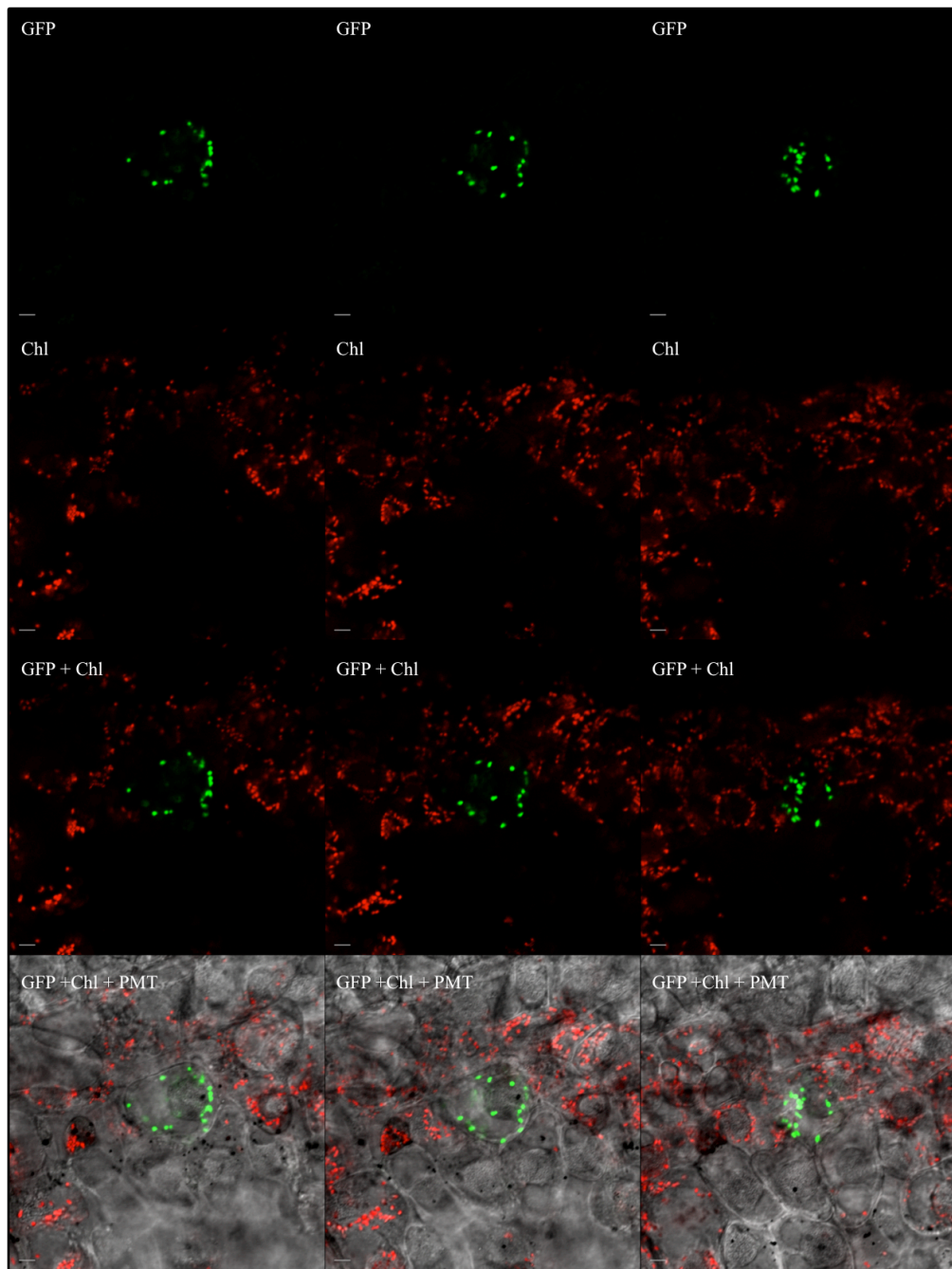
Fig 5.10 Transient GFP expression in bombarded piLS tissue. Continuation from Fig 5.9. Each column of images represents a section through a specific location in a bombarded piLS piece. GFP, green fluorescent protein fluorescence; Chl, chlorophyll auto fluorescence; PMT, photomultiplier tube (background). Scale bar represents 5μm.

5.3.5 Bombardment of IEC for stable chloroplast transformation

A total of 3860 IEC were bombarded in my investigations, and the different parameters for each bombardment study is detailed in table 5.1. Chloroplast transformation vector pRRes14mF2-WPrrn was used for all bombardments.

	Experiment	Explant no.	Age when bombarded (days)	CI Selection (mg/L)	Days on Selective CI	R selection (mg/L)
**	1	1260	21	0	0	500
	2	800	14	250	7	500
	3	800	14	400	14	500
*	4	500	21	400	14	500
*	5	500	14	400	14	500

Table 5.1 Bombarded IEC for chloroplast transformation. CI, callus induction, conducted on M(9%) supplemented with varying concentrations of streptomycin and hormones. R, Regeneration, conducted on R regeneration media (see) supplemented with streptomycin and hormones. * These calli were callus induced on WLS media.

For all bombardment experiments, small green cell masses/shoots were observed on 1/100 IEC while on selective R media (Fig 5.11). These cell masses were analysed for GFP expression using a confocal microscope, however, while chlorophyll auto fluorescence was observed, GFP fluorescence was not. When post bombardment incubation on non-selective callus induction media was conducted (See ** in table 5.1), large shoots were observed also forming while on selective R media, although prolonged exposure to selection eventually lead to bleaching (Fig 5.11f). No GFP expression was observed from any green cell mass or shoot that formed from bombarded IEC on selective R media. Some bombarded IEC were able to form roots, however after 6-8 weeks on selective R media, the IEC would undergo cell death. IEC would eventually turn yellow and then brown when undergoing cell death. None of the bombardment experiments produced any stable chloroplast transformation events.
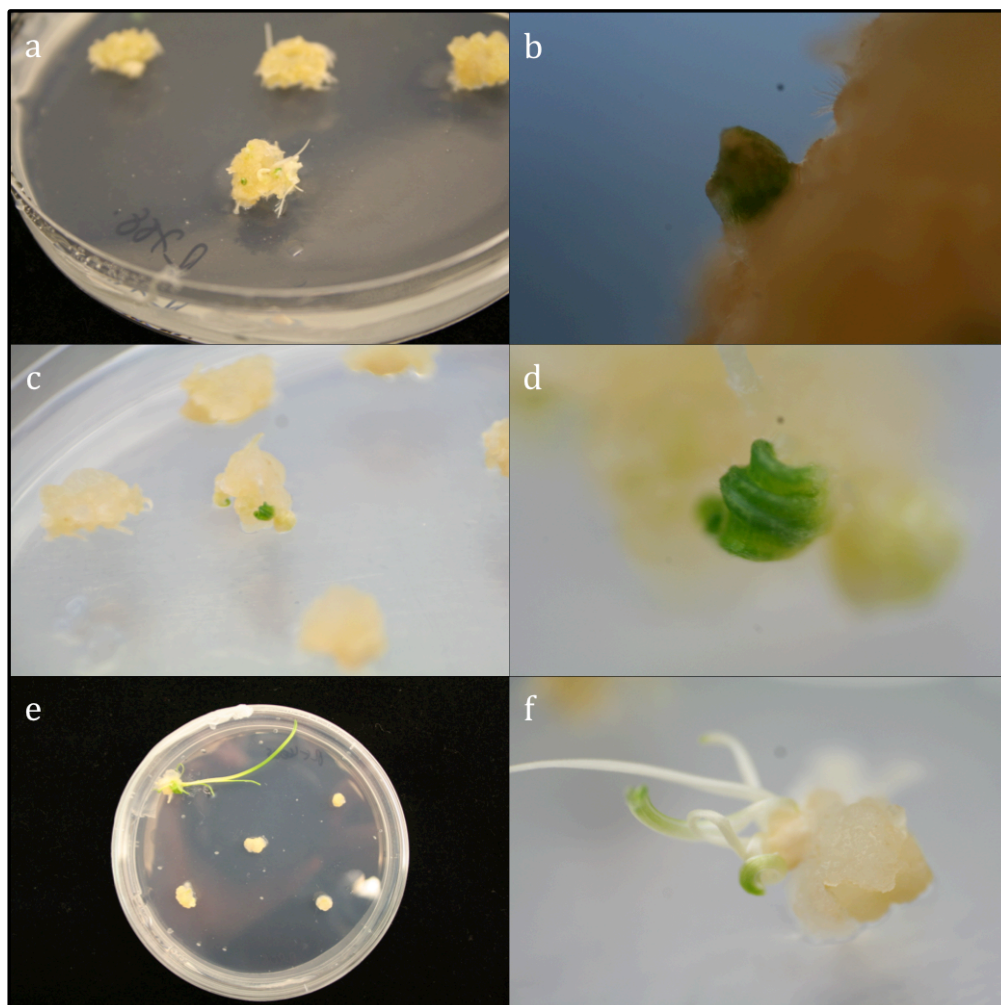
Fig 5.11 Green shoot formation on bombarded IEC. In all bombardment studies, small green cell masses/shoots (a-d) and large shoots (e-f) were observed when transferred to selective R regeneration media.

5.3.6   Bombardment of piLS for stable chloroplast transformation

A total of 1212 piLS were bombarded in my investigations, and the different parameters for each bombardment study is detailed in table 5.2. Chloroplast transformation vector pRRes14mF2-WPrrn was used for all bombardments.

Experiment	Explant no.	CI Selection	Days on Selective CIM	R selection
1	702	200	25-30	200
2	410	400	25-30	400
3	100	400	60	400

Table 5.2. Bombardment of piLS for chloroplast transformation. CI, CIM supplemented with varying concentrations of streptomycin and hormones. R, Regeneration, conducted on piLS R regeneration media supplemented with various concentrations of streptomycin and hormones.

Following bombardment and subsequent selection on CIM, some piLS pieces formed what appeared to be callus (Fig 5.12). Frequency of callus formation was greater with 200mg/L streptomycin selection than 400mg/L selection. Transferring these on to selective R media did not result in any green shoot formation from callus pieces, except for a single event that eventually bleached. The majority of callus pieces would die after 4 weeks on selective R media, and those pieces that continued to survive selection would form roots. When incubation on selective CIM was increased to 60 days, callus that formed appeared larger and more embryogenic. When placed on selective R media, callus failed to develop any resistant shoots, while some formed small roots/root hairs, and others died after 4 weeks. None of the bombardment experiments produced any stable chloroplast transformation events.
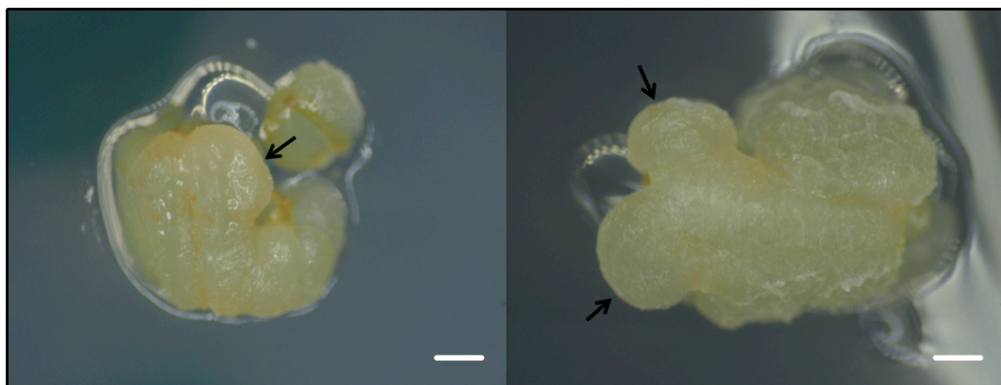
Fig 5.12 bombarded piLS pieces following 25-30 days incubation on selective CIM. Black arrows indicate possible embryoids. Scale bars represent 1mm.

5.4   Discussion

Progress of plastid transformation in the monocot species has been very limited, especially when compared to plastid transformation in the dicot species. Part of my investigation was to identify the bottlenecks that hinder the use of the chloroplast transformation technology in wheat. One of these bottlenecks is the absence of suitable explants for bombardment. In chapter 4, I detailed a previously undescribed tissue that contains abundant chloroplasts, the leaf sheath, and demonstrated a robust protocol for the regeneration of the primary leaf sheath (piLS). In this chapter, I have demonstrated that delivery of exogenous DNA via particle bombardment into piLS cells is not a bottleneck, and that a bombardment pressure of 1100 is sufficient to deliver gold particles into close proximity of chloroplasts. GFP fluorescence from plastids following bombardment of piLS with pRRes14mF2-TPrrn seen in piLS (Fig 5.9 and 5.10) was originally believed to be transient GFP expression, emanating from chlorophyll absent plastids. These plastids are located within one or two cells, which contain no chlorophyll expressing plastids. It is unclear whether all the plastids within this cell/these cells are expressing GFP, although when further magnified, plastids of similar size and shape that are not producing GFP or chlorophyll are also observable (Fig 5.13). Numerous repeats of this bombardment experiment did not produce any similar events, and therefore another explanation for the GFP expression is necessary.

Other members of the research group also use the biolistic bombardment gun used in this experiment. Although thorough cleaning and sterilisation of equipment takes place before and after each experiment, it is possible that the GFP expression is the result of contamination with a previously used vector. Looking through the gun usage history, 10 days prior to my bombardment experiment where GFP expression was observed, a vector containing a nuclear transformation cassette that targeted GFP to the chloroplast was used. The gun was also used three more times with vectors that do not contain GFP elements before I used it. Although it is highly unlikely that a gold particle carrying this vector DNA could have remained on a stopping screen or in the carrier chamber, or that vector DNA could have persisted despite numerous washes, the possibility of contamination being the cause of GFP expression cannot be ruled out, especially in the absence of a successful repeat. It is for this reason that I believe the transient GFP expression I have observed to be inconclusive.
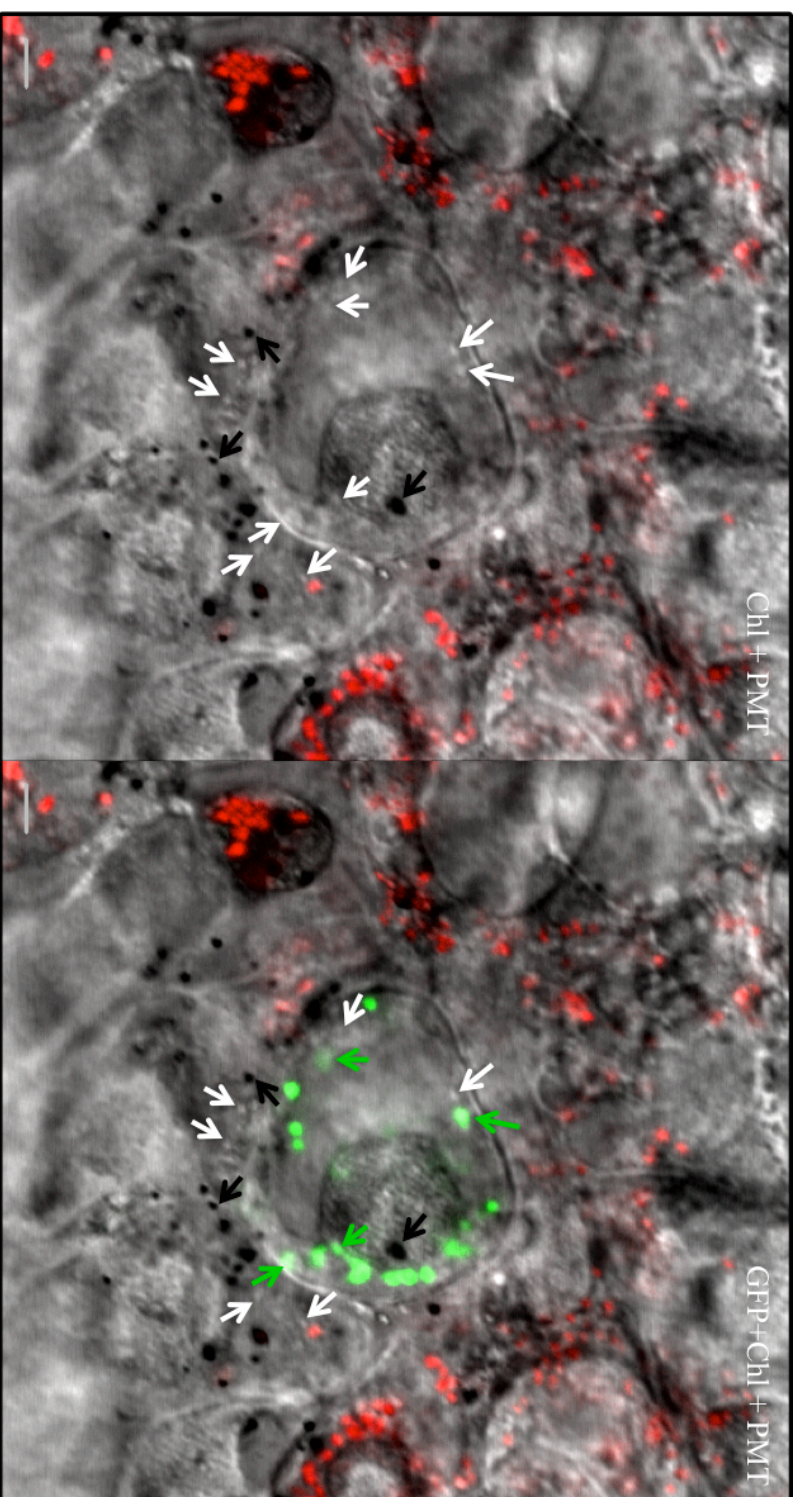
Fig 5.13 GFP excitation in chlorophyll absent plastids.. plastids (white arrows) of similar size and shape to chloroplasts (red) in adjacent cells show GFP expression (right, green arrows). Gold particles (black arrows) can be clearly observed. Scale bar represents 5μm.

Selection of transformed chloroplasts presents another bottleneck, which I have attempted to address in this chapter. Both IEC and piLS were tested for sensitivity to streptomycin instead of spectinomycin, as cereals such as wheat, are resistant to spectinomycin (Fromm et al., 1987). I was encouraged by the heteroplasmic transformation achieved in rice chloroplast transformation studies (Khan and Maliga, 1999, Lee et al., 2006), which used streptomycin for selection at a maximum concentration of 300mg/L. Exposure of IEC to streptomycin did not appear to have a significant cytotoxic effect, even at concentrations as high as 500mg/L. Callus tissue were able to grow and proliferate, and presented slightly less compact and watery callus formation. When 21 day old IEC were transferred to selective R regeneration medium, green shoot formation was completely inhibited at 500mg/L streptomycin. At these concentrations clear shoots formed from approximately 50% of callus pieces, although the density or shoot formation was less than IEC on non-selective R regeneration media. IEC pieces would eventually show cell death after 2 months on selective R regeneration media. Although IEC were still capable of growing and proliferating on selective R regeneration media, I believed that streptomycin demonstrated sufficient inhibition of green shoot formation and wheat plant regeneration to be used as a selection agent for cells containing transformed chloroplasts. Initially, 21 day old IEC were bombarded and moved directly onto selective R regeneration media. This did not produce any chloroplast transformation event, which suggested two things. First, 21 day old IEC are at a developmental stage where embryoids are ready to regenerate. Second, the time on selection following bombardment was not sufficient enough, and more time was needed to allow bombarded callus with transformed chloroplasts to proliferate. To test these hypotheses, in IEC bombardment experiments 2 and 3, I introduced bombarded IEC to selective M (9%) callus induction media for a period of time prior to transferring to selective R regeneration media. These conditions also did not produce any chloroplast transformation events, suggesting two things. First, IEC require exposure to selective callus induction for longer periods, and second, streptomycin does not provide sufficient selection to provide an advantage to transformed chloroplasts. To test the first of these hypotheses, I conducted IEC bombardment experiments 4 and 5, which increased the incubation time on selective callus induction media. Immature embryos cannot be callused on M (9%) for longer than 4 weeks, as by this point embryoids have reached optimum maturity and begin to form shoots (even if kept on callus

induction media), and recalcitrance increases. In order to keep callus incubated on selective callus induction for longer periods, I opted to use WLS media (Table 5.1) for callus induction. WLS media allows immature embryos to remain on callus induction up to 6 weeks. In experiments 4 and 5, IEC was formed on WLS, and following bombardment were transferred to selective WLS media for a period of time prior to transferring to selective R regeneration media. These conditions also did not produce any chloroplast transformation events, which suggests three things. First, bombarded callus needs a longer period of time on selective callus induction, second, streptomycin is not a robust enough selection agent for transformed chloroplasts, and third, IEC is not a suitable explant type. I believe it is mostly a combination of the first two, which is preventing chloroplast transformation in wheat from occurring. Given that a single chloroplast transformation even was not observed from 3860 bombarded embryos, strongly indicates that streptomycin is not a robust enough selection agent. Furthermore, the observations that IEC can still grow and proliferate on high amounts of streptomycin, in addition to the inability of streptomycin to completely inhibit shoot formation and cause a more rapid cell death, correlates with streptomycin not being an appropriate selective agent for chloroplast transformation in IEC.

Although streptomycin does not appear to be an appropriate selection agent for transformed chloroplasts in the IEC, it did inhibit callus formation of piLS. This provided encouragement that transformed chloroplasts would be positively selected for in bombarded piLS. In experiment 1 (Table 5.2), following bombardment and incubation on selective CIM for 25-30 days, some piLS pieces did produce what appeared to be callus, although these did not resemble the embryogenic callus normally observed in piLS tissue culture. These pieces were moved to selective R regeneration media, however resistant shoots did not develop. Callus either died or formed short roots/root hairs. Increasing the concentration of streptomycin to 400mg/L did result in an observable reduction in piLS pieces forming callus, however on R regeneration media, callus pieces produced the same results as observed in experiment 1. Due to the lack of embryogenic like formation from bombarded piLS following incubation on selective CIM, I hypothesised that the duration of time on CIM may not have been enough for resistant cells to form embryogenic callus. To test this, in experiment 3 (Table 5.2) I incubated bombarded piLS on CIM for 60 days, and observed callus formation that appeared embryogenic. These were transferred to selective R media, and also failed to produce resistant shoots.

134

Apogee piLS were also tested for their sensitivity to Kanamycin, as it has previously been demonstrated to be an effective selection agent for tobacco chloroplast transformation. I have demonstrated that kanamycin inhibits callus formation in piLS at 100mg/L. Unfortunately there was not sufficient time left in the project to conduct chloroplast transformation experiments using kanamycin as a selection agent.

In chapter 3, I described the construction of a number of chloroplast transformation vectors, and the successful use of the transformation cassettes in these vectors in transforming tobacco chloroplasts. These results indicated that all transformation vectors constructed were functional. I chose to only use one of these vectors, pRRes14mF2-WPrrn (containing the wheat Prrn), for two reasons. First, there was not sufficient time to test all the vectors, and doing so would also be inefficient as they were all functional and differed only in the promoter, and second, to maximise the chances of transgene expression, I used the transformation vector that contained the Prrn specific to wheat. Given the functionality of the WPrrn in tobacco chloroplasts, the transformation cassette is not a reason for the inability to achieve chloroplast transformation in wheat, and can therefore be used for further investigation.

5.5 Future investigations

IEC are highly embryogenic tissue, and do contain pro-plastids. However the inability to take IEC through numerous rounds of selection over several weeks is likely to prevent selection of transformed chloroplasts. In the wheat regeneration system, there is currently no cell line or protocol where an immature embryo can be continually proliferated through callus induction for several weeks. In maize, the Hi II Type II callus system provides such an option (Frame et al., 2000). Replicating such a protocol or cell line in wheat would be a wise option for further investigation of chloroplast transformation in wheat.

In my bombardment studies with piLS, I was unable to achieve chloroplast transformation. This could be due to a number of factors, first, streptomycin is not an appropriate selection agent, and second, incubation time on selective CIM was not sufficient enough to encourage growth of resistant callus. I have demonstrated that Kanamcyin significantly inhibits callus formation in piLS, and given its function in tobacco chloroplast transformation, should be tested in follow up experiments with piLS.

piLS are the only chloroplast abundant explant tissue that can be regenerated from, and should continue to be considered for chloroplast transformation studies. To increase the tissue's ability to undergo chloroplast transformation, increasing its regeneration capabilities is important. In the tobacco transformation system, every section of the young tobacco leaf can form a new plant. In comparison, the current regeneration protocol of apogee piLS used in our bombardment studies shows a regeneration capacity of approximately 20%. While this is a significant improvement from the situation prior to this work, it presents obvious limitations. In chapter 4, I have demonstrated that moving regeneration from R media to MS media supplemented with the same concentration of hormones increases the regeneration to approximately 25%. Further manipulation of hormone levels (0.25mg/L zeatin and 0.1mg/L 2,4D) indicated an increase of regeneration to approximately 40%. Unfortunately this improved protocol could not be tested for chloroplast transformation of wheat, as there was not sufficient time left to conduct further bombardment studies. However, this demonstrates the potential that exists within piLS callus to regenerate, and that further optimisation of regeneration from apogee piLS could result in higher levels of regeneration, and thus increasing the possibility of chloroplast transformation.

6 Improving photosynthesis in tobacco using the *ict*B gene

6.1 Introduction

6.1.1 The problem

Feeding an ever growing, meat hungry, human population presents many challenges. It is arguably the biggest threat to the longevity of our species, and thus food security has become a global priority. A change in human dietary behavior would ease this burden, and calls for a transition from meat heavy diets to ones that target lower trophic levels is taking place in developed countries. However with the members of the developing world now demanding more red meat as a result of greater economic prosperity, and this trend set to increase over the following decades, solutions to increase grain yield is necessary. With insecurity surrounding water and fertilizer availability, climate change, and the reduced availability of arable land further compounding the issue, the toolbox to meet future demands needs to be extensive. This toolbox will require GM technologies.

Wheat is produced on over 200 million hectares of land, and accounts for one fifth of the world's calorie intake. The gains that were once experienced during the green revolution are no longer being realized, and global yield increases have ground to a halt, while decreasing in some areas. This is not ideal given that the global demand for wheat is predicted to increase at a faster rate (Rosegrant and Cline, 2003) than the gains being realised (Miralles and Slafer, 2007, Fischer, 2007, Shearman et al., 2005).

6.1.2   **Possible solution**

To increase yield, we need to increase grain biomass, and for this we would therefore need to increase photosynthesis. Total crop photosynthesis is dependent on three factors, 1, the amount of light captured by the canopy, 2, the duration of light capture, and 3, photosynthetic capacity and efficiency. Agronomic crops such as wheat which are grown through high-input systems, and therefore light capture from the canopy has ultimately been optimised, leaving few improvements to be made. A way to increase the duration of light capture would be to either encourage earlier flag leaf growth, or to extend the photosynthetic period using an "evergreen" phenotype. However, given the inefficiencies observed in crop photosynthesis, the greatest improvements are likely to be attained by optimisation of photosynthetic capacity and efficiency.

As mentioned in chapter 1, the most distinct difference between crop photosynthesis (C_3) and more efficient models (C_4) is the CO_2 fixation pathway, catalyzed by the bi-functional carboxylase enzyme called RubisCO. C_4 plants have several advantages over C_3 plants. Firstly, by concentrating CO_2 in C_4 leaves, more CO_2 is fixed per photon absorbed due to an increase in RubisCO carboxylation (Skillman, 2008). Increased carbon concentration also increases the partial pressure of CO_2 around RubisCO, which results in the enzyme functioning at a maximal catalytic rate. This in turn ensures a smaller protein investment into RubisCO at any given CO_2 assimilation rate, hence increasing the CO_2 assimilation rate per unit of leaf nitrogen. Secondly, PEP carboxylase in C_4 plants uses bicarbonate formed by carbonic (CA) rather than CO_2 in C_3 plants. Because of this, PEP carboxylase can satisfy the C_4 pump at intercellular carbon concentrations lower than that in C_3 plants, consequently leading to C_4 plant having greater transpiration efficiencies. Thus, a combination of all the above attributes results in C_4 plants able to fix more carbon per unit of light, nitrogen, and water. Wheat is a C_3 plant. Therefore in theory, if wheat can be modified to photosynthesise with more C_4 type characteristics, yield can be increased.

Targets for improving photosynthesis have been discussed in chapter… One of these targets is intercellular carbon concentration. As discussed above, increasing the partial pressure of CO_2 surrounding RubisCO results in an increase in carbon fixation. Introducing a carbon concentrating mechanism (CCM) into the chloroplast of wheat, would in theory increase carbon fixation and biomass accumulation. C_3 crops grown at elevated carbon concentrations in the field demonstrate an increase in yield, however

at a far lesser increase than is theoretically possible (a third in some instances, (Long et al., 2006a)

6.1.3 Cyanobacterial carbon concentrating mechanisms

Extant cyanobacterial species and chloroplasts share common ancestry. Due to their evolutionary relationship, research into cyanobacterial photosynthesis has advanced our understanding of photosynthesis significantly. Cyanobacterial carbon fixation provides a rich source for information when devising biotechnological ideas for improving photosynthesis in C_3 plants. One aspect of the CCM involves active and passive transporters of inorganic carbon (HCO_3^-). Cyanobacteria exist in aquatic environments where the solubility of bicarbonate ions is much higher than that of CO_2, with the natural hydration/dehydration equilibrium favouring the formation of HCO_3^-. It comes as no surprise that cyanobacteria have thus evolved several mechanisms in the form of transporters for the import of HCO_3 along with CO_2. Once transported into the cyanobacteria, these inorganic carbons are fixed into 3-phosphoglycerate (3-PGA) in the carboxysomes, specialised semi-permeable protein shells that concentrate CO_2 around the encapsulated RubisCO. Plant chloroplasts do not contain carboxysomes, instead RubisCO is free within the stroma. However, one can theorise that if inorganic carbon transporters can be introduced into plant chloroplast membranes, thus increasing the concentration of CO_2 surrounding RubisCO following conversion of bicarbonate via stromal carbonic anhydrase, this would likely increase the rate of photosynthetic carbon fixation. One such protein, designated as inorganic carbon transporter B (IctB), discovered in *Synechococcus elongatus* PCC 7942, was originally thought to be a HCO_3^- transporter (Bonfil et al., 1998).

6.1.4 ictB

Inorganic carbon transporter B (ictB) is a protein first discovered in *S. elongatus* PCC 7942. Cyanobacteria, such as *S. elongatus* PCC 7942, possess an inducible mechanism to concentrate inorganic carbon (iC) within the cells. A high CO_2 requiring mutant of *S. elongtus* PCC 7942 was isolated and found to exhibit inhibited HCO_3^- uptake, which was attributed to a mutation in the *ictB* gene (ORF467)(Bonfil et al., 1998). ictB mutants grew almost as wild type (WT) under normal CO_2 concentrations, however were unable to grow under low CO_2 due to the deficiency in HCO_3^- transport. This combined with the ictB protein having 12 predicted membrane spanning domains, a high homology to several transporter proteins, and it being located on the inner membrane of the cell, ictB was believed to be a HCO_3^- transporter. Although the correlation between ictB expression and iC accumulation in *S.elongatus* PCC 7942 is clear, and ictBs importance in iC uptake in *Sunechocystis sp.* PCC 6803 has been demonstrated, how it achieves this is not yet fully understood (Shibata et al., 2002), and the likelihood of ictB functioning as a HCO_3^- transporter is highly unlikely (Xu et al., 2008). Despite the lack of evidence supporting ictB's function as a HCO_3^- transporter, expression of ictB in *Arabidopsis thaliana* and *Nicotiana tabacum* resulted in a photosynthetic rate phenotype that was faster than that observed in the WT under limiting conditions, but not under saturating conditions (Lieman-Hurwitz et al., 2003). Both *A. thaliana* and *N. tabacum* ictB transgenics also showed a lower CO_2 compensation point (5-9 ppm) compared to WT, suggesting a higher accumulation of CO_2 in close proximity to RubisCO in the transgenics. Even if ictB is not functioning as a HCO_3^- transporter, it clearly has a carbon concentrating mechanism, even when expressed in the nucleus and targeted to the chloroplasts (Lieman-Hurwitz et al., 2003). The question we have asked in this chapter, is what effects on photosynthesis would ictB have when expressed in the chloroplasts of a C_3 plant, in this case, *N. tabacum*.

6.1.5 Aims

- Produce homoplasmic tobacco plants containing the cyanobacterial *ictB* gene.
- Analyse the effect of *ictB* expression on internal carbon concentration, photosynthesis, and growth.

6.2 Material and methods

6.2.1 Chloroplast transformation of tobacco via Biolistic bombardments

For information on explant preparation, bombardment, and selection parameters, see chapter 2.1

6.2.2 pBNG1

pBNG1 is a tobacco chloroplast transformation vector (Fig 6.1)
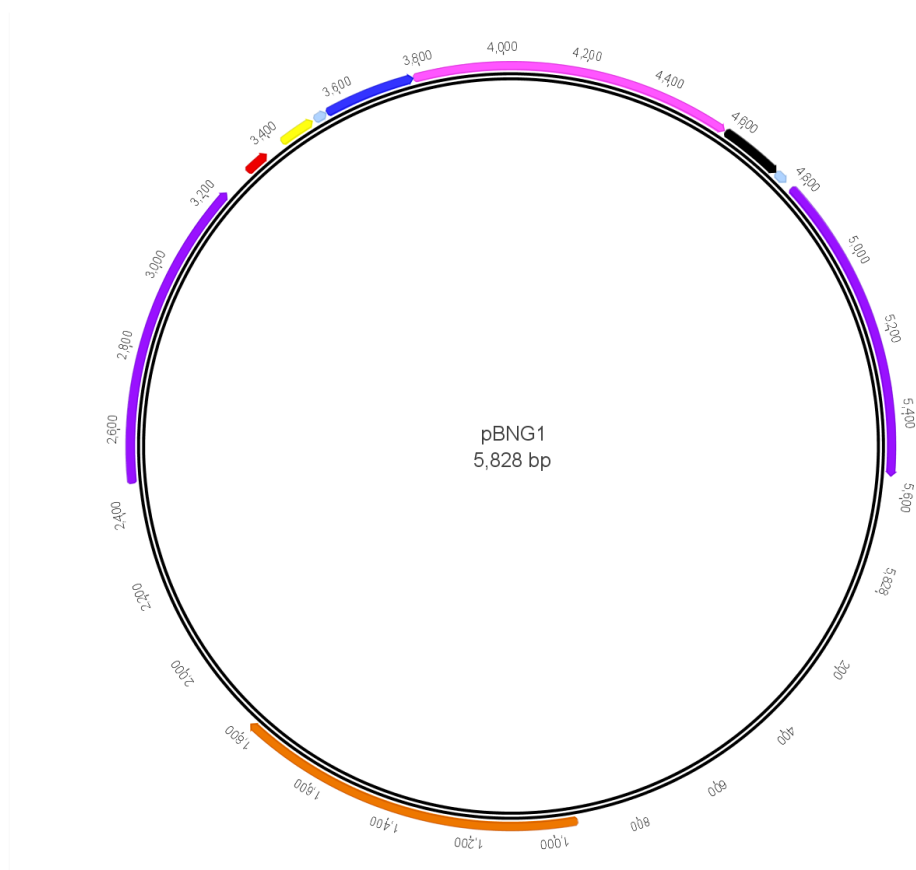
Fig 6.1 pBNG1. Gift from Prof Maureen Hansen (Cornell University, Ithaca, NY, USA). A chloroplast transformation vector targeted to the *trnI/trnA* gene region (purple flanking arms), with ampicillin resistance (orange). Transformation cassette contains a T7g10 5'UTR (red), tobacco psbA 3'UTR (yellow), loxP sites (light blue), tobacco psbA promoter (blue), *aadA* gene (pink), and the trps16 3'UTR (black). A multiple cloning site is located between the T7g10 5'UTR and tobacco psbA 3'UTR.

6.2.3   Cloning of pBNG1-ictB

6.2.3.1   ictB amplification

The ictB gene was amplified from the nuclear transformation vector pRRes_ictb (Dr Steven Driever) using PCR as described in Chapter 2. Primers used for amplification are described in Table 6.1 PCR primers were designed to add an *Nde*I and *Not*I restriction endonuclease site to the 5' and 3' end of the amplicon respectively.

Primer	Sequence	Restriction site	Tm °C
ictB F1	CATATGACTGTCTGGCAAACTCTGACTTTTGC	Nde I	60
ictB R1	GCGGCCGCCTACATTTTTTCGTCTGAATGCTC	Not I	

Table 6.1 Primers used for the amplification of ictB from pRRes_ictb. Red bases indicate engineered restriction sites. Expected amplicon size is 1418bp.

6.2.3.2   DNA extraction of ictB amplicon from agarose gel

Using ultraviolet light, a DNA band was first identified in the agarose gel, and then excised with a clean sharp blade. As much as possible of the agarose was removed at the time of excision. The excised band was then placed in a 2ml eppendorf, and DNA extracted from the band using a Wizard® SV gel and PCR clean up kit (Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA), following manufacturer's instructions.

6.2.3.3   Cloning into the pGEM®-T easy vector and pBNG-1.

Poly-A tailing of ictB amplicon was conducted as described in 2.3.1. Cloning of poly-A tailed ictB into pGEM®-T easy vector was conducted as per manufacturer's instructions, or as described in Chapter 2.3.

6.2.4   Molecular characterisation of transplastomics

To determine successful integration of transgenes into tobacco chloroplasts, DNA from leaves from resistant plantlets were extracted as described in 2.2.1. PCR was then conducted as described in 2.2.2 and 2.2.3. Primers for determining successful integration are detailed in table…

Primer pair	Sequence (5'-3')	Tm°C	Band size (bp) w/o insert	Band size (bp) with insert
16s rRNA F1 pBNG1-ictB **R1**	CGAATCCTCTTGAAAGA CTGTAGGTGAGGATCAGA	52	0	2480
16s rRNA F1 **Tob 23s R1**	CGAATCCTCTTGAAAGA CTAGGTATCCACGTAAG	53	2653	5476

Table 6.2 Primer pairs used to determine successful integration of exogenous DNA into the tobacco plastome.

6.2.5 RNA isolation, cDNA synthesis, and qPCR

Total RNA was extracted from 100mg of snap-frozen leaf tissue from *N. tabacum* WT and transplastomic lines using the Ribopure™ Kit (Ambion®) according to the manufacturer's instructions. RNA was quantified using a Nanodrop spectrophotometer and integrity of RNA was visualised using denaturing agarose gel electrophoresis (Sambrook *et al.* 2000). DNA was removed using RQ1 RNase-free DNase (Promega). cDNA was synthesised using SuperScript® III First-Strand Synthesis System (ThermoFisher Scientific) using 2ug of total RNA and oligodT primers according to the manufacturer's instructions. Gene expression was quantified using SYBR Green chemistry on a Real-Time PCR system 7500 (Applied Biosystems). Total reaction size was 20µl containing 10µl SYBR® Green Jumpstart™ Taq ReadyMix™ (Sigma Aldrich), 2µl cDNA and 0.5mM primers. PCR used an initial denaturation stage of 95°C for 2 mins, followed by 40 cycles of 95°C for 15s (denaturation), 60°C for 1 min (annealing and extension). The specificity of products was confirmed by performing a temperature gradient analysis of products at temperatures ranging from 55°C to 95°C at 0.5°C increments. Two technical replicates were completed for each sample. Data was normalised using the nuclear 18s rRNA gene, and the chloroplast 16s rRNA gene. Primers were designed using Geneious software, and are described in Table 6.3

Primer pair	Sequence (5'-3')	Tm°C	Band size (bp)
ictB qPCR			
ictB-qPCR F1	GTGTCTACGGCCTCAACCAA	60	255
ictB-qPCR R1	CGCGACTGTAGGTGAGGATC		
ictB-qPCR F2*	GTGGCTTCTTGCTTGCTGTC	60	264
ictB-qPCR R2*	CCGCTAGGGCAAAAACCAAC		
16s qPCR			
16srRNA-qPCR F1	CTGAACAGACTGCCGGTGAT	60	219
16srRNA-qPCR R1	GTATGGCTGACCGGCGATTA		
18s rRNA qPCR			
18srRNA-qPCR F1	GTGCAACAAACCCCGACTTC	60	254
18srRNA-qPCR R1	CCTTGGATGTGGTAGCCGTT		

Table 6.3. qPCR primers. * Alternative primer pair tested but not used for qPCR.

6.2.6 **Measurement of photosynthesis**

Leaf photosynthesis, stomatal conductance, transpiration, and intercellular carbon concentration measurements were made simultaneously using a portable leaf gas exchange and fluorescence system (LI-6400XT; LI-Cor, Lincoln, NE, USA), as per manufacturers instruction.

The measurements were made during the daylight hours of 9am-3pm. Fully expanded fourth leaves were clamped on the 2cm^2 chamber. Leaves were allowed to stabilize for 2 mins at these following conditions in the cuvette: Cooler block temperature (Tblock) of 20 °C, Flow set to 200 µmol s^{-1}, Chamber fan speed at 5, CO_2 concentration in the cuvette (C_a) of between 200-400 µmol mol^{-1}, and Photosynthetic Active radiation (PAR) of 1500 µmol m^{-2} s^{-1}. Statistics on data was carried out using a students t-test. Significance was determined as p=0.05.

6.2.7 **Measurement of plant height, biomass, leaf length, and leaf width**

Heights of transplastomic *N. tabacum* and WT plants were measured using a tape measure, starting from the base of the plant, to the tip. The fourth expanded leaf was measured for leaf length and width. Leaf lengths were determined by running string from the leaf base to the blade tip, and then measuring the string. Leaf widths were measured at the widest point of the leaf blade using a 30cm ruler. Biomass was calculated by drying transplastomic and WT plants in an oven at 80°C until completely dry, then weighed on a scientific balance. Statistics on data was carried out using a students t-test. Significance was determined as $p=0.05$.

6.3 Results

6.3.1 Production of pBNG1-ictB

The *ictB* gene (derived from cyanobacterium Synechococcus PCC 7942) was amplified from a monocot codon-optimised sequence located in the pRRes_ictb nuclear transformation vector. PCR products were run on an agarose gel (Fig 6.2, left), and ictB amplicons were gel extracted, poly-A tailed, and cloned into the pGEM®-T easy vector to give pG-ictB. The pG-itcB vector was amplified and DNA extracted using a plasmid mini-prep kit. Isolated pG-ictB was double digested with *nde*I and *not*I to release the ictB gene (Fig 6.2, centre). The ictB gene, with the engineered 5' and 3' ends, was gel extracted and cloned into linearised pBNG1 (also double digested with *nde*I and *not*I) to give pBNG1-ictB (Fig 6.3). A diagnostic restriction digest with *pvu*II demonstrated successful integration of ictB into pBNG1 (Fig 6.2 right). Sequencing also confirmed sequence integrity.
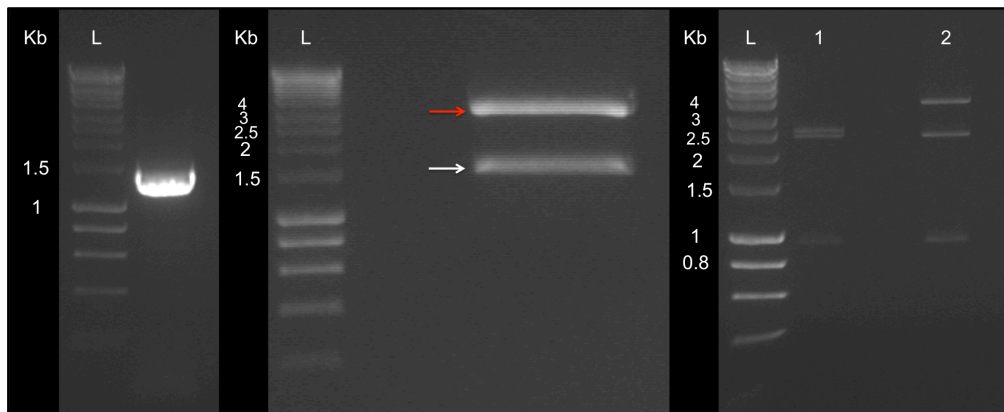
Fig 6.2 Construction of pBNG1-ictB. Left, amplification of ictB from pRRes_ictb. Expected amplicon is 1418 bp in size. Centre, pG-ictB digested with *nde*I and *not*I to release the ictB gene (white arrow, 1418bp) from the pGEM®-T easy vector backbone (red arrow, 3015bp). Right, digestion with *pvu*II of pBNG1 (Lane 1) to give 3 bands of 939, 2364, and 2525bp, and pBNG1-ictB (Lane 2) to give 3 bands of 939, 2364, and 3654bp.
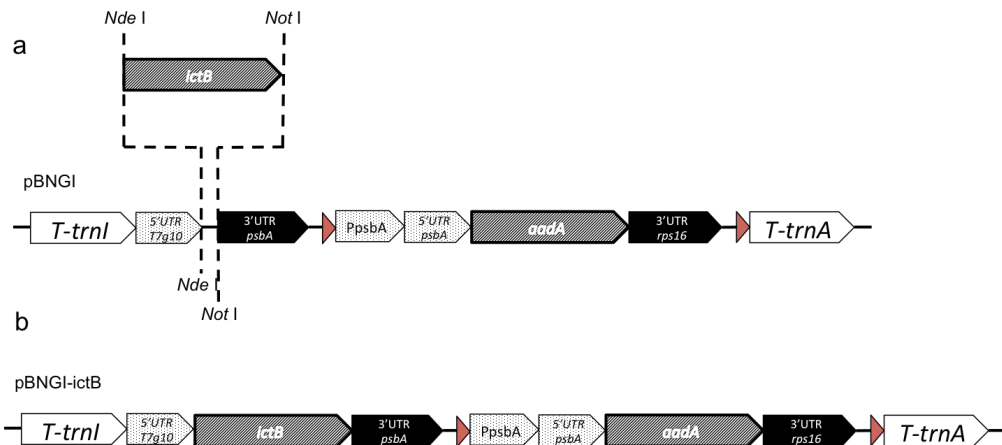
Fig 6.3 The cloning of ictB into pBNG1 (a) to give (b) pBNG1-ictB. *T-trnI*, tobacco trnI gene; *T-trnA*, tobacco trnA gene; PpsbA, Tobacco psbA promoter. pBNG1 is described in…

6.3.2 Production of ictB transplastomic tobacco plants

Tobacco leaves were bombarded with the pBNG1-ictB chloroplast transformation vector. Two individual bombardment studies were conducted, with each study containing 10 bombarded plates (1 leaf/plate). Following approximately 4-6 weeks on selective RMOP, shoots from callus started to form (Fig 6.4). During the same period of time, resistant cell masses formed from callus (Fig. 6.5), however some of these did not produce shoots. Diagnostic PCR on total DNA from resistant shoots/cell masses with appropriate primers determined successful integration of exogenous transformation vector DNA into plastome. Total resistant shoots/cell masses were cultured further and grown into plants (Fig 6.6). Five successful transplastomic lines, designated ictB-1, ictB-2, ictB-3, ictB-4, and ictB-5, were grown to seed. Seeds were extracted from dried seed pods, and allowed to dry further for 2 weeks.
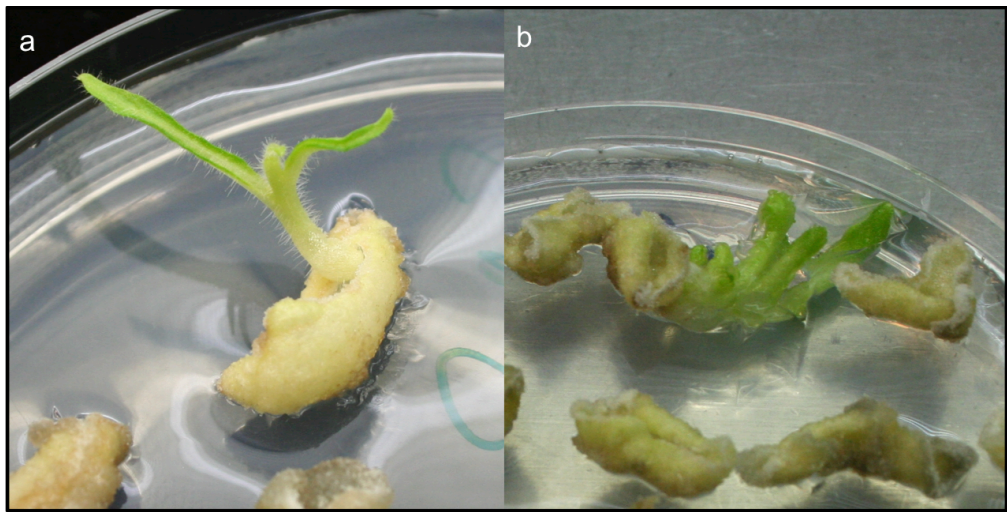
Fig 6.4 Resistant shoots forming from calli after a, 4 weeks and b, 6 weeks on selective RMOP.
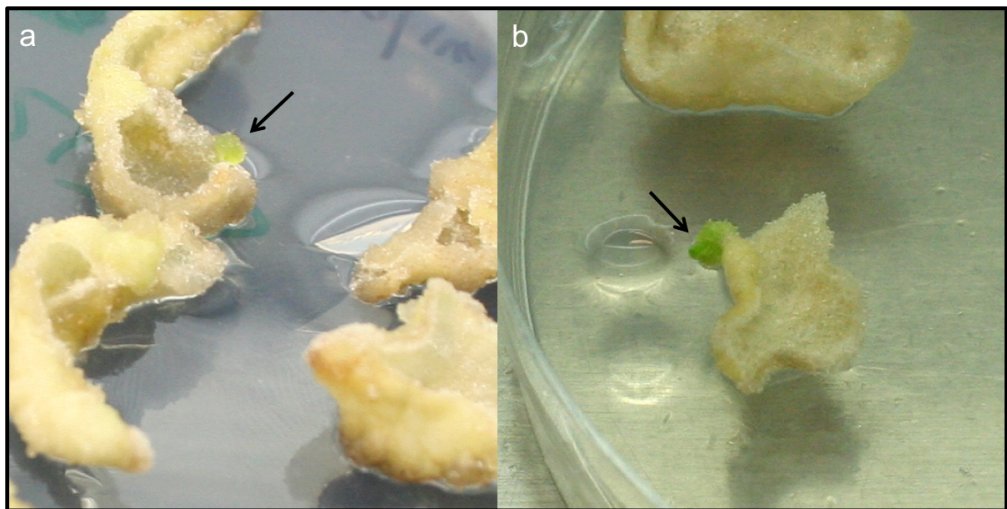
Fig 6.5 Resistance cell masses (black arrows) developing from callus on selective RMOP. Some masses (a) did not go on to produce regenerated plants, whereas others (b) did.
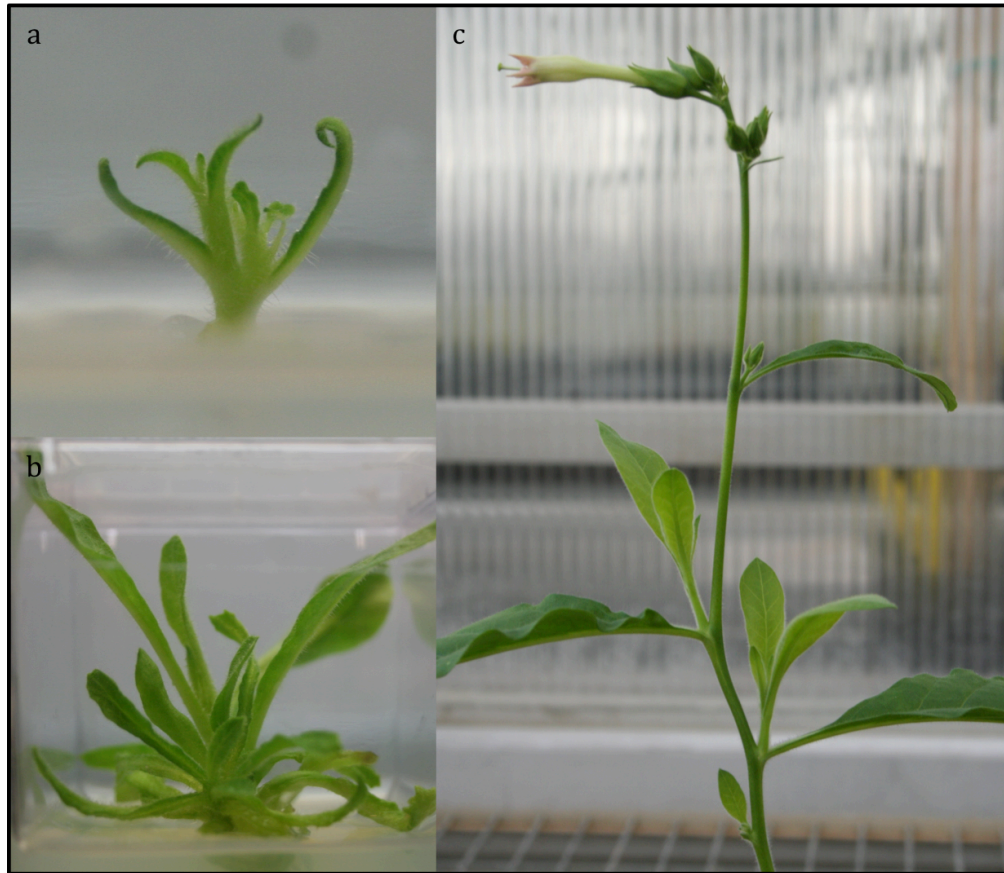
Fig 6.6 Regeneration of transplastomic plants from resistant shoots. After identification of resistant shoots/cell masses on selective RMOP, they were separated from the callus and cultured further on selective RMOP for up to 2 weeks before being transferred to a magenta box containing selective RMOP (a). Confirmation of successful cassette integration was conducted by extracting DNA from shoot sections, and performing PCR with primers described in Table 6.2. Once the shoot has developed into a plantlet (b), it was transferred to selective MS to encourage root formation. When sufficient roots were formed, the transplastomic plantlet was transferred to soil and allowed to develop into a plant (c).

6.3.3 Molecular characterisation of transplastomics

The seeds from the 5 transplastomic lines were sown, and the plantlets of these were tested for successful integration of pBNG1-ictB vector DNA. The forward primer (16s rRNA F1) binds to a location outside of the left flanking arm, while the reverse primer (pBNG1-ictB R1) binds to a location within the ictB gene itself. From the results (Fig 6.7), it is clear that the cassette has successfully integrated into the plastome.
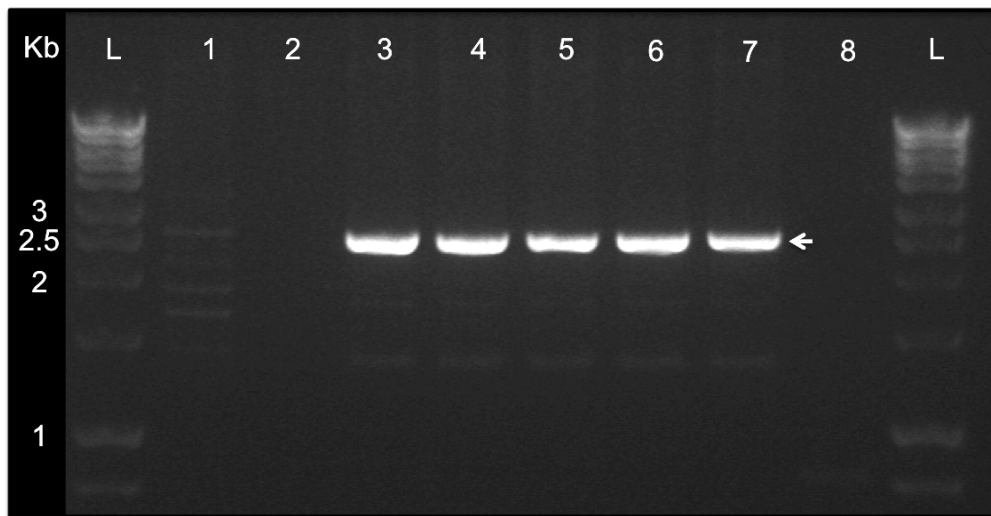
Fig 6.7 PCR analysis of DNA extractions from the 5 transplastomic lines. White arrow indicates a band at the expected 2480bp, confirming integration of the chloroplast transformation cassette. L, 1 Kb ladder; 1, WT; 2, blank; 3, ictB-1; 4, ictB-2; 5, ictB-3; 6, ictB-4; 7, ictB-5; 8, blank.

To determine homoplasmy (that all plastomes have been transformed), a second PCR was conducted with the forward primer (16s rRNA F1) and the reverse primer (Tob 23s R1) both binding outside of the region of the chloroplast transformation flanking arms, but within the area of IR region (Fig 6.8). Here we can once again confirm that the transformation cassette has successfully integrated within the plastome of all lines. However, as amplification also produced a band at approximately 2653bp, which is present in WT, we cannot definitively say that the transplastomics are homoplasmic.
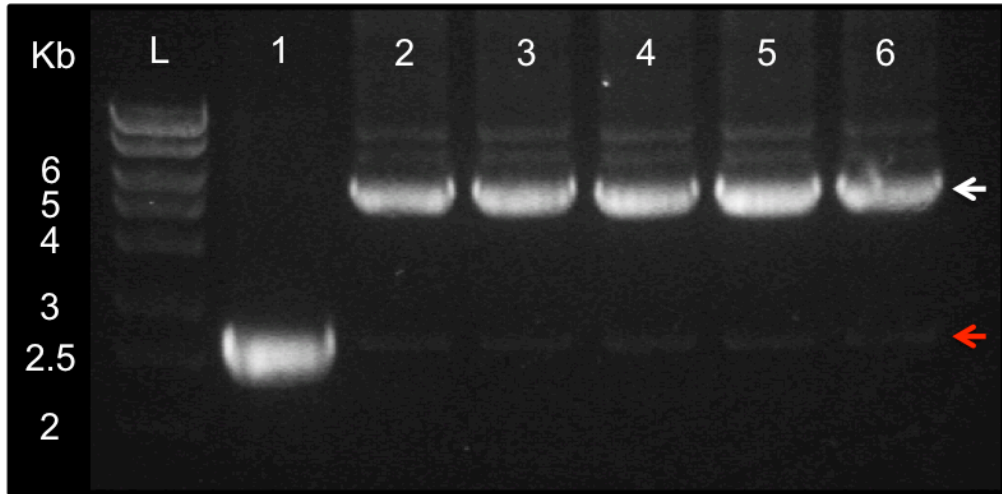
Fig 6.8 PCR analysis of DNA extractions from the 5 transplastomic lines. White arrow indicates a band at the expected 5476bp, confirming integration of the chloroplast transformation cassette. Red arrow indicates a band at the expected 2653bp following amplification of WT. L, 1 Kb ladder; 1, WT; 2, ictB-1; 3, ictB-2; 4, ictB-3; 5, ictB-4; 6, ictB-5.

6.3.4 qPCR for the expression levels of ictB

Primers to be used for qPCR analysis were first tested on DNA extractions from ictB-1 (Fig 6.9). All primer pairs produced bands of expected sizes.
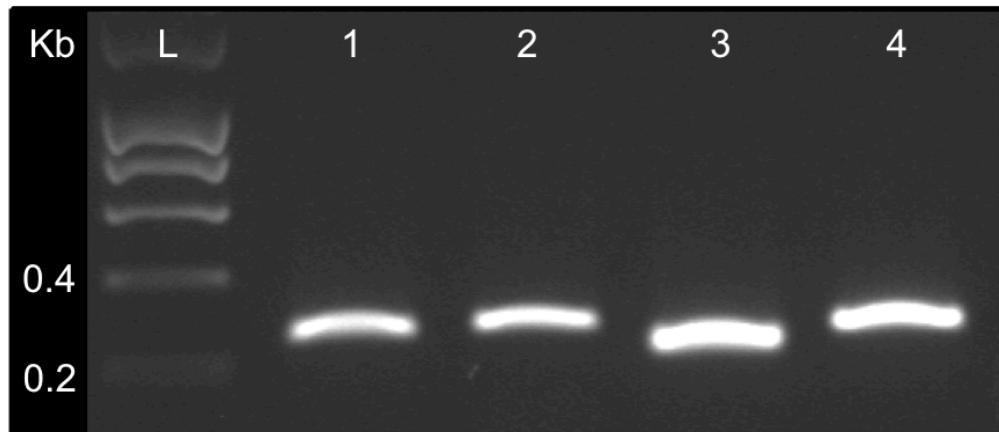
Fig 6.9 Testing of qPCR primers. DNA extractions of ictB-1 were used as the template to test the qPCR primer pairs 1, ictB-qPCR F1/ictB-qPCR R1; 2, ictB-qPCR F2/ictB-qPCR R2; 3, 16srRNA-qPCR F1/16srRNA-qPCR R1; and 4, 18srRNA-qPCR F1/18srRNA-qPCR R1. L, 1 Kb ladder. See Table 6.3 for corresponding amplicon sizes.

From the qPCR data (Fig 6.10), when compared to expression levels of the nuclear 18s rRNA and plastid 16s rRNA genes, it is clear that ictB is being expressed within the chloroplasts of each transplastomic line.
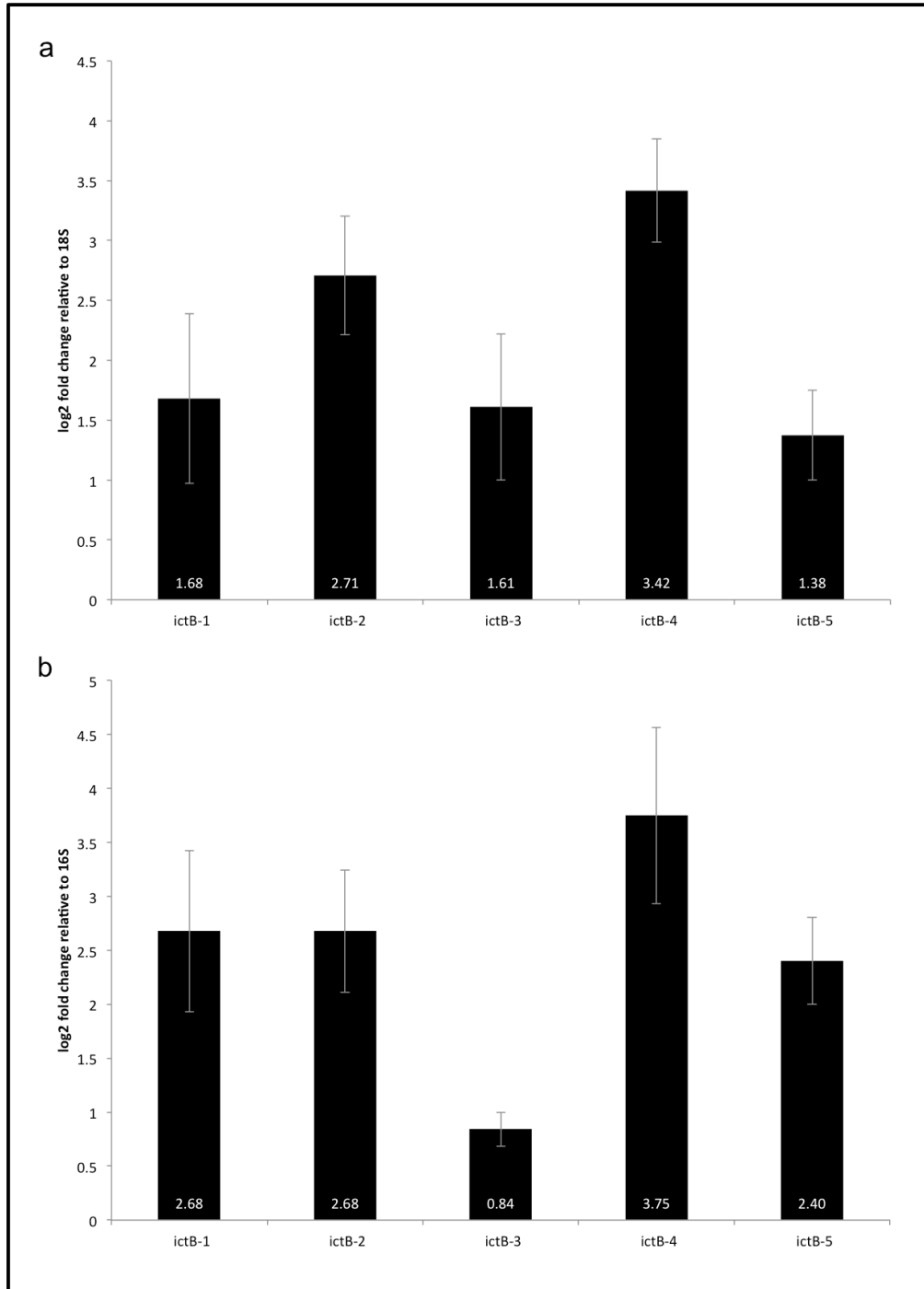
Fig 6.10 Relative expression of ictB in transplastomic lines compared to a, 18s rRNA and b, 16s rRNA transcript levels.

When compared to 18s rRNA expression, ictB-2 and 4, appear to have the greatest level of ictB expression, and ictB-1, 3, and 5 have similar levels of expression. When compared to 16s rRNA expression, ictB-4 shows greatest ictB expression, while ictB-3 shows the least ictB expression.

6.3.5 Phenotype of transplastomic ictB plants

The confirmed expression of ictB within transplastomic lines indicates the successful transcription of ict. To see what affect this has upon the phenotype of transplastomic lines compared to WT, I looked at changes to photosynthetic parameters and growth characteristics.

6.3.5.1 Effects of ictB on photosynthesis

The photosynthetic rates of all ictB transplastomic lines were lower than that for WT, with the greatest decrease in rate seen in ictB-1 at both ambient (400ppm) and low (200ppm) CO_2 concentrations (Fig 6.11 and Fig 6.12). On average, photosynthetic rates in ictB lines were 1.658 (7.5%) and 0.69 (6.5%) μmol CO_2 m^{-2} s^{-1} lower than that for WT at ambient and low CO_2 concentrations respectively.
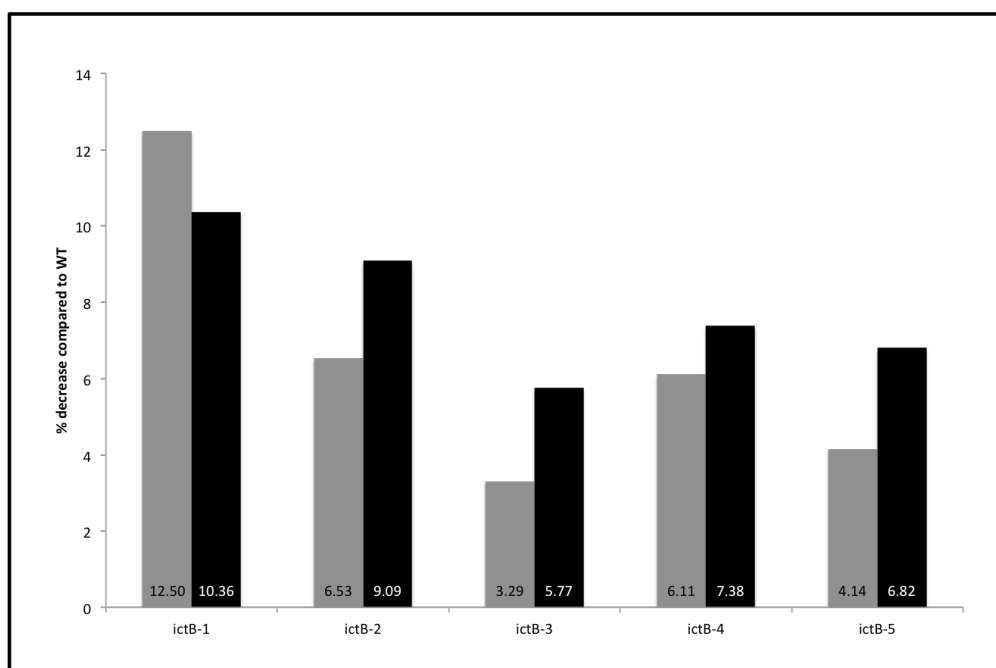
Fig 6.11 Percentage decrease of photosynthetic rates in ictB lines compared to WT at 200ppm (grey) and 400ppm (black) concentrations of CO_2. 6 biological replicates and 4 technical replicates per measurement.
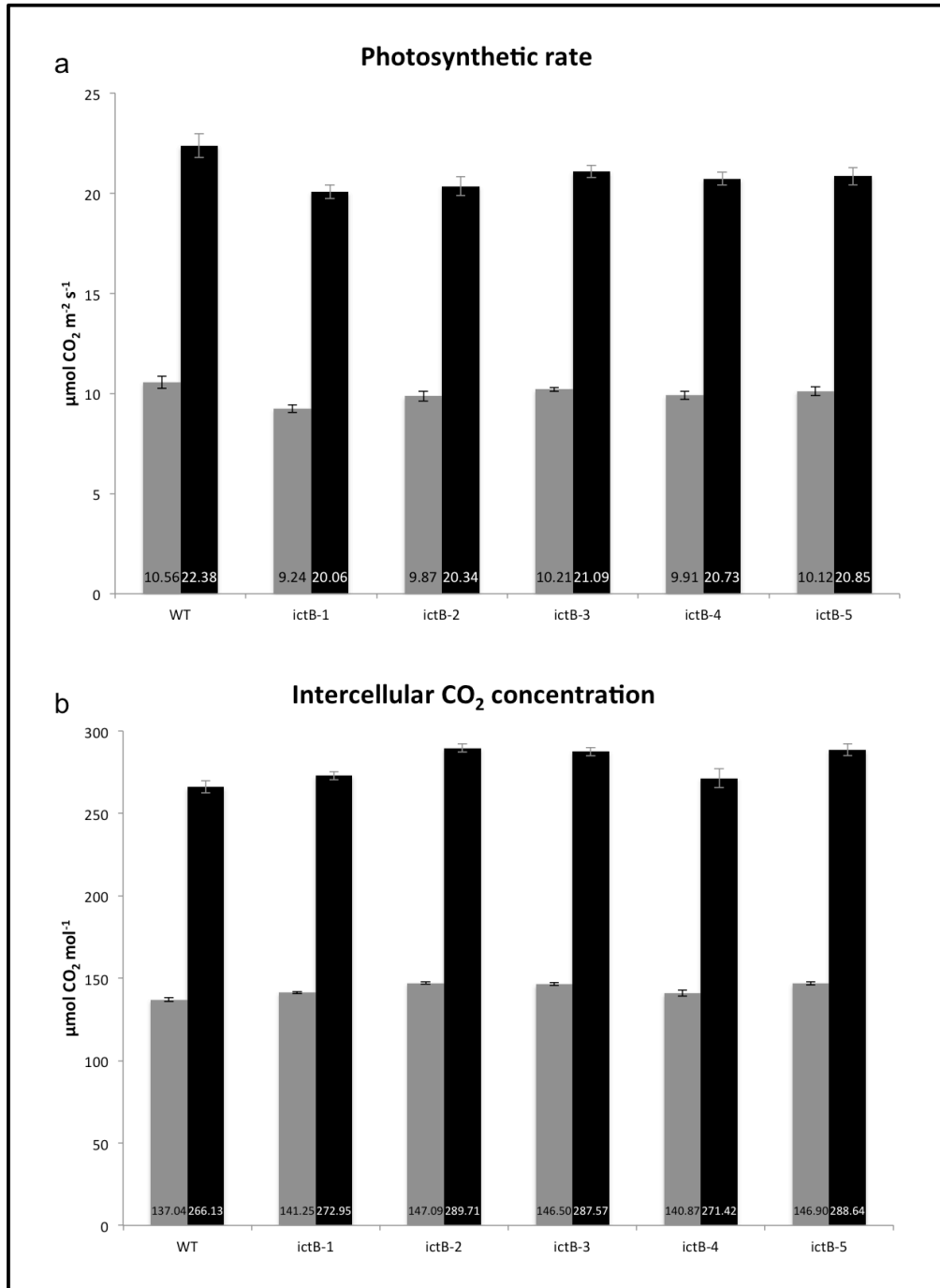
Fig 6.12 Photosynthetic (a) and transpiration (b) rates of WT and ictB lines at 200 (grey) and 400ppm (black) CO_2. 6 biological replicates and 4 technical replicates per measurement.

Intercellular carbon concentrations (Ci) are elevated in all transplastomic lines compared to WT at both 200 and 400ppm CO_2 (Fig 6.12). On average, ictB lines demonstrated increased Ci of 5.46% and 5.99% compared to WT at 200 and 400ppm CO_2 respectively. Transplastomic lines ictB-2, 3, and 5 show the largest increases in Ci at both 200 and 400ppm CO_2 (Fig 6.13). Both ictB-1 and ictB-4 showed lowest increases in Ci at 200ppm than at 400ppm. This was opposite for ictB-2,3 , and 5, which showed the highest increases in Ci for 200ppm than at 400ppm CO_2.
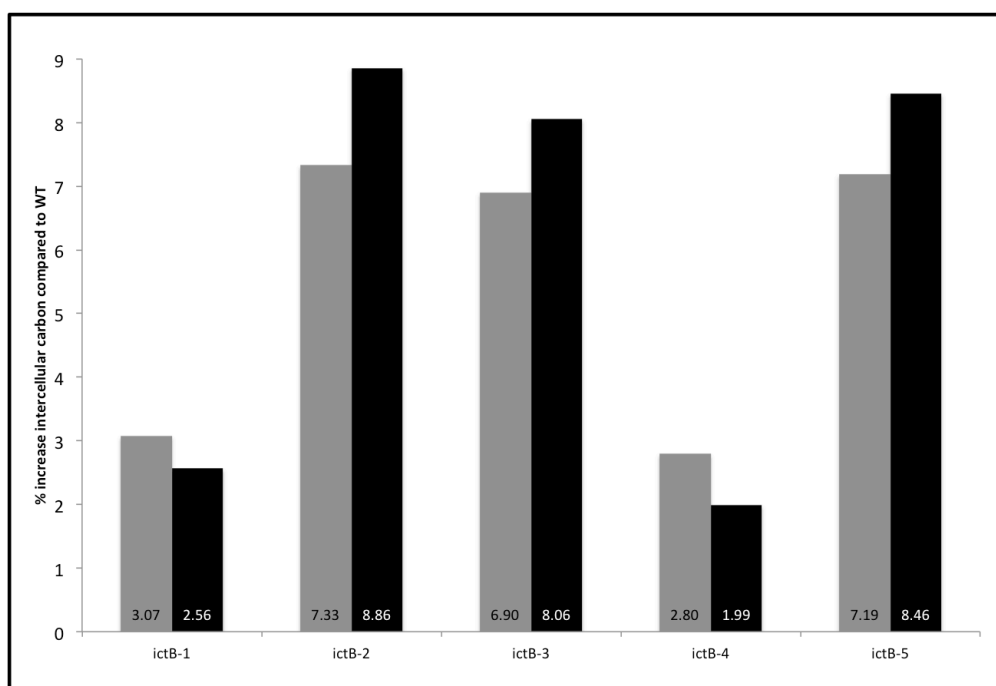
Fig 6.13 Percentage increase in intercellular CO_2 concentration of ictB lines compared to WT at 200 (grey) and 400 ppm (black) CO_2. 6 biological replicates and 4 technical replicates per measurement.

Transpiration rates and stomatal conductance (Fig6.14) share the same trend, with ictB-2 and 3 showing the greatest elevation of transpiration at both 200 and 400ppm CO_2, and ictB-2, 3, and 5 showing the greatest increases in stomatal conductance at both 200 and 400ppm CO_2.
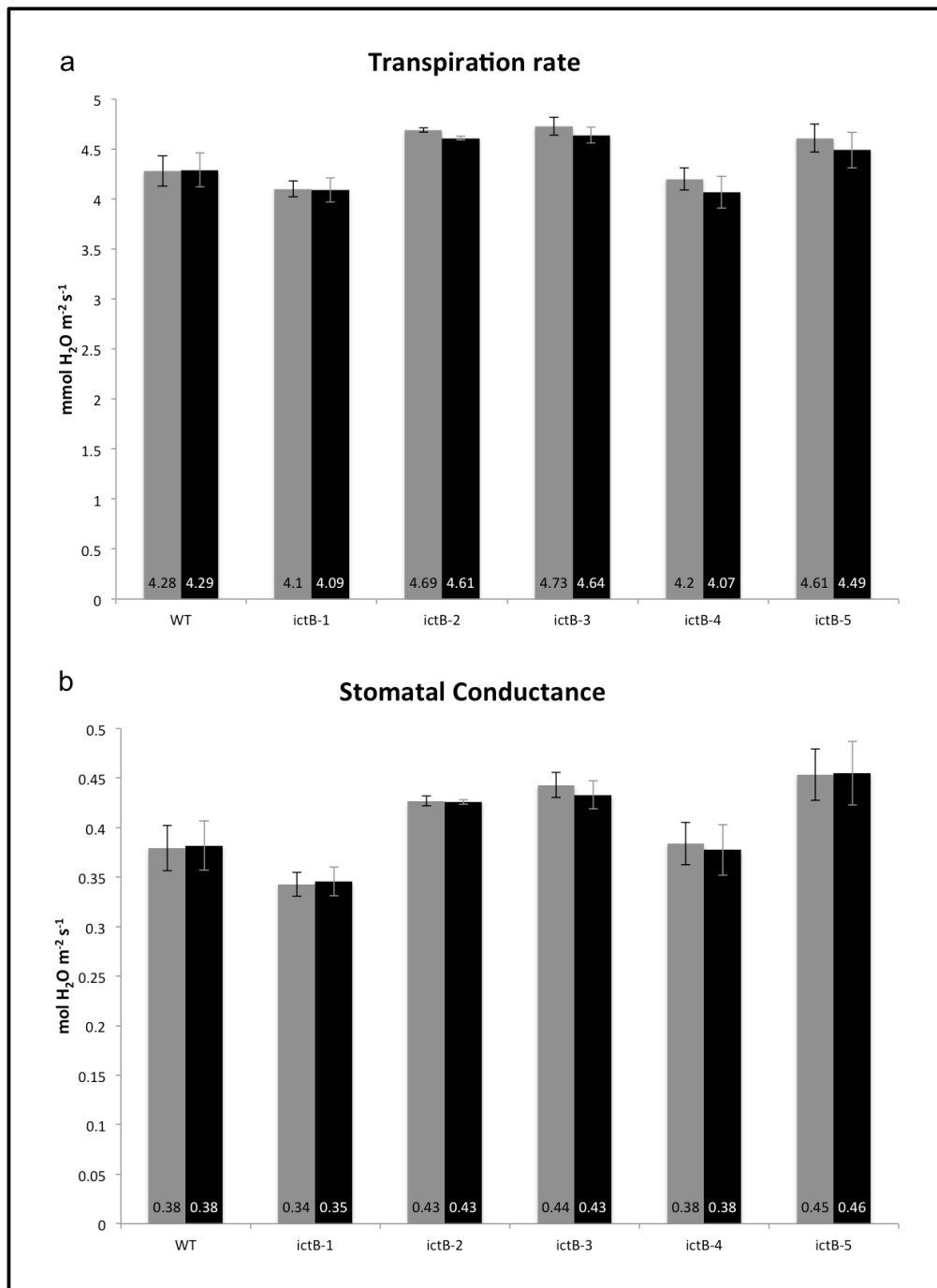
Fig 6.14 Intercellular carbon concentrations (a) and stomatal conductance (b) of WT and ictB lines at 200 (grey) and 400ppm (black) CO_2. 6 biological replicates and 4 technical replicates per measurement.

6.3.5.2 Effects of ictB on plant growth

All ictB transplastomic lines were smaller in height than WT, and produced a lower biomass than WT (Fig 6.15 and 6.16). On average, ictB lines were 32.3% shorter than WT, and produced 43.8% less biomass, with ictB-4 being the tallest and creating the greatest amount of biomass out of all ictB lines. Variation in physiological characteristics between different transplastomic lines would be expected.

Fig 6.15 Effect of ictB on plant growth. From left to Right, WT, ictB-1, ictB-2, ictB-3, ictB-4, ictB-5.
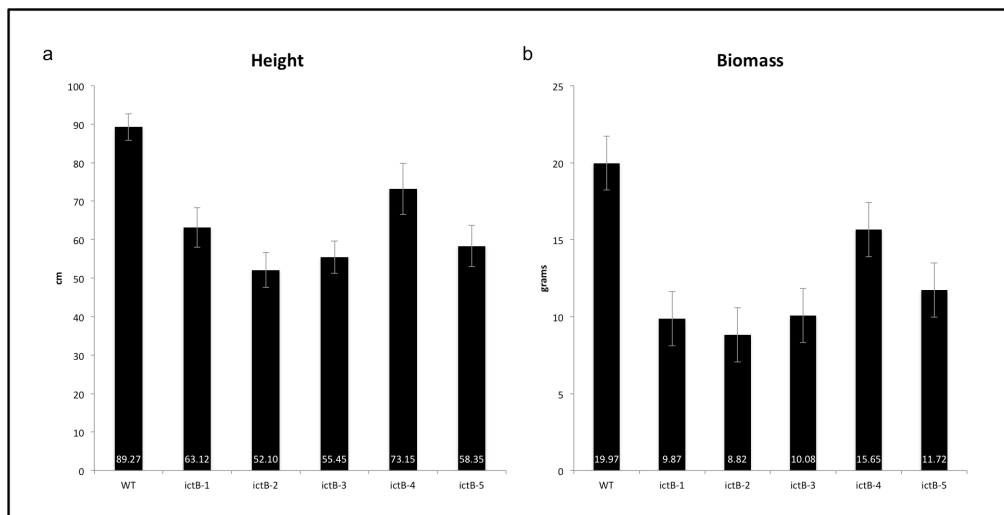
Fig 6.16 Effect of ictB on plant height (a) and biomass (b) in ictB lines compared to WT. 6 biological replicates per measurement.

On average, ictB lines had a 1.6% shorter leaf length, and a 10.13% increase in leaf width. The ictB line with the longest leaf length compared to WT was ictB-3, and the lines with the widest leaves compared to WT were ictB-3, 4, and 5 (Fig 6.17).
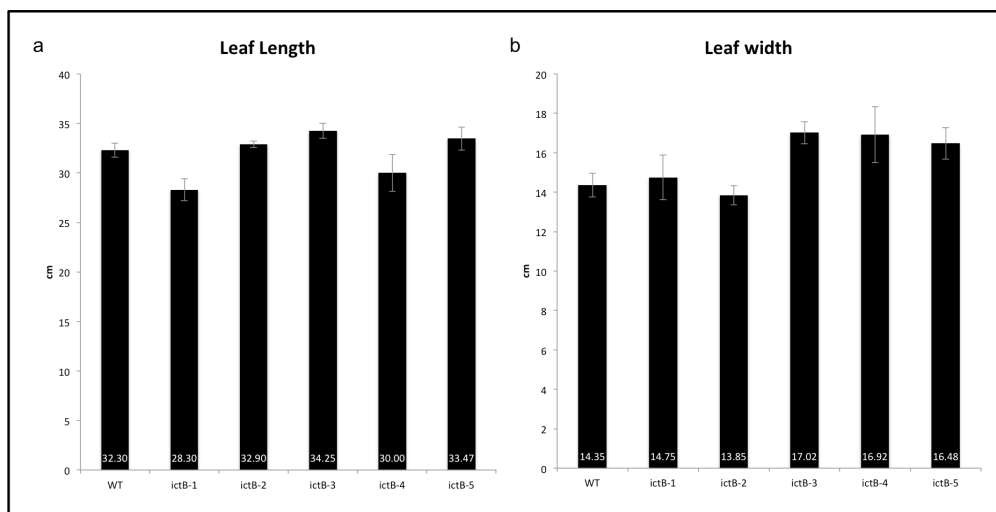
Fig 6.17 Effect of ictB on leaf length (a) and leaf width (b) in ictB lines compared to WT. 6 biological replicates per measurement.

6.3.5.3   Other observations

All ictB lines started to develop seed pods on average 7 days earlier than WT, with ictB-2 and ictB-5 producing seed pods up to 10 days prior to WT.

6.4   Discussion

In this chapter I have demonstrated the successful chloroplast transformation of *N*
.tabacum with a cyanobacterial gene, *ictB* encoding a carbon concentrating function.
This is the first example of the integration of a cyanobacterial CCM gene into the
chloroplast of a higher plant. PCR data cast some doubt on the homoplasmic nature of
these transplastomic lines. However the PCR data does suggest the vast majority of IR
regions of the plastome does contain the transformation cassette. In addition, qPCR
data shows the expression of ictB within all ictB lines. Although correct transcript
processing was not confirmed in this study, the clear phenotype difference suggests
that the ictB gene is being successfully transcribed and translated into functional
protein.

From the above results, the expression of ictB appears to slightly reduce the overall
photosynthetic rate for all ictB lines, however, none of these results were significant.
There was also no significant difference between stomatal conductance between WT
and ictB lines at both 200 (low) and 400 (ambient) ppm CO_2. When comparing
transpiration rates, ictB-2 was the only line to show a significant increase compared to
WT at both low and ambient CO_2. Given that the ictB lines were expressing a carbon
concentrator in the plastid, I would expect to see an increase in intercellular
concentrations of CO_2. This is indeed what I observed for all transplastomic lines, at
both low and ambient CO_2. However, the difference from control plants was
significant only for lines ictB-2, 3, and 5 at 200ppm and ictB-2 and 3 at 400ppm CO_2.
There was a strong correlation between increases in intercellular carbon
concentrations and the levels of transgene expression at both low and ambient carbon
concentrations. The data shows that the increased intercellular carbon in ictB-2, 3, and
5, is correlated with an increase in transpiration rate and stomatal conductance (Fig
6.18 and Fig 6.19). This is not surprising as the most likely source of inorganic carbon
(iC) is CO_2 diffusion from the atmosphere via the stomata. However, the mechanisms
that link ictB expression and increased stomatal conductance remain unclear.

ictB lines showed significant differences in height, and biomass, with ictB-2, 3, and 5
on average having a 38.1% decrease in height and a 48.1% decrease in biomass. There
was no significant difference seen in the leaf length for any of the ictB lines compared
to WT, however leaf width was significantly greater in ictB-3 (18.6%) and ictB-5
(14.8%).
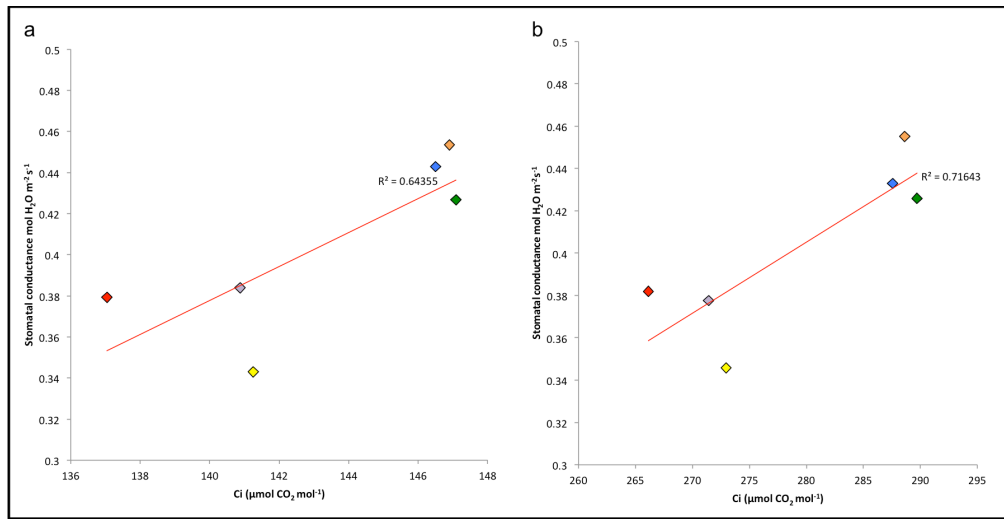
Fig 6.18 Correlation between Ci (intercellular CO_2 concentrations) and stomatal conductance between WT (red) and ictB transplastomic lines, ictB-1 (yellow), ictB-2 (green), ictB-3 (blue), ictB-4 (lilac) and ictB-5 (orange) at 200 (a) and 400 (b) ppm CO_2.
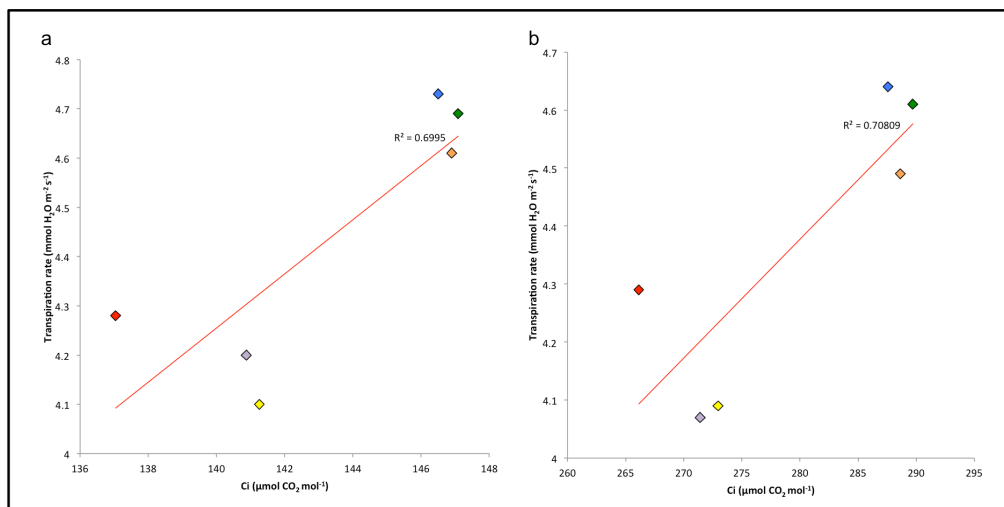
Fig 6.19 Correlation between Ci (intercellular CO_2 concentrations) and transpiration rate between WT (red) and ictB transplastomic lines, ictB-1 (yellow), ictB-2 (green), ictB-3 (blue), ictB-4 (lilac) and ictB-5 (orange) at 200 (a) and 400 (b) ppm CO_2.

160

The questions posed by results for ictB-2, 3, and 5 are (a) what mechanisms link the increased expression of ictB and increase in stomatal conductance/transpiration, (b) if the extra intercellular carbon is not driving carbon assimilation (net photosynthesis), then where is it going, and (c) how can we explain the physical differences observed in plant growth between ictB transplastomics and WT,

The exact function of *ictB* remains unclear. Originally the gene product was reported as a likely HCO_3^- transporter, following data obtained from a CO_2 requiring mutant of *Synechococcus* sp. strain PCC 7942, IL-2, derived from an inactivation library. Genomic analysis showed a single crossover recombination in ORF467, designated *ictB*, which resulted an impaired HCO_3^- transport phenotype (Bonfil et al., 1998). Despite subsequent research determining that ictB is unlikely to be a HCO_3^- transporter (Shibata et al., 2002, Price et al., 2004, Xu et al., 2008), transgenic *N. tabacum* and *A. thaliana* overexpressing *ictB* produced a 5-9ppm decrease in the CO_2 compensation point, and in Arabidopsis showed a higher relative growth rate in comparison to WT (Lieman-Hurwitz et al., 2003). The hydrophobic structure and 12 membrane spanning domains of ictB certainly implies its function as a membrane protein. In 3 of the 5 ictB transplastomic lines tested, the intercellular concentrations of carbon are significantly higher than controls. This most certainly implicates ictB as a carbon concentrator, however without knowing its exact location within the plastid, or analysing HCO_3^- uptake into plastids from the cytosol, the mechanisms by which ictB increases intercellular CO_2 and thus stomatal conductance, remains to be elucidated.

To answer (b), we must first understand the internal carbon environment of a C_3 leaf cell, and the initial carboxylation reactions that begin carbon assimilation. C_3 plants have significant diffusion resistance for CO_2, that is, the prevention of CO_2 diffusion from the air, through the stomata, cell walls and cytoplasm, and eventually through to the chloroplast (Evans et al., 2009). This creates a large drawdown or deficit in the steady state CO_2 concentration in the chloroplast relative to ambient air, despite evolutionary adaptations aiming to reduce this (Evans and vonCaemmerer, 1996). This (relatively) low intercellular carbon concentration results in reduced RubisCO mediated plant photosynthetic carbon metabolism. Plant photosynthetic carbon metabolism is composed of two connected pathways, (1) the C_3/Calvin cycle (reductive), and (2) the C_2/photorespiratory pathway (oxidative). Both are dependent

on RubisCO converting RuBP into 3-phosphoglycerate (3-PGA). However, oxygenation of RuBP results in the formation of 3-PGA and of 2-phosphoglycolate (2-PGA) (Fig 6.20. It must be noted that the ratio of 3-PGA : 2-PGA is determined by CO_2/O_2 ratio in the chloroplast and the CO_2/O_2 specificity factor of RubisCO). The photorespiratory cycle then recycles 2-PGA into 3-PGA, with the loss of 1 molecule of ATP and CO_2 per conversion. Both cyanobacterial and C4 crops reduce the effect of photorespiration by increasing the concentration of carbon surrounding RubisCO. For an in-depth analysis of photorespiration, see Maurino and Peterhansel (2010).
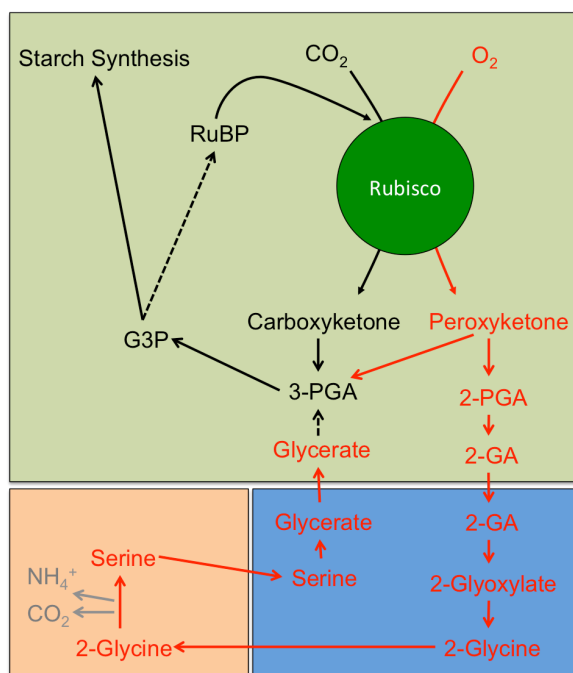
Fig 6.20 A simplified diagram of reductive photosynthesis (black) and photorespiration (red). 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycolate; 2-GA, 2 Glycoltate, G3P, Glyceraldehyde 3-phosphate, RuBP, Ribulose-1,5-bisphosphate. Grey arrows and text indicate losses from system, dashed arrows indicate reactions that use up one molecule of ATP. Green square, chloroplast; blue square, peroxisome; orange square, mitochondrion.

The ictB lines 2, 3 and 5 all have a significantly higher concentration of intercellular carbon in comparison to WT. Presumably this is because ictB is increasing the transport of HCO_3^- within the chloroplast, and stromal carbonic anhydrase is converting HCO_3^- into CO_2. In theory, as the pumping of iC into the chloroplasts addresses the natural drawdown observed in C_3 plant cells, this should increase the

concentration of carbon surrounding RubisCO, and thus reducing carbon, nitrogen, and energy loss via photorespiration (Parry et al., 2011, Hibberd et al., 2008). This would lead to an increased level of photosynthetic carbon assimilation, and hence overall photosynthetic rate and plant growth. However, our results do not show this. We see no significant change to overall photosynthetic rate. One reason for this may be that, although there is an increase in carbon concentration within the cell, we do not know whether this concentration is occurring around RubisCO. One large gap in our current knowledge is the diffusion properties of the chloroplast envelope and plasma membrane/cell wall (Evans et al., 2009). It could be that the excess carbon is simply diffusing away from the site of RubisCO and out of the chloroplast. The reason why cyanobacteria are able to maintain the concentration of CO_2 around RubisCO is because they have a multi-level carbon concentrating mechanism (CCM). The initial level is constituted by the iC pumps/symporters/transporters, such as ictB. As demonstrated in the ictB lines, iC transporters increase the concentration of HCO_3^- within the chloroplast. Cyanobacteria also have well placed CA. CA can rapidly convert HCO_3^- into CO_2, and vice versa (as it is a reversible reaction), and if located within the cytosol of the cyanobacteria, would rapidly catalyse the forward reaction of HCO_3^- into CO_2, allowing CO_2 to diffuse away into the external medium (Price and Badger, 1989). However, as the CA is located within the carboxysome and thus disconnected from the cytosol, this coupled with the thylakoid membrane (located in the cytosol) converting CO_2 into HCO_3^-, maintains a large concentration of HCO_3^- within cyanobacteria and CO_2 in close proximity to RubisCO (also located within the carboxysome). This represents a second level to the CCM. Thus, introducing iC transporters into C_3 plants, as with the ictB lines, may not be enough to increase photosynthetic rates. Compartmentalising CA and RubisCO into a carboxysome within C_3 plants is also necessary, which would be the next step of engineering better C_3 photosynthesis. Although the expression of structurally correct α-carboxysomes within *E. coli* has been demonstrated (Bonacci et al., 2012), construction of a carboxysome in higher proves to be problematic (Kanevski et al., 1999). Indeed, the process of introducing a CCM within higher plants has been considered and been planned out (Price et al., 2013), with the engineering of iC transporters and carboxysomes into C_3 plants considered as phase 1a and phase 1b respectively. For further steps on engineering cyanobacterial CCM into C_3 chloroplasts, please refer to Price et al, 2013.
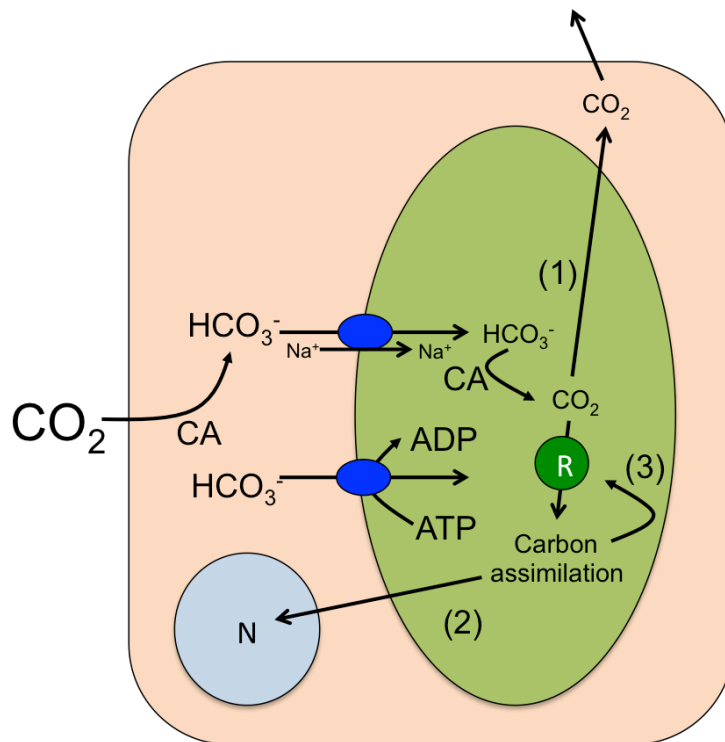
Fig 6.21 Summary of what might be happening to imported CO_2. CO_2 is readily converted to HCO_3^- in the cytosol by carbonic anhydrase (CA), and pumped into the chloroplast via ictB (could be using a sodium symport mechanism, or ATP). HCO_3^- is rapidly converted to CO_2, thus increasing the carbon concentration within the plastid. (1) Excess CO_2 diffuses out of the chloroplast and out of the cell. (2) Alterntively, CO_2 is utilised by RubisCO (R) for carbon assimilation. However due to the increased efficient, signals via assimilated carbon signal/feed back to the nucleus (N) to reduce *rbcS* transcription, and (3) reduce plastid transcription/translation of *rbcL*.

Another hypothesis for the absence of photosynthetic increase in the presence of elevated carbon may be due to a reduction in RubisCO transcription/translation. Up to 25% of leaf nitrogen is invested in RubisCO formation (Evans, 1989). If, at elevated internal CO_2 concentrations, the RubisCO is working more efficiently, the cell may choose to reduce RubisCO translation, as it does not require the energy and nitrogen investment. Long term exposure to high levels of atmospheric CO_2 does result in reduced RubisCO protein accumulation, and reduced transcription of *rbcL* and *rbcS*, as well as other key enzymes of reductive photosynthesis (Cheng et al., 1998, Drake et al., 1997). Although, overexpression of *ictB* in tobacco and arabidopsis showed no

change in amounts of RubisCO (Lieman-Hurwitz et al., 2003), in order to make a conclusive hypothesis, transcript levels of RubisCO subunits and final protein RubisCO accumulation levels would need to be analysed in ictB transplastomic lines.

All ictB lines were smaller and of lower biomass compared to WT. Apart from the significant difference in height, plants appeared phenotypically normal and healthy. The observation that ictB lines produced seed pods up to 10 days earlier than WT is intriguing. This would need to be repeated again in order to confirm such an important and significant phenotype, especially as similar results were not observed in nuclear overexpression of plastid-targeted ictB (Lieman-Hurwitz et al., 2003) where enhanced photosynthesis and growth only occurred under limiting conditions. However, my results may indicate that an increase in efficiency of RubisCO activity is occurring. Rather than investing carbon in producing taller plants, assimilated carbohydrates may get pushed to achieve faster plant maturation and thus seed formation (possibly due to a change in the normal sink-source relationship). Plants had the same number of leaves as compared WT, and leaves analysed were of similar length. However there was a significant observation of wider leaves in ictB-3 and 5. Changes of internal structure of leaves can occur when grown under elevated CO_2 levels (Pritchard et al., 1999). Although results vary, larger leaf size is likely to be due to increased cell expansion rather than increased cell division. Without conducting further investigation into the anatomy of the leaves from ictB transplastomic lines, and performing more accurate measurements on total leaf area and leaf thickness, we cannot form a robust mechanistic explanation for this observation.

7   Concluding Remarks

Developing chloroplast transformation in any new species is a considerable endeavour. Achieving this technology in the monocots is made even harder due to the recalcitrance of adult tissues containing chloroplasts. In my thesis, I have demonstrated a robust method for the plant regeneration of wheat from a previously undescribed, metabolically active, chloroplast rich tissue, the primary inflorescence leaf sheath (piLS). Although streptomycin appears unsuitable as a selectable marker, others such as kanamycin can be tested in the future. I have also demonstrated the regeneration of plants from piLS in two varieties of wheat, Apogee and Cadenza. Through optimisation, it is likely that this protocol can be made more effective for Apogee and Cadenza, as well as other wheat varieties. Achieving chloroplast transformation in wheat will require a number of novel tissue targets, and selectable markers. I have provided one such target in this thesis (piLS), however a more embryogenic target would increase the possibility of achieving chloroplast transformation. For example, having a cell line like the maize Hi II Type II embryogenic callus, which can be taken through tissue culture for a considerable length of time.

If one day, chloroplast transformation is routinely made possible in the monocots, plastid biotechnology can be used to attempt "supercharging" photosynthesis in the cereals. In terms of improving photosynthesis in C_3 crops, the results from my thesis suggest that a single gene solution is unlikely to achieve the desired results. Adapting an effective CCM into C_3 plants will most likely require a multi gene approach, whether it is by incorporating a carboxysome into the plastids along with inorganic carbon (iC) transporters, or by introducing "Kranz" like C_4 anatomy. An interesting advancement from my work would be to cross, once produced, a nuclear transformed tobacco line expressing a functional carboxysome, with one of the highest iC transporting ictB transplastomic tobacco lines described in this thesis. This will then provide a greater indication of the feasibility for improving photosynthesis in C_3 crops. It must be noted that I did not perform any experiments on the ictB tobacco transplastomic lines under limiting conditions, which the literature indicates are the conditions where ictB provides a growth advantage compared to WT. Thus in a world where growing conditions are becoming more hostile, having such genes incorporated

into the chloroplasts of our agronomic crops, albeit via the nucleus, may still provide a growth advantage.

Finally, plastid biotechnology is an exciting area for the production of exogenous proteins. Being able to produce a number of proteins from a polycistron has a number of advantages, however, repetition of promoters increases the likelihood of loss of genetic information through rearrangement. In my thesis I have demonstrated the first use of a monocot plastid promoter, the wheat Prrn (WPrrn), in a dicot plastid. The WPrrn differs in sequence with its tobacco (TPrrn) counterpart by approximately 30%, which is sufficiently different to prevent rearrangement should both the WPrrn and TPrrn be included in a polycistronic plastid transformation cassette. The strength of the Wprrn in comparison to Tprrn is yet to be elucidated, however from my work (results not included) the number of transplastomic plants recovered per bombardment using the Wprrn was greater than those using Tprrn. This suggests that the Wprrn is at least as efficient as the Tprrn, and can be considered for use in future monocistronic or polycistronic plastid biotechnology studies.

I would like to thank the examiners for taking time to review the last four years of my work. I hope they have found the experimental results interesting, and are a positive contribution to the research area.

8   References

ADL, S. M., SIMPSON, A. G. B., FARMER, M. A., ANDERSEN, R. A., ANDERSON, O. R., BARTA, J. R., BOWSER, S. S., BRUGEROLLE, G., FENSOME, R. A., FREDERICQ, S., JAMES, T. Y., KARPOV, S., KUGRENS, P., KRUG, J., LANE, C. E., LEWIS, L. A., LODGE, J., LYNN, D. H., MANN, D. G., MCCOURT, R. M., MENDOZA, L., MOESTRUP, O., MOZLEY-STANDRIDGE, S. E., NERAD, T. A., SHEARER, C. A., SMIRNOV, A. V., SPIEGEL, F. W. & TAYLOR, M. 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *Journal of Eukaryotic Microbiology,* 52**,** 399-451.

ALLISON, L. A., SIMON, L. D. & MALIGA, P. 1996. Deletion of rpoB reveals a second distinct transcription system in plastids of higher plants. *Embo Journal,* 15**,** 2802-2809.

ALONSO, H., BLAYNEY, M. J., BECK, J. L. & WHITNEY, S. M. 2009. Substrate-induced Assembly of Methanococcoides burtonii D-Ribulose-1,5-bisphosphate Carboxylase/Oxygenase Dimers into Decamers. *Journal of Biological Chemistry,* 284**,** 33876-33882.

AMMIRATO, P. V. 1983. The regulation of somatic embryo development in plant-cell cultures - suspension-culture techniques and hormone requirements. *Bio-Technology,* 1**,** 68-74.

ANDERSSON, I. & BACKLUND, A. 2008. Structure and function of Rubisco. *Plant Physiology and Biochemistry,* 46**,** 275-291.

ANDRES, C., AGNE, B. & KESSLER, F. 2010. The TOC complex: Preprotein gateway to the chloroplast. *Biochimica Et Biophysica Acta-Molecular Cell Research,* 1803**,** 715-723.

ANDREWS, T. J. & WHITNEY, S. M. 2003. Manipulating ribulose bisphosphate carboxylase/oxygenase in the chloroplasts of higher plants. *Archives of Biochemistry and Biophysics,* 414**,** 159-169.

AZEVEDO, J., COURTOIS, F., HAKIMI, M.-A., DEMARSY, E., LAGRANGE, T., ALCARAZ, J.-P., JAISWAL, P., MARECHAL-DROUARD, L. & LERBS-MACHE, S. 2008. Intraplastidial trafficking of a phage-type RNA polymerase is mediated by a thylakoid RING-H2 protein. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 9123-9128.

BALLY, J., BELGHAZI, M., JOB, C. & JOB, D. 2011. Metabolic Adaptation in Transplastomic Plants Massively Accumulating Recombinant Proteins. *Figshare,* 1.

BALLY, J., NADAI, M., VITEL, M., ROLLAND, A., DUMAIN, R. & DUBALD, M. 2009. Plant Physiological Adaptations to the Massive Foreign Protein Synthesis Occurring in Recombinant Chloroplasts. *Plant Physiology,* 150**,** 1474-1481.

BARKAN, A. 2011. Expression of Plastid Genes: Organelle-Specific Elaborations on a Prokaryotic Scaffold. *Plant Physiology,* 155**,** 1520-1532.

BARKAN, A. & GOLDSCHMIDT-CLERMONT, M. 2000. Participation of nuclear genes in chloroplast gene expression. *Biochimie,* 82**,** 559-572.

BARNES, D., FRANKLIN, S., SCHULTZ, J., HENRY, R., BROWN, E., CORAGLIOTTI, A. & MAYFIELD, S. P. 2005. Contribution of 5 '- and 3 '-untranslated regions of

plastid mRNAs to the expression of Chlamydomonas reinhardtii chloroplast genes. *Molecular Genetics and Genomics,* 274**,** 625-636.

BAUER, J., HILTBRUNNER, A. & KESSLER, F. 2001. Molecular biology of chloroplast biogenesis: gene expression, protein import and intraorganellar sorting. *Cellular and Molecular Life Sciences,* 58**,** 420-433.

BENDICH, A. J. 2004. Circular chloroplast chromosomes: The grand illusion. *Plant Cell,* 16**,** 1661-1666.

BEVAN, M. 1984. Binary agrobacterium vectors for plant transformation. *Nucleic Acids Research,* 12**,** 8711-8721.

BIRKY, C. W. 1995. Uniparental inheritance of mitochondrial and chloroplast genes - mechanisms and evolution. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 11331-11338.

BLOWERS, A. D., BOGORAD, L., SHARK, K. B. & SANFORD, J. C. 1989. Studies on chlamydomonas chloroplast transformation - foreign dna can be stably maintained in the chromosome. *Plant Cell,* 1**,** 123-132.

BOCK, R. 2000. Sense from nonsense: How the genetic information of chloroplasts is altered by RNA editing. *Biochimie,* 82**,** 549-557.

BOCK, R. 2015. Engineering Plastid Genomes: Methods, Tools, and Applications in Basic Research and Biotechnology. *Annual Review of Plant Biology, Vol 66,* 66**,** 211-241.

BOCK, R. & KHAN, M. S. 2004. Taming plastids for a green future. *Trends in Biotechnology,* 22**,** 311-318.

BOCK, R., KOSSEL, H. & MALIGA, P. 1994. Introduction of a heterologous editing site into the tobacco plastid genome - the lack of rna editing leads to a mutant phenotype. *Embo Journal,* 13**,** 4623-4628.

BOCK, R. & TIMMIS, J. N. 2008. Reconstructing evolution: gene transfer from plastids to the nucleus. *Bioessays,* 30**,** 556-566.

BOHMERT-TATAREV, K., MCAVOY, S., DAUGHTRY, S., PEOPLES, O. P. & SNELL, K. D. 2011. High Levels of Bioplastic Are Produced in Fertile Transplastomic Tobacco Plants Engineered with a Synthetic Operon for the Production of Polyhydroxybutyrate. *Plant Physiology,* 155**,** 1690-1708.

BONACCI, W., TENG, P. K., AFONSO, B., NIEDERHOLTMEYER, H., GROB, P., SILVER, P. A. & SAVAGE, D. F. 2012. Modularity of a carbon-fixing protein organelle. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 478-483.

BONFIL, D. J., RONEN-TARAZI, M., SULTEMEYER, D., LIEMAN-HURWITZ, J., SCHATZ, D. & KAPLAN, A. 1998. A putative HCO(3)(-) transporter in the cyanobacterium Synechococcus sp. strain PCC 7942. *Febs Letters,* 430**,** 236-240.

BOYNTON, J. E., GILLHAM, N. W., HARRIS, E. H., HOSLER, J. P., JOHNSON, A. M., JONES, A. R., RANDOLPHANDERSON, B. L., ROBERTSON, D., KLEIN, T. M., SHARK, K. B. & SANFORD, J. C. 1988. Chloroplast transformation in chlamydomonas with high-velocity microprojectiles. *Science,* 240, 1534-1538.

CARMO-SILVA, A. E., KEYS, A. J., ANDRALOJC, P. J., POWERS, S. J., ARRABACA, M. C. & PARRY, M. A. J. 2010. Rubisco activities, properties, and regulation in three different C-4 grasses under drought. *Journal of Experimental Botany,* 61**,** 2355-2366.

CARMO-SILVA, A. E., POWERS, S. J., KEYS, A. J., ARRABACA, M. C. & PARRY, M. A. J. 2008. Photorespiration in C(4) grasses remains slow under drought conditions. *Plant Cell and Environment,* 31**,** 925-940.

CARRER, H., HOCKENBERRY, T. N., SVAB, Z. & MALIGA, P. 1993. Kanamycin resistance as a selectable marker for plastid transformation in tobacco. *Molecular & General Genetics,* 241**,** 49-56.

CARRER, H. & MALIGA, P. 1995. Targeted insertion of foreign genes into the tobacco plastid genome without physical linkage to the selectable marker gene. *Bio-Technology,* 13**,** 791-794.

CAVALIER-SMITH, T. 2000. Membrane heredity and early chloroplast evolution. *Trends in Plant Science,* 5**,** 174-182.

CAVALIER-SMITH, T. 2010. Deep phylogeny, ancestral groups and the four ages of life. *Philosophical Transactions of the Royal Society B-Biological Sciences,* 365**,** 111-132.

CAVALIERSMITH, T. 1982. THE ORIGINS OF PLASTIDS. *Biological Journal of the Linnean Society,* 17**,** 289-306.

CHAKRABARTI, S. K., LUTZ, K. A., LERTWIRIYAWONG, B., SVAB, Z. & MALIGA, P. 2006. Expression of the cry9Aa2 B.t. gene in tobacco chloroplasts confers resistance to potato tuber moth. *Transgenic Research,* 15**,** 481-488.

CHEN, X. M., KINDLE, K. L. & STERN, D. B. 1995. The initiation codon determines the efficiency but not the site of translation initiation in chlamydomonas chloroplasts. *Plant Cell,* 7**,** 1295-1305.

CHENG, L., LI, H.-P., QU, B., HUANG, T., TU, J.-X., FU, T.-D. & LIAO, Y.-C. 2010. Chloroplast transformation of rapeseed (Brassica napus) by particle bombardment of cotyledons. *Plant Cell Reports,* 29**,** 371-381.

CHENG, S. H., MOORE, B. D. & SEEMANN, J. R. 1998. Effects of short- and long-term elevated CO2 on the expression of ribulose-1,5-bisphosphate carboxylase/oxygenase genes and carbohydrate accumulation in leaves of Arabidopsis thaliana (L) Heynh. *Plant Physiology,* 116**,** 715-723.

CHIN, J. C. & SCOTT, K. J. 1977. Studies on formation of roots and shoots in wheat callus-cultures. *Annals of Botany,* 41**,** 473-&.

CHIYODA, S., LINLEY, P. J., YAMATO, K. T., FUKUZAWA, H., YOKOTA, A. & KOHCHI, T. 2007. Simple and efficient plastid transformation system for the liverwort Marchantia polymorpha L. suspension-culture cells. *Transgenic Research,* 16**,** 41-49.

CHUMLEY, T. W., PALMER, J. D., MOWER, J. P., FOURCADE, H. M., CALIE, P. J., BOORE, J. L. & JANSEN, R. K. 2006. The complete chloroplast genome sequence of Pelargonium x hortorum: Organization and evolution of the largest and most highly rearranged chloroplast genome of land plants. *Molecular Biology and Evolution,* 23**,** 2175-2190.

DANIELL, H., CHEBOLU, S., KUMAR, S., SINGLETON, M. & FALCONER, R. 2005. Chloroplast-derived vaccine antigens and other therapeutic proteins. *Vaccine,* 23**,** 1779-1783.

DANIELL, H., DATTA, R., VARMA, S., GRAY, S. & LEE, S. B. 1998. Containment of herbicide resistance through genetic engineering of the chloroplast genome. *Nature Biotechnology,* 16**,** 345-348.

DANIELL, H., KRISHNAN, M. & MCFADDEN, B. F. 1991. Transient expression of beta-glucuronidase in different cellular compartments following biolistic delivery of foreign dna into wheat leaves and calli. *Plant Cell Reports,* 9**,** 615-619.

DANIELL, H., VIVEKANANDA, J., NIELSEN, B. L., YE, G. N., TEWARI, K. K. & SANFORD, J. C. 1990. Transient foreign gene-expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proceedings of the National Academy of Sciences of the United States of America,* 87**,** 88-92.

DATTA, S. K. & WENZEL, G. 1987. Isolated microspore derived plant formation via embryogenesis in tricum-aestivum l. *Plant Science,* 48**,** 49-54.

DAVARPANAH, S. J., JUNG, S. H., KIM, Y. J., PARK, Y.-I., MIN, S. R., LIU, J. R. & JEONG, W. J. 2009. Stable Plastid Transformation in Nicotiana benthamiana. *Journal of Plant Biology,* 52**,** 244-250.

DAY, A. & GOLDSCHMIDT-CLERMONT, M. 2011. The chloroplast transformation toolbox: selectable markers and marker removal. *Plant Biotechnology Journal,* 9**,** 540-553.

DE COSA, B., MOAR, W., LEE, S. B., MILLER, M. & DANIELL, H. 2001. Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. *Nature Biotechnology,* 19**,** 71-74.

DEBLOCK, M., SCHELL, J. & VANMONTAGU, M. 1985. Chloroplast transformation by agrobacterium-tumefaciens. *Embo Journal,* 4**,** 1367-1372.

DRAKE, B. G., GONZALEZMELER, M. A. & LONG, S. P. 1997. More efficient plants: A consequence of rising atmospheric CO2? *Annual Review of Plant Physiology and Plant Molecular Biology,* 48**,** 609-639.

DRECHSEL, O. & BOCK, R. 2011. Selection of Shine-Dalgarno sequences in plastids. *Nucleic Acids Research,* 39**,** 1427-1438.

DUFOURMANTEL, N., DUBALD, M., MATRINGE, M., CANARD, H., GARCON, F., JOB, C., KAY, E., WISNIEWSKI, J. P., FERULLO, J. M., PELISSIER, B., SAILLAND, A. & TISSOT, G. 2007. Generation and characterization of soybean and marker-free tobacco plastid transformants over-expressing a bacterial 4-hydroxyphenylpyruvate dioxygenase which provides strong herbicide tolerance. *Plant Biotechnology Journal,* 5**,** 118-133.

DUFOURMANTEL, N., PELISSIER, B., GARCON, F., PELTIER, G., FERULLO, J. M. & TISSOT, G. 2004. Generation of fertile transplastomic soybean. *Plant Molecular Biology,* 55**,** 479-489.

DUNWELL, J. M. 1985. ANTHER AND OVARY CULTURE. *Bright, S. W. J. and M. G. K. Jones (Ed.). Advances in Agricultural Biotechnology: Cereal Tissue and Cell Culture. Viii+304p. Martinus Nijhoff/Dr W. Junk Publishers: Dordrecht, Netherlands; Boston, Mass., USA (Dist. by Kluwer Academic Publishers: Hingham, Mass., USA; Lancaster, England; Kluwer Academic Publishers Group: Dordrecht, Netherlands). Illus***,** 1-44.

EBERHARD, S., DRAPIER, D. & WOLLMAN, F. A. 2002. Searching limiting steps in the expression of chloroplast-encoded proteins: relations between gene copy number, transcription, transcript abundance and translation rate in the chloroplast of Chlamydomonas reinhardtii. *Plant Journal,* 31**,** 149-160.

ECONOMOU, C., WANNATHONG, T., SZAUB, J. & PURTON, S. 2014. A simple, low-cost method for chloroplast transformation of the green alga Chlamydomonas reinhardtii. *Methods in molecular biology (Clifton, N.J.),* 1132**,** 401-11.

EIBL, C., ZOU, Z., BECK, A., KIM, M., MULLET, J. & KOOP, H. U. 1999. In vivo analysis of plastid psbA, rbcL and rpl32 UTR elements by chloroplast transformation: tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. *Plant J,* 19**,** 333-45.

ELGHABI, Z., RUF, S. & BOCK, R. 2011. Biolistic co-transformation of the nuclear and plastid genomes. *The Plant Journal,* 67**,** 941-948.

EMS, S. C., MORDEN, C. W., DIXON, C. K., WOLFE, K. H., DEPAMPHILIS, C. W. & PALMER, J. D. 1995. Transcription, splicing and editing of plastid RNAs in the nonphotosynthetic plant Epifagus virginiana. *Plant Molecular Biology,* 29**,** 721-733.

ESPOSITO, D., HICKS, A. J. & STERN, D. B. 2001. A role for initiation codon context in chloroplast translation. *Plant Cell,* 13**,** 2373-2384.

EVANS, J. R. 1989. Photosynthesis and nitrogen relationships in leaves of c-3 plants. *Oecologia,* 78**,** 9-19.

EVANS, J. R., KALDENHOFF, R., GENTY, B. & TERASHIMA, I. 2009. Resistances along the CO2 diffusion pathway inside leaves. *Journal of Experimental Botany,* 60**,** 2235-2248.

EVANS, J. R. & VONCAEMMERER, S. 1996. Carbon dioxide diffusion inside leaves. *Plant Physiology,* 110**,** 339-346.

FENG, L., WANG, K., LI, Y., TAN, Y., KONG, J., LI, H., LI, Y. & ZHU, Y. 2007. Overexpression of SBPase enhances photosynthesis against high temperature stress in transgenic rice plants. *Plant Cell Reports,* 26**,** 1635-1646.

FISCHER, R. A. 2007. Understanding the physiological basis of yield potential in wheat. *Journal of Agricultural Science,* 145**,** 99-113.

FRAME, B. R., ZHANG, H. Y., COCCIOLONE, S. M., SIDORENKO, L. V., DIETRICH, C. R., PEGG, S. E., ZHEN, S. F., SCHNABLE, P. S. & WANG, K. 2000. Production of transgenic maize from bombarded type II callus: Effect of gold particle size and callus morphology on transformation efficiency. *In Vitro Cellular & Developmental Biology-Plant,* 36**,** 21-29.

FROMM, H., EDELMAN, M., AVIV, D. & GALUN, E. 1987. The molecular-basis for ribosomal-rna-dependent spectinomycin resistance in nicotiana chloroplasts. *Embo Journal,* 6**,** 3233-3237.

FUKAYAMA, H., UEGUCHI, C., NISHIKAWA, K., KATOH, N., ISHIKAWA, C., MASUMOTO, C., HATANAKA, T. & MISOO, S. 2012. Overexpression of Rubisco Activase Decreases the Photosynthetic CO2 Assimilation Rate by Reducing Rubisco Content in Rice Leaves. *Plant and Cell Physiology,* 53**,** 976-986.

GAO, L., SU, Y. J. & WANG, T. 2010. Plastid genome sequencing, comparative genomics, and phylogenomics: Current status and prospects. *Journal of Systematics and Evolution,* 48**,** 77-93.

GATENBY, A. A., CASTLETON, J. A. & SAUL, M. W. 1981. Expression in Escherichia-Coli of Maize and Wheat Chloroplast Genes for Large Subunit of Ribulose Bisphosphate Carboxylase. *Nature,* 291**,** 117-121.

GISBY, M. F., MUDD, E. A. & DAY, A. 2012. Growth of Transplastomic Cells Expressing D-Amino Acid Oxidase in Chloroplasts Is Tolerant to D-Alanine and Inhibited by D-Valine. *Plant Physiology,* 160**,** 2219-2226.

GOLDSCHMIDTCLERMONT, M. 1991. Transgenic expression of aminoglycoside adenine transferase in the chloroplast - a selectable marker for site-directed transformation of chlamydomonas. *Nucleic Acids Research,* 19**,** 4083-4089.

GOULD, S. B., WALLER, R. R. & MCFADDEN, G. I. 2008. Plastid evolution. *Annual Review of Plant Biology.* Palo Alto: Annual Reviews.

GRAY, B. N., AHNER, B. A. & HANSON, M. R. 2009. High-Level Bacterial Cellulase Accumulation in Chloroplast-Transformed Tobacco Mediated by Downstream Box Fusions. *Biotechnology and Bioengineering,* 102**,** 1045-1054.

GRAY, B. N., YANG, H., AHNER, B. A. & HANSON, M. R. 2011. An efficient downstream box fusion allows high-level accumulation of active bacterial beta-glucosidase in tobacco chloroplasts. *Plant Molecular Biology,* 76**,** 345-355.

GREEN, B. R. 2011. Chloroplast genomes of photosynthetic eukaryotes. *Plant Journal,* 66**,** 34-44.

GREVELDING, C., FANTES, V., KEMPER, E., SCHELL, J. & MASTERSON, R. 1993. Single-copy t-dna insertions in arabidopsis are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf-disks. *Plant Molecular Biology,* 23**,** 847-860.

HAGEMANN, R. 2002. Milestones in plastid genetics of higher plants. *In:* ESSER, K., LUETTGE, U., BEYSCHLAG, W. & HELLWIG, F. (eds.) *Progress in Botany. Genetics: Physiology: Ecology.* Springer-Verlag GmbH and Co. KG, Heidelberger Platz 3, D-14197, Berlin, Germany; Springer-Verlag New York Inc., 175 Fifth Avenue, New York, NY, 10010-7858, USA.

HAJDUKIEWICZ, P. T. J., ALLISON, L. A. & MALIGA, P. 1997. The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. *Embo Journal,* 16**,** 4041-4048.

HANSON, M. R., GRAY, B. N. & AHNER, B. A. 2013. Chloroplast transformation for engineering of photosynthesis. *Journal of Experimental Botany,* 64**,** 731-742.

HEDTKE, B., LEGEN, J., WEIHE, A., HERRMANN, R. G. & BORNER, T. 2002. Six active phage-type RNA polymerase genes in Nicotiana tabacum. *Plant Journal,* 30**,** 625-637.

HERRIN, D. L. & NICKELSEN, J. 2004. Chloroplast RNA processing and stability. *Photosynth Res,* 82**,** 301-14.

HERZ, S., FUSSL, M., STEIGER, S. & KOOP, H. U. 2005. Development of novel types of plastid transformation vectors and evaluation of factors controlling expression. *Transgenic Research,* 14**,** 969-982.

HESS, W. R., PROMBONA, A., FIEDER, B., SUBRAMANIAN, A. R. & BORNER, T. 1993. Chloroplast rps15 and the rpob/c1/c2 gene-cluster are strongly transcribed in ribosome-deficient plastids - evidence for a functioning non-chloroplast-encoded rna-polymerase. *Embo Journal,* 12**,** 563-571.

HIBBERD, J. M., LINLEY, P. J., KHAN, M. S. & GRAY, J. C. 1998. Transient expression of green fluorescent protein in various plastid types following microprojectile bombardment. *Plant Journal,* 16**,** 627-632.

HIBBERD, J. M., SHEEHY, J. E. & LANGDALE, J. A. 2008. Using C-4 photosynthesis to increase the yield of rice - rationale and feasibility. *Current Opinion in Plant Biology,* 11**,** 228-231.

HOU, B. K., ZHOU, Y. H., WAN, L. H., ZHANG, Z. L., SHEN, G. F., CHEN, Z. H. & HU, Z. M. 2003. Chloroplast transformation in oilseed rape. *Transgenic Research,* 12**,** 111-114.

HOUTZ, R. L., MAGNANI, R., NAYAK, N. R. & DIRK, L. M. A. 2008. Co- and post-translational modifications in Rubisco: unanswered questions. *Journal of Experimental Botany,* 59**,** 1635-1645.

IAMTHAM, S. & DAY, A. 2000. Removal of antibiotic resistance genes from transgenic tobacco plastids. *Nature Biotechnology,* 18**,** 1172-1176.

JARVIS, P. 2008. Targeting of nucleus-encoded proteins to chloroplasts in plants. *New Phytologist,* 179**,** 257-285.

JARVIS, P., DORMANN, P., PETO, C. A., LUTES, J., BENNING, C. & CHORY, J. 2000. Galactolipid deficiency and abnormal chloroplast development in the Arabidopsis MGD synthase 1 mutant. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 8175-8179.

JEONG, S. W., JEONG, W. J., WOO, J. W., CHOI, D. W., PARK, Y. I. & LIU, J. R. 2004. Dicistronic expression of the green fluorescent protein and antibiotic resistance genes in the plastid for selection and tracking of plastid-transformed cells in tobacco. *Plant Cell Reports,* 22**,** 747-751.

JONES, H. D. 2005. Wheat transformation: current technology and applications to grain development and composition. *Journal of Cereal Science,* 41**,** 137-147.

KAHLAU, S., ASPINALL, S., GRAY, J. C. & BOCK, R. 2006. Sequence of the tomato chloroplast DNA and evolutionary comparison of solanaceous plastid genomes. *Journal of Molecular Evolution,* 63**,** 194-207.

KAHLAU, S. & BOCK, R. 2008. Plastid transcriptomics and translatomics of tomato fruit development and chloroplast-to-chromoplast differentiation: Chromoplast gene expression largely serves the production of a single protein. *Plant Cell,* 20**,** 856-874.

KANEVSKI, I. & MALIGA, P. 1994. Relocation of the plastid rbcl gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco chloroplasts. *Proceedings of the National Academy of Sciences of the United States of America,* 91**,** 1969-1973.

KANEVSKI, I., MALIGA, P., RHOADES, D. F. & GUTTERIDGE, S. 1999. Plastome engineering of ribulose-1,5-bisphosphate carboxylase/oxygenase in tobacco to form a sunflower large subunit and tobacco small subunit hybrid. *Plant Physiology,* 119**,** 133-141.

KEBEISH, R., NIESSEN, M., THIRUVEEDHI, K., BARI, R., HIRSCH, H.-J., ROSENKRANZ, R., STAEBLER, N., SCHOENFELD, B., KREUZALER, F. & PETERHAENSEL, C. 2007. Chloroplastic photorespiratory bypass increases photosynthesis and biomass production in Arabidopsis thaliana. *Nature Biotechnology,* 25**,** 593-599.

KHAN, M. S. 2007. Engineering photorespiration in chloroplasts: a novel strategy for increasing biomass production. *Trends in Biotechnology,* 25**,** 437-440.

KHAN, M. S. & MALIGA, P. 1999. Fluorescent antibiotic resistance marker for tracking plastid transformation in higher plants. *Nature Biotechnology,* 17**,** 910-915.

KLEIN, T. M., WOLF, E. D., WU, R. & SANFORD, J. C. 1987. High-velocity microprojectiles for delivering nucleic-acids into living cells. *Nature,* 327**,** 70-73.

KLEINE, T., MAIER, U. G. & LEISTER, D. 2009. DNA Transfer from Organelles to the Nucleus: The Idiosyncratic Genetics of Endosymbiosis. *Annual Review of Plant Biology.* Palo Alto: Annual Reviews.

KOBAYASHI, T., TAKAHARA, M., MIYAGISHIMA, S. Y., KUROIWA, H., SASAKI, N., OHTA, N., MATSUZAKI, M. & KUROIWA, T. 2002. Detection and localization of a chloroplast-encoded HU-like protein that organizes chloroplast nucleoids. *Plant Cell,* 14**,** 1579-1589.

KRAUSE, K., MAIER, R. M., KOFER, W., KRUPINSKA, K. & HERRMANN, R. G. 2000. Disruption of plastid-encoded RNA polymerase genes in tobacco: expression of only a distinct set of genes is not based on selective transcription of the plastid chromosome. *Molecular and General Genetics,* 263**,** 1022-1030.

KUMAR, A., LI, C. & PORTIS, A. R., JR. 2009. Arabidopsis thaliana expressing a thermostable chimeric Rubisco activase exhibits enhanced growth and higher rates of photosynthesis at moderately high temperatures. *Photosynthesis Research,* 100**,** 143-153.

KUMAR, S., DHINGRA, A. & DANIELL, H. 2004. Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. *Plant Molecular Biology,* 56**,** 203-216.

KURODA, H. & MALIGA, P. 2001a. Complementarity of the 16S rRNA penultimate stem with sequences downstream of the AUG destabilizes the plastid mRNAs. *Nucleic Acids Research,* 29**,** 970-975.

KURODA, H. & MALIGA, P. 2001b. Sequences downstream of the translation initiation codon are important determinants of translation efficiency in chloroplasts. *Plant Physiol,* 125**,** 430-6.

KUROIWA, T. 1991. The replication, differentiation, and inheritance of plastids with emphasis on the concept of organelle nuclei. *International Review of Cytology-a Survey of Cell Biology,* 128**,** 1-&.

KUSUMI, K., YARA, A., MITSUI, N., TOZAWA, Y. & IBA, K. 2004. Characterization of a rice nuclear-encoded plastid RNA polymerase gene OsRpoTp. *Plant and Cell Physiology,* 45**,** 1194-1201.

LEE, S. M., KANG, K., CHUNG, H., YOO, S. H., XU, X. M., LEE, S.-B., CHEONG, J.-J., DANIELL, H. & KIM, M. 2006. Plastid transformation in the monocotyledonous cereal crop, rice (Oryza sativa) and transmission of transgenes to their progeny. *Molecules and Cells,* 21**,** 401-410.

LEFEBVRE, S., LAWSON, T., ZAKHLENIUK, O. V., LLOYD, J. C. & RAINES, C. A. 2005. Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. *Plant Physiology,* 138**,** 451-460.

LEGEN, J., KEMP, S., KRAUSE, K., PROFANTER, B., HERRMANN, R. G. & MAIER, R. M. 2002. Comparative analysis of plastid transcription profiles of entire plastid chromosomes from tobacco attributed to wild-type and PEP-deficient transcription machineries. *Plant Journal,* 31**,** 171-188.

LELIVELT, C. L. C., MCCABE, M. S., NEWELL, C. A., DESNOO, C. B., VAN DUN, K. M. P., BIRCH-MACHIN, I., GRAY, J. C., MILLS, K. H. G. & NUGENT, J. M. 2005. Stable plastid transformation in lettuce (Lactuca sativa L.). *Plant Molecular Biology,* 58**,** 763-774.

LI, W., LIAO, Y., HUANG, T. & LI, H. 2012. Construction of a polycistronic expression vector for site-specific integration into rapeseed chloroplast. *Genomics and Applied Biology,* 31**,** 460-466.

LIEMAN-HURWITZ, J., RACHMILEVITCH, S., MITTLER, R., MARCUS, Y. & KAPLAN, A. 2003. Enhanced photosynthesis and growth of transgenic plants that express ictB, a gene involved in HCO3- accumulation in cyanobacteria. *Plant Biotechnology Journal,* 1**,** 43-50.

LIERE, K. & BOERNER, T. 2007. Transcription and transcriptional regulation in plastids. *Topics in Current Genetics,* 19**,** 121-174.

LIERE, K., KADEN, D., MALIGA, P. & BORNER, T. 2004. Overexpression of phage-type RNA polymerase RpoTp in tobacco demonstrates its role in chloroplast transcription by recognizing a distinct promoter type. *Nucleic Acids Research,* 32**,** 1159-1165.

LIERE, K., WEIHE, A. & BORNER, T. 2011. The transcription machineries of plant mitochondria and chloroplasts: Composition, function, and regulation. *Journal of Plant Physiology,* 168**,** 1345-1360.

LISITSKY, I. & SCHUSTER, G. 1995. Phosphorylation of a chloroplast RNA-binding protein changes its affinity to RNA. *Nucleic Acids Res,* 23**,** 2506-11.

LIU, C.-W., LIN, C.-C., CHEN, J. J. W. & TSENG, M.-J. 2007. Stable chloroplast transformation in cabbage (Brassica oleracea L. var. capitata L.) by particle bombardment. *Plant Cell Reports,* 26**,** 1733-1744.

LOBELL, D. B. & FIELD, C. B. 2007. Global scale climate - crop yield relationships and the impacts of recent warming. *Environmental Research Letters,* 2.

LOHSE, M., DRECHSEL, O., KAHLAU, S. & BOCK, R. 2013. OrganellarGenomeDRAW-a suite of tools for generating physical maps of plastid and mitochondrial genomes and visualizing expression data sets. *Nucleic Acids Research,* 41**,** W575-W581.

LONG, S. P., AINSWORTH, E. A., LEAKEY, A. D. B., NOSBERGER, J. & ORT, D. R. 2006a. Food for thought: Lower-than-expected crop yield stimulation with rising CO2 concentrations. *Science,* 312**,** 1918-1921.

LONG, S. P., ZHU, X. G., NAIDU, S. L. & ORT, D. R. 2006b. Can improvement in photosynthesis increase crop yields? *Plant Cell and Environment,* 29**,** 315-330.

LU, C. Y. & VASIL, I. K. 1982. Somatic embryogenesis and plant-regeneration in tissue-cultures of panicum-maximum jacQ. *American Journal of Botany,* 69**,** 77-81.

LUTZ, K. A., AZHAGIRI, A. K., TUNGSUCHAT-HUANG, T. & MALIGA, P. 2007. A guide to choosing vectors for transformation of the plastid genome of higher plants. *Plant Physiology,* 145**,** 1201-1210.

LYSENKO, E. A. 2007. Plant sigma factors and their role in plastid transcription. *Plant Cell Reports,* 26**,** 845-859.

LYSENKO, E. A. & KUSNETSOV, V. V. 2005. Plastid RNA polymerases. *Molecular Biology,* 39**,** 762-775.

MAGNUSSON, I. & BORNMAN, C. H. 1985. Anatomical observations on somatic embryogenesis from scutellar tissues of immature zygotic embryos of triticum-aestivum. *Physiologia Plantarum,* 63**,** 137-145.

MAIER, A., FAHNENSTICH, H., VON CAEMMERER, S., ENGQVIST, M. K. M., WEBER, A. P. M., FLUEGGE, U.-I. & MAURINO, V. G. 2012. Transgenic introduction of a

glycolate oxidative cycle into A-thaliana chloroplasts leads to growth improvement. *Frontiers in Plant Science,* 3.

MALIGA, P. 2001. Plastid engineering bears fruit - The tomato has been engineered to express in its plastids high levels of a recombinant protein. *Nature Biotechnology,* 19**,** 826-827.

MALIGA, P. 2002. Engineering the plastid genome of higher plants. *Current Opinion in Plant Biology,* 5**,** 164-172.

MALIGA, P. 2003. Progress towards commercialization of plastid transformation technology. *Trends in Biotechnology,* 21**,** 20-28.

MALIGA, P. 2004. Plastid transformation in higher plants. *Annual Review of Plant Biology,* 55**,** 289-313.

MANUELL, A. L., QUISPE, J. & MAYFIELD, S. P. 2007. Structure of the chloroplast ribosome: novel domains for translation regulation. *PLoS Biol,* 5**,** e209.

MARIN-NAVARRO, J., MANUELL, A. L., WU, J. & S, P. M. 2007. Chloroplast translation regulation. *Photosynth Res,* 94**,** 359-74.

MARTIN, W. 2003. Gene transfer from organelles to the nucleus: Frequent and in big chunks. *Proceedings of the National Academy of Sciences of the United States of America,* 100**,** 8612-8614.

MARTIN, W., STOEBE, B., GOREMYKIN, V., HANSMANN, S., HASEGAWA, M. & KOWALLIK, K. V. 1998. Gene transfer to the nucleus and the evolution of chloroplasts. *Nature,* 393**,** 162-165.

MAURINO, V. G. & PETERHANSEL, C. 2010. Photorespiration: current status and approaches for metabolic engineering. *Current Opinion in Plant Biology,* 13**,** 249-256.

MCFADDEN, G. I. 1999. Plastids and protein targeting. *Journal of Eukaryotic Microbiology,* 46**,** 339-346.

MCFADDEN, G. I. & VAN DOOREN, G. G. 2004. Evolution: Red algal genome affirms a common origin of all plastids. *Current Biology,* 14**,** R514-R516.

MELKONIAN, M. 1990. PHYLUM CHLOROPHYTA INTRODUCTION TO THE CHLOROPHYTA. *Margulis, L., Et Al. (Ed.). Jones and Bartlett Series in Life Sciences: Handbook of Protoctista: the Structure, Cultivation, Habitats and Life Histories of the Eukaryotic Microorganisms and Their Descendants Exclusive of Animals, Plants and Fungi: a Guide to the Algae, Ciliates, Foraminifera, Sporozoa, Water Molds, Slime Molds and the Other Protoctists. Xli+914p. Jones and Bartlett Publishers, Inc.: Boston, Massachusetts, USA. Illus***,** 597-599.

MEURER, J., LEZHNEVA, L., AMANN, K., GODEL, M., BEZHANI, S., SHERAMETI, I. & OELMULLER, R. 2002. A peptide chain release factor 2 affects the stability of UGA-containing transcripts in Arabidopsis chloroplasts. *Plant Cell,* 14**,** 3255-3269.

MIRALLES, D. J. & SLAFER, G. A. 2007. Sink limitations to yield in wheat: how could it be reduced? *Journal of Agricultural Science,* 145**,** 139-149.

MIYAGAWA, Y., TAMOI, M. & SHIGEOKA, S. 2001. Overexpression of a cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase in tobacco enhances photosynthesis and growth. *Nature Biotechnology,* 19**,** 965-969.

MOGENSEN, H. L. 1996. The hows and whys of cytoplasmic inheritance in seed plants. *American Journal of Botany,* 83**,** 383-404.

MORDEN, C. W., DELWICHE, C. F., KUHSEL, M. & PALMER, J. D. 1992. Gene phylogenies and the endosymbiotic origin of plastids. *Biosystems,* 28**,** 75-90.

MOTOHASHI, R., YAMAZAKI, T., MYOUGA, F., ITO, T., ITO, K., SATOU, M., KOBAYASHI, M., NAGATA, N., YOSHIDA, S., NAGASHIMA, A., TANAKA, K., TAKAHASHI, S. & SHINOZAKI, K. 2007. Chloroplast ribosome release factor 1 (AtcpRF1) is essential for chloroplast development. *Plant Molecular Biology,* 64**,** 481-497.

MUHLBAUER, S. K. & KOOP, H. U. 2005. External control of transgene expression in tobacco plastids using the bacterial lac repressor. *Plant Journal,* 43**,** 941-946.

MULLET, J. E. & KLEIN, R. R. 1987. Transcription and rna stability are important determinants of higher-plant chloroplast rna levels. *Embo Journal,* 6**,** 1571-1579.

NEWMAN, S. M., BOYNTON, J. E., GILLHAM, N. W., RANDOLPHANDERSON, B. L., JOHNSON, A. M. & HARRIS, E. H. 1990. Transformation of chloroplast ribosomal-rna genes in chlamydomonas - molecular and genetic-characterization of integration events. *Genetics,* 126**,** 875-888.

NISHIMURA, K., OGAWA, T., ASHIDA, H. & YOKOTA, A. 2008. Molecular mechanisms of RuBisCO biosynthesis in higher plants. *Plant Biotechnology,* 25**,** 285-290.

NUGENT, G. D., TEN HAVE, M., VAN DER GULIK, A., DIX, P. J., UIJTEWAAL, B. A. & MORDHORST, A. P. 2005. Plastid transformants of tomato selected using mutations affecting ribosome structure. *Plant Cell Reports,* 24**,** 341-349.

OEY, M., LOHSE, M., KREIKEMEYER, B. & BOCK, R. 2009. Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. *Plant Journal,* 57**,** 436-445.

OMATA, T., PRICE, G. D., BADGER, M. R., OKAMURA, M., GOHTA, S. & OGAWA, T. 1999. Identification of an ATP-binding cassette transporter involved in bicarbonate uptake in the cyanobacterium Synechococcus sp strain PCC 7942. *Proceedings of the National Academy of Sciences of the United States of America,* 96**,** 13571-13576.

ONEILL, C., HORVATH, G. V., HORVATH, E., DIX, P. J. & MEDGYESY, P. 1993. Chloroplast transformation in plants - polyethylene-glycol (peg) treatment of protoplasts is an alternative to biolistic delivery systems. *Plant Journal,* 3**,** 729-738.

OZIASAKINS, P. & VASIL, I. K. 1982. Plant-regeneration from cultured immature embryos and inflorescences of triticum-aestivum l (wheat) - evidence for somatic embryogenesis. *Protoplasma,* 110**,** 95-105.

PARRY, M. A. J., KEYS, A. J., MADGWICK, P. J., CARMO-SILVA, A. E. & ANDRALOJC, P. J. 2008. Rubisco regulation: a role for inhibitors. *Journal of Experimental Botany,* 59**,** 1569-1580.

PARRY, M. A. J., MADGWICK, P. J., CARVALHO, J. F. C. & ANDRALOJC, P. J. 2007. Prospects for increasing photosynthesis by overcoming the limitations of Rubisco. *Journal of Agricultural Science,* 145**,** 31-43.

PARRY, M. A. J., REYNOLDS, M., SALVUCCI, M. E., RAINES, C., ANDRALOJC, P. J., ZHU, X. G., PRICE, G. D., CONDON, A. G. & FURBANK, R. T. 2011. Raising yield potential of wheat. II. Increasing photosynthetic capacity and efficiency. *Journal of Experimental Botany,* 62**,** 453-467.

PRICE, G. D. 2011. Inorganic carbon transporters of the cyanobacterial CO2 concentrating mechanism. *Photosynthesis Research,* 109**,** 47-57.

PRICE, G. D. & BADGER, M. R. 1989. Expression of human carbonic-anhydrase in the cyanobacterium synechococcus pcc7942 creates a high co2-requiring phenotype - evidence for a central role for carboxysomes in the co2 concentrating mechanism. *Plant Physiology,* 91**,** 505-513.

PRICE, G. D., BADGER, M. R., WOODGER, F. J. & LONG, B. M. 2008. Advances in understanding the cyanobacterial CO(2)-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants. *Journal of Experimental Botany,* 59**,** 1441-1461.

PRICE, G. D., PENGELLY, J. J. L., FORSTER, B., DU, J., WHITNEY, S. M., VON CAEMMERER, S., BADGER, M. R., HOWITT, S. M. & EVANS, J. R. 2013. The cyanobacterial CCM as a source of genes for improving photosynthetic CO2 fixation in crop species. *Journal of Experimental Botany,* 64**,** 753-768.

PRICE, G. D., WOODGER, F. J., BADGER, M. R., HOWITT, S. M. & TUCKER, L. 2004. Identification of a SulP-type bicarbonate transporter in marine cyanobacteria. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 18228-18233.

PRITCHARD, S. G., ROGERS, H. H., PRIOR, S. A. & PETERSON, C. M. 1999. Elevated CO2 and plant structure: a review. *Global Change Biology,* 5**,** 807-837.

PRZIBILLA, E., HEISS, S., JOHANNINGMEIER, U. & TREBST, A. 1991. SITE-SPECIFIC MUTAGENESIS OF THE D1 SUBUNIT OF PHOTOSYSTEM-II IN WILD-TYPE CHLAMYDOMONAS. *Plant Cell,* 3**,** 169-174.

QUESADA-VARGAS, T., RUIZ, O. N. & DANIELL, H. 2005. Characterization of heterologous multigene operons in transgenic chloroplasts. Transcription, processing, and translation. *Plant Physiology,* 138**,** 1746-1762.

RAINES, C. A. 2006. Transgenic approaches to manipulate the environmental responses of the C(3) carbon fixation cycle. *Plant Cell and Environment,* 29**,** 331-339.

RAVI, V., KHURANA, J. P., TYAGI, A. K. & KHURANA, P. 2008. An update on chloroplast genomes. *Plant Systematics and Evolution,* 271**,** 101-122.

REUMANN, S., INOUE, K. & KEEGSTRA, K. 2005. Evolution of the general protein import pathway of plastids (Review). *Molecular Membrane Biology,* 22**,** 73-U20.

REZNIKOFF, W. S., SIEGELE, D. A., COWING, D. W. & GROSS, C. A. 1985. The regulation of transcription initiation in bacteria. *Annu Rev Genet,* 19**,** 355-87.

ROSEGRANT, M. W. & CLINE, S. A. 2003. Global food security: Challenges and policies. *Science,* 302**,** 1917-1919.

ROTT, R., DRAGER, R. G., STERN, D. B. & SCHUSTER, G. 1996. The 3' untranslated regions of chloroplast genes in Chlamydomonas reinhardtii do not serve as efficient transcriptional terminators. *Mol Gen Genet,* 252**,** 676-83.

ROTT, R., LIVEANU, V., DRAGER, R. G., STERN, D. B. & SCHUSTER, G. 1998. The sequence and structure of the 3'-untranslated regions of chloroplast transcripts are important determinants of mRNA accumulation and stability. *Plant Mol Biol,* 36**,** 307-14.

RUF, S., HERMANN, M., BERGER, I. J., CARRER, H. & BOCK, R. 2001. Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nature Biotechnology,* 19**,** 870-875.

RUF, S., KARCHER, D. & BOCK, R. 2007. Determining the transgene containment level provided by chloroplast transformation. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 6998-7002.

SALVUCCI, M. E. & CRAFTS-BRANDNER, S. J. 2004. Inhibition of photosynthesis by heat stress: the activation state of Rubisco as a limiting factor in photosynthesis. *Physiologia Plantarum,* 120**,** 179-186.

SANFORD, J. C. 1990. Biolistic plant transformation. *Physiologia Plantarum,* 79**,** 206-209.

SATO, N., ALBRIEUX, C., JOYARD, J., DOUCE, R. & KUROIWA, T. 1993. DETECTION AND CHARACTERIZATION OF A PLASTID ENVELOPE DNA-BINDING PROTEIN WHICH MAY ANCHOR PLASTID NUCLEOIDS. *Embo Journal,* 12**,** 555-561.

SCHLEIFF, E., EICHACKER, L. A., ECKART, K., BECKER, T., MIRUS, O., STAHL, T. & SOLL, J. 2003. Prediction of the plant beta-barrel proteome: A case study of the chloroplast outer envelope. *Protein Science,* 12**,** 748-759.

SCHMITZ, A. J., GLYNN, J. M., OLSON, B., STOKES, K. D. & OSTERYOUNG, K. W. 2009. Arabidopsis FtsZ2-1 and FtsZ2-2 Are Functionally Redundant, But FtsZ-Based Plastid Division Is Not Essential for Chloroplast Partitioning or Plant Growth and Development. *Molecular Plant,* 2**,** 1211-1222.

SCHMITZ-LINNEWEBER, C., KUSHNIR, S., BABIYCHUK, E., POLTNIGG, P., HERRMANN, R. G. & MAIER, R. M. 2005. Pigment deficiency in nightshade/tobacco cybrids is caused by the failure to edit the plastid ATPase alpha-subunit mRNA. *Plant Cell,* 17**,** 1815-1828.

SCHWEER, J., TUERKERI, H., KOLPACK, A. & LINK, G. 2010. Role and regulation of plastid sigma factors and their functional interactors during chloroplast transcription - Recent lessons from Arabidopsis thaliana. *European Journal of Cell Biology,* 89**,** 940-946.

SCHWENKERT, S., SOLL, J. & BOLTER, B. 2011. Protein import into chloroplasts-How chaperones feature into the game. *Biochimica Et Biophysica Acta-Biomembranes,* 1808**,** 901-911.

SEARS, R. G. & DECKARD, E. L. 1982. Tissue-culture variability in wheat - callus induction and plant-regeneration. *Crop Science,* 22**,** 546-550.

SERINO, G. & MALIGA, P. 1997. A negative selection scheme based on the expression of cytosine deaminase in plastids. *Plant Journal,* 12**,** 697-701.

SEVERINOV, K., MUSTAEV, A., KUKARIN, A., MUZZIN, O., BASS, I., DARST, S. A. & GOLDFARB, A. 1996. Structural modules of the large subunits of RNA polymerase - Introducing archaebacterial and chloroplast split sites in the beta and beta' subunits Escherichia coli RNA polymerase. *Journal of Biological Chemistry,* 271**,** 27969-27974.

SHARWOOD, R. E., VON CAEMMERER, S., MALIGA, P. & WHITNEY, S. M. 2008. The catalytic properties of hybrid Rubisco comprising tobacco small and sunflower large Subunits mirror the kinetically equivalent source Rubiscos and can support tobacco growth. *Plant Physiology,* 146**,** 83-96.

SHARWOOD, R. E. & WHITNEY, S. M. 2010. Engineering the Sunflower Rubisco Subunits into Tobacco Chloroplasts: New Considerations. *In:* REBEIZ, C. A., BENNING, C., BOHNERT, H. J., DANIELL, H., HOOBER, J. K., LICHTENTHALER, H. K., PORTIS, A. R. & TRIPATHY, B. C. (eds.) *Chloroplast: Basics and Applications.*

SHEARMAN, V. J., SYLVESTER-BRADLEY, R., SCOTT, R. K. & FOULKES, M. J. 2005. Physiological processes associated with wheat yield progress in the UK. *Crop Science,* 45**,** 175-185.

SHI, L. X. & THEG, S. M. 2013. The chloroplast protein import system: From algae to trees. *Biochimica Et Biophysica Acta-Molecular Cell Research,* 1833**,** 314-331.

SHIBATA, M., KATOH, H., SONODA, M., OHKAWA, H., SHIMOYAMA, M., FUKUZAWA, H., KAPLAN, A. & OGAWA, T. 2002. Genes essential to sodium-dependent bicarbonate transport in cyanobacteria - Function and phylogenetic analysis. *Journal of Biological Chemistry,* 277**,** 18658-18664.

SHIINA, T., ISHIZAKI, Y., YAGI, Y. & NAKAHIRA, Y. 2009. Function and evolution of plastid sigma factors. *Plant Biotechnology,* 26**,** 57-66.

SHIINA, T., TSUNOYAMA, Y., NAKAHIRA, Y. & KHAN, M. S. 2005. Plastid RNA polymerases, promoters, and transcription regulators in higher plants. *International Review of Cytology - a Survey of Cell Biology, Vol 244,* 244**,** 1-68.

SHIMADA, T., SASAKUMA, T. & TSUNEWAK.K 1969. IN VITRO CULTURE OF WHEAT TISSUES .I. CALLUS FORMATION, ORGAN REDIFFERENTIATION AND SINGLE CELL CULTURE. *Canadian Journal of Genetics and Cytology,* 11**,** 294-&.

SHINOZAKI, K., OHME, M., TANAKA, M., WAKASUGI, T., HAYASHIDA, N., MATSUBAYASHI, T., ZAITA, N., CHUNWONGSE, J., OBOKATA, J., YAMAGUCHISHINOZAKI, K., OHTO, C., TORAZAWA, K., MENG, B. Y., SUGITA, M., DENO, H., KAMOGASHIRA, T., YAMADA, K., KUSUDA, J., TAKAIWA, F., KATO, A., TOHDOH, N., SHIMADA, H. & SUGIURA, M. 1986. THE COMPLETE NUCLEOTIDE-SEQUENCE OF THE TOBACCO CHLOROPLAST GENOME - ITS GENE ORGANIZATION AND EXPRESSION. *Embo Journal,* 5**,** 2043-2049.

SIDOROV, V. A., KASTEN, D., PANG, S. Z., HAJDUKIEWICZ, P. T. J., STAUB, J. M. & NEHRA, N. S. 1999. Stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. *Plant Journal,* 19**,** 209-216.

SILHAVY, D. & MALIGA, P. 1998. Mapping of promoters for the nucleus encoded plastid RNA polymerase (NEP) in the iojap maize mutant. *Current Genetics,* 33**,** 340-344.

SKILLMAN, J. B. 2008. Quantum yield variation across the three pathways of photosynthesis: not yet out of the dark. *Journal of Experimental Botany,* 59**,** 1647-1661.

SPALDING, M. H. 2008. Microalgal carbon-dioxide-concentrating mechanisms: Chlamydomonas inorganic carbon transporters. *Journal of Experimental Botany,* 59**,** 1463-1473.

SPRENGART, M. L., FATSCHER, H. P. & FUCHS, E. 1990. The initiation of translation in E. coli: apparent base pairing between the 16srRNA and downstream sequences of the mRNA. *Nucleic Acids Res,* 18**,** 1719-23.

SPRENGART, M. L., FUCHS, E. & PORTER, A. G. 1996. The downstream box: An efficient and independent translation initiation signal in Escherichia coli. *Embo Journal,* 15**,** 665-674.

STAUB, J. M. & MALIGA, P. 1993. Accumulation of D1 polypeptide in tobacco plastids is regulated via the untranslated region of the psbA mRNA. *EMBO J,* 12**,** 601-6.

STAUB, J. M. & MALIGA, P. 1995. Expression of a chimeric uidA gene indicates that polycistronic mRNAs are efficiently translated in tobacco plastids. *Plant J,* 7**,** 845-8.

STEINER, J. M., YUSA, F., POMPE, J. A. & LOFFELHARDT, W. 2005. Homologous protein import machineries in chloroplasts and cyanelles. *Plant Journal,* 44**,** 646-652.

STERN, D. B., GOLDSCHMIDT-CLERMONT, M. & HANSON, M. R. 2010. Chloroplast RNA Metabolism. *Annual Review of Plant Biology, Vol 61,* 61**,** 125-155.

STERN, D. B. & GRUISSEM, W. 1987. Control of plastid gene-expression - 3' inverted repeats act as messenger-rna processing and stabilizing elements, but do not terminate transcriptioN. *Cell,* 51**,** 1145-1157.

STERN, D. B., HIGGS, D. C. & YANG, J. J. 1997. Transcription and translation in chloroplasts. *Trends in Plant Science,* 2**,** 308-315.

STOPPEL, R. & MEURER, J. 2012. The cutting crew - ribonucleases are key players in the control of plastid gene expression. *Journal of Experimental Botany,* 63**,** 1663-1673.

STUDIER, F. W., ROSENBERG, A. H., DUNN, J. J. & DUBENDORFF, J. W. 1990. Use of T7 Rna-Polymerase to Direct Expression of Cloned Genes. *Methods in Enzymology,* 185**,** 60-89.

SUGIURA, M. 2008. RNA editing in chloroplasts. *In:* GORINGER, H. U. (ed.) *Nucleic Acids and Molecular Biology.*

SUGIURA, M., HIROSE, T. & SUGITA, M. 1998. Evolution and mechanism of translation in chloroplasts. *Annual Review of Genetics,* 32**,** 437-459.

SVAB, Z., HAJDUKIEWICZ, P. & MALIGA, P. 1990. STABLE TRANSFORMATION OF PLASTIDS IN HIGHER-PLANTS. *Proceedings of the National Academy of Sciences of the United States of America,* 87**,** 8526-8530.

SVAB, Z. & MALIGA, P. 1993. High-frequency plastid transformation in tobacco by selection for a chimeric aada gene. *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 913-917.

SVAB, Z. & MALIGA, P. 2007. Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 7003-7008.

SWIATECKA-HAGENBRUCH, M., LIERE, K. & BOERNER, T. 2007. High diversity of plastidial promoters in Arabidopsis thaliana. *Molecular Genetics and Genomics,* 277**,** 725-734.

TAMOI, M., NAGAOKA, M., MIYAGAWA, Y. & SHIGEOKA, S. 2006. Contribution of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase to the photosynthetic rate and carbon flow in the Calvin cycle in transgenic plants. *Plant and Cell Physiology,* 47**,** 380-390.

TCHERKEZ, G. G. B., FARQUHAR, G. D. & ANDREWS, T. J. 2006. Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 7246-7251.

THOMAS, E., KING, P. J. & POTRYKUS, I. 1979. Improvement of crop plants via single cells invitro - assessment. *Zeitschrift Fur Pflanzenzuchtung-Journal of Plant Breeding,* 82**,** 1-30.

TILLER, N. & BOCK, R. 2014. The Translational Apparatus of Plastids and Its Role in Plant Development. *Molecular Plant, 7*, 1105-1120.

VASIL, I. K., AHUJA, M. R. & VASIL, V. 1979. Plant tissue cultures in genetics and plant breeding. *Caspari, E. W. (Ed.). Advances in Genetics, Vol. 20. Ix+464p. Academic Press: New York, N.Y., USA; London, England. Illus*, P127-216.

VASIL, V. & VASIL, I. K. 1980. Isolation and culture of cereal protoplasts .2. embryogenesis and plantlet formation from protoplasts of pennisetum-americanum. *Theoretical and Applied Genetics, 56*, 97-99.

VERHOUNIG, A., KARCHER, D. & BOCK, R. 2010. Inducible gene expression from the plastid genome by a synthetic riboswitch. *Proceedings of the National Academy of Sciences of the United States of America, 107*, 6204-6209.

WANG, L., OUYANG, M., LI, Q., ZOU, M., GUO, J., MA, J., LU, C. & ZHANG, L. 2010. The Arabidopsis chloroplast ribosome recycling factor is essential for embryogenesis and chloroplast biogenesis. *Plant Molecular Biology, 74*, 47-59.

WANI, S. H., HAIDER, N., KUMAR, H. & SINGH, N. B. 2010. Plant Plastid Engineering. *Current Genomics, 11*, 500-512.

WEI, Z. M. 1982. POLLEN CALLUS-CULTURE IN TRITICUM-AESTIVUM. *Theoretical and Applied Genetics, 63*, 71-73.

WERNICKE, W. & MILKOVITS, L. 1984. Developmental gradients in wheat leaves - response of leaf segments in different genotypes cultured invitro. *Journal of Plant Physiology, 115*, 49-58.

WHITNEY, S. M. & ANDREWS, T. J. 2001a. Plastome-encoded bacterial ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) supports photosynthesis and growth in tobacco. *Proceedings of the National Academy of Sciences of the United States of America, 98*, 14738-14743.

WHITNEY, S. M. & ANDREWS, T. J. 2001b. The gene for the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) small subunit relocated to the plastid genome of tobacco directs the synthesis of small subunits that assemble into Rubisco. *Plant Cell, 13*, 193-205.

WHITNEY, S. M., BALDETT, P., HUDSON, G. S. & ANDREWS, T. J. 2001. Form I Rubiscos from non-green algae are expressed abundantly but not assembled in tobacco chloroplasts. *Plant Journal, 26*, 535-547.

WHITNEY, S. M., HOUTZ, R. L. & ALONSO, H. 2011. Advancing Our Understanding and Capacity to Engineer Nature's CO2-Sequestering Enzyme, Rubisco. *Plant Physiology, 155*, 27-35.

WHITNEY, S. M., KANE, H. J., HOUTZ, R. L. & SHARWOOD, R. E. 2009. Rubisco Oligomers Composed of Linked Small and Large Subunits Assemble in Tobacco Plastids and Have Higher Affinities for CO2 and O-2. *Plant Physiology, 149*, 1887-1895.

WHITNEY, S. M. & SHARWOOD, R. E. 2008. Construction of a tobacco master line to improve Rubisco engineering in chloroplasts. *Journal of Experimental Botany, 59*, 1909-1921.

WILKINSON, M. J., SWEET, J. & POPPY, G. M. 2003. Risk assessment of GM plants: avoiding gridlock? *Trends in Plant Science, 8*, 208-212.

XU, M., BERNAT, G., SINGH, A., MI, H., ROEGNER, M., PAKRASI, H. B. & OGAWA, T. 2008. Properties of Mutants of Synechocystis sp Strain PCC 6803 Lacking

Inorganic Carbon Sequestration Systems. *Plant and Cell Physiology,* 49**,** 1672-1677.

YANG, H., GRAY, B. N., AHNER, B. A. & HANSON, M. R. 2013. Bacteriophage 5 ' untranslated regions for control of plastid transgene expression. *Planta,* 237**,** 517-527.

YE, G. N., COLBURN, S. M., XU, C. W., HAJDUKIEWICZ, P. T. J. & STAUB, J. M. 2003. Persistence of unselected transgenic DNA during a plastid transformation and segregation approach to herbicide resistance. *Plant Physiology,* 133**,** 402-410.

YE, G. N., HAJDUKIEWICZ, P. T. J., BROYLES, D., RODRIGUEZ, D., XU, C. W., NEHRA, N. & STAUB, J. M. 2001. Plastid-expressed 5-enolpyruvylshikimate-3-phosphate synthase genes provide high level glyphosate tolerance in tobacco. *Plant Journal,* 25**,** 261-270.

ZARZYCKI, J., AXEN, S. D., KINNEY, J. N. & KERFELD, C. A. 2013. Cyanobacterial-based approaches to improving photosynthesis in plants. *Journal of Experimental Botany,* 64**,** 787-798.

ZHANG, X.-H., WEBB, J., HUANG, Y.-H., LIN, L., TANG, R.-S. & LIU, A. 2011. Hybrid Rubisco of tomato large subunits and tobacco small subunits is functional in tobacco plants. *Plant Science,* 180**,** 480-488.

ZHANG, X. H., BROTHERTON, J. E., WIDHOLM, J. M. & PORTIS, A. R. 2001. Targeting a nuclear anthranilate synthase alpha-subunit gene to the tobacco plastid genome results in enhanced tryptophan biosynthesis. Return of a gene to its pre-endosymbiotic origin. *Plant Physiology,* 127**,** 131-141.

ZHU, C., ZHANG, N., XIE, W., NIU, Y., YANG, W., LIU, J. & LI, H. 2011. Foreign protein expression in chloroplast transformation of Phaeodactylum tricornutum. *Journal of Tropical and Subtropical Botany,* 19**,** 267-272.

ZOSCHKE, R., LIERE, K. & BOERNER, T. 2007. From seedling to mature plant: Arabidopsis plastidial genome copy number, RNA accumulation and transcription are differentially regulated during leaf development. *Plant Journal,* 50**,** 710-722.

ZOUBENKO, O. V., ALLISON, L. A., SVAB, Z. & MALIGA, P. 1994. EFFICIENT TARGETING OF FOREIGN GENES INTO THE TOBACCO PLASTID GENOME. *Nucleic Acids Research,* 22**,** 3819-3824.