An Investigation into Transformation and RNA Editing in the *Lolium* Plastome

A thesis submitted by

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Abbreviations:

A:	adenosine
aadA:	aminoglycoside 3"-adenylyltransferase
aphA-6:	3'-aminoglycoside phosphotransferase
APX:	ascorbate peroxidase
ATP:	adenosine-5'-triphosphate
BAP:	6-benzylaminopurine
BSA:	bovine serum albumin
C:	cytosine
$CaCl_2$:	calcium chloride
CAT:	catalase
cDNA:	complementary DNA
CIM:	callus induction medium
CMM:	callus maintenance medium
CO_2:	Carbon dioxide
Cv:	cultivar
2,4-D:	2,4-dichlorophenoxyacetic acid
DHAR:	dehydroascorbate reductase
DIG:	digoxigenin
DNA:	deoxyribonucleic acid
dNTP:	deoxynucleotide triphosphate
DW:	dry weight
EDTA:	ethylenediaminetetra acetic acid
EU:	European union
ETC:	electron transport chain
FW:	fresh weight
g:	gram
G-418:	geneticin
gDNA:	genomic DNA
GFP:	green fluorescent protein
GM:	genetically modified

GM:	germination medium
gRNA:	guide RNA
GR:	glutathione reductase
GRIN:	Germplasm Resources Information Network
GST:	glutathione S-transferase
GUS:	β-glucoronidase
H_2O_2:	hydrogen peroxyide
hpt:	hygromycin phosphotransferase
I:	inosine
IR:	inverted repeat
K:	kilo
L:	litre
LB:	Luria broth
LSC:	large single copy
μ:	micro
m:	milli
M:	molar
mRNA:	messenger RNA
MS:	Murashige and Skoog
mtDNA:	mitochondrial DNA
NAA:	α-Naphtalene acetid acid
NADPH:	nicotinamide adenine dinucleotide phosphate
NaOH:	sodium hydroxide
NDH complex:	NADH dehydrogenase complex
NEB:	New England Biolabs
NEP:	nuclear-encoded RNA polymerase
nptII:	neomycin phosphotransferase II
$^{\bullet}O_2^-$:	superoxide
$^1O_2^-$:	singlet oxide
$^{\bullet}OH$:	Hydroxyl radical
PCR:	polymerase chain reaction

PEG:	polyethylene glycol
PEP:	plastid-encoded RNA polymerase
PPE:	poison primer extension
PPR:	pentatricopeptide repeat
PQ:	Plastoquinone
PS I:	photosystem I
PS II:	photosystem II
Psi:	pounds per square inch
ptDNA:	plastid DNA
rbs:	ribosomal binding site
RGM:	regeneration medium
RM:	rooting medium
RNA:	ribonucleic acid
ROS:	reactive oxygen species
Rpm:	revolutions per minute
rRNA:	ribosomal RNA
RT:	reverse transcription
Rubisco:	Ribulose-1,5-biphosphate carboxylase/oxygenase
RWC:	relative water content
SSC:	small single copy
SD:	Shine-Dalgarno
SDS:	sodium dodecyl sulphate
slRNA:	spliced leader RNA
SNP:	single nucleotide polymorphism
SOD:	superoxide dismutase
T:	thymine
T7g10:	gene 10 leader sequence of the T7 phage
5'TCR:	5' translated coding region
Tm:	melting temperature
tRNA:	transfer RNA
TSP:	total soluble protein

TW:	turgor weight
U:	uracil
UTR:	untranslated region
3'UTR:	3' untranslated region
5'UTR:	5' untranslated region
UV:	ultraviolet
X-gluc:	5-bromo-4-chloro-3-indolyl β-D-glucuronide

Abstract

Ireland's agricultural land area comprises of 91% grassland, which predominantly consists of perennial ryegrass (*Lolium perenne* L.). Owing to the predicted climate change, grasslands in the east of Ireland will be severely affected over the next hundred years. Due to this prediction, strategies to circumvent this problem need to be addressed. The present study is focused on RNA editing (post-transcriptional nucleotide modifications resulting in altered transcripts) within plastidial transcripts of the NDH complex (NADH dehydrogenase complex) in relation to the drought response of several accessions of perennial ryegrass. Previous studies have shown that the NDH complex is involved with countering oxidative stress during environmental stresses like drought. Due to the nature of RNA editing within this complex, the RNA editing machinery could potentially play a role in regulating the activity of the NDH complex. In this study a difference was observed in editing behaviour between accessions of *Lolium perenne* L., however a direct relationship between editing behaviour and drought response could not be confirmed. Despite the lack of correlation, a possible role of RNA editing in regulation of the NDH complex cannot be completely disregarded. To expand the investigation concerning the role of RNA editing, plastid transformation can be utilised. Due to the absence of a protocol for plastid transformation of perennial ryegrass, a study was dedicated to design such a protocol. However despite extensive evaluation of all the involved factors in the transformation process, a working protocol was not established. Nevertheless small adjustments to this protocol in the future could potentially lead to an extension of the plastid transformation technology to this species.

Publications and presentations during this project

Publication

Diekmann, K., Hodkinson, T. R., Wolfe, K. H., van den Bekerom, R., Dix, P. J. and Barth, S. (2009). "Complete chloroplast genome sequence of a major allogamous forage species, perennial ryegrass (*Lolium perenne* L.)." DNA Res **16**(3): 165-76.

Presentations:

Van Den Bekerom, R. J.M., Barth, S., Dix, P.J. (2008) Development of a regeneration system for plastid transformation of perennial ryegrass (*Lolium perenne* L.) cv. 'Cashel'. Irish Plant Biologist Association Meeting (IPSAM), NUIM Maynooth 26th-28th March 2008

Diekmann, K., Hodkinson, T.R., Bekerom, R.v.d., Dix, P.J., Wolfe, K., & Barth, S. (2008) Complete chloroplast genome sequence of perennial ryegrass (*Lolium perenne* L.). Irish Plant Biologist Association Meeting (IPSAM), NUIM Maynooth 26th-28th March 2008

Van Den Bekerom, R.J.M., Barth, S., Dix P.J. (2008) The Development of Genetically Modified Perennial Ryegrass, using Chloroplast Transformation. Science Speak 2008, 4th April 2008

Van Den Bekerom, R.J.M., Barth, S., Diekmann, K and Dix, P.J. (2009) The development of a protocol for nuclear and plastid transformation of perennial ryegrass (*Lolium perenne* L.), Walsh Fellowships Seminar, 11th November, 2009

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Declaration of Originality

This thesis has not been submitted in whole or in parts, to this, or any other University for any degree, and is, except where otherwise stated the original work of the author.

Signed: ……

Rob J.M. van den Bekerom

Chapter 1:

General introduction

1.1 Importance of grasses in Irish agriculture

The most important crop in Ireland is grass: 91% of the agricultural land area is dedicated to grasslands. Grasslands contain a wide range of plants, including productive grasses, clovers and also weed species. Perennial ryegrass (*Lolium perenne* L.) dominated pasture is the basis for livestock production in Ireland and perennial ryegrass makes up 95% of the grass seed sales in Ireland (DAFF 2010a). Grass is the main feedstock for the livestock industry for production of beef, dairy and sheep (Holden and Brereton 2002), In 2009 it was estimated that the agriculture sector of Ireland exported livestock and livestock products worth approximately €7.2 billion (DAFF 2010b).

1.1.1 Impact of climate change on yield and viability of *Lolium perenne* L.

As a consequence of estimated climate change over the next hundred years in Europe and particularly in Ireland, the production of forage grasses will become a problem due to changes in temperature and rainfall (Holden and Brereton 2002). Climate change will affect grassland agriculture in a number of ways. Forage and livestock production will be influenced by changing seasonal patterns of weather, for example summer drought stress would lead to less grass production and a shift towards alternative forage crops will be needed. This will result in changes in grazing patterns and rotational management on the farm. An extensive study was performed on the effect of climate change on yield of forage grasses (Holden and Brereton 2002). These researchers estimated that in the east of Ireland, yield would decrease because of summer drought stress, to such an extent that grasses may not be viable anymore in this region, unless artificially irrigated. In the west of Ireland on the other hand, yield of grasses is estimated to increase. By improving the

drought tolerance of cultivars of perennial ryegrass, the impact of climate change can be countered. This could be accomplished by several different means, either by traditional breeding or genetic engineering.

1.2 Plastids

Plants are eukaryotes, however a plant cell has distinct differences compared to other eukaryotic cells. A higher plant cell lacks centrioles, lysosomes, intermediate filaments, cilia, and flagella. On the other hand a plant cell has specialised organelles that are lacking in other eukaryotic cells, like a vacuole, plasmodesmata and plastids, including chloroplasts (Campbell 1996).

Chloroplasts are the energy factories of plant cells, where photosynthesis takes place. In the thylakoids, the photosynthetic membranes are organised in grana containing an internal lumen. These membranes contain the four main protein complexes involved in the light reactions of photosystem I and II, the cytochrome b_6/f complex and ATP synthase. The thylakoids are surrounded by a liquid matrix, the stroma. This is the site for carbon fixation by Ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco) (Hinshaw and Miller 1989). Other functions of plastids include the biosynthesis of starch, fatty acids, pigments and amino acids from inorganic nitrogen (Neuhaus and Emes 2000).

There are several distinct plastid types (see Fig. 1.1), each derived, directly or indirectly from proplastids in meristematic cells of shoot, root, embryo's or endosperm. Proplastids are undifferentiated plastids that are variable in shape and are colourless. On the basis of their structure, pigment composition, metabolism and function, plastids are classified into

different groups (Fig. 1.1) (Lopez-Juez and Pyke 2005). Chloroplasts can be found in leaf tissue, the outer layers of unripe fruits, cotyledons and embryos. Chloroplasts are green and are the site for photosynthesis. Chromoplasts are located in fruits and petals and contain relatively high levels of carotenoids, which give rise to the yellow, orange and red pigmentation of petals, fruits and senescing leaves. Leucoplast is a general term applied to colorless plastids, but unlike proplastids these are not progenitors of other plastids. This group of plastids include two types of plastids, the first are amyloplasts, which are found in roots and storage tissues like cotyledons and the endosperm. These plastids contain large quantities of starch granules. The other type of leucoplast is the elaioplast, oil-containing plastids which can be found in the epidermis of some monocotyledonous species. The last type of plastid is the etioplast, which can be found in dark-grown leaf tissue. These appear yellow due to the presence of protochlorophyll. They can develop into chloroplasts upon exposure to light (Neuhaus and Emes 2000).

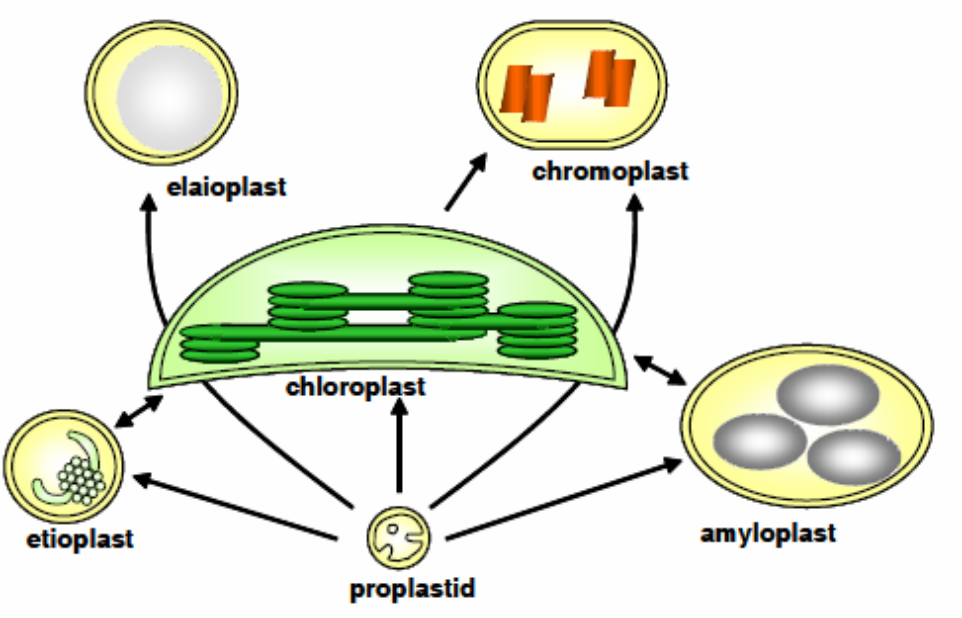

Fig. 1.1: Diversity of plastid types and their interconversions.
Source: (Lopez-Juez and Pyke 2005)

The genetic information of plants is located in three different compartments of the cell, within the nucleus (gDNA), the mitochondria (mtDNA) and the plastid (ptDNA) (Bock 2001). The plastid genome molecules can be circular or linear, mono- or multimeric, but the genome can be represented by a monomeric circular map containing two copies of an inverted repeat (IR) region and two singly copy regions, the small single copy (SSC) and the large single copy (LSC) region (See Fig. 1.2) (Diekmann et al. 2009). The genome size ranges from 120 kb to 160 kb in flowering plants, largely due to variable sizes of the inverted repeat regions.

In the case of perennial ryegrass, the plastid genome, which was recently sequenced (Diekmann et al. 2009), consists of 110 genes, of which 76 are protein-coding genes, 30 are tRNA (transfer RNA) genes and four are rRNA (ribosomal RNA) genes (Diekmann et al. 2009). Plastid genes with related functions are often transcribed as polycistronic clusters within vascular plants, similar to bacterial operons (Stern et al. 1997). This clustering allows separate regulations of different functions, as subunits within a complex can be transcribed at similar rates.

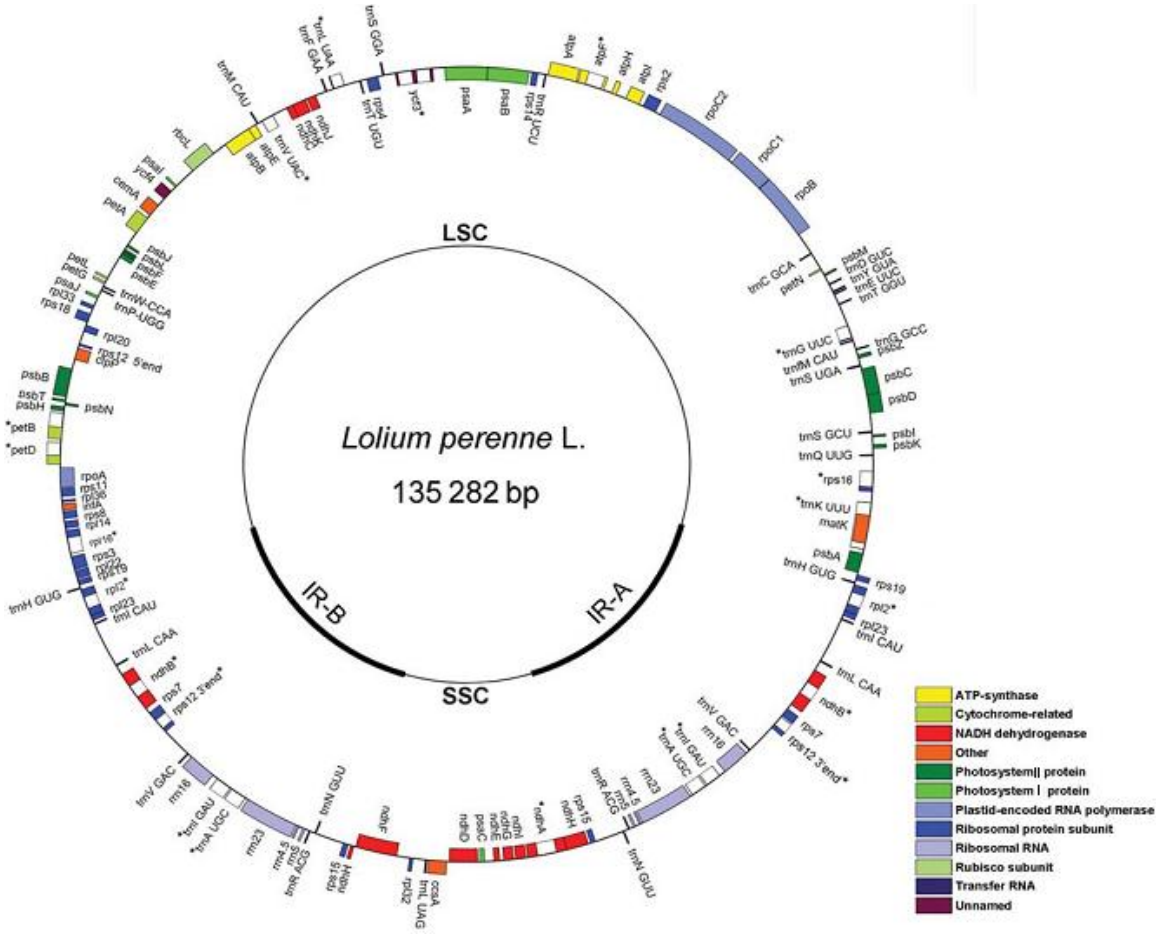

Fig 1.2: Map of the plastid genome of *Lolium perenne* L.. Annotated genes are colour coded according to their function. Source: Diekmann et al. (2009).

The plastid DNA is associated as nucleoids with the inner layer of the double membrane of the organelle (Lopez-Juez and Pyke 2005). Plastids contain multiple copies of the genome, ranging from twenty copies in proplastids to a hundred copies in chloroplasts (10.000 genome copies per cell) (Sugiura 1992).

1.2.1 Evolution of plastids

It is generally accepted that plastids were derived from an ancestral photosynthetic prokaryote related to cyanobacteria. This endosymbiotic theory was first mentioned by Mereschkowski in 1905. This was based on information that plastids always arise by

division of pre-existing plastids and the ability of plastids to stay alive for a while in the absence of the nucleus in the cell. This suggested that plastids had been acquired from the outside, through foreign bodies or symbionts (Mereschkowsky 1905). The symbiotic theory basically implies that a eukaryotic cell captured a prokaryotic cell, after which the prokaryotic cell got integrated functionally within the eukaryotic cell (see Fig 1.3, primary endosymbiosis). Confirmation of this theory has been reported in recent decades. Molecular analyses of chloroplast 5S rRNA and 16S rRNA genes revealed high similarities to those found in the cyanobacterium *Anacystis nidulans* (Dyer and Bowman 1979; Tomioka and Sugiura 1983). Comparable similarities for protein-coding genes were later found, confirming the link. Nevertheless it long remained a point of discussion if all plastids were derived from a single endosymbiotic event, or if more independent endosymbiotic events occurred. Some algae contain plastids with three or four membranes, this phenomenon could have occurred by the engulfment of algae containing plastids by other algae, this is called secondary symbiosis (Fig .1.3) (Gibbs 1981).

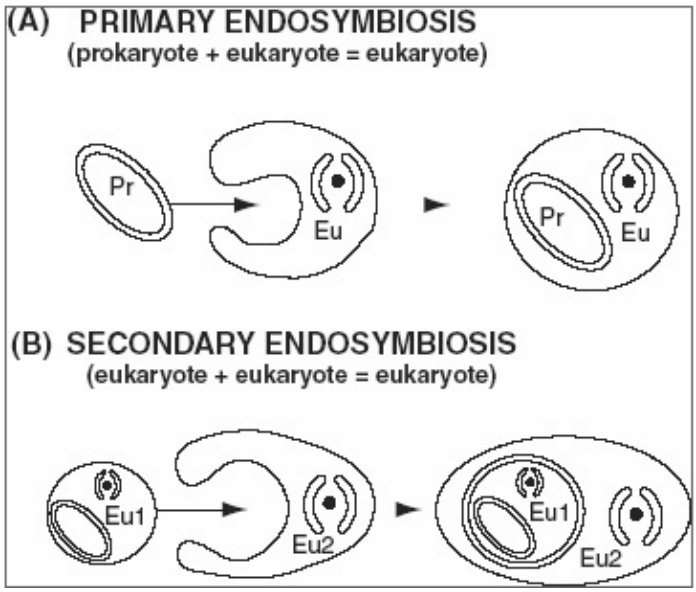

Fig. 1.3: Illustration of primary and secondary endosymbiosis.
Source: (McFadden 2001)

Most of the present data suggest a single acquisition event of plastids by a primary host, out of which other organisms evolved. There is however a number of species with plastids which appear to be different, but little is known about their evolution. An example is the amoeba *Paulinella chromatophora*, which contain plastids that may be derived from an independent primary endosymbiosis, however more research may shed a light upon the origin of the plastid (McFadden 2001).

The acquisition of plastids is not only restricted to plants, one animal species has been identified that has the ability to integrate plastids into their cells. This occurs after feeding upon algae. The plastids are able to retain their function for up to nine months within the animal cells. This has only been observed in sea-slugs of the taxon Sacoglossa (Handeler et al. 2009).

Over the course of time the majority of the genes within the plastid genome have been transferred to the nucleus. This is evident after comparison of the ancestral cyanobacterial genomes and the current plastid genomes, which indicates a genome reduction to about 5% to 10% of the original size (Sugiura et al. 1998)

1.2.2 Transcriptional and translational regulation of plastid protein expression

1.2.2.1 Promoters

Plastid genes are transcribed by at least two different RNA polymerases, which can bind to specific sequence elements within promoters. One of these polymerases is encoded by the *rpo* complex within the plastid genome (Serino and Maliga 1998). This polymerase is

therefore called the plastid-encoded RNA polymerase (PEP). This polymerase is related to the eubacterial α_2 ββ' RNA polymerases (lgloi and Kössel 1992). PEP promoters are similar in structure to the eubacterial σ^{70}-promoters, in such a way that they contain two conserved blocks of hexameric sequences, corresponding to the eubacterial -35 and -10 promoter elements. The accumulation of transcripts depends on the conservation of these elements, in conjunction with upstream activators, as is the case with the light-responsive promoter of the *psbD* gene (Shiina et al. 1998).

The other type of RNA polymerase active within plastids, is encoded in the nuclear genome and transferred to the plastid compartment, through a translated signal peptide. Therefore this polymerase is called nuclear-encoded plastid polymerase (NEP). The existence of this polymerase, was first suggested when the plastid genome of *Epifagus virginia*, a non-photosynthetic species, was sequenced. The plastome lacked all but one of the PEP genes, however transcription was maintained at a number of plastid genes (Morden et al. 1991). There is a consensus motif from different NEP promoters from both dicots and monocots that binds the NEP (Hübschmann and Börner 1998).

Plastid promoters can have PEP binding sites, NEP binding sites or both. NEP promoters are usually active within proplastids and meristematic tissue, where housekeeping genes are mostly transcribed. PEP promoters generally take over transcription of housekeeping genes and initiates transcription of photosynthetic-related genes in developing chloroplasts (Toyoshima et al. 2005).

Transcription from different promoters is regulated at various levels. For example the *rbcL* gene, encoding the large subunit of ribulose-1,5-bisphosphate carboxylase, which has a PEP promoter. This promoter has a 10-fold higher transcription rate in light-grown leaves compared to dark-grown leaves. (Shiina et al. 1998). Another example is the plastid rRNA operon promoter Prrn, on which the transcription rates vary considerably when grown in the light or the dark (DuBell and Mullet 1995). Furthermore the transcription rates varied 50-fold in barley in response to developmental and environmental cues (Baumgartner et al. 1993). There have been two reports where dicotyledonous plastid promoters were successfully applied in monocots. In one they showed transient expression of the *uidA* gene in wheat (*Triticum aestivum* L.) using the PpsbA promoter from spinach. In this case only transient expression was achieved (Daniell et al. 1991). In the second paper, plastid transformation was achieved in rice (*Oryza sativa)* with an active Prrn promoter from tobacco (*Nicotiana tabacum*) (Khan and Maliga 1999). The reverse was also successfully demonstrated, where the barley promoter from *psbD* was analysed in tobacco. (Thum et al. 2001). This indicates that the transcription systems between the two classes are similar and interchangeable, when applied during plastid transformation.

1.2.2.2 Expression signals: 5' Untranslated region (5'UTR) and 5' translation coding region (5'TCR)

5'untranslated regions (5'UTR) are regions upstream of the start-codon of the gene. These regions are involved in the post-transcriptional control of gene-expression and in translation efficiency. Plastid gene expression is mainly regulated at a post-transcriptional

level, induced by developmental changes where *cis* elements within the mRNA, interact with nuclear encoded *trans*-factors (Sugita and Sugiura 1996). Although the transcription rate is mainly influenced by the promoter, the 5'UTR influences the mRNA turnover. An example is the *rbcL* 5'UTR that helps maintain a steady-state of *rbcL* mRNA levels in the dark (Shiina et al. 1998).

In prokaryotes, mRNAs usually contain a ribosomal-binding site (SD (Shine-Dalgarno) sequence) between four and twelve nucleotides upstream of the initiation codon (Shine and Dalgarno 1975). Although 90% of the plastid 5'UTR's contain SD-like sequences, the location of these elements is much more variable. As a result identification of these elements has been difficult. Furthermore it was shown that certain regions of the 5'UTR of *psbA*, were the target for the 16S rRNA, while the SD-like element in this 5'UTR was dispensable (Hirose and Sugiura 1996). At the 5'UTR of *psbA* in *Chlamydomonas reinhardtii* a stem-loop formation is the binding site for a protein complex, under light-regulation, indicating another pathway can be responsible for translation initiation (Katz and Danon 2002). Another region responsible for translational regulation, is the region directly downstream of the initiation codon, called the 5' translation coding region (5'TCR). A study showed that when point mutations were inserted downstream of the AUG start codon in a bacterial antibiotic resistance gene neomycin phosphotransferase II (*nptII*) transcript fused with the N-terminus of *rbcL* and *atpB*, protein accumulation was reduced significantly in the resulting transplastomic tobacco plants (Kuroda and Maliga 2001b). Another study showed that complementary sequences to the 16S rRNA, directly downstream of the AUG, destabilised transcripts (Kuroda and Maliga 2001c).

1.2.2.3 Expression signals: 3' untranslated region (3'UTR)

Like their bacterial counterparts, chloroplasts 3'untranslated regions (3'UTR) contain in almost all cases inverted repeats (IR). These repeats can form stem-loop structures, where mRNA associated proteins can bind. This interaction is involved in RNA processing and RNA stability. This is an important feature, as transcription termination is very inefficient at the IRs, resulting in extensive read-through (Monde et al. 2000). When the 3'UTR of *atpB* in *Chlamydomonas reinhardtii* was substituted with the 3'UTR of *petD* from spinach the mRNA processing was not affected, indicating that the IR structure is necessary, but not an absolute sequence (Stern et al. 1991). The 3'UTR also seems to be involved in translation regulation, by interacting with the 5'UTR. It was shown that proteins bound to the 5'UTR of *psbA* from *C. reinhardtii* had more affinity, when the 5'UTR was *cis* linked with the 3'UTR (Katz and Danon 2002).

1.3 Plastid transformation

Plastid transformation involves the integration of foreign DNA into a single plastid genome copy followed by replication of the transplastome, and the segregation of transformed and wild-type gene copies under selective pressure that eventually yields homoplasmic lines (Svab et al. 1990). An expression cassette containing plastid signal sequences surrounding the transgene are flanked by two targeting sequences that allow homologous integration into the plastid genome (Svab and Maliga 1993). The primary transformation event only leads to one or a few genome copies in the transformed cell alongside the majority of wild-type genome copies. These cells are heteroplasmic and

therefore unstable for transgene integration. To obtain stable transformants the remaining wild-type genome copies have to be eliminated. This is accomplished by multiple rounds of regeneration under the selection pressure. The number of rounds of regeneration depends on the species, typically for the model species tobacco this would involve two to four rounds of regeneration (Svab and Maliga 1993).

1.3.1 Advantages of plastid transformation

Plastids harbour a large number of metabolic pathways such as CO_2 fixation, starch, fatty acids, pigment and hormone synthesis. Furthermore photoreduction of nitrogen and sulphur for amino acid production occurs within plastids (Lopez-Juez and Pyke 2005). By utilising plastid transformation, genes involved with these pathways can be altered or disrupted, allowing investigation of the pathways by reverse genetics (Rochaix 1997; Heifetz 2000). Furthermore due to the possibility to express transgenes to a high level, the prospect to produce pharmaceuticals within plastids is appealing. There are several other advantages of plastid transformation, in comparison to nuclear transformation, including gene containment, lack of gene-silencing or positioning effects and the ability to have targeted integration.

1.3.1.1 Gene containment

One of the main concerns about Genetically Modified (GM) crops is the spread of transgenes into the environment. During the creation of transgenic plants, along with the gene of interest, a resistance gene is necessary to select for transgenic tissue. This can be an antibiotic resistance gene or a herbicide resistance gene.

This raises the risk that weed species could become tolerant to herbicides with consequences for organic crops grown nearby, or wild relatives. Another implication of lack of containment of transgenes is the demand of the public to be able to distinguish between GM food and non-GM food. In the European Union (EU) there is a regulation that all products that are called non-GM must have less than 0.9% GM content (EU Regulation No 1829/2003). When transgenes are transmitted to nearby fields, the ability to guarantee non-GM content is nearly impossible.

In the case of the traditional nuclear transformants, the transgenes are inherited biparentally according to Mendel's rules. The advantage of plastid transformation is that in most angiosperms, plastids are maternally transmitted to the progeny, pollen therefore does not contain plastids. When the plastid genome of a plant is genetically modified, the transgene will not be transmitted to the progeny through the pollen (Maliga 2004). However several publications show that there is a small fraction of the pollen that do contain plastids, this happens at a frequency of about $1.0\ 10^{-5}$ (Ruf et al. 2007; Svab and Maliga 2007). This means that plastids can be transmitted through the pollen, but at a very low frequency.

1.3.1.2 High accumulation of recombinant protein

Unlike nuclear transformation, plastid transformation allows accumulation of extremely high levels of recombinant protein. This is of particular interest for the production of pharmaceutical proteins. In general, levels of recombinant proteins produced in nuclear transformants are less then 1% total soluble protein (TSP). For the production of

pharmaceutical proteins this is generally less than is required to make the system commercially viable, especially in view of losses during subsequent purification steps (Rybicki 2009). To achieve higher levels of recombinant proteins, plastid transformation can be applied. Due to the high copy number of plastid genomes within each cell, accumulation levels up to 70% TSP has been achieved (Oey et al. 2009). Plant-based systems are more economical then bioreactor systems. Furthermore the purification steps can be minimised if a food crop is used so the processing only needs to provide crude extracts. There are even prospects for directly supplying these edible vaccines directly as fresh food products (Daniell et al. 2001).

1.3.1.3 Lack of positioning effects

Plastids have a mechanism for DNA repair, involving a RecA-mediated recombination machinery inherited from their prokaryotic ancestors (Cerutti et al. 1992). This mechanism prevents the random insertion of transgenes that characterises nuclear transformation, and allows targeted integration of expression cassettes within the plastid genome. Integration involved homologous recombination and can be directed to any part of the plastome, simply by flanking the insert with sequences homologous to the chosen site of integration. Generally expression cassettes are targeted to intergenic regions, although it is possible to target expression cassettes to an existing operon. Some of the favoured sites used to date are located in the LSC region, whereas others are within the IR region (See Table 1.1) (Maliga 2004).

Due to the ability for targeted integration associated with plastid transformation, this system avoids the positioning effects that are commonly observed within nuclear

transformants. In nuclear transformation, random integration of the transgene within the nuclear genome can result in gene silencing and undesired gene disruptions (Birch 1997). While for plastid transformation targeted integration could be used for desired gene disruptions.

Insertion site	Species	Reference
trnH/psbA	*N. tabacum*	(Carrer and Maliga 1995)
Ycf3/trnS	*N. tabacum*	(Huang et al. 2002)
trnG/trnfM	*N. tabacum* *L. esculentum*	(Bock and Maliga 1995; Ruf et al. 2001)
rbcL/accD	*N. tabacum*	(Svab and Maliga 1993)
petA/psbJ	*N. tabacum*	(Bock et al. 1994)
5'rps12/clpP	*N. tabacum*	(Shikanai et al. 2001; Kuroda and Maliga 2003)
petD/rpoA	*N. tabacum*	(Suzuki and Maliga 2000)
ndhB/rps7	*B. napus*	(Hou et al. 2003)
3'rps12/trnV	*N. tabacum* *A. thaliana* *O. sativa* *L. fendleri*	(Staub and Maliga 1992) (Sikdar et al. 1998) (Khan and Maliga 1999) (Skarjinskaia et al. 2003)
trnV/rrn16	*N. tabacum*	(Staub and Maliga 1993)
rrn16/trnI	*N. tabacum*	(Svab et al. 1990)
trnI/trnA	*N. tabacum*	(Daniell et al. 1998; Muhlbauer et al. 2002)
trnN/trnR	*N. tabacum*	(Zou et al. 2003)
Rpl32/trnL	*N. tabacum*	(Koop et al. 1996; Thum et al. 2001)

Table 1.1: Regions of the plastid genome (plastome) used for targeted integration of expression cassettes.

1.3.1.4 Transgene stacking

Many chloroplast genes are located within clusters on the plastid genome, and are transcribed as polycistronic mRNAs. This phenomenon can be mimicked by insertion of multiple genes of interest within an expression cassette into the plastome, thereby allowing gene-stacking. The advantage of this strategy is the possibility to insert multiple genes, with just one transformation event, thereby avoiding the need for multiple selectable markers (Bock 2001).

1.3.2 DNA delivery into the plastids

Plastid transformation requires that foreign DNA is delivered to the plastid compartment through the cell wall, the plasma membrane and the plastid double membrane. Three different methods have been developed to accomplish this: Biolistics, PEG (polyethylene glycol)-mediated transformation and femto-injection. The type of transformation system has to be chosen carefully, as they may not work equally well depending upon the species to be transformed. For instance, if an efficient regeneration system of intact tissue is absent for a specific species, but a functional protoplast regeneration protocol is present, then the PEG-mediated transformation system is prefered (Kofer et al. 1998).

1.3.2.1 Biolistics

Biolistics consists of a process where nano-sized tungsten or gold particles coated with DNA are bombarded into tissue. Modern devices use a high pressure helium pulse to deliver these particles into plant tissue at a high-velocity in a vacuum chamber (see Fig. 1.4) (Sanford 1990), after which transgenic shoots can be obtained under selective

pressure. The development of biolistics for nuclear transformation was a major breakthrough in genetic engineering of plants due to the limitations of plant species that were not amendable to *Agrobacterium*-mediated transformation. Transient expression in onion epidermal cells using biolistics was first demonstrated in 1987 (Klein et al. 1987), and soon followed by the first plastid transformation event using this technique in *Chlamydomonas* (Boynton et al. 1988). Since then biolistics has been applied to a wide range of species including *Lolium perenne* L. for nuclear transformation, although at various efficiencies. The advantage of this approach in plastid transformation is the relative high efficiency compared to other methods, and its simplicity, so it is by far the most widely used approach (Bock 2001). The main drawback is that it requires special instrumentation (Sanford 1990).

Fig 1.4: PDS-1000/He Gun (Biorad)

1.3.2.2 PEG mediated transformation

PEG-mediated transformation in plants involves the delivery of DNA into protoplasts. Protoplasts are obtained by enzymatic removal of the cell walls, after which the protoplasts are treated with a solution containing polyethylene glycol (PEG), ions and DNA. The PEG disrupts the plasma membrane to allow the entry of DNA into the cytoplasm. Whether the PEG is also involved with penetrating the chloroplast membranes remains however unclear (Kofer et al. 1998). The first successful use of polyethylene glycol (PEG) for genetic modification was achieved in yeast (Hinnen et al. 1978). This was followed a few years later with genetic modification of tobacco protoplasts (Krens et al. 1982). In 1993 this approach was extended to plastid transformation, where a ptDNA fragment containing a point mutation for spectinomycin resistance was introduced into tobacco plastids (O'Neill et al. 1993). Since then this approach has been successfully applied to achieve plastid transformants in lettuce (Lelivelt et al. 2005) and cauliflower (Nugent et al. 2006).

1.3.2.3 Femtoinjection

A third approach to introduce foreign DNA into plastids is called femto-injection, which involves the direct injection of DNA into chloroplasts through a glass capillary with a 0.1μm diameter. The injection is accomplished by expansion of an alloy within the capillary due to warming, to drive the DNA into the plastid (Knoblauch et al. 1999). This method has been used to transform cyanobacteria, however only transient expression was achieved within plastids. Since then, no reports were published using this technique for plastid transformation.

1.3.3 Selection strategies

In order to achieve genetic modification in plants, a procedure is necessary to select for transgenic tissue. Selection strategies are usually based on inclusion of an antibiotic resistance gene which allows proliferation of transformed cells on antibiotic containing medium on which wild type cells are unable to grow (Joersbo and Okkels 1996).

1.3.3.1 Heterologous genes

Selection with heterologous genes is based on a gene insertion approach. This means using a plasmid containing an expression cassette with a selectable marker gene, flanked by a specific promoter and transcription/translation signals. This expression cassette can then be integrated into the respective genome, by various methods. Selection with the appropriate antibiotic is then utilized to select for transformed tissue.

Different selection markers can be used for nuclear transformation, in comparison with plastid transformation. This is mainly due to the mode of action of the corresponding antibiotic and the type of selection that is needed for each type of transformation. (e.g. achieving homoplasmy in plastid transformation, as opposed to the relatively quick full integration within the nuclear genome). For nuclear transformation several types of antibiotics can be used, with the corresponding antibiotic resistance genes. Most of these antibiotics are aminoglycosides, of which several subgroups exist. One of the most used aminoglycoside in nuclear transformation experiments is the aminocyclitol hygromycin B produced by *Streptomyces hygroscopicus*. This antibiotic inhibits protein synthesis by interfering with ribosomal translocation and aminoacyl-tRNA recognition, furthermore it

causes misreading of mRNAs (Zheng et al. 1991). This antibiotic has a lethal effect on plant tissue at higher concentrations. So far this antibiotic has not been successfully used to produce transplastomic plants, possibly due to a mode of action that causes lethality on tissue during selection. The detoxifying protein acting on this antibiotic is encoded by the hygromycin phosphotransferase gene (*hpt*) from *Escherichia coli*.

A second aminoglycoside group contains antibiotics like kanamycin, gentamycin, paromomycin and geneticin (G-418). These antibiotics bind to the 16S rRNA and to some extent to the 30S subunits of the prokaryotic ribosome, causing inhibition of protein synthesis and misreading of initiated translation. Furthermore they stimulate membrane leakage. Visually this results in bleaching of plant tissue. All these antibiotics have a lethal effect on plant tissue at higher concentrations. However kanamycin has a less stringent effect, compared to paromomycin during selection in several plant species (Mauro et al. 1995; Wang et al. 2005). Several enzymes can be used to detoxify these antibiotics, one of these is encoded by the neomycin phosphotransferase II gene (*nptII)* derived from the transposon Tn5 from *Escherichia coli.* This gene is widely used, to generate nuclear transformed plants. Another protein that can detoxify these antibiotics is encoded by the 3'-aminoglycoside phosphotransferase gene (*aphA-6)* from *Acinetobacter baumannii* (Shaw et al. 1993). This last marker gene, was successfully used to generate transplastomic plants in several species, including tobacco (Huang et al. 2002), *Chlamydomonas* (Bateman and Purton 2000) and cotton (Kumar et al. 2004).

A last group of aminoglycosides includes spectinomycin from *Streptomyces spectabilis*. This antibiotic binds to the 30S subunit of ribosomes, causing inhibition of protein synthesis, but in contrast to other aminoglycosides, it does not cause mistranslation. As a result it inhibits growth and causes bleaching, but is not lethal (Maliga 2004). This antibiotic is routinely used to select for transplastomic tissue. The detoxifying protein acting against spectinomycin is encoded by the aminoglycoside 3"-adenylyltransferase gene (*aadA*).

Recently the chloramphenicol acetyltranferase (*cat*) gene was used to generate transplastomic tobacco using chloramphenicol selection. The development of this new selection system for plastid transformation can potentially lead to an extension of the range of species amendable to plastid transformation (Li et al. 2010).

1.3.3.2 "Binding-type" markers

Several point mutations were identified within the genes 16S rRNA and *rps12* responsible for spectinomycin and streptomycin resistance, while mutations in the 23S rRNA gene conferred resistance to lincomycin (Kavanagh et al. 1994). The first report where these mutations were utilised to create stable plastid transformants, was with *Chlamydomonas reinhardtii* (Newman et al. 1990). This selection strategy was shortly afterwards also applied to tobacco (Svab et al. 1990). These "binding type" markers are desirable, as this system does not require dominant bacterial antibiotic resistance genes and therefore allays public concerns that the use of the bacterial genes might interfere with medical applications of the antibiotics (Conner et al. 2003).

1.3.3.3 Limitation of spectinomycin selection

Due to the presence of point mutations conferring spectinomycin resistance within monocotyledonous species (Fromm et al. 1987), including *Lolium perenne* L., the most utilised selection marker *aadA* can not be used in conjunction with spectinomycin selection for plastid transformation of perennial ryegrass.

1.4 Drought stress

1.4.1 Drought stress response

'Drought' is a general term, as it can have many meanings concerning crop production in different kind of aspects like time frame, severity of drought and the effect of drought on the crop. The effect of water deficit is looked upon differently depending on the type of person that is asked. For farmers the effect of water deficit is secondary to many other considerations like weeds, diseases and insects, that may affect yield much more than the water supply (Passioura 2006a). Furthermore a farmer takes into account a time frame of months to a few years. Plant physiologists on the other hand usually tend to look at drought stress over a relative short period of days up to a few months, while they explore the rate of survival of plants under water-deficit, rather then yield. Molecular biologists work with a timeframe of hours, where attention is paid to the molecular processes involved with water deficit. This includes the production of reactive oxygen species and the pathways involved in countering these molecules (Passioura 2006a).

1.4.2 Oxidative stress

Water deficit causes oxidative stress, as a result of a serious imbalance between the production of Reactive Oxygen Species (ROS) and the presence of the antioxidant defences (Møller 2001). ROS consist of superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and singlet oxide ($^1O_2^-$). Superoxide and hydrogen peroxide are produced at high rates within chloroplasts, even under optimal conditions. These compounds are produced by several enzyme systems (plasmalemma-bound NADPH (nicotinamide adenine dinucleotide phosphate)-dependent superoxide synthase and superoxide dismutase (SOD)). Although the toxicity of these compounds is relatively low, their detrimental effect resides in the ability to initiate cascade reactions that result in the production of hydroxyl radicals and other destructive molecules (Noctor and Foyer 1998). However, ROS can also play a role in signalling, for instance during pathogen infection, a plasma membrane NADPH oxidase is activated, which initiates the production of superoxide radicals, which in turn are converted to H_2O_2 by SOD. The high levels of H_2O_2 kill both the pathogen and the plant cells surrounding the infection site, thus preventing the spread of the pathogen to neighbouring cells (Grant and Loake 2000).

1.4.2.1 Antioxidant system

To counter the accumulation of these species, several ROS scavenging enzymes are produced within different compartments of the cells. These compounds quench ROS without undergoing conversion themselves to a destructive radical. The enzymes either catalyse reactions or are involved in the direct processing of ROS. Known ROS scavenging enzymes are glutathione S-transferase (GST), ascorbate peroxidase (APX),

dehydroascorbate reductase (DHAR), glutathione reductase (GR), Catalase (CAT) and superoxide dismutase (SOD) (Noctor and Foyer 1998).

1.4.2.2 Chlororespiration

The discovery of the plastid NDH-complex (NADH dehydrogenase complex) provided molecular evidence of the existence in chloroplasts of chlororespiration (Burrows et al. 1998), which is defined as a respiratory electron transport chain (ETC). Increased activity of the NDH complex has been observed under various stress conditions (Casano et al. 2001). This pathway is believed to supply electrons, which can be cycled around photosystem I. This is used to drive ATP (adenosine-5'-triphosphate) synthesis, and is considered as an important system for extra ATP production when exposed to stress conditions which cause photoinhibition (Bendall and Manasse 1995). The second function is believed to involve compensating stromal overreduction through mediation of cyclic electron transfer, which prevents the formation of ROS (Wang et al. 2006).

1.4.3 Ways to overcome the impact of climate change

To achieve improved tolerance to drought stress, three approaches can be exploited. Firstly, plant physiology has provided new insights and developed new tools to understand the complex network of drought-related traits. Secondly molecular genetics has revealed many Quantitave trait loci (QTL) affecting yield under drought stress and detected expression of drought tolerance-related traits. Finally molecular biology has provided genes, useful either as candidate sequences to dissect QTL or for improving stress tolerance through a transgenic approach (Cattivelli et al. 2008).

Despite the need for studies to counter drought stress in perennial ryegrass, most studies regarding drought response are performed on model species, like *Arabidopsis* and maize. This is due to the availability of full genome sequences of these species, which allows the use of extensive micro-array data. Unfortunately in perennial ryegrass, public accessible micro-array data are not available. Therefore only a few gene-expression studies have been performed on this species to identify genes involved with countering water deficit (Foito et al. 2009). The molecular analysis results obtained with one species cannot always be applied to a different species, so more studies are needed with perennial ryegrass (Foito et al. 2009).

1.5 RNA editing

RNA editing alters the nucleotide sequence of an RNA molecule so that it deviates from the sequence of its DNA template (Tillich et al. 2006). There are several different types of RNA editing, which include insertions, deletions and conversion of nucleotides. RNA editing is particularly associated with the organellar genomes and has been observed within molecules of messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA) and spliced leader RNA (slRNA) within all organellar compartments.

The majority of editing events take place within mRNA molecules. These events can result in an altered protein product, or the alteration of the start codon or stop codon. Nevertheless not all editing sites change the amino acid sequence, as editing events have also been observed within both introns and untranslated regions (UTRs). These editing sites may be involved in the stabilization of the transcripts (Maier et al. 1996).

Editing of tRNA creates essential structural elements at the primary, secondary, and tertiary levels. A tRNA molecule transfers a specific amino acid to a growing polypeptide chain at the ribosomal site of protein synthesis during translation. One instance of tRNA editing has been identified in plastids, namely adenosine (A) to inosine (I) editing within the $tRNA^{Arg}$ (Pfitzinger et al. 1990; Karcher and Bock 2009).

Editing of rRNA appears to be less frequent than alterations in mRNAs and tRNAs, and has not been observed in plastids. Nevertheless rRNA editing was shown to be important for maturation of the mitochondrial small subunits of 17S rRNA of *Physarum polycephalum* (Mahendran et al. 1994).

Editing in slRNA was found in *Leptomonas collosoma*. slRNAs are small RNA molecules that are added as leader sequences to all mRNAs by *trans*-splicing in trypanosomes (Ben-Shlomo et al. 1999).

1.5.1 RNA editing in plastids

RNA editing in plastids consists mostly of C to U conversions, with the exception of U to C conversions in the bryophyte *Anthoceros formosae* (Kugita et al. 2003). So far 21 to 37 editing sites have been identified in plastid genomes of seed plants depending on the species. In closely related taxa, common editing sites can range from 23 sites within the Poaceae branch up to 30 sites within the Solanaceae branch. Editing occurs all along the plastid genome, however editing sites appear to be clustered mostly within the *rpoB*, *ndhB* and *ndhA* transcripts. The hypothesis is that editing occurs mostly in transcripts,

where transitory loss of function can be tolerated (Fiebig et al. 2004). The NDH protein complex is believed to play a role in cyclic electron flow around photosystem I, a function that is compatible with the absence of significant phenotypic effects of *ndhB* gene inactivation under normal growth conditions (Horvath et al. 2000). However under various stress conditions this complex did indeed play a role. Under water deficit, which causes a limitation of CO_2 availability, it was shown that in *ndh* mutants were compromised in its capability to quench fluorescence non-photochemically in an early stage of light induction, furthermore it was shown that the NDH complex optimizes the induction of photosynthesis under water stress conditions (Burrows et al. 1998; Horvath et al. 2000). A second study showed that the NDH complex was involved with reduction of the plastoquinone pool (PQ) in the dark in response to heat stress (Sazanov et al. 1998). In a third study it was shown that under illumination of strong light, *ndh* mutants exhibited more photodamage, which indicated the importance of the NDH complex in countering photodamage resulting from the stromal overreduction. This was thought to be accomplished by mediating the cyclic electron transport to supply ATP for the calvin cycle (Endo et al. 1999). Similar effects on *ndh* mutants were observed when cold stress was applied (Li et al. 2004)

1.5.2 Evolution of RNA editing in plastids

RNA editing has been observed in plastid genomes of all lands plants tested, except in the liverwort *Marchantia polymorpha* (Freyer et al. 1997). The frequency of RNA editing events differs immensely within the plant kingdom. Within angiosperms, RNA editing occurs at about 30 separate sites, whereas within the bryophyte hornwort *Anthoceros*

formosae 941 editing sites have been detected and within the fern *Adiantum capillus-veneris* 350 editing sites were detected (Wolf et al. 2004). In comparison with *Anthoceros formosae* and *Adiantum capillus-veneris*, it was found that, 18 out of 85 editing sites found in seed plants, are shared with at least one of these taxa. These editing sites could be remnants of the original RNA editing system of land plants (Tillich et al. 2006). On the other hand, new editing sites have emerged or disappeared over a relative short time, this is evident when editing sites were compared within closely related species (Freyer et al. 1997). The reason for the emergence and disappearance of editing sites can be linked to the mutation rate on the DNA level. However the efficiency of the RNA editing machinery itself, seems to be more of a driving factor for the evolution of editing sites (Duff and Moore 2005).

1.5.3 *Cis*-acting elements

Cis acting elements are stretches of sequence surrounding editing sites that facilitate binding of specific proteins or guide RNAs (gRNAs), also called *trans*-factors, to induce RNA editing. Although a specific sequence consensus has not been found for plastidial RNA editing, it has been shown that sequences -20 to -5 upstream of the editing site are critical for binding of the *trans*-factors. Sequences at positions -5 and -1 upstream of the editing site are responsible to a lesser degree for interaction with the *trans*-factor (Hayes et al. 2006).

1.5.4 *trans*-factors

The mechanism of the RNA editing reaction involves *trans*-factors. These are molecules that interact with *cis* acting elements to induce editing. In mitochondrial RNA editing, it was found that small RNA molecules (gRNAs) were acting as *trans*-factors. For this reason, initially it was believed similar gRNA molecules would be responsible for editing site recognition within plastids (Gray and Covello 1993). However when a putative gRNA stretch was found within the plastid genome (ptDNA) that was complementary to the *psbL* transcript, this hypothesis was tested by altering the 14 nucleotide RNA molecule by plastid transformation (Bock and Maliga 1995). Despite this alteration the editing efficiency within the *psbL* transcript was not abolished. Since no other sequences were found that were complementary to the *psbL* editing site, it was hypothesized that either the gRNA molecule would have little complementarity to the editing site, or it would be encoded in the nuclear genome (Bock and Maliga 1995).

In 2001 a study showed that RNA-binding proteins could be involved in RNA editing, instead of gRNAs (Hirose and Sugiura 2001). Recently an increasing number of *trans*-factors have been identified. The first protein identified as being involved with RNA editing in the plastid genome was the cp31 protein, this chloroplast ribonucleoprotein was required for editing of a range of editing sites (Hirose and Sugiura 2001). All the other identified proteins involved with RNA editing are pentatricopeptide repeat (PPR) containing proteins (Kotera et al. 2005; Okuda et al. 2007; Chateigner-Boutin et al. 2008; Cai et al. 2009; Hammani et al. 2009; Okuda et al. 2009; Robbins et al. 2009; Yu et al. 2009; Zhou et al. 2009). This protein family consists of 400 members in rice and

Arabidopsis. Unlike cp31, these proteins are involved with editing at specific sites (Robbins et al. 2009). Only one of the identified PPR proteins was involved in more then one site, namely the CLB19 protein (Chateigner-Boutin et al. 2008; Kobayashi et al. 2008).

Robbins et al 2009 utilised a novel approach to identify more PPR proteins involved with RNA editing. With the use of bioinformatics, eight orthologs of PPR proteins with chloroplast signals were identified that were present in *Arabidopsis* but absent in rice. It was also known, that eight editing sites were present in *Arabidopsis*, but absent in rice. With this knowledge one of the PPR proteins, namely 'RACE1' was knocked out in *Arabidopsis*, after which editing malfunction was observed within one of the editing sites that was absent in rice. This strategy could be employed on other species, where genome resources are available (Robbins et al. 2009).

1.5.5 RNA editing regulation

RNA editing does not always happen at 100% efficiency. The efficiency of editing is determined by the availability of the corresponding *trans*-factor in conjunction with the number of editable transcripts present. When there are insufficient molecules of *trans*-factor available for the number of transcripts then the editing will be incomplete, resulting in transcripts being partially edited. There are several possible consequences of partial editing in coding sequences. Some editing sites create start or stop codons, if editing at these sites is absent in some transcripts, then translation will not occur correctly (Kudla et al. 1992; Neckermann et al. 1994; Lopez-Serrano et al. 2001). In other cases,

editing results in amino acid changes after translation, by translation of unedited coding sequences. This could impair the proteins' function (Zito et al. 1997; Sasaki et al. 2001).

1.5.5.1 Tissue specificity

RNA editing efficiency can be tissue specific, as was shown in tobacco, spinach and *Arabidopsis*, where editing efficiencies were reduced in root tissue compared to leaf tissue for editing sites within the NDH complex (Chateigner-Boutin and Hanson 2003). Furthermore editing efficiency of sites in different transcripts differed between various tissues in maize (Peeters and Hanson 2002).

1.5.5.2 Environmental specificity

RNA editing can be influenced by both endogenous and abiotic stimuli. For example, editing of the editing site III within the *ndhB* gene in *Nicotiana tabacum* could be completely blocked under heat shock conditions and treatment of the plants with spectinomycin, which impairs prokaryotic translation within plastids (Karcher and Bock 1998). It is likely that the production of the corresponding *trans*-factor is regulated by environmental conditions, therefore influencing the editing function (Hirose and Sugiura 1997).

1.5.6 RNA editing in *Lolium perenne* L.

Recently the plastid genome of *Lolium perenne* L. has been sequenced and during this study 31 RNA editing sites were identified (Diekmann et al. 2009). This study revealed five RNA editing sites unique to perennial ryegrass and previously not observed among

other species. Furthermore partial editing was observed in eight editing sites, although the number of unedited transcripts was small, with the exception of the two RNA editing sites located within the *matK* and the *psbL* transcripts (Diekmann et al. 2009).

1.6 Project aims and objectives

The aim of this project was to develop a plastid transformation protocol for perennial ryegrass (*Lolium perenne* L.) which could be employed to engineer the plastome to improve stress tolerance.

As target for investigation into drought tolerance, the editing machinery was evaluated to assess differences in editing behaviour of accessions that had various tolerances to drought stress. The information gathered from this investigation, could then be used to design a strategy to further assess the role of RNA editing in relationship with drought response by plastid engineering, and by investigating genes consisting of the NDH complex. This could be accomplished by several different approaches: 1. Over-expression of these genes; 2. Gene disruptions by replacing the gene-of-interest with the selectable marker; 3. Substitution of the endogonous genes with modified versions that are uneditable, but result in a dysfunctional protein; 4. Substitution of the endogonous genes with modified versions that are uneditable, but would result in a functional protein.

Chapter 2:

Transformation

2.1 Introduction

In order to achieve plastid transformation in *Lolium perenne* L. several aspects have to be optimized. Each component is equally important, especially in a species that is recalcitrant to transformation procedures (Bock 2007). Despite the increasing range of dicotyledonous species where plastid transformation has been applied, it is only routinely used in tobacco (De Marchis et al. 2009). Furthermore plastid transformation has only been achieved in one monocotyledonous species, rice and even in that case the fertile plants remained heteroplasmic (Khan and Maliga 1999; Lee et al. 2006). The lack of protocols for plastid transformation within monocotyledonous species indicates that these species are highly recalcitrant to plastid transformation.

2.1.1 Regeneration systems

Tissue culture for transformation experiments is based on the ability of single cells to proliferate after gene transfer into fully fertile plants (Birch 1997).

One of the limitations for achieving genetic modification in monocotyledonous species is their less than optimal regeneration system. In monocotyledonous species regeneration take place through an “indirect organogenesis”, often referred to as embryogenesis. During this type of regeneration, explants undergo an extensive proliferation before developing shoots or roots, unlike the “direct organogenesis” within dicotyledonous species, where explants may undergo minimum proliferation before forming shoots or roots (Ovecka et al. 2000). One important difference between monocotyledonous species and dicotyledonous species is that the former do not have a comparable wound reaction.

Once the cell has stopped dividing and is committed to a particular function in the plant, there is no stimulation of cell division following wounding (Dale 1983). In *Lolium multiflorum* the mitotic activity is restricted to tissue close to the apical meristem and the mitotic activity decreases rapidly in older leaves. As a consequence, cell division and callusing potential is restricted to a small region close to the apical meristem (Joarder et al. 1986). Calli can be induced from leaf-base explants, consisting of either the apical meristem or leaf tissue in close proximity to the apical meristem, as has been demonstrated in several studies (Dalton et al. 1999; Newell and Gray 2005; Bajaj et al. 2006). Another source for induction of regenerable callus in monocotyledonous species is mature embryos. Induction of calli derived from this source tissue has been utilized in order to achieve nuclear transformation in several previous studies (Maas et al. 1994; Spangenberg et al. 1995; Altpeter et al. 2000; Wu et al. 2005). In all cases the callus induction media consisted of full-strength Murashige and Skoog (MS) medium, with 30 g L^{-1} maltose or sucrose, supplemented with 5 mg L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), occasionally including 0.1 to 0.2 mg L^{-1} BAP (6-benzylaminopurine). This common callus induction medium across different projects suggests that this medium may be optimal for callus induction of *Lolium perenne* L. Furthermore in all of the mentioned studies, the callus induction was conducted in the dark. In contrast to the callus induction medium several different culture media were used for shoot regeneration in previous studies, as shown in Table 2.1. The major differences in regeneration media indicate that an optimal culture medium has not been developed yet for the induction of shoots Furthermore the optimal regeneration medium is likely to be cultivar dependent

(Altpeter and Posselt 2000). Significant genotypic variation for tissue culture response is well documented for cereals (Lazar et al. 1983; Lührs and Lörz 1987).

In this project, the standard callus induction media was utilized to induce callus (full strength MS medium supplemented with 30 g L^{-1} maltose and 5 mg L^{-1} 2,4-D), while two different regeneration protocols were tested for their efficiency for shoot regeneration on various cultivars of *Lolium perenne* L. (Protocol I and II, see Table 2.1.).

Table 2.1: Different regeneration media used for *Lolium perenne L.* transformation experiments.

Regeneration media	*Publications*
MS-medium + 0.2 mg L^{-1} kinetin	(Spangenberg et al. 1995; Dalton et al. 1999; Hiroko and Tadashi 2006)
MS medium + 0.1 mg L^{-1} 2,4-D + 0.1 mg L^{-1} BAP	(Altpeter et al. 2000)
Protocol I: MS medium + 5 mg L^{-1} 2,4-D + 0.1 mg L^{-1} BAP, followed by MS medium + 0.1 mg L^{-1} 2,4-D + 0.25 mg L^{-1} BAP	(Altpeter and Posselt 2000; Newell and Gray 2005)
Protocol II: MS medium + 1 mg L^{-1} BAP	(Bajaj et al. 2006)

2.1.2 Expression of recombinant genes

The second limitation to obtain transplastomic tissue in monocots is the low protein synthesis rate within the proplastids of non-green tissue. This leads to poor expression of the selectable marker from a transgene cassette, needed to detoxify the selectable agent. To overcome this problem during plastid transformation experiments, a strong promoter and suitable plastid signals have to be used within the expression cassette to ensure sufficient expression of the selectable marker gene (Daniell et al. 2002).

During this study, two different plastid transformation vectors were constructed. The *Lolium perenne* L. plastid transformation vectors pIAPRvdB4 and pIAPRvdB5 contain the *trnI-trnA* region of the *Lolium perenne* L. plastid genome to facilitate targeted integration of an expression cassette within the intergenic region between the *trnI* and *trnA* genes. Both pIAPRvdB4 and pIAPRvdB5 contain a dicistronic cassette containing the reporter gene soluble modified green fluorescent protein (*smGFP*), for visualisation of transgenic tissue and the selectable marker *aphA-6*, which detoxifies kanamycin, paromomycin, geneticin and neomycin (Shaw et al. 1993). The difference between these two vectors lies in the expression signals within the expression cassette. In the vector pIAPRvdB4, these genes are under regulation of the full-length Prrn promoter including the binding sites for nuclear-encoded polymerases (NEP) and plastid-encoded polymerases (PEP), whereas the mRNA stability and processing are regulated by the 5'UTR of *rbcL* and the 3'UTR of *rps16* of N*icotiana tabacum*, as described by (Svab and Maliga 1993). In pIAPRvdB5 these genes are transcribed by a truncated Prrn promoter fused with the *gene 10* leader sequence of the T7 phage, which is known to facilitate high expression levels (Kuroda and Maliga 2001b). Furthermore the cassette contains the 3'UTR of *rps16* from *Nicotiana tabacum* for transcript stabilisation.

2.1.3 Selection regimes

The third limitation involves the selection regime in the dark. In monocotyledonous species, the proliferation of calli is usually restricted under conditions in the dark. Although this might be quite manageable for nuclear transformation, this poses a problem for plastid transformation, because the aminoglycoside antibiotics, like spectinomycin,

streptomycin and kanamycin used for selection of plastid transformation are mostly ineffective when selection is performed in the dark (Bock 2007).

2.1.4 Gene delivery

The last limitation is the delivery of foreign DNA into the double membrane chloroplast. DNA delivery to plant cells of monocotyledonous species can be accomplished by several different methods; 1. *Agrobacterium*-mediated transformation (Wu et al. 2005; Hiroko and Tadashi 2006); 2. PEG-mediated transformation (Folling et al. 1998); 3. Biolistic particle bombardment (Dalton et al. 1999; Altpeter et al. 2000); 4. Silicon carbide whisker-mediated transformation (Dalton et al. 1998). However for plastid transformation, only two of the above methods have been used successfully. These are biolistic particle bombardment (biolistics) and the PEG-mediated transformation. The preferred method for plastid transformation is biolistics, mainly for its efficiency in comparison with PEG-mediated transformation (Daniell et al. 2002). In conjunction to the choice of gene delivery method, the choice of target tissue is equally important. The tissue should be able to proliferate readily and selection on the tissue should be possible to develop transgenic tissue after transgene delivery.

2.1.5 Aim and objectives

The aim of this study was to achieve plastid transformation of *Lolium perenne* L..

In order to maximize the chances to achieve DNA modification of *Lolium perenne* L. (both plastid transformation and nuclear transformation) all the above mentioned aspects had to be optimized, as the transformation efficiency was expected to be very low. This is

based on the fact, that only one other monocotyledonous species has ever been successfully transformed within the plastid genome (Khan and Maliga 1999; Lee et al. 2006) and monocot species remain relatively recalcitrant even to nuclear transformation. Firstly an efficient transformation vector was constructed based on data obtained from previous studies at the time of the start of the project. Secondly an optimal regeneration protocol was developed. This step is critical due to the limitations of a regeneration system for *Lolium perenne* L. conducted in the light. Light is essential for an efficient selection procedure for plastid transformation. Thirdly, an optimal selection regime was assessed and determined. Several antibiotics were analysed for feasibility for both plastid and nuclear transformation. Fourthly, gene delivery conditions were tested for their efficiency in a transient expression test. The DNA-Gold coating protocol, target distance and shooting pressure were assessed for their effect on transient expression. Finally transformation experiments were conducted, and putative transformants were subsequently characterised.

2.2 Materials and methods

2.2.1 Molecular techniques

2.2.1.1 Minipreparations of plasmid DNA using alkaline lysis

2 ml of overnight bacterial cultures containing plasmids were spun down in 1.5ml Eppendorf tubes. The pellet was resuspended in 100 µl lysis buffer (50mM Glucose; 10mM ethylenediaminetetra acetic acid (EDTA); 25mM Tris; 1 µl 100 mg ml^{-1} RNase) with vortexing. After 10 minutes at room temperature, 200 µl of fresh solution 2 (0.2M sodium hydroxide (NaOH); 1% sodium dodecyl sulphate (SDS) was added. The Eppendorf tubes were placed on ice for 10min, before 200 µl 3M sodium acetate, pH 5.2, was added. The Eppendorf tubes were placed on ice for a further 10 minutes, before they were spun down at max revolutions per minute (rpm) for 5 minutes. The supernatant was transferred to new Eppendorf tubes, and spun down again. 2 volumes of 96% ethanol were added to the Eppendorf tubes, which were then placed at -20^{o}C for 30 minutes. The samples were spun down at max rpm for 10 minutes, and the DNA pellets were washed with 300 µl 70% ethanol. After another 3 minute spin, the ethanol was removed and the pellet was air-dried in the laminar flow hood. Finally the DNA was dissolved in 50 µl of sterile MilliQ. DNA samples were stored at -20^{o}C.

2.2.1.2 Total genomic DNA (gDNA) isolation of plant tissue

100 mg of plants tissue was collected into an Eppendorf tube and frozen in liquid nitrogen, prior to grinding up the tissue into powder. The plant cells were subsequently lysed with 500 µl lysis buffer 1 (20mM Tris pH 8; 20mM EDTA; 2M sodium chloride).

Tubes were heated for 5 minutes at 85°C and cooled for 5 minutes on ice. This step was repeated twice. The suspension was spun at max rpm for 10 minutes, and the supernatant was transferred to a new tube. This was done twice. To each tube 1 µl 100 mg ml^{-1} RNase was added, and incubated at 37°C for 30 minutes, after which 1/10 volume of 3M sodium acetate pH 5.2 and 1 volume 100% isopropanol was added. Tubes were incubated at -20°C for 30 minutes, and the precipitated DNA was spun down at max rpm for 10 minutes. The DNA-pellet was washed with 500 µl 70% ethanol and spun at max rpm for 3 minutes. The supernatant was removed, and the pellet was air-dried. Finally the DNA-pellet was dissolved in 50 µl MilliQ.

2.2.1.3 Polymerase Chain Reaction (PCR)

All PCR reactions were conducted with REDTaq® Genomic DNA Polymerase (Sigma cat. no. D8312). The standard PCR mixture was as shown in Table 2.2 and the PCR cycles are given in Table 2.3.

Table 2.2. Standard PCR mixes

Reaction mix	*Volume (µl)*
DNA (1µg $µl^{-1}$)	1.0
Primer 1 (0.1µM $µl^{-1}$)	1.0
Primer 2 (0.1µM $µl^{-1}$)	1.0
REDTaq® Genomic DNA Polymerase (1unit $µl^{-1}$)	2.5
10x REDTaq buffer	5.0
dNTP (10mM)	2.0
MilliQ ddH_2O	37.5
Total reaction mix	50.0

Table 2.3: PCR cycle:

Steps	*Temperature*	*Time*
Step1 Denaturation	94°C	5 min
Step 2 Denaturation	94°C	30 seconds
Step 3 Annealing	5°C below the lowest melting temperature of both primers	30 seconds
Step 4 Extension	72°C	1kb per minute
Step 5 Repeat cycles	*Repeat step 2-4*	*30 times*
Step 6 Final extension	72°C	10 min
Step 7 End of program	4°C	Indefinite storage

2.2.1.4 Gel extraction of PCR fragments

PCR fragments were run on a 0.8% agarose gel, after which the fragment was cut out of the gel with a clean scalpel. The fragment was subsequently purified from the gel with the "QIAquick® Gel Extraction Kit", as specified by the manufacturer's protocol (Qiagen cat. No. 28704).

2.2.1.5 Preparation of chemically competent TOP10 *Escherichia coli* cells.

Commercial TOP10 heat-shock competent *Escherichia coli* cells (Invitrogen) were plated out on solid Luria broth (LB) plates. The next day a tube containing 5 ml LB was inoculated with a colony from the LB plates for overnight growth at 225 rpm and 37°C. 5ml of the overnight culture was inoculated into 200 ml LB in a 1 litre flask. This culture was grown at 37°C at 225 rpm until an OD_{600} value of 0.3 was reached. The culture was spun down inside a pre-cooled sterile centrifuge tube at 5,000 rpm and 4°C for 10min. The pellet was resuspended in 100ml ice-cold 100mM calcium chloride ($CaCl_2$) and incubated on ice for 20 minutes, after which the cells were spun down at 4,000 rpm and

4°C for 10min. The pellet was resuspended in 20ml ice-cold 100mM $CaCl_2$ and the culture was incubated for 1 hour on ice. 3.87 ml 100% glycerol was added to the centrifuge tube to give a final concentration of 15% glycerol and 100mM $CaCl_2$. The culture was mixed gently and 200 µl was aliquoted to cold 0.5 ml Eppendorf tubes. Tubes were snap frozen in liquid nitrogen and stored at -80°C for future use.

2.2.1.6 Ligation of PCR fragments into the pCR2.1 topo vector

PCR fragments were cloned into the TA cloning vector pCR2.1 (Invitrogen, cat. no. K2020-20) using the manufacturer's protocol. The vector with insert was then transfected into chemically competent TOP10 cells.

2.2.1.7 Heat-shock of plasmid DNA into chemically competent TOP10 cells

1 µl of 1µl μl^{-1} plasmid DNA was added to 50 µl of ice-cold competent cells. The cells were then heat-shocked at 42°C for a duration of 45 seconds. Afterwards the tube was directly placed on ice for 2 minutes. Subsequently 200 µl LB was added, and the culture was incubated for 1 hour at 37°C at 200 rpm. Finally 75 µl and 150µl of cultures were plated on solid LB with the appropriate antibiotic.

2.2.1.8 Restriction digests of plasmid DNA

To facilitate cloning, plasmids were digested with appropriate restriction endonucleases ordered from Promega as summarised in Table 2.4.

Table 2.4: General restriction digestion overview

Components	*Volumes or units*
Plasmid DNA for backbone fragment	5 µg (usually 5 µl)
Restriction endonuclease I	1 unit if single digest
	2 units if double digest
Restriction endonuclease II	1 unit if single digest
	2 units if double digest
Appropriate restriction buffer (10x)	2 µl
BSA (10x) (if needed)	2 µl if needed
MilliQ ddH_2O	Add until final volume is 20 µl
Total mix	20 µl

Components	*Volumes or units*
plasmid DNA for creating an insert	10 µg (usually 10 µl)
Restriction endonuclease I	1 unit if single digest
	2 units if double digest
Restriction endonuclease II	1 unit if single digest
	2 units if double digest
Appropriate restriction buffer (10x)	2 µl
BSA (10x) (if needed)	2 µl if needed
MilliQ ddH_2O	Add until final volume is 20 µl
Total mix	20 µl

2.2.1.9 Ligation of inserts and vectors

Digested inserts and vectors were ligated together using T4 DNA Ligase (Invitrogen, cat. no. 15224-017), according to the manufacturer's protocol.

2.2.1.10 Southern blot analysis

Southern blot analysis was performed according to the method of McCabe et al (1997).

2.2.1.10.1 PCR DIG-probe labelling

A dNTP labelling mix was used from the digoxigenin (DIG) DNA labelling kit (Roche cat. No. 11175033910) in conjunction with GoTaq polymerase (Promega cat. No. M8301). The PCR reaction was performed in a 0.2 ml Eppendorf tube, containing 1 µl of 0.1µg μl^{-1} template DNA, 0.2 µl GoTaq polymerase, 10 µl 5x buffer, 6 µl 2.5 M $CaCl_2$, 1 µl 10 mM forward primer, 1 µl 10 mM reverse primer, 5 µl dNTP labelling mix and 25.8 µl MilliQ. A control reaction with unlabeled PCR reaction was also set up as a reference. The PCR cycle consisted of 35 cycles with the annealing temperature five degrees Celsius below the Tm of the used primers. 5 µl of the reaction mix was analysed on a 0.8% agarose gel. The control product would show at the expected size, whereas the labelling reaction's product would migrate slower due to the integrated dUTPs. DIG-labelled probes were stored at -20^0C until use.

2.2.1.10.2 Sample digestion and electrophoresis

For testing plastid putative transformants 1 µg of total DNA was digested with *SacII*, while for nuclear putative transformants 12 µg of total DNA was digested with *SacII*. As control, 0.1 µg plasmid DNA was digested with *SacII* or *KpnI* depending on the plasmid. Digests were setup containing 1 µl restriction enzyme, 2 µl of appropriate restriction enzyme buffer, 2 µl 10x BSA, an x amount of DNA depending on the concentration and MilliQ to added to gain a final volume of 20 µl. The reactions were incubated at 37^0C for a minimum of 16 hours. The reaction mixes were ran on a 0.8% agarose gel for five hours at 60 volts and visualised using UV.

2.2.1.10.3 Southern blotting and hybridisation with probe

The DNA was blotted onto a Hybond N^+ membrane by capillary pull overnight using 0.4M NaOH as transfer buffer (McCabe et al. 1997). The capillary blot was dismantled and the membrane was neutralized by washing in 2x SSC (0.3M sodium chloride, 30 mM sodium citrate, pH 7) for 5 minutes. The membrane was subsequently baked for 30 minutes at 120^0C, after which the membrane was transferred into a roller tube containing 20 ml pre-hybridisation solution (DIG easy hyb solution (Roche cat. No. 11603558001)) and incubated for a minimum of 1 hour at 37^0C. Meanwhile DIG labelled probe was denatured for 10min at 95^0C, of which 25 μl was added to 10 ml of DIG easy hyb solution, resulting in the hybridisation solution. The pre-hybridisation solution was poured off, and replaced with the hybridisation solution. The roller tube was placed back in the hybaid oven at 42^0C for 16 hours. The pre-hybridisation solution can be stored at -20^oC and be reused three times. The membrane was washed twice in 50ml of wash solution A (2x SSC, 0.1% SDS) at 20^0C for five minutes and twice with 50 ml of wash solution B (0.5x SSC, 0.1% SDS) at 68^0C for 15 minutes.

2.2.1.10.4 Chemiluminescent detection

The membrane was rinsed briefly in buffer 1A (0.1M Maleic acid, 3M sodium chloride, pH 8, autoclaved, 0.3% tween-20). The membrane was blocked by incubation in 50ml of 0.75% blocking solution (Roche cat No. 11096176001 dissolved in buffer 1A) for one hour. The solution was poured in a tube, and anti-DIG-AP antibody was prepared by dilution 1:10.000 (v/v) in the blocking solution. The membrane was incubated in this solution for 30 minutes. The unbound anti-DIG-AP was removed by washing the

membrane five times in buffer 1A for 10 minutes. The blot was incubated in 50 ml buffer 3 (0.1M Tris/Cl, pH 9.5, 0.1M sodium chloride) for five minutes, after which buffer 3 was replaced with buffer 3 containing 0.24 mM CDP-star (Roche cat No. 12041677001) for five minutes. The blot was drained of excess solution and sealed between two acetate sheets, the hybridisation signals were detected by exposing the filter to Kodak X-ray film for 1 to 16 minutes.

2.2.2 Vector construction pIAPRvdB4 and pIAPRvdB5

2.2.2.1 Primers used for construction of pIAPRvdB4 and pIAPRvdB5

The following primers were used for construction of the *Lolium perenne* L. plastid transformation vectors pIAPRvdB4 and pIAPRvdB5.

P1trnI: aagcttttagggtgaagtaagacc

Underlined sequence is the *HindIII* restriction site.

P2trnI: ttaattaaggtacctgcttcttctattcttttcc.

Underlined sequences are the *PacI* and *KpnI* restriction sites respectively

P1trnA: ttaattaatctgactctttcatgcatac

Underlined sequence is the *PacI* restriction site

P2trnA: ccatgaatgcgaaatcatag.

P1Prrn: Ggtaccgctcccccgccgtcgttcaa

Underlined sequence is the *KpnI* restriction site

P2Prrn-RbcL: gagctcccctccctacaactgtatcca

Underlined sequence is the *SacI* restriction site

P1smGFP: gagctcatgagtaaaggagaagaact

Underlined sequence is the *SacI* restriction site

P2smGFP: Ggccggccccctcccttatttgtatagttcatccatgc

Underlined sequence is the *FseI* restriction site, the sequence in red is the ribomosal binding site from *RbcL* gene derived from *Nicotiana tabacum*

P1aphA: ggccggccatgaccatggaattaccaaa

Underlined sequence is the *FseI* restriction site

P2aphA: gcggccgctcaattcaattcatcaagtt

Underlined sequence is the *NotI* restriction site

P1rps16: gcggccgcaccgaaattcaattaaggaa

Underlined sequence is the *NotI* restriction site

P2rps16: ctcgag*ttaattaa*agaacacggaattcaatgga

Underlined sequences are the *XhoI* and *PacI* restriction sites respectively

P1nheI- smGFP: gctagcagtaaaggagaagaact

Underlined sequence is the *NheI* restriction site

P2T7g10-ndeI-nheI: gctagccatatgtatatctccttctt

Underlined sequences are the *NheI* and *NdeI* restriction sites respectively

2.2.2.2 Construction of the *Lolium perenne* transformation vector pIAP

The flanking regions *trnI* and *trnA* were amplified from total DNA isolated from *Lolium perenne* accession *'Shandon'* with the primers P1trnI, P2trnI, P1trnA and P2trnA (see 2.2.2.1). The PCR products were ligated into pCR2.1, and subsequently sequenced at John Lester's DNA sequencing facility at Cambridge using the M13 sequencing primers, which bind on the pCR2.1 vector for confirmation. Positive clones of fragments trnI and

trnA were ligated together with the use of restriction sites *HindIII* and *PacI.* This resulted in the transformation vector pIAP with a *KpnI-PacI* cloning site for integration of an expression cassette. The construction of this vector is illustrated in Fig. 2.1

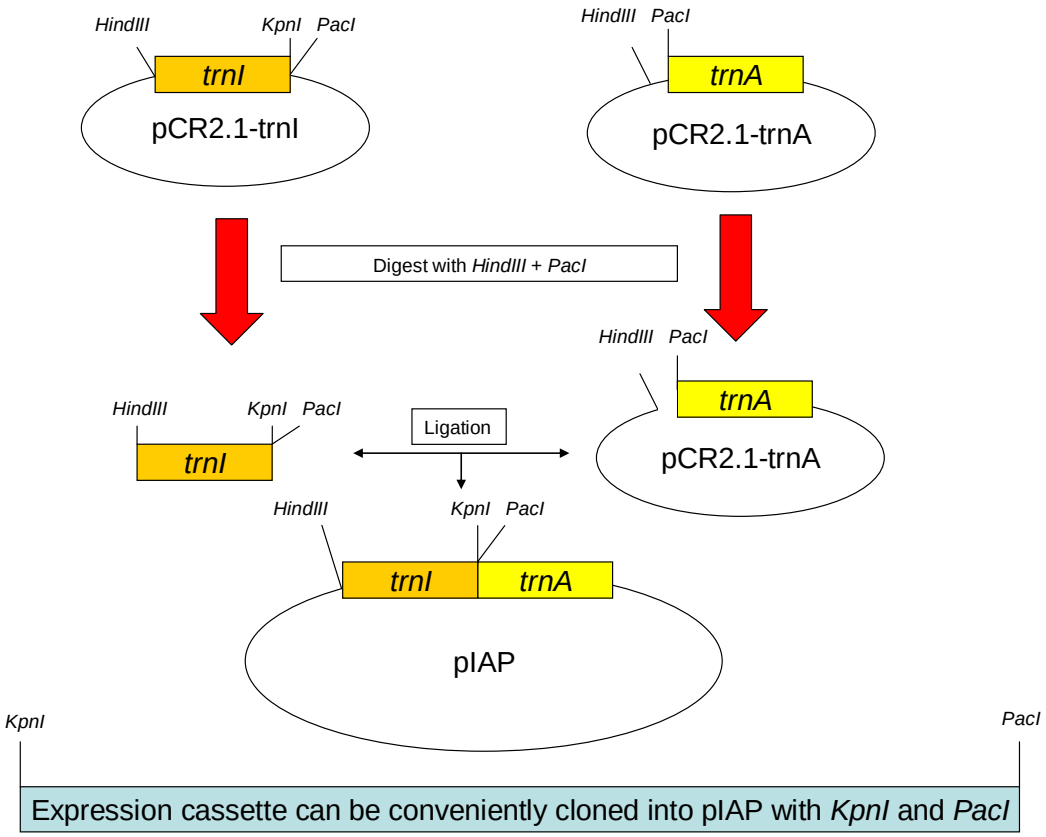

Fig. 2.1: Illustration of construction of the *Lolium perenne* L. plastid transformation vector pIAP. Flanking sequences homologous to the *Lolium perenne* L. plastid region *trnI (tRNA^{ile})* and *trnA* (*tRNA^{ala}*).

2.2.2.3 Construction of expression cassette RvdB4:

The following fragments for pRvdB4 were amplified:

Full length Prrn promoter + *rbcL* 5'UTR from pZS197(Svab et al. 1990), with the following primers P1Prrn and P2Prrn-Rbcl (see 2.2.2.1). *smGFP* (soluble modified *Green Fluorescent Protein*) from the plasmid smGFP (Davis and Vierstra 1998) with the following primers, P1smGFP and P2smGFP (see 2.2.2.1). *AphA-6* (*3'-aminoglycoside phosphotransferase*) from pSK.KmR (Bateman and Purton 2000), with the following primers; P1aphA, and P2aphA (see 2.2.2.1). *rps16* 3'UTR from total DNA isolated from *Nicotiana tabacum* Cv. 'Petit Havanna', with the following primers; P1rps16 and P2rps16 (see 2.2.2.1). Amplified fragments were cloned into pCR2.1 and sequenced for confirmation using M13 primers which bind to the pCR2.1. Positive clones were selected and used to construct the vector pRvdB4. An overview of the steps that were involved in this process, are illustrated in Fig. 2.2a and Fig. 2.2b.

pCR2.1-aphA-6 and pCR2.1-Trps16, were digested with *NotI* and *XhoI*. The rsp16 fragment was subsequently cloned downstream of the *aphA-6* gene. This resulted in the pCR2.1-aphA-6-Trps16 construct. This vector and the pCR2.1-smGFP were digested with *SacI* and *FseI*. After which the smGFP fragment was cloned upstream of the *aphA-6* gene, resulting in the pCR2.1-smGFP-aphA-6-Trps16, finally this vector and the pCR2.1-Prrn-Rbcl 5'UTR were digested with *KpnI* and *SacI*. The Prrn-Rbcl 5'UTR fragment was cloned upstream of the *smGFP* gene, resulting in the expression vector pRvdB4.

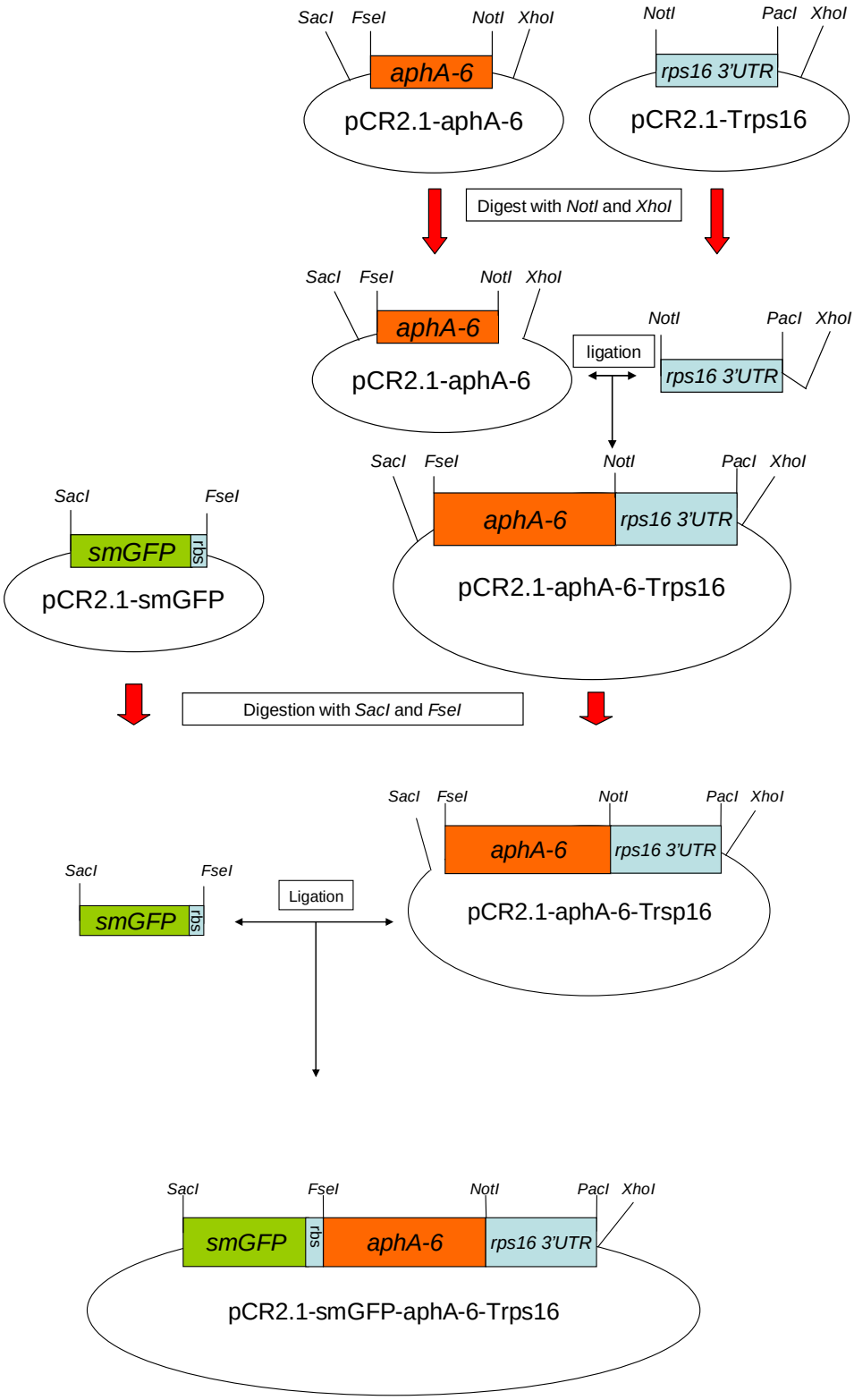

Fig. 2.2a: Part I of stepwise illustration of the construction of expression vector pRvdB4

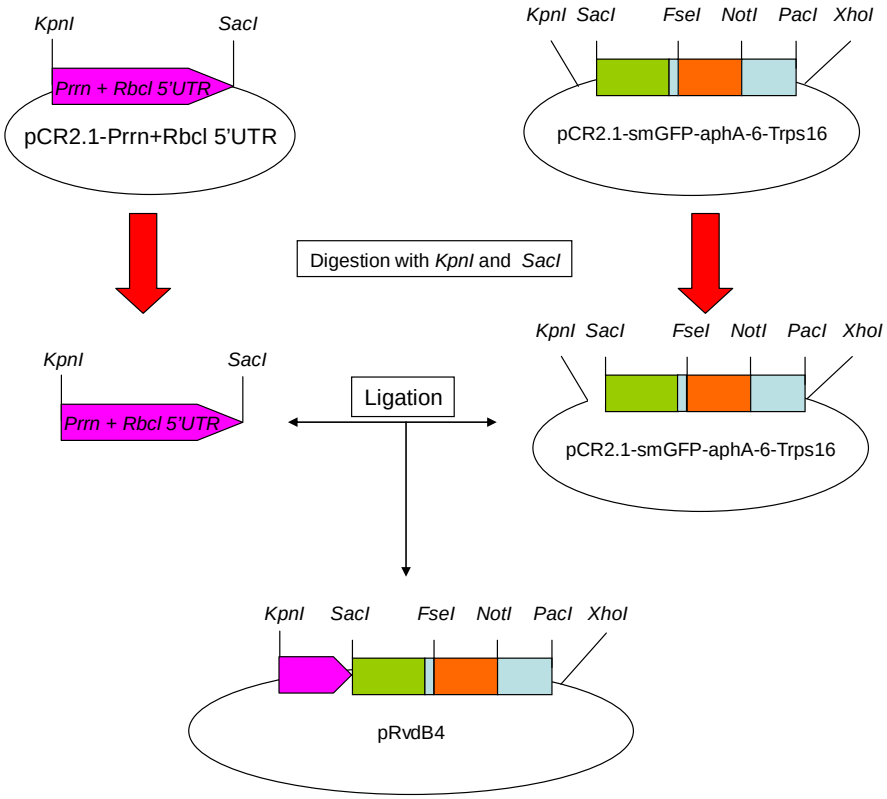

Fig. 2.2b: Part II of stepwise illustration of the construction of expression vector pRvdB4. Prrn: Promoter from the *rrn* operon in *Nicotiana tabacum*, *rbcL: rubisco large subunit, smGFP: soluble modified Green Fluorescent Protein*, aphA-6: *3'-aminoglycoside phosphotransferase* , *rps16*: *ribosomal protein S16.*

2.2.2.4 Construction of expression cassette RvdB5:

The expression cassette RvdB5 contains the truncated Prrn promoter with the binding sites for the PEP (Plastid-Encoded Polymerase), fused with the *T7g10* (*gene* 10 of the T7 phage) leader for high expression of the transgene. The Prrn-*T7g10* fragment was amplified from pHK20 (Kuroda and Maliga 2001b) using the following primers; P1Prrn and P2T7g10-ndeI-nheI (see 2.2.2.1). The *smGFP* fragment was amplified from pRvdB4 using the following primers; P1ndeI- smGFP and P2smGFP (see 2.2.2.1). The cloning steps are illustrated in Figs. 2.3 and 2.4.

pCR2.1-Prrn-T7g10 5'UTR and pRvdB4 were digested with *KpnI* and *SacI.* The Prrn T7g10 5'UTR fragment was cloned into the excised region from pRvdB4, replacing the Prrn Rbcl 5'UTR. The resulting vector was pRvdB4/5 intermediate, as shown in Fig. 2.3. The pRvdB4/5 intermediate vector can be used to construct a tricistronic vector. An extra gene can be cloned between the *T7g10* leader and the *smGFP.* However if done, an extra ribosomal binding site (RBS), has to be amplified between the new gene and the *smGFP*.

Finally the pRvdB4/5 intermediate and the pCR2.1-nheI-smGFP vectors were digested with *NheI* and *FseI*. The NheI-smGFP-FseI fragment was cloned into the excised region of the pRvdB4/5 intermediate, replacing the *smGFP* fragment from pRvdB4. This resulted in the pRvdB5 expression vector. The maps of the completed vectors are shown in Fig. 2.5.

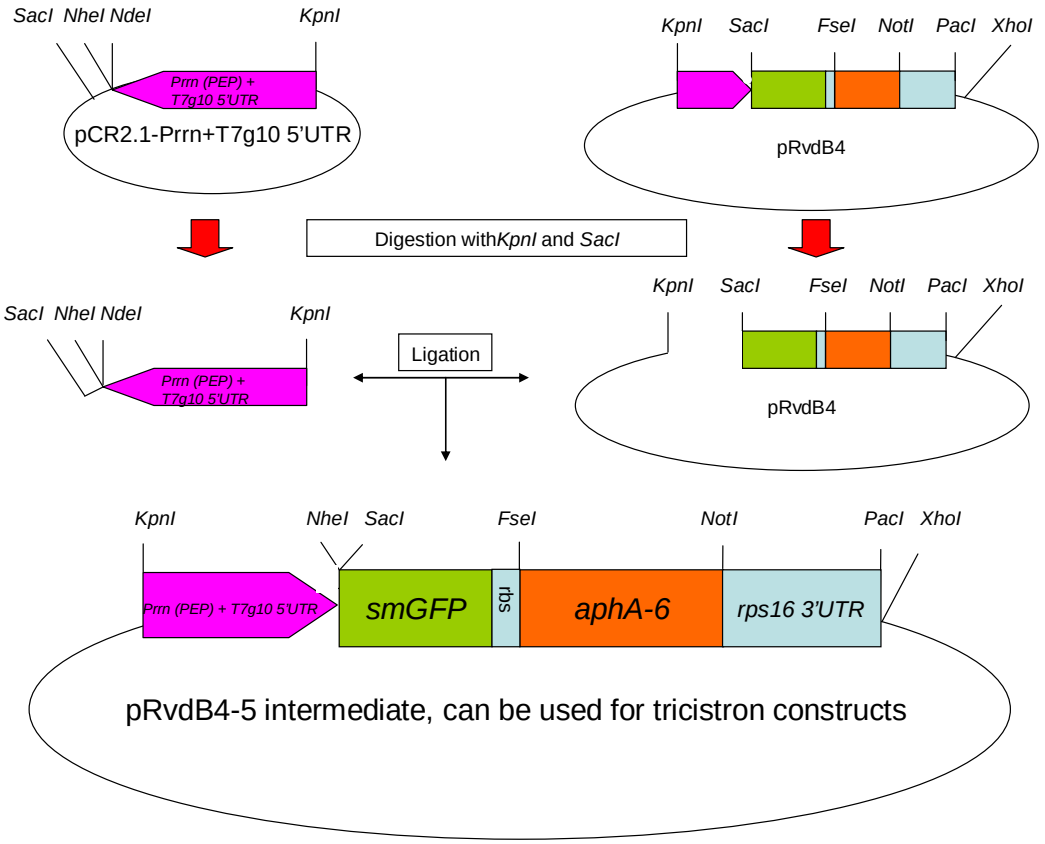

Fig. 2.3: Illustration of construction of the intermediate vector pRvdB4/5, Prrn (PEP): *Prrn(PEP):* Truncated promoter of the *rrn* operon including the PEP binding sites. *T7g10* 5'UTR: *gene 10* of the T7 phage 5'UTR. *smGFP: soluble modified Green Fluorescent Protein*, aphA-6: *3'-aminoglycoside phosphotransferase* , *rps16*: *ribosomal protein S16*

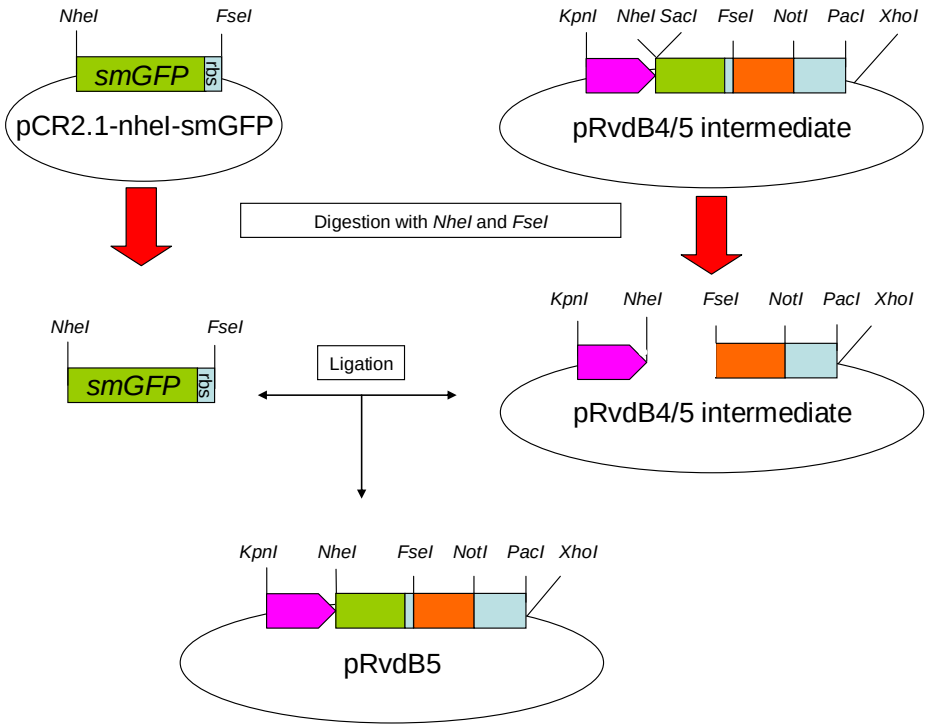

Fig. 2.4: Final step of construction of expression vector pRvdB5

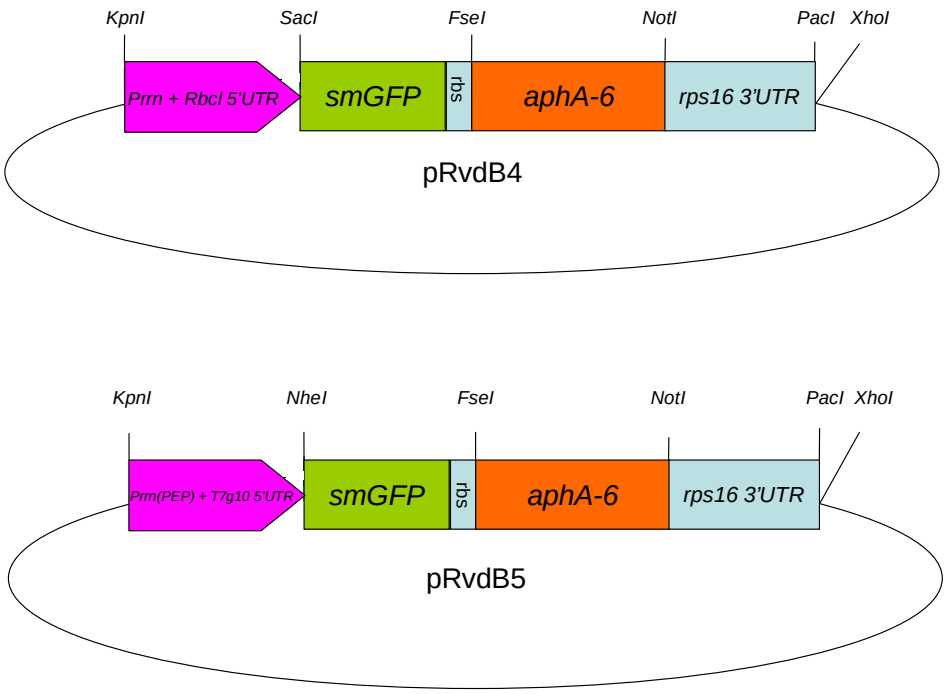

Fig. 2.5: Complete map of pRvdB4 and pRvdB5, (illustrated fragment sizes do not represent actual sizes)
Prrn: Full length promoter from the *rrn* operon of *Nicotiana tabacum*, *rbcL: rubisco large subunit, Prrn(PEP):* Truncated promoter of the *rrn* operon of *Nicotiana tabacum* including the PEP binding sites. *T7g10* 5'UTR: *gene 10* of the T7 phage. *smGFP: soluble modified Green Fluorescent Protein*, aphA-6: *3'-aminoglycoside phosphotransferase* , *rps16*: *ribosomal protein S16.*

2.2.2.5 Construction of the transformation vectors pIAPRvdB4 and pIAPRvdB5

The transformation vector pIAPRvdB4 and pIAPRvdB5 was constructed by digesting both the pIAP and pRvdB4/5 with *KpnI* and *PacI.* The excised expression cassette was ligated into pIAP resulting in respectively pIAPRvdB4 and pIAPRvdB5 as illustrated in Fig. 2.6.

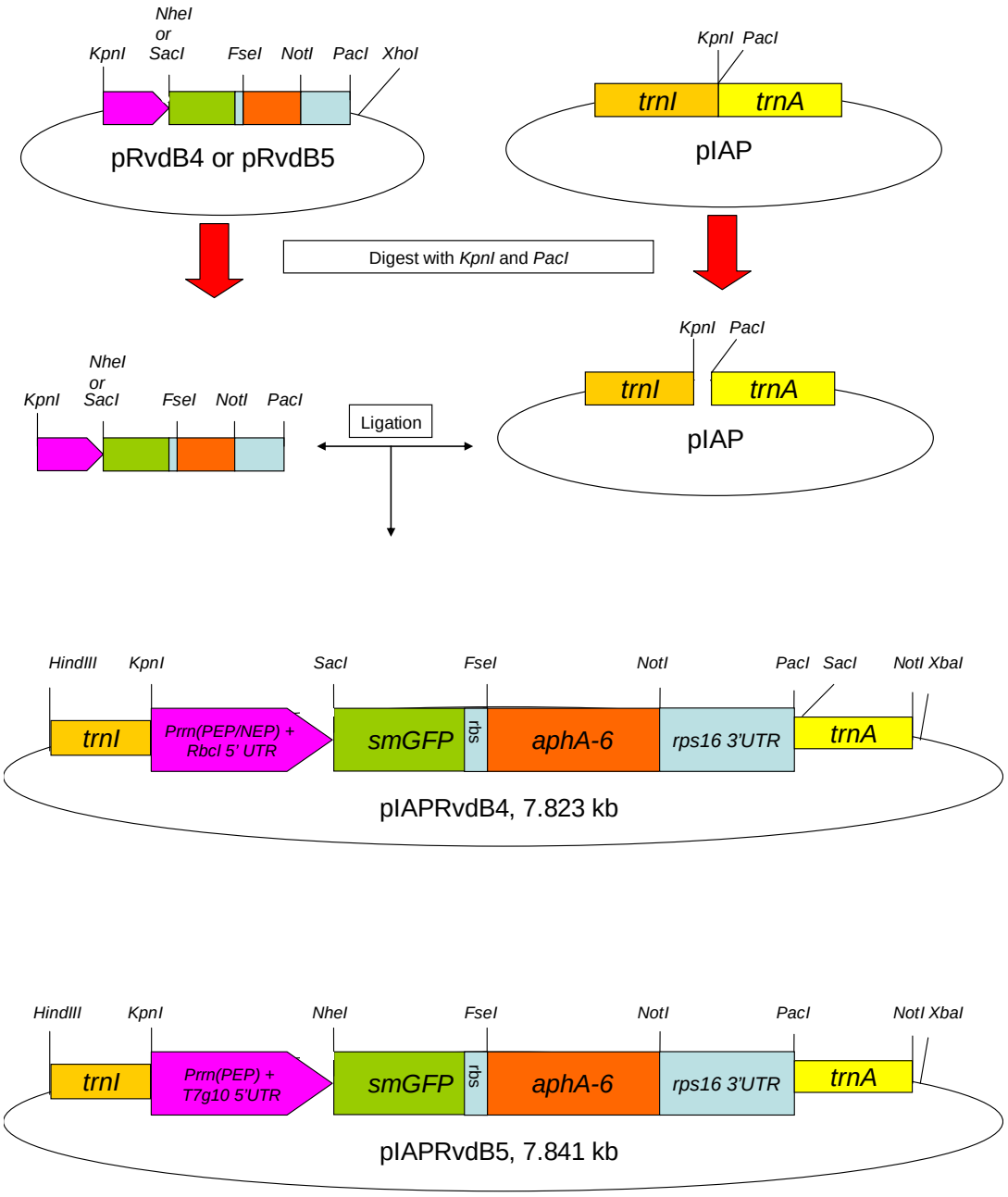

Fig. 2.6: Illustration of introduction of RvdB4 and RvdB5 cassettes into the *Lolium perenne* L. plastid transformation vector pIAP, resulting in plastid transformation vectors pIAPRvdB4 and pIAPRvdB5.

2.2.2.6 Testing functionality of pIAPRvdB4 and pIAPRvdB5

2.2.2.6.1 Testing for GFP accumulation in *Escherichia coli*

Escherichia coli stain TOP10 was transfected with either plastid transformation vector pIAPRvdB4, pIAPRvdB5 or an empty transformation vector pIAP, as described in paragraph 2.1.1.7.

Transfected bacteria, were grown overnight in 3 ml of LB, after which centrifuged bacteria could be assessed for GFP accumulation using a tabletop UV-light.

2.2.2.6.2 Testing for functionality of the *aphA-6* gene conferring resistance to G-418 and paromomycin

To test if the *aphA*-6 gene was correctly translated, and to test if G-418 and paromomycin could be used as antibiotic, the expression cassette was ligated into the pUC19 vector, which lacked a backbone containing the *nptII* gene. This step was necessary, due to the *nptII* gene within the backbone of the transformation vector pIAPRvdB4 and pIAPRvdB5. Bacteria containing pUC19-RvdB4, pUC19-RvdB5 or pUC19 were plated on LB + 50 mg L^{-1} paromomycin and LB + 50 mg L^{-1} G-418 and allowed to grow overnight at 37^0C. Growth was assessed by looking at the number of colonies that developed for each construct.

2.2.3 *In vitro* culture methods

2.2.3.1 Plant material:

Seed from six accessions of *Lolium perenne* L. were provided by Teagasc, Oak Park, Carlow. These were 'Cashel', 'Shandon', 'Greengold' (4n), 'S23', 'Cancan' and 'Limes'. Another two accessions were acquired, namely cultivars 'Action' and 'Telstar' (kindly provided by Dr. Christian Sig Jensen, DLF-Trifolium A/S, Denmark)

2.2.3.2 Aseptic techniques

All tissue culture experiments were carried out in a laminar flow cabinet using aseptic techniques. Equipment was sterilized, by dipping in 70% ethanol, prior to flaming. All media were sterilized by autoclaving for 20 minutes at 120^{o} Celsius. Heat labile chemical solutions were filter sterilized using Millex GP filter units (pore size 0.22μm)

2.2.3.3 Culture media

The media used for regeneration studies of *Lolium perenne* L are described in Table 2.5.

Table 2.5. Composition of media used for tissue culture.

Name of media ---------- *Ingredients*	*CIM (Callus Induction Medium)*	*CMM (Callus Maintenance Medium)*	*RGM1 (Regeneration medium 1)*	*RGM2 (Regeneration medium 2)*	*GM (Germination/ Rooting medium)*
MS + vitamins (Murashige & Skoog, 1962) (including vitamins)	4.4 g L^{-1}	4.4 g L^{-1}	4.4 g L^{-1}	4.4 g L^{-1}	2.2 g L^{-1}
Sucrose					30 g L^{-1}
Maltose*	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	-
2,4-D*	5 mg L^{-1}	5 mg L^{-1}	-	0.1 mg L^{-1}	-
BAP*	-	0.25 mg L^{-1}	1 mg L^{-1}	0.25 mg L^{-1}	-
micro agar	7 g L^{-1}	7 g L^{-1}	7 g L^{-1}	7 g L^{-1}	7 g L^{-1}
pH	pH 5.7	pH 5.7	pH 5.7	pH 5.7	pH 5.7

*: Filter sterilised, and added after autoclaving

2.2.3.4 Surface sterilization of seeds

Seeds were placed in 1.5ml eppendorf tubes, with 1 ml of 5% sodium hypochlorite (undiluted Domestos) solution 1 hour. The seeds were three times rinsed with sterile water, after which they were transferred to a new sterile eppendorf tube and kept overnight at 4^{o}C. The next day the seeds were treated with 0.5% sodium hypochloride for 10min, after which they were rinsed three times with sterile water and left to dry on a sterile filter paper in the laminar flow unit before use.

2.2.3.5 Induction of callus cultures

2.2.3.5.1 Induction of calli from apical meristem and leaf-base explants

Sterile seedlings were grown for four weeks on 150 ml of Germination Medium (GM) (see Table 2.5.) in polypropylene tubs of 90mm in diameter and 140mm in height. After four weeks apical meristems and 2mm sections of leaf-base explants adjacent to the apical meristem were removed from the seedlings under a compound microscope (Cambridge Instruments, model Z30 E) see Fig. 2.7. The explants were arranged on the Petri dishes according to leaf number and distance from the apical meristem. From these pieces, calli were induced on CIM (see Table 2.5.) as described by (Newell and Gray 2005) (see Fig. 2.7). Callus was induced for four weeks in the dark at 22^{o}C.

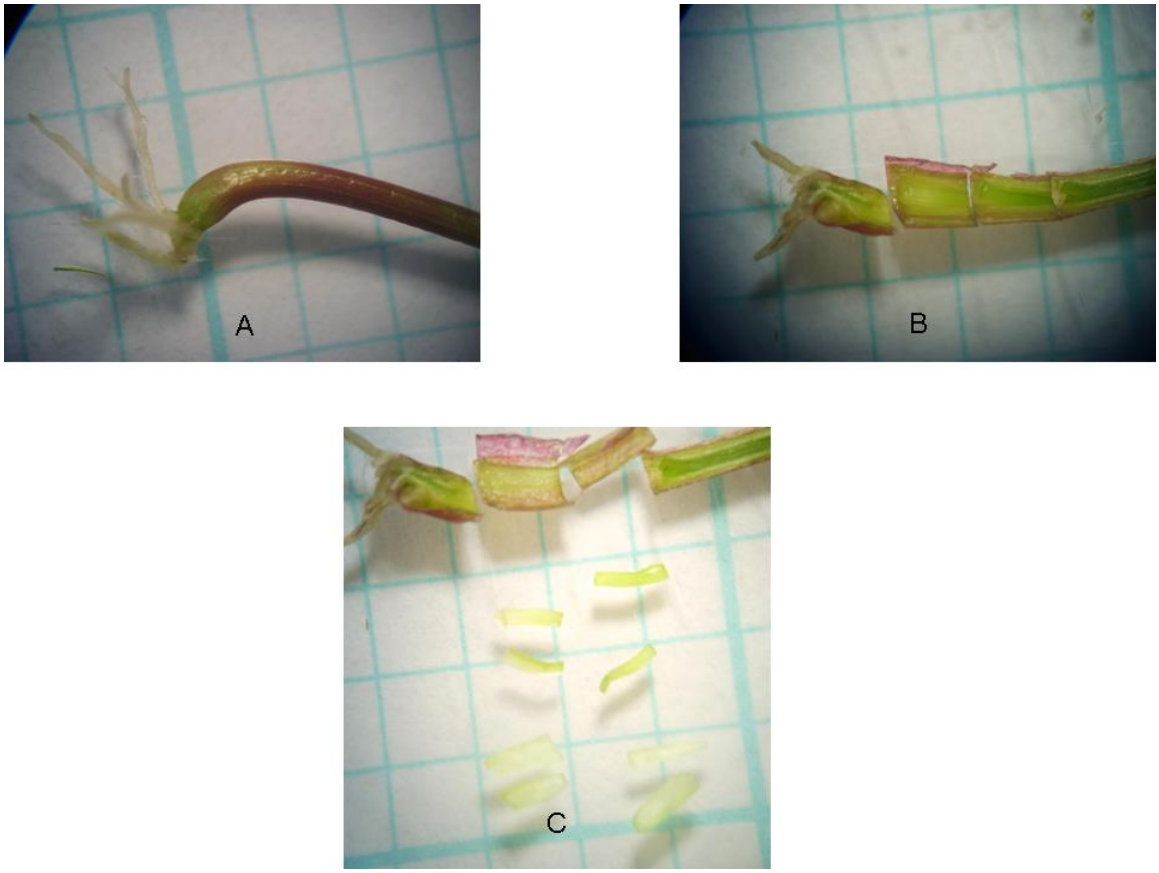

Fig. 2.7: Preparation of source tissue for callus induction;
A: 4 week old base of a seedling, B: longitudinal section of leaf base, C: excision of 2mm leaf-base explants adjacent to the apical meristem. Small squares on roster paper represent 2mm by 2mm.

2.2.3.5.2 Induction of calli from mature embryos

Sterilized seed were soaked in sterile water overnight at 4^{o}C to facilitate embryo excision. The next day, the embryos were aseptically excised from the seed under a compound microscope (Cambridge Instruments, model Z30 E) with the use of a seeker (see Fig. 2.8). The embryos were gently squeezed with a seeker to prevent germination before placement on CIM (see Table 2.5) in the dark at 22^{o}C for callus induction.

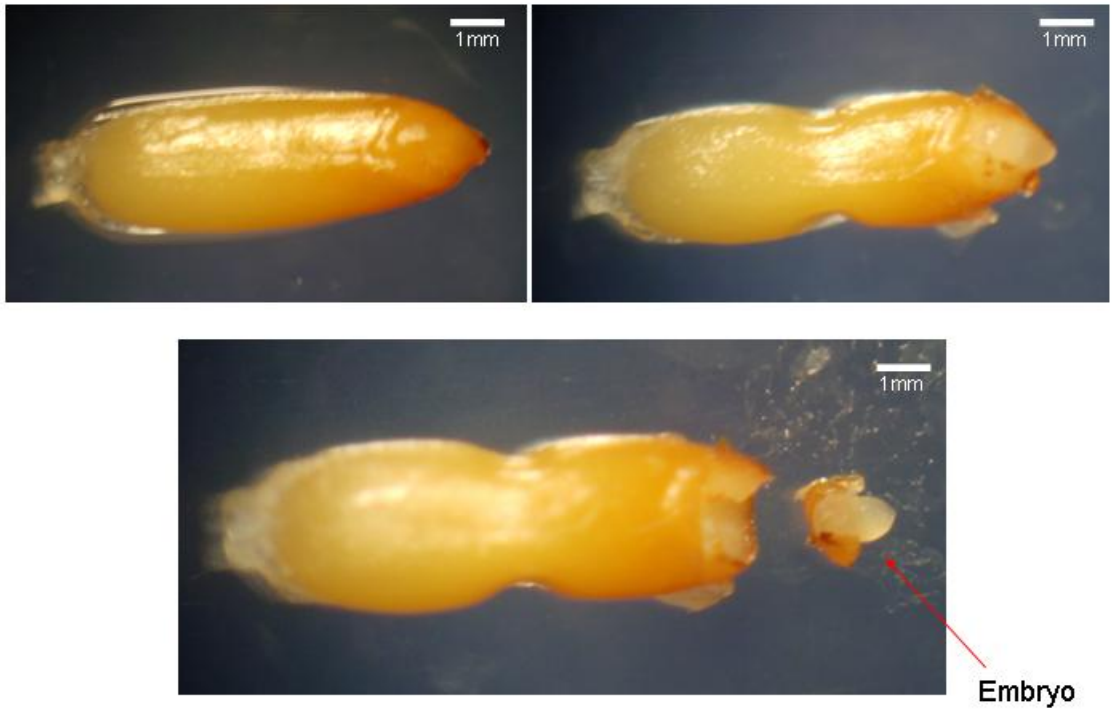

Fig 2.8: Mature embryo excision from sterile seeds

2.2.3.6 Shoot regeneration

2.2.3.6.1 Direct shoot regeneration

This protocol consisted of direct shoot regeneration, after callus induction (Bajaj et al. 2006). Four week old calli were placed on Regeneration medium 1 (RGM1) (see Table 2.5) at 22^{o}C with a 16 hour photoperiod. Calli were transferred to new RGM1 media biweekly.

2.2.3.6.2 Delayed shoot regeneration

This protocol consisted of a callus maintenance phase, which allows the calli to differentiate further into the embryogenic stage using a Callus Maintenance Medium (CMM, see Table 2.4.) in the dark at 22^{o}C. Calli which produced shoots were transferred to Regeneration Medium 2 (RGM2) (see Table 2.5) at 22^{o}C with a 16 hour photoperiod, while the remaining calli were sub cultured on CMM at a 22^{o}C with a 16 hour photoperiod. Calli were sub cultured biweekly. The percentage of calli that produced shoots was recorded.

2.2.3.7 Root induction from shoots

Small plantlets were transferred to tubs with 150 ml of Rooting Medium (RM) (Table 2.5) for root development.

2.2.4 Determination of conditions for antibiotic-resistance selection

2.2.4.1 Antibiotic stock solutions

Antibiotics were added to freshly prepared medium from filter-sterilised 50 mg ml^{-1} stock solutions.

2.2.4.2 Testing effect of antibiotics on callus initiation and growth

To test if leaf-base explants could be used for the transformation experiments, leaf-base explants of Cv. 'Cashel' were placed on CIM (see Table 2.5) supplemented with various antibiotics at various concentrations in the dark at 22^{o}C for four weeks. The percentage of explants producing regenerable calli was assessed, for each treatment twenty to thirty leaf-base explants were used.

2.2.4.3 Testing effect of antibiotics on callus differentiation and shoot formation

Four week old calli were placed on CMM (see Table 2.5) in a 16h photoperiod at 22^{0}C with various concentrations of several antibiotics. The callus morphology and the frequency of shoot development were recorded for each treatment. For each treatment 20 calli were used.

2.2.5 Nuclear and plastid transformation experiments

2.2.5.1 Vectors used

For transient expression experiments and nuclear transformation experiments the vectors pGUSHYG (Fig. 2.9) (Nugent et al. 2006) and pCambia1305.1 (Fig. 2.10) (http://www.cambia.org) were used. Both contained the *uidA* gene, for visualisation of

cells containing the GUS protein (β-glucoronidase). Furthermore both contained the *hygromycin phosphotransferase* (*hpt)* gene for hygromycin selection in plants. The differences between the vectors were the expression signals and promoters. The pCambia 1305.1 vector contained introns within the *uidA* gene.

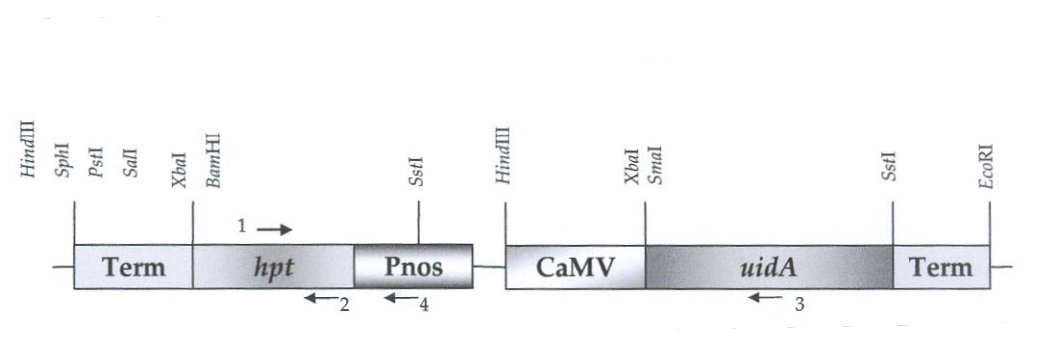

Fig. 2.9: Nuclear transformation vector pGUSHYG. It contains the *GUS* reporter gene, encoding β-glucuronidase under the control of the Cauliflower Mosaic Virus 35S promoter (CaMV) and terminator (Term). Furthermore it contains the selectable marker gene, hygromycin phosphotransferase (*hpt*) under regulation of the nopaline synthase promoter (Pnos) and termination (Term).

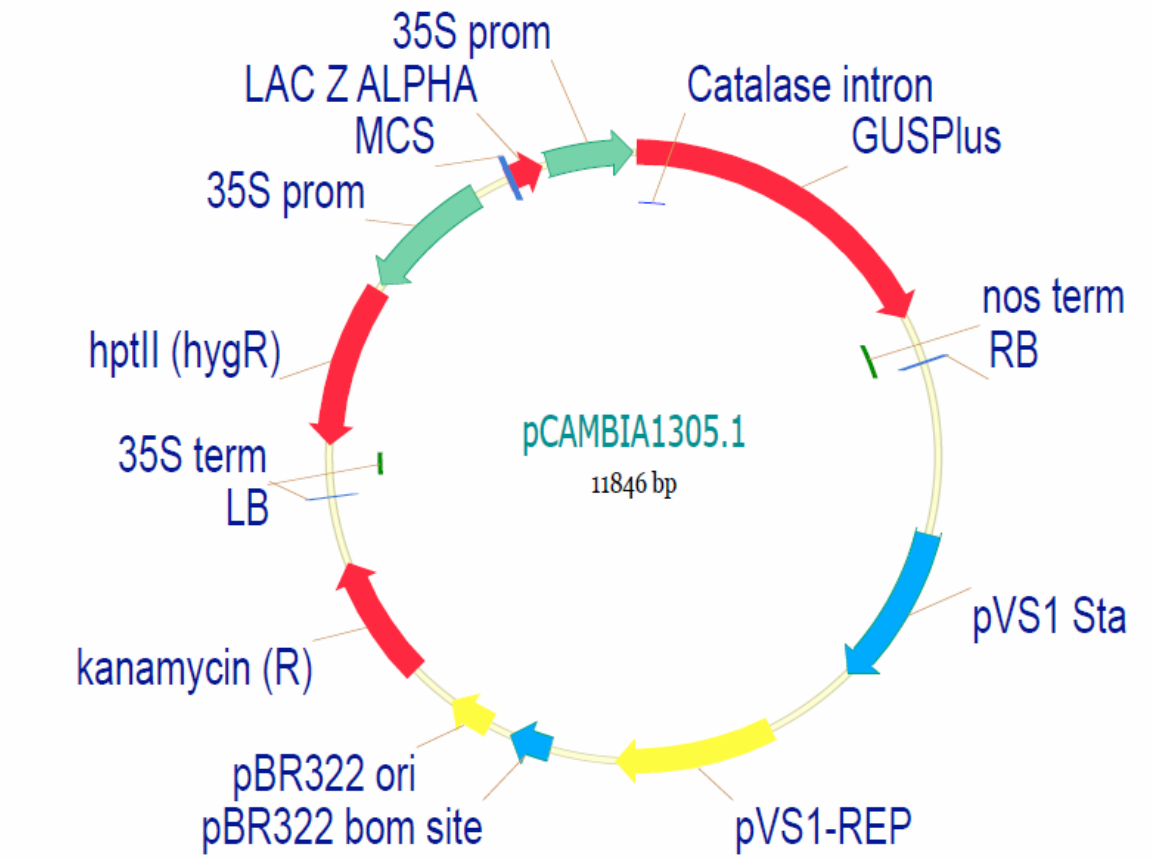

Fig. 2.10: Nuclear transformation vector pCambia 1305.1. Containing the hygromycin phosphotransferase (*hpt*) gene under regulation of the Cauliflower Mosaic Virus promoter (35S prom) and terminator (35S term). Furthermore it contains a modified *GUS* reporter gene including introns for optimized expression under regulation of the 35S promoter and nopaline synthase terminator. This vector also contains left and right border regions (LB, RB) for use in conjunction with A*grobacterium*-mediated transformation.

For the plastid transformation experiments the vector pIAPRvdB5 was used (Fig. 2.6).

2.2.5.2 Biolistic delivery of DNA

2.2.5.2.1 Stock solutions for biolistical delivery

0.1M Spermidine stock solution: 1g ampoule spermidine was heated in a water bath at 37^{o}C for 5 min until liquid. 750 µl spermidine was transferred to a falcon tube, after which 47.5 ml of MilliQ was added. The solution was filter-sterilized and divided into 200 µl aliquots in 500 µl Eppendorf tubes. 0.1 M spermidine stocks were stored at -80^{o}C.

2.5M Calcium chloride stock solution: 3.86 g $CaCl_2.2H_2O$ was dissolved in 10 ml MilliQ using a vortex. The solution was divided into aliquots of 1 ml in 1.5ml Eppendorf tubes and stored at -20^{o}C. 0.6 Micron Gold particle preparation; 36 mg of 0.6 micron Gold (Biorad) was transferred into a 1.5 ml Eppendorf tube. 1 ml of 96% ethanol was added to the tube and sonicated for 2 minutes, after which the tube was spun at 11,000 rpm for 5 seconds. The supernatant was removed, and the step was repeated twice. After this the pellet was washed with 1 ml of sterile MilliQ, followed by a spin at 13,000 rpm for 5 seconds. The supernatant was removed, and the step was repeated twice. After the last spin, the pellet of gold was resuspended in 600 µl 50% sterile glycerol. Aliquots of 30 µl were made from this suspension in 1.5 ml Eppendorf tubes. Each tube contained 1.8 mg gold and could be used for 10 biolistical shots. The tubes were stored at -20^{o}C.

2.2.5.2.2 Leaf-base explants preparation for biolistics

2 mm leaf-base explants were excised from four-week old seedlings, and placed on CIM + 64 g L^{-1} mannitol for pre-osmotic treatment 1 day prior to biolistic delivery, as illustrated in Fig. 2.11.

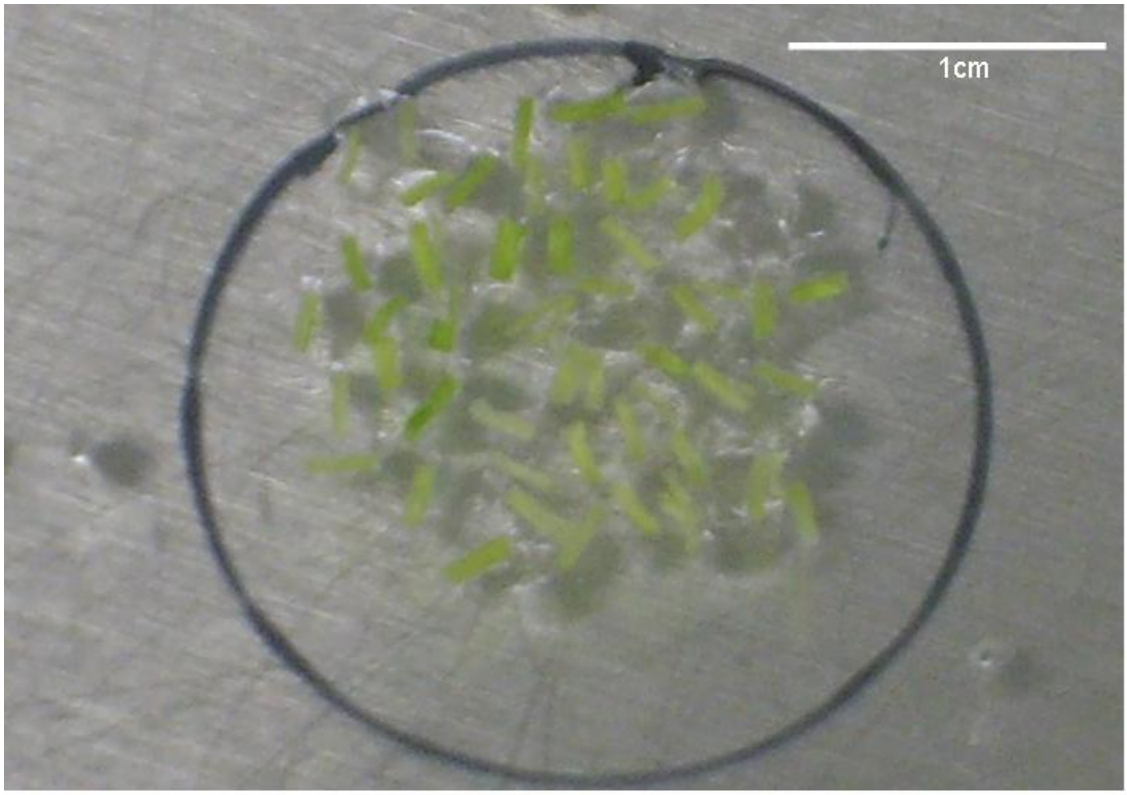

Fig. 2.11: Arrangement of leaf-base explants for biolistic delivery.

2.2.5.2.3 Callus preparation for biolistics

Four week-old calli (both embryogenic and non-embryogenic) were broken up into 0.25 cm2 pieces and placed in the middle of a Petri dish containing CIM + 64 g L-1 mannitol for pre-osmotic treatment 1 day prior to biolistic delivery, as illustrated in Fig. 2.12.

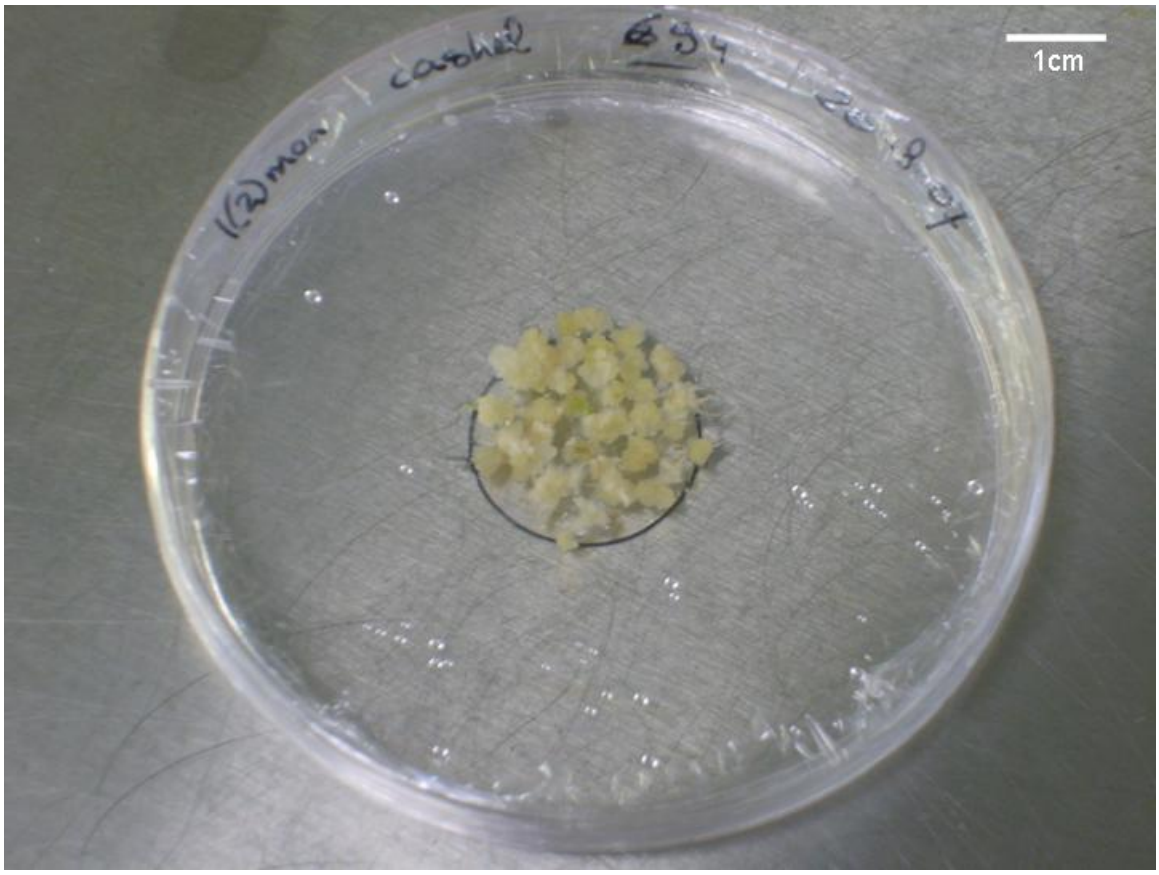

Fig. 2.12: Arrangement of calli for biolistic delivery

2.2.5.2.4 Testing for efficiency of DNA coating on gold particles

1.8 mg of 0.6 micron Gold was sonicated for 2 minutes. 15 µl 1 µg μl^{-1} plasmid DNA was added to the gold and mixed 5 seconds using a vortex at speed setting 3. Next, 20 µl 0.1M spermidine and 50 µl 2.5M CaCl2 was added to the lid of the tube and mixed. The lid was closed and the whole suspension was vortexed for either 3 minutes (protocol I) or 15 seconds (protocol II) at speed setting 3, followed by a spin at 5,000 rpm for 15

seconds. The supernatant was removed and stored (Solution B) and the pellet was washed with 140 µl 96% ethanol. The resulting suspension was spun at 3,000 rpm for 1 min, after which the supernatant was removed and stored (Solution C). The pellet consisting of DNA-coated gold particles was resuspended in 100 µl 96% ethanol, and spun down at 5,000 rpm for 15 seconds. The supernatant was removed and stored (Solution D). DNA coated to the gold particles was dissolved in 20 µl Milli-Q. (Solution A).

DNA from solutions B, C and D was precipitated, by adding 1/10 volume of 3 M sodium acetate pH 5.2 and 1 volume of 96% ethanol. The solutions were incubated at -20^{o}C for 30 minutes and spun down at max. rpm for 10 minutes. The DNA pellets were washed with 300 µl 70% ethanol, followed by another spin at max. rpm for 3 minutes. The pellet was resuspended in 20 µl MilliQ. The DNA originated from the gold (A) and precipitated DNA from B, C and D were run on a 0.8% agarose gel, for visualisation of DNA retained at each step during coating.

2.2.5.2.5 Particle preparation for bombardment

1.8 mg of 0.6 micron Gold was sonicated for 2 minutes. 15 µl 1 µg µl^{-1} plasmid DNA was added to the gold and mixed for 5 seconds using a vortex at speed setting 3. Next, 20 µl 0.1M spermidine and 50 µl 2.5M CaCl2 was added to the lid of the tube and mixed. The lid was closed and the whole suspension was vortexed for 15 seconds at speed setting 3, followed by a spin at 5,000 rpm for 15 seconds. The supernatant was removed and the pellet was washed with 140 µl 96% ethanol. The suspension was spun at 3,000

rpm for 1 min, after which the supernatant was replaced with 100 µl 96% ethanol. 10 µl of gold-DNA suspension was used per shot.

2.2.5.2.6 Preparation for biolistics

Transformation experiments were conducted through biolistics. For these experiments the "Biolistic® PDS-1000/He Particle Delivery System" from Bio-Rad was used, as described by (Svab and Maliga 1993), and (Johnston 1990).

The assembly for the holder of the macrocarriers, the retaining cap for rupture disks and the tray for the Petri dishes were sprayed with 70% ethanol and dried prior to use. The macrocarriers were soaked in 96% ethanol and left to dry. The stopping screens were submerged in 96% ethanol, flamed and left on a sterile cardboard plate in the laminar flow hood. The macrocarrier holder was submerged in 96% ethanol and flamed before being placed on a sterile cardboard plate. Dried macrocarriers were inserted into the macrocarrier holder. The rupture discs were submerged in 70% isopropanol prior to use.

2.2.5.2.7 Biolistic delivery process

10 µl of the DNA coated gold particles were pipetted onto the macrocarrier and left to dry for 15 minutes. When ready the gene gun was turned on, along with the vacuum pump. The helium tank was opened at a pressure of 200 PSI over the shooting pressure. The shooting chamber was sprayed with 70% ethanol before use. Rupture disks were placed in the retaining cap, and securely fastened in the top of the shooting chamber. The macrocarriers containing the microcarriers with the gold, were added to the holder assembly device, and placed directly beneath the retaining cap. Lastly the Petri dish with

the calli or explants was placed on the Petri dish tray and located at the desired shooting distance from the holder assembly device. The chamber was closed, and a vacuum was created at 28 in Hg. When the right vacuum pressure was reached, the shots were performed by holding down the "fire" button until the rupture disc burst. Lastly the vacuum was released by pressing the "vent" button. The Petri dish was removed and wrapped with Parafilm. The contents of the chamber were reassembled with a new rupture disk and a new macrocarrier. The process was repeated for the remaining shots.

2.2.5.2.8 Treatment of calli and leaf-base explants after biolistics

Bombarded calli and leaf-base explants remained on CIM + 64 g L^{-1} mannitol for two more days, before transferral to other media.

2.2.5.2.9 Optimization of biolistic delivery regarding shooting parameters

Several different parameters (shooting distances and shooting pressures) were tested (Table 2.6) to find the optimal conditions for biolistics on calli of *Lolium perenne* L. They were evaluated by testing for GUS activity after biolistics with pCambia 1305.1, containing the *uidA* gene.

Table 2.6: Parameters tested for biolistical delivery of DNA

Shooting distance (cm)	*Shooting pressure (PSI)*
6	900
6	1100
6	1350
9	900
9	1100
9	1350

2.2.5.3 Analysis of transient expression of *uidA* gene by GUS assay

Calli bombarded with pGUSHYG and pCAMBIA 1305.1 were tested for transient expression two days after biolistic delivery of DNA. GUS activity was tested histochemically by submerging the explants in the GUS incubation solution (100mM sodium phosphate buffer, pH 7; 10mM EDTA; 0.5mM potassium ferricyanide; 0.5mM potassium ferrocyanide; 1mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc); 0.1%(v/v) triton X-100) at 37^{o}C overnight. The number of blue spots, indicating GUS activity, was recorded the next day.

2.2.5.4 Fluorescent and confocal microscopy for GFP detection

Putative transformants could be analysed by checking for GFP presence using a fluorescent microscope or confocal microscope. Tissue is illuminated with light of specific wavelengths, which can be absorbed by GFP molecules, causing them to emit light of longer wavelengths. This light is passed through a specific filter, allowing visualisation of GFP within the tissue. As positive control, transplastomic tobacco plants expressing GFP within the plastids were used. Initial checks were performed with the fluorescent microscope “Olympus BX51” with a mercury lamp “Olympus U-RFL-T”.

Confirmation of GFP presence and better resolution could be accomplished by using the confocal microscope "Olympus Fluoview 1000", with which photographs could be taken for subsequently analysis using the program "Olympus Fluoview version 1.6b".

2.2.6 Transformation

Initially biolistic delivery was performed on leaf-base tissue, but after re-evaluation of the strategy, the target tissue was changed to four week calli as described in section 2.2.5.2.3. All biolistical experiments were performed on cultivar 'Cashel', with the exception of the last few plastid transformation experiments, which were performed on Cultivar 'Action'

2.2.6.1 Nuclear transformation

A total of 175 biolistic shots were performed on four week old calli (±20 calli per shot). Forty of these shots were performed with the nuclear transformation vector pGUSHYG (see map at Fig. 2.9). This vector contained the *hpt* selectable marker gene for hygromycin selection under regulation of the CaMV 35S promoter and the *uidA* gene as visual marker gene under regulation of the *nopaline synthase* promoter.

Another 135 shots were performed with the nuclear transformation vector pCAMBIA 1305.1 (see map at Fig.2.10), containing the *hpt* selectable marker gene and *uidA* visual marker gene, both under regulation of the CaMV 35S promoter.

2.2.6.2 Selection regime for nuclear transformation

Calli bombarded with pGUSHYG and pCAMBIA 1305.1 were moved to CIM two days after biolistics. After another five days, the calli were transferred to CIM + 75 mg L^{-1}

hygromycin B for two weeks, followed by two weeks on CIM + 150 mg L^{-1} hygromycin B. Selection was continued after this on CMM + 150 mg L^{-1} hygromycin B with biweekly sub culturing, until shoot development. Once shoots developed, calli with shoots were transferred to RGM2 + 150 mg L^{-1} hygromycin B. Remaining calli were subculture on CMM + 150 mg L^{-1} hygromycin B.

2.2.6.3 Plastid transformation

A total of 339 shots were performed on four week old calli (±20 calli per shot). All the shots were performed with the *Lolium perenne* L. plastid transformation vector pIAPRvdB5. The first series of shots (102 shots), the calli were exposed to G-418 selection two days post-biolistics. The second series of shots (162 shots), the calli were exposed to paromomycin selection two days post-biolistics. The third series of shots (75 shots), the calli were exposed to paromomycin selection 14 days post-biolistics. Of which 27 shots were performed on Cv. 'Action' instead of Cv. 'Cashel'.

2.2.6.4 Selection regime for plastid transformation

2.2.6.4.1 Selection with geneticin (G-418)

Calli bombarded with pIAPRvdB4 and pIAPRvdB5 were transferred to CIM two days after biolistic delivery of DNA. After another five days the calli were transferred to CIM + 75 mg L^{-1} G-418 for two weeks, followed by a two week period on CIM + 100 mg L^{-1} G418. All calli were sub cultured thereafter biweekly on CMM + 100 mg L^{-1} G-418 until shoot development. Calli with shoots were moved to RGM2 + 100 mg L^{-1} G-418. When

green shoots were over 2 cm in size they were transferred to tubs with RM + 100 mg L^{-1} G-418 for rooting.

2.2.6.4.2 Selection with paromomycin

Calli bombarded with pIAPRvdB4 and pIAPRvdB5 were transferred to CIM two days after biolistics. After another five days the calli were transferred to CIM + 75 mg L^{-1} paromomycin for two weeks, followed by a two week period on CIM + 150 mg L^{-1} paromomycin. All calli were sub cultured thereafter biweekly on CMM + 150 mg L^{-1} paromomycin until shoot development. Calli with shoots were moved to RGM2 + 150 mg L^{-1} paromomycin. When green shoots were over 2 cm in size they were transferred to tubs with RM + 150 mg L^{-1} paromomycin for rooting.

2.2.6.4.3 Delayed selection with paromomycin

Calli bombarded with pIAPRvdB4 and pIAPRvdB5 were transferred to CIM two days after biolistics. After 14 days the calli were transferred to CIM + 100 mg L^{-1} paromomycin for two weeks, followed by a two week period on CIM + 150 mg L^{-1} paromomycin. All calli were sub cultured thereafter biweekly on CMM + 150 mg L^{-1} paromomycin until shoot development. Calli with shoots were moved to RGM2 + 150 mg L^{-1} paromomycin. When green shoots were over 2 cm in size they were transferred to tubs with RM + 150 mg L^{-1} paromomycin for rooting.

2.2.6.5 Characterisation of putative transformants

Nuclear putative transformed tissue could be analysed using several methods, the easiest and fastest method involved a GUS assay, to assess transgene expression of the *uidA*

gene (see section 2.2.5.3). However due to gene-silencing, tissue could show up negative with the assay, while the tissue could actually be transformed. Therefore other methods were employed to assess transgene integration. These include PCR analysis and southern blots

Plastid putative transformed tissue could be analysed using several methods. The fastest method would be to use a fluorescent microscope, which can visualise GFP presence in transformed tissue (See section 2.2.5.4). Other methods were utilised to assess transgene integration, these include gene specific PCR analysis, long-range PCR analysis and southern blots.

2.2.6.5.1 PCR analysis of putative transformants

2.2.6.5.2 Primers used

Primers used for the PCR analysis of putative transformants were:

P1hpt CAMBIA: cctgcctgaaaccgaactgcccgct

(primer to assess nuclear transformants)

P2hpt CAMBIA: gatgttggcgacctcgtatt

(primer to assess nuclear transformants)

P1extern IA: tggatcacctccttttcagg

(external primer to assess putative plastid transformants)

P2 extern IA: gcaagcctttcctcttttga

(external primer to assess putative plastid transformants)

P2T7g10-ndeI-nheI: gctagccatatgtatatctccttctt

(Primer to assess putative plastid transformants) Underlined sequences are *NheI* and *NdeI*

P1aphA*:* ggccggccatgaccatggaattaccaaa

(Primer to assess putative plastid transformants) Underlined sequence is *FseI*

P2aphA: gcggccgctcaattcaattcatcaagtt

(Primer to assess putative plastid transformants) Underlined sequence is the *NotI* restriction site

2.2.6.5.3 PCR analysis on putative transformants

All PCR reactions were carried about with RedAccuTaq® LA DNA Polymerase mix (Sigma, cat no. D4937). The PCR mix used is shown in Table 2.7 and the PCR cycle regime in Table 2.8.

Table 2.7: general PCR mix

Reaction mix	*Volumes (µl)*
gDNA (1µg μl^{-1})	1.0
Primer 1 (0.1µM μl^{-1})	1.0
Primer 2 (0.1µM μl^{-1})	1.0
REDTaq® Genomic DNA Polymerase (1unit μl^{-1})	2.5
10x REDaccuTaq buffer	5.0
dNTP (10mM)	2.0
MilliQ ddH_2O	37.5
Total reaction mix	50.0

Table 2.8: PCR cycle

Steps	*Temperature*	*Time*
Step1 Denaturation	94^{o}C	5 min
Step 2 Denaturation	94^{o}C	30 seconds
Step 3 Annealing	5^{o}C below the lowest melting temperature of both primers	30 seconds
Step 4 Extension	68^{o}C	1kb per minute
Step 5 Repeat cycles	*Repeat step 2-4*	*30 times*
Step 6 Final extension	68^{o}C	10 min
Step 7 End of program	4^{o}C	Indefinite storage

2.2.6.5.4 Southern blot analysis of putative transformants

Southern blotting was performed according to the method of (McCabe et al. 1997) as described in section 2.2.1.10.

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2.2.7 Statistical analysis

For the tissue culture evaluation, replicates represented separate experiments conducted on different days. Arcsine transformation and *t*-tests were conducted in 'Microsoft Excel', whereas chi square tests and the variance tests were performed in the program 'Minitab Solutions 15'. All data sets were initially tested for sampling distribution using the chi-square test and analysed for equal variance using the Levene test. Subsequently *t*-tests were performed on arcsine transformed percentage values on the datasets to determine statistical differences, the differences were grouped accordingly.

2.3 Results

2.3.1 Construction of plastid transformation vectors pIAPRvdB4 and pIAPRvdB5

2.3.1.1 pIAPRvdB4

The vector pIAPRvdB4 has a total size of 7.823 kb (complete sequence in Appendix A). To show the vector was correctly assembled as described in section 2.2.2, the vector was digested with various restriction enzymes. This resulted in the following restriction pattern, which was confirmed as illustrated in Fig. 2.13.

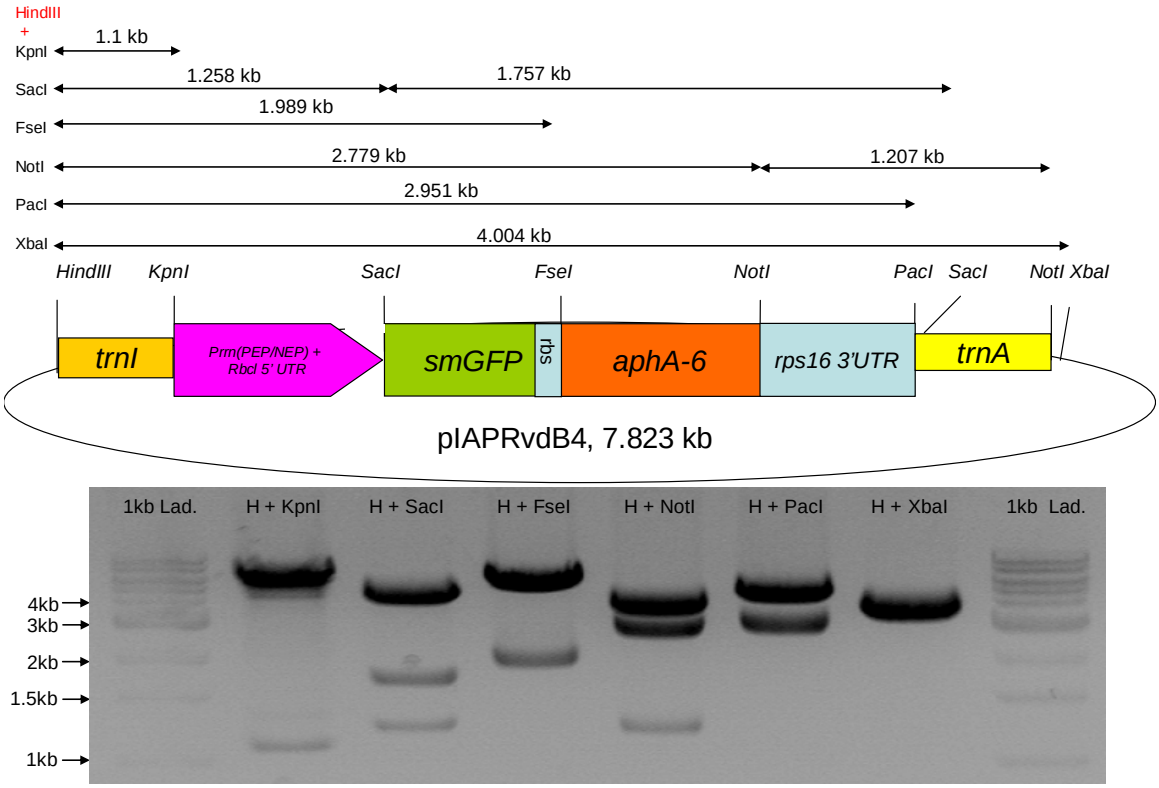


Fig. 2.13: Illustration restriction digest pattern of pIAPRvdB4. The expected restriction pattern is shown at the top for *HindIII* and a second enzyme (*KpnI, SacI, FseI, NotI, PacI* and *XbaI*). Below are the separate digests on pIAPRvdB4 separated on a 0.8% agarose gel.

2.3.1.2 pIAPRvdB5

The vector pIAPRvdB5 had a total size of 7.841 kb (complete sequence in Appendix A). To show the vector was correctly assembled as described in section 2.2.2, the vector was digested with various restriction enzymes. This resulted in the following restriction pattern, which was confirmed as illustrated in Fig. 2.14.

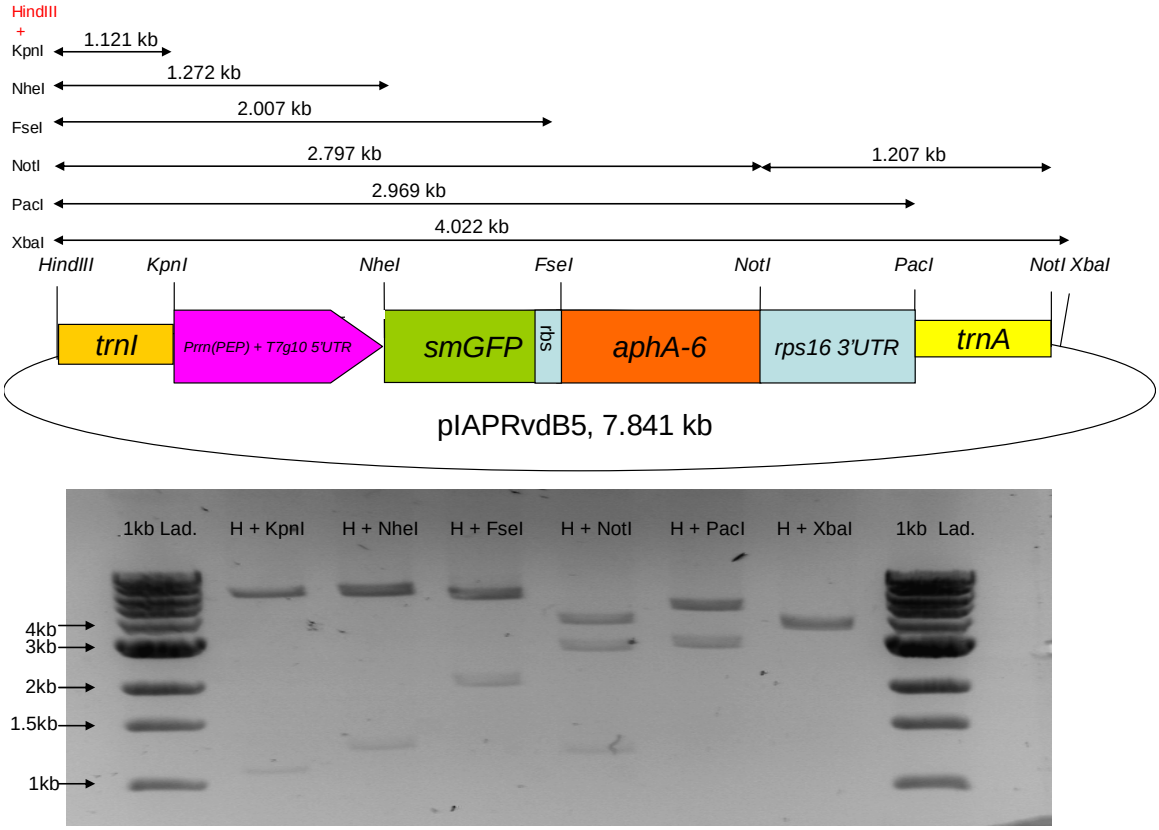


Fig. 2.14: Illustration restriction digest pattern of pIAPRvdB5. The expected restriction pattern is shown at the top for *HindIII* and a second enzyme (*KpnI, NheI, FseI, NotI, PacI* and *XbaI*). Below are the separate digests on pIAPRvdB5 separated on a 0.8% agarose gel.

2.3.1.3 Functionality test for pIAPRvdB4 and pIAPRvdB5

Because of the prokaryotic nature of plastids, the expression cassette is also expected to be active in *Escherischia coli.* To show that the plasmids pIAPRvdB4 and pIAPRvdB5 were correctly assembled and functional, the expression cassettes were tested in *Escherischia coli*.

2.3.1.4 GFP accumulation

Both constructed vectors contain an expression cassette including the *smGFP* gene. Therefore if the bacteria contained these plasmids, they would fluorescence green under UV-light. As shown in Fig. 2.15 this was the case for bacteria containing either pIAPRvdB4 or pIAPRvdB5. The bacteria containing vector pIAPRvdB5 had a higher expression, as was expected due to the nature of the expression cassette.

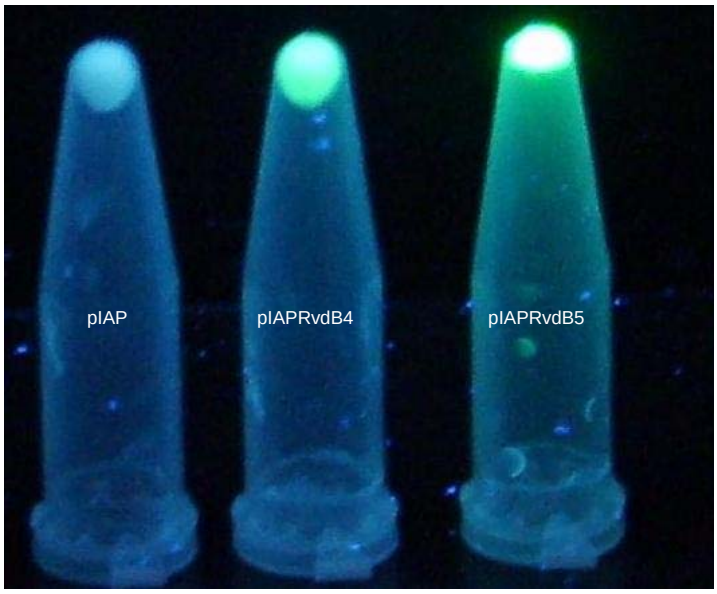


Fig. 2.15: Green Fluorescent Protein presence in *Escherischia coli* containing pIAPRvdB4 and pIAPRvdB5 (vector containing smGFP) and pIAP (control without smGFP).

2.3.1.5 Functionality of the *aphA-6* gene conferring resistance to G-418 and paromomycin

Bacteria transfected with pUC19-RvdB4 and pUC19-RvdB5 grew on LB + 50 mg L^{-1} paromomycin and LB + 50 mg L^{-1} G-418, whereas bacteria with an empty pUC19 failed to grow on this medium. Moreover pUC19-RvdB4 and pUC19-RvdB5 fluorescent green under UV light, indicating the functionality of the *smGFP* gene within these constructs.

2.3.2 *In vitro* culture methods

2.3.2.1 Seed germination

The seed germination rate influences the amount of starting material which can be used to induce callus. In Table 2.9 the germination rates are shown of all the tested cultivars.

Table 2.9: Germination rate of cultivars used

Cultivars	*Germination rate in %*
Cv. 'Shandon'	100
Cv. 'Cashel'	98
Cv. 'Greengold'	26
Cv. 'Cancan'	66
Cv. 'Limes'	7
Cv. 'Action'	~75
Cv. 'Telstar'	~75

2.3.2.2 Callus induction

During this study, four different types of induced calli were observed; of which three types were able to induce shoots at a later stage (see Fig. 2.16, B, C and D). The fourth type was unable to initiate differentiation (see Fig. 2.16, A). More often then not, different types of calli could be observed within 1 cluster. To record the callus induction rate for each cultivar, regenerable callus type B, C and D were pooled together. These were recorded for their frequency of occurrence during the callus induction phase in relationship to the total number of explants used. Most calli were induced from the edges of the leaf-base explants, therefore when leaf-base explants were to be used as target tissue for transformation experiments, the chances of obtaining transgenic tissue would be restricted as the majority of the target cells would not be able to proliferate.

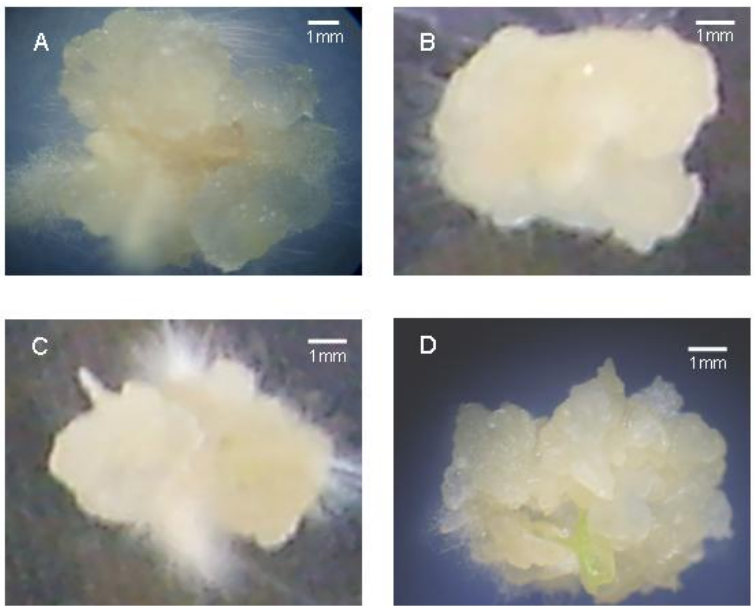


Fig. 2.16.: Types of calli induced from leaf-base explants. A: watery callus, B: Friable callus, C: Friable callus with root hairs, D: Embryogenic callus

2.3.2.2.1 Callus induction from apical meristems and adjacent leaf-base explants

In this study calli were induced from apical meristems and leaf-base explants adjacent to the apical meristems (see Fig. 2.17) on Callus Induction Media (see Table 2.5).

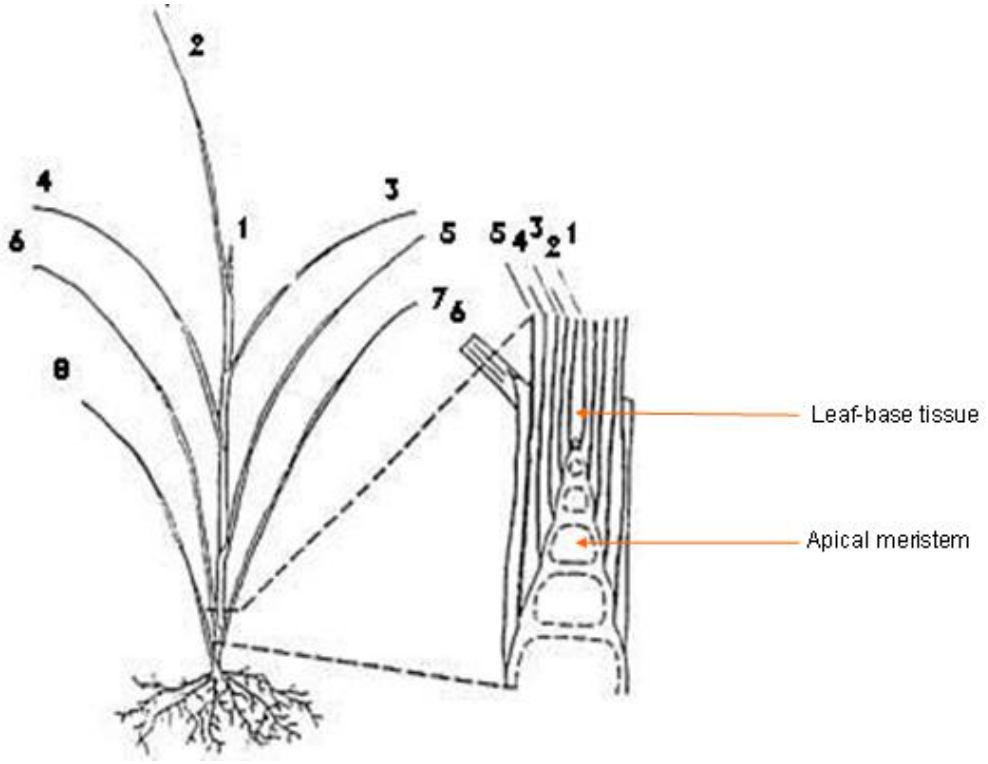


Fig. 2.17: Illustration of growth of a typical monocot plant. Numbers represent the order of leaves according to age, where 1 is the youngest and 8 is the oldest. Generally the numbering is reversed, but for the nature of this tissue culture study, the shown order was chosen.
Source: Modified from Colorado State University Extension no. 6.108: Grass Growth and Response to Grazing by M.J. Trlica

The leaf tissue adjacent to the apical meristems had the highest callus induction response. Tissue further away from the meristem had a significant decrease in callus induction rate. Furthermore older leaves proved to be less efficient in callus induction (Fig. 2.18 and Fig. 2.19). Differences in callus induction response between cultivars were observed. They were arranged in groups that were significantly different, separated for each origin of

explants as shown in Table 2.10 and 2.11. Furthermore *in vitro* culture in the light had a significantly negative effect on callus induction rate in the cultivar 'Cashel' (16h photoperiod), compared to growth conditions in the dark (see Table 2.10 and Table 2.11). All the results were combined to rank the different cultivars for their overall efficiency in callus induction, as shown in Table 2.12. Cultivars 'Cashel' and 'Shandon' were ranked as most responsive to callus induction, whereas cultivar 'Cancan' was ranked the least responsive.

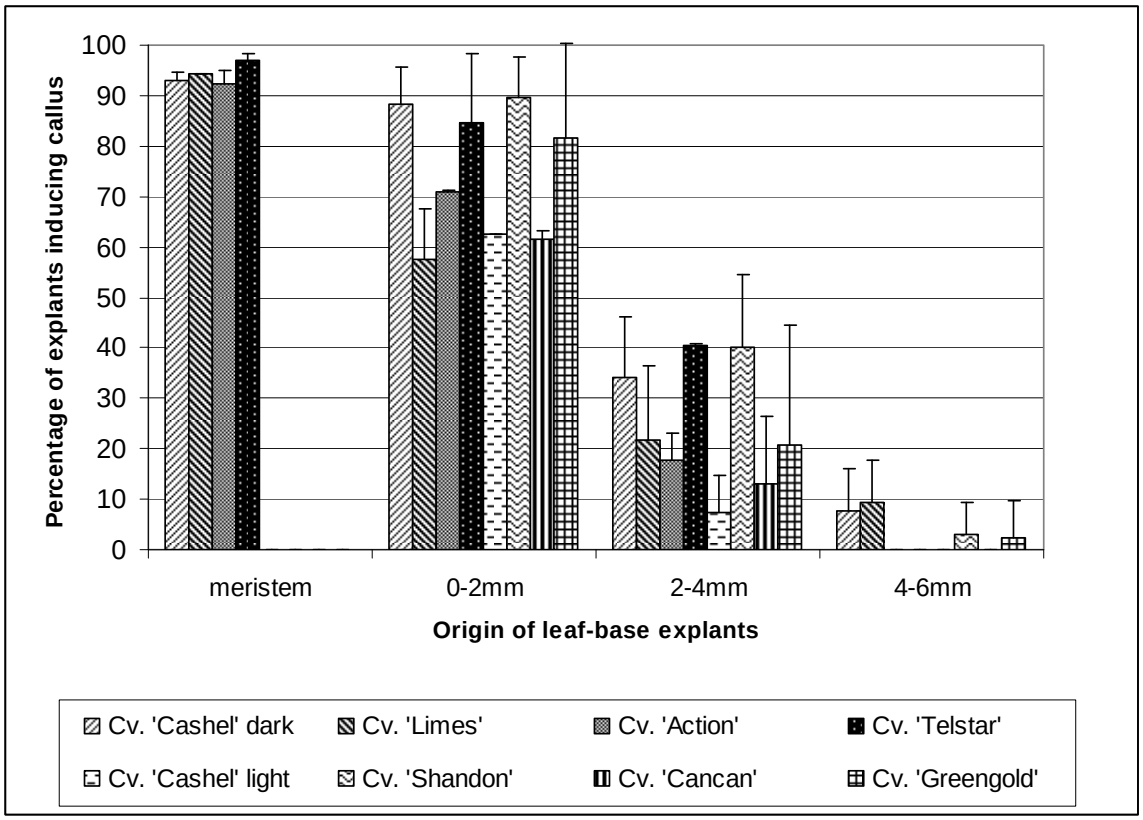


Fig. 2.18: The percentage of explants derived from the youngest leaf, producing regenerable callus. n = experimental replicates 'Cashel' dark n=15, 'Limes' n=6, 'Action' n=4, 'Telstar' n=4, 'Cashel' light n=2, 'Shandon' n=12, 'Cancan' n=3, 'Greengold' n=10. Error bars represent the standard deviation of the mean.

Table 2.10: Percentage of leaf explants that induce regenerable callus, separated into different cultivars and source tissue. Between brackets are given the standard deviation of the means. Means within a column followed by different letters are significantly different according to the student *t*-test (two-tailed distribution, unequal variance done on arcsine transformed percentages of combined replicates) with a statistical difference at $P<0.05$. n.a. = not analysed.

origin of explants	*meristem*	*Leaf 1, 0-2mm*	*Leaf 1 2-4mm*	*Leaf 1 4-6mm*
Cv 'Cashel' dark	93% (±1.5) [a]	88.3%(± 7.4) [a]	34.1%(±12.0) [a]	7.8%(±8.3) [a]
Cv. 'Limes'	94.4%(±0.0) [a]	57.5%(±10.0) [b]	21.9%(±14.6) [a,b]	9.5%(±8.2) [a]
Cv. 'Action'	92.2%(±2.9) [a]	70.8%(± 0.6) [c]	17.6%(± 5.6) [a,b]	n.a.
Cv. 'Telstar'	97.1%(±1.3) [a]	84.6%(±13.6) [a,b]	40.6%(± 0.3) [b]	n.a.
Cv. 'Cashel' light	n.a.	62.5%(± 0.0) [b]	7.5% (± 7.1) [a,b]	n.a.
Cv. 'Shandon'	n.a.	89.5%(± 8.0) [a]	40.2%(±14.3) [a,b]	3.1%(±6.3) [a]
Cv. 'Cancan'	n.a.	61.7%(± 1.4) [b]	13.1%(±13.0) [a,b]	n.a.
Cv. 'Greengold'	n.a.	81.5%(±18.7) [a,b]	20.7%(±23.9) [a,b]	2.5%(±7.1) [a]

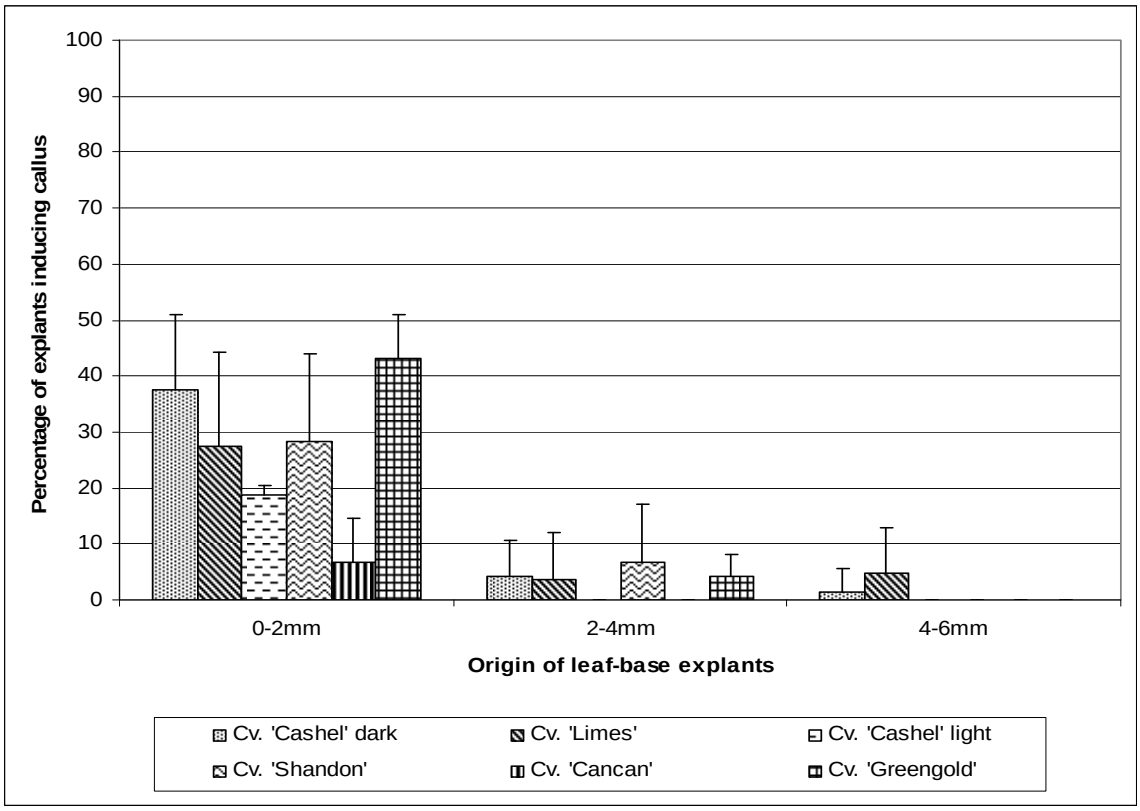


Fig. 2.19: The percentage of explants derived from the second youngest leaf, producing regenerable callus. n = number of experimental replicates 'Cashel' dark n=15, 'Limes' n=6, 'Action' n=4, 'Telstar' n=4, 'Cashel' light n=2, 'Shandon' n=12, 'Cancan' n=3, 'Greengold' n=10. Error bars represent the standard deviation of the mean.

Table 2.11: Percentage of leaf explants inducing regenerable callus. Between brackets are given the standard deviation of the means. Means within a column followed by different letters are significantly different according to the student *t*-test (two-tailed distribution, unequal variance done on arcsine transformed percentages of combined replicates) with a statistical difference at $P<0.05$.
[1] = replicates were too variable according to a chi-square test to do statistical analysis.
n.a. = not analysed.

Origin of explants	*Leaf 2 0-2mm*	*Leaf 2 2-4mm*	*Leaf 2 4-6mm*
Cv 'Cashel' dark	37.5%(±13.5) [b,c,d]	4.1%(± 6.5) [b]	1.3%(±4.3) [1]
Cv. 'Limes'	27.5%(±16.7) [b,c,d,e]	3.8%(± 8.4) [a,b]	4.8%(±8.2) [1]
Cv. 'Action'	n.a.	n.a.	n.a.
Cv. 'Telstar'	n.a.	n.a.	n.a.
Cv. 'Cashel' light	18.8%(± 1.8) [a,c,e]	0.0%(± 0.0) [b]	n.a.
Cv. 'Shandon'	28.4%(±15.6) [b,c,e]	6.8%(±10.3) [a]	0.0%(±0.0) [1]
Cv. 'Cancan'	6.7%(± 7.8) [a,e]	n.a.	0.0%(±0.0) [1]
Cv. 'Greengold'	43.2%(± 7.7) [b,d]	4.1%(± 4.1) [a,b]	0.0%(±0.0) [1]

Table 2.12.: Responses regarding callus induction of different cultivars, arranged in groups according to statistical differences.
Average rating: rating after pooling of all separate ratings.
Ranking: Ranking for best callus induction responses overall.

explant position / Cultivar	*Leaf 1 0-2mm*	*Leaf 1 2-4mm*	*Leaf 1 4-6mm*	*Leaf 2 0-2mm*	*Leaf 2 2-4mm*	*Average Rating*	*Ranking*
Cv 'Cashel' dark	1	2	1	1	1	1.2	1
Cv. 'Shandon'	1	1	1	2	1	1.2	1
Cv. 'Telstar'	2	1	-	-	-	1.5	2
Cv. 'Greengold'	2	3	1	1	2	1.8	3
Cv. 'Limes'	4	4	1	2	2	2.6	4
Cv. 'Action'	3	3	-	-	-	3	5
Cv. 'Cancan'	4	4	-	3	3	3.5	6

2.3.2.2.2 Callus induction from mature embryos

Callus was induced from excised mature embryos on CIM in the dark at 22^0C. Results show that cultivars ‘Telstar’ had the highest induction rate, whereas cultivars ‘Limes’ and ‘Action’ had a significantly lower induction rate. Cultivar ‘Shandon’ showed no significant difference with the three aforementioned cultivars. Cultivars ‘Cancan’, ‘Cashel’ and ‘Greengold’ appeared to perform the worst, however no statistical analysis could be performed on those cultivars, due to limited replicates (see Table 2.13 and Fig. 2.20).

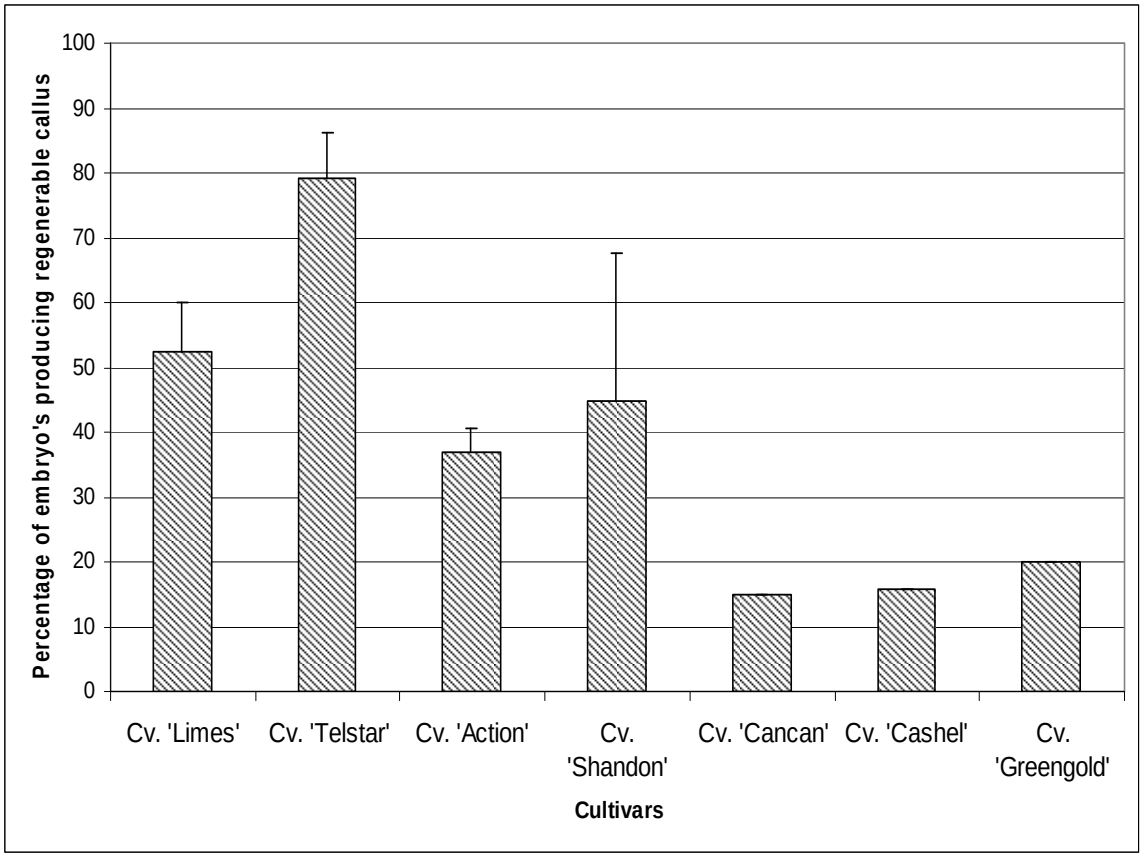


Fig 2.20: Percentage of mature embryo's that induced regenerable callus in the tested cultivars. Error bars represent the standard deviation of the mean. n = number of replicates, 'Limes' n=3, 'Telstar' n=2, 'Action' n=3, 'Shandon' n=2, 'Cancan' n=1, 'Cashel' n=1, 'Greengold' n=1.

Table 2.13: Percentage of mature embryo's inducing regenerable callus. Between brackets are given the standard deviation of the means. Means within a column followed by different letters are significantly different according to the student *t*-test (two-tailed distribution, equal variance done on arcsine transformed percentages of combined replicates) with a statistical difference at $P<0.05$.
[1] = too few replicates to perform statistical analysis.

Cultivar	Percentage of explants producing regenerable callus
Cv. 'Limes'	52.3(± 7.7) [b]
Cv. 'Telstar'	79.1(± 7.1) [a]
Cv. 'Action'	36.9(± 3.6) [c]
Cv. 'Shandon'	44.7(± 22.8) [a,b,c]
Cv. 'Cancan'	15.0(± 0.0) [1]
Cv. 'Cashel'	15.8(± 0.0) [1]
Cv. 'Greengold'	20.0(± 0.0) [1]

2.3.2.3 Callus differentiation and shoot induction

Two different protocols were tested for their regeneration efficiency. Protocol I consisted of a direct regeneration phase after a four week callus induction period, whereas protocol II consisted of a callus differentiation phase, followed by a regeneration period (See Table 2.1). Shoots could be induced from friable callus (Fig. 2.21) and embryogenic callus (Fig. 2.22). Shoot induction results were scored by taking the percentage of calli producing shoots per replicate. The means and standard deviation were calculated based on the results obtained in each replicate.

There were no statistical differences between Protocol I (direct regeneration), and Protocol II (callus differentiation, followed by shoot induction) in all tested cultivars 'Cashel', 'Shandon' and 'Limes' (see Table 2.14 and Fig. 2.23). When Protocol II was further tested with various cultivars, it showed that there was no statistical difference in response between cultivars, except Cv. 'Greengold' which had no shoot induction whatsoever under these conditions (see Table 2.14 and Fig. 2.23). Induction of calli at a 16h photoperiod, prior to induction of shoots, seemed to improve the shoot induction potential in cultivar 'Cashel', however this could not be statistically proven. Moreover, the callus induction rate was significantly lower, resulting in a lower amount of starting material that could potentially be used for transformation experiments (See Table 2.10 and Fig. 2.18).

Cv. 'Action' and Cv. 'Telstar' were acquired at a very late stage of the project. Because of the known good regeneration response (Patent WO/2004/035797; Patent

WO/2003/076612) and the limited timeframe they were subjected to the transformation experiments after callus induction, without having tested the regeneration potential.

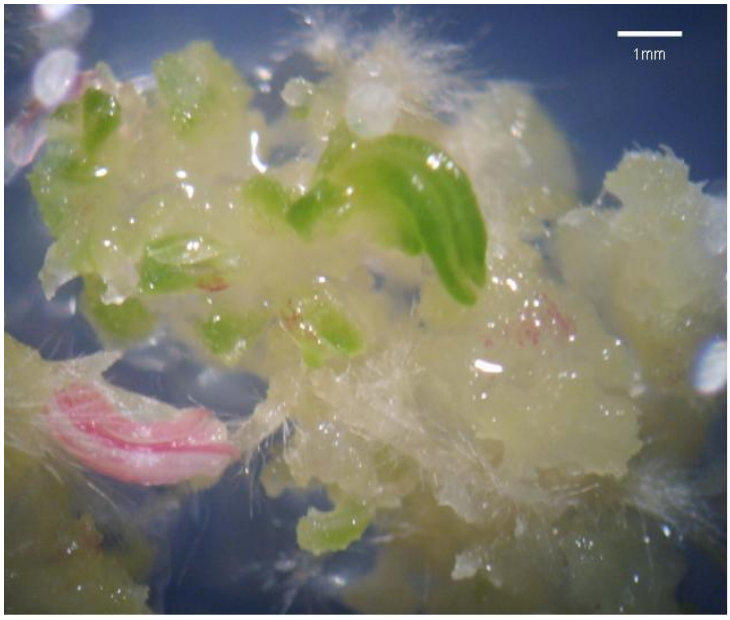


Fig. 2.21: Shoot induction from friable callus in *Lolium perenne* L. Cv. 'Cashel'

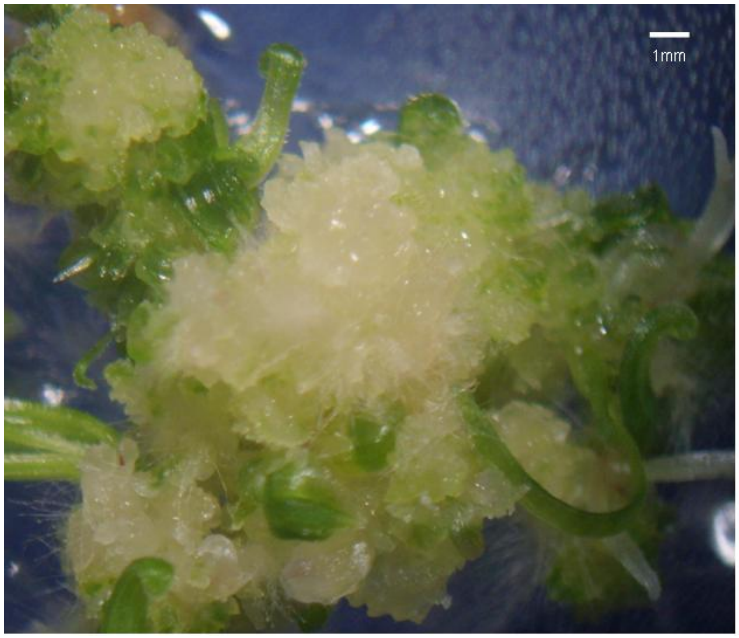


Fig. 2.22: Shoot induction from embryogenic callus in *Lolium perenne* L. Cv. 'Cashel'

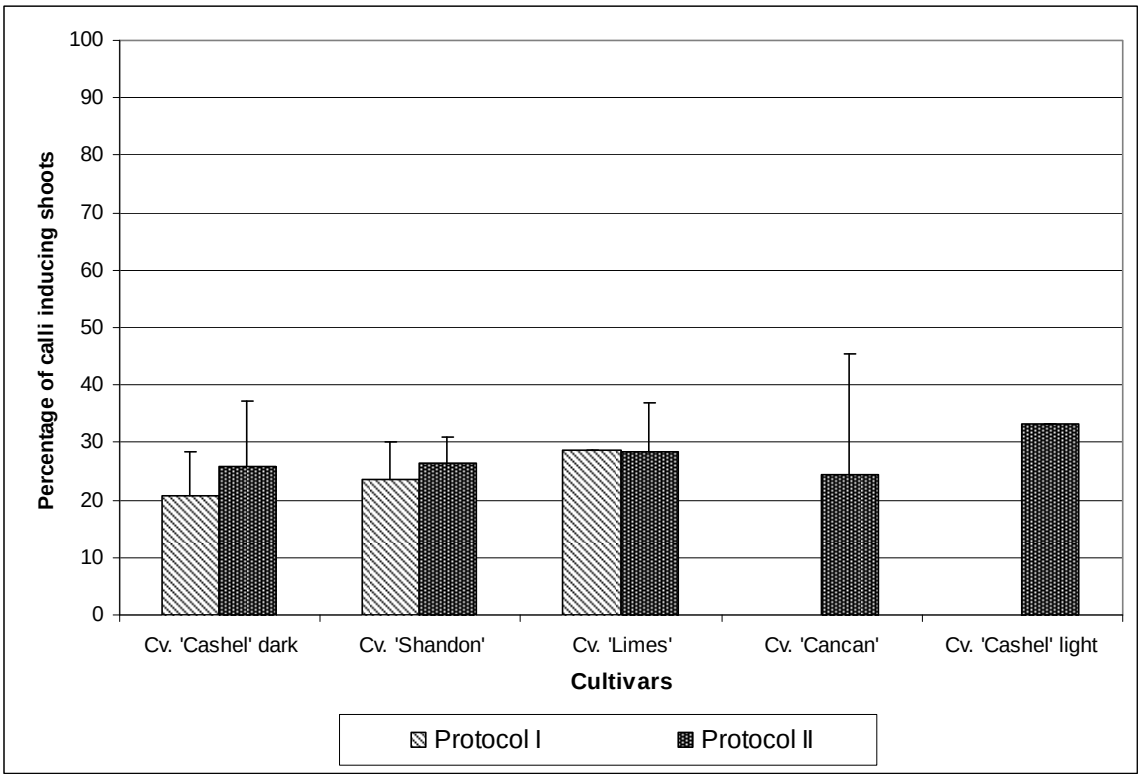


Fig. 2.23: Regeneration efficiency of both protocols. n = number of experimental replicates. Protocol I 'Cashel' dark n=3, 'Shandon' n=3, 'Limes' n=1, Protocol II 'Cashel' dark n=5, 'Shandon' n=2, 'Limes' n=3, 'Cancan' n=4, 'Cashel' light n=1. Error bars represent the standard deviation of the mean.

Table 2.14: Percentage of calli inducing shoots for both regeneration protocols. Between brackets are given the standard deviation of the means. Means within a column followed by different letters are significantly different according to a *t*-test (two-tailed distribution, unequal variance) on arcsine transformed percentages of combined replicates with a statistical difference at $P<0.05$.
[1] = too few replicates to perform statistical analysis.
n.a. = not applicable

Cultivars	*Protocol I*	*Protocol II*
Cv 'Cashel' dark	20.7(± 7.8) [a]	25.9(± 11.3) [a]
Cv. 'Shandon'	23.6(± 6.5) [a]	26.4(± 4.6) [a]
Cv. 'Limes'	28.6(± 0.0) [1]	28.4(± 8.5) [a]
Cv. 'Cancan'	n.a.	24.4(± 21.2) [a]
Cv. 'Cashel' light	n.a.	33.3(± 0.0) [1]
Cv. 'Greengold'	n.a.	0.0(± 0.0) [b]

2.3.3 Determination of conditions for antibiotic-resistance selection

2.3.3.1 Effect of antibiotics on callus induction

The response of callus induction to hygromycin B is shown in Table 2.15, to kanamycin in Table 2.16, to geneticin in Table 2.17, to streptomycin in Table 2.18 and to paromomycin in Table 2.19 and Fig. 2.24. Callus induction response was scored after four weeks on CIM supplemented with antibiotics.

Table 2.15: Effect of hygromycin B on callus induction. Percentages represent the amount of leaf-base explants at specific distances from the apical meristem, producing regenerable callus.

Hygromycin concentrations in mg L^{-1}	*Percentage of explants inducing calli at 0-2mm from the meristem*	*Percentage of explants inducing calli at 2-4mm from the meristem*	*Percentage of explants inducing calli at 4-6mm from the meristem*
0	60	20	n.a.
10	20	0	n.a.
20	10	0	n.a.
30	0	0	n.a.
50	0	0	n.a.

Table 2.16: Effect of kanamycin on callus induction. Percentages represent the amount of leaf-base explants at specific distances from the apical meristem, producing regenerable callus.

Kanamycin concentrations in mg L^{-1}	*Percentage of explants inducing calli at 0-2mm from the meristem*	*Percentage of explants inducing calli at 2-4mm from the meristem*	*Percentage of explants inducing calli at 4-6mm from the meristem*
0	67	10	27
10	50	17	0
25	60	0	0
50	50	0	0
100	20	0	0

Table 2.17: Effect of geneticin (G-418) on callus induction. Percentages represent the amount of leaf-base explants at specific distances from the apical meristem, producing regenerable callus.

G-418 concentrations in mg L^{-1}	*Percentage of explants inducing calli at 0-2mm from the meristem*	*Percentage of explants inducing calli at 2-4mm from the meristem*	*Percentage of explants inducing calli at 4-6mm from the meristem*
0	67	10	27
10	40	0	10
25	0	0	0
50	0	0	0
75	0	0	0

Table 2.18: Effect of streptomycin on callus induction. Percentages represent the amount of leaf-base explants at specific distances from the apical meristem, producing regenerable callus.

Streptomycin concentrations in mg L^{-1}	*Percentage of explants inducing calli at 0-2mm from the meristem*	*Percentage of explants inducing calli at 2-4mm from the meristem*	*Percentage of explants inducing calli at 4-6mm from the meristem*
0	67	20	n.a.
1000	62	23	n.a.
5000	13	8	n.a.
10000	0	0	n.a.

Table 2.19: Effect of paromomycin on callus induction. Percentages represent the amount of leaf-base explants at specific distances from the apical meristem, producing regenerable callus.

Paromomycin concentrations in mg L-1	*Percentage of explants inducing calli at the meristem*	*Percentage of explants inducing calli at 0-2mm from the meristem*	*Percentage of explants inducing calli at 2-4 mm from the meristem*
0	91	75	11
25	4	0	0
50	0	0	0
75	0	0	0
100	0	0	0
125	0	0	0

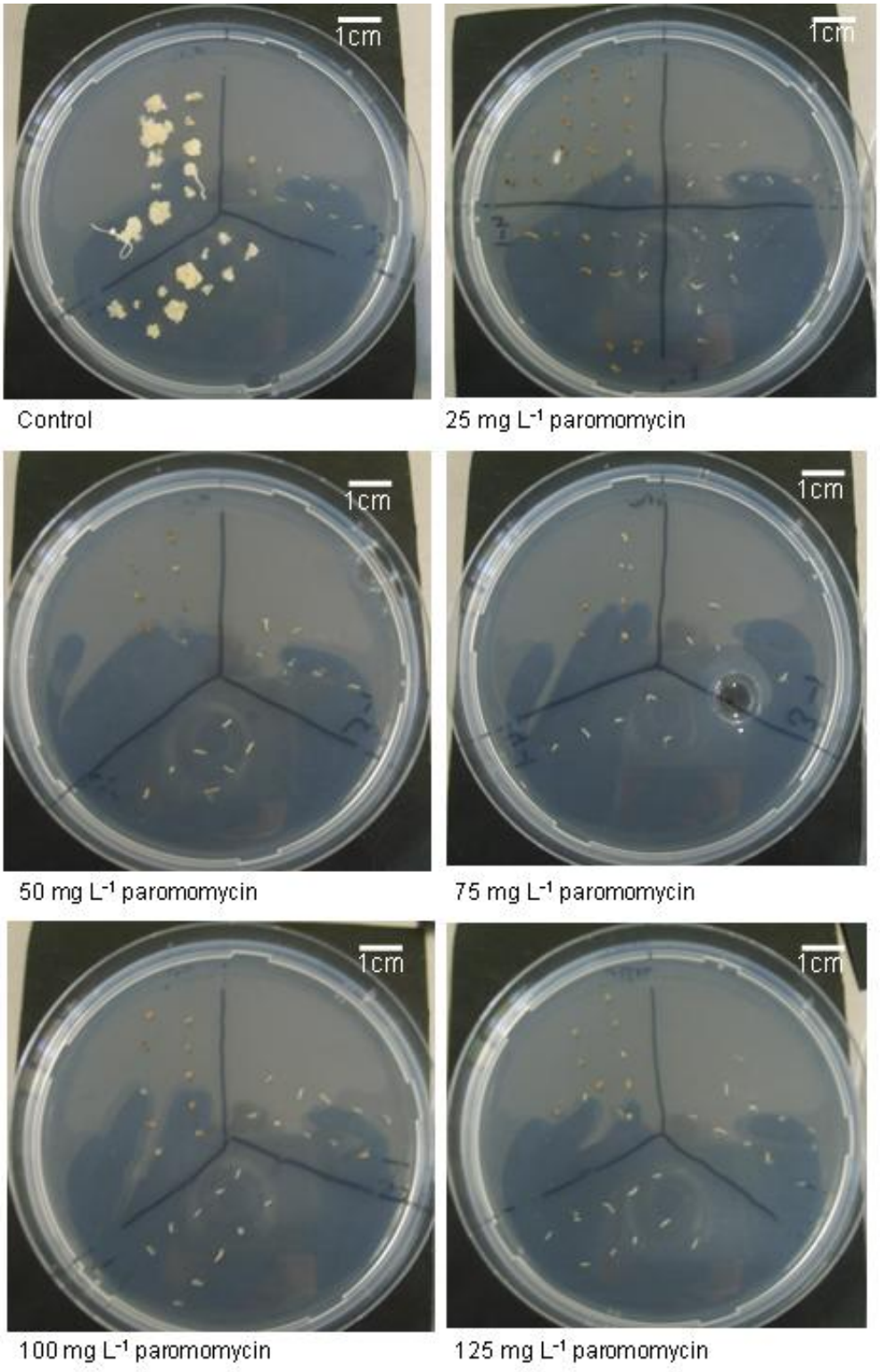


Fig. 2.24: Paromomycin selection after four weeks on CIM supplemented with various concentrations of paromomycin**.** Each Petri dish was divided into sections representing origin of the explants.

Selection on streptomycin was ineffective. Even at antibiotic concentrations of 5000 mg L^{-1} calli were able to develop (See Table 2.18). This is due to the nature of callus induction, as this had to be performed in the dark, while streptomycin blocks protein synthesis of photosynthetic genes in the light.

Kanamycin selection was also fairly inefficient. Even at a concentration of 100 mg L^{-1} 20% of the leaf-base explants were able to induce regenerable callus (see Table 2.16).

Selection with geneticin (see Table 2.17), paromomycin (Table 2.19 and Fig. 2.24) and hygromycin B (See Table 2.15) at 50 mg L^{-1} was sufficient to prevent callus development completely. Furthermore all explants showed necrosis at this concentration with these antibiotics. It can be noted that these concentrations may have to be lowered to allow transgenic tissue to proliferate, as the selection pressure may be too stringent. The downside is the increased possibility of escapes.

2.3.3.2 Effect of antibiotics on callus differentiation and shoot induction

The shoot induction response and callus growth during the regeneration phase to hygromycin B is shown in Table 2.20, to geneticin in Table 2.21, to kanamycin in Table 2.22, and to paromomycin in Table 2.23 and Fig. 2.25. Callus development and shoot induction was scored after six weeks on CIM2 supplemented with antibiotics.

Table 2.20: Effect of hygromycin on callus growth and shoot induction

hygromycin concentrations in mg L-1	*Percentage of calli producing shoots*	*Percentage of calli, where growth was inhibited within 6 weeks*
0	20	0
10	20	20
25	17	17
50	25	17
75	0	78
150	2	95

Table 2.21: Effect of geneticin (G-418) on callus growth and shoot induction

G-418 concentrations in mg L-1	*Percentage of calli producing shoots*	*Percentage of calli, where growth was inhibited within 6 weeks*
0	47	0
25	20	53
50	25	73
75	48	76
100	23	92
125	30	95
150	23	95

Table 2.22: Effect of kanamycin on callus growth and shoot induction

Kanamycin concentrations in mg L-1	*Percentage of calli producing shoots*	*Percentage of calli, where growth was inhibited within 6 weeks*
0	20	0
10	20	20
25	17	17
50	25	17
75	0	78
100	0	75

Table 2.23: Effect of paromomycin on callus growth and shoot induction

Paromomycin concentrations in mg L-1	*Percentage of calli producing shoots*	*Percentage of calli producing white shoots*	*Percentage of calli, where growth was inhibited within 6 weeks*
0	20	0	0
50	7	0	80
75	13	0	93
100	0	0	87
125	0	0	75
150	5	5	91

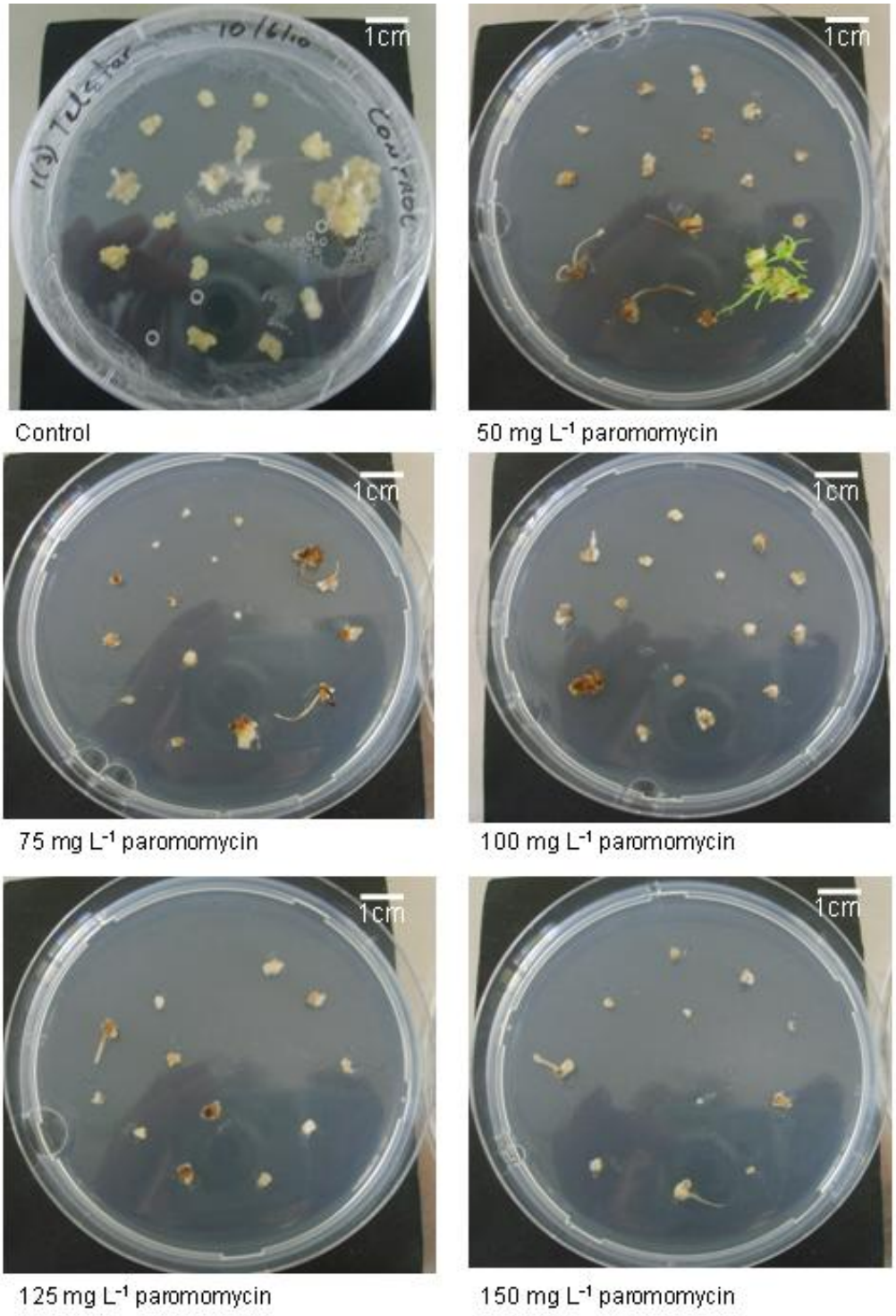


Fig. 2.25: Paromomycin selection after six weeks on CIM2 supplemented with various concentrations of paromomycin.

Selection with hygromycin B up to 50 mg L^{-1} resulted in a low efficiency for growth inhibition. At 75 mg L^{-1} 78% of the calli stopped developing, at 150 mg L^{-1} this was 95.4%. Shoot induction could not completely be inhibited even at 150 mg L^{-1}.

For geneticin necrosis was evident from concentration of 75 mg L^{-1} and upwards, but even at 150 mg L^{-1} 5% of the calli remained viable. Furthermore shoot induction was not inhibited even at 150 mg L^{-1}.

Kanamycin had a mild effect on callus survival, although more than 75% of the calli stopped developing at concentrations higher then 75 mg L^{-1}, the callus itself appeared healthy. Green healthy shoots stopped developing at concentrations of 75 mg L^{-1}.

Paromomycin selection was quite rigorous, as the percentage of viable callus decreased rapidly to about 10% at antibiotic concentrations of 75 mg L^{-1} upwards. However complete callus growth inhibition was not accomplished even at 150 mg L^{-1}. Shoots that developed in the first week turned brown/white within a week on concentrations higher then 100 mg L^{-1}, this was mainly evident at the meristematic regions of the induced shoots (the apical meristem).

2.3.4 Choosing the target tissue for transformation experiments

The choice of target tissue is of utmost importance to create a successful transformation protocol. The tissue should be able to proliferate readily and selection on the tissue should be possible to develop transgenic tissue after DNA delivery by biolistics. Based

on the regeneration studies and the selection regimes tested, the choice of target tissue is therefore restricted to four week old induced calli. This is because initial callus induction from leaf-base explants is mostly limited to the wounded edges of the leaf-explants. Furthermore selection in the dark will be difficult when attempting to create transplastomic tissue, due to the low expression levels in the dark of the selectable marker gene. Selection in the light would therefore be favourable, which can be accomplished when using four week old induced calli.

2.3.5 Gene delivery

Biolistical particle bombardment was the method used during this project. In order to use biolistics for *Lolium perenne* L., all the parameters involved with biolistics had to be assessed and optimized. To test these parameters, a GUS assay was utilized to test for transient expression of the *uidA* gene. The nuclear transformation vector pCAMBIA1305.1 containing the *uidA* gene, was used for this study. The *uidA* gene produces an enzyme that can convert X-Gluc to a blue dye during a GUS-assay. The efficiency of DNA delivery could be calculated by counting the blue spots (foci of GUS-stained cells) two days after biolistical delivery of the transgene.

2.3.5.1 Testing for efficiency of DNA coating on gold particles for biolistics

Two different protocols were tested for their efficiency at coating DNA on gold particles. Protocol I consisted of a 3 minute spin to bind the DNA to the gold particles, while protocol II consisted of a 15 second spin to bind the DNA to the gold particles (see 2.2.5.2.4). The waste solutions of each step during the coating procedure were run on an

agarose gel to visualise the presence of DNA contained within each waste solution (see Fig. 2.26). Solution A (A_2 and A_{15}) contained the DNA that was attached to the gold particles after the coating procedure, the DNA present in this solution indicates the DNA actually delivered to the tissue during biolistics. All the other lanes in Fig. 2.26 show the DNA lost in the respective washing solutions. The presence of DNA in solution A indicates that protocol II proved to be much more efficient in binding the DNA to gold particles then protocol I.

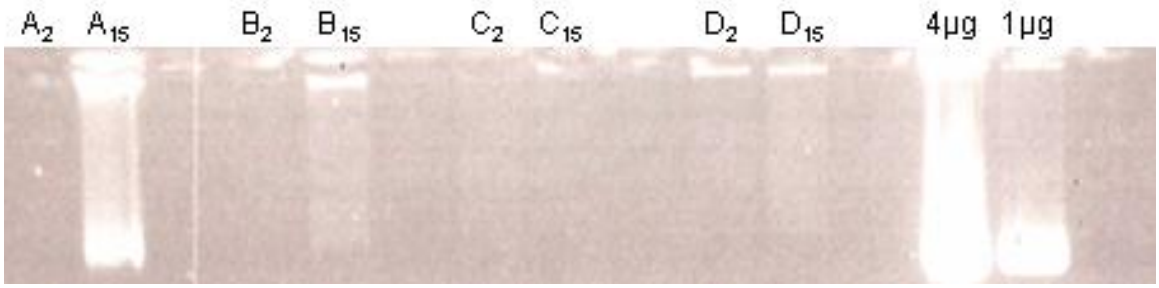


Fig. 2.26: A 0.8% agarose gel visualising the DNA contained within each washing solution, and the DNA coated to the gold particles.
A_2: DNA redissolved into water from gold particles prepared with protocol I
A_{15}: DNA redissolved into water from gold particles prepared with protocol II
B_2: Waste solution B from protocol I
B_{15}: Waste solution B from protocol II
C_2: Waste solution C from protocol I
C_{15}: Waste solution C from protocol II
D_2: Waste solution D from protocol I
D_{15}: Waste solution D from protocol II
4µg: 4 µg DNA
1µg: 1µg DNA

2.3.5.2 Optimization of biolistic delivery regarding shooting parameters using transient expression of the *uidA* gene in the cytosol.

The shooting pressure and shooting distance were evaluated for their influence on transgene delivery. This was tested by introducing the nuclear transformation vector pCAMBIA 1305.1, which contains the *uidA* gene, into calli of *Lolium perenne* L. Cv. 'Cashel'. After a histochemical GUS assay, the blue spots were counted (see Fig. 2.27) and compared for each shooting parameter and different DNA coating protocol. Initial results obtained with DNA coating protocol I, suggested that a shooting pressure of 1100 PSI and a shooting distance of six cm was optimal (see Fig. 2.28). Further transient expression tests were narrowed down to fewer parameters and were conducted using the DNA coating protocol II. The number of expressing foci increased dramatically, furthermore there did not seem any clear difference in delivery efficiency between the shooting distance parameter (See Fig. 2.28 and Fig. 2.29). However when a shooting distance of three centimetre was used, the target area got reduced substantially, therefore the use of six to nine centimetre was determined preferential.

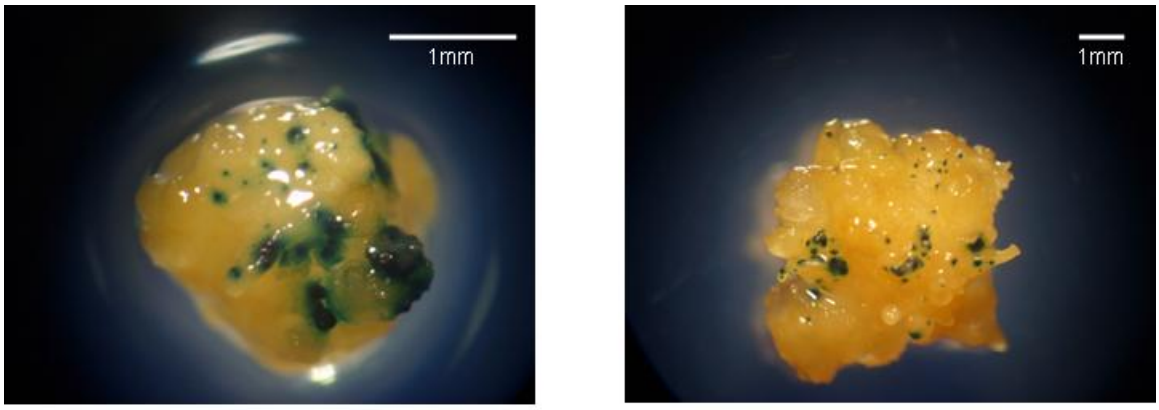


Fig 2.27: GUS expression after biolistic delivery of pCAMBIA 1305.1, histochemically visualised by a GUS assay, in calli 2 days post-biolistics

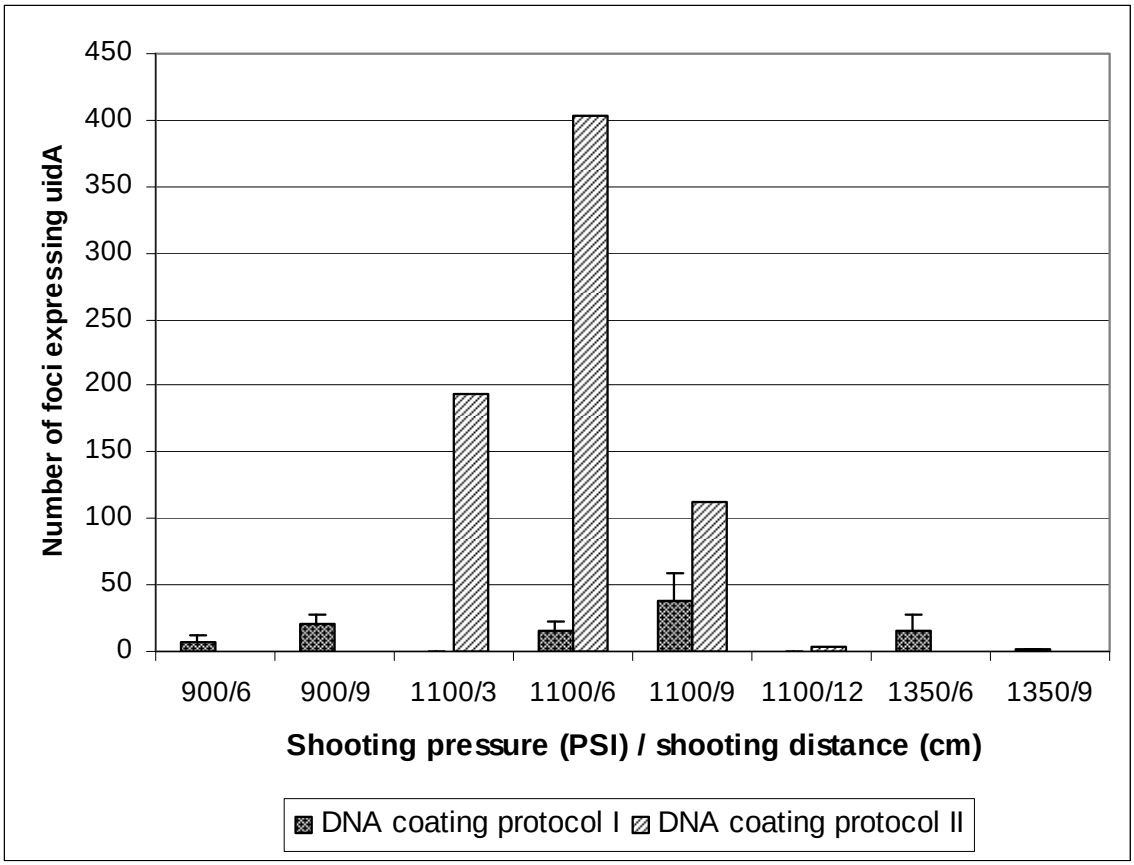


Fig. 2.28: Number of foci expressing the *uidA* gene after biolistic delivery using various parameters. DNA preparation protocol I: n=3, DNA preparation protocol II: n=1. Error bars represent the standard deviation of the mean.

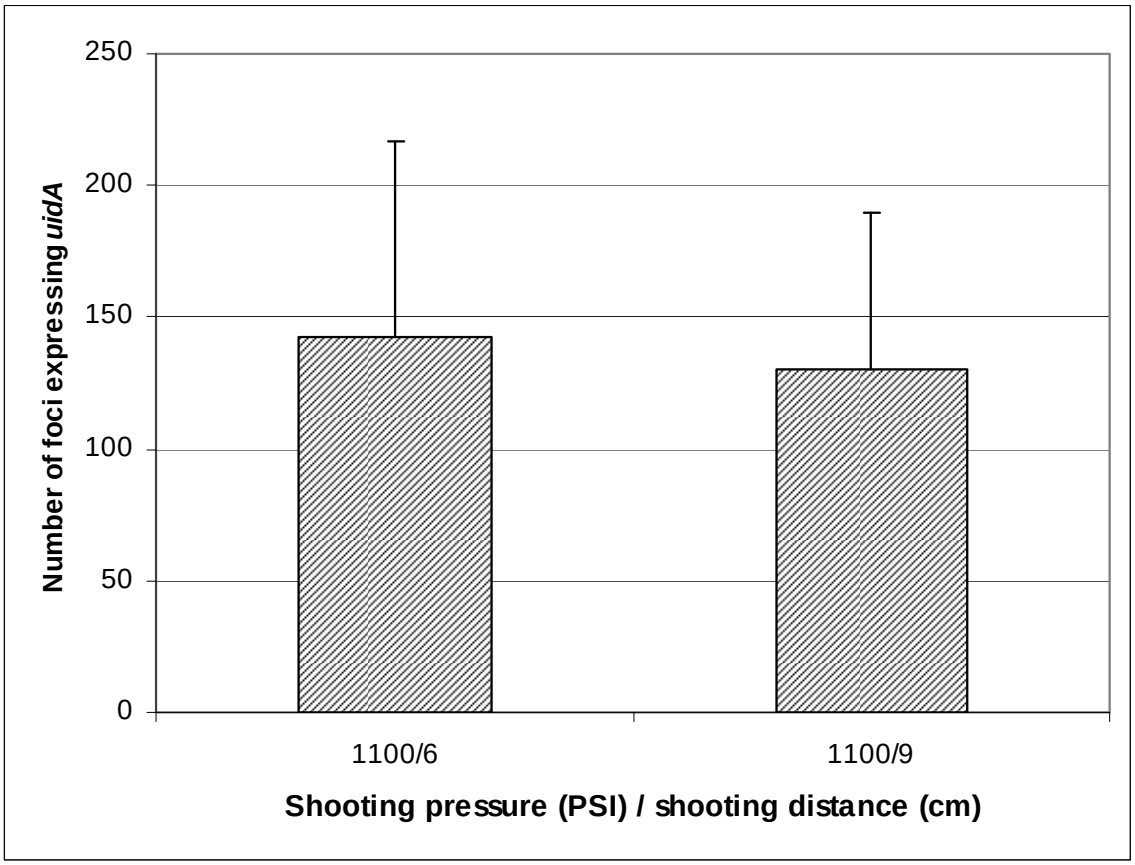


Fig. 2.29: Number of foci expressing the *uidA* gene after biolistic delivery using various parameters with DNA preparation protocol II: n=5. Error bars represent the standard deviation of the mean.

After assessment of the parameters, it could be concluded that the optimal conditions for DNA delivery were the following.

The utilisation of DNA-Gold coating protocol II.

For biolistical delivery:

- Six or nine cm target distance
- 1100 PSI shooting pressure.

Although the DNA delivery efficiency was relatively low, compared to earlier published results, the efficiency was greatly increased when Cv. 'Action' was used at a later stage

in the project. Nevertheless the optimal conditions obtained with the tests conducted on Cv. 'Cashel' could be applied to other cultivars of *Lolium perenne* L..

2.3.5.3 Transient expression assessment in plastids of leaf-tissue bombarded with pIAPRvdB5.

To test if the *Lolium perenne* L. vector pIAPRvdB5 could be successfully used for plastid transformation, transient expression experiments were conducted. Leaf tissue was bombarded with a shooting pressure of 1100 PSI and a shooting distance of nine cm or six cm. After a two day cultivation, leafs were assessed for green fluorescent protein located within the plastids, using a confocal microscope with UV-light. As positive control, a *Nicotiana tabacum* leaf was used with GFP expressed within the chloroplasts (kindly provided by Aisling Dunne). As negative control a wild-type *Lolium perenne* L. leaf was used.

The assessment was complicated by a strong background signal, making it nearly impossible to locate the true GFP emissions (See Fig. 2.30 and Fig. 2.31); this is due to the low number of GFP emitting plastids that were expected.

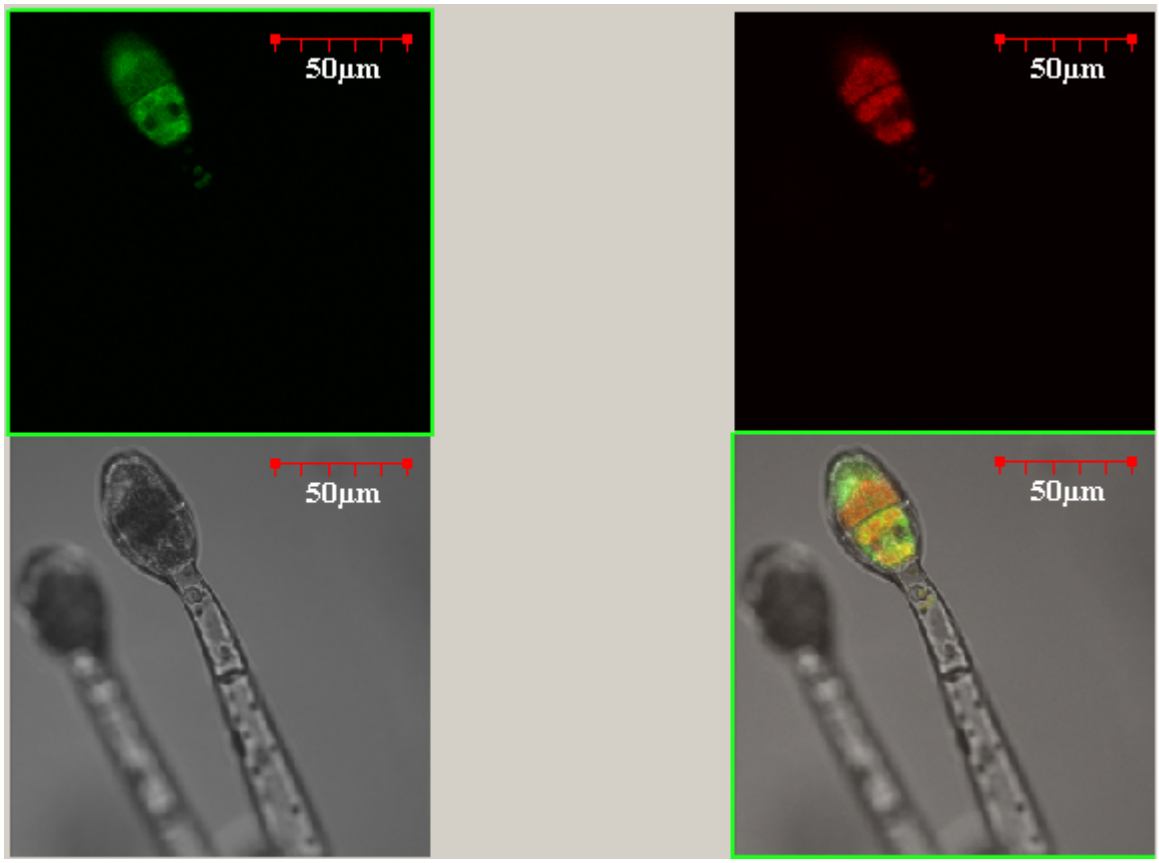


Fig. 2.30: Positive control (plastid transformant of tobacco expressing GFP, kindly supplied by Aisling Dunne) in a tricome containing chloroplasts, top left GFP filter, top right auto-fluorescence, bottom left no filter, bottom right merged.

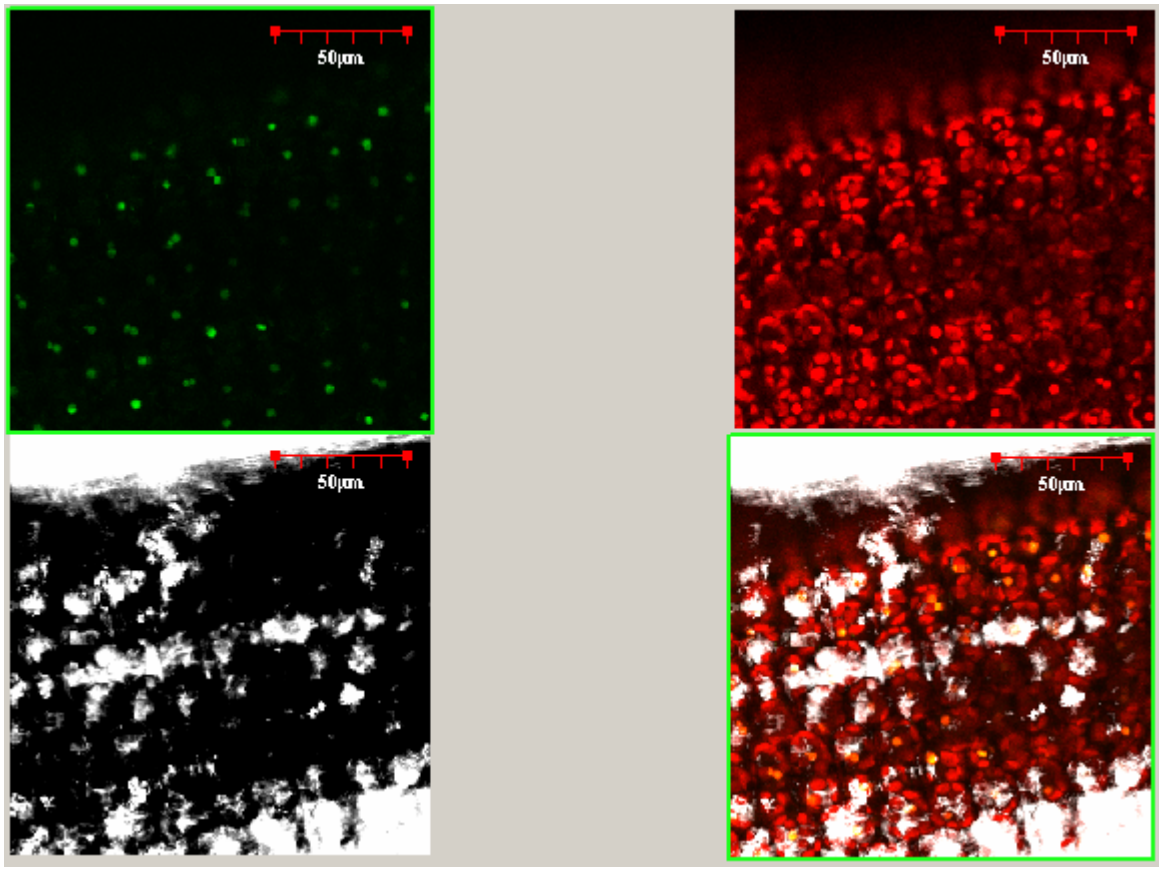


Fig. 2.31: Negative control, leaf tissue of wild-type *Lolium perenne* L.. Top left: GFP filter, top right: auto-fluorescence, bottom left: no filter, bottom right: merged.

Afterwards a DAPI stain on leaf tissue of wild-type *Lolium perenne* L. was conducted to find the location of this background signal, this showed that the signal was outside the chloroplasts and in proximity of the nucleus.

2.3.6 Analysis of putative transformants

2.3.6.1 Analysis of putative nuclear transformants

Three separate calli continued to develop slowly after delivery of pCAMBIA 1305.1, nevertheless shoot induction did not occur. These calli were analysed using a GUS-assay, PCR analysis and southern blot.

2.3.6.1.1 GUS assay

None of the three proliferated calli during selection showed GUS expression.

2.3.6.1.2 PCR analysis

Gene specific PCR was carried out on gDNA isolated from putative plastid transformants with an *hpt* gene specific primer pair. The expected fragment size was 391bp (see Fig. 2.32 lane 6). A false positive band was amplified from wild-type DNA (see Fig 2.32, lane 5). Different primer pairs were tested, but all resulted in false positives in the negative control.

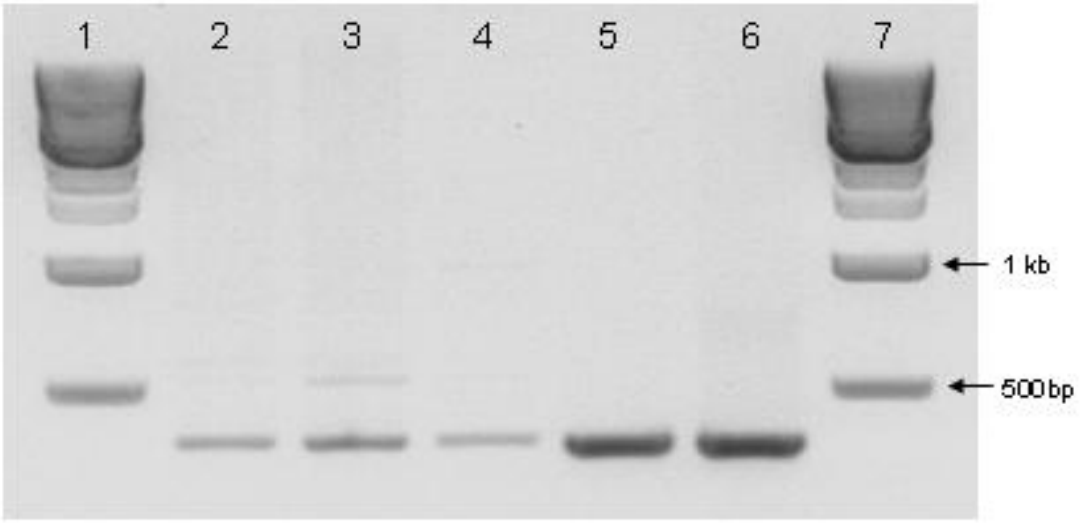
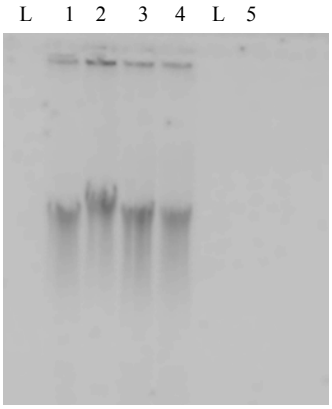


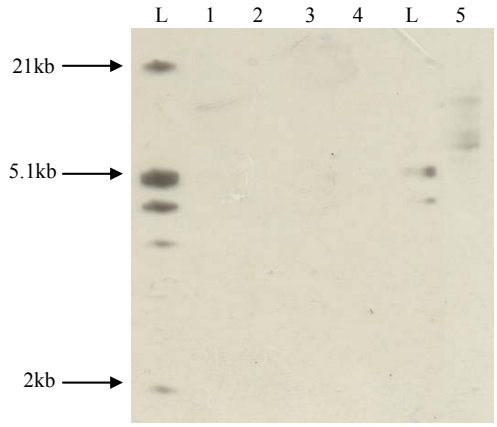
Fig. 2.32: PCR analysis of putative transformants using *hpt* specific primers.
Lane 1 + 7: 1kb ladder
Lane 2-4: Putative transformants
Lane 5: negative control, wild-type *Lolium perenne* L. Cv. 'Cashel'
Lane 6: Positive control, pCAMBIA1305.1

2.3.6.1.3 Southern blot

A Probe was designed to hybridise with the *hpt* gene. During hybridization with *SacII* digested wild-type gDNA, no fragment should be visualised. If the expression cassette of vector pCAMBIA 1305.1 got integrated, a fragment of any size should be visualised due to random integration, possibly more fragments could be visualised if there were more then one integration event. For all three putative transformants no fragment appeared after hybridisation, indicating that in all putative transformants the expression cassette was absent (See Fig. 2.33).

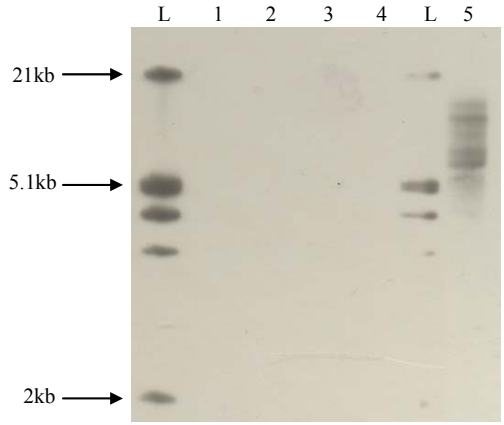


A: DNA ran on a 0.8% agarose gel



B: Three minutes exposure

Lanes:
L: DNA molecular weight marker III, digoxigenin-labeled
1-3. Putative transformants
4. Wild-type *Lolium perenne* L.
5. pCAMBIA 1305.1



C: Seven minutes exposure

Fig. 2.33: Southern blot with hpt probe, pCAMBIA 1305.1 plasmid DNA digested with *EcoRI* and *SacII.* gDNA from putative transformants and wild-type *Lolium perenne* L. digested with *SacII.* **A**: Digested DNA ran on a 0.8% agarose gel, **B**: Southern blot with a Three minutes exposure time, **C**: Southern blot with a seven minutes exposure time

2.3.6.2 Analysis of putative plastid transformants

Nine small shoots developed during delayed paromomycin selection. These shoots were green at first, but slowly started to exhibit detrimental effects under paromomycin selection, including a lack of root growth. Nevertheless these were analysed with fluorescent microscopy to detect GFP accumulation, PCR analysis and southern blots.

2.3.6.2.1 Confocal microscopy

No GFP was detected in regenerated leaf tissue.

2.3.6.2.2 PCR analysis

2.3.6.2.2.1 Gene-specific PCR

Gene specific PCR was carried out on gDNA isolated from putative plastid transformants with various primer pairs. However for every primer pair, a false positive band appeared at the negative control with similar sizes to the expected band. However with a primer pair designed on the *aphA-6*, a slight difference in size was observed, so that positive bands (865 bp) could be distinguished from false positive bands (see Fig. 2.34). All putative transformants appeared negative for transgene integration.

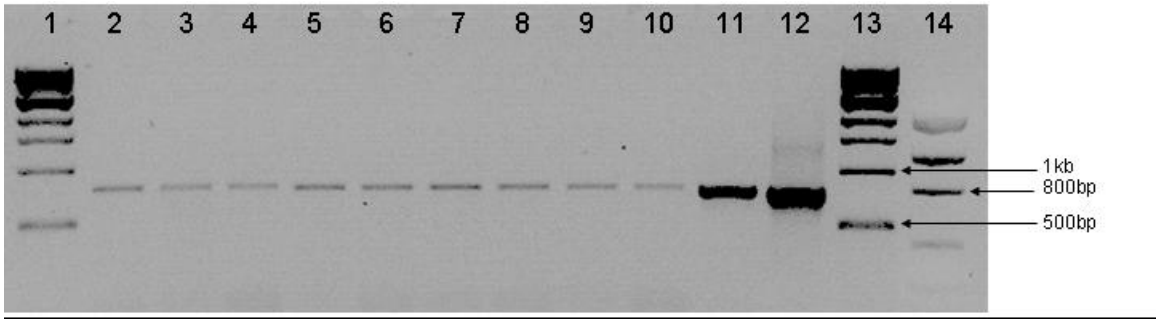


Fig. 2.34: PCR analysis of putative transformants using *aphA-6* specific primers.
Lane 1 + 13: 1kb ladder
Lane 14: low mass ladder
Lane 2-10: Putative transformants
Lane 11: negative control, wild-type *Lolium perenne* L. Cv. 'Cashel'
Lane 12: Positive control, pIAPRvdB5

2.3.6.2.2.2 Long-range PCR

Long-range PCR was carried out on gDNA isolated from putative plastid transformants with a primer pair designed on the plastid region external of the homologous integration region. A wild-type PCR product of 2.589 kb was expected, with a possible PCR fragment of 4.2kb size, indicating transgene integration. All putative transformants only contained the wild-type band as shown in Fig. 2.35.

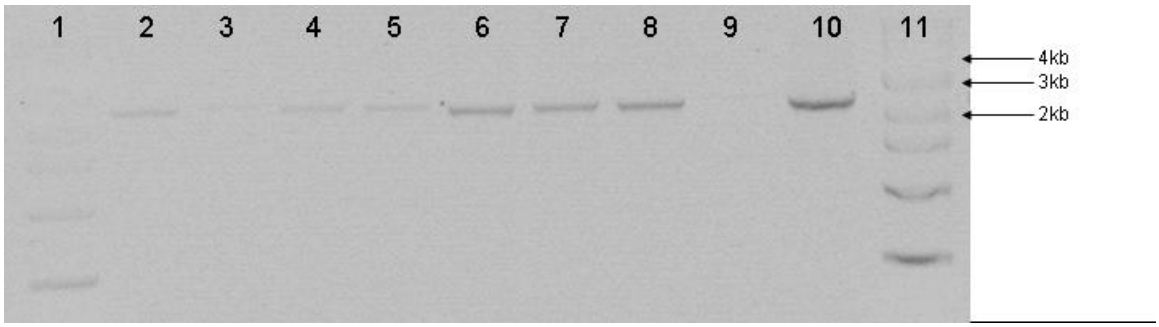


Fig. 2.35: PCR analysis of putative transformants using primers external of the integration cassette
Lane 1 + 11: 1kb ladder
Lane 2-9: Putative transformants
Lane 10: negative control, wild-type *Lolium perenne* L. Cv. 'Cashel'

2.3.6.2.3 Southern blot

Two probes were designed to hybridise with the *trnI* and the *aphA-6* gene. During hybridization with *SacII* digested wild-type gDNA, a fragment of 5615 bp should be visualised with the *trnI* probe. If the expression cassette RvdB5 is integrated within the plastid genome a fragment of 7471bp would be visualised for both probes (See Fig. 2.36). For all the putative transformants strictly the wild-type fragment was present, indicating that none of the putative transformants were positive for targeted integration (See Fig. 2.37). Furthermore hybridisation with probe designed on the *aphA-6* gene, showed no signal in all putative transformants, indicating the absence of the expression cassette (see Fig. 2.38).

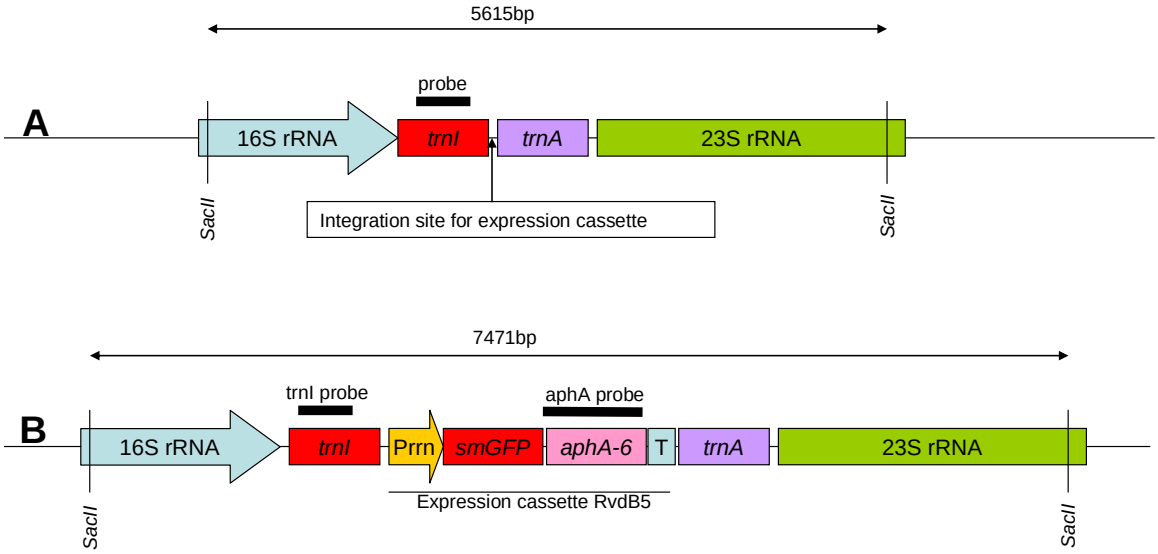
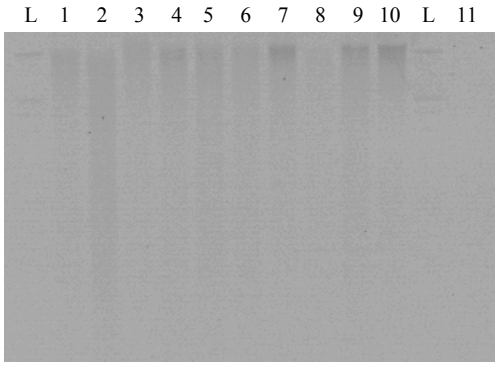
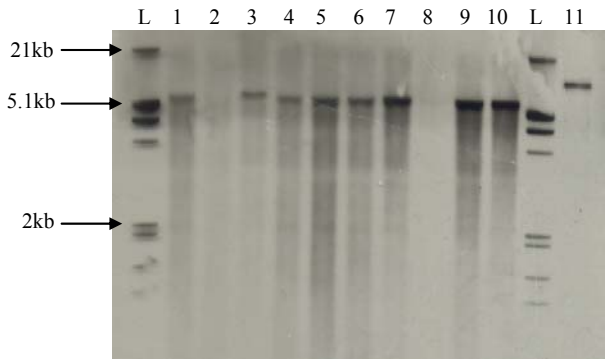


Fig.2.36: Map of the plastid genome region of *Lolium perenne* L with the insertion site for targeted integration of the expression cassette RvdB5. **A**: Map of plastid genome region without integration of the expression cassette (WT), dig labelled probe for the *trnI* gene is shown. The trnI probe hybridises to a fragment of 5615bp **B**: Plastid genome region with targeted integration with expression cassette RvdB5. Both the trnI and aphA probe hybridise to a fragment of 7471bp.

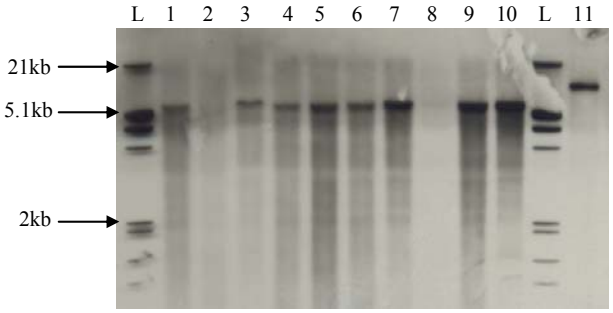


A: DNA ran on a 0.8% agarose gel

Lanes:
L: DNA molecular weight marker III, digoxigenin-labeled
1-9: Putative transformants
10: Wild-type *Lolium perenne* L.
11: pIAPRvdB5

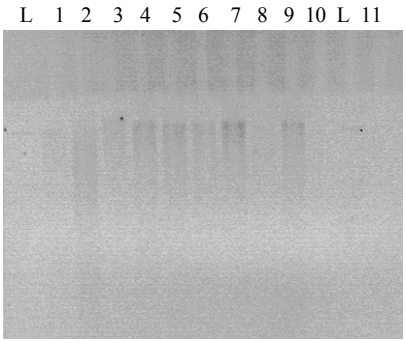


B: One minute exposure

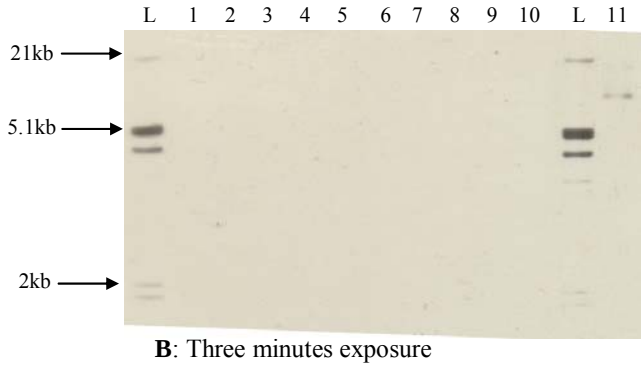


C: Three minutes exposure

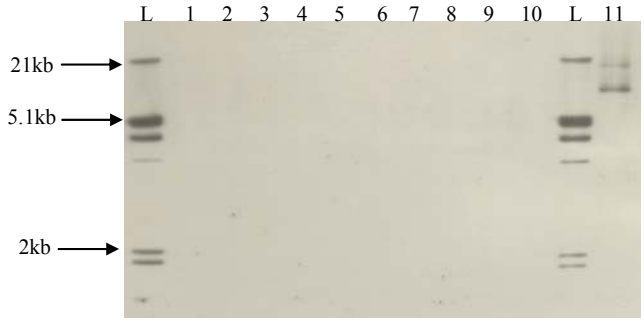
Fig. 2.37: Southern blot using trnI probe, all gDNA samples were digested with *SacII*, pIAPRvdB5 was digested with *KpnI*, **A**: Digested DNA ran on a 0.8% agarose gel, **B**: Southern blot with a one minute exposure time, **C**: Southern blot with a three minutes exposure time.



A: DNA ran on a 0.8% agarose gel



B: Three minutes exposure



C: Six minutes exposure

Lanes:
L: DNA molecular weight marker III, digoxigenin-labeled
1-9: Putative transformants
10: Wild-type *Lolium perenne* L.
11: pIAPRvdB5

Fig. 2.38: Southern blot using aphA probe, all gDNA samples were digested with *SacII*, pIAPRvdB5 was digested with *KpnI*, **A**: Digested DNA ran on a 0.8% agarose gel, **B**: Southern blot with a three minutes exposure time, **C**: Southern blot with a Six minutes exposure time.

2.4 Discussion

During this study, all the known factors involved with plastid and nuclear transformation through biolistics were assessed and accordingly optimized.

The *Lolium perenne* L. plastid transformation vectors pIAPRvdB4 and pIAPRvdB5 were correctly assembled; both could be used for plastid transformation experiments. Vector pIAPRvdB5 was designed to achieve a higher protein accumulation, which could be beneficial due to lower expected gene-expression within callus tissue. Vector pIAPRvdB4 contained the full-length Prrn promoter with both the NEP and PEP binding sites, which is active in both light and dark growth conditions albeit at lower levels, facilitating selection regimes in the dark.

A suitable tissue culture regime was established. As source tissue for callus induction, two types were assessed for efficiency in callus induction. Both leaf-base explants and mature embryos successfully induced regenerable callus for all cultivars tested. However there were differences in response between cultivars. When leaf-base explants were used as source tissue, cultivars ‘Cashel’ and ‘Shandon’ had the highest induction rates, while cultivar ‘Telstar’ had the highest induction rate when mature embryos were used. The advantage of using mature embryos as source tissue is the time-frame at which the callus can be obtained. Unlike leaf-base explants as source tissue, this protocol does not require a four-week tissue culture period prior to callus induction. However for plastid transformation, to obtain homoplasmic tissue, the tissue has to go through several rounds of regeneration. To achieve this, leaf-base explants are the obvious choice for callus

induction, as this will be readily available after obtaining the primary transformants. There are two problems with combining the two aforementioned techniques. A specific genotype (seed) might be efficient in regenerable callus induction from mature embryos, but might not be efficient for callus induction from leaf-base explants. This could prove to be a bottleneck to obtain homoplasmy during plastid transformation. On balance it was felt that leaf-base explants were the preferred choice as source tissue for transformation experiments.

An overview about the order of responses for callus induction from leaf-base explants and shoot induction is given in Table 2.24., with an overall score of both combined in column 4 of Table 2.24. The cultivars 'Cashel' and 'Shandon' responded overall the best, for both callus induction and shoot regeneration. As a consequence these two cultivars were prime candidates to continue the transformation experiments with. However due to a sister project where the plastid genome of Lolium perenne L. Cv. 'Cashel' was sequenced (Diekmann et al. 2009), this cultivar was ultimately chosen to continue with. It should be noted that when calli were transferred in 16h light conditions, the first shoots appeared within a week. This could pose a problem during selection, where callus selection would be preferential, due to the unwanted possibility of obtaining chimeric shoots.

Table 2.24: The overall response for callus induction and regeneration and the combined ranking of cultivars by taking both responses into consideration.

Cultivar	*Callus induction ranking*	*shoot induction ranking*	*Combined response ranking*
Cv 'Cashel' dark	1	1	1
Cv. 'Shandon'	1	1	1
Cv. 'Limes'	4	1	2
Cv. 'Cancan'	6	1	3
Cv. 'Greengold'	3	2 (no shoot induction)	4
Cv. 'Telstar'	2	n.a.	*
Cv. 'Action'	5	n.a.	*

A selection regime was developed for both nuclear transformation and plastid transformation. For nuclear transformation experiments selection at 75 mg L^{-1} hygromycin B should be sufficient to select primary transformants, although a fair number of initial escapes are to be expected. For plastid transformation, paromomycin seems to be the best choice, with selection pressures between 100 to 150 mg L^{-1} paromomycin. Several options as to when selection should be initiated to obtain transplastomic tissue remains a question that has yet to be answered.

Lastly, the gene-delivery protocol was optimized for the use on calli from Lolium perenne L. Cv. ‘Cashel’. Although the DNA delivery efficiency was relatively low, the efficiency was greatly increased when Cv. ‘Action’ was used at a later stage in the project. Despite this, the ratio obtained with the tests conducted on Cv. ’Cashel’ could be applied to other cultivars of *Lolium perenne* L..

Despite the thorough assessment and optimisation of cultivars, explant source, tissue culture parameters, biolistic delivery parameters, and selection protocol, transgenic tissue was not recovered. The reason for the failure to achieve genetic modification can be due to several independent aspects. The most likely bottle neck to acquire any nuclear or plastid transformants, could be due to the choice of cultivar. Despite the fact that Cv. 'Cashel' proved to be the best candidate based on the tissue culture conditions, this does not exclude the possibility that another untested cultivar would perform better. Furthermore gene-delivery proved to be more efficient when the Cv. 'Action' was used. Unfortunately this cultivar was obtained at a late stage of the project, therefore limited number of transformation experiments were conducted on this cultivar.

There are several other likely problems in acquiring plastid transformants. For instance, in deciding what kind of vector would be most suitable for plastid transformation of *Lolium perenne* L., you are restrained by the available data. To test the efficiency of an expression cassette in *Lolium perenne* L., a working plastid transformation protocol has to be developed first. So basically you end up in a vicious circle. To determine "A" you need to have done "B" and vice versa.

Theoretically the expression cassette used within pIAPRvdB5 should express sufficient APHA-6 to inactivate the antibiotic paromomycin. The functionality was tested and confirmed in *Escherichia coli*, by introducing the expression cassette into the pUC19 vector, which lacked the *nptII* gene within the backbone. Bacteria containing the expression cassette were resistant to paromomycin and G-418. Furthermore high

expression levels with similar constructs were recorded in previous studies (Kuroda and Maliga 2001b). Moreover similar expression signals were used to achieve plastid transformants in the monocot *Oryza sativa* (Khan and Maliga 1999). Nevertheless there is no guarantee the expression cassette would be as efficient in *Lolium perenne* L. as it was in other systems. Every species and every tissue has a different expression profile, this was demonstrated by comparing expression profiles within chromoplast and chloroplasts in tomato (Kahlau and Bock 2008). Therefore it is quite possible that this expression cassette would not be active enough in callus tissue within *Lolium perenne* L., to provide enough APHA-6 to inactivate the selectable agent.

Another likely barrier to obtain transgenic tissue is the selection regime. After biolistical delivery, the expression cassette has to be integrated within the plastid genome. Furthermore sufficient accumulation of the detoxifying protein has to be present for transgenic cells to proliferate, this can pose a problem as it was shown that the Prrn PEP promoter activity in rice proplastids was seven fold lower compared to the activity in chloroplasts (Silhavy and Maliga 1998). As a result, when selection is commenced too early, the transgenic cells may not be able to proliferate. If selection is commenced too late, the transgene might have looped out already. The timing to induce selection could therefore be of vital importance to obtain transplastomic tissue.

Taking all this into account, it is possible that a working protocol for plastid transformation in this species will be difficult to obtain, as has proven the case with

monocots in general. On the other hand, a small alteration to this protocol could well prove to be sufficient to achieve plastid transformation within *Lolium perenne* L..

Chapter 3:

RNA-Editing

3.1 Introduction

In this study, the editing efficiencies of various plastid editing sites were evaluated under drought stress in various genotypes of perennial ryegrass. The hypothesis was that it may be that drought tolerance could in part be regulated directly or indirectly by editing events within the plastid genome, in particular within the NDH complex (NADH dehydrogenase complex). Previous reports indicate that the NDH complex optimizes the induction of photosynthesis under conditions of water stress in the light (Burrows et al. 1998) and that the plastidial NDH complex activity increases under stressed conditions (Ibanez et al. 2010). More specifically when the *ndhB* gene was inactivated in transplastomic plants, the dark reduction in the plastoquinone pool was impaired and an enhanced growth retardation was observed under humidity stress conditions (Horvath et al. 2000). Potentially the functionality of the NDH complex could be impaired by the lack of RNA editing, therefore decreasing the tolerance to oxidative stress caused by water deficit.

RNA editing alters the nucleotide sequence of an RNA molecule so that it deviates from the sequence of its DNA template (Tillich et al. 2006). Different RNA editing systems exist and each is thought to have evolved independently (Brennicke et al. 1999). RNA editing in chloroplasts belongs to the conversion system, where exclusively C to U substitutions occur, with the exception of U to C substitutions in the bryophyte *Anthoceros formosae* (Kugita et al. 2003). mRNA editing usually results in the restoration of codons for conserved amino acids (Bock et al. 1994).

3.1.1 Assessment of RNA editing efficiency

RNA editing efficiency can be investigated by several different methods. The most trustworthy method is a colony screen (Roberson and Rosenthal 2006). This involves the amplification of a fragment derived from complementary DNA (cDNA) within a transcript where editing occurs. The fragment is introduced into a sequencing vector, after which the vector is transfected into *Escherichia coli*. Subsequently the editing efficiency can be determined, by screening separate colonies of bacteria for editing sites within the fragment in the vector. By screening a large number of clones, the editing efficiency can be calculated. Although this method is very accurate, it is a labour intensive method, furthermore it is a costly endeavour, due to the large number of sequencing reactions involved. A second method to analyse the RNA editing efficiency is the poison primer extension (PPE) technique (Peeters and Hanson 2002). This assay is based on using a polymerase to extend a $_{32}$P end-labeled primer through the editing site in the presence of three deoxynucleotide triphosphates and one dideoxynucleotide triphosphate. The dideoxynucleotide, or chain terminator, normally contains the same base as the edited nucleotide. Therefore, if a transcript is edited, the extension stops at the editing site. If not, it extends to the next instance of the edited nucleotide in the chain. To estimate editing efficiency, reactions can be separated on an acrylamide gel and the relative proportions of the two extension products are quantified. The run through can be as low as 5%, although in some cases it was 70%, depending on the polymerase and terminators used (Peeters and Hanson 2002; Roberson and Rosenthal 2006). This method is quite accurate, although the optimization can be major drawback to get it to work efficiently. However once optimized it is cost- and labour effective. The last method

available is the trace-file method (Nakae et al. 2008). This technique involves direct sequencing of specific PCR products containing editing sites, amplified from cDNA. The trace files from the sequencing results can be analysed by comparing peaks representing edited and unedited editing sites. The ratio determines the editing efficiency. The accuracy of this method was found to be 5%, although in general a lower accuracy in the range of 20% is mentioned (Nakae et al. 2008). This method is labor efficient and fairly cost effective (Nakae et al. 2008).

3.1.2 Systems for mimicking drought stress conditions for plants

Several systems for assessing drought susceptibility or tolerance have been employed in previous studies. However none of the systems is considered optimal, for various reasons, which are discussed below (Munns et al. 2010). The system mostly used, is plants grown in pots under controlled conditions. If the plants are grown in soil, the induction of drought can be accomplished by withholding water for a specific duration of time. Uniformity of soil is however difficult to maintain, resulting in a variable water potential throughout the soil profile, this can result in a mixed nutrient transmission (Nye and Tinker 1977). Furthermore soil saturation at the bottom can happen easily when pots are not sufficiently deep, resulting in 'control' plants not growing as well as moderately stressed plants (Passioura 2006b). To avoid saturation, inorganic 'soils', like calcined clay can be used. However artificial material with large particles may have little root contact, resulting in decreased growth. For example, vermiculite caused a greater reduction in root growth at low water potential than did liquid medium at the same water potential at least up to –1.6 MPa (Verslues et al. 1998).

Alternatively hydroponics can be used to circumvent the problems with heterogeneity, drainage and variable water potential. Several non-ionic osmotica have been used to decrease the media water potential. Examples are mannitol, melibiose and sorbitol. Despite the osmotic properties of these chemicals, these components are not suitable for the induction of drought stress, because the cell membranes are permeable to these small molecules. Therefore entry of these chemicals within the roots occurs, resulting in their movement within the xylem towards the shoots. In the case of mannitol it was shown that cell elongation occurs, causing a problem for interpretation of growth differences between stressed and control conditions (Hohl and Schopfer 1991). Besides these low-molecular-weight molecules, high-molecular-weight polyethylene glycol (PEG) was used to mimic drought stress. The advantage of this chemical is the inability to penetrate the cell membrane; so it does not cause any unintended adverse effects on growth. However due to the viscous nature of a PEG solution, the poor supply of oxygen to the roots can pose a problem (Mexal et al. 1975). To overcome this problem, aerating of the roots is necessary. However too rigorous bubbling can cause damage to roots through shear stress, so a gentle aeration is desirable (Mexal et al. 1975) . To improve oxygen supply, it was shown that bubbling with oxygen instead of air increased root growth (Verslues et al. 1998). Despite the inability of PEG to penetrate the cell membrane, the change of solutions during experiments can lead to entry of PEG into the roots, by lateral breaks of the roots. This was shown to cause necrosis, by blocking pathways used for transpiration (Lawlor 1970).

Besides hydroponics and soil based systems, another system to mimic drought stress was developed by Van Der Weele et al. (2000) using a nutrient-agar medium supplemented with PEG-8000. This system is called the "PEG-infused *in vitro* system" It involved dissolving PEG in a nutrient medium, after which the PEG medium was allowed to diffuse into an agar medium. *Arabidopsis* seedlings were subsequently placed on top of the PEG-agar medium. By placing the Petri dishes vertically, the supply of oxygen to the roots was guaranteed (van der Weele et al. 2000).

3.1.3 Assessment of drought stress response of plants

To test for drought tolerance, several different tests can be performed. Firstly the Relative water content (RWC) can be assessed. This is a method for determining the water content within tissue under different environmental conditions. It was first described by (Barrs and Weatherley 1962). Under optimal conditions leaf tissue has a RWC of 85-95%, however when exposed to drought conditions this value decreases. The extent of this reduction is an indication how well it copes with its ability to retain water by limiting transpiration and maintaining water uptake (Volaire and Lelièvre 2001). Another indicator of drought tolerance is root growth. In previous studies it was shown that drought tolerant plants had an increase in root development under moderate stress conditions. Most likely, this reflects an adaptive response involving an increase in root length to reach water deeper in the soil (van den Berg and Zeng 2006). Other strategies for determining drought tolerance are analyses of stomatal conductance by imaging analyses (Grant et al. 2006; Leinonen et al. 2006) or determination of the photosynthesis rate (Hu et al. 2010). A relationship between the RWC and photosynthesis has been

demonstrated in previous studies (Lawlor and Cornic 2002). A decreased RWC slowed down the CO_2 assimilation rate and at RWC values lower then 75%, metabolic inhibition occurred.

3.1.4 Aim and objectives

Previous studies have shown that the RNA editing pattern is developmentally influenced (Peeters and Hanson 2002; Chateigner-Boutin and Hanson 2003), furthermore environmental control was also shown to influence the editing pattern as temperatures of 37^0C was shown to inhibit editing of specific editing sites within tobacco (Karcher and Bock 2002). The aim of this study was to determine if RNA editing was directly or indirectly involved with drought response. In order to investigate this, the RNA editing efficiencies of various editing sites were analysed in accessions of *Lolium perenne* L. that had different responses to drought stress. In a previous study only two cultivars were characterized for drought response (Foito et al. 2009), these cultivars were initially assessed for RNA editing patterns (see section 3.3.1). After which the study was expanded to a range of other accessions, which had to be characterized first for their drought response.

Initially the "*In vitro* PEG-induced system" was used to detect differences in drought response between accessions of *Lolium perenne* L. obtained from the GRIN database (Germplasm Resources Information Network in the United States of America). The choice of this system relied on the ability of this protocol to assess a large number of accessions simultaneously. The results obtained with this system are described in section

3.3.2. To confirm the drought response of these accessions, another drought test was performed to verify the findings and to obtain sufficient amount of tissue for RNA editing analyses. For this a hydroponics system with PEG (*In vivo* PEG-induced system) was utilized. The results are described in section 3.3.3.

Once the drought response of the tested accessions was determined, the RNA editing behaviour of these genotypes was assessed; these results are described in section 3.3.4

After analyses of the data, the relationship between drought response and RNA editing could be evaluated, this is shown in section 3.3.5.

Finally another feature in plastid DNA was analysed, namely single nucleotide polymorphisms (SNPs). These are single nucleotide polymorphisms that can in some cases change the amino-acid sequence of a transcript. There are two different types of SNPs, either transition or transversion mutations. Transition mutations are C – T and A – G combinations, whereas transversion are C – G and A – T combinations. These combinations co-exist within the same plant, making up different haplo-types. These SNP's can be used to design markers to assess population genetics and phylogenetic studies (Diekmann et al. 2009). Despite the identification of SNP's within the plastid genome, it has not been tested whether these SNP's could potentially be converted by the RNA editing machinery. This is shown in chapter 3.3.6

3.2 Materials and methods

3.2.1 Plant material

Lolium perenne L. cultivar 'Cashel' was obtained Teagasc, Oak Park, Carlow.

Other accessions of *Lolium perenne* L. were acquired from the Germplasm Resources Information Network (GRIN) in the United States of America as shown in Table 3.1.

Table 3.1: Accessions ordered from GRIN database.

Accessions - Origin		
PI462336 New Zealand	PI 418717 Italy	PI 577270 Norway
PI 598433 Italy	PI 610803 Norway	PI 440475 Russia
PI 610806 Romania	PI 418723 Luxembourg	PI 598434 Italy
PI 502412 Russia	PI 418701 Former Yugoslavia	PI 610802 Norway
PI 610820 Romania	PI 201187 Netherlands	PI 610825 Switzerland
PI 577266 Romania	PI 512321 Spain	PI 632538 Italy
PI 628717 Bulgaria	PI 619554 Wales	PI 632553 Italy
PI 611044 Russia	PI 223178 Greece	PI 632575 Italy
PI 598453 Romania	W6 11256 Turkey	PI 632590 Morocco
NK 200 USA	PI 229476 Iran	PI 634278 USA
PI 231565 Libya	PI 577270 Norway	

These accessions had been tested for their winter hardiness (Hulke et al. 2007; Hulke et al. 2008), and accessions used in this study were chosen over a scale of good to bad response to low temperatures.

3.2.2 cDNA synthesis from total RNA

A drought stress experimental series has been carried out at Oak Park by Dr. Stephen Byrne. For this experiment clones were vegetatively propagated from single tillers from two different cultivars, Cv. 'Cashel' (drought susceptible) and Cv. 'New Zealand' PI462336 (drought tolerant).

Total RNA was extracted from leafs at different time points during the stress test, both from control conditions and stressed conditions, a total of 28 samples were prepared.

This RNA was used to synthesize cDNA, using Superscript III Reverse transcriptase following the manufacturer's instructions (Invitrogen cat. No. 18080-400).

3.2.3 Primers used for RNA-editing

All the primers used for RNA editing are listed in Table 3.2.

Table 3.2: Primers designed to amplify regions containing known RNA-editing sites.

Fragments	*Editing site / SNP location within the plastid genome*	*Primer pair*	*Primer 1*	*Primer 2*
psbJ	61111	66	ctggtccctccgatt	gataaaatgtggaggaaagt
petB	72259	7	ggttctaatgatgatcctg	aattctttatgatatgcctt
ndhB-1	85696	8	gaaagaaatagacctagcag	ccttttcatcaatggact
	86347	8		
	86341	8		
ndhB-2	87155	9	tccttcgtagacgtcag	ttggatgcagttactaattc
	87188	9		
	87281	9		
	87306	9		
	87425	9		
	87743	9		
ndhD	107165	17	cattaatagaccggactggt	ggaagcgcattatagtacatg
rps8	76422	31	gaattcgggtactatagccacagc	gaaaaatttggaggaacctagaattaga
rpoC2	28731	34	gcagctaaaccttatttggccac	tcctttatgtatccttaactcgtataaatgg
atpA	35112	89	taccaatagttgagactcaat	aacaagaaagagtagacgtg
ndhA	111250	23	agaagaaattagaaaaaccagaaa	gggaagttgatcgttgaaa
	112355	23		
	112778	23		
ndhG	109624	12	tttgcaattcttataatttttactcttt	tgcctttcgttgggattag
rpl2	82031 (133217)	11	tgctgctctagctaattgc	ctattccacttctagatagagaaaa
rpl20	66009	29	gggaattcggtttttattat	attagttattcattaaggttaatttg
ndhF	103515	88	ggagctagtaaccaatccca	agtaaaaattgcaatttcttttc
	103675	88		
ndhK	49245	86	tgaacattccccctgtaata	atggcgaaaaggagcctt
	49367	86		
rpoB	19737	13	taagattaagatgctccgg	caagattaagcctccga
	19815	13		
	19830	13		
	19560	13		
ycf	42700	30	ttatgaaaaagaaggagcgtggtc	ggttgggaattatgcctagatcc
	43599	30		
psbL	61339	44	tccctccgattactatagagatgaa	ccaacgataaacaaaattccaac
psaB	37506	psaB	tcaaagatcttttagaagcgca	gccaataaaaagtaacccat
rps18	65631	rps18	ctaaacaacctttttcttaaa	aacttaagttccgattgttg

Red annotated genome locations are known SNPs.

3.2.4 PCR to amplify fragments from cDNA containing the editing-sites

Fragments were amplified using Taq polymerase (New England Biolabs, Inc., Ipswich, MA, USA). The reaction mixture is shown in Table 3.3 and the PCR cycles are given in Table 3.4.

Table 3.3: Standard PCR mix

Reaction mix	*Volume (µl)*
cDNA (1µg μl^{-1})	1.0
Primer 1 (0.1µM μl^{-1})	1.0
Primer 2 (0.1µM μl^{-1})	1.0
Taq Polymerase (1unit μl^{-1})	1.0
10x Thermo buffer	2.0
dNTP (10mM)	1.0
ddH_2O	13.0
Total reaction mix	20.0

Table 3.4: PCR cycle:

Steps	*Temperature*	*Time*
Step1 Denaturation	95°C	5 min
Step 2 Denaturation	95°C	1 min
Step 3 Annealing	50°C	1 min
Step 4 Extension	72°C	1 min
Step 5 Repeat cycles	*Repeat step 2-4*	*35 times*
Step 6 Final extension	72°C	10 min
Step 7 End of program	4°C	Indefinite storage

3.2.5 Analyses of RNA editing patterns using the trace-file method

All PCR products were sent to AGOWA (Germany) for PCR purification and sequencing. The returned trace files were analysed with the program 'Chromas Lite' for RNA-editing sites and the corresponding efficiency. To analyse the efficiency of editing, the height of the peaks at an editing location within the trace files were measured, and compared to each other as illustrated in Fig. 3.1. The editing efficiency was calculated with the following formula;

$$\frac{\text{Height edited peak}}{\text{Height edited peak} + \text{Height unedited peak}} x100\%$$

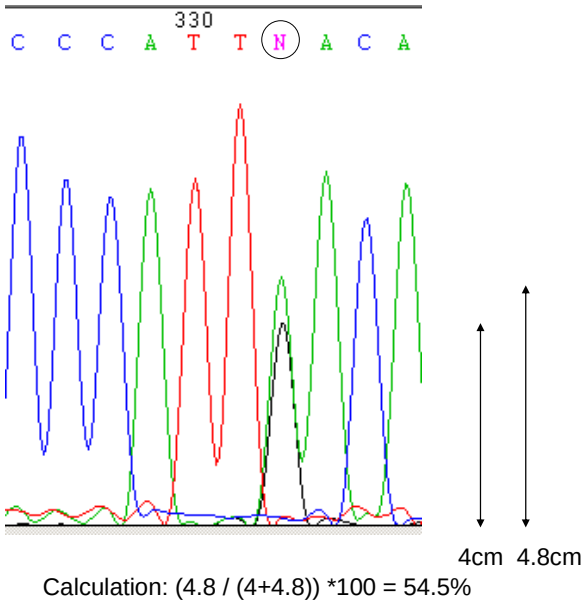


Fig. 3.1: Calculation of editing efficiency using the trace-file method**.** The circle around the nucleotide represents the editing site.

3.2.6 Verification of the trace-file method, using colony screens

To determine if the trace-file method can be used confidently, a colony screen was performed. This involved setting up PCR reactions of randomly selected samples (see Table 3.5.) using primers designed for the *ndhB* and *ndhF* fragments (see Table 3.2, primer-sets 9 and 88).

Table 3.5: Samples used for colony screen experiment

PCR-product	*Samples*	*RNA editing sites at genome position within the plastid genome of Lolium perenne L.*
ndhB	17 (Cashel)	87188, 87281, 87306, 87425, 87743
ndhB	23 (New Zealand)	87188, 87281, 87306, 87425, 87743
ndhF	23 (Cashel)	103675

These PCR-products were sent off for sequencing at John Lester's, sequencing facility at the department of biochemistry, University of Cambridge, United Kingdom. Peaks in the resulting trace files were analysed using the formula stated in section 3.2.5. The same PCR-products were cloned into the cloning-vector pCR2.1-TOPO, and subsequently introduced into *E.coli* strain TOP10 as described in paragraph 2.2.1.

Agar stab-cultures were made in 96-well plates, each well containing a separate clone, derived from the *E.coli* with pCR2.1-*ndhB* and *E.coli with* pCR2.1-*ndhF*. The clones containing the *ndhB* fragment were sequenced in both directions, whereas the clones containing the *ndhF* fragment was only sequenced in one direction. The sequencing was done at GATC Biotech AG, Konstanz, Germany.

The editing efficiency was calculated as the percentage of clones containing the edited site, in comparison to the total number of clones. If the colony screen gave similar results, compared to the trace-file method, then it could be assumed the trace-file method was trustworthy.

3.2.7 Evaluation of accessions of *Lolium perenne* L. on drought stress susceptibility using an in vitro PEG-induced system

3.2.7.1 Plant material preparation

Surface sterilised seeds of all accessions were germinated on germination medium (see Table 2.4). When the hypocotyls of the seedlings reached a length of 1 cm, the drought stress experiment was initiated.

3.2.7.2 Preparation of PEG-infused plates

Polyethylene glycol (PEG)-infused plates were made as described by (van der Weele et al. 2000; Verslues et al. 2006). The water potential of plates was lowered by addition of various amounts of PEG-8000 (Sigma) to the culture medium (see Table 3.6). Culture medium (half-strength MS, 1.2 g L^{-1} MES, pH 5.7) was prepared. For base medium, 15 g L^{-1} agar was added and autoclaved at 120^{o}C for 20 minutes. For the overlay solution, no agar was added, prior to autoclaving. Desired amounts of PEG-8000 were dissolved in the medium after autoclaving, without filter sterilisation. To set up the PEG-infused plates, 20 ml of base media was poured into 9 cm Petri dishes. When solidified, 30 ml of overlay solution was added on top. The PEG in the overlay solution was allowed to diffuse into the agar overnight. After which the remaining liquid on top, was gently

removed by decanting. Plates were used immediately, or stored while wrapped in cling-film until use.

Table 3.6: Final water potential of the medium, when using specific amounts of PEG in overlay solution.

Final water potential of the PEG-infused plate (MPa)	*PEG added per litre of medium to make the overlay solution (g)*
- 0.25	0
- 0.5	250
- 0.7	400
- 1.2	550
- 1.7	700

Source: (Verslues et al. 2006).

3.2.7.3 Drought stress experiment

Five to seven seedlings, 1 cm in length were placed on the surface of each Petri dish, both the PEG-infused and the control plates. The plates were then placed vertically in a growth chamber at 22^{o}C with a 16 hour photoperiod. The seedlings were exposed to the stress treatment for 13 days, prior to assessment.

3.2.7.4 Analyses of seedlings exposed to drought stress

Photographs were taken before and after the drought treatment of each Petri dish on top of a 1 mm roster paper; these images were used to measure the shoot lengths of each seedling. In addition the total dry biomass and the Relative Water Content (RWC) were measured as described by (Barrs and Weatherley 1962). The Fresh weight (FW) was measured by weighing whole seedlings on a fine scale (Ohaus, PA-114). The seedlings were submerged in distilled water for 3 hours, after which the seedlings were blotted dry

and the Turgor Weight (TW) was measured of these seedlings on a fine scale. Subsequently, the seedlings were placed in a tube to dry in an oven at 70^{o}C, after which the Dry Weight (DW) was measured.

The following formula was used to calculate the RWC;

$$\frac{(FW - DW)}{(TW - DW)} \times 100\% = RWC$$

FW: Fresh weight, DW: Dry weight, TW: Turgor weight, RWC: Relative Water Content

3.2.8 Evaluation of accessions of *Lolium perenne* L. for drought stress susceptibility using an *in vivo* PEG-induced system

3.2.8.1 Plant material

Accessions were chosen based on the results from the *in vitro* PEG induced system. Seeds of each accession were germinated, after which at least ten clones from a single seedling per accession were propagated vegetative, by continuous tillering. All clones were genotypically identical unlike the seedlings in the *in vitro* experiment.

3.2.8.2 Experimental setup

Plants were allowed to establish in a hydroponics system, supplemented with 4.4 g L^{-1} Gamborg + vitamins B5 (Gamborg et al. 1968). The system was aerated by an aquatic pump, to supply oxygen in the solution as illustrated in Fig. 3.2. Two separate systems were setup. After one week the solution was refreshed, to prevent depletion of nutrients. The second week after experimental setup, the drought stress experiment was initiated by replacing the solutions in both systems, in the first system the solution was replaced with

4.4 g L^{-1} MS, this system acted as the control. In the second system the solution was replaced with a solution comprising of 4.4 g L^{-1} MS supplemented with 20% PEG-6000 (Duchefa cat. No. P0805) for the induction of drought stress. This concentration of PEG results in a water potential of about -0.45 MPa (Michel and Kaufmann 1973). The experiment was performed in a controlled glass house at Teagasc, Oak Park, Carlow with a mean daily temperature of 22^{o}C and supplemented with lighting (PAR = 650 microeinsteins $m^{-2}s^{-1}$) for 16hrs. After two weeks in these conditions, samples were taken for analysis.

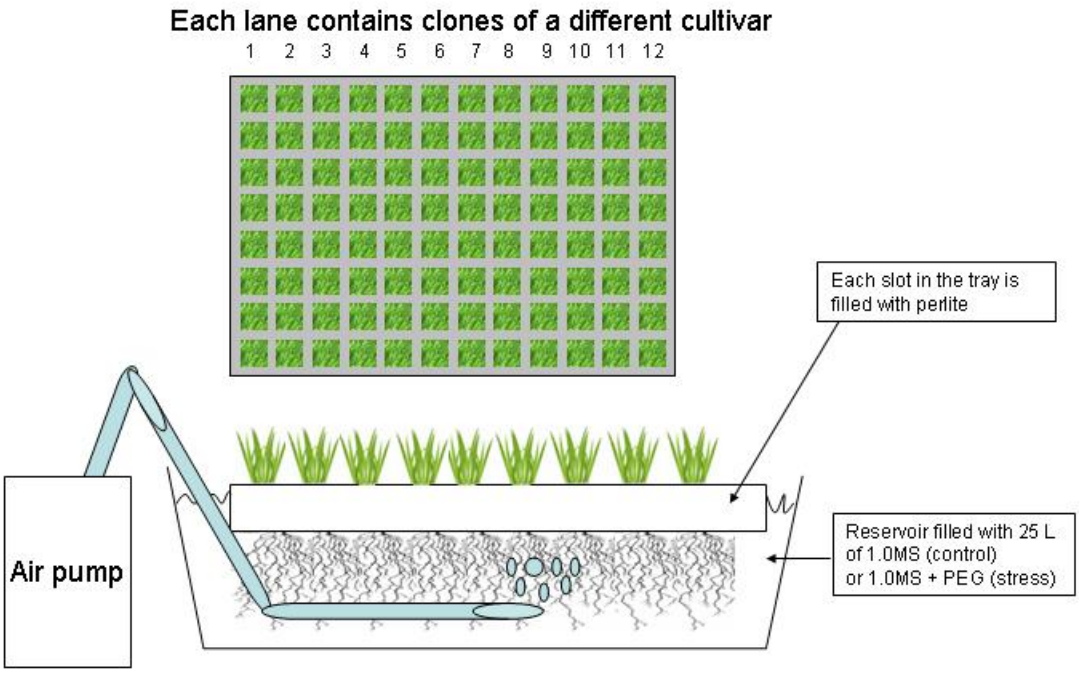


Fig. 3.2: Experimental set-up of the hydroponics system for assessing drought stress.

3.2.9 Analyses of plants exposed to drought stress

Three different methods were used, to determine the response to drought stress.

3.2.9.1 Pixel detection to record growth differences

Photographs were taken of the plants after the completion of the experiment and analysed using Adobe Photoshop 5.5. Pixel detection of leaf tissue and root tissue was performed (See Fig. 3.3), revealing the number of pixels in each photo consisting of either leaf (Fig. 3.3 C) or root tissue (Fig. 3.3 D), thereby indicated the amount of tissue present after each treatment.

Because the photos were not all taken from the same distance, the number of pixels could not be compared directly. To circumvent this problem the data had to be converted, this was achieved by using a reference area in each photograph to detect the number of pixels therein (See Fig. 3.3B), the ratio of pixels between each photo for this reference area represented the difference in photograph size. Although these data cannot be converted to biomass, the ratio of pixels between treatments/accessions could be analysed and provide a reliable measure of relative growth. Pixel detection of leaf tissue within individual plants could not be performed, due to overlapping tissue of separate clones. Therefore the number of pixels within leaf tissue of individual plants was predicted by taking the total number of pixels consisting of leaf tissue, divided by the number of plants in the photograph. For pixel detection of root tissue, individual plants could be assessed, due to the fact that roots tissue did not overlap between separate clones.

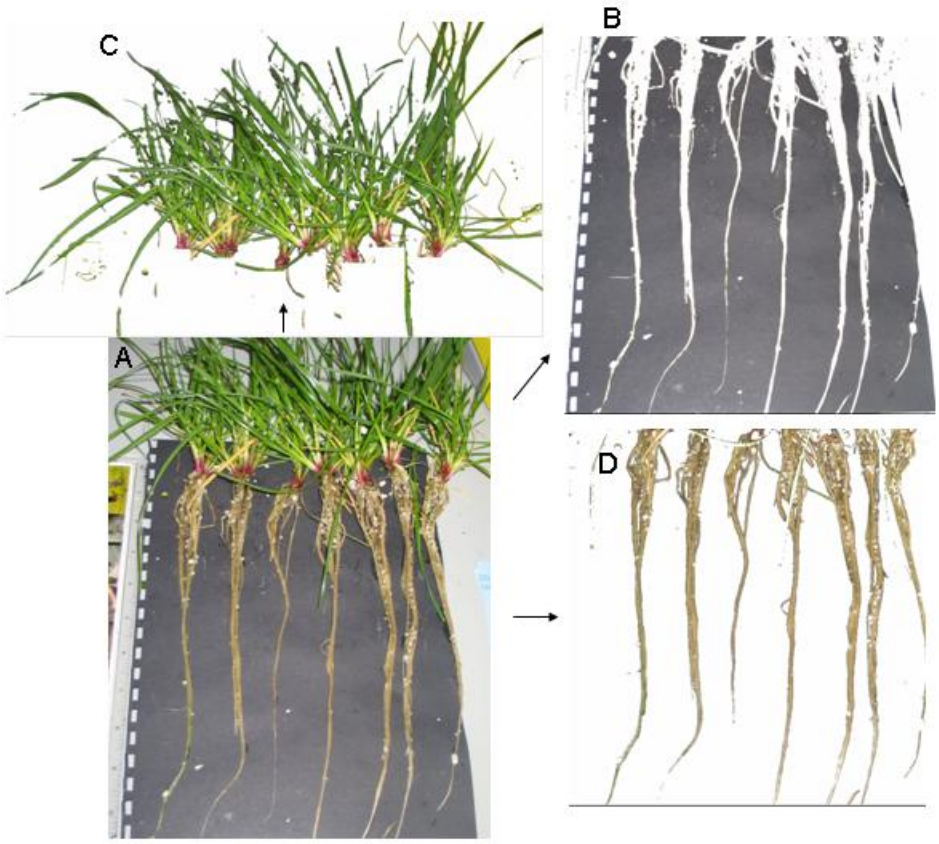


Fig. 3.3: Analyses of amounts of different tissues using pixel detection.
A: Complete photograph
B: Pixels consisting of background
C: Pixels consisting of leaf tissue
D: Pixels consisting of root tissue

3.2.9.2 Relative water content analyses

The RWC was calculated for each plant, by taking a two cm leaf piece from the middle of the plant and weighing the Fresh Weight (FW), Turgor Weight (TW) and the Dry weight (DW) of these leaf tissues as described in paragraph 3.2.7.4.

3.2.9.3 Total dry root biomass analyses

The total roots from each plant were harvested, wrapped in tin foil and dried in an oven at 70^0C for three days. The total root dry weight was afterwards recorded for each separate

plant. The leaf dry weight could not be recorded, as the total leaf tissue was required to extract total RNA for RNA editing analyses.

3.2.10 Molecular analyses

3.2.10.1 Total RNA isolation from leaf tissue

Total RNA from three plants per treatment, per accession was extracted using the RNeasy® Plant Mini Kit from Qiagen Cat. No.74903. mRNA extraction was performed using the manufacturer's instructions.

3.2.10.2 cDNA synthesis

cDNA was synthesized using Superscript III Reverse transcriptase following the manufacturer's instructions (Invitrogen cat. No. 18080-400).

3.2.10.3 PCR to amplify *ndhB* and *ndhF* fragments from cDNA and gDNA

For the *ndhB* fragment primerset 9 was used, while for the *ndhF* fragment primerset 88 was used (see Table 3.2). Fragments were amplified using GoTaq polymerase (Promega cat. No. M8301). The reaction mixture was as shown in Table 3.7, and the PCR cycles were as shown in Table 3.8.

Table 3.7. PCR reaction mix

Reaction mix	*Volume (µl)*
cDNA / gDNA (1µg μl^{-1})	1.0
Primer 1 (0.1µM μl^{-1})	1.0
Primer 2 (0.1µM μl^{-1})	1.0
GoTaq polymerase (5unit μl^{-1})	0.2
5x GoTaq flexi buffer	6.0
dNTP (10mM)	2.0
$MgCl_2$ (25 mM)	3.6
ddH_2O	15.2
Total reaction mix	30.0

Table 3.8: PCR cycle:

Steps	*Temperature*	*Time*
Step1 Denaturation	95^oC	5 min
Step 2 Denaturation	95^oC	1 min
Step 3 Annealing	45^oC	1 min
Step 4 Extension	72^oC	1 min
Step 5 Repeat cycles	*Repeat step 2-4*	*35 times*
Step 6 Final extension	72^oC	10 min
Step 7 End of program	4^oC	Indefinite storage

3.2.11 Statistical analyses

The arcsine transformation and *t*-tests were conducted in 'Microsoft Excel', whereas the variance tests were performed in the program 'Minitab Solutions 15'.All data sets were analysed for equal variance using the Levene test, results obtained determined if *t*-tests were performed with equal variance or unequal variance.

For the RWC analyses, arcsine transformation of the values was necessary to obtain a normal distribution to do statistical analyses, as percentages cannot be used directly for comparison studies. These values were subsequently tested for statistical differences using the *t*-test with a one-tailed distribution and equal variance. A one-tailed distribution was chosen, as the hypothesis was that stressed plants would exhibit lower values compared to the plants in control conditions.

For the total dry weight analyses, the results were tested for a normal distribution and subsequently tested for statistical differences using the *t*-test with a two-tailed distribution and unequal variance. A two-tailed distribution was chosen on the hypothesis that the root biomass could be either higher or lower for the stressed conditions compared to the control conditions.

For the editing efficiency, the values were transformed into Arcsine values. These values were subsequently tested for statistical differences using the *t*-test with a two-tailed distribution and unequal variance. A two-tailed distribution was chosen, based on the hypothesis that efficiency could be either lower or higher for plants in stressed conditions compared to plants in control conditions.

3.3 Results

3.3.1 Editing analyses

3.3.1.1 Assessment of the Trace-file method to detect RNA editing efficiency in comparison with the conventional colony screen method.

cDNA samples were randomly selected for analyses of RNA editing. A colony screen was performed as described in section 3.2.6. Results obtained from the colony screen were compared to results obtained from the trace-file method (described in section 3.2.5) as shown in Table 3.9. The highest difference observed between methods, was a 10.8% difference in editing, whereas the lowest difference observed was 0.8%. The corresponding trace-files can be found in appendix B.

The editing efficiencies themselves have no relevance, as this was just to test the methodology.

Table 3.9: The difference in editing efficiencies obtained by the Colony-screen method and the Trace-file method derived from specific samples for several different editing sites within the *ndhB* and *ndhF* transcript.

Editing site – cDNA sample	*Colony screen derived editing efficiency in percentage*	*Trace-file method derived editing efficiency in percentage*	*Difference observed between methods in percentage*
ndhB 87188 – sample 17	93.6%	88.2%	5.4%
ndhB 87188 – sample 23	18.1%	16.1%	2.0%
ndhB 87281 – sample 17	92.2%	87.5%	4.7%
ndhB 87281 – sample 23	85.7%	74.9%	10.8%
ndhB 87306 – sample 17	94.4%	84.3%	10.1%
ndhB 87306 – sample 23	83.7%	73.4%	10.3%
ndhB 87425 – sample 17	96.6%	91.8%	4.8%
ndhB 87425 – sample 23	83.9%	83.1%	0.8%
ndhB 87743 – sample 17	92.1%	89.3%	2.8%
ndhB 87743 – sample 23	81.3%	75.6%	5.7%
ndhF 103675 – sample 23	21.5%	27.3%	5.8%

3.3.1.2 The editing efficiency within various transcripts in cultivars 'Cashel' and 'New Zealand'

Initially the RNA editing events were examined in two genotypes of *Lolium perenne* L. that were known to differ in their response to drought. The drought responses of Cv. 'Cashel' and Cv. 'New Zealand' were previously determined using the *in vivo* PEG-induced drought stress system (Foito et al. 2009). This study showed that these specific genotypes showed a clear difference in response, namely Cv. 'Cashel' was classified as drought susceptible and Cv. 'New Zealand' as drought tolerant.

Leaf samples of these plants were taken at 0 hours, 4 hours, 24 hours and 168 hours after initiation of drought stress, after which mRNAs were extracted, for subsequent use in this study. A range of 28 editing sites were analysed, however only 16 editing sites exhibited partial editing. The editing sites shown in Table 3.10 showed 100% editing in all samples tested. These sites were not used for any further analysis.

Table 3.10: Editing sites that showed complete editing in all samples tested.

Editing site: transcript – genome position			
petB – 72259	*rps8* – 76422	*ndhA* – 112355	*ycf* - 43599
ndhB – 85696	*rpoC2* – 28731	*ndhA* – 112778	
ndhB – 86347	*atpA* – 35112	*rpl2* – 82031 (133217)	
ndhB – 86341	*ndhA* – 111250	*ycf* – 42700	

3.3.1.3 Editing pattern differences between drought stressed and non-stressed plants

The efficiency of partially edited editing sites were analysed for difference between drought stress treated genotypes and non-stressed treated genotypes using the trace-file method. A representative example of editing over time is shown in Fig. 3.4, there were no obvious differences observed over time for RNA editing efficiency. There were some fluctuations, but they appeared to be random. Furthermore no difference was observed overall between treatments for all editing sites for either cultivar tested, except for the RNA editing site at genome position 49245 within the *ndhK* transcript in cultivar 'Cashel' (see Fig. 3.5). There was a statistical difference observed, however this seems to be attributed to coincidental low standard deviation (Cv. 'Cashel' see Fig. 3.5 and Table 3.11, Cv. 'New Zealand' see Fig. 3.6 and Table 3.12).

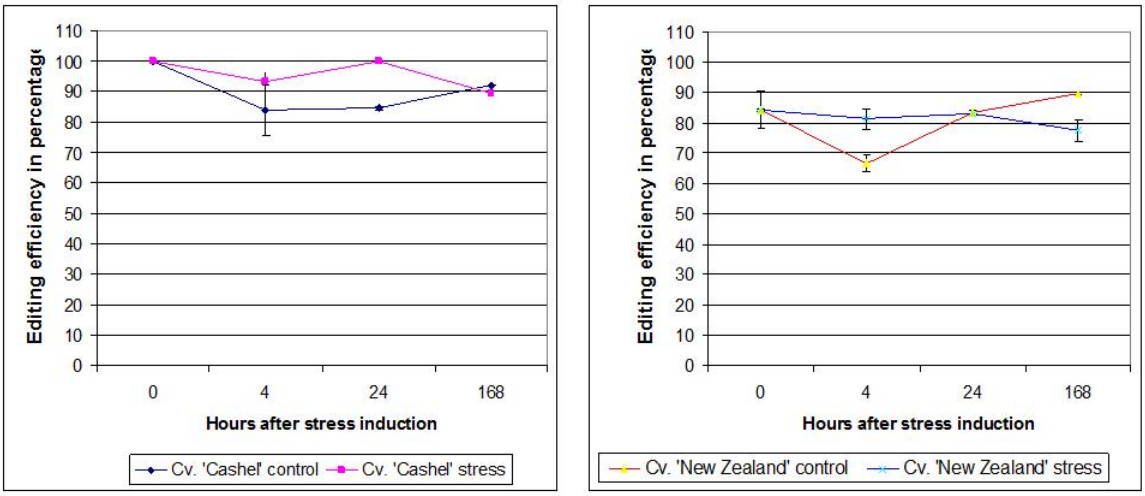


Fig. 3.4: Editing efficiency over time after stress induction for editing site 87281 within the *ndhB* transcript.

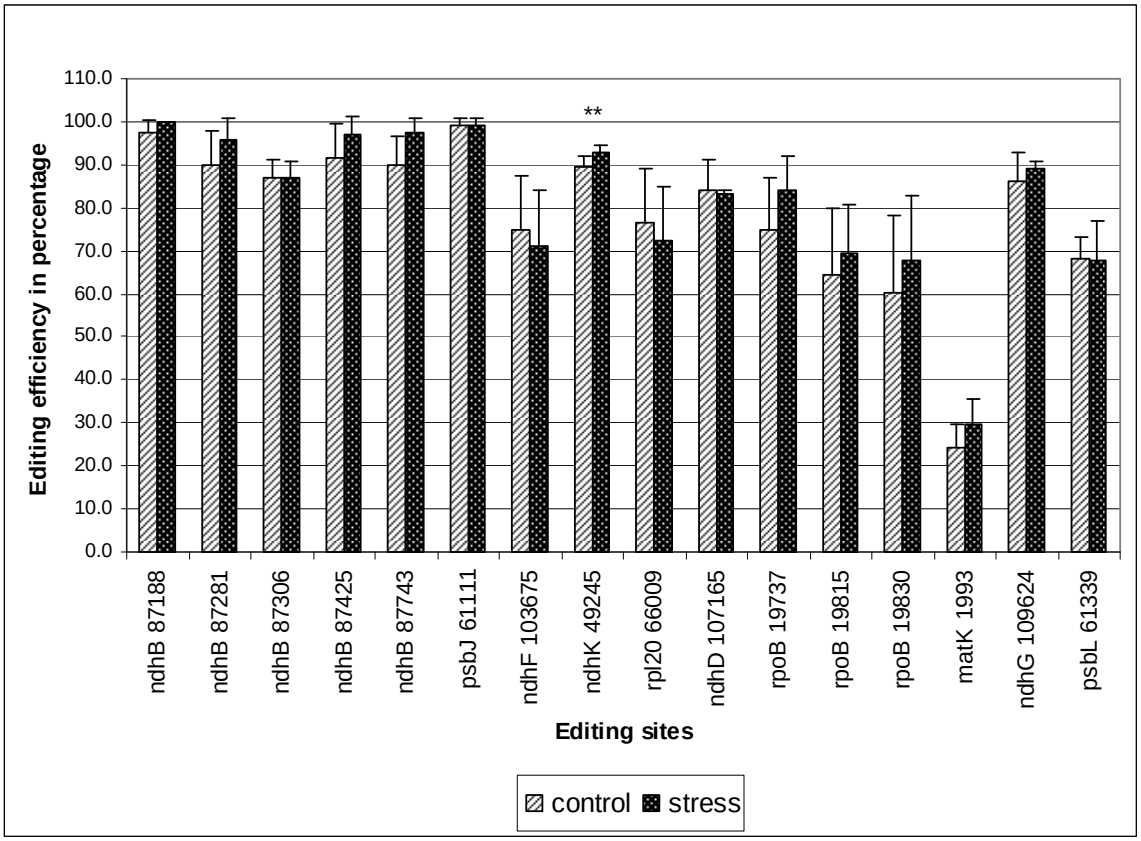


Fig. 3.5: Editing efficiency of sites in different transcripts in cultivar 'Cashel' for drought stressed and non-stressed plants. Error bars represent the standard deviation of the mean. Statistical differences in editing efficiency between stressed versus non-stressed plants calculated with a *t*-test (two-tailed distribution, equal variance, on arcsine transformed values). **: Editing sites with a statistical difference at $P<0.05$.

Table 3.11: Editing efficiency of sites in different transcripts in cultivar 'Cashel' for drought stressed and non-stressed plants. The standard deviation is shown between brackets. P-values are based on a *t*-test (two-tailed distribution, equal variance) on arcsine transformed editing percentages.
** = statistically difference at P<0.05

Editing site (gene and genome position)	*Non-stressed plants*	*Drought stressed plants*	*P-values*
ndhB 87188	97.3 (± 3.2)	100 (± 0.0)	0.15
ndhB 87281	90.1 (± 7.6)	95.7 (± 5.2)	0.13
ndhB 87306	86.9 (± 4.3)	86.9 (± 3.6)	1.00
ndhB 87425	91.5 (± 8.2)	97.2 (± 3.9)	0.17
ndhB 87743	89.8 (± 6.9)	97.3 (± 3.4)	0.05
psbJ 61111	99.3 (± 1.4)	99.1 (± 1.8)	0.93
ndhF 103675	74.7 (±12.9)	71.1 (±12.8)	0.61
NdhK 49245	89.4 (± 2.8)	92.8 (± 1.6)	0.01**
rpl20 66009	76.5 (±12.4)	72.4 (±12.4)	0.67
ndhD 107165	84.0 (± 7.0)	83.2 (± 1.0)	0.78
rpoB 19737	74.8 (±12.4)	84.0 (± 7.9)	0.27
rpoB 19815	64.5 (±15.3)	69.4 (±11.5)	0.68
rpoB 19830	60.1 (±18.2)	67.6 (±15.0)	0.59
matK 1993	24.4 (± 5.3)	29.9 (± 5.7)	0.20
ndhG 109624	86.3 (± 6.6)	89.1 (± 1.8)	0.48
psbL 61339	68.4 (± 4.8)	67.9 (± 9.3)	0.95

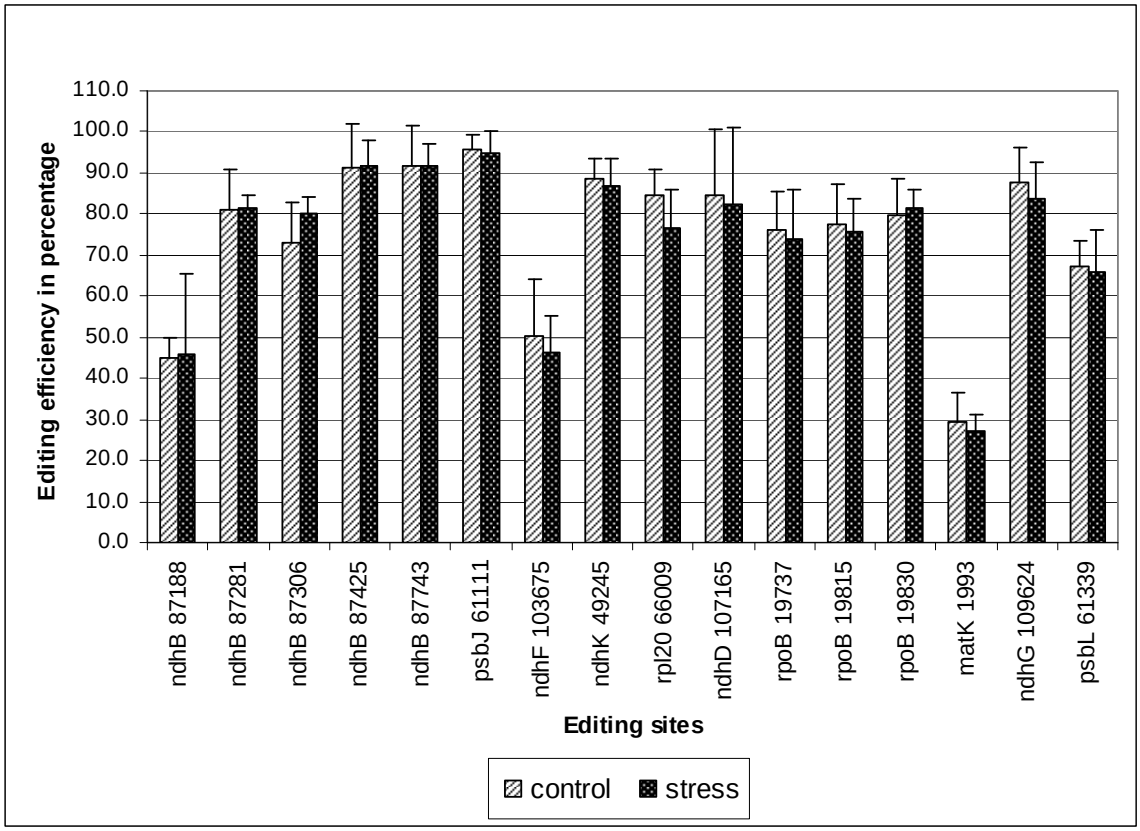


Fig. 3.6: Editing efficiency of sites in different transcripts in cultivar 'New Zealand' for drought stressed and non-stressed plants. Error bars represent the standard deviation of the mean. Statistical differences in editing efficiency between stressed versus non-stressed plants calculated with a *t*-test (two-tailed distribution, equal variance, on arcsine transformed values).

Table 3.12: Editing efficiency of sites in different transcripts in cultivar 'New Zealand' for drought stressed and non-stressed plants. The standard deviation is shown between brackets. P-values are based on a *t*-test (two-tailed distribution, equal variance) on arcsine transformed editing percentages.

Editing site (gene and genome position)		*Non-stressed plants*	*Drought stressed plants*	*P-values*
ndhB	87188	45.1 (± 4.7)	46.0 (±19.6)	0.70
ndhB	87281	81.0 (±10.1)	81.5 (± 2.9)	0.89
ndhB	87306	72.9 (±10.0)	80.4 (± 3.9)	0.43
ndhB	87425	91.1 (±10.7)	91.8 (± 6.0)	0.81
ndhB	87743	91.6 (±10.0)	91.9 (± 5.3)	0.81
psbJ	61111	95.9 (± 3.5)	94.7 (± 5.5)	0.85
ndhF	103675	50.1 (±14.1)	46.4 (± 8.8)	0.61
NdhK	49245	88.8 (± 4.9)	86.6 (± 6.8)	0.48
rpl20	66009	84.6 (± 6.3)	76.6 (± 9.2)	0.20
ndhD	107165	84.6 (±16.1)	82.5 (±18.8)	0.89
rpoB	19737	76.4 (± 9.0)	73.8 (±12.0)	0.81
rpoB	19815	77.5 (± 9.9)	75.8 (± 7.8)	0.76
rpoB	19830	79.5 (± 9.2)	81.4 (± 4.4)	0.78
matK	1993	29.5 (± 7.0)	27.3 (± 3.9)	0.60
ndhG	109624	87.8 (± 8.4)	83.9 (± 8.9)	0.50
psbL	61339	67.3 (± 6.3)	65.9 (±10.3)	0.84

3.3.1.4 Editing pattern differences between cultivars 'Cashel' and 'New Zealand'

Editing sites were analysed using the trace-file method, to determine differences in editing between the drought tolerant cultivar 'New Zealand' and drought susceptible cultivar 'Cashel' as shown in Fig. 3.7 and Table 3.13. The RNA editing efficiency for four different editing sites was significantly different between the two cultivars at $P<0.01$. All of these editing sites were within genes, which are part of the NDH complex. These were the editing sites on genome positions 87188, 87281 and 87306 within the *ndhB* transcript, and genome position 103675 within the *ndhF* transcript. Another two RNA editing sites were statistically different but to a lesser extent ($P<0.05$), these are the sites

at genome position 49245 within the *ndhK* transcript, and position 19830 within the *rpoB* transcript.

Partial editing of these particular sites was observed before in root and callus tissue in *Zea mays*, however not in leaf tissue (Peeters and Hanson 2002). The occurrence of partial editing in the transcripts, which encode part of the NDH complex is interesting, because of the involvement of the NDH complex in response to stress (Burrows et al. 1998; Ibanez et al. 2010).

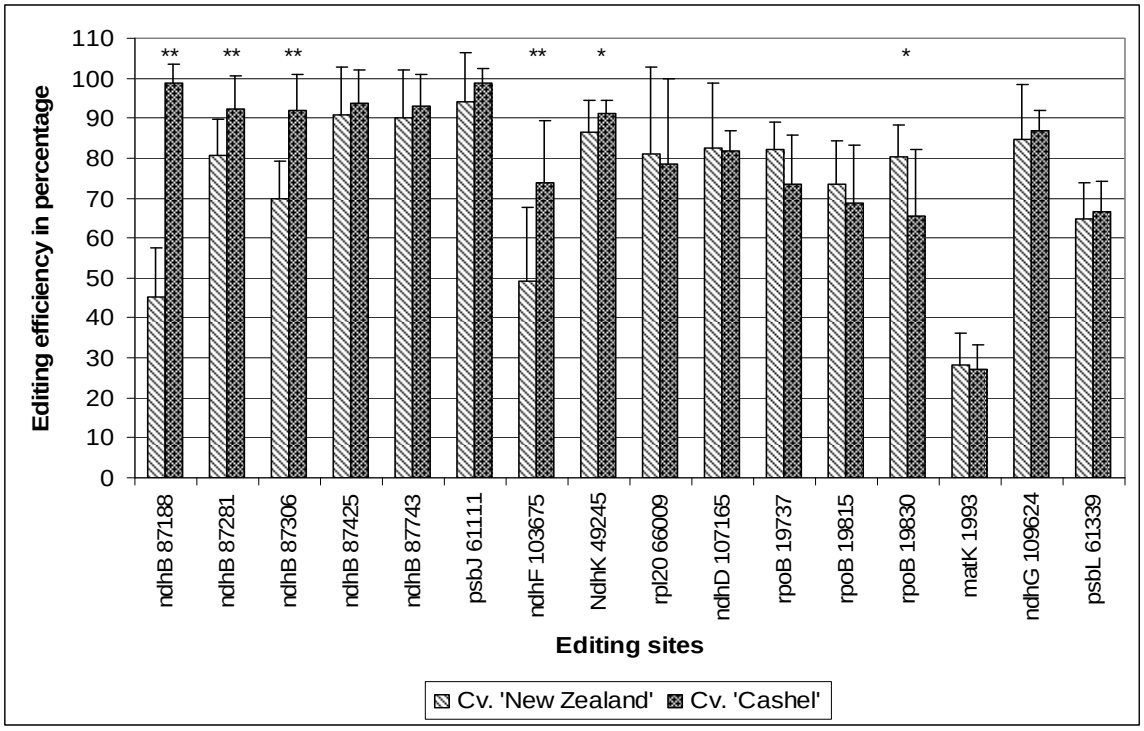


Fig. 3.7: Editing efficiency of sites in different transcripts in cultivars 'New Zealand' and 'Cashel'. Statistical differences in editing efficiency between cultivars 'Cashel' and 'New Zealand' were calculated with a *t*-test (two-tailed distribution, unequal variance, on arcsine values) Error bars represent the standard deviation of the mean.
* : Editing sites with a statistical difference at $P<0.05$
**: Editing sites with a statistical difference at $P<0.01$

Table 3.13: Editing efficiencies for editing sites within various genes for cultivar 'Cashel' and cultivar 'New Zealand'. The standard deviation is shown between brackets. P-values are based on a *t*-test (two-tailed distribution, unequal variance, on arcsine transformed values).
*: Editing site with a statistical difference at P<0.05
**: Editing sites with a statistical difference at P<0.01

Editing sites (gene, genome position)		*Cv. 'Cashel'*	*Cv. 'New Zealand'*	*P-values*
ndhB	87188	98.6 (± 4.8)	45.2 (±12.4)	<0.01**
ndhB	87281	92.2 (± 8.3)	80.7 (± 9.1)	<0.01**
ndhB	87306	91.8 (± 9.4)	69.9 (± 9.5)	<0.01**
ndhB	87425	93.8 (± 8.4)	90.7 (±12.2)	0.51
ndhB	87743	93.1 (± 7.7)	90.3 (±11.8)	0.57
psbJ	61111	99.0 (± 3.6)	94.1 (±12.4)	0.05
ndhF	103675	73.8 (±15.6)	49.2 (±18.3)	<0.01**
NdhK	49245	91.1 (± 3.2)	86.5 (± 8.1)	0.01*
rpl20	66009	78.6 (±21.1)	80.9 (±22.0)	0.63
ndhD	107165	81.6 (± 5.3)	82.6 (±16.3)	0.55
rpoB	19737	73.3 (±12.6)	82.3 (± 6.9)	0.68
rpoB	19815	68.7 (±14.4)	73.3 (±10.9)	0.68
rpoB	19830	65.3 (±16.8)	80.3 (± 7.9)	0.02*
matK	1993	27.2 (± 6.1)	28.3 (± 7.9)	0.45
ndhG	109624	86.8 (± 5.1)	84.5 (±13.8)	0.9
psbL	61339	66.4 (± 7.7)	64.9 (± 8.8)	0.67

3.3.2 *In vitro* PEG-induced system to evaluate drought tolerance

29 accessions of *Lolium perenne* L. were ordered from the GRIN database, based on differences in their winter hardiness response (Hulke et al. 2007; Hulke et al. 2008). Five to seven separate seedlings (each a distinct genotype) of each accession were exposed to stressed conditions (both -0.75 MPa and -0.9 MPa) and non stressed conditions (-0.25 MPa) as described in section 3.2.7.

3.3.2.1 Evaluation of accessions of *Lolium perenne* L. on drought tolerance using an *in vitro* PEG-induced system.

After 13 days of treatment, the RWC (Fig. 3.8, Table 3.14 and Fig. 3.9, Table 3.15) and total dry weights (Fig. 3.10, Table 3.16 and Fig. 3.11, Table 3.17) were determined for each seedling, these results were pooled together to obtain a ranking in response to drought stress. The results obtained reflected the population of the accession, rather then a single genotype within a cultivar.

3.3.2.2 Relative water content

The RWC values under control conditions were in the range of 73% to 99%, whereas under stressed conditions these values dropped as was expected under drought stress (Fig. 3.8, Table 3.14 and Fig. 3.9, Table 3.15). The extent of reduction reflected how well the plants coped with water deficit. The lowest RWC value, which was observed under stressed conditions, was 45.7% in accession PI223565 at a -0.75 MPa water potential of the medium (Fig. 3.8, Table 3.14). Unexpectedly the RWC values under stressed conditions at -0.9 MPa water potential of the medium, was less dramatic (Fig. 3.9, Table 3.15). If an accession has no significant reduction in relative water content (RWC) under stressed conditions compared to control conditions, this accession can be considered drought tolerant. On the other hand, if there was a significant reduction in RWC then the accession can be considered drought susceptible.

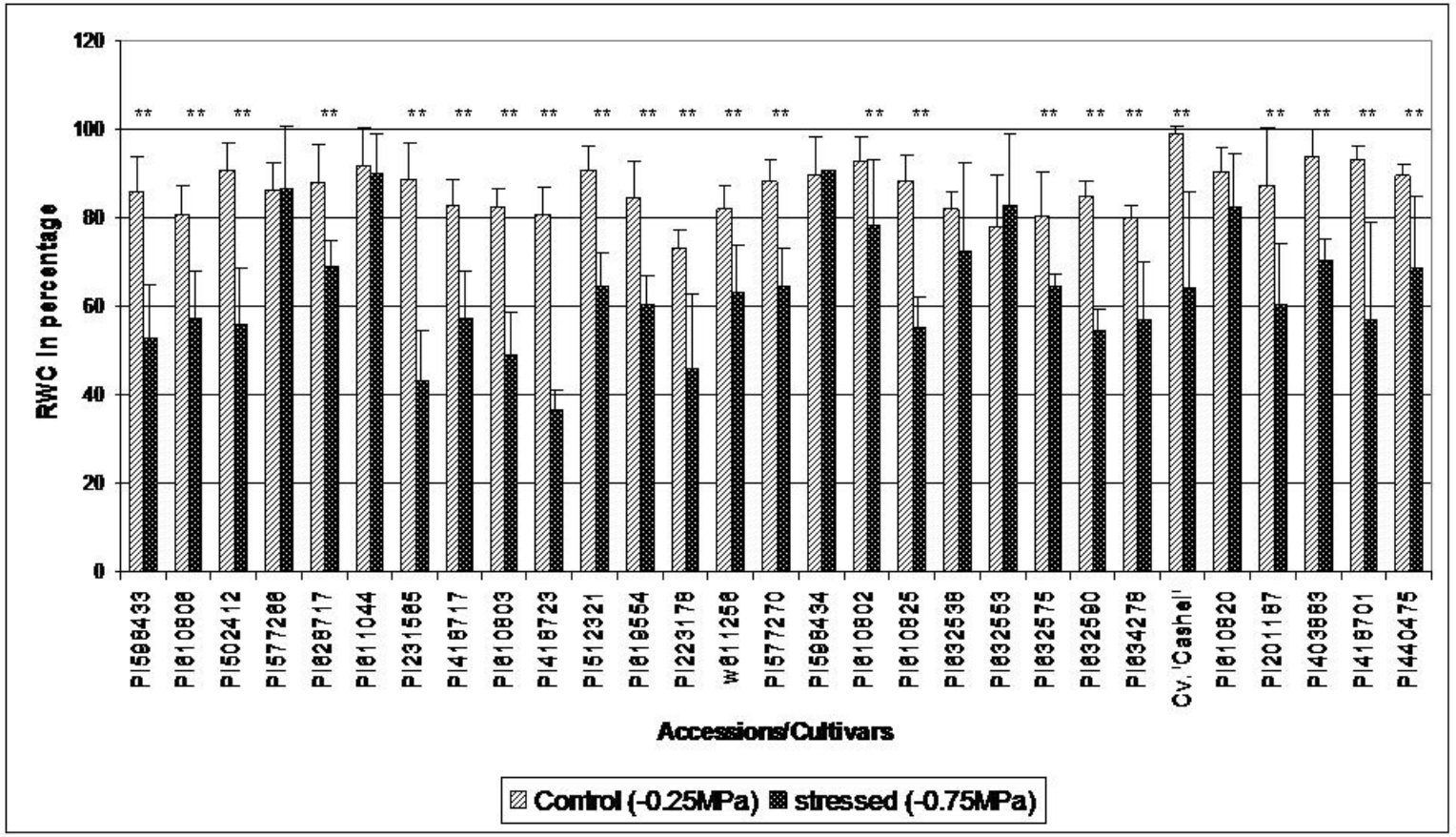


Fig. 3.8: Relative water content (RWC) of seedlings grown under control conditions (-0.25 MPa) versus drought stressed conditions (-0.75 MPa). Error bars represent the standard deviation of the mean.
** Accessions that were statistically different (t-test, one-tailed distribution, equal variance, on arcsine transformed values, at $P<0.05$)

Table 3.14: Relative water content of seedlings subjected to control conditions (-0.25 MPa) and stressed conditions (-0.75MPa). Between brackets are given the standard deviations. P-values are given based on a *t*-test (one-tailed distribution, equal variance) on arcsine transformed values.
[1]: Too few samples to perform a *t*-test.
[**]: RWC in stressed conditions was significantly lower then RWC in control conditions at P<0.05.

Accession	*Control (-0.25MPa)*	*Stress (-0.75MPa)*	*P-value*
PI598433	85.7(± 8.3)	52.7(±12.2)	<0.01**
PI610806	80.8(± 6.7)	57.4(±10.4)	<0.01**
PI502412	90.7(± 6.3)	55.9(±13.1)	<0.01**
PI577266	86.4(± 6.1)	86.6(±14.2)	0.34
PI628717	88.0(± 8.8)	69.0(± 6.1)	0.01**
PI611044	91.8(± 8.7)	90.1(± 9.0)	0.40
PI231565	88.6(± 8.6)	43.5(±11.0)	<0.01**
PI418717	82.9(± 5.7)	57.4(±10.4)	<0.01**
PI610803	82.7(± 4.1)	49.0(± 9.5)	<0.01**
PI418723	81.0(± 6.2)	36.8(± 4.4)	<0.01**
PI512321	90.8(± 5.5)	64.5(± 7.6)	<0.01**
PI619554	84.6(± 8.2)	60.6(± 6.7)	0.01**
PI223178	73.2(± 4.2)	45.7(±17.4)	0.01**
w611256	82.1(± 5.3)	63.2(±10.8)	<0.01**
PI577270	88.2(± 5.0)	64.5(± 8.7)	<0.01**
PI598434	89.7(± 8.6)	90.7(± [1])	[1]
PI610802	92.9(± 5.7)	78.5(±14.9)	0.05
PI610825	88.5(± 5.9)	55.4(± 6.6)	<0.01**
PI632538	82.2(± 3.8)	72.7(±19.7)	0.28
PI632553	77.8(±11.8)	82.9(±16.1)	0.18
PI632575	80.6(±10.0)	64.5(± 3.2)	0.01**
PI632590	84.9(± 3.5)	54.5(± 5.2)	<0.01**
PI634278	80.1(± 3.0)	57.1(±12.9)	0.01**
Cv. 'Cashel'	99.2(± 1.8)	64.0(±22.0)	<0.01**
PI610820	90.4(± 5.5)	82.5(±12.0)	0.13
PI201187	87.7(±12.7)	60.3(±13.7)	0.01**
PI403883	93.8(± 6.1)	70.5(± 4.8)	0.02**
PI418701	93.4(± 2.7)	56.9(±22.0)	0.01**
PI440475	89.8(± 2.2)	68.7(±16.3)	0.02**

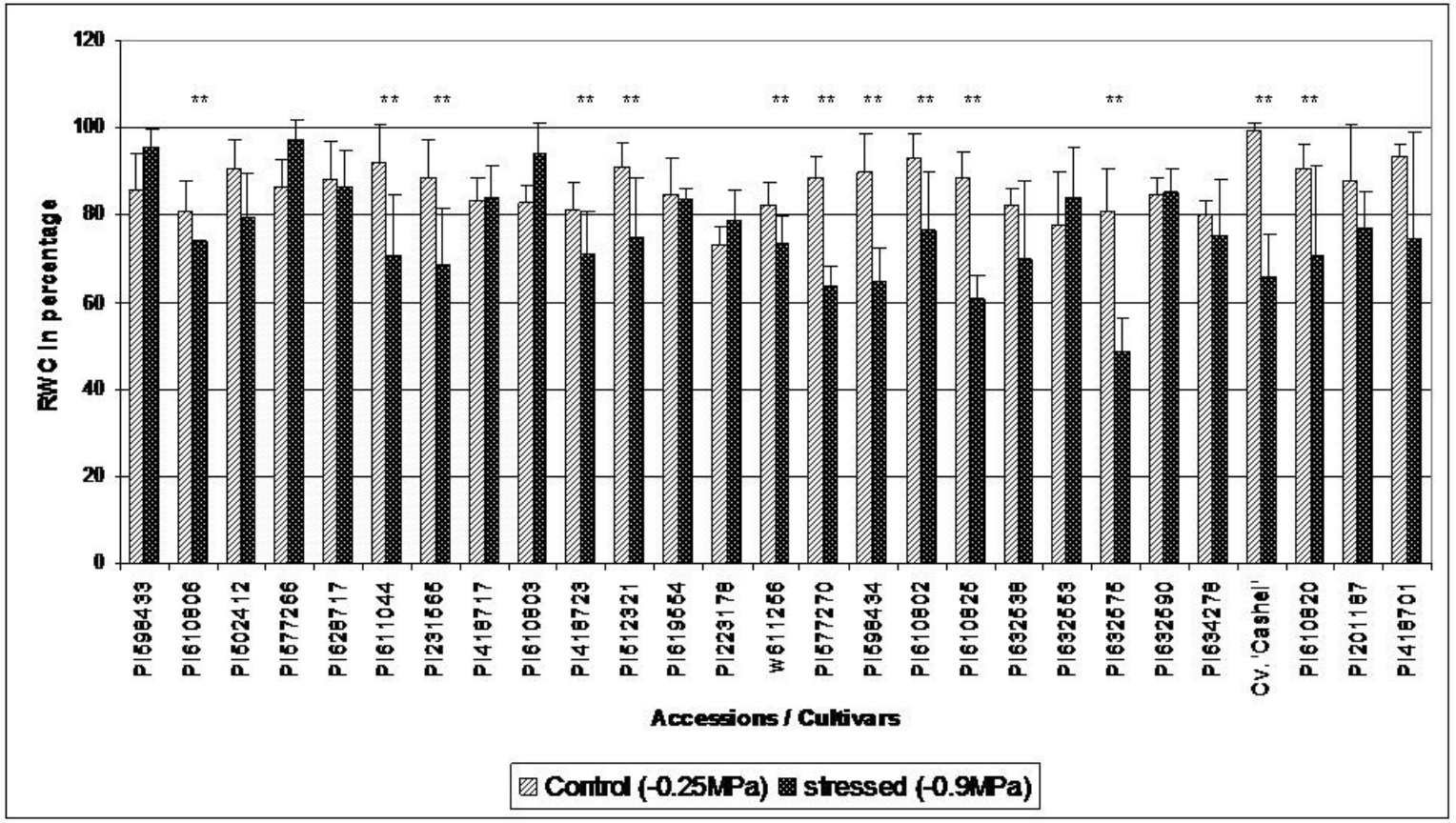


Fig. 3.9: Relative water content (RWC) of seedlings grown under control conditions (-0.25 MPa) versus drought stressed conditions (-0.9 MPa). Error bars represent the standard deviation of the mean.
** Accessions that were statistically different (t-test, one-tailed distribution, equal variance, on arcsine transformed values, at $P<0.05$)

Table 3.15: Relative water content of seedlings subjected to control conditions (-0.25 MPa) and stressed conditions (-0.9 MPa). Between brackets are given the standard deviations. P-values are given based on a *t*-test (one-tailed distribution, equal variance) on arcsine values.
[1]: Too few samples to perform a *t*-test.
[**]: RWC in stressed conditions was significantly lower then RWC in control conditions at P<0.05.

Accessions	*Control (-0.25MPa)*	*Stress (-0.9MPa)*	*P-value*
PI598433	85.7(± 8.3)	95.4(± 4.1)	0.04**
PI610806	80.8(± 6.7)	73.9(± [1])	[1]
PI502412	90.7(± 6.3)	79.3(±10.1)	0.05
PI577266	86.4(± 6.1)	97.2(± 4.7)	0.01**
PI628717	88.0(± 8.8)	86.4(± 8.5)	0.46
PI611044	91.8(± 8.7)	70.6(± 7.6)	0.01**
PI231565	88.6(± 8.6)	68.4(±12.9)	0.01**
PI418717	82.9(± 5.7)	83.7(± 7.5)	0.42
PI610803	82.7(± 4.1)	93.9(± 7.1)	0.01**
PI418723	81.0(± 6.2)	71.0(± 9.7)	0.05
PI512321	90.8(± 5.5)	74.7(±13.6)	0.02**
PI619554	84.6(± 8.2)	83.6(± 2.3)	0.34
PI223178	73.2(± 4.2)	78.5(± 6.9)	0.10
w611256	82.1(± 5.3)	73.5(± 6.3)	0.01**
PI577270	88.2(± 5.0)	63.7(± 4.4)	<0.01**
PI598434	89.7(± 8.6)	64.9(± 7.5)	<0.01**
PI610802	92.9(± 5.7)	76.4(±13.4)	0.03**
PI610825	88.5(± 5.9)	60.6(± 5.6)	<0.01**
PI632538	82.2(± 3.8)	69.9(±17.7)	0.14
PI632553	77.8(±11.8)	83.7(±11.9)	0.19
PI632575	80.6(±10.0)	48.7(± 7.5)	<0.01**
PI632590	84.9(± 3.5)	85.1(± 5.3)	0.45
PI634278	80.1(± 3.0)	75.1(±13.2)	0.31
Cv. 'Cashel'	99.2(± 1.8)	65.5(±10.2)	<0.01**
PI610820	90.4(± 5.5)	70.4(±20.8)	0.03**
PI201187	87.7(±12.7)	76.6(± 8.5)	0.08
PI418701	93.4(± 2.7)	74.5(±24.5)	0.07

3.3.2.3 Total dry biomass

The effect of water deficit was overall reflected by a reduction of total dry biomass under stressed conditions. Differences in the reduction of dry biomass were observed between accessions, some had a significant reduction illustrating drought susceptibility, whereas other accessions had no clear reduction in dry biomass, indicating drought tolerance (see Fig. 3.10 and Table 3.16, Fig. 3.11 and Table 3.17).

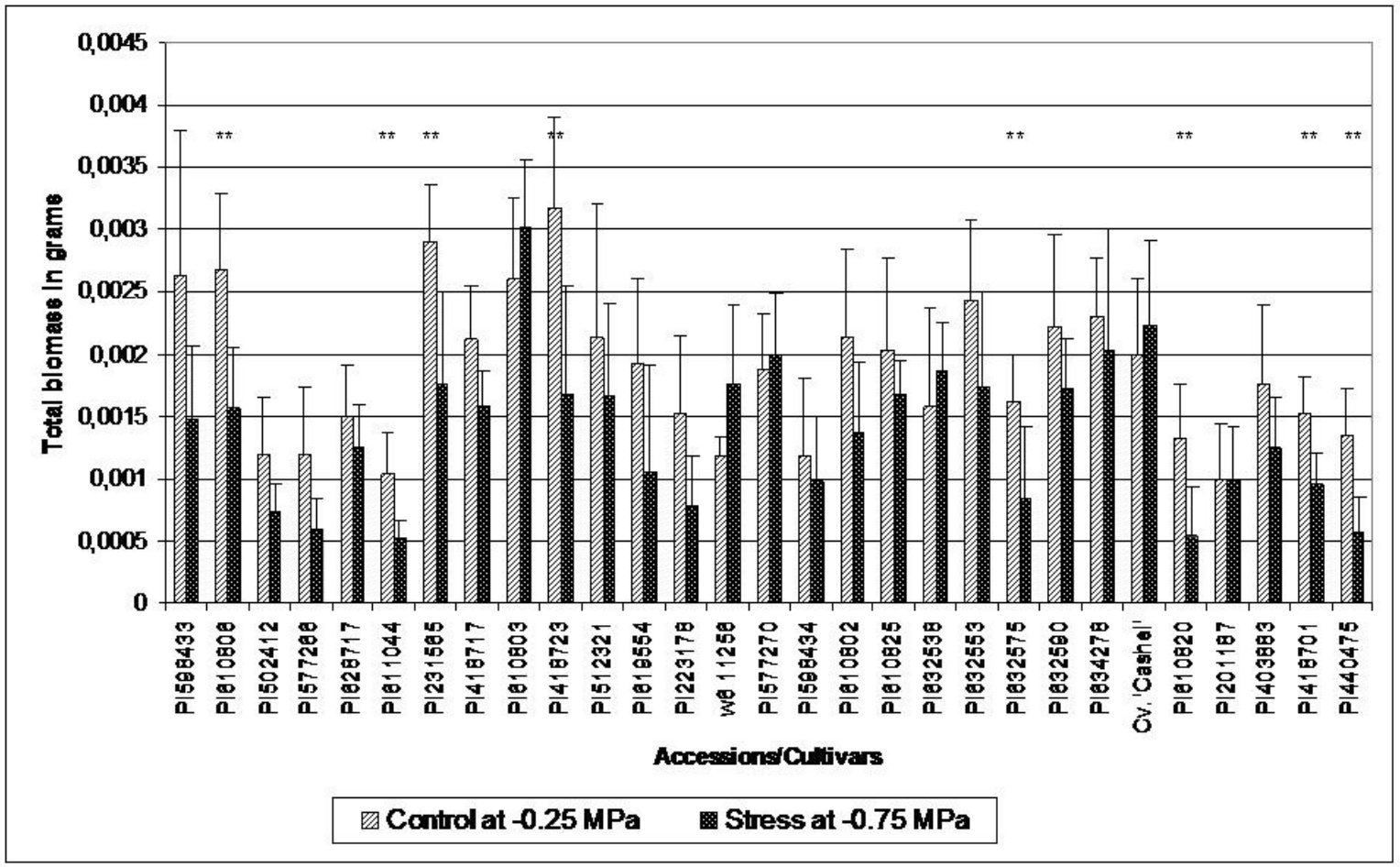


Fig. 3.10: Total Dry biomass of different accessions, under stressed (-0.75 MPa) and control conditions (-0.25 MPa). Error bars represent the standard deviation of the mean.
**: statistical differences between treatments (t-test, two-tailed distribution, unequal variance, $P<0.05$).

Table 3.16: Total Dry biomass of different accessions, subjected to control conditions (-0.25 MPa) and stressed conditions (-0.75 MPa). Between brackets are given the standard deviations. P-values are given base on a *t*-test (two-tailed distribution, unequal variance). ** = Total dry biomass in stressed conditions was significantly lower then in control conditions at P<0.05.

Accession / Cultivar	*Dry biomass of plants after control conditions (-0.25MPa) (g)*	*Dry biomass of plants after stress conditions (-0.9MPa) (g)*	*P values*
PI598433	0.00264(±0.0012)	0.00148(±0.0006)	0.10
PI610806	0.00268(±0.0006)	0.00156(±0.0005)	0.01**
PI502412	0.00120(±0.0005)	0.00074(±0.0002)	0.09
PI577266	0.00120(±0.0005)	0.00060(±0.0002)	0.07
PI628717	0.00150(±0.0004)	0.00126(±0.0003)	0.34
PI611044	0.00104(±0.0003)	0.00052(±0.0001)	0.02**
PI231565	0.00290(±0.0005)	0.00176(±0.0007)	0.02**
PI418717	0.00212(±0.0004)	0.00158(±0.0003)	0.05
PI610803	0.00260(±0.0007)	0.00302(±0.0005)	0.31
PI418723	0.00318(±0.0007)	0.00168(±0.0009)	0.02**
PI512321	0.00214(±0.0011)	0.00166(±0.0008)	0.44
PI619554	0.00192(±0.0007)	0.00106(±0.0008)	0.12
PI223178	0.00152(±0.0006)	0.00078(±0.0004)	0.06
w6 11256	0.00118(±0.0002)	0.00176(±0.0006)	0.11
PI577270	0.00188(±0.0004)	0.00200(±0.0005)	0.70
PI598434	0.00118(±0.0006)	0.00098(±0.0005)	0.60
PI610802	0.00214(±0.0007)	0.00136(±0.0005)	0.09
PI610825	0.00204(±0.0007)	0.00168(±0.0003)	0.35
PI632538	0.00158(±0.0008)	0.00186(±0.0004)	0.51
PI632553	0.00244(±0.0006)	0.00174(±0.0008)	0.16
PI632575	0.00162(±0.0004)	0.00084(±0.0006)	0.04**
PI632590	0.00222(±0.0007)	0.00172(±0.0004)	0.23
PI634278	0.00231(±0.0005)	0.00204(±0.0010)	0.59
Cv. 'Cashel'	0.00200(±0.0006)	0.00224(±0.0007)	0.63
PI610820	0.00132(±0.0004)	0.00054(±0.0004)	0.02**
PI201187	0.00100(±0.0004)	0.00098(±0.0004)	0.94
PI403883	0.00176(±0.0006)	0.00126(±0.0004)	0.21
PI418701	0.00153(±0.0003)	0.00095(±0.0003)	0.03**
PI440475	0.00135(±0.0004)	0.00058(±0.0003)	0.02**

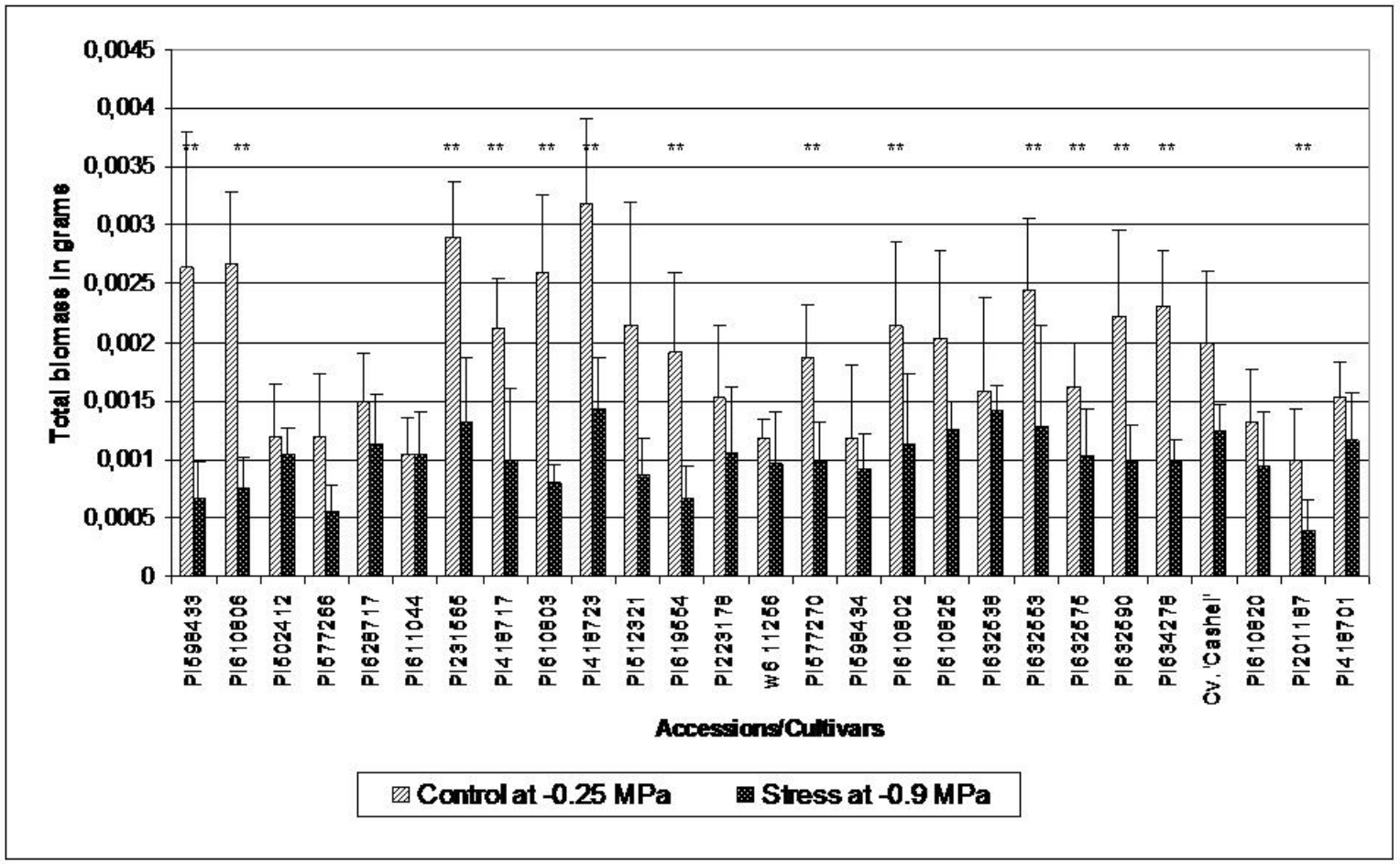


Fig. 3.11: Total Dry biomass of different accessions, under stressed (-0.9 MPa) and control conditions (-0.25 MPa). Error bars represent the standard deviation of the mean.
**: statistical differences between treatments (t-test, two-tailed distribution, unequal variance, $P<0.05$).

Table 3.17: Total Dry biomass (in g) of different accessions, subjected to control conditions (-0.25 MPa) and stressed conditions (-0.9 MPa). Between brackets are given the standard deviations. P-values are given base on a *t*-test (two-tailed distribution, unequal variance).
** = Total dry biomass after stressed conditions was significantly lower then after control conditions at P<0.05.

Accession / Cultivar	*Dry biomass of plants during control conditions (-0.25MPa) (g).*	*Dry biomass of plants during stress conditions (-0.9MPa) (g)*	*P values*
PI598433	0.00264(±0.0012)	0.00066(±0.0003)	0.02**
PI610806	0.00268(±0.0006)	0.00076(±0.0003)	<0.01**
PI502412	0.00120(±0.0005)	0.00104(±0.0002)	0.51
PI577266	0.00120(±0.0005)	0.00056(±0.0002)	0.05
PI628717	0.00150(±0.0004)	0.00114(±0.0004)	0.20
PI611044	0.00104(±0.0003)	0.00104(±0.0004)	1.00
PI231565	0.00290(±0.0005)	0.00132(±0.0006)	<0.01**
PI418717	0.00212(±0.0004)	0.00100(±0.0006)	0.01**
PI610803	0.00260(±0.0007)	0.00080(±0.0002)	<0.01**
PI418723	0.00318(±0.0007)	0.00144(±0.0004)	<0.01**
PI512321	0.00214(±0.0011)	0.00086(±0.0003)	0.05
PI619554	0.00192(±0.0007)	0.00066(±0.0003)	0.01**
PI223178	0.00152(±0.0006)	0.00106(±0.0006)	0.26
w6 11256	0.00118(±0.0002)	0.00096(±0.0005)	0.35
PI577270	0.00188(±0.0004)	0.00098(±0.0003)	0.01**
PI598434	0.00118(±0.0006)	0.00092(±0.0003)	0.44
PI610802	0.00214(±0.0007)	0.00114(±0.0006)	0.04**
PI610825	0.00204(±0.0007)	0.00126(±0.0002)	0.08
PI632538	0.00158(±0.0008)	0.00142(±0.0002)	0.69
PI632553	0.00244(±0.0006)	0.00128(±0.0009)	0.04**
PI632575	0.00162(±0.0004)	0.00102(±0.0004)	0.04**
PI632590	0.00222(±0.0007)	0.00098(±0.0003)	0.02**
PI634278	0.00231(±0.0005)	0.00098(±0.0002)	<0.01**
Cv. 'Cashel'	0.00200(±0.0006)	0.00124(±0.0002)	0.15
PI610820	0.00132(±0.0004)	0.00094(±0.0005)	0.22
PI201187	0.00100(±0.0004)	0.00040(±0.0002)	0.04**
PI418701	0.00153(±0.0003)	0.00117(±0.0004)	0.27

3.3.2.4 Overview of results for drought response, based on both RWC and total dry biomass evaluation

In table 3.18, an overview of the results is given, separated by accession and test. Based on the results obtained with the PEG-infused *in vitro* system, accessions were selected that were drought susceptible, drought tolerant or intermediate in response (see Table 3.18, red annotated accessions). These cultivars were subsequently assessed with an *in vivo* drought stress experiment to confirm these findings.

Table 3.18: Overview of accessions and their response to drought stress, based on RWC and total dry biomass.
+ = result indicating drought tolerance, - = result indicating drought susceptibility
Red annotated accessions were chosen for an *in vivo* drought stress experiment.

Cultivar	*RWC -0.75 MPa*	*Dry weight -0.75 MPa*	*RWC -0.9 MPa*	*Dry weight -0.9 MPa*	*Overall response*
PI 577266	+	+	+	+	Drought tolerant
PI 632538	+	+	+	+	Drought tolerant
PI 502412	-	+	+	+	Moderately drought tolerant
PI 628717	-	+	+	+	Moderately drought tolerant
PI 223178	-	+	+	+	Moderately drought tolerant
PI 598434	+	+	-	+	Moderately drought tolerant
PI 632553	+	+	+	-	Moderately drought tolerant
PI 632590	-	+	+	-	Intermediate
PI 634278	-	+	+	-	Intermediate
Cv. 'Cashel'	-	+	-	+	Intermediate
PI 598433	-	+	+	-	Intermediate
PI 610820	+	-	-	+	Intermediate
PI 611044	+	-	-	+	Intermediate
PI 418717	-	+	+	-	Intermediate
PI 610803	-	+	+	-	Intermediate
PI 418701	-	-	+	+	Intermediate
PI 201187	-	+	+	-	Intermediate

Table 3.18 continued..

Cultivar	*RWC -0.75 MPa*	*Dry weight -0.75 MPa*	*RWC -0.9 MPa*	*Dry weight -0.9 MPa*	*Overall response*
PI 512321	-	+	-	+	Intermediate
PI 619554	-	+	+	-	Intermediate
W6 11256	-	+	-	+	Intermediate
PI 610825	-	+	-	+	Intermediate
PI 610802	-	+	-	-	Moderately drought susceptible
PI 577270	-	+	-	-	Moderately drought susceptible
PI 440475	-	-	n.a.	n.a.	Drought susceptible
PI 610806	-	-	-	-	Drought susceptible
PI 231565	-	-	-	-	Drought susceptible
PI 418723	-	-	-	-	Drought susceptible
PI 632575	-	-	-	-	Drought susceptible

n.a.: not analysed

3.3.3 *In vivo* PEG-induced system to evaluate drought tolerance

3.3.3.1 Evaluation of accessions of *Lolium perenne* L. on drought stress response using an *in vivo* PEG-induced system.

Eleven accessions were selected based on initial results from the *in vitro* PEG-infused system (see Table 3.18, red annotated accessions), another cultivar 'New Zealand' was added to this experiment as this was described as a drought tolerant accession (Foito et al. 2009). Unfortunately not all accessions managed to propagate enough tillers, to accumulate a sufficient number of clones, therefore in the end nine accessions were assessed with this system as described in section 3.8.2. These accessions and cultivars

were Cv. ‘New Zealand’, Cv. ‘Cashel’, PI231565, PI610825, PI201187, PI418701, PI611044, PI632553 and PI223178.

After the exposure to the stress treatment, three factors were assessed to determine drought response. Firstly the growth was recorded using pixel detection (see 3.3.3.2), secondly the relative water content (RWC) was recorded (see 3.3.3.3) and thirdly the root dry weights were analysed (3.3.3.4). The results were subsequently analysed for statistical differences.

3.3.3.2 Pixel detection to record growth differences between plants exposed to different treatments

After two weeks in stressed or non-stressed conditions, plants were removed and assessed for their phenotypical differences. Photographs were taken (see Fig. 3.12 – 3.14), and subsequently analysed for root and shoot development using the pixel detection method with the help of Adobe Photoshop 5.5 (see section 3.2.9.1). Differences in shoot and root development were documented and shown in sections 3.3.3.2.1 and 3.3.3.2.2

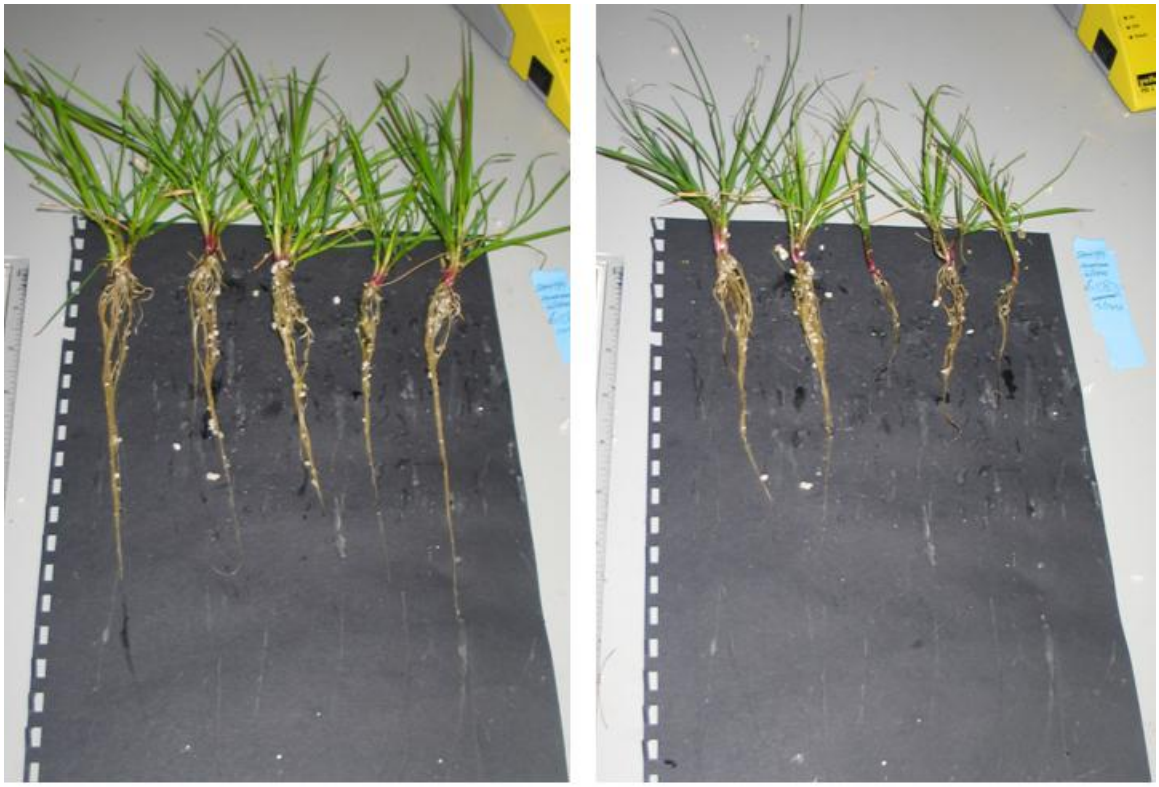


Fig. 3.12: Photographs taken of accession PI 610825 after different treatments (left: hydroponics without addition of PEG to mimic optimal conditions, right: hydrophonics with additions of PEG to mimic drought stress conditions)

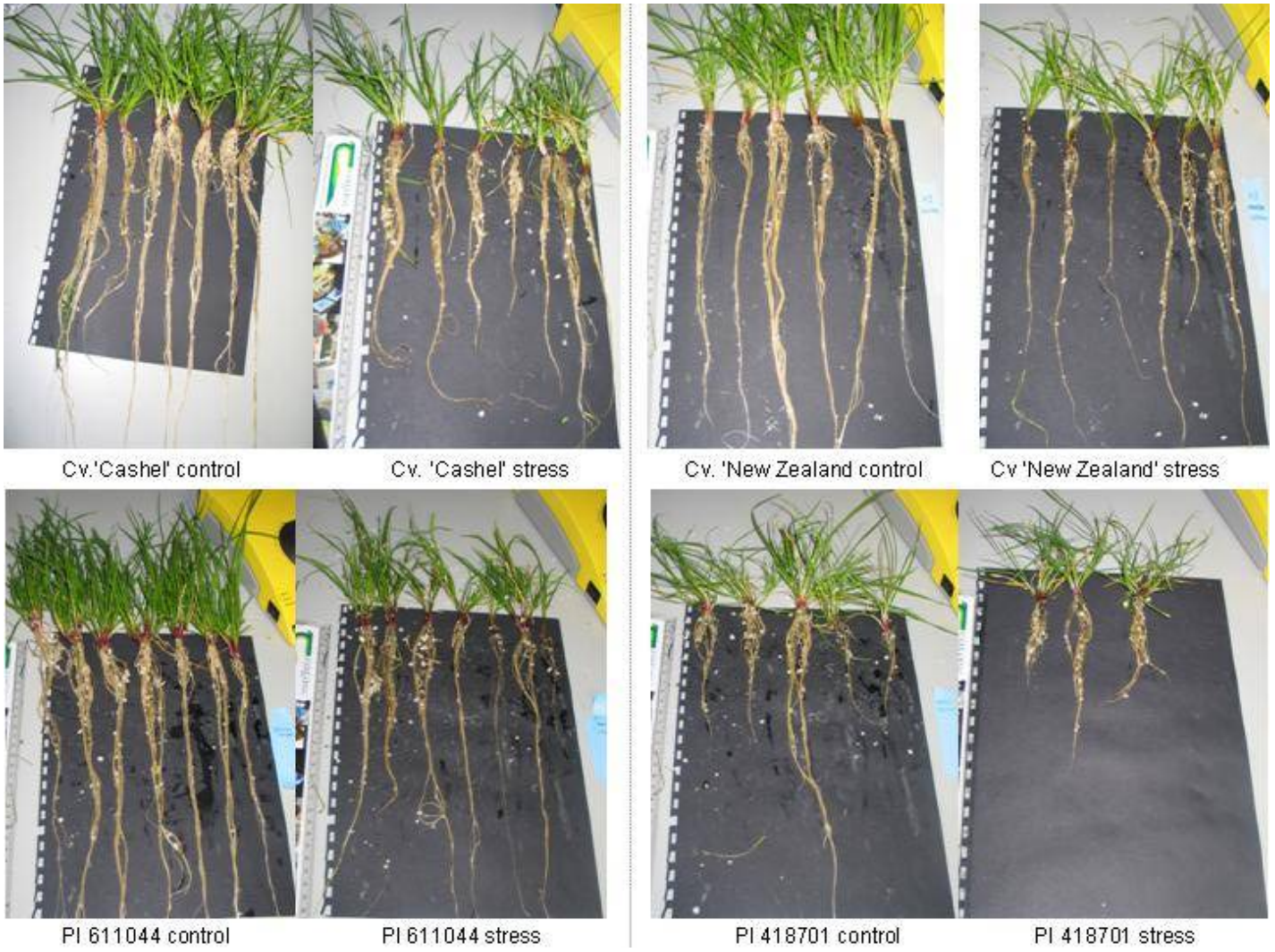


Fig. 3.13: Photographs taken of Cv. 'Cashel', Cv. 'New Zealand', accession PI 611044 and accession PI 418701 after different treatments (left: hydroponics without addition of PEG to mimic optimal conditions, right: hydrophonics with additions of PEG to mimic drought stress conditions)

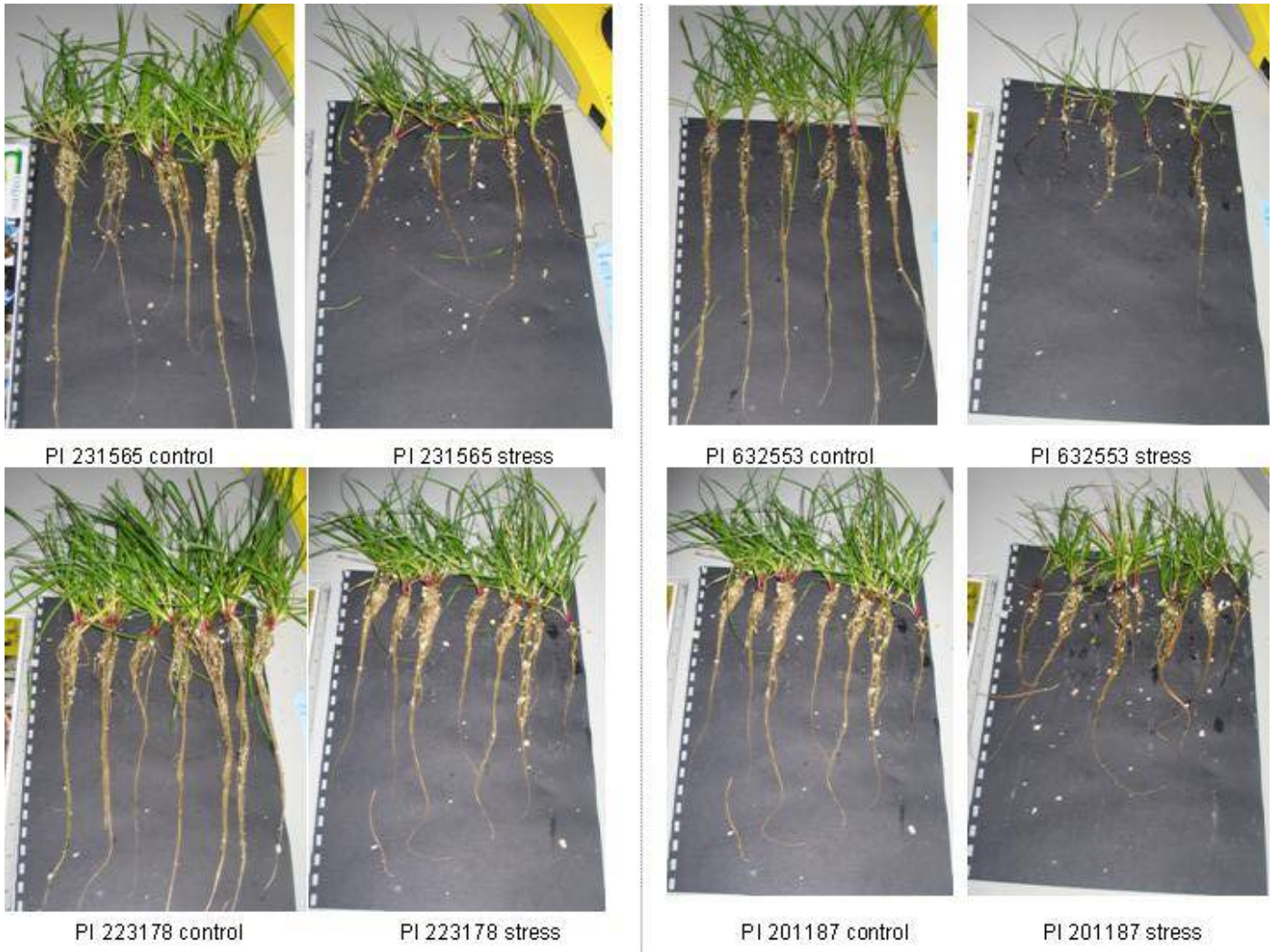


Fig. 3.14: Photographs taken of accession PI 231565, accession PI 632553, accession PI 223178 and accession PI 201187 after different treatments (left: hydroponics without addition of PEG to mimic optimal conditions, right: hydrophonics with additions of PEG to mimic drought stress conditions)

3.3.3.2.1 Root development differences after exposure to drought stress and control conditions

Photographs were analysed using pixel detection. The increase in root biomass under drought stress reflects an adaptive response involving an increase in root length to reach water deeper in the soil (van den Berg and Zeng 2006). Accessions PI611044, PI632553, PI223178, PI201187 and PI231565 showed a significant decrease in root development after exposure to drought stress compared to control conditions, indicating that these accessions are drought susceptible. Accession PI418701 seemed to have a slight increase in root growth during drought stress, compared to control conditions, suggesting a drought tolerance response. However this could not be proven statistically. Accession PI610825 and cultivars 'Cashel' and 'New Zealand' showed an apparent reduction in root development during stress, but this was not statistically significant (See Fig. 3.15 and Table 3.19).

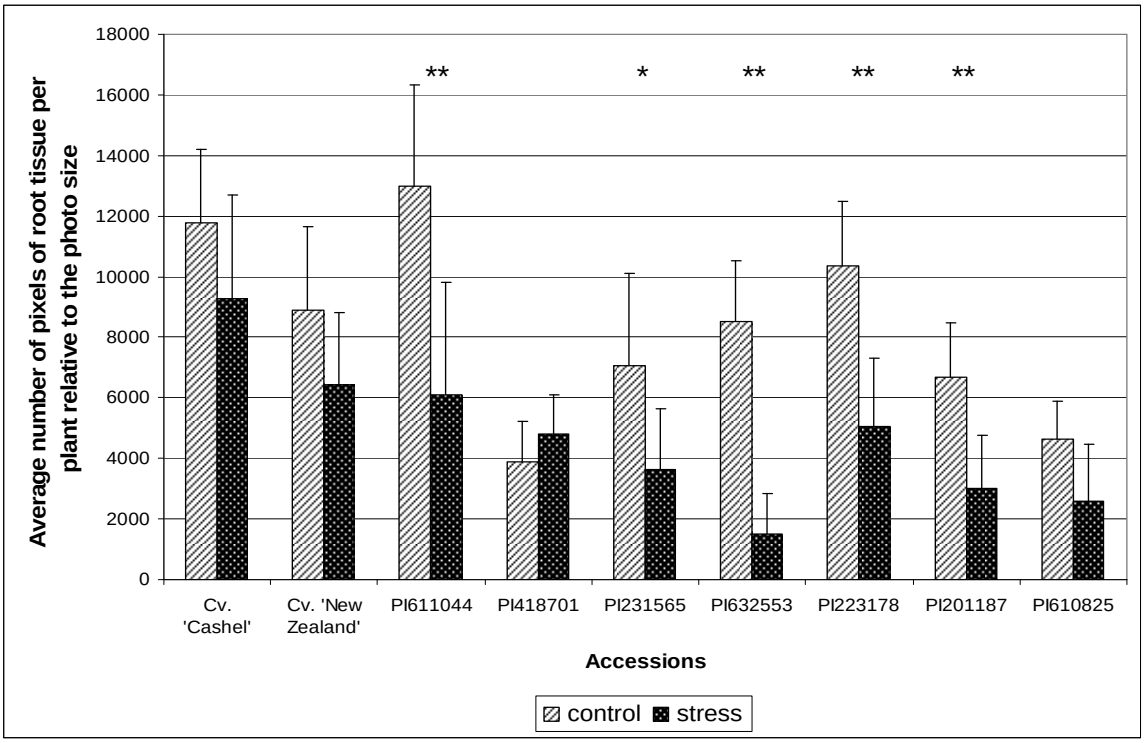


Fig. 3.15: Mean root biomass per plant based on pixel detection separated per accession. Statistical differences in number of pixels between stressed and unstressed treatments were calculated according to a *t*-test (two-tailed distribution, unequal variance) Error bars represent the standard deviation of the mean.
**: statistical differences at P<0.01,
*: statistical difference at P<0.05.

Table 3.19: The mean root biomass per plant based on pixel detection; between brackets the standard deviations are presented. The pixel number has been converted to circumvent photo size differences. P-values are given for the *t*-test (two-tailed distribution, unequal variance).
** : Statistical difference between control and stressed conditions at P<0.01
* : Statistical difference between control and stressed conditions at P<0.05.

Accession/Cultivar	*Mean root biomass based on pixel values under control condition (pixels)*	*Mean root biomass based on pixel values under stress conditions (pixels)*	*P value*
Cv. 'Cashel'	11769(± 2450)	9281(± 2392)	0.07
Cv. 'New Zealand'	8879(± 2776)	6440(± 1758)	0.15
PI611044	12988(± 3343)	6107(± 3688)	<0.01 **
PI418701	3880(± 1343)	4810(± 1268)	0.40
PI231565	7068(± 3042)	3621(± 1997)	0.02 *
PI632553	8526(± 1997)	1508(± 1343)	<0.01 **
PI223178	10366(± 2122)	5047(± 2246)	<0.01 **
PI201187	6676(± 1794)	3005(± 1758)	<0.01 **
PI610825	4616(± 1285)	2608(± 1872)	0.09

3.3.3.2.2 Shoot development differences after exposure to drought stress and control conditions

The pixel detection method was used to determine the leaf biomass difference between accessions. However this could not be applied to separate clones within accessions, as the leaf material overlapped with each other. The average leaf biomass based on pixels per plant was calculated by taking the total number of pixels divided by the number of plants. For this reason no statistical analyses could be performed. Results show that accession PI418701 and cultivar 'New Zealand' had a slight decrease in leaf development under stress conditions, indicating a drought tolerant response for this specific clone within this accession or cultivar. All the other accessions and cultivars had a clear decrease in biomass production during stress conditions (see Fig. 3.16 and Table 3.20), conferring a drought susceptible response. This type of response was previously observed to correlate with drought response (Foito et al. 2009).

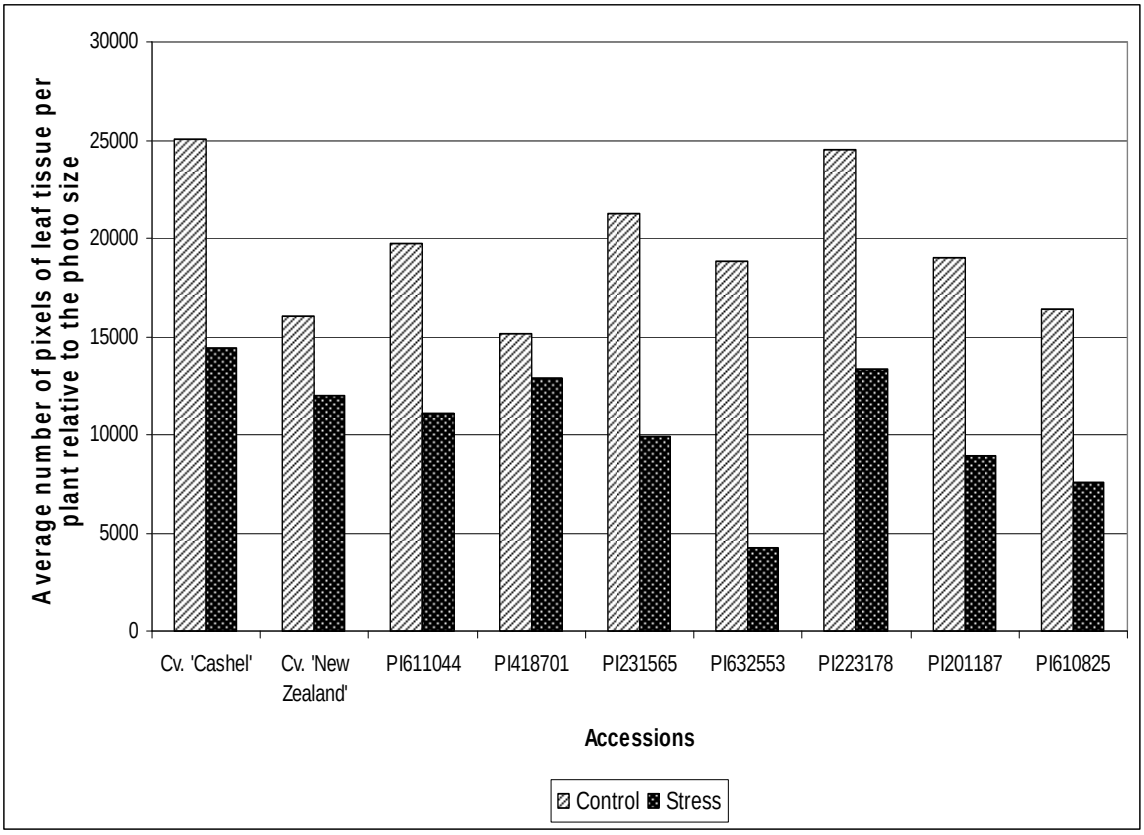


Fig. 3.16: Average leaf biomass per plant, derived from total leaf biomass, based on pixel detection per cultivar/accession. No statistical analyses could be performed.

Table 3.20: Average leaf biomass per plant, based on pixel detection. The pixel number has been converted to circumvent photo size differences.

Accession/Cultivar	*Mean leaf biomass based on pixel values under control conditions*	*Mean leaf biomass based on pixel values under stress conditions*
Cv. 'Cashel'	25076	14433
Cv. 'New Zealand'	16029	12015
PI611044	19708	11086
PI418701	15123	12915
PI231565	21273	9925
PI632553	18820	4275
PI223178	24541	13374
PI201187	19025	8883
PI610825	16440	7556

3.3.3.3 Results of drought response using the RWC and Root dry biomass.

3.3.3.3.1 Relative water content

There was a statistical difference in relative water content between stressed and non-stressed plants for Cv. 'Cashel, Cv. 'New Zealand', accession PI231565 and accession PI632553 (see Fig. 3.17 and Table 3.21). This indicates that these particular clones of these accessions were susceptible to drought stress.

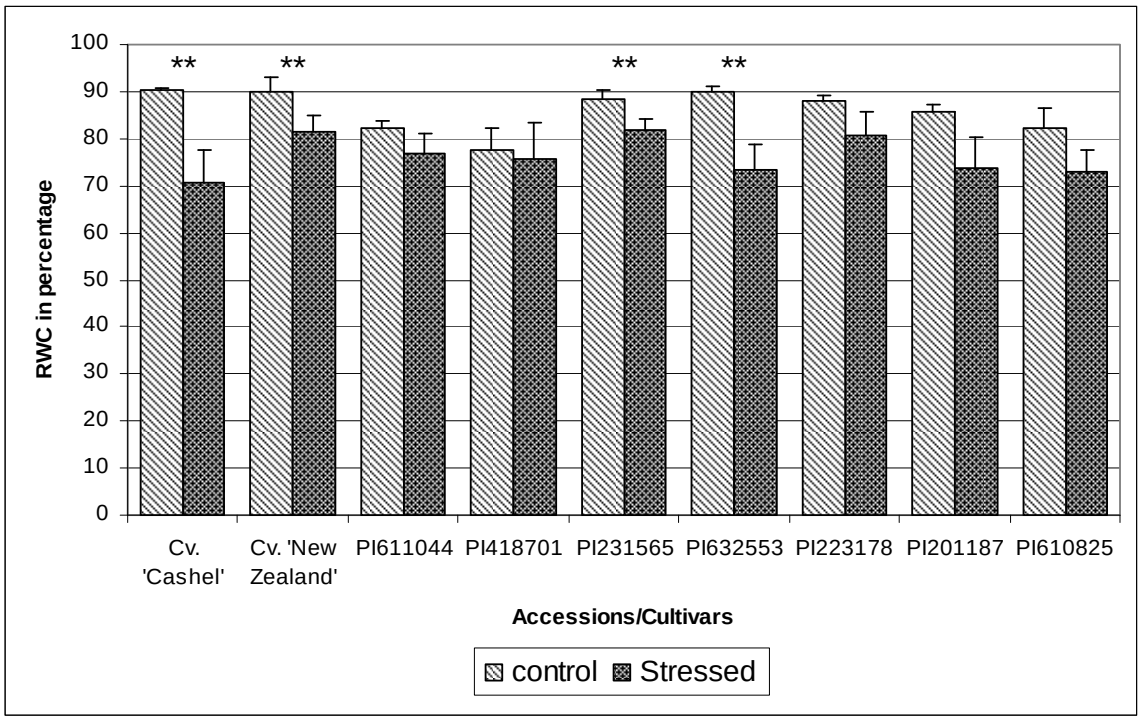


Fig. 3.17: Relative water content after two weeks exposure to drought stress compared to control conditions. Error bars represent the standard deviation of the mean.
**: statistical difference between stressed and unstressed treatments according to a *t*-test (one-tailed distribution, equal variance on arcsine transformed values) at $P<0.05$.

Table 3.21: Relative water content (RWC) values derived from plants subjected to control and stressed conditions. P values are based on a *t*-test (one-tail distribution, equal variance) on arcsine transformed values.
**: statistical difference between control and stressed plants at P<0.05.

Cultivar / Accession	*RWC Control treatment*	*RWC stress treatment*	*P values*
Cv. 'Cashel'	90.3(±0.4)	70.8(±6.7)	<0.01**
Cv. 'New Zealand'	90.0(±3.0)	81.5(±3.3)	0.04**
PI611044	82.1(±1.9)	76.7(±4.2)	0.14
PI418701	77.8(±4.6)	75.7(±7.8)	0.41
PI231565	88.4(±2.1)	82.0(±2.2)	0.02**
PI632553	90.1(±1.3)	73.4(±5.6)	<0.01**
PI223178	87.9(±1.5)	80.7(±5.0)	0.08
PI201187	85.6(±1.7)	73.7(±6.8)	0.05
PI610825	82.1(±4.2)	73.1(±4.5)	0.09

3.3.3.3.2 Root dry biomass

There was a statistical difference in root dry biomass between treatments for accession PI632553 and accession PI223178 (See Fig. 3.18 and Table 3.22). This indicates that clones of these accessions were more susceptible to drought then the other accessions. Accession PI418701 had a slight increase in root dry biomass however this could not be statistically proven.

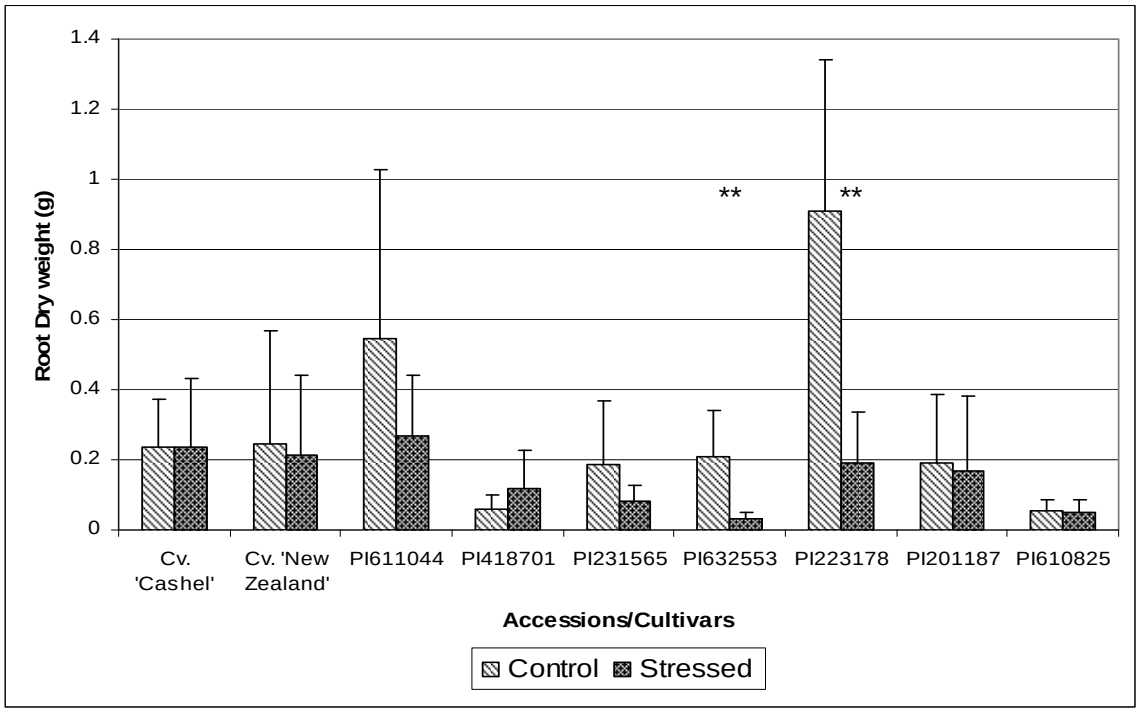


Fig. 3.18: The mean root dry biomass per accession. Error bars represent the standard deviation of the mean.
**: Accessions, which had a statistical difference in dry root biomass (*t*-test, two tailed distribution, unequal variance $P<0.05$) between stressed and non-stressed conditions.

Table 3.22: Root dry biomass (in g) for both treatments for different accessions / cultivars. P values are based on a *t*-test (two-tailed distribution, unequal variance) between treatments.
**: Statistical difference between control conditions and stresses conditions at P<0.05.

Cultivars / Accessions	*Root dry biomass under after control conditions (g)*	*Root dry biomass under after stress conditions (g)*	*P values*
Cv. 'Cashel'	0.24(±0.14)	0.24(±0.20)	1.00
Cv. 'New Zealand'	0.25(±0.32)	0.21(±0.23)	0.84
PI611044	0.55(±0.48)	0.27(±0.17)	0.19
PI418701	0.06(±0.04)	0.12(±0.11)	0.47
PI231565	0.19(±0.18)	0.08(±0.04)	0.22
PI632553	0.21(±0.13)	0.03(±0.02)	0.02**
PI223178	0.91(±0.43)	0.19(±0.14)	<0.01**
PI201187	0.19(±0.20)	0.17(±0.21)	0.86
PI610825	0.06(±0.03)	0.05(±0.04)	0.79

The reduction or increase in root dry biomass under stress conditions could also be visualized differently, Fig.3.19 and Table 3.23 show that accession PI418701 had an almost 100% increase in root biomass compared to control conditions, whereas accession PI632553 had the most dramatic reduction in root biomass.

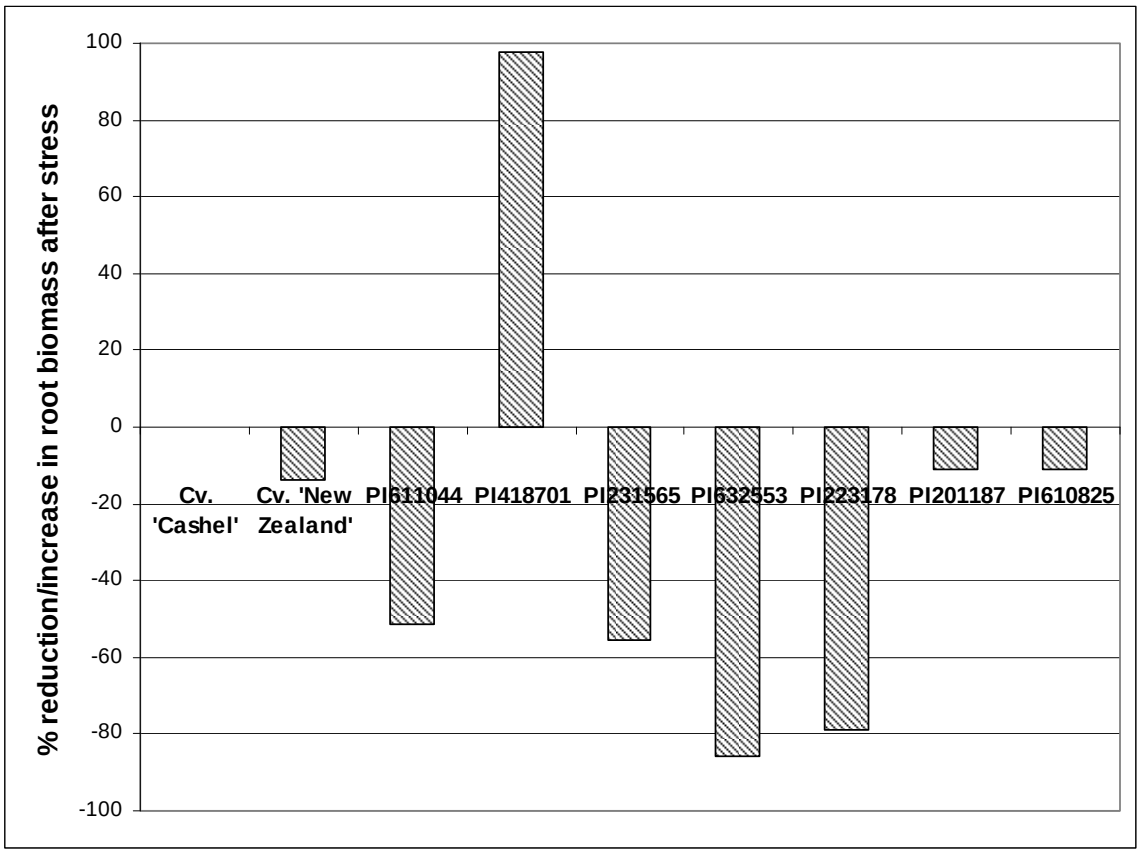


Fig. 3.19: Reduction/Increase of root dry biomass after stress, compared to the control.

Table 3.23: Reduction/increase of root dry biomass after stress, in comparison to control conditions in percentage.

Cultivars / Accessions	*Reduction/increase of root dry biomass after stress, in comparison to control conditions in percentage.*
Cv. 'Cashel'	0
Cv. 'New Zealand'	- 14
PI611044	- 51
PI418701	+ 98
PI231565	- 55
PI632553	- 86
PI223178	- 79
PI201187	- 11
PI610825	- 11

3.3.3.4 Overview of results after analyses of plants exposed to drought stress with the *in vivo* PEG induced system.

The results obtained from the phenotypical analyses and the analytical analyses were combined to determine how each accession was affected by drought stress. The responses are ranked in Table 3.24.

Accession PI418701 was not subject to negative effects under drought stress, so the clones of this accession could be considered drought tolerant. Cultivar 'New Zealand' was only mildly affected by drought. Accession PI610825 had an intermediate negative response under drought stress. The following cultivars and accessions had an increasing drought susceptible response, Cv. 'Cashel', PI231565, PI201187, PI611044, PI223178 and PI632553.

Table 3.24: Overall review of the results for the *in vivo* drought stress experiment.
+ = No difference in response between stressed and non-stressed conditions
I = Moderate difference in response between stressed and non-stressed conditions
- = Distinct difference in response between stressed and non-stressed conditions
Overall response was scored by taking the average of +'s, I's and –'s.

Accession/Cultivar	*Phenotypical assessment of shoot development*	*Phenotypical assessment of root development*	*Relative water content*	*Root dry biomass*	*Overall*
Accession PI418701	+	+	I	+	+ 3
Cv. 'New Zealand'	+	+	-	I	+ 1
Accession PI610825	-	+	I	I	0
Cv. 'Cashel'	-	+	-	I	- 1
Accession PI231565	-	I	-	I	- 2
Accession PI201187	-	-	I	I	- 2
Accession PI611044	-	-	I	I	- 2
Accession PI223178	-	-	I	-	- 3
Accession PI632553	-	-	-	-	- 4

3.3.4 Assessment of RNA editing efficiency within the *ndhB* and *ndhF* transcripts of accessions with various drought tolerance responses.

Tissue derived from the *in vivo* PEG induced system were analysed for RNA editing events using the trace-file method.

3.3.4.1 RNA editing efficiency in the *ndhB* trancript of accessions of *Lolium perenne* L. tested in the *in vivo* PEG-induced drought stress experiment

There were no statistical significant differences in RNA editing within accessions between stressed and non-stressed clones for any of the analysed editing sites. However there were differences between accessions for editing efficiencies. For the editing sites

within the *ndhB* transcript the editing efficiencies of Cv. 'Cashel', Cv. 'New Zealand', accessions PI611044, PI223178, PI201187 and PI610825 were statistically different ($P<0.05$) in comparison with the editing efficiencies of accessions PI418701, PI231565 and PI632553. (See Fig. 3.20 and Table 3.25). The observed differences were dramatic. Some accessions showed almost complete editing, while other accessions almost completely lacked editing at these sites.

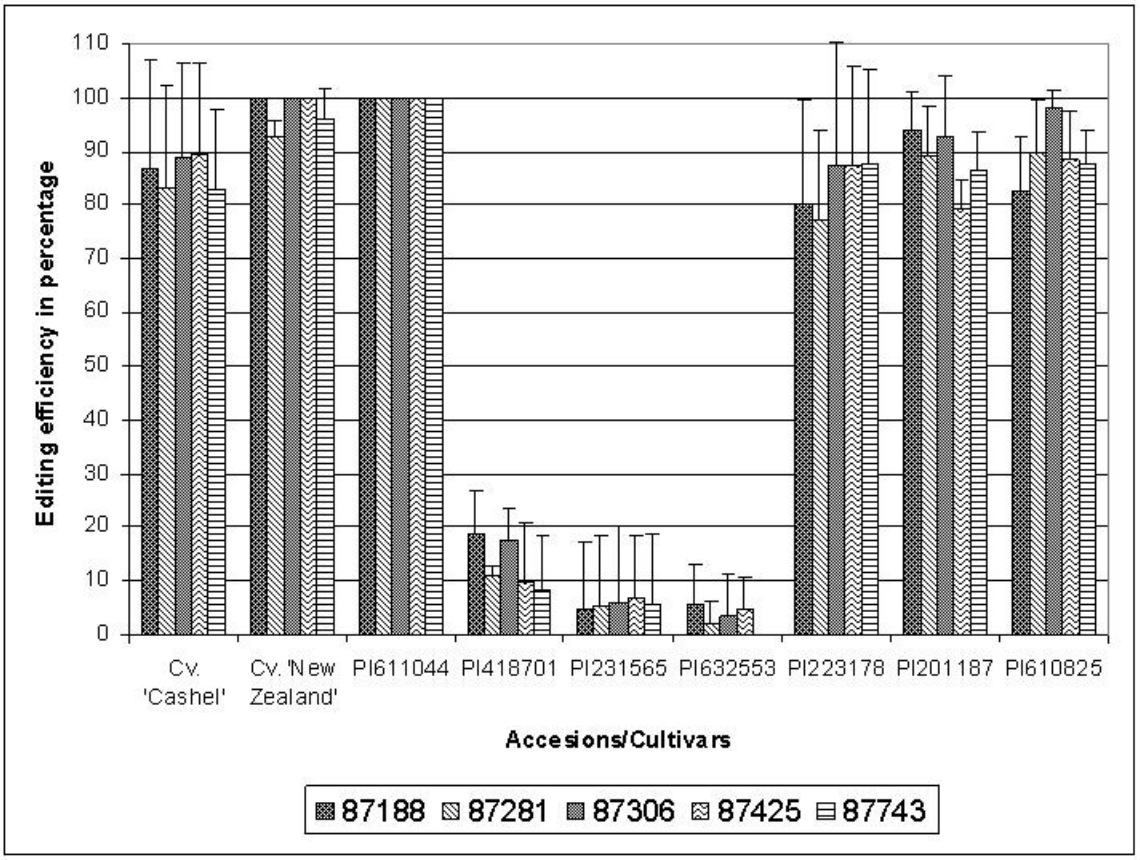


Fig 3.20: RNA-editing efficiency within the *ndhB* transcript of editing sites located on plastid genome positions 87188, 87281, 87306, 87425 and 87743. Error bars represent the standard deviation of the mean.

Table 3.25: Editing efficiency in percentage of editing sites within the *ndhB* transcript, including grouping for statistical differences based on a *t*-test (two-tailed distribution, equal variance, on arcsine transformed values at P<0.05).
[1] = not enough replicates to do statistical analyses.

Cultivar - Editing site	*Editing efficiency at site: 87188 in %*	*Editing efficiency at site: 87281 in %*	*Editing efficiency at site: 87306 in %*
Cv. 'Cashel'	86.8(±20.5) [a,b]	83.4(±19.0) [a]	89.0(±17.7) [a]
Cv. 'New Zealand'	100.0(± 0.0) [a]	92.7(± 3.2) [a]	100.0(± 0.0) [a]
Accession PI611044	100.0(± 0.0) [1]	100.0(± 0.0) [1]	100.0(± 0.0) [1]
Accession PI418701	18.6(± 8.2) [c]	11.2(± 1.5) [b]	17.5(± 6.1) [b,c]
Accession PI231565	5.0(±12.2) [d]	5.3(±13.0) [b,c]	5.9(±14.4) [c]
Accession PI632553	5.5(± 7.6) [d]	2.0(± 4.4) [c]	3.5(± 7.8) [c,d]
Accession PI223178	80.1(±19.5) [b]	77.5(±16.6) [a]	87.3(±22.9) [a,b]
Accession PI201187	93.9(± 7.0) [a,b]	89.3(± 9.2) [a]	92.7(±11.3) [a]
Accession PI610825	82.7(±10.0) [b]	89.8(± 9.7) [a]	98.3(± 2.9) [a]

Cultivar - Editing site	*Editing efficiency at site: 87425 in %*	*Editing efficiency at site: 87743 in %*
Cv. 'Cashel'	89.4(±17.0) [a]	83.0(±14.9) [a]
Cv. 'New Zealand'	100.0(± 0.0) [a]	96.2(± 5.4) [a]
Accession PI611044	100.0(± 0.0) [1]	100.0(± 0.0) [1]
Accession PI418701	9.7(± 9.7) [b]	8.5(± 9.8) [b]
Accession PI231565	6.9(± 11.4) [b]	5.4(± 13.2) [b]
Accession PI632553	4.9(± 5.9) [b]	0.0(± 0.0) [b]
Accession PI223178	87.5(± 18.3) [a]	87.7(± 17.3) [a]
Accession PI201187	79.4(± 5.1) [a]	86.5(± 7.1) [a]
accession PI610825	88.3(± 9.0) [a]	87.8(± 6.3) [a]

3.3.4.2 RNA editing efficiency in the *ndhF* transcript of accessions of *Lolium perenne* L. tested in the *in vivo* PEG-induced drought stress experiment

The known editing site at genome position 103675 within the *ndhF* transcript showed a similar difference between accessions as was evident for the *ndhB* editing sites, however the editing efficiency of accessions PI223178 and PI610825 were not statistically different ($P<0.05$) from the efficiency of accession PI418701 (See Fig. 3.21 and Table 3.26).

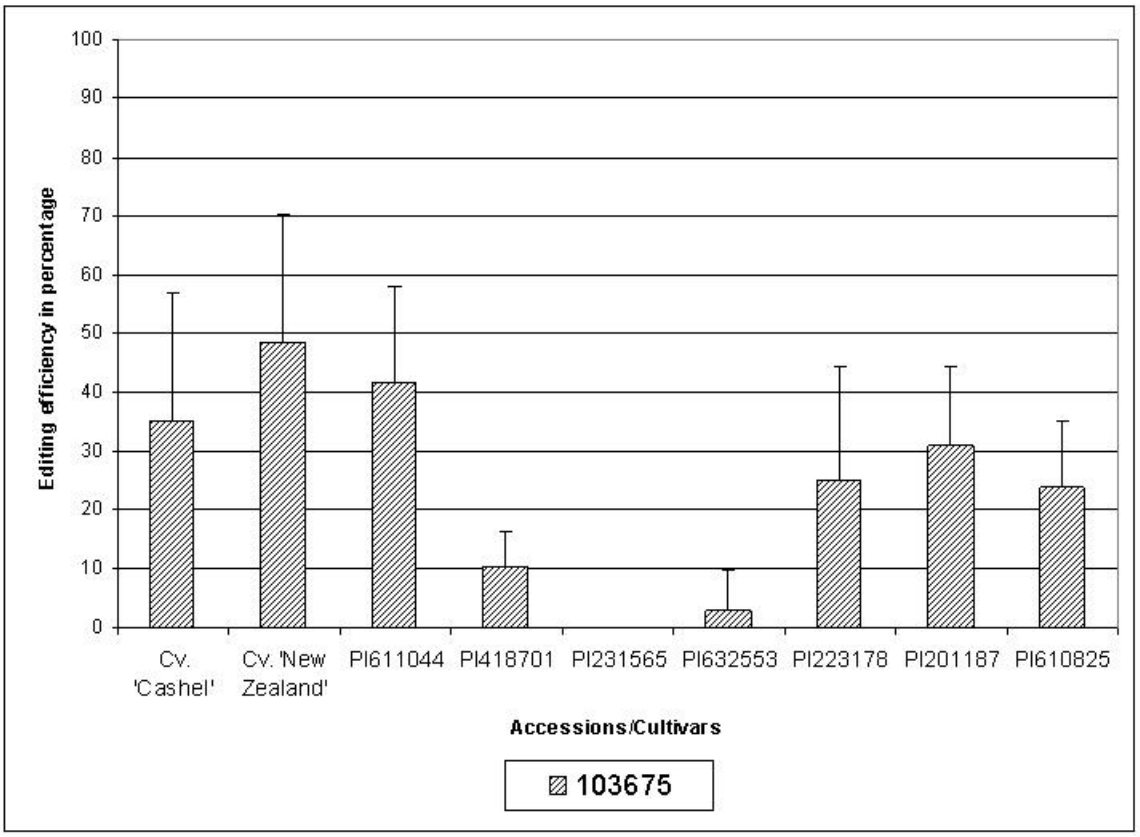


Fig. 3.21: RNA-editing efficiency of the editing site within the *ndhF* transcript located on plastid genome position 103675. Error bars represent the standard deviation of the mean.

Table 3.26: Editing efficiency in percentages of editing site 103675 within the *ndhF* transcript, including grouping for statistical differences based on a *t*-test (two-tailed distribution, equal variance, on arcsine transformed values at P<0.05).

Cultivar - Editing site	*Editing efficiency at site 103675 in %*
Cv. 'Cashel'	35.3(±21.6) [a]
Cv. 'New Zealand'	48.5(±21.7) [a]
Accession PI611044	41.8(±16.1) [a]
Accession PI418701	10.1(± 6.3) [b,c]
Accession PI231565	0.0(± 0.0) [d]
Accession PI632553	2.8(± 6.8) [c,d]
Accession PI223178	25.1(±19.2) [a,b]
Accession PI201187	30.9(±13.6) [a]
Accession PI610825	23.9(±11.1) [a,b]

3.3.5 Assessment of relationship between drought stress response and RNA editing efficiency within the *ndhB* and *ndhF* transcripts

There was no correlation between drought tolerance and editing efficiencies for editing sites within the *ndhB* and the *ndhF* genes (See Table 3.27). For example, accessions PI418701 and PI632553 had similar editing patterns, while their drought response was distinctly different. The opposite was shown for two different clones (genotypes) of cultivar 'New Zealand', these showed a distinctly different editing pattern, while the drought stress response was similar (See Table 3.27).

Table 3.27: Editing efficiency in relationship to drought tolerance for the tested clones of different accessions.

Accessions / Cultivars (specific genotypes)	*Editing efficiency of editing sites within the ndhB gene*	*Editing efficiency of editing sites within the ndhF gene*	*Drought stress response*
Accession PI418701	Low	Moderately low	Tolerant
Cv. 'New Zealand' (Foito et al. 2009)	Moderately high	High	Tolerant
Cv. 'New Zealand'	High	High	Tolerant
Accession PI610825	High	Moderately high	Moderately tolerant
Cv. 'Cashel'	High	High	Intermediate
Accession PI231565	Low	Low	Intermediate
Accession PI201187	High	High	Intermediate
Accession PI611044	High	High	Intermediate
Accession PI223178	High	Moderately high	Moderately susceptible
Accession PI632553	Low	Low	Susceptible
Cv. 'Cashel' (Foito et al. 2009)	High	High	Susceptible

3.3.6 RNA editing of *Lolium perenne* L. accession-specific Single nucleotide polymorphism (SNP)

During analyses of the trace-files for the *ndhF* fragment a transition single nucleotide polymorphism (SNP) appeared for some accessions at genome position 103515. The conserved gDNA nucleotide was a thymine (T) in Cv. 'Cashel' and accessions PI418701, PI231565 and PI610825. However in Cv. 'New Zealand' and accessions PI611044, PI632553, PI223178 and PI201187 the thymine nucleotide at genome position 103515 was present in conjunction with a cytosine (C) (See Table 3.28 and Fig. 3.22). The SNP results in an amino-acid change from Serine to Proline. However at the mRNA level, this nucleotide was converted back to the Uracil (U), reconstituting the amino-acid Serine.

Table 3.28: The ratio of the Single nucleotide polymorphism on position 103515 within the plastid genome, for different accessions/cultivars of *Lolium perenne* L.

Accession / Cultivar	*Origin*	*Nucleotide 1*	*Nucleotide 2*	*Percentage nucleotide 1 at the gDNA level*	*Percentage nucleotide 1 at the cDNA level*
Cv. 'Cashel'	Ireland	T	C	100%	100%
Cv. 'New Zealand'	New Zealand	T	C	31%	100%
Accession PI611044	Russia	T	C	21%	100%
Accession PI418701	Former Yugoslavia	T	C	100%	100%
Accession PI231565	Libya	T	C	100%	100%
Accession PI632553	Italy	T	C	20%	100%
Accession PI223178	Greece	T	C	15%	100%
Accession PI201187	Netherlands	T	C	15%	100%
Accession PI610825	Switzerland	T	C	100%	100%

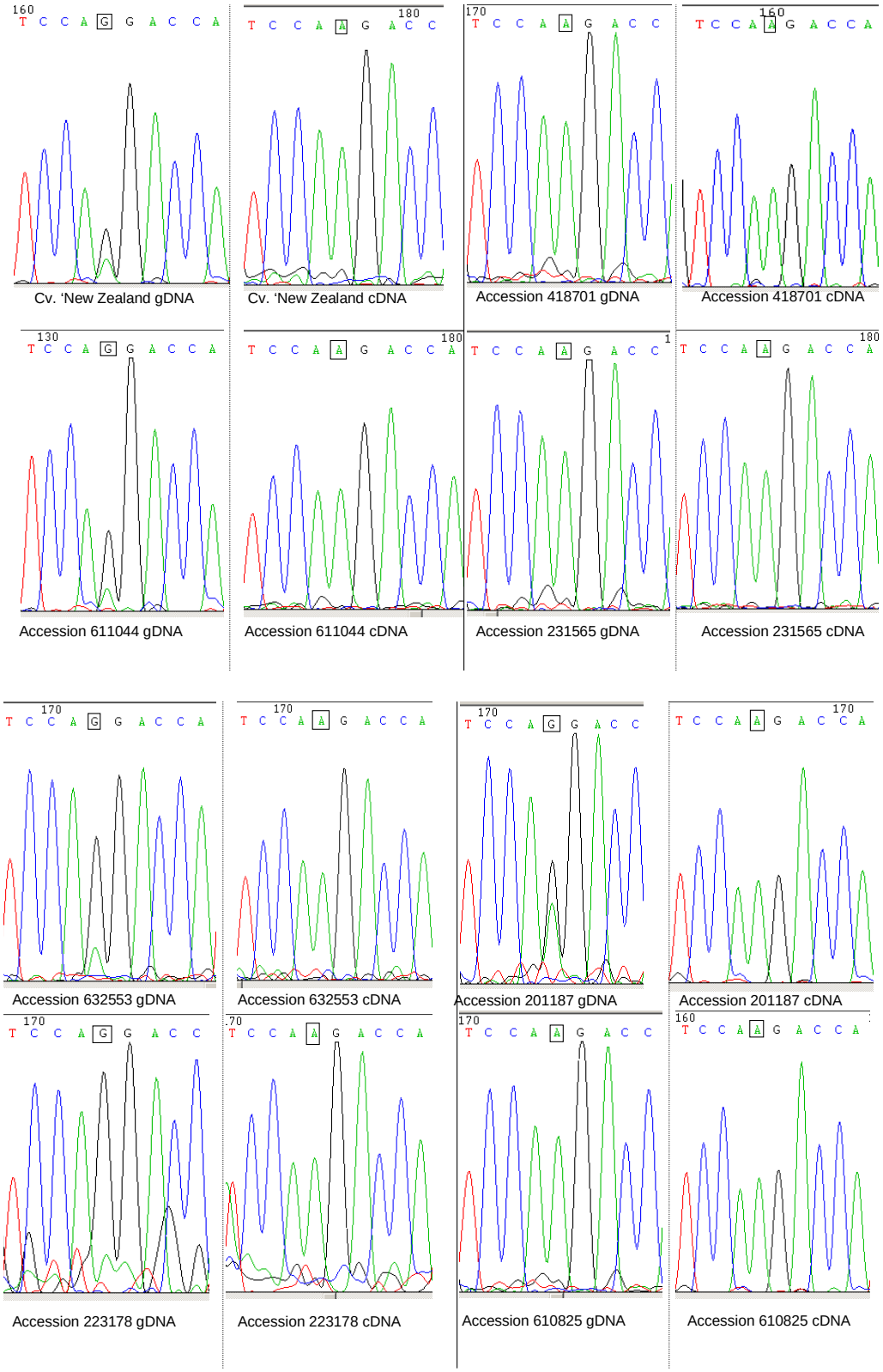


Fig. 3.22: Trace-files of gDNA and cDNA of tested accessions, showing the single nucleotide polymorphism (SNP) on genome position 103515 marked by a box, which is subsequently edited back.

Diekmann et al. (2009) found seven other SNP's within the plastid genome of *Lolium perenne* L. that could potentially be editing sites. Three of these were located within coding sequences, these were the SNP at genome position 65631 within the *rps18* transcript, the SNP at genome position 37506 within the *psaB* transcript and the SNP at genome position 19560 within the *rpoB* transcript. All three sites were assessed for SNP presence within each accession / cultivar. Only for the SNP within the *psaB* transcript a difference in composition within and between accessions was observed. Cultivar 'New Zealand' only contained the Thymine nucleotide, whereas accession PI223178 contained both the nucleotides thymine and cytosine at this genome position, all remaining accessions exclusively showed the cytosine nucleotide (See Table 3.29). However when RNA editing was assessed on any of these sites, only the conserved nucleotide observed within the gDNA was present, indicating the absence of RNA editing at any of these SNP's.

Table 3.29: Reported SNP's and their presence within different accessions / cultivars

Cultivars / accessions	*SNP at 19560 within rpoB*		*SNP at 65631 within rps18*		*SNP at 37506 within psaB*	
	T	C	T	C	T	C
Cv. 'Cashel'	100%	0%	0%	100%	0%	100%
Cv. 'New Zealand'	100%	0%	0%	100%	100%	0%
Accession PI611044	100%	0%	0%	100%	0%	100%
Accession PI418701	100%	0%	0%	100%	0%	100%
Accession PI231565	100%	0%	0%	100%	0%	100%
Accession PI632553	100%	0%	0%	100%	0%	100%
Accession PI223178	100%	0%	0%	100%	58%	42%
Accession PI201187	100%	0%	0%	100%	0%	100%
Accession PI610825	100%	0%	0%	100%	0%	100%

3.4 Discussion

To test the accuracy of the trace-file method for determining the editing efficiency, the results obtained from the trace-file method were compared to the results obtained from the established colony-screen method. The difference in observed editing between these methods was in the range of 0.8% to 10.8%, while the average difference between methods was 5.7%. These results indicate that the trace-file method can be used confidently to distinguish differences in editing efficiencies between samples.

Editing efficiency experiments conducted on specific clones from cultivars 'Cashel' and 'New Zealand' showed there was no significant difference in editing efficiency in respect to treatment within each cultivar. However editing efficiency comparisons conducted on specific clones from cultivars 'Cashel' and 'New Zealand', showed a clear difference among both genotypes in editing efficiency at editing sites within the *ndhB* gene at genome positions 87188, 87281 and 87306. Another editing site within the *ndhF* gene showed a clear difference in editing efficiency at genome position 103675, between cultivars 'New Zealand' and 'Cashel'. These results are based on clones of a single seed, which were either drought tolerant (Cv. 'New Zealand'), or drought susceptible (Cv. 'Cashel'). Perennial ryegrass is an outbreeding species leading to high heterogeneity and heterozygosity within populations. Therefore these results may not represent the editing efficiency for the entire range of genotypes within each cultivar.

To verify if the editing efficiency was related to the drought tolerance, two different drought stress experiments were conducted on a number of accessions of *Lolium perenne*

L. obtained from the GRIN database. The initial drought stress experiment (*in vitro* PEG induced system), showed clear differences in drought response between accessions, and based on these results a range of accessions were selected to verify these findings. To confirm the drought response of these accessions, clones were propagated and tested under drought stress using the *in vivo* PEG induced system. The results obtained from the second test (*in vivo* PEG induced system) differed from those of the first test (*in vitro* PEG induced system), this could be explained by genotypic variation, due to the use of clones in the *in vivo* experiment compared to a range of seedlings within each accession used in the *in vitro* experiment. Nevertheless to explore the relationship between RNA editing behaviour and drought tolerance, the results obtained from the *in vivo* PEG induced system could be used, by using these specific clones for RNA editing analyses.

The editing efficiency for the editing sites within the *ndhB* and *ndhF* were evaluated within the accessions tested for drought tolerance, and subsequently compared to the respective drought tolerance of these clones. These results indicate there is no correlation between drought tolerance and editing efficiency within the *ndh* genes. The findings of this study are in contrast to another study (Casano et al. 2000) where the NDH complex was involved in drought stress resistance. Casano et al. (2000) demonstrated that expression levels of plastid NDH complex genes were up regulated during drought stress (a situation which causes photo oxidative stress) and play a role in reducing plastoquinone (PQ) in conjunction with superoxide dismutase (SOD) and hydroquinone peroxidise (Casano et al. 2000; Abdeen et al. 2010). This information, in concurrence with this new data, suggests that although the NDH complex is involved in circumventing

oxidative stress, the RNA editing alone of the involved transcripts is not the determining factor for regulation of this complex. Nevertheless very low editing efficiencies were observed within certain accessions, the lowest editing efficiency was a mere 5%. This may indicate that these genes are upregulated, in such a way that the editing *trans*-factors are not able to edit the majority of the transcripts. Despite this, there are apparent sufficient functional transcripts available for correct assembly of the complex to counter oxidative stress.

Another explanation could be that another pathway is more prominently involved with countering oxidative stress. This pathway could be the PGR5/PGRL1-dependent route, also known as the non-NDH pathway (Rumeau et al. 2007; Suorsa et al. 2009). The involvement of this pathway with cyclic electron transfer was shown to be important under near optimal conditions in *Arabidopsis* (Munekage et al. 2004). A recent publication showed that components of the PGR5/PGRL1 route were upregulated during drought stress, whereas a component of the NDH complex *ndhH* was not affected during drought stress in *Arabidopsis*, indicating the importance of the PGR5/PGRL1 pathway (Lehtimäki et al. 2010).

The observed differences in RNA editing efficiencies are most likely due to different expression levels of the proteins involved in the editing of these specific editing sites. These could potentially be genotype specific and unrelated to environmental stimuli. Another possibility could be the difference in amount of transcripts of *ndhB* and *ndhF*

available for editing. If there are fewer transcripts available, then the editing efficiency might increase. Both these explanations could contribute to the observed effects.

Other studies have identified certain *trans*-factors that are essential for editing of certain sites, however this does not exclude the possibility that other proteins may be involved in the editing machinery, as is implied by Chateigner-Boutin et al. (2008). The editing machinery can be limited by the least available protein within that editing complex. This was demonstrated when chimeric RNA was expressed containing the editing site of *psbL* in tobacco chloroplasts, this led to a significant decrease in the editing efficiency of the endogenous *psbL* RNA. This competitive effect of the transgene was specific to the *psbL* gene, with other editing sites being properly edited, indicating depletion of the *psbL*-specific *trans*acting factor (Chaudhuri et al. 1995).

Some proteins which bind to specific *cis*-factors surrounding the editing sites have been identified. These belong to the pentatricopeptide repeat protein (PPR) family. This large family of proteins is believed to be involved in RNA maturation in plastids and mitochondria (Shikanai 2006). Other unknown proteins within the editing complex could have a general function, and if knocked-out, could potentially impair the whole editing machinery. An example is the CP31 protein in tobacco, when this protein was knocked out, editing within the *psbL* transcript was completely absent, while editing in the *ndhB* gene was partially impaired. Unfortunately other proteins comprising of the editing complex acting on plastid transcripts have yet to be identified. Due to the lack of information regarding all the *trans*-factors involved within the *ndhB* and *ndhF* genes and

the lack of sequence data to find homologs within perennial ryegrass of known *trans*-factors, the regulation and accumulation of these proteins could not be tested during this project.

Lastly a novel single nucleotide polymorphism was found at plastid genome position 103515 within the *ndhF* gene in certain accessions, which was post-transcriptionally edited back to the conserved nucleotide. To our knowledge this phenomenon has not been observed previously, although these editing events could easily have been missed, due to the low percentage of secondary nucleotides in these specific genome locations at the DNA level. Within the *Lolium perenne* L. plastid genome, seven other SNP's were identified before, which could potentially be editing sites as well, three of these sites reside within coding regions, namely at genome position 19560 within the *rpoB* transcript, position 37506 within the *psaB* transcript, and position 65631 within the *rps18* transcript. (Diekmann et al. 2009). After analyses of nine accessions and cultivars for these SNPs, no subsequent RNA editing was observed. Therefore it indicates that within the perennial ryegrass plastid genome only the SNP within the *ndhF* coding region was edited, although the possibility exists that other not tested accessions could exhibit editing in the three other analysed sites. The occurrence of this phenomenon could be an evolutionary intermediate, where the loss of an editing site is in progress due to a reversion of a point mutation. However due to the polyploidy of the plastid genome in each cell, the chances of getting a complete reversion of the point mutation are small, and might take numerous generations to achieve, similar to population curves. Once achieved, the RNA editing

mechanism for this site is not required anymore, resulting in a gradual loss of editing capacity.

Chapter 4:

General discussion

Due to the predicted climate change, the production of forage grasses may become a problem in Europe. Specifically in the east of Ireland the yield will decrease due to summer drought, unless artificial irrigation is implemented (Holden and Brereton 2002). To counter the detrimental effects of drought stress, it is paramount that studies are performed to improve in the long term drought tolerance in grasses.

One of the complexes that is known to be involved with drought response is the plastidial NDH complex (Casano et al. 2000; Quiles 2006; Ibanez et al. 2010). The NDH complex is one of the components of the chlororespiration pathway that interacts with the photosynthetic electron transport, involving the non-photochemical reduction and oxidation of the plastoquinone pool (Bennoun 1982). This complex consists of eleven subunits that are encoded by the plastome (*ndhA-ndhK)* and three putative nuclear-encoded subunits (Casano et al. 2004). Two of the plastid-encoded subunits, *ndhB* and *ndhF* harbour several RNA editing sites. In this study it was shown that the editing efficiencies of some of these sites vary greatly within accessions of the species *Lolium perenne* L.. As a consequence in some accessions this would lead to an accumulation of dysfunctional subunits of the NDH complex. Several previous studies showed that inactivation of one of the subunits would result in the disappearance of the entire NDH complex (Burrows et al. 1998; Horvath et al. 2000). A similar effect could be expected when unedited transcripts are translated. Therefore a low efficiency of RNA editing in a NDH subunit would suggest a reduced NDH activity. However a direct relationship between RNA editing within the *ndhB* and *ndhF* transcripts and drought response was not observed.

The interpretation of these results can be multifaceted concerning the involvement of RNA editing in drought tolerance. Some accessions of perennial ryegrass with a drought tolerant response could have an overall elevated transcript accumulation for this complex, resulting in a larger number of transcripts targeted by the corresponding RNA editing *trans*-factor, which could potentially result in a lower efficiency in RNA editing. Other accessions with a similar drought response, could have less transcript accumulation, resulting in a higher editing efficiency. These alternatives can lead to an equal amount of edited transcripts used for translation. Essentially RNA editing can have a regulatory function in respect to accumulation of functional transcripts. For instance if the accumulation of transcripts involved with the NDH complex is too high, RNA editing efficiency could be lowered to reduce the number of edited transcripts, hence affecting the accumulation of correct subunits of the NDH complex. Alternatively, the effect could be in the opposite direction: stimulating the production of functional NDH complex, by increasing the RNA editing efficiency. The observed differences of RNA editing efficiencies in this study could also be explained by different expression levels of the *trans*-factors. The expression levels of these *trans*-factors could essentially be caused by growth conditions or different parameters like endogenous genetic differences between accessions. Despite the increasing number of *trans*-factors being identified, many *trans*-factors are still unknown (Hammani et al. 2009). Furthermore the mechanism of targeting of these *trans*-factors to specific editing sites is not entirely clear, although a limited range of sequences surrounding the editing sites play an important role in targeting (Hayes et al. 2006). The possibility of RNA editing being an important factor in drought

tolerance can therefore not be excluded. Because of the many parameters involved with the RNA editing machinery, more studies need to be performed to assess these possibilities.

It is also possible that any effect of the efficiency of RNA editing in the NDH transcripts on drought response is masked by other factors which have a more pronounced role in the defence against drought stress. These factors could include scavenging enzymes that counter oxidative stress (Mittler et al. 2004), or a NDH independent pathway, like the PGR5/PGRL1-dependent route (Rumeau et al. 2007; Suorsa et al. 2009). Under near optimal growth conditions this pathway was shown to be important for cyclic electron transfer in *Arabidopsis* (Munekage et al. 2004). Lehtimäki et al. (2010) found in *Arabidopsis,* components of the PGR5/PGRL1 route that were upregulated during drought stress, whereas a component of the NDH-complex *ndhH* was not influenced by the same stress, indicating the importance of the PGR5/PGRL1 pathway under certain conditions (Lehtimäki et al. 2010).

During this study one other feature was observed, namely a SNP that was edited. Both SNPs and RNA editing are common in plastids, however the occurrence of both mechanisms at the same position was a novel discovery. The occurrence of this combination could be an evolutionary intermediate, where the loss of an editing site is in progress due to a reversion of a point mutation. However due to the polyploidy of the plastid genome in each cell, the chances of getting a complete reversion of the point mutation are small, and might take numerous generations to achieve. Once this is

achieved, the RNA editing mechanism for this site is not required anymore, which could result in a gradual loss of editing capacity for this site.

During this project an attempt was made to develop a protocol for plastid transformation of *Lolium perenne* L.. However no transformants were recovered despite extensive evaluation of all the involved parameters. There can be several causes that prevented the recovery of transplastomic plantlets. These include the possible low expression of the selectable marker gene *aphA-6* in conjunction with a less then optimal selection regime. Furthermore the choice of genotype could have a large impact on the efficiency of the transformation process. Nevertheless further adjustments to the presented protocol could provide the means to achieve plastid transformation in this species. Graminaceous species remain particularly recalcitrant to plastid transformation, and at least two other teams experienced in plastid transformation have invested effort in the development of a procedure for *Lolium* species without success (Gray, personal communication, Altpeter, personal communication)

Further investigations by means of plastid transformation regarding the involvement of RNA editing on drought stress could not be performed in *Lolium perenne* L.. However in future projects it would be intriguing to test the relevance of RNA editing in relationship with drought by plastid transformation; this could be performed in other species where readily available plastid transformation protocols exist, like tobacco (Svab and Maliga 1993). *Arabidopsis* would also be an interesting target species, because of the available genome data present for this species in conjunction with known *trans*-factors; however

the plastid transformation efficiency for this species was reported to be very low (Sikdar et al. 1998).

Plastid transformation could be used to elucidate the involvement of RNA editing in the functionality of the NDH complex. This can be tested with a gene-replacement approach as illustrated in Fig. 4.1. Transcripts derived from unedited mRNA would result in a dysfunctional protein (Fig. 4.1A), while transcripts derived from edited mRNA would result in functional protein (Fig. 4.1B). Both situations can be mimicked in such a way, without the interference of RNA editing. For instance, the endogenous *ndhB* gene can be substituted by a modified version containing specific point mutations, the resulting transcripts would be rendered uneditable, while being translated into dysfunctional protein (see Fig 4.1C). On the other hand a modified version of this gene can be used, which would contain point mutations resulting in a protein structure similar to an edited protein (See Fig 4.1D). The absence of one functional component of the NDH complex tends to result in the absence of the entire complex (Horvath et al. 2000). If this "unedited" version would result in absence of the complex, it would show the importance of editing and its possible regulatory function.

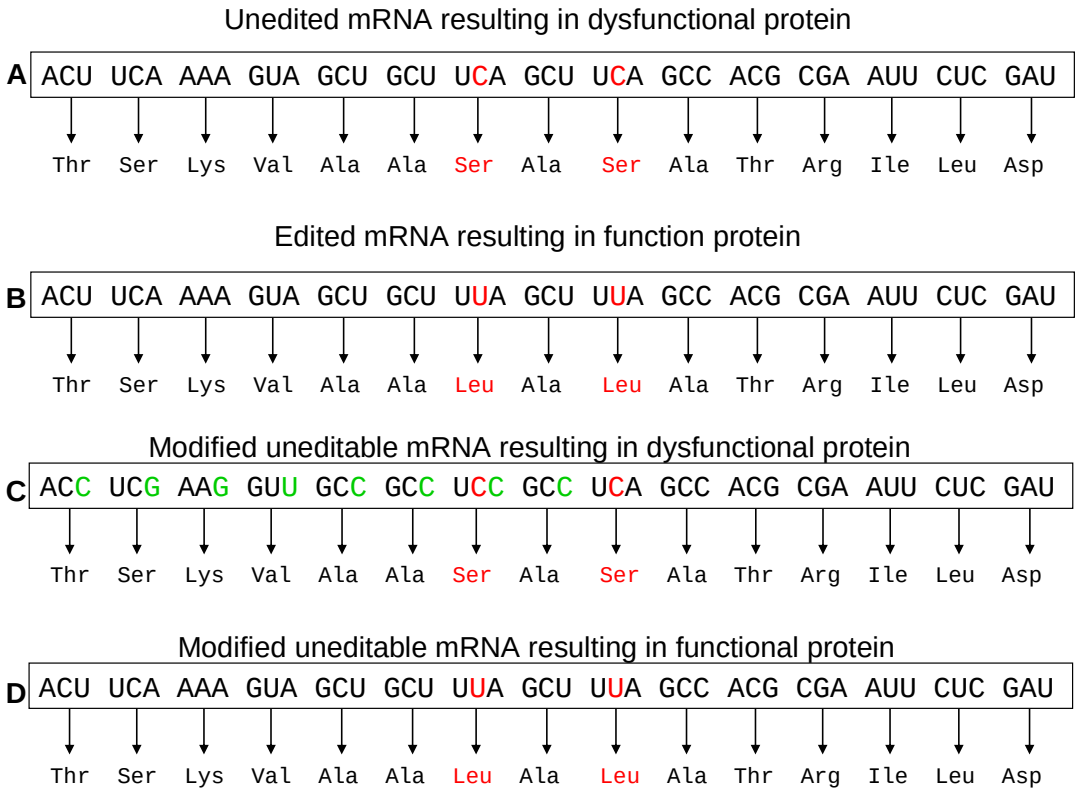


Fig. 4.1: An overview of four possible transcripts parts within the *ndhB* gene **A**: Unedited form of mRNA, translatable into dysfunctional protein **B**: Edited form of mRNA, translatable into functional protein **C**: mRNA from an uneditable modified *ndhB* gene, resulting in a dysfunctional protein, **D**: mRNA from an uneditable modified *ndhB* gene, resulting in a functional protein. Red annotated nucleotides are RNA editing sites, green annotated nucleotides are modifications to prevent editing in that transcript.

Having a working protocol for plastid transformation for perennial ryegrass would also open up a powerful avenue for improving environmental stress tolerance, by overexpressing ROS scavenging enzymes within the plastid compartment. The effectiveness of this approach was recently demonstrated in our laboratory with the model species tobacco (Le Martret et al. submitted). This study demonstrated that overexpression of the ROS scavenging enzymes GR, DHAR and GST provided cold and salt tolerance, while co-expression of DHAR:GR or GST:GR conferred paraquat tolerance.

Beside the possibility to counter environmental stresses by plastid engineering, this technology also offers the possibility to improve other traits. For instance perennial ryegrass can be a prime candidate in Ireland as crop for biofuel production (Smyth et al. 2009). At the moment most biofuels are produced in maize and sugarcane, however biofuels have had bad press coverage in recent years. This is mainly due to habitat destruction in tropical countries and the negative effect on food production. In Ireland beef and sheep production is highly self sufficient, therefore the effect on food production is not a direct concern if grasses were to be used for biofuel production. Furthermore habitat destruction is not an issue in this case (Smyth et al. 2009). Perennial ryegrass has a significant yield and biomethanol has a very good energy balance, however improvements in this would benefit the biofuel industry. To accomplish this, genetic engineering can be used, for instance by increasing the polysaccharide content or by increasing the overall biomass production (Sticklen 2008). These properties might be elevated by means of plastid transformation.

Development of a plastid transformation protocol for perennial ryegrass could have a large impact on the improvement of this crop, whether in stress tolerance or for other applications. The prime advantage of gene containment in this outbreeding crop, alongside the possibility to engineer pathways by gene-stacking and over expression of transgenes, would make it an invaluable new technology to assist in breeding programs for improvements in this crop.

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Appendix A: Complete sequences of *Lolium perenne* L. plastid transformation vectors pIAPRvdB4 and pIAPRvdB5

pIAPRvdB4 complete sequence

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   1  AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGC
  61  ACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGC
 121  TCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAA
 181  TTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCTTT
 241  TTAGGGTGAAGTAAGACCAAGCTCATGAGCTTATTATCCTAGGTCGGAACAAATTAGTTG
 301  ATAGTGATAGGATCCCTTTTTTGACGTCCCCATGTCCCCCCCCCTGTGGTGTGGCGGCAT
 361  GGGGATGTCAAAAGGAAAGGGATGGAGTTTTTCTCGCTTTTGGCGTAGCAGGCCTCCCTT
 421  TCTTTGGGAGGCCCGCGCGACGGGCTATTAGCTCAGTGGTAGAGCGCGCCCCTGATAATT
 481  GCGTCGTTGTGCCTGGGCTGTGAGGGCTCTCAGCCACATGGATAGTTCAATGTGCTCATC
 541  AGCGCCTGACCCGAAGATGTGGATCATCCAAGGCACATTAGCATGGCGTACTCCTCCTAT
 601  TTGAATCGGAGTTTGAAACCAAACAAACTTCTCCTCAGGAGGATAGATGGGGCGATTCAG
 661  GTGAGATCCCATGTAGATCTAACTTTCTATTCACTCGTGGGATCCGGGCGGTCCGGGGAG
 721  GGTCCACCACGGCTCCTCTCTTCTCGAGAATCCATACATCCCTTATCAGTGTATGGAGAG
 781  CTATCTCTCGAGCACAGGTTGAGGTTCGTCCTCAATGGGAAAATGGAGCACCTAACAACG
 841  CATCTTCACAGACCAAGAACTACGAGATCACCCCTTTCATTCTGGGGTGACGGAGGGATC
 901  GTACCATTCGAGCCTTTTTTTTCATGCTTTTCCCGGCGGTCTGGAGAAAGCAGCAATCAA
 961  TAGGACTTTCCTAATCCTCCCTTACTTTCAGGAAGAACGTGAAATTCTTTTTCCTTAAAT
1021  GGGAGCAGAGCAGGTTTGAAAAAGGATCTTAGAGTGTCTAGGGTTGGGCCAGGAGGGTCT
1081  CTTAACGCCTTCCTTTTTCTGCCCATCGGAGTTATTTCCCAAGGACTTGCCATGGTAAGA
1141  GGGAGAAGGGGGAAAAAGCACACTTGAAGAGCGCAGTACAACGGGGAGTTGTATGCTGCG
1201  TTCGGGAAGGATGAATCGCTCCCGAAAAGGAGTCTATTGATTCTCTTCCAATTGGTTGGA
1261  TCGTAGGGGCGATGATTTACTTCACGGGCGAGGTCTCTGGTTCAAGTCCAGGATGGCCCA
1321  GCTGCGCCAGGGAAAAGAATAGAAGAAGCAGGTACCGCTCCCCCGCCGTCGTTCAATGAG
1381  AATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAG
1441  CGAACTCCGGGCGAATACGAAGCGCTTGGATACAGTTGTAGGGAGGGGAGCTCATGAGTA
1501  AAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTA
1561  ATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGAAAACTTA
1621  CCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTA
1681  CTTTCTCTTATGGTGTTCAATGCTTTTCAAGATACCCAGATCATATGAAGCGGCACGACT
1741  TCTTCAAGAGCGCCATGCCTGAGGGATACGTGCAGGAGAGGACCATCTCTTTCAAGGACG
1801  ACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAGGGAGACACCCTCGTCAACAGGA
1861  TCGAGCTTAAGGGAATCGATTTCAAGGAGGACGGAAACATCCTCGGCCACAAGTTGGAAT
1921  ACAACTACAACTCCCACAACGTATACATCACGGCAGACAAACAAAAGAATGGAATCAAAG
1981  CTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATC
2041  AACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCA
2101  CACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGT
2161  TTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAATAAGGGAGGGGGC
2221  CGGCCATGACCATGGAATTACCAAATATTATTCAACAATTTATCGGAAACAGCGTTTTAG
2281  AGCCAAATAAAATTGGTCAGTCGCCATCGGATGTTTATTCTTTTAATCGAAATAATGAAA
2341  CTTTTTTTCTTAAGCGATCTAGCACTTTATATACAGAGACCACATACAGTGTCTCTCGTG
2401  AAGCGAAAATGTTGAGTTGGCTCTCTGAGAAATTAAAGGTGCCTGAACTCATCATGACTT
2461  TTCAGGATGAGCAGTTTGAATTCATGATCACTAAAGCGATCAATGCAAAACCAATTTCAG
2521  CGCTTTTTTTAACAGACCAAGAATTGCTTGCTATCTATAAGGAGGCACTCAATCTGTTAA
2581  ATTCAATTGCTATTATTGATTGTCCATTTATTTCAAACATTGATCATCGGTTAAAAGAGT
2641  CAAAATTTTTTATTGATAACCAACTCCTTGACGATATAGATCAAGATGATTTTGACACTG
2701  AATTATGGGGAGACCATAAAACTTACCTAAGTCTATGGAATGAGTTAACCGAGACTCGTG
2761  TTGAAGAAAGATTGGTTTTTTCTCATGGCGATATCACGGATAGTAATATTTTTATAGATA
2821  AATTCAATGAAATTTATTTTTTAGATCTTGGTCGTGCTGGGTTAGCAGATGAATTTGTAG
2881  ATATATCCTTTGTTGAACGTTGCCTAAGAGAGGATGCATCGGAGGAAACTGCGAAAATAT
2941  TTTTAAAGCATTTAAAAAATGATAGACCTGACAAAAGGAATTATTTTTTAAAACTTGATG
3001  AATTGAATTGAGCGGCCGCACCGAAATTCAATTAAGGAAATAAATTAAGGAAATACAAAA
3061  AGGGGGGTAGTCATTTGTATATAACTTTGTATGACTTTTCTCTTCTATTTTTTTGTATTT
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3121  CCTCCCTTTCCTTTTCTATTTGTATTTTTTTTATCATTGCTTCCATTGAATTCCGTGTTCT
3181  TTAATTAATCTGACTCTTTCATGCATACTCCACTTGGCTCGGGGGGGATATAGCTCAGTT
3241  GGTAGAGCTCCGCTCTTGCAATTGGGTCGTTGCGATTACGGGTTGGCTGTCTAATTGTCC
3301  AGGCGGTAATGATAGTATCTTGTACCTGAACCGGTGGCTCACTTTTTCTAAGTAATGGGG
3361  AAGAGGACTGAAACATGCCACTGAAAGACTCTACTGAGACAAAAAGATGGGCTGTCAAAA
3421  AGGTAGAGGAGGTAGGATGGGCAGTTGGTCAGATCTAGTATGGATCGTACATGGACGATA
3481  GTTGGAGTCGGCGGCTCTCCTAGGCTTCCCTCATCTGGGATCCCTGGGGAAGAGGATCAA
3541  GTTGGCCCTTGCGAATAACTTGATGCACTATCTCCCTTCAACCCTTTGAGCGAAATGTAG
3601  CAAAAGGAAGGAAAATCCATGGACCGACCCCATTGTCTCCACCCCGTAGGAACTACGAGA
3661  TCACCCCAAGGACGCCTTCGGCGTCCAGGGGTCACGGACCGACCATAGACCCTGTTCAAT
3721  AAGTGGAACACATTAGCCGTCCGCTCTCTGGTTGGGCAGTAAGGGTCGGAGAAGGGCAAT
3781  CACTCGTTCTTAAAACCAGCATTCTTAAGTTTAAGATCAAAGAGTCGGGCGGAAAAAGGG
3841  GAGATCTCCCCGTTCCTGGTTCTCCTGTAGCTGGATTCCCCGGAACCACAAGAATCCTTA
3901  GAATGGGATTCCAACTCAGCACCTTTTGTTTTGAGATTTTGAGAAGAGTTGCTCTTTGGA
3961  GAGCACAGTACGATGAAAGTTGTAAGCTGTGTTCGGGGGGGAGTTATTGTCTATCGTTGG
4021  CCTCTATGGTAGAACCCGTCGGGGAGGCCTGAGAGGCGGTGGTTTACCCTGTGGCGGATG
4081  TCAGCGGTTCGAGTCCGCTTATCTCCAGCCCGTGAACTTAGCGGATACTATGATAGCACC
4141  GAATTTTGCCAATTCAGCAGTTCGATCTATGATTTCGCATTCATGGAAGGGCGAATTCTG
4201  CAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTA
4261  TAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCC
4321  TGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAG
4381  CGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGAC
4441  GCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCT
4501  ACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACG
4561  TTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGT
4621  GCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCA
4681  TCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGA
4741  CTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAA
4801  GGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAAC
4861  GCGAATTTTAACAAAATTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAG
4921  CCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAA
4981  GGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGC
5041  TAGACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTG
5101  GTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGAT
5161  GGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAAC
5221  AAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACT
5281  GGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGC
5341  GCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGG
5401  CAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTG
5461  TCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGT
5521  CATCCCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGC
5581  ATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAG
5641  CACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGG
5701  GGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATC
5761  TCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTT
5821  CTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGG
5881  CTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTT
5941  ACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCT
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6061  TTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGA
6121  TGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAA
6181  GATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCT
6241  GCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCAT
6301  ACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGA
6361  TGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGC
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6721  TGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCC
6781  CTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAG
6841  ACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTA
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6961  GATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGC
7021  GTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAAT
7081  CTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGA
7141  GCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGT
7201  TCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATA
7261  CCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTAC
7321  CGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGG
7381  TTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCG
7441  TGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAG
7501  CGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCT
7561  TTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTC
7621  AGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTT
7681  TTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCG
7741  TATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGA
7801  GTCAGTGAGCGAGGAAGCGGAAG
```

Red annotated: *trnI* homologous region of the *Lolium perenne* L. plastid genome.

Dark blue annotated: full length *16S Prrn* promoter from *Nicotiana tabacum* with the 5'UTR of *rbcL* of *Nicotiana tabacum*

Green annotated: *smGFP* – gene

Purple annotated: Ribosomal binding site from the *rbcL* gene of *Nicotiana tabacum*

Orange annotated: *aphA-6* – gene

Light blue annotated: 3'UTR of the *rps16* gene of *Nicotiana tabacum*

Brown annotated: *trnA* homologous region of the *Lolium perenne* L. plastid genome

Black annotated: pCR2.1 backbone vector

pIAPRvdB5 complete sequence

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   1  AGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGC
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 241  TTAGGGTGAAGTAAGACCAAGCTCATGAGCTTATTATCCTAGGTCGGAACAAATTAGTTG
 301  ATAGTGATAGGATCCCTTTTTTGACGTCCCCATGTCCCCCCCCCTGTGGTGTGGCGGCAT
 361  GGGGATGTCAAAAGGAAAGGGATGGAGTTTTTCTCGCTTTTGGCGTAGCAGGCCTCCCTT
 421  TCTTTGGGAGGCCCGCGCGACGGGCTATTAGCTCAGTGGTAGAGCGCGCCCCTGATAATT
 481  GCGTCGTTGTGCCTGGGCTGTGAGGGCTCTCAGCCACATGGATAGTTCAATGTGCTCATC
 541  AGCGCCTGACCCGAAGATGTGGATCATCCAAGGCACATTAGCATGGCGTACTCCTCCTAT
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 661  GTGAGATCCCATGTAGATCTAACTTTCTATTCACTCGTGGGATCCGGGCGGTCCGGGGAG
 721  GGTCCACCACGGCTCCTCTCTTCTCGAGAATCCATACATCCCTTATCAGTGTATGGAGAG
 781  CTATCTCTCGAGCACAGGTTGAGGTTCGTCCTCAATGGGAAAATGGAGCACCTAACAACG
 841  CATCTTCACAGACCAAGAACTACGAGATCACCCCTTTCATTCTGGGGTGACGGAGGGATC
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 961  TAGGACTTTCCTAATCCTCCCTTACTTTCAGGAAGAACGTGAAATTCTTTTTCCTTAAAT
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1201  TTCGGGAAGGATGAATCGCTCCCGAAAAGGAGTCTATTGATTCTCTTCCAATTGGTTGGA
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1321  GCTGCGCCAGGGAAAAGAATAGAAGAAGCAGGTACCGCTCCCCCGCCGTCGTTCAATGAG
1381  AATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAG
1441  GGAGACCACAACGGTTTCCCACTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAC
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1861  ACACCCTCGTCAACAGGATCGAGCTTAAGGGAATCGATTTCAAGGAGGACGGAAACATCC
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2101  ACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACC
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2281  TCGGAAACAGCGTTTTAGAGCCAAATAAAATTGGTCAGTCGCCATCGGATGTTTATTCTT
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3001  ATTTTTTAAAACTTGATGAATTGAATTGAGCGGCCGCACCGAAATTCAATTAAGGAAATA
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3121  TTCTATTTTTTTGTATTTCCTCCCTTTCCTTTTCTATTTGTATTTTTTTATCATTGCTTC
3181  CATTGAATTCCGTGTTCTTTAATTAATCTGACTCTTTCATGCATACTCCACTTGGCTCGG
3241  GGGGGATATAGCTCAGTTGGTAGAGCTCCGCTCTTGCAATTGGGTCGTTGCGATTACGGG
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3361  TTTTTCTAAGTAATGGGGAAGAGGACTGAAACATGCCACTGAAAGACTCTACTGAGACAA
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3481  GATCGTACATGGACGATAGTTGGAGTCGGCGGCTCTCCTAGGCTTCCCTCATCTGGGATC
3541  CCTGGGGAAGAGGATCAAGTTGGCCCTTGCGAATAACTTGATGCACTATCTCCCTTCAAC
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3721  CCATAGACCCTGTTCAATAAGTGGAACACATTAGCCGTCCGCTCTCTGGTTGGGCAGTAA
3781  GGGTCGGAGAAGGGCAATCACTCGTTCTTAAAACCAGCATTCTTAAGTTTAAGATCAAAG
3841  AGTCGGGCGGAAAAAGGGGAGATCTCCCCGTTCCTGGTTCTCCTGTAGCTGGATTCCCCG
3901  GAACCACAAGAATCCTTAGAATGGGATTCCAACTCAGCACCTTTTGTTTTGAGATTTTGA
3961  GAAGAGTTGCTCTTTGGAGAGCACAGTACGATGAAAGTTGTAAGCTGTGTTCGGGGGGGA
4021  GTTATTGTCTATCGTTGGCCTCTATGGTAGAACCCGTCGGGGAGGCCTGAGAGGCGGTGG
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7561  GGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG
7621  ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTT
7681  TTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCC
7741  TGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCG
7801  AACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAG
```

Red annotated: *trnI* homologous region of the *Lolium perenne* L. plastid genome.

Dark blue annotated: Truncated *16S Prrn* promoter from *Nicotiana tabacum* with the 5'TCR of *gene 10* of the T7 phage

Green annotated: *smGFP* – gene

Purple annotated: Ribosomal binding site from the *rbcL* gene of *Nicotiana tabacum*

Orange annotated: *aphA-6* – gene

Light blue annotated: 3'UTR of the *rps16* gene of *Nicotiana tabacum*

Brown annotated: *trnA* homologous region of the *Lolium perenne* L. plastid genome

Black annotated: pCR2.1 backbone vector

Appendix B: Trace files for comparison with the colony screen

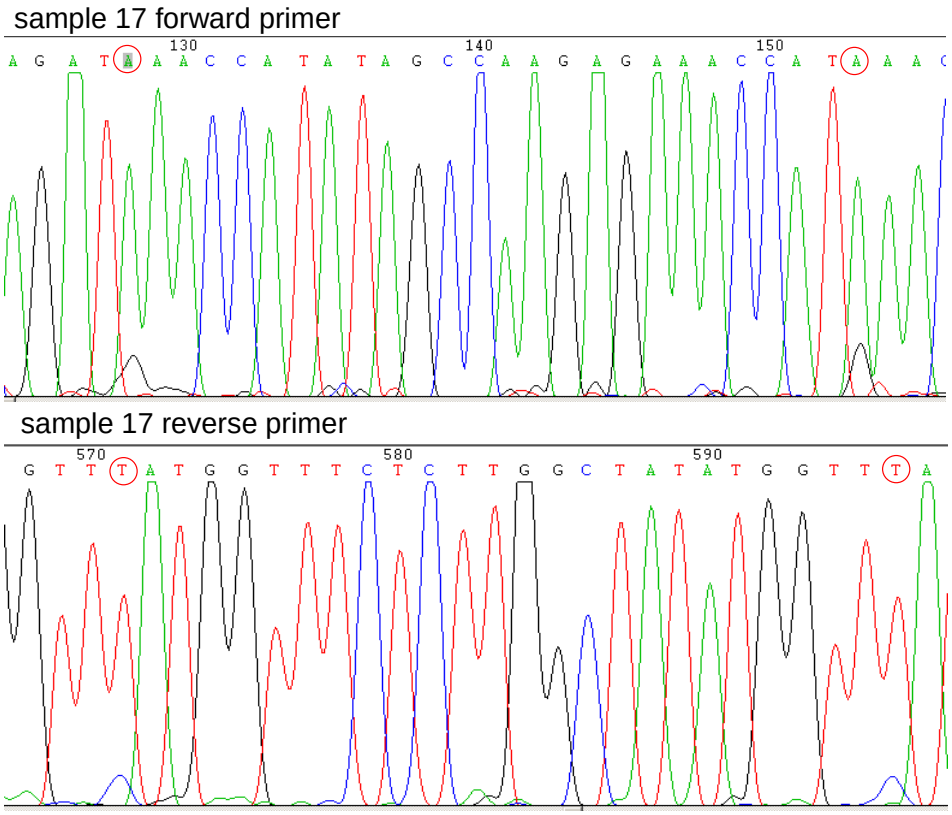

Fig. 1: Trace files of editing sites 87306 and 87281 in sample no. 17

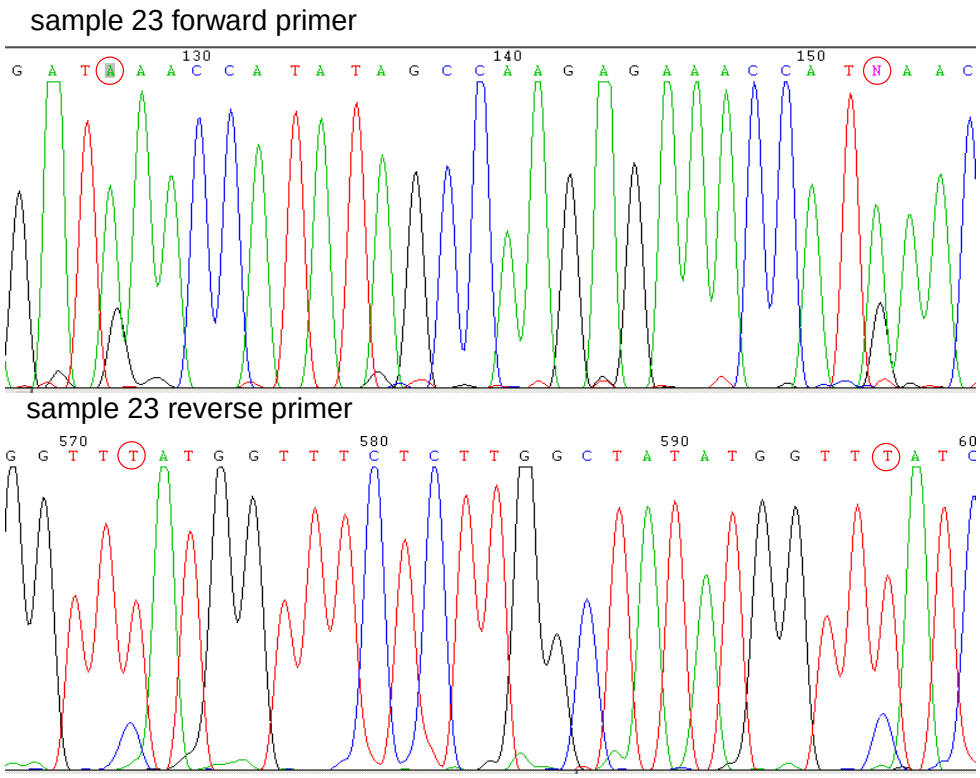

Fig. 2: Trace files of editing sites 87306 and 87281 in a sample no. 23

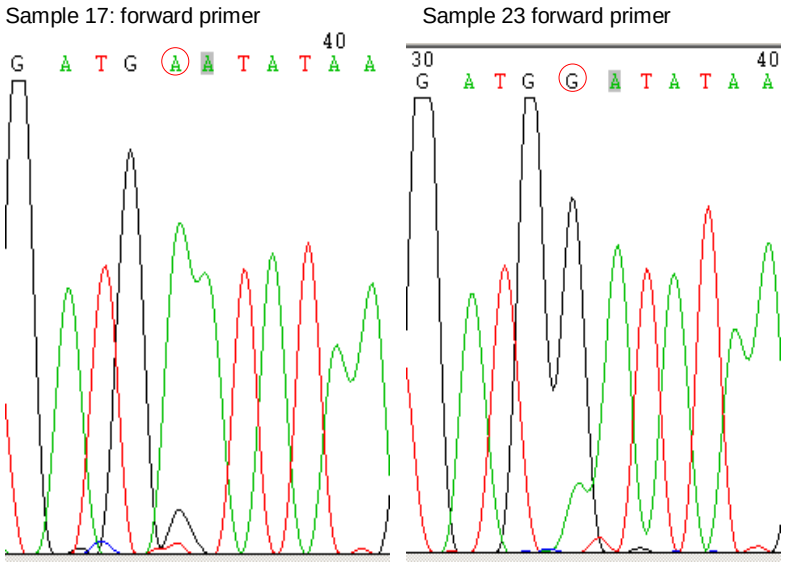

Fig. 3: Trace-files of editing site 87188 in samples no. 17 and 23

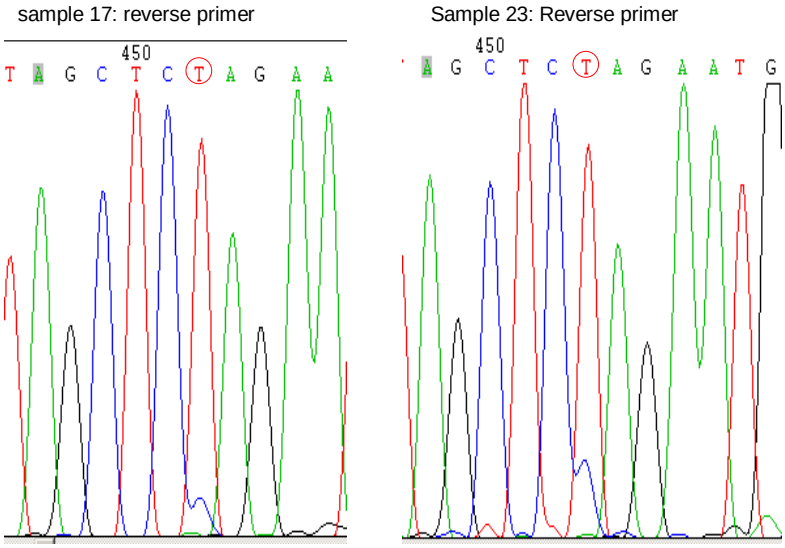

Fig. 4: Trace-files of editing site 87425 in samples no. 17 and 23

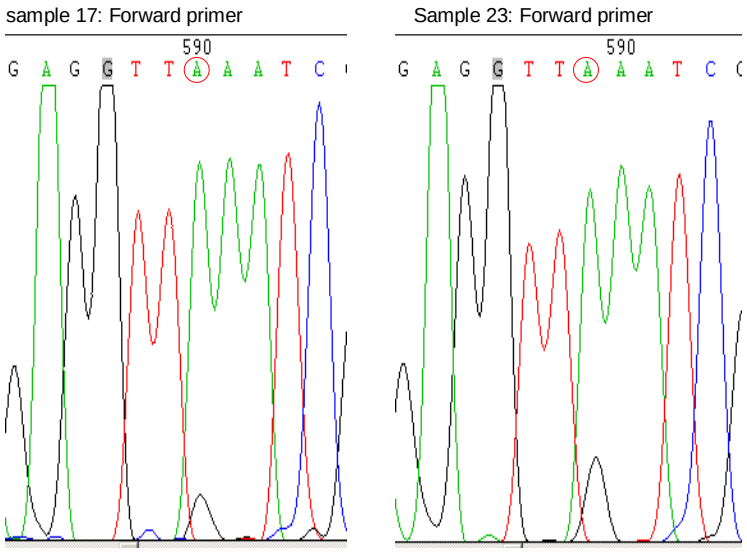

Fig. 5: Trace-files of editing site 87743 in samples no. 17 and 23.

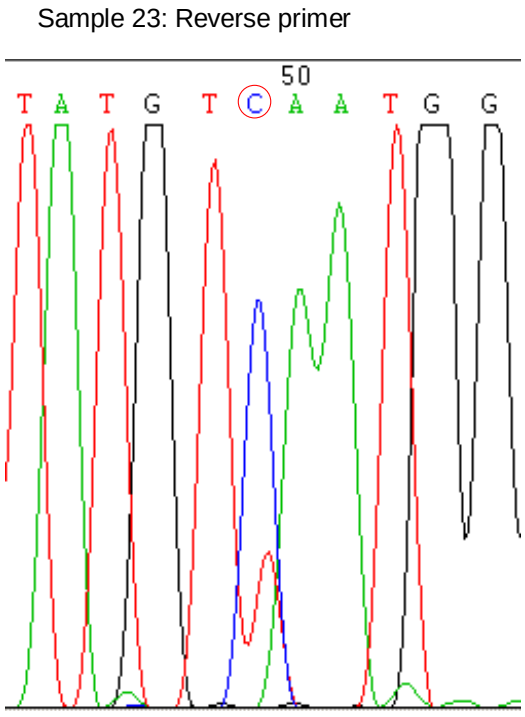

Fig. 6: Trace-file of editing site 103675 in sample no. 17