Evaluation of two viral vectors for virus-induced gene silencing in pearl millet

by

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Declaration

I, Irene Schoeman, declare that the thesis submitted herewith for the degree Magister Scientiæ to the University of Pretoria, contains my own work and has not been submitted for any degree at any other tertiary institute.

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Preface

The study presented in this thesis was aimed at evaluating the use of two existing monocotyledonous viral vectors, barley stripe mosaic virus (BSMV) and brome mosaic virus (BMV), to conduct gene silencing in the non-model cereal crop, *Pennisetum glaucum* (L.) R. Br. (pearl millet). Several defence related genes have been identified in pearl millet (Crampton et al. 2009) and the development of a virus-induced gene silencing (VIGS) system would provide a means to functionally characterize these candidates. The thesis is presented as a compilation of five chapters that jointly present the research findings with regards to the above mentioned aim.

Chapter 1 Literature review

This chapter presents a review of published literature relevant to the cereal crop pearl millet. Methods of gene silencing such as transformation, mutation based applications and virus-induced gene silencing (VIGS) are discussed. The two viral vectors evaluated in the study are reviewed comprehensively with regards to their applications in monocot gene silencing. The molecular mechanism of VIGS, namely post-transcriptional gene silencing (PTGS) and experimental considerations when developing a gene silencing system are also reviewed in this chapter.

Chapter 2 Methods and materials

This chapter comprises a description of all materials used and experimental procedures conducted throughout the study.

Chapter 3 BSMV results

The results obtained in conducting gene silencing with a BSMV VIGS vector are reported in this chapter. The VIGS vector analysed was a BSMV construct where the coat protein has been deleted from the viral genome called BSMVΔCP derived from the BSMV strain ND-18. An RNAγ based BSMV VIGS vector for *Pds* silencing, BSMV:Pds was generated by cloning a 365 bp fragment of the expressed pearl millet *Pds* gene in antisense orientation downstream

of a T7 promoter region in the vector. A control construct, BSMV:GFP which bears a full-length green fluorescent protein gene in place of the *Pds* insert was used as control of viral infection symptoms. Wheat cv. Tugela was used as a positive control in the experiments, as wheat is a known host for the BSMV viral vector. Pearl millet seedlings infected with the BSMV constructs did not display any virus or gene-silencing symptoms. Virus-derived RNAs were identified in systemic leaves of the positive control wheat seedlings, but not in pearl millet seedlings infected with BSMV-based VIGS vectors.

Chapter 4 BMV results

The results of evaluating the BMV VIGS vector in pearl millet are presented in this chapter. An RNA 3 based BMV *Pds* silencing vector, BMV:Pds, was created by cloning a 300 bp fragment of the pearl millet *Pds* gene in an antisense orientation downstream of a T3 promoter region in the vector. An unmodified hybrid BMV construct, H-BMV, was used as control of viral symptoms and barley cv. Morex and *Nicotiana benthamiana* were used as positive controls in the experiments. Pearl millet seedlings infected with the BMV-based VIGS vector did not display any virus or gene-silencing associated symptoms. BMV RNAs were present in systemic leaves of positive control species but not in the pearl millet seedlings. The ability of the BMV VIGS vector to infect two maize accessions is presented.

Chapter 5 Discussion

A concluding discussion about the results obtained in this study is presented in this final chapter. Recommendations towards future work are made and the discussion finishes with a short summary of the research findings made throughout the study.

Abstract

Pearl millet [*Pennisetum glaucum* L. Br.] is an agriculturally important cereal farmed in the marginal semi-arid regions of sub-Saharan Africa and India. The crop shows remarkable tolerance to both biotic and abiotic stress and several genetic components involved in these traits have already been identified. To date little progress has been made towards the development of a molecular technique to functionally characterise such candidate genes in pearl millet. One such potential method is virus-induced gene silencing (VIGS), a nucleotide sequence homology dependent technique used to down-regulate expression of selected genes using a recombinant viral vector. VIGS has been applied with great success in dicotyledonous and more recently monocotyledonous plant species. The objective of this study was to evaluate two viruses, barley stripe mosaic virus (BSMV) and brome mosaic virus (BMV), as VIGS vectors for pearl millet. Our hypothesis was that these viruses, which have successfully been used to conduct VIGS in other monocotyledonous species, could potentially be transferred to perform gene silencing in pearl millet. The viral vectors were modified to target a phenotypic marker gene, Phytoene desaturase (*Pds*), in pearl millet. *Triticum aestivum* (wheat), a known host for BSMV and *Hordeum vulgare* (barley), a known host for BMV and BSMV, were used as positive controls of viral infection. No visual *Pds* silencing symptoms were observed in the two pearl millet breeding lines, ICML12 and ICMB96222. Phenotypic symptoms associated with *Pds* silencing were observed in wheat seedlings infected with the *Pds* silencing BSMV construct. No significant distinction could be made between viral symptoms and potential *Pds* silencing in barley indicating that the pearl millet *Pds* insert in the BMV construct was insufficient to induce visual *Pds* silencing in barley. The presence of the viruses in systemic pearl millet leaves was evaluated using reverse transcription PCR targeted to all three genomic subunits of the respective viruses. Virus-specific products could be amplified from the barley and wheat accessions used as positive controls but it was found that neither of the viruses was present in pearl millet. This indicated that the VIGS vectors; BSMV and BMV were not amenable to conduct gene silencing in pearl millet under the conditions tested in this study. Although this study did not identify a VIGS vector, it does present some advances made towards the identification of a gene silencing system in pearl millet.

Abbreviations

µg	microgramme
µl	Microlitre
µM	micromolar
BMV	brome mosaic virus
bp	base pairs
BSMV	barley stripe mosaic virus
C-BMV	chimeric BMV
cDNA	complementary DNA
cv.	cultivar
DCL	DICER-LIKE
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
dpi	days post-infection
dsRNA	double-stranded RNA
GFP	green fluorescent protein
H-BMV	hybrid BMV
hpRNA	hairpin RNA
IPTG	isopropyl-β-d-thiogalactopyranoside
kb	kilobase
L3	third systemic leaf
LB	Luria Bertani
M	molar
min	minute
miRNA	microRNA
ml	millilitre
mm	millimetre
ng	nanogramme
nt	nucleotides
PCR	polymerase chain reaction
Pds	phytoene desaturase
PTGS	Post-transcriptional gene silencing

qPCR	quantitative PCR
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription PCR
SAS	systemic acquired silencing
siRNA	small interfering RNA
UV	ultraviolet
VIGS	virus-induced gene silencing
WT	wild type
X-GAL	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside

Chapter 1 Literature review

1.1 Pearl millet

The grass family, *Poaceae*, contains more than 10 000 species and includes important cereal crops such as rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum*). Pearl millet, one of the oldest cultivated food crops known, is an important food and feed crop in semi-arid and arid areas of the developing world and is located at position six in the global ranking of cereal hectarage with 29 million hectares grown annually (www.faostat.org). This cereal was domesticated in western Africa 5000 years ago and spread to India 2000 years later. The small, hard seeds are traditionally milled to a fine powder and used to make porridges, flat breads, and beverages (Andrews and Kumar, 1992). Pearl millet grain has a high nutritional value and, apart from food applications of the grain, pearl millet forage is also used for fencing, thatching and fuel (Andrews and Kumar, 1992).

Despite the importance of this cereal, especially as food and feed crop in the developing world, pearl millet is known as one of the so-called orphan crops that have received little public or private investment to date. Millet production in Africa has increased by 25% since 1970 and its place in domestic diets is steadily on the increase (www.icrisat.org). Climate change has also sparked renewed interest in this hardy crop in terms of food security and as a source of renewable energy. Pearl millet is well adapted to semi-arid and arid regions due to its C4 metabolism and well-developed root system. The grain can be used as a supplement or even substitute for maize as cattle feed or for bio-ethanol production in regions where droughts and low-fertility soils occur. Agricultural pearl millet residues, such as stalks, can be used for second generation biofuel production by fermenting ethanol from the lignocellulosic biomass (Rajvanshi et al. 2007). The US Department of Energy's Joint Genome Institute (JGI) undertook the sequencing of foxtail millet *(Setaria italica)* (Doust et al. 2009) in the interest of biofuel development. Foxtail millet (2n=2x=18) is taxonomically most closely related to pearl millet (2n=2x=14) (Devos and Gale, 1997). The haploid genome size of pearl millet is 2350 Mb (Bennett et al. 2000) compared to foxtail millet 490 Mb, and maize (2n=2x=20) 2,300 - 2,700 Mb (www.gramene.org). The taxonomic relationships between members of the *Poaceae* family are depicted in Figure 1.1.

Furthermore, efforts by multinational agricultural research institutes such as the International Crop Research Institute in the Semi-Arid Tropics (ICRISAT) and the Consultative Group on International Agricultural Research (CGIAR) to conduct research on and develop molecular resources for pearl millet, indicates the growing interest in this crop. In 2006 the "Alliance for a green revolution" was established with the aim to develop 100 new crop varieties in five years to double or treble yields within the next 20 years and pearl millet is one of the targeted crops (Blaustein 2008).
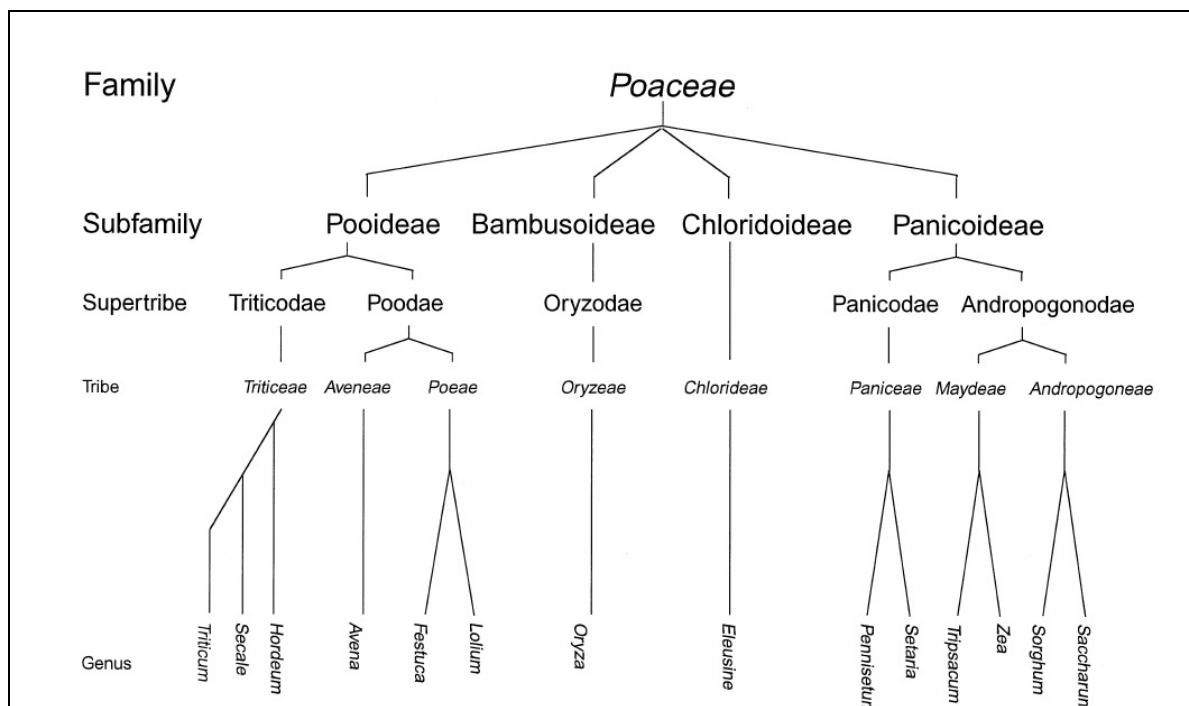
Figure 1.1 Taxonomic relationships between pearl millet and other members of the Poaceae family. Figure from Devos and Gale, 1997.

Biotic stresses limit potential pearl millet production globally. Several pathogens such as bacteria, fungi, nematodes and viruses have an effect on pearl millet yields. Bacterial diseases include bacterial spot caused by *Pseudomonas syringae* pv. *syringae* van Hall and bacterial leaf streak caused by *Xanthomonas campestris* (Pammel) Dowson pv. *pennamericanum*. Fungi that infect pearl millet include *Sclerospora graminicola* (Sacc.) Schroet., causal agent of downy mildew, *Claviceps* species, causal agent of ergot, and *Puccinia substriata* causal agent of pearl millet leaf rust (Wilson 2000).

Resistance (*R*) genes are the primary method of rust control in cereals including pearl millet (Webb and Fellers, 2006). Rust resistant germplasm lines have long been used in pearl millet breeding programmes (Singh et al. 1997, Morgan et al. 1998). The *Rr1* resistance gene was the original source of rust resistance in pearl millet breeding programmes and came from a wild subspecies of pearl millet from Senegal. However, the resistance has since been overcome by a new race or races of rust (Morgan et al. 1998) and increasingly advanced breeding approaches are used to combat rust disease. Quantitative disease-resistance and *R* gene pyramids are employed to attain more durable resistance (Ayliffe et al. 2008). At present, researchers at ICRISAT are screening elite breeding lines to develop advanced rust resistant hybrids. The use of such advanced breeding lines in resistance breeding is more effective and less time consuming than introgressing resistance from wild germplasm (Sharma et al. 2009).

The availability of genetic and molecular resources for pearl millet is limited despite the great potential of this hardy cereal as a food crop. A genetic map with several markers and phenotypic traits has been published (Devos et al. 2006) and several abiotic stress-induced ESTs have been isolated and characterised in pearl millet (Mishra et al. 2007). Microsatellite markers have been used to describe the phylogenetic relationship between several wild and cultivated pearl millet accessions (Oumar et al. 2008) and expressed sequenced tags simple sequence repeat (EST-SSR) markers are now used by ICRISAT for diversity assessment and marker-aided breeding programs (Senthilvel et al. 2008, Yadav et al. 2008). Thousands of pearl millet ESTs have been identified to date and are available on the public database GenBank which could be mined for potential *R* gene homologs (RGHs). The ESTs include several pearl millet genes involved in drought resistance (Mishra et al. 2007) and defence response (Crampton et al. 2009), but functional characterisation thereof remains elusive.

Pearl millet breeding lines ICML12 and ICMB96222 were used in this study due to their respective known susceptibility and resistance against *Puccinia substriata*, the causal agent of leaf rust in pearl millet (C.T. Hash, personal communication). Treatment with salicylic acid was shown to induce rust resistance in the rust susceptible line ICML12 (Crampton et al. 2009) whereas ICMB96222 is a rust resistant breeding line (Sharma et al. 2009). Genes that are differentially expressed upon salicylic acid treatment and rust infection have been identified in ICML12 (van den Berg et al. 2004, Crampton et al. 2009) and it is therefore important to develop a gene silencing system in these two specific pearl millet breeding lines to functionally characterise induced resistance candidate genes.

1.2 Methods for functional characterisation of genes

Recent advances in genome sequencing techniques have identified large numbers of plant genes to which no function has yet been ascribed. In many instances, the classic approach of forward genetics, which investigates the genetic basis for a desired trait, is not sufficient when dealing with large quantities of functionally poorly characterised genes. Reverse genetics, on the other hand, investigates what phenotype or phenotypes arise from specific genes. This approach allows functional characterisation in the absence of other biological information, which is often more difficult to obtain. Several techniques have been developed and refined to annotate and functionally characterise plant genes during the past decade and a half (Gilchrist and Haughn, 2010).

Transformation techniques can either overexpress or inhibit the expression of a target gene while mutagenesis techniques disrupt the nucleic acid sequence of the target or its promoter. Stably transformed plants that display a modified gene expression pattern can be analysed to elucidate the function of the target gene. Alternatively, transient gene function analyses techniques such as transient-induced gene silencing (Schweizer et al. 1999) or virus-induced gene silencing (Benedito et al. 2004), present a fast alternative for plant species where transformation is complicated or unreliable.

Selected approaches to functionally characterise candidate genes, and the applicability thereof in pearl millet, will be discussed in the section below.

1.2.1 Transformation based methods

The ability to express foreign genetic material in a plant was first reported in 1984 (De Block et al. 1984). Since then, transformation has become one of the core techniques used in plant biotechnology. Genetic material is introduced into the host's genome by means of *Agrobacterium tumefaciens* mediated or biolistic transformation to generate a transgenic plant. Target genes can be overexpressed in a specific plant and the phenotype analysed to infer the function of the gene or the role thereof in a biological pathway. For example, *NPR1* is an important regulator of systemic acquired resistance in Arabidopsis (Cao et al. 1997). Transgenic rice plants that overexpress this gene displayed enhanced resistance against the bacterial rice blight pathogen thereby providing evidence for a disease-resistance pathway in monocotyledonous plants comparable to that in the Arabidopsis model system (Chern et al. 2001).

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In contrast to overexpressing a target gene to infer its function, transcription or translation of the gene can be inhibited to generate a transgenic knock-down. A transgenic plant that expresses an RNA interference (RNAi) construct targeted to a specific gene will display a knock-down phenotype of that gene (Waterhouse and Helliwell, 2003). RNAi has become a popular method to functionally characterise genes in recent years (Hannon 2002, Gilchrist and Haughn, 2010). During RNAi, double-stranded RNA (dsRNA) molecules direct degradation of messenger RNA (mRNA), a process that is initiated in a sequence specific manner (Waterhouse et al. 1998). RNAi is activated by introducing dsRNA, or short single-stranded RNA molecules complimentary to the target transcript into plants. These RNA silencing inducers can be delivered by bombarding plants with nucleic-acid-coated particles such as gold or tungsten or by infiltrating plant cells with transgene-carrying *A. tumefaciens* (Waterhouse and Helliwell, 2003). Typically, RNAi transgenes encode either hairpin forming RNAs (hpRNA) or antisense RNAs complimentary to the target genes (Baulcombe 2004).

Transformation based functional characterisation techniques have successfully been employed in cereals such as rice (Miki and Shimamoto, 2004) and wheat (Travella et al. 2006) for which reliable transformation and tissue culture protocols have been established. For example; after sequencing the rice genome the function of several sequences identified as putative genes were established using antisense transformation (Miki and Shimamoto, 2004).

Stable pearl millet transformation has been achieved by means of particle bombardment in a few selected breeding lines. Short term callus culture and morphogenesis of pearl millet were first reported in 1997 (Mythili et al. 1997) and efficient explant regeneration was achieved independently by Mythili et al. and Oldach et al. in 2001. Establishment of *in vitro* plant regeneration protocols laid the foundation for producing transgenic pearl millet and the first transgenic hygromycin resistant plants were reported in 2000 (Lambe et al. 2000). In 2002, Girgi et al. published successful integration of two marker genes, the β-glucuronidase reporter gene, *gus*, and the herbicide resistance gene, *bar*, in four pearl millet breeding lines under the control of the CaMV 35S or the maize Ubiquitin1 promoter. Presence of the two transgenes in tranformants was confirmed by a histochemical GUS assay and resistance against BASTA herbicide, as well as PCR and Southern blot analysis of genomic DNA (Girgi et al. 2002). The positive selectable gene; phosphomannose isomerase (*pmi)*, was shown to be a superior choice as marker gene for pearl millet transformation (O'Kennedy et al. 2004).

Agrobacterium can also be used to generate transgenic plants that carry insertion mutations across its genome by T-DNA insertion. The insertion mutations can be traced by PCR screening with a gene-specific primer used in collaboration with an insertion, or T-DNA based primer (Stepanova and Alonso, 2006). A transgenic rice population of T-DNA insertion lines (Jeong et al. 2006) offer valuable resources for functional analyses of cereal genes. In general, Agrobacterium based transformation methods are reserved for dicotyledonous species and only super-virulent Agrobacterium strains have proven useful in some monocotyledonous plants. In addition, Agrobacterium mediated transformation systems are highly cultivar specific and a susceptible pearl millet breeding line has not been identified to date. In general, pearl millet explant regeneration has proven to be slow and somewhat challenging (Kothari et al. 2005, Ceasar and Ignacimuthu, 2009)

Thus, transformation based techniques, such as overexpression and RNAi and T-DNA mutation, have been successfully applied to confirm the function of candidate genes in some of the economically important cereals but not yet in pearl millet. Transient expression or silencing systems, that circumvent the need for stable transformation, present an attractive alternative to the challenging, breeding line dependent and time consuming process of establishing stable transformation in pearl millet.

1.2.2 Mutation based methods

Another approach to functionally characterise genes is to produce gene disruptions by means of chemical or physical gene mutation. A whole population of plants that carry genome wide mutations can be generated and subsequently screened for genes of interest. Such mutant populations are used as a resource to identify the phenotype of gene mutations. For example, a 20 000 M_2 mutant barley cv. Optic population has been created with the chemical mutagen, ethyl methanesulfonate (EMS) to be used for functional genomics in this cereal (Caldwell et al. 2004). A web accessible database of some of the M_3 mutant phenotypes were developed to be used in forward genetic screens (Caldwell et al. 2004).

The reverse genetics technique TILLING (Targeting Induced Local Lesions IN Genomes) utilize a mismatch specific endonuclease to identify single-base-pair variations in a target gene (Till et al. 2004a). A TILLING population is produced by treating seeds with a chemical mutagen, such as EMS, to induce widespread, randomly distributed mutations in the

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genome. PCR amplification of specific genes from pooled DNA of the TILLING population is analysed electrophoretically in polyacrylamide gels to identify multiple individuals in the TILLING population that have a mutation in the gene of interest (Gilchrist and Haughn, 2005). Offspring from the individual mutants can be used to observe the phenotype resulting from a mutation in the target gene. TILLING populations for five cereals crops namely: barley (Caldwell et al. 2004), maize (Till et al. 2004b), wheat (Slade et al. 2005), rice (Till et al. 2007) and sorghum (Xin et al. 2008) have been developed as reverse genetics tools.

A TILLING population for pearl millet is currently being developed. The IP144499 breeding line has been used to generate a mutant TILLING population by ICRISAT (C.T. Hash, personal communication) and this population will be used to functionally characterise candidate genes.

1.2.3 Virus-induced gene silencing (VIGS)

VIGS is a technique in which a recombinant plant virus induces silencing of a specific host target gene in a homology dependent manner (van Kammen 1997, Baulcombe 1999, Burch-Smith et al. 2004). VIGS exploits the natural, RNA-mediated, antiviral defence mechanism of plants. Van Kammen first used the term VIGS to describe viral infection recovery (van Kammen 1997) but the term has since been used exclusively for the technique of sequence-specific gene silencing directed by recombinant viruses. This technique can be used to determine the biological functional of candidate genes, discover the role of genes in biological pathways (Becker and Lange, 2010) and also to characterise host genes involved in viral pathogenicity (Zhu and Dinesh-Kumar, 2008).

In comparison to other functional genomics approaches, VIGS is rapid, avoids the need for stable transformation as well as regeneration and can overcome functional redundancy by targeting specific gene-family members (Robertson 2004, Purkayastha and Dasgupta, 2009). VIGS can be used to study otherwise lethal genes and validate the phenotype before generating a stable knock-down mutant (Becker and Lange, 2010). The technique has already demonstrated a lot of potential in both forward and reverse genetic characterisation of genes, but also has limitations. For example; silencing symptoms are transient and the duration thereof varies between species. The appearance of symptoms can last for as little as one to two weeks in barley (Holzberg et al. 2002) or as long as 16 weeks in the ornamental

flowering plant *Eschscholzia californica* (Wege et al. 2007). Silencing is mostly limited to specific plant organs and often fails to extensively penetrate all tissues (Wege et al. 2007). VIGS is not suitable for all plant species due to incompatibility between certain plants and viruses. Another limiting factor in developing a VIGS system could be the availability of appropriate biosafety measures to ensure containment of the recombinant virus (Purkayastha and Dasgupta, 2009).

1.2.3.1 Natural gene silencing in plants: Post-transcriptional gene silencing

RNA silencing is an evolutionary conserved process which has been termed quelling in fungi (Cogoni et al. 1996), RNAi in animal systems (Fire et al. 1998) and post-transcriptional gene silencing (PTGS) in plants (Kumagai et al. 1995). PTGS was first observed when over-expressing the chalcone synthase gene in Petunia induced the unexpected suppression thereof (Napoli et al. 1990). This phenomenon was originally referred to as co-suppression (Napoli et al. 1990, van der Krol et al. 1990). In 1995, Kumagai et al. reported that a recombinant RNA virus inhibited the carotenoid biosynthetic pathway in tobacco by means of repressing endogenous gene expression. The potential of viruses to suppress a targeted gene, by means of the sequence specific mRNA degradation process of PTGS, was soon recognised as a tool to analyse gene function (Baulcombe 1999).

RNA silencing is induced by small RNA molecules and mediates the cellular processes of chromatin modification, mRNA degradation and translational repression (Sunkar and Zhu, 2007, Brodersen et al. 2008). PTGS has been studied extensively in model plants and many of the genes involved in the biogenesis, processing and mode of action of small RNAs in plants have been identified in Arabidopsis and rice (Vaucheret 2006, Sunkar and Zhu, 2007). In terms of the underlying mechanism, small RNAs are loaded into an RNA-induced silencing complex (RISC), bound to the ARGONAUT1 (AGO1) proteins and used to induce PTGS by targeting complementary, or partly complementary, mRNAs for degradation or translational repression (Baulcombe 2004, Hammond 2005).

Small RNA species can be divided into two major classes: microRNAs (miRNA) and small interfering RNAs (siRNAs). miRNAs are non-coding 20-22 nt RNAs derived from single transcripts encoded by genomic miRNA genes that form imperfect hairpins. Precursor double-stranded or hairpin transcripts of the miRNA genes are processed by an RNase III

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enzyme named DICER LIKE-1 (DCL1) and associated proteins to form miRNAs (Hammond 2005, Vaucheret 2006). Various aspects of plant development, auxin signalling and stress response are regulated by miRNAs (Jones-Rhoades et al. 2006, Navarro et al. 2006, Sunkar and Zhu, 2007). In Arabidopsis, other members of the DCL family, DCL-2, DCL-3 and DCL-4, process double-stranded RNA (dsRNA) to form siRNAs. These 21-23 nt siRNAs are formed from endogenous, transgene or viral derived dsRNA (Waterhouse et al. 2001, Hammond 2005, Vaucheret 2006).

Antiviral immunity in plants is mediated by virus derived siRNAs and results in the specific silencing of viruses by RISC (reviewed in Ding and Voinnet, 2007). The dsRNA are produced by RNA-dependent RNA polymerases (RdRP) during the replication of RNA viruses. Furthermore, double-stranded RNA replication intermediates of DNA viruses and highly structured regions of viral genomes are targeted by the cellular PTGS machinery (Benedito et al. 2004, Moissiard and Voinnet, 2006, Shao et al. 2008).

The current model for PTGS divides the process into three phases namely: triggering/initiation, maintenance and signal amplification/dissemination (Nishikura 2001, Shao et al. 2008). The process is summarized in Figure 1.2. Initiation (Figure 1.2, A) involves the formation of viral derived dsRNAs which trigger the PTGS process (Shao et al. 2008). Viral derived dsRNAs are targeted and cleaved by the DCL-1 enzyme to produce siRNAs which are typically in plants between 21 and 23 nucleotides long (Bernstein et al. 2001, Benedito et al. 2004). During the maintenance phase (Figure 1.2, B) siRNA molecules, also referred to as guide RNAs, are incorporated into the RISC protein complex (Hammond et al. 2000, Hammond 2005). RISC has exo- and endonuclease activities, RNA homology searching activity and helicase activity to unwind double-stranded RNA. siRNAs are unwound by RISC and these single-stranded sequences are used for identification and degradation of complementary transcripts (Benedito et al. 2004).

Silencing is amplified and the signal disseminated throughout the plant during the last phase (Figure 1.2, C). Cellular RdRP use siRNAs from the maintenance phase to prime dsRNA synthesis from complementary transcripts. The resulting dsRNAs are recognized by DCL1 in the initiation phase, which leads to amplification of silencing along the transcript (Waterhouse et al. 2001, Benedito et al. 2004, Purkayastha and Dasgupta, 2009). The secondary siRNAs therefore extend regions targeted for degradation.

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Systemic dispersal of the silencing signal was demonstrated by grafting experiments and is referred to as systemic acquired silencing (SAS) (Palauqui et al. 1997). SAS allows the RNA degradation process, which starts out on a local, cellular level, to spread systemically via the mobile silencing signal (Vance and Vaucheret, 2001, Shaharuddin et al. 2006). The silencing signal is virus-derived and 21 nt siRNAs are believed to act as the short range silencing signal whilst RNA-dependent RNA polymerase6 (RDR6) is necessary for long range signal transportation (Ding and Voinnet, 2007, Kalantidis et al. 2008). It has been shown that the silencing signal moves via the phloem from source to sink (Tournier et al. 2006).

1.2.3.2 Methodology of VIGS

In order to conduct a VIGS experiment, a viral vector that carries a fragment of the host target gene is used to induce gene silencing by means of PTGS. Thus, the plant defence mechanism that is normally used to suppress virus accumulation is now directly targeting the host plant gene itself. There are several factors to take into consideration during the experimental design concerning the choice of e.g. viral vectors, candidate gene inserts, delivery to the host species, a reporter gene and the environmental conditions for the experiment. These factors will be discussed in further detail.
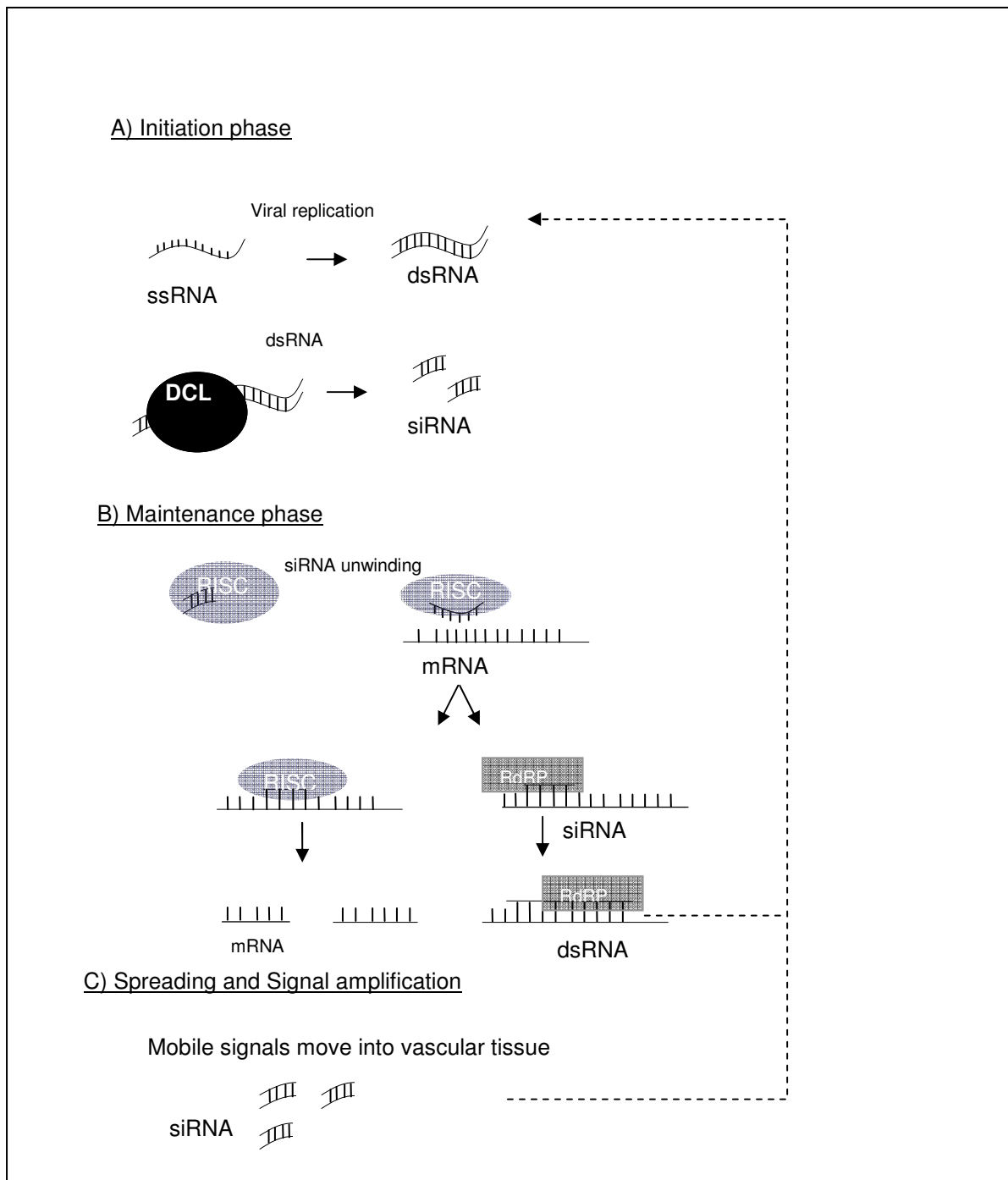
Figure 1.2 Model for post-transcriptional gene silencing in plants induced by single-stranded RNA viruses. During the initiation phase, when a single-stranded RNA (ssRNA) virus reproduces, double-stranded RNA (dsRNA) is generated from a viral encoded RNA-dependent RNA polymerase (RdRP). The dsRNA is recognized by DICER LIKE (DCL) enzymes and cut into 21-23 nucleotide fragments called small interfering RNAs (siRNAs). During the maintenance phase, the RNA induced silencing complex (RISC) uses siRNAs to find complimentary RNA and cleaves it by means of RNase activity. In addition, siRNAs prime dsRNA synthesis by RdRP which in turn amplifies the silencing signal along the transcript in a feedback fashion. The systemic spread of silencing occurs when the short siRNAs moves to other plant parts (Benedito et al 2004, Shao et al. 2008).

1.2.3.2.1 Reporter gene

It is important to establish an optimal virus inoculation protocol, stable infection levels and plant growth conditions to obtain reproducible silencing results in a VIGS experiment (Burch-Smith et al. 2004). Environmental factors such as temperature, humidity and photoperiod have been found to influence the efficacy of VIGS in tomato (Fu et al. 2005), *Arabidopsis thaliana* (Meza et al. 2001) and barley (Bruun-Rasmussen et al. 2007). A reporter gene with a clear, reproducible silencing phenotype allows optimization of experimental variables. Several genes such as phytoene desaturase (*Pds*), actin, β7 subunit of the 20S proteasome complex (20S-β7) and a subunit of the magnesium-protoporphyrin chelatase complex (*ChlH*) involved in chlorophyll biosynthesis, have visual silencing phenotypes and have been used as reporter genes in developing VIGS in a range of plant species (Table 1).

Pds has frequently been used to evaluate efficacy of viruses as new VIGS vectors (Kumagai et al. 1995, Ratcliff et al. 2001, Holzberg et al. 2002, Ding et al. 2006). Phytoene desaturase is an enzyme that converts the substrate phytoene to ζ-carotene. Carotenoids protect chlorophyll from UV damage and plants with ζ-carotene deficiency that are exposed to light, develop symptoms called photobleaching due to degradation of chlorophyll (Garcia-Asua et al. 1998). Symptoms of *Pds* virus-induced gene silencing vary slightly in different host/virus systems and are often confined to the narrow stripes parallel to leaf veins in monocotyledonous plants (Scofield et al. 2005, Ding et al. 2006). The symptoms are generally lost by the fourth systemically infected leaf (Holzberg et al. 2002, Scofield et al. 2005, Ding et al. 2006).

Gene silencing can precede and outlast the occurrence of phenotypic symptoms. A 65% reduction of *Pds* transcripts could be measured before any photobleaching appeared and a 48% reduction outlasted the lack of visual phenotypes in barley (Bruun-Rasmussen et al. 2007). These results emphasize the importance of molecular techniques to verify silencing phenotypes. Transcript reduction can be analysed electrophoretically by means of semi-quantitative reverse transcription PCR (Burch-Smith et al. 2004) or more precisely by quantitative reverse transcription PCR (Rotenberg et al. 2006). Another molecular technique to verify silencing of the reporter gene is high performance liquid chromatography (HPLC). HPLC allows quantification of specific chemical compounds and has been used to confirm *Pds* silencing by measuring the amount of the enzyme's substrate, phytoene, in tissue

(Holzberg et al. 2002). A pure solution of phytoene is used to determine the standard elution time graph which is subsequently used to quantify phytoene in control and silenced tissue.

Table 1 Visual silencing phenotypes of plant genes that have been used as reporter genes in virus-induced gene silencing experiments.

Reporter gene	Abbreviation	Silencing phenotype	Description	Reference
H subunit of the magnesium chelatase enzyme	*ChlH*		Prevents chlorophyll synthesis	Tai et al. (2005)
Actin	Actin		Stunting	Ding et al. (2006)
Phytoene desaturase	*Pds*		Photobleaching	Holzberg et al. (2002)
β7 subunit of the 20S proteasome complex.	*20S-β7*		Spontaneous Programmed cell death	Tai et al. (2005)

1.2.3.2.2 Insert design and orientation

The next consideration in developing a VIGS system is the sequence, design and orientation of the insert in the virus vector. One or more at least 23 nt long fragments with 100% similarity to the target gene are required to act as a silencing trigger (Benedito et al. 2004). The sequence of this insert should be unique to the target gene to prevent off-target silencing of any other host transcripts. Silencing a green fluorescent protein (GFP) transgene in *Nicotiana benthamiana* was achieved with a 23 nt insert using potato virus X (PVX) (Thomas

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et al. 2001). However, in general, insert lengths between 100 and 800 bp are used in VIGS experiments (Robertson 2004).

Plant viruses have a strong propensity to lose non-viral sequences inserted into their genomes (Pogue et al. 2002) and thus stability of the insert in the virus is one of the main challenges in VIGS. Large inserts are unstable and easily lost from the recombinant virus and optimal insert sizes have been defined for several of the established VIGS vectors (Avesani et al. 2007, Bruun-Rasmussen et al. 2007, Cakir and Tör, 2010). For example; BSMV carrying fragments from 128 to 584 bp can induce silencing in barley (Bruun-Rasmussen et al. 2007) whereas inserts smaller than 120 bp are significantly less effective for BSMV VIGS (Scofield et al. 2005, Bruun-Rasmussen et al. 2007). Insert length have been found to influence insert stability but not silencing efficiency in BSMV mediated VIGS in barley (Bruun-Rasmussen et al. 2007).

Silencing of target genes is influenced, not only by the length of the insert, but also by its orientation and design in the virus vector. The production of dsRNA, and subsequent transcript cleavage, is enhanced considerably in virus vectors that contain inverted-repeat sequences of the target gene (Lacomme et al. 2003). Such hairpin-forming inserts have been shown to produce stronger and more robust silencing of trans- or endo- genes in different host/virus systems. Inserts designed as palindromic sequences of target genes produce dsRNA from intra-, but mostly intermolecular hybridization thereby enhancing the occurrence of VIGS (Waterhouse et al. 1998, Lacomme et al. 2003). Hairpin or inverted-repeat inserts reduced transcript levels significantly more than inserts in the sense or antisense orientation in tobacco mosaic virus (TMV) mediated VIGS in tobacco and BSMV VIGS in barley (Lacomme et al. 2003).

Another factor that has to be taken into account when designing the insert is the downstream verification of silencing. For this, an oligonucleotide primer pair that targets a fragment out-of the region of the silenced gene has to be identified. The primers can quantify transcript degradation in silenced plants compared to uninfected or control plants with semi-quantitative or quantitative reverse transcription PCR but avoid detection of viral derived transcription of the target gene.

During the process of PTGS many siRNAs can be derived from the cleavage of dsRNA by DCL (Benedito 2004) (Figure 1.2, A). RdRP produce many secondary siRNAs which amplify and expand the silencing signal to non-target transcripts (Xu et al. 2006) as seen in

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Figure 1.2, B. Analysis of any unintended silencing of additional targets (off-targets) is necessary to design an optimal insert. A software programme called "siRNA Scan" (http://bioinfo2.noble.org/RNAiScan.htm) identifies secondary off-target silencing of an insert in a selected data set. The program can be used to identify an insert that causes no, or minimal, secondary silencing of additional host genes for fully sequenced organisms. Sequence specifications for silencing inserts have been proposed (Reynolds et al. 2004) but these criteria do not necessarily apply to all host organisms.

1.2.3.2.3 Delivery method

Once the target insert has been identified and cloned into a suitable viral vector, the host plant species can be infected with the recombinant virus to conduct VIGS. There are several methods to introduce the VIGS vector into the host, namely mechanical inoculation with *in vitro* transcribed RNA or a crude sap extract prepared from infected leaf material, agroinoculation, or microprojectile bombardment.
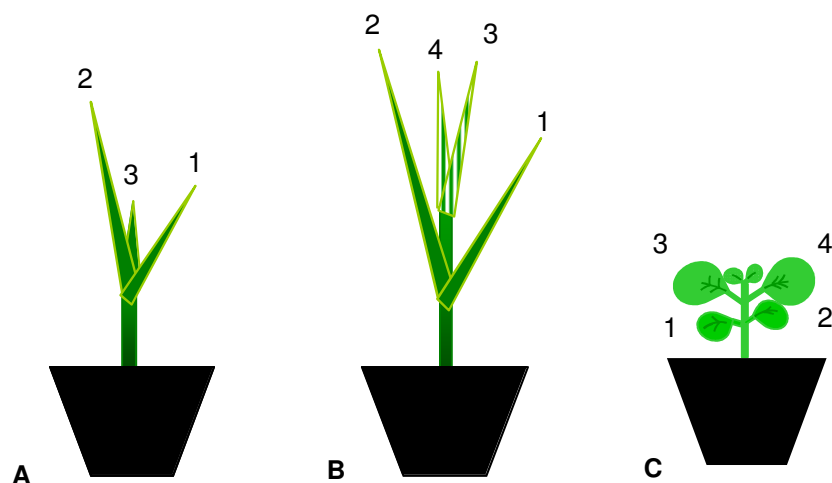
Figure 1.3 Schematic representation of growth stages suitable for mechanical inoculation and VIGS symptoms. A) Leaf one (cotyledon) and leaf two (the first true leaf) in monocotyledonous plants are typically inoculated at the growth stage where the third leaf is just emerging. B) Photobleaching symptoms are expected in the third and fourth leaf from 10 days after infection. C) Leaf three and four of dicotyledonous plant such as *Nicotiana benthamiana* are infected when the leaves are approximately 1.5 to 2 cm in diameter.

Mechanical inoculation is the recommended method to initially establish infections for certain VIGS vectors (X.S. Ding, personal communication). In monocotyledonous species the cotyledon (referred to as leaf 1 or L1) and first true leaf (L2) of seedlings are infected

with a mixture of viral RNA and buffer at the growth stage when the third leaf (L3) is emerging (Figure 1.3, A). In dicotyledonous species the first two true leaves (Leaf 3 and 4) are infected when the leaves are approximately 2 cm in diameter as indicated in Figure 1.3, C. Although time consuming and expensive, mechanical inoculation has been reported to increase silencing efficiency in certain hosts (Ratcliff et al. 2001). In the case of RNA viruses, infectious RNA to be used for mechanical inoculation can be transcribed, *in vitro* using DNA-dependent RNA polymerase from DNA plasmids, linearized downstream of a cDNA copy of the viral RNA (Petty et al. 1989). This technique has been used for both BSMV and BMV mediated VIGS (Holzberg et al. 2002, Scofield et al. 2005, Ding et al. 2006).

A crude sap extract from *N. benthamiana* infected with a BMV VIGS vector was used to infect barley, rice and maize to induce silencing of a target gene (Ding et al. 2006). The sap extract is typically derived from the first and second leaves that emerge after infection, since stability of the insert has been shown to diminish as the virus continues to replicate in the host (Pogue et al. 2002).

Agrobacterium based vectors can be used to shuttle the VIGS virus into the host plant and such vectors have been developed for both DNA and RNA viruses. Agrobacterium is used to introduce an expression cassette, which contains the VIGS virus genome, into the host species by T-DNA transfer. Viral RNA transcribed by host RNA polymerases, initiate the RNA silencing pathway (Robertson 2004). This method of viral delivery is more popular with dicotyledonous hosts due to the fact that the natural host range of the bacterium excludes certain monocotyledonous plants, but has been used as a delivery method of the rice tungro bacilliform virus (RTBV) VIGS vector in rice (Purkayashta et al. 2010).

Alternatively, viral DNA in plasmid form under control of a CaMV 35S promoter can be bombarded into plant cells. Successful gene silencing using such a BSMV construct has been achieved in barley using microprojectile bombardment (Hu et al. 2009). This method avoids the need for expensive *in vitro* transcription and does not depend on Agrobacterium-susceptibility of the host. However, biolistic equipment that is suited to bombard whole plants or intact leaves instead of explant material such as callus or protoplast are required if biological assays will be conducted subsequently to VIGS.

1.2.3.2.4 Virus-host compatibility

Many viruses have been used to derive VIGS vectors for several plant species during the past decade and a half (reviewed in Robertson 2004, Shoa et al. 2008, and Purkayastha and Dasgupta, 2009). These include, but are not limited to, RNA viruses such as, TMV used in *Nicotiana benthamiana* (Kumagai et al. 1995), PVX used in *N. benthamiana* and potato (Ruiz et al. 1998, Faivre-Rampant et al. 2004), and tobacco rattle virus (TRV) used in *N. benthamiana* and tomato (Ratcliff et al. 2001, Liu et al. 2002), DNA viruses such as tomato golden mosaic virus (TGMV) used in *Arabidopsis thaliana* (Peele et al. 2001) and the RNA satellite virus named satellite tobacco mosaic virus (STMV) used in *N. tabacum* (Gossele et al. 2002).

A suitable VIGS vector must be able to infect its host without causing lethal or obscuring symptoms and the host should tolerate substantial viral accumulation to form dsRNA molecules, which induce silencing (Voinnet 2001). A virus is not suitable to conduct VIGS if it has the ability to suppress host defence processes by distinct interactions with the host gene silencing machinery (Voinnet et al. 1999, Voinnet 2005). Viruses have evolved a wide range of mechanisms to suppress or evade PTGS and several of the suppressors encoded by plant viruses have been identified (Ding and Voinnet, 2007). Suppressors target specific parts of the RNA silencing pathway and two main kinds of suppression can occur (Roth et al. 2004). The first kind of suppression targets the small RNA metabolism. For example; tombusvirus encoded protein P19 binds specifically to siRNA, preventing association with RISC (Voinnet 2005). The second kind of suppression targets systemic silencing. One of the first suppressors identified was Potyvirus encoded helper component proteinase HcPro which has been shown to enhance replication of TMV, PVX and cucumber mosaic virus (CMV) in *N. benthamiana* (Pruss et al. 1997). HcPro inactivates the RNA silencing pathway by interaction with a calmodulin-like protein, regulator of gene silencing CaM (*rgsCaM*) thereby preventing systemic silencing (Anandalakshmi et al. 2002, Roth et al. 2004).

The severity of host infection is not only determined by viral suppression of silencing but also by the balance between virus replication and spread within the host and host defence mechanisms (Hiriart et al. 2003). The presence of viral resistance genes in hosts render VIGS vectors ineffective if the virus carries the cognate *avr* gene (Kang et al. 2005). For example, the toll and the interleukin-1 receptor (TIR) nucleotide binding (NB), leucine rich repeat (LRR) resistance gene *N* in tobacco confers resistance to TMV (Whitham et al. 1994)

and potato genes *Rx1* and *Rx2* control resistance to PVX (Bendahmane et al. 2000). Identification of *R* genes in monocotyledonous species has largely been limited to the major crop species, such as rice, wheat and barley (Kang et al. 2005). For example, the *Bdv1* allele confers resistance to barley yellow dwarf virus (BYDV) in wheat (Singh 1993). Viral symptoms in any specific plant cultivar/accession must therefore be examined and positive/negative control plants, which can be inoculated with the wild type or virus that contains a control insert, should be included in experimental design to account for susceptibility/resistance against or virus induced phenotypes in the host respectively (Burch-Smith et al. 2004).

Only four viruses have been modified and applied for VIGS in monocotyledonous species: barley stripe mosaic virus (BSMV), which has been used in barley (Holzberg et al. 2002), wheat (Scofield et al. 2005, Tai et al. 2005), ginger (Renner et al. 2009) and *Brachypodium distachyon* (Demirican and Akkaya, 2010). Brome mosaic virus (BMV) has been used in barley, rice and a specific cultivar of maize (Ding et al. 2006, van der Linde et al. 2011). The third virus, cymbidium mosaic virus (CymMV), was the first VIGS vector used in a non-crop monocotyledonous species and has been used to conduct gene silencing in orchids (Lu et al. 2007). The fourth virus, rice tungro bacilliform virus (RTBV), which is also the first VIGS vector derived from a DNA virus, has been developed for rice (Purkayastha et al. 2010). Apart from the four aforementioned viruses (Table 2), several other cloned viruses have been identified as potential gene silencing vectors in monocotyledonous species (Scofield and Nelson, 2009). Pearl millet is a natural host for several viruses (reviewed in Wilson 2000, see Table 3) and two such viruses have already been isolated and cloned, and could potentially be developed as VIGS vectors (Scofield and Nelson, 2009). It would, however, be valuable to evaluate the transferability of one of the existing vectors to pearl millet before investing resources into developing a new virus VIGS vector.

Table 2 Recombinant virus vectors with established ability to conduct gene silencing in monocotyledonous species.

Virus	Type	Host Species	Reference
BSMV	RNA/*Hordeivirus*	*Hordeum vulgare*	Holzberg et al. (2002) Bruun-Rasmussen et al. (2007), Hein et al. (2005), Oikawa et al. (2007), Meng et al. (2009), Hu et al. (2009)
		Triticum aestivum	Scofield et al. (2005), Tai et al. (2005), Cakir and Scofield (2008)
		Brachypodium distachyon	Demirican and Akkaya (2010)
		Haynaldia villosa	Wang et al. (2010)
		Zingiber officinale	Renner et al. (2009)
BMV	RNA/*Bromovirus*	*H. vulgare*	Ding et al. (2006)
		Oryza sativa	
		Zea mays	
CymMV	RNA/*Potexvirus*	*Phalaenopsis* sp.	Lu et al. (2007)
RTBV	DNA/*Tungrovirus*	*O. sativa*	Purkayastha et al. (2010)

BSMV, Barley stripe mosaic virus; BMV, Brome mosaic virus; CymMV, Cymbidium mosaic virus; RTBV; Rice tungro bacilliform virus

Table 3 Viruses reported to cause disease in pearl millet (compiled from Wilson 2000).

Virus	Clone availability	Symptoms	Reference
Black-streaked dwarf virus	No	Stunting and dwarfing	Luttrell (1954)
Guinea grass mosaic virus	No	Striped mosaic, eyespots dwarfing	Kukla et al. (1984)
Indian peanut clump virus	No	None described in literature	Reddy et al. (1998)
Maize streak virus	Yes	Non-severe chlorotic streaks	Mesfin et al. (1992)
Panicum mosaic virus	No	Mild chlorotic mottle	Day et al. (1994)
Satellite panicum mosaic virus	No	Severe chlorotic mottle	Qui et al. (1998)
Wheat streak mosaic virus	Yes	Non-severe chlorotic streaks	Seifers et al. (1996)

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1.2.3.2.5 Environmental conditions

Environmental factors such as temperature, humidity and photoperiod have been found to influence the efficacy of VIGS in tomato (Fu et al. 2005) and *Arabidopsis thaliana* (Meza et al. 2001), barley (Bruun-Rasmussen et al. 2007) and wheat (Cakir and Tör, 2010). Infection temperatures influence BSMV mediated *Pds* silencing significantly in barley (Bruun-Rasmussen et al. 2007) and wheat (Cakir and Tör, 2010). In barley, low temperatures of 16°C delayed onset and reduced the extent of *Pds* silencing whilst high temperatures of 28°C retarded plant growth and limited silencing. The optimal infection temperature was found to be at 22°C (Bruun-Rasmussen et al. 2007). In wheat, the highest accumulation of small RNAs occurs in the second leaf at low temperatures (18°C) and the best *Pds* silencing in wheat, as with barley, occurred at 22°C (Cakir and Tör, 2010). These findings indicate that optimal temperatures for VIGS are virus/host specific and should be established for a specific virus/host combination.

1.2.3.2.6 Cultivar specificity

VIGS phenotypes of the same gene can differ in cultivars of the same plant species. Differences in the silencing phenotype have been observed in several petunia cultivars where the carotenoid biosynthetic gene, *chalcone synthase* (CHS), was silenced using a TRV VIGS vector (Chen et al. 2004). A similar result was observed in the barley cultivar Golden Promise where BSMV-induced viral symptoms and *Pds* silencing were reduced and delayed if compared to the cultivar Black Hulless (Holzberg et al. 2002).

As mentioned before, host resistance towards viruses influence the extent of gene silencing that occurs and resistance could differ between cultivars of the same species. Therefore, it is important when developing a VIGS system, to consider the effect of the VIGS vector in the specific cultivar of interest, especially if a cultivar-specific biological assay is to be performed on silenced plants.

1.2.3.3 Barley stripe mosaic virus VIGS vector development and use

BSMV is a member of the *Hordeivirus* genus. It can be mechanically transmitted to a broad host range of cereals including barley, wheat and oats (Jackson and Lane, 1981). Symptoms

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can be latent (asymptomatic infection) or include yellow and white mottling and necrosis. Moderate stunting can occur and infected plants bear poorly developed ears (Kassanis and Slykhuis, 1959).

The BSMV genome consists of three single-stranded RNA molecules (Petty et al. 1989). The genome, as seen in Figure 1.4, is organized in three capped genomic RNAs namely RNAα, RNAβ and RNAγ and three subgenomic RNAs (sgRNA). RNAα encodes the αa protein which is required for viral replication. RNA β contains five ORFs: βa, TGB1, TGB2, TGB3 and TGB2'. The coat protein βa is translated directly from genomic RNA. The overlapping triple gene block (TGB) contains proteins required for virus movement and are expressed from two subgenomic RNAs. TGB1, encoded by sgRNAβ1, has RNA binding activity and TGB2 and TGB3 are small hydrophobic proteins that target TGB1 to plasmodesmata. A small translational read through protein, TGB2' is dispensable for infection. RNA γ encodes two proteins, γa which is the polymerase subunit of the replicase and the γb protein. γb is expressed from the sgRNAγ and is involved in pathogenesis (Jackson et al. 2009).
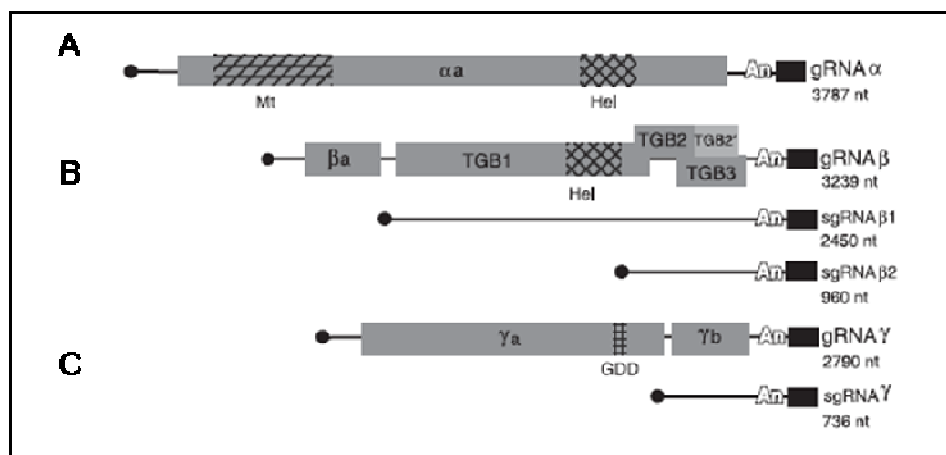

Figure 1.4 Schematic representation of the tripartite BSMV genome. The filled circle on the end of each genomic (gRNA) and subgenomic RNA (sgRNA) represents the 5' cap structure. On the 3' end following a polyadenylate sequence (An) the solid rectangles represents a 238 nt terminus. Solid grey blocks represent open reading frames and domains are indicated in checked lines. A) RNA α. Encodes the αa protein. B) RNA β contains five ORFs: βa, three overlapping genes in the triple gene block (TGB), TGB1, TGB2, TGB3, and a small translational read through protein, TGB2'. C) RNA γ encodes two proteins: γa and γb. Figure and text adapted from Bragg and Jackson (2004).

Full length cDNA clones of the BSMV strain ND18 (Petty et al. 1989) were used to develop a suitable VIGS vector. Foreign genes were successfully expressed by cloning them in the RNAγ subunit (Holzberg et al. 2002). Restriction enzyme recognition sites were inserted

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behind the γb gene in RNAγ, to allow directional cloning of target gene fragments. The *Pds* gene was used to evaluate silencing due to its readily discernable knock-down phenotype which involved streaks and patches of unusually white tissue in L4 and occasional small faint streaks in L3 near the leaf base in plants where L1 and L2 were infected (see Figure 1.3) (Holzberg et al. 2002). White photobleached tissue was morphologically indistinguishable from healthy green tissue when viewed under a transmission microscope. Silenced patches were larger and confluent in L5 and L6 when fully expanded but after 21 days post-inoculation (dpi) emerging leaves showed no more silencing symptoms (Holzberg et al. 2002). Barley cv. Black Hulless plants infected with this RNAγ-based BSMV VIGS vector, expressing a barley *Pds* gene fragment, displayed photobleaching by 8 to 10 days after infection in systemically infected tissues. Symptoms of the infected plants were similar to plants treated with the chemical inhibitor of Pds, norflurazon. Phytoene, the substrate of the Pds enzyme, was shown to accumulate to higher amounts in the infected compared to uninfected barley seedlings, thereby confirming down-regulation of the targeted *Pds* gene. This was the first report of VIGS in a monocotyledenous plant (Holzberg et al. 2002). Deleting the CP from BSMV RNAβ resulted in enhanced silencing in barley. This CP-deletion vector was named BSMVΔCP (Holzberg et al. 2002) and used subsequently to functionally characterise genes involved in powdery mildew resistance in barley (Hein et al. 2005, Dagdas et al. 2009) and also to classify the involvement of the P23k gene in secondary cell wall formation in barley leaves (Oikawa et al. 2007).

In wheat, BSMV CP-deletion induced increased necrosis which obscured the true silencing phenotype. Wild type RNAβ instead of the CP deletion RNAβ were therefore used to optimize the BSMV VIGS vector for wheat (Scofield et al. 2005). Again, *Pds* was used as a reporter gene to establish BSMV as a VIGS vector, which was subsequently used to characterise Lr21 mediated leaf rust resistance in wheat (Scofield et al. 2005). Mosaics and chlorosis, characteristic of BSMV infection in barley, were also found to be milder in wheat than in barley (Scofield et al. 2005).

Another BSMV VIGS vector has been developed to conduct gene silencing in wheat (Tai et al. 2005). This vector differs from BSMVΔCP only in that expression of the silencing suppressor γb (Bragg and Jackson, 2004) is blocked. Three reporter genes *Pds*, *ChlH* and *20Sβd7* were silenced using this vector (Tai et al. 2005). The same vector was used to transiently express the ToxA protein, a fungal toxin involved in wheat tan spot resistance (Tai et al. 2007).

A BSMV VIGS vector that can silence two wheat genes simultaneously was developed by Cakir and Scofield (2008). Two gene fragments, *Pds* used as a visual marker gene and *Sgt1* a defence-related gene, were cloned in tandem downstream of the γb protein in RNAγ. Quantification of the marker and target gene transcripts indicated that both genes were down-regulated. The visual *Pds* silencing phenotype could conveniently indicate where the target gene was likely to be silenced (Cakir and Scofield, 2008). The efficiency of gene silencing by a vector with two target inserts is, however, lower than a vector carrying a single target gene (Cakir and Tör, 2010). Bruun-Rassmussen et al. (2007) determined that BSMV loses or partially deletes inserts that exceed a certain length. A strategy to circumvent the loss of large fused reporter and target gene inserts from the viral genome has been published by Campbell and Huang (2010). Wheat plants were infected with a mixture of BSMV constructs containing single inserts of a visual reporter and a target gene respectively. Both genes could be silenced simultaneously and as efficiently as targeting a single gene. Co-silencing with the visual marker gene *Pds* provided an effective internal indication of the Lr21 rust resistance target gene silencing (Campbell and Huang, 2010).

Recently, BSMV VIGS, targeting *Pds*, has successfully been performed in the emerging monocotyledonous model crop *Brachypodium distachyon* (Demircan and Akkaya, 2010). *B. distachyon* is increasingly seen as a favourable alternative model crop to *Oryzae sativa* (rice), due to its comparably small genome size and the additional small plant size, short lifecycle and simple growth requirements. BSMV does not infect rice and the development of BSMV to functionally characterise crop genes in a model plant is a valuable addition in understanding monocotyledonous genetics. To date, BSMV is the most extensively used monocotyledonous VIGS vector and factors that affect stability of the viral vector have been studied extensively (Bruun-Rasmussen et al. 2007, Scofield and Nelson, 2009, Cakir and Tör, 2010).

1.2.3.4 Brome mosaic virus VIGS vector development and use

Brome mosaic virus (BMV), a member of the genus *Bromovirus*, has a broad monocotyledonous host range including wheat, barley, oat, rye, sorghum and several other grasses (McKinney 1944). This tripartite positive strand RNA virus has been well studied as a model of viral processes such as replication, movement and virion formation. The virus has been reported to naturally infect several grasses in Europe, North America, Asia and Africa

(Koa and Sivakumaran, 2000). BMV is mainly spread by mechanical transmission and two beetle species, *Chaerocnema aridula* and *Oulema melanopus*, are reported to be natural vectors for BMV transmission (Panarin 1978).

Barley is often used as host plant to study BMV infectivity. Symptoms in susceptible barley varieties include mosaic symptoms from seven to ten days after infection (Rao and Grantham, 1995). *Nicotiana benthamiana*, a small Solanaceous plant often used in the development of VIGS vectors (Baulcombe 1999, Voinnet 2001) is also a host for BMV (Ford et al. 1970). BMV infection in *N. benthamiana* is symptomless but has been shown to produce symptoms when a spontaneous mutation in the movement protein gene of BMV results in vein chlorosis (Rao and Grantham, 1995). BMV infection in tall fescue (*Festuca arundinacea* Schreb.) is reported to cause stunting and low vigour in addition to mosaic symptoms (Rouf Mian et al. 2005).

The BMV genome consists of three capped genomic RNA segments known as RNA 1 (GenBank NC_002026), RNA 2 (GenBank NC_002027) and RNA 3 (GenBank NC_002026) (Figure 1.5) as well as one subgenomic RNA, RNA 4. Non-structural proteins 1a and 2a enable viral replication and are encoded by RNA 1 and RNA 2 respectively. The 1a protein has m7G methyltransferase activity and contains helicase motifs in the C-terminal half (Yi et al. 2009). This protein mediates replication by assembling the RdRP and viral RNAs to form a reconfigured membrane mini-organelle called a spherule in which RNA replication can occur (Yi et al. 2009). BMV RdRP is encoded by the 2a coding sequence from RNA 2. The 5' half of the bicistronic RNA 3 encodes a movement protein and the 3' half encodes the coat protein. The 30 kDa movement protein allows cell-to-cell movement of the virus and the coat protein enables host specific cell-to-cell movement and virion formation (Ahlquist 1999). The coat protein has also been implicated in regulating viral accumulation during BMV infection by inhibiting the accumulation of replication protein 1a (Yi et al. 2009). RNA 4 shows homology to the 3' half of RNA 3 and is transcribed from a promoter within the intergenic region of RNA 3 during infection (Ahlquist 1992). Each of the BMV genomic RNAs are packaged in a separate capsid, to form three equally sized nucleocapsids. Sub-genomic RNA 4 is packed with the smaller RNA 3 and thus adds up to approximately 3000 nucleotides, similar to RNA1 and RNA 2 (Wojtkowiak et al. 2002).

RNA 3 is dispensable for viral replication but the proteins RNA 3 encodes, a movement protein and the capsid protein, are required for systemic movement of infection (Ahlquist

1992). This finding indicated that RNA 3 of BMV could potentially be modified to obtain a suitable VIGS vector and to insert target gene sequences (Wojtkowiak et al. 2002). A BMV virus vector, able to introduce foreign RNA into barley plant cells, was constructed by cloning cDNA copies of all three BMV viral RNAs. The coat protein was deleted from RNA 3 and a multiple cloning site was created to allow insertion of foreign sequences (Wojtkowiak et al. 2002). The recombinant RNA 3 could be identified by means of reverse transcription PCR in mechanically infected plants. The highest accumulation occurred at five days after infection and the presence of the recombinant fragment diminished by 15 days after infection (Wojtkowiak et al. 2002). This BMV vector, however, was not analysed for efficiency to silence targeted genes.

A virus isolated from tall fescue grass was identified as BMV (Rouf Mian et al. 2005) and this isolate, referred to as F-BMV, was cloned and modified as a vector for gene silencing in barley, rice and a specific maize cultivar (Ding et al. 2006). RNA 1 and RNA 2 of F-BMV were shown to be the genetic determinants that controlled systemic infection in rice. RNA 3 from a Russian strain of BMV (R-BMV) was cloned and modified to accept inserts. Infection with a hybrid virus (H-BMV), consisting of RNA 1 and RNA 2 from F-BMV and RNA 3 from R-BMV, which expressed a maize *Pds* gene fragment induced visual photobleaching symptoms in barley, maize and rice. Decreased *Pds* transcript levels corresponded with the phenotypic photobleaching symptoms thereby verifying the silencing result and thus the suitability of the H-BMV vector for VIGS (Ding et al. 2006). H-BMV infection causes light yellow chlorosis in barley (Figure 1.6, far left) and photobleaching could be distinguished from the viral symptoms as distinct, pronounced yellow and white streaks induced by the H-BMV *Pds* vector (Figure 1.6, middle) (Ding et al. 2006). Photobleaching did not extend over the entire leaf blades in barley, maize and rice infected with *Pds* BMV
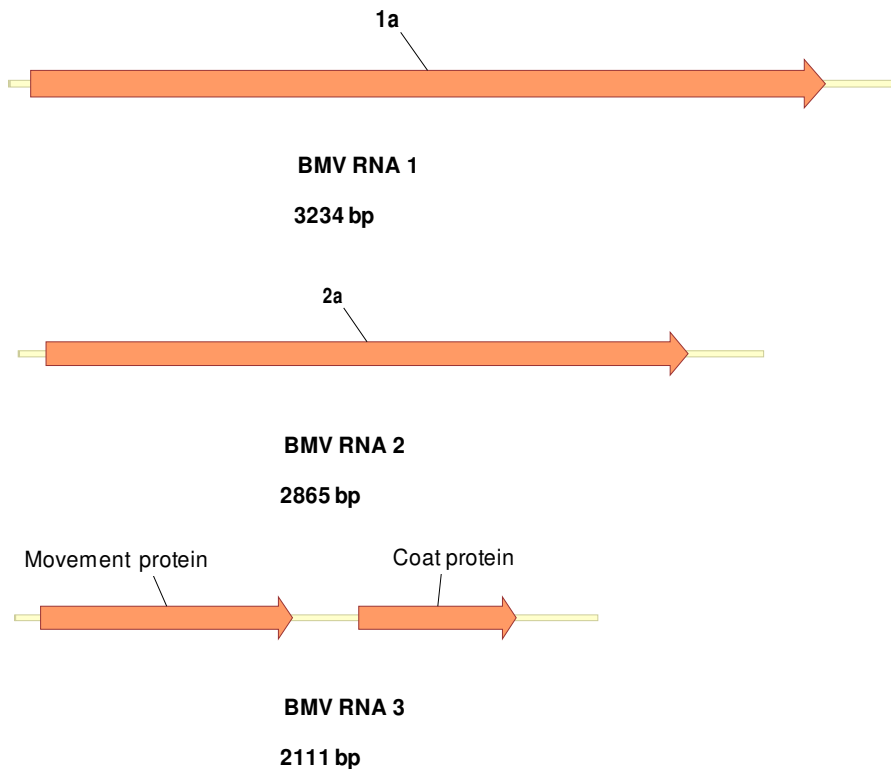
1a

BMV RNA 1

3234 bp

2a

BMV RNA 2

2865 bp

Movement protein   Coat protein

BMV RNA 3

2111 bp

Figure 1.5 A schematic representation of the BMV genome. The brome mosaic virus consists of three genomic RNAs. RNA 1 and RNA 2 encode protein 1a and 2a respectively whilst the movement protein and coat protein is encoded by RNA 3. A subgenomic RNA, RNA 4, is encoded from RNA 3 (not shown).
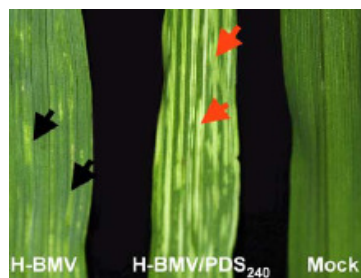

Figure 1.6 Silencing *Pds* in barley with the hybrid brome mosaic virus (H-BMV) results in photobleaching. Barley infected with H-BMV without an insert (H-BMV) and the hybrid BMV virus containing a 240 bp *Pds* fragment (H-BMV/PDS240) and uninfected plants (Mock) were compared to distinguish photobleaching from viral symptoms. Black arrows indicate the viral symptoms and red arrows indicate photobleaching which results from *Pds* silencing. Figure from Ding et al. 2006.

To optimize the BMV silencing vector further, modifications were made to the F-BMV RNA 3 to create a chimeric virus (C-BMV$_{A/G}$) (Ding et al. 2006). The intergenic region of F-BMV was replaced with an intergenic region from R-BMV, which has been shown to increase R-BMV 3 and 4 replication by 2 to 2.5 fold. The C-BMV$_{A/G}$ vector, which consists of F-BMV's RNA 1, 2 and the modified RNA 3, caused milder symptoms in infected plants whilst

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maintaining high levels of virus accumulation compared to F-BMV or H-BMV, improving the suitability of this virus for use in VIGS studies (Ding et al. 2006). Transcript reductions of 94% were achieved using the optimized C-BMV$_{A/G}$ vector to silence rice ribulose-1,5-bisphosphate carboxylase oxidase. This C- BMV$_{A/G}$ vector was shown to be useful for VIGS in barley and, importantly, rice and maize for which no VIGS system has previously been reported (Ding et al 2006, Ding et al. 2007). There are no published reports of a BMV based VIGS vector being used in pearl millet.

Chapter 2 Materials and methods

2.1 Plant materials and growth conditions

Pearl millet seeds of the ICML12 and ICMB96222 breeding lines were obtained from the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India. *Triticum aestivum* (wheat) cv. Tugela and *Hordeum vulgare* (barley) cv. Morex seeds were obtained from Small Grains Institute, Bethlehem, RSA. *Zea mays* lines X and Y were acquired from the international maize and wheat improvement center, CIMMYT (Mexico, 2006). Seeds were planted in 15 cm pots in a 1:4 peat:vermiculite soil mixture and fertilized with Multifeed® and Nitrosol® biweekly. Temperature in the barley, wheat and *N. benthamiana* glasshouse varied between 20 and 24°C during the day and night, whereas pearl millet and maize seedlings were grown at 26°C ± 4°C. Infections were conducted in a contained glasshouse at 22°C with 16 hours daylight. Natural light was complemented with high pressure sodium and mercury lamps. The average, daily maximum light intensity in the glass house was recorded between 700 and 900 µmol/m^2/s^2. Relative humidity in the glasshouse varied between 20-70% throughout the trials. Two or three plants were used per experiment and at least two independent replicates of each experiment were conducted, unless otherwise stated.

2.2 Norflurazon treatment

Seedlings at the three-leaf stage were watered with a 3 µM solution of Norflurazon (Sigma Aldrich, Irvine, UK) once a week for the duration of the experiments.

2.3 Fluorescence visualization

A hand held long wave UV radiation black light (UVP, Upland, California, USA) was used to assess GFP fluorescence in wheat cv. Tugela and barley cv. Morex seedlings infected with BSMV:GFP and BMV:GFP respectively. Infected seedlings were placed in a dark room and illuminated with UV light. Seedlings infected with non-GFP containing viral constructs were

also assessed for comparison. GFP visualization was performed at 48 hours post-infection and again at seven days after infection.

2.4 BSMV VIGS

2.4.1 Construction of a BSMV RNAγ based VIGS vector for silencing *Pds*

2.4.1.1 Pearl millet RNA isolation

The first two leaves of three week old pearl millet breeding line 842B seedlings were harvested and immediately frozen in liquid nitrogen. RNA isolation was performed using the RNAeasy® Minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was removed by digestion with 1 unit DNaseI (Fermentas, Burlington, Canada) and purified using an RNeasy® Minelute cleanup kit (Qiagen, Hilden, Germany). The RNA concentration was determined with a spectrophotometer (ND-1000 Spectrophotometer, Nanodrop®) at 260 nm and the integrity was evaluated on a 1.5% agarose gel.

2.4.1.2 Synthesis of cDNA

The pure RNA was used to synthesize first strand cDNA using 200 units of Superscript™ III Reverse Transcriptase (Invitrogen, California, USA); 500 ng oligo(dT)12-18 (Invitrogen), 1 µg RNA, dNTPs at a final concentration of 0.5 mM, 0.25 µM DDT, 40 units RNaseOUT™ in a 20 µl reaction according to manufacturer's protocol.

2.4.1.3 Amplification of a pearl millet *Pds* fragment

A *Zea mays Pds* nucleotide sequence obtained from GenBank (accession number L39266) was used as a template to design primers with Primer Designer software 4 version 4.2 (Scientific & Educational software, 2000) to amplify a pearl millet *Pds* gene fragment. Forward primer 2 (F2) (5' AGCTGCTTGGAAGGATGAAG 3') was combined with Reverse primer 2 (R2) (5' TTTGGGTCATAGTATTCCTTGC 3') to amplify a fragment of the expressed pearl millet 842B *Pds* gene from cDNA. Each 25 µl PCR reaction contained 0.5 µM of the forward and reverse primers, 0.2 mM dNTP mix, 1x NH_4 reaction buffer, 2.5 mM

MgCl$_2$, 1 unit BIOTAQ™ DNA polymerase (Bioline, London, UK). The reactions were denatured at 94°C for 5 min followed by 30 cycles (94°C 20 sec, 60°C 30 sec, 72°C 45 sec) and a 72°C 5 min hold. The PCR products were precipitated by addition of 1/10 volume 3 M NaOAc (pH5.2) and 2.5 volumes 100% ethanol, followed by 30 min centrifugation at 1000×g and two 70% ethanol washes. The PCR products were analysed on a 1.5% agarose gel. A GeneAmp® PCR system 2700 (Applied Biosystems, Foster City, USA) was used for all thermocycling reactions. The 900 bp PCR product amplified with the primer pair F2R2 was sequenced by Inqaba Biotechnological Industries (Pty) Ltd.

2.4.1.4 Cloning the pearl millet *Pds* fragment into the pGEM®-T Easy vector system

The 900 bp pearl millet *Pds* amplicon was ligated into the pGEM®-T Easy vector (Promega, Madison, USA) according to the manufacturer's protocol to generate the construct ppm*Pds*. Ligation reactions, with a final volume of 5 µl, containing 1x T4 DNA ligase buffer, 50 ng pGEM®-T Easy vector DNA, 220 ng PCR product was incubated overnight at 4°C. Transformation was performed using 2 µl of the ligation reaction products and 25 µl *Escherichia coli* JM109 competent cells (Promega). DNA uptake was achieved by heat shock for 48 sec in a 42°C water bath. Cells were grown in Luria Bertani (LB) liquid medium for 20 min at 37°C, centrifuged at 1000×g for 10 min and suspended in 200 µl LB liquid medium. 100 µl of the cells were plated on LB plates containing 100 mg/ml ampicillin (Sigma Aldrich), 50 mg/ml X-GAL (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) (Fermentas) and 0.1M IPTG isopropyl-β-d-thiogalactopyranoside (Fermentas). The plates were incubated at 37°C overnight. Positive clones were selected based on blue-white screening and verified by PCR screening using the F2R2 primer pairs as described above. A white colony diluted in 100 µl sterile distilled water was use as template DNA. The initial denaturation was performed for 10 min at 94°C. Positive colonies were stored in glycerol at -80°C.

2.4.1.5 Plasmid isolation and sequencing of the pearl millet *Pds* fragment

Plasmid DNA was isolated using the Qiaprep® Spin Miniprep kit (Qiagen). The plasmid DNA was analysed on a 1.5% agarose gel. The concentration of the plasmid DNA was determined using a spectrophotometer (ND-1000 Spectrophotometer, Nanodrop®). Cycle

sequencing was performed and each 10 µl reaction contained 2 µl of the Big Dye terminator v3.1 ready reaction mix as well as 1x sequencing buffer (Applied Biosystems), 90 ng of the plasmid DNA and either 10 pM M13+ (5' GGTTTTCCCAGTCACGAC 3') or M13– (5' AGCGGATAACAATTTCACAC 3') primers. The reactions were denatured at 96°C for 5 min followed by 25 cycles (96°C 10 sec, 50°C 5 sec, 60°C 4 min). Ethanol/sodium acetate precipitation was performed as described above to precipitate the sequencing products. The samples were sequenced at the African Centre for Gene Technologies (ACGT), sequencing facility in the Department of Genetics at the University of Pretoria.

2.4.1.6 Identification and production of a 385 bp antisense construct

The nucleotide sequence of the 900 bp pearl millet *Pds* gene fragment was analysed using Vector NTI Advance™ 9.1.0 (Invitrogen). Similarity searches were made with BLAST programs (Altschul et al. 1990) at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/). An oligonucleotide primer pair used to amplify a 385 bp *Pac*I and *Not*I flanked fragment from the pearl millet *Pds* clone, 300PdsF (5'-aa**GCGGCCGC**ATGGTCTAACGGTTTCAG-3') and 664PdsR (5'-gc**TTAATTAA**TGCCGCACAAACATAAGC -3'), was identified using Primer Designer 4 version 4.2. The restriction enzyme recognition sites are indicated in bold typeface. The amplification reactions contained 1 µl of a 1:10 dilution of the plasmid DNA as template, 1x NH_4 reaction buffer, 2.5 mM $MgCl_2$, 0.5 µM of both the forward and reverse primers, 1 unit BIOTAQ™ DNA polymerase (Bioline) and 0.2 mM dNTP mix. The PCR program was as follows: 94°C for 5 min, 25x (94°C 20 sec, 60°C 30 sec, 72°C 30 sec), 72°C for 5 min. PCR products were purified by sodium acetate/ethanol precipitation and the concentration thereof was determined with a spectrophotometer at 260 nm. The products were analysed on a 1.5% agarose gel.

2.4.1.7 Cloning the antisense pearl millet *Pds* construct

An *Escherichia coli* DH5α culture 1 ml aliquot with an optical density of 0.94 at 600 nm was treated with 80 mM $CaCl_2$ and 50 mM $MgCl_2$ to make the cells competent for DNA uptake. The 385 bp pearl millet *Pds* gene fragment was ligated into the pGEM®-T Easy vector system (Promega) according to manufacturer's protocol to generate ppmPdsNP. Competent *E. coli*

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cells were transformed with the ligation reaction by heat-shock for 37ºC for 5 min. Cells were cultured in LB liquid media and plated onto LB plates containing 100 mg/ml ampicillin (Sigma), 50 mg/ml X-GAL and 0.1M IPTG (Fermentas). The plates were incubated overnight at 37ºC. Presence of the insert in bacterial colonies was confirmed by PCR using M13+ and M13- primers. A dilution made from a positive colony was used as template DNA for the PCR. Restriction enzyme digestion, using *Not*I and *Pac*I verified integrity of the restriction enzyme recognition sites in the clone. Each digestion reaction contained 400 ng of plasmid DNA, 1 unit *Pac*I restriction enzyme (New England Biolabs, Ipswich, USA), 1 x reaction buffer and 100 µg/ml BSA. The reaction was incubated for 2 hours at 37ºC. Linearized plasmid DNA was precipitated and used as template for a secondary digest with 1 unit *Not*I restriction enzyme (New England Biolabs) in the presence of a suitable reaction buffer. The reaction was incubated overnight at 37ºC.

The recombinant plasmid was named ppmPdsNP and sequenced with the Big Dye terminator v3.1 kit (Applied Biosystems). SP6 (5'- TACGATTTAGGTGACACT -3') and T7 (5'- GTAATACGACTCACTATA -3') primers were used respectively to perform the cycle sequencing as described above. The samples were sequenced by the ACGT sequencing facility in the Department of Genetics at the University of Pretoria, aligned and analysed using Vector NTI Advance ™ 9.1.0 (Invitrogen).

2.4.1.8 Directional cloning of pearl millet *Pds* fragment into BSMV RNAγ

The 385 bp pm*Pds* fragment was isolated from the ppmPdsNP vector using *Not*I and *Pac*I restriction enzymes and cloned in an antisense orientation into the *Pac*I/*Not*I restriction site of the pγ42 plasmid (Petty et al. 1989) which contains an infectious BSMV RNAγ cDNA clone under control of a T7 promoter. The resulting plasmid was named pBSMVγPds_as. Cloning was done at the Scottish Crop Research Institute (SCRI) by standard cloning procedures as described by Holzberg et al. (2002).

2.4.2 BSMV inoculation procedure

The BSMVΔCP vector plasmids pBSMVα, pBSMVβΔCP, pBSMVγGFP and the above mentioned pBSMVγPds_as were obtained from SCRI under licence from Large Scale

Biology Corporation, USA. Plasmids were linearized with appropriate enzymes to transcribe infectious RNAs from the cDNA clones. pBSMVα were linearized with *Mlu*I (Roche, Mannheim, Germany), pBSMVβΔCP with *Spe*I (Roche); pBSMVγGFP with *Smi*I and pBSMVγPds_as with *Bss*HII (Fermentas).

Linear plasmids were precipitated by adding one tenth the volume of 3M NaOAc (pH 4.6) and 2.5 volumes of 90% ethanol. The mixtures were cooled for 1 hour and precipitated by centrifugation and subsequently cleaned with two 70% ethanol wash steps. The pellet was resuspended in sterile distilled H_2O. One microgram of each of the linear plasmids was used as template for *in vitro* transcription using the mMessage mMachine T7 *in vitro* transcription kit (Ambion, Austin, Texas) according to the manufacturer's protocol. The three viral genome components were mixed in a 1:1:1 molar ratio, mixed with 27 μl FES buffer (Pogue et al. 1998) and used to infect the first and second leaves (L1 and L2) of 15 day old pearl millet seedlings by rub inoculation. The leaf was held between the forefinger and thumb of gloved hands and 30 μl of the infectious RNA/buffer mixture was pipetted onto the adaxial leaf surface. Fingers gently squeezed and rubbed the leaf from the base toward the leaf tip repeatedly taking care not to break the leaf off.

Wheat cv. Tugela seedlings at the three leaf growth stage were infected using the same method as a positive control for pearl millet *Pds* silencing.

2.4.3 BSMV diagnostic RT-PCR

The nucleotide sequences of all three the BSMV genomic RNAs were obtained from GenBank (accession numbers NC_003469, NC_003481 and NC003478) and were used as templates to design primers using Primer Designer 4 version 4.2 (Scientific & Educational Software, 2000). Experimental leaf samples were harvested and immediately frozen in liquid nitrogen. Total RNA was isolated from 500 mg finely ground leaf material using a miniEasy Plant RNA extraction kit (Qiagen) according to the manufacturer's protocol. An on-column DNaseI (Qiagen) digestion was performed to remove all contaminating genomic DNA. Integrity of the RNA was analysed on a 0.9% RNA formaldehyde containing agarose gel. First strand cDNA was synthesized from 2 μg of each RNA sample using Superscript III (Invitrogen) following the manufacturer's protocol. The cDNA synthesis reaction was verified by amplifying the pearl millet actin housekeeping gene. This PCR would also

indicate any genomic DNA contamination. A 1:10 dilution of the cDNA was used as template in each 25 μl reaction. Each reaction contained: 1 μl cDNA, 1x Buffer, 1.5 mM MgCl$_2$, 1 mM dNTP's, 0.25 μM of ActinF (5'-ACCGAAGCCCCTCTTAACCC-3') and ActinR (5'-GTATGGGTGACACCATCACC -3'), 1 unit Taq DNA polymerase (Bioline). Samples were denatured at 94°C for 5 minutes followed by 30 cycles of denaturing at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds.

The cDNA was subsequently used as template in three PCR reactions with primer pairs specific to each of the BSMV RNAs. PCR conditions were as follows: each 25 μl reaction contained 1x Buffer, 2 mM MgCl$_2$, 0.2 mM dNTP's, 0.5 μM of either BSMVαF (5'-GATGTCACCGCAGCAATGTA-3'), and BSMVαR (5'-TCCTTCTCACGCAGTTGCTA -3'), or BSMVβF (5'-AGTTCCTGGACCTGAGTTGA-3'), and BSMVβR (5'-AGGCTCTGAGTCTTCGTTGT -3'), or BSMVγF(5'-CCAACGGAGGTTATGTGATG -3') and BSMVγR(5'-GATTCCAAGTACCGGCAAGT -3') and 1 unit Taq DNA polymerase (Bioline). PCR samples were denatured at 94°C for 5 minutes followed by 30 cycles of denaturing at 94°C for 30 seconds, primer annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds.

2.5. BMV VIGS

2.5.1 Construction of a BMV RNA 3 based VIGS vector

Cloned cDNA copies of the BMV genome were obtained from The Samuel Roberts Noble Foundation (Ardmore, USA). The vector consists of three plasmids. The plasmids pF1-11 and pF2-2 contain RNA 1 and RNA 2 respectively which was cloned from a strain of BMV isolated from *Festuca arundinacea* Schreb. (Ding et al. 2006). The third plasmid, pB3m, contains RNA 3 from a Russian strain of BMV (Ding et al. 2006). Together, these three plasmids, pF1-11, pF2-2 and PB3m constitute the hybrid BMV (H-BMV) VIGS vector which has been used to conduct VIGS in rice (Ding et al. 2006). Another copy of RNA 3 fused with green fluorescent protein was cloned in a plasmid called pF13m-GFP which can be used with pF1-11 and pF2-2 to make the BMV:GFP control vector. The plasmids were transformed into competent JM109 *E. coli* cells by a standard heat shock transformation protocol. For the reaction, 2 μl of the suspended plasmids were mixed with 20 μl of competent cells. After a 30 min incubation period on ice the cells were heat shocked at 42°C for 90 sec. Thereafter

200 µl of LB broth was added and allowed to grow for 30 minutes before plating aliquots of the culture on LB plates supplemented with 100 mg/ml ampicillin (Sigma Aldrich). One colony of each plasmid was cultured and used to make glycerol stocks of each of the events and stored at -80°C for future use.

A fragment of pearl millet *Pds* was amplified from cloned cDNA using extension primers to facilitate cloning into the multiple cloning site of pB3m. The primer pair AvrIIPdsF 5'-gg**CCTAGG**TTCCTGATCGAGTGAATGA-3' and NcoIPdsR 5'-aa**CCATGG**CGGTAAGTGCGAAGTGTT-3' (restriction sites indicated in bold) amplified a 301 bp fragment from 1 ng of the cDNA clone in 25 cycles of PCR. Each reaction contained: 1x Buffer, 1.5 mM MgCl$_2$, 1 mM dNTPs, 0.2 µM of each of the primers and 1 unit Taq DNA polymerase (Bioline). Samples were denatured at 94°C for 5 minutes followed by 25 cycles of denaturing at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds.

The 301 bp *Pds* fragment was cloned into the pTZ57R/T InsTA clone™ PCR cloning vector (Fermentas) and purified using the MSB Spin PCRapace kit (Invitec, Berlin, Germany). The purified PCR products and pB3m were digested with *Nco*I and *Avr*II to create compatible overhangs. Ligations were performed overnight at 4°C using 3:1 ratios of insert:vector and 1 unit of T4 DNA ligase (Promega). A 5 µl aliquot of the ligation reactions was transformed into 100 µl competent JM109 cells as described earlier. Colonies that formed after incubation at 37°C were screened for the presence of the 301 bp *Pds* fragment with PCR using the primers AvrIIPdsF and NcoIPdsR. The recombinant plasmid was named pB3mPds301. Cloning was verified by nucleotide sequencing done by Inqaba Biotechnological Industries (Pty) Ltd.

2.5.2 Preparation of BMV RNA for plant inoculations

BMV vector plasmids were linearized before *in vitro* transcription. A 2 µg aliquot of pF1-11 was linearized with *Spe*I and 2 µg of each pF2-2, pB3mPds300, pB3mGFR and pB3m were linearized with *Psh*AI. Restricted plasmids were analysed electrophoretically and precipitated subsequently. Each digestion reaction was mixed with an equal volume phenol:chloroform and vortexed for 1 minute. Samples were centrifuged at 15000 x g for 5 minutes. The upper liquid phase was mixed with one tenth the volume of 3 M sodium acetate

(pH 4.6) and 100 µl ethanol. Tubes were kept at -80°C for 30 minutes and thereafter centrifuged at full speed for 15 minutes. The precipitate was washed with 70% ethanol, dried and suspended in nuclease-free water. Approximately 1 µg of each of the clean linear plasmids was used as template for RNA transcription.

Transcription was performed according to manufacturer's protocol using the mMessage mMachine T3 *in vitro* transcription kit (Ambion). Reactions were incubated for two hours at 37°C and RNA yield subsequently analysed in a 1% agarose gel.

2.5.3 BMV inoculations procedure

2.5.3.1 Direct RNA infection

RNA transcripts of all three BMV genomic RNAs were mixed in a 1:1:1 ratio for plant inoculations. The BMV:Pds inoculation mixture consisted of 1µl of each of the RNA transcription reactions of F1-11, F2-2 and B3mPds301 and the control construct BMV:GFP which contained B3mGFP instead of B3mPds301. The RNA was mixed with 17 µl FES buffer (Pogue et al. 1998). A 10 µl aliquot of the inoculation mixture was applied to each plant. The mixture was carefully rubbed onto the first and second leaf blades of each plant between the thumb and forefinger of gloved hands similarly to the BSMV inoculation.

2.5.3.2 Indirect sap infection

The first two systemically infected leaves of symptomatic BMV-infected *H. vulgare* cv. Morex plants were used to prepare infective plant sap for indirect infections. Leaves were harvested and immediately frozen in liquid nitrogen. The frozen samples were ground with an RNase free mortar and pestle to a fine powder. Approximately 0.3 g of ground tissue was mixed with 1.5 ml 0.1 M phosphate buffer (pH 7) in a 1:5 w/v ratio. A small amount (approximately 0.5% w/v) of sterile celite (Sigma Aldrich) was added to the sap mixture. Infective sap was kept on ice prior to inoculation. The first two leaves of seedlings at the L3 growth stage were infected with 20 µl of the infective sap. A pair of scissors treated with RNase Away™ (Molecular BioProducts, San Diego, CA) was used to cut 2 mm from the front of a filter tip to allow efficient pipetting of the crude plant sap onto the leaf blades. The infective sap was gently rubbed into the leaf using the forefinger and thumb of gloved hands.

2.5.4. BMV diagnostic RT-PCR

The nucleotide sequences of all three BMV genomic RNAs were obtained from GenBank (accession numbers DQ_530423, DQ_530424 and DQ_530425) and were used as template to design primers with Primer Designer 4 version 4.2 (Scientific & Educational software, 2000). These primer pairs were used to identify any BMV RNA in systemically infected leaves of seedlings.

Systemically infected leaves were harvested and immediately frozen in liquid nitrogen. Total RNA was isolated from 500 mg finely ground leaf material using an RNeasy® Plant RNA extraction kit (Qiagen). Integrity of the RNA was analysed on a 0.9% RNA formaldehyde containing agarose gel. First strand cDNA was synthesized from 2 μg of each RNA sample using Superscript III (Invitrogen, California, USA) following the manufacturer's protocol. The cDNA synthesis reaction was verified by amplifying the pearl millet and *N. benthamiana* actin housekeeping gene or the small ribosomal subunit (*sRs*) gene in the case of barley. A 1:10 dilution of the cDNA was used as template in each 25 μl reaction. Each reaction contained: 1 μl cDNA 1x Buffer, 1.5 mM $MgCl_2$, 1 mM dNTP's, 0.25 μM of ActinF (5'-ACCGAAGCCCCTCTTAACCC-3') and ActinR (5'-GTATGGGTGACACCATCACC -3'), or HvsRs (5'- ATGGCTTCGTCGGCTACC-3') and HvsRs (5'-AAGGCGATGAAGCTGACG-3) 1 unit Taq DNA polymerase (Bioline). Samples were denatured at 94°C for 5 minutes followed by 30 cycles of denaturing at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds.

The same cDNA was subsequently used as template in three PCR reactions with primer pairs specific to each of the BMV RNAs. PCR conditions were as follows: each 25 μl reaction contained 1x Buffer, 2 mM $MgCl_2$, 0.2 mM dNTP's, 0.5 μM of either BMV1F (5'-GACACGGAGCCATGAACATGAAGG -3'), and BMV1R (5'-TCCTTCTCACGCAGTTGCTA -3'), or BMV2F (5'-GAGTGCTCAGCGAAGAGTTG-3'), and BMV2R (5'-AGGCTCTGAGTCTTCGTTGT -3'), or BMV3F (5'-CACCAGTTCTGCGTTGGAAG -3') and BMV3R(5'-ACAGATGACGCGGATACAGA -3'') and 1 unit Taq DNA polymerase (Bioline). Samples were denatured at 94°C for 5 minutes followed by 25 cycles of denaturing at 94°C for 30 seconds, primer annealing at 62°C for 30 seconds and extension at 72°C for 30 seconds.

Chapter 3 BSMV results

3.1 Generating a BSMV RNAγ based VIGS vector.

RNA was isolated from pearl millet seedlings (Figure 3.1) and subjected to reverse transcription PCR to amplify an approximately 900 bp fragment of the pearl millet *Pds* gene (Figure 3.2). The maize *Pds* sequence (GenBank accession gi_642623) was used to design the primers, due to the documented close relationship of these two species (Devos and Gale, 1997; Figure 1.1). This pearl millet *Pds* gene fragment was cloned into the pGEM®-T Easy vector system (Appendix Figure 1) to generate a plasmid named ppmPds (Figure 3.3). A smaller 365 bp fragment of this *Pds* gene fragment was amplified from the ppmPds plasmid using restriction enzyme targeted extension primers that added a *Pac*I (5'-end) and *Not*I (3'-end) site for directional cloning into the BSMV RNAγ at a later stage. This smaller fragment was initially cloned into the pGEM®-T Easy vector and referred to as ppmPdsNP (Figure 3.4). Restriction enzyme digestion confirmed cloning of the 365 bp pearl millet *Pds* gene fragment in the vector plasmid (Figure 3.5). The pGEM®-T Easy vector (Promega), used as cloning vector, has two *Not*I restriction sites at 43 bp and 77 bp respectively and span the site of fragment insertion. The expected restriction product size was 410 or 406 bp depending on the restriction enzymes used. Double digestion with *Not*I and *Pac*I (Figure 3.5, lane 5) produced a band slightly smaller than single digestion with *Not*I only (Figure 3.5, lane 4) which indicated that both restriction enzyme sites were incorporated and that the 385 bp *Pds* gene fragment was inserted successfully to create ppmPdsNP. This amplicon is 20 bp longer than the original 365 bp fragment due to incorporation of restriction enzyme recognition sites and protective terminal nucleotides.

The ppmPdsNP vector was used to cut out the insert and clone it in an antisense orientation into the *Not*I/*Pac*I restriction site of the BSMV RNAγ vector plasmid to generate the BSMV RNYγ based VIGS vector. This recombinant plasmid was named pBSMVγPds_as (Figure 3.6).

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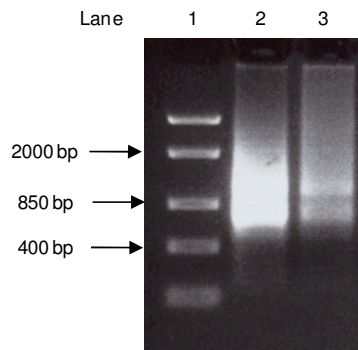
Figure 3.1 Formaldehyde agarose gel electrophoresis of total RNA isolated from pearl millet leaves. Lane 1: FastRuler^{TM} DNA ladder, Middle Range (Fermentas); lane 2: RNA isolated from one leaf sample; lane 3, RNA isolated from a second leaf sample.
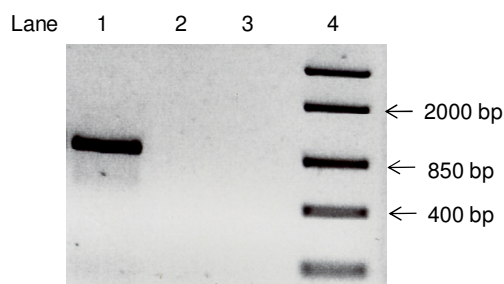

Figure 3.2 RT-PCR amplification of a pearl millet gene fragment from leaf mRNA. mRNA samples were subjected to RT-PCR to amplify a fragment of the pearl millet *Pds* gene sequence. Lane 1: *Pds* amplification product of approximately 900 bp; lane 2: no template negative control; lane 3: open; lane 4: FastRuler^{TM} DNA Ladder Middle Range (Fermentas).
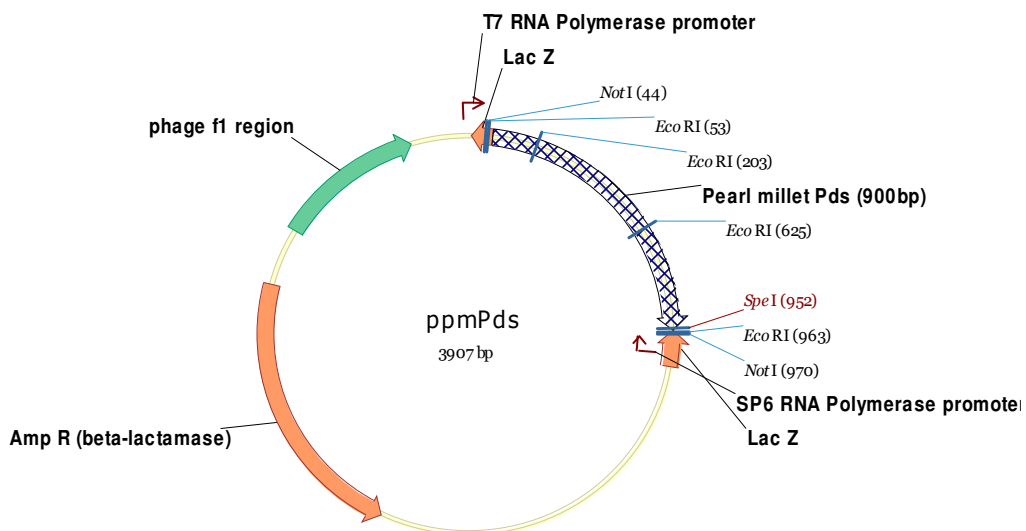

Figure 3.3 Plasmids ppmPds with selected restriction sites indicated. An approximately 900 bp fragment of the pearl millet *Pds* gene was cloned into the pGEM®-T Easy vector (Promega) to generate the ppmPds vector.

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Wheat is a natural host of BSMV (Jackson and Lane, 1981) and was used as a positive control of BSMV infections and *Pds* silencing in this study. Nucleotide sequencing revealed that this pearl millet *Pds* gene fragment is 87% identical to a partial wheat *Pds* nucleotide sequence (GenBank accession BT009315). Furthermore, there is one stretch of more than 23 consecutive identical nucleotides within these two partial gene sequences that are sufficient to trigger PTGS of the endogenous wheat *Pds* gene (Figure 3.7). pBSMVγPds_as was combined with pBSMVα and pBSMVβΔCP to make up the BSMVΔCP VIGS vector as seen in Figure 3.8.
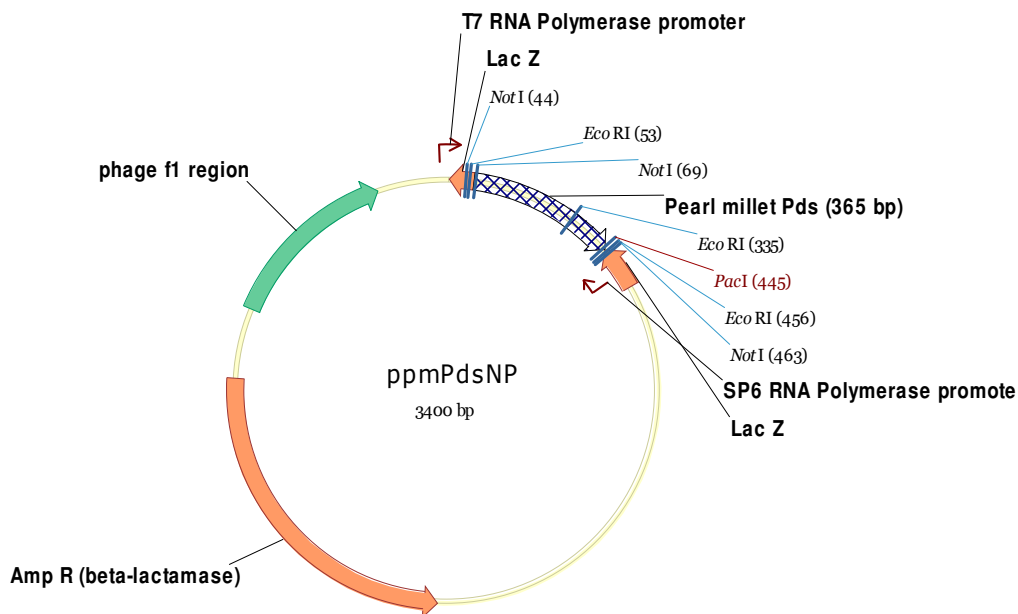

Figure 3.4 Plasmid ppmPdsNP with selected restriction sites indicated. A 365 bp pearl millet *Pds* gene fragment with added *Not*I/*Pac*I restriction sites was cloned into the pGEM®-T Easy vector (Promega) backbone to generate the ppmPdsNP vector.
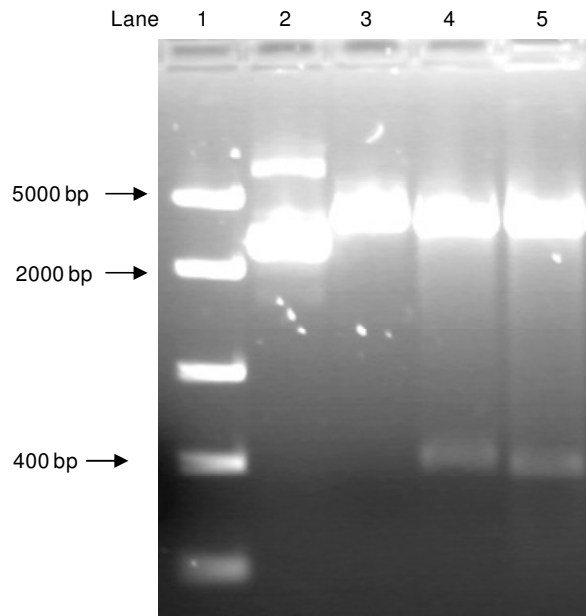
Figure 3.5 A *Not*I and *Pac*I restriction enzyme digestion of recombinant pearl millet *Pds* vector plasmid ppmPdsNP. Lane 1: Fast Ruler^TM DNA Ladder Middle Range (Fermentas); lane 2: circular recombinant vector plasmid ppmPdsNP; lane 3: ppmPdsNP digested with *Pac*I; lane 4: ppmPdsNP digested with *Not*I; lane 5: ppmPdsNP digested with *Not*I and *Pac*I. The presence of a band slightly smaller than the *Not*I restriction indicated that both restriction enzyme sites are intact and that the 385 bp fragment was inserted successfully to create pmPdsNP.
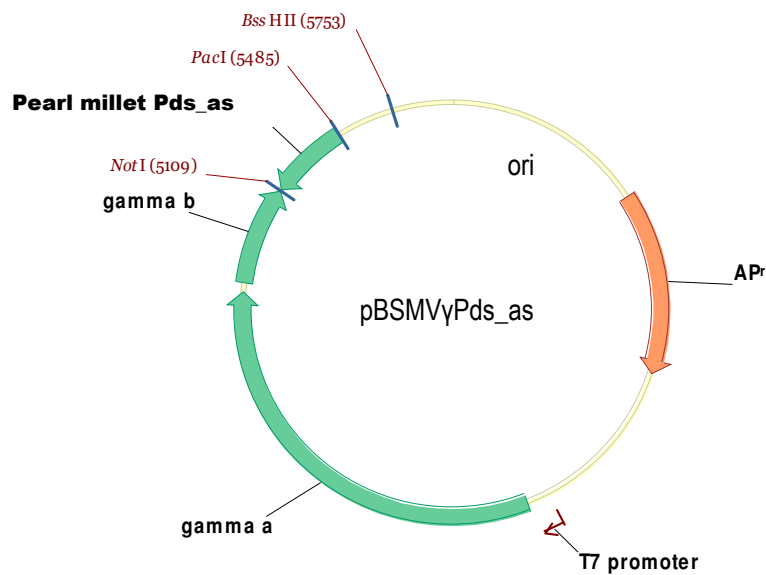

Figure 3.6 Recombinant BSMV RNAγ plasmid; pBSMVγPds_as with selected restriction sites indicated. A 365 bp fragment of the pearl millet *Pds* gene is cloned in antisense orientation into the *Not*I/*Pac*I restriction enzyme recognition site, immediately downstream of the γb open reading frame. The selectable ampicillin resistance gene (AP^r) also occurs within this vector. The total vector size is 6030 bp.

Section 1

		1	10	20	30	40	50	61
Pearl millet	(1)	-ATGGTCTAACGGTTTCAGAGTGGATGAAAAAGCAGGGTGTTCCTGATCGAGTGAATGATG						
Wheat	(1)	GATGGCTTAACTGTTTCAGAATGGATGGAAAAGCAGGGTGTTCCTGATCGGGTCAACGACG						

Section 2

		62	70	80	90	100	110	122
Pearl millet	(61)	AAGTTTTTATTGCAATGTCCAAGGCACTCAATTTCATCAATCCTGATGAGCTATCCATGCA						
Wheat	(62)	AGGTTTTTATTGCAATGTCCAAGGCACTCAATTTCATAAACCCTGACGAGTTATCCATGCA						

Section 3

		123	130	140	150	160	170	183
Pearl millet	(122)	GTGCATTTTGATTGCTTTGAACCGATTTCTTCAGGAGAAGCATGGTTCCAAAATGGCATTC						
Wheat	(123)	GTGCATTCTGATTGCTCTAAACCGATTTCTCCAGGAGAAGCATGGCTCGAAAATGGCATTC						

Section 4

		184	190	200	210	220	230	244
Pearl millet	(183)	TTGGATGGCAATCCACCTGAAAGGCTGTGCATGCCTATTGTTGATCACATTCGGTCTAGGG						
Wheat	(184)	TTGGATGGTAATCCTCCTGAAAGGCTATGCATGCCTATTGTTAACCACATTCAGTCTTTGG						

Section 5

		245	250	260	270	280	290	305
Pearl millet	(244)	GGGGTGAAGTCCGCCTGAATTCTCGTATTAAAAAGATTGAGTTGAATCCTGATGGAACTGT						
Wheat	(245)	GTGGTGAGGTCCGGCTGAATTCTCGTATTCAGAAAATTGAACTGAACCCGGACGGAACAGT						

Section 6

		306	320	330	340	350	366
Pearl millet	(305)	AAAACACTTCGCACTTACCGACGGAACTCAAATAACTGGAGATGCTTATGTTTGTGCGGCA						
Wheat	(306)	GAAGCACTTTGCACTTACTGATGGGACTCAAATAACTGGAGATGCATATGTTTTTGCAGCA						

Section 7

Figure 3.7 Nucleotide sequence alignment of the partial pearl millet and wheat *Pds* gene sequences. The 365 bp fragment of the pearl millet *Pds* gene cloned in the pBSMVγPds_as vector is 87% identical to a fragment of the wheat *Pds* gene (GenBank accession BT009315). Non-similar residues are shown in black, whereas identical residues are in red typeface, highlighted in yellow. The black box indicates stretches with 23-nt or more similarity.
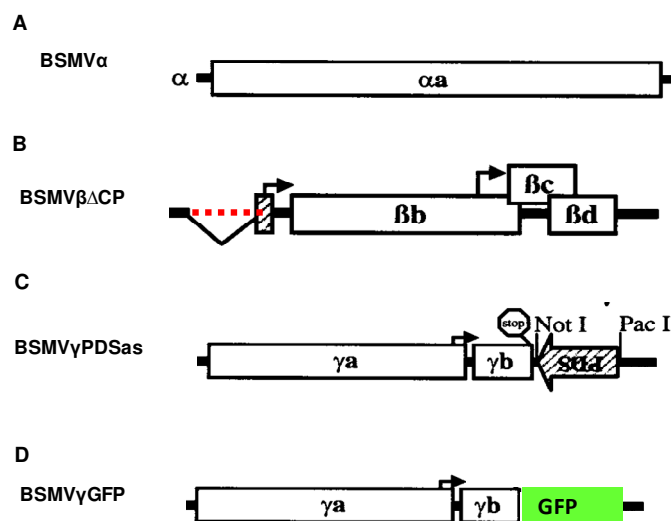
A
BSMVα

B
BSMVβΔCP

C
BSMVγPDSas

D
BSMVγGFP

Figure 3.8 Genomic organisation of the BSMV RNAγ based BSMVΔCP VIGS vector. (A-C) Genomic RNAs of BSMV, strain ND18 indicating the modifications made to facilitate VIGS. A) No changes were made to the RNAα, which encodes the αa protein. B) The coat protein was deleted from RNAβ, indicated by red dotted line to generate BSMVβΔCP. C) BSMVγPdsas contains an untranslatable fragment of the pearl millet *Pds* gene in an antisense orientation behind the γb gene. D) BSMVγGFP contains a green fluorescent protein (GFP) gene (green box) downstream of the γb protein. Clear boxes represent ORF's and dashed boxes indicate untranslatable OFRs. Arrows indicate subgenomic promoters. Figure adapted from Holzberg et al. (2002).

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3.2 Norflurazon treatment

Norflurazon treatments were conducted on a subset of wheat cv. Tugela and pearl millet ICML12 and ICMB96222 plants to get a reference of Pds inhibition for comparison with *Pds* silencing results. Norflurazon, a chemical inhibitor of the Pds enzyme, causes severe whitening due to photobleaching in treated plants. Symptoms were visible from five days after the first application, initiating in the leaf base and progressing toward the leaf apex. Photobleaching occurred in all leaves that emerged after the treatment was applied (Figure 3.9). Plants became weak, necrotic and eventually died after prolonged norflurazon treatment.
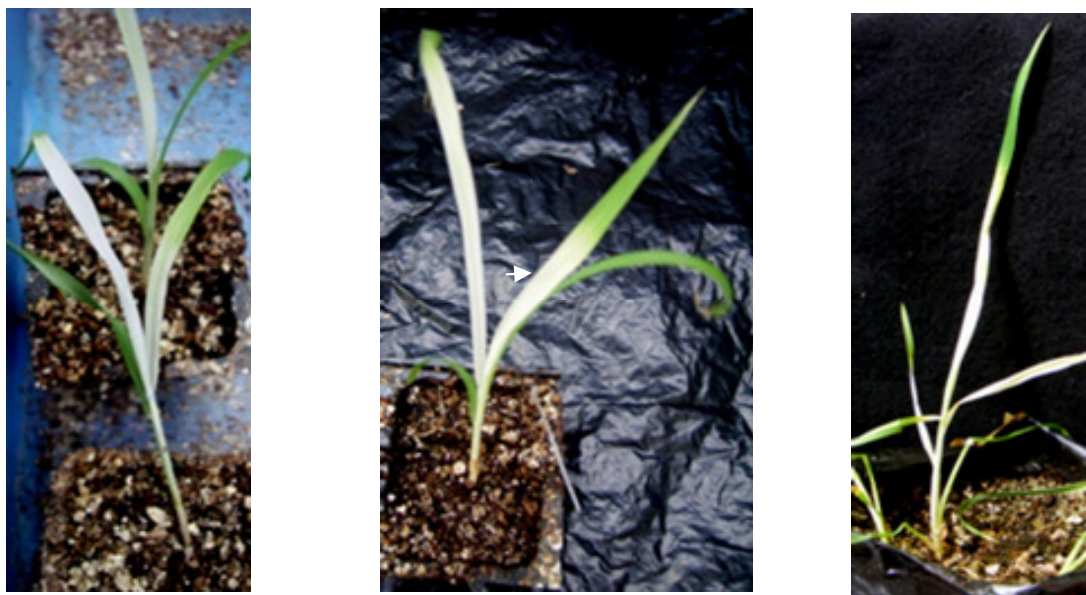
A) *P. glaucum* ICMB96222   B) *P. glaucum* ICML12   C) *T. aestivum* cv. Tugela

Figure 3.9 Norflurazon-induced photobleaching in pearl millet and wheat. Homogenous photobleaching of newly emerging leaves in pearl millet. A) ICMB96222, B) ICML12 and C) wheat cv. Tugela. Photobleaching started from the leaf base and progressed towards the apex. Pictures were taken eight days after initiating norflurazon treatment.

3.3 BSMVΔCP based VIGS experiments

3.3.1 BSMVΔCP RNA vector linearization

The individual BSMVΔCP plasmids that contain the RNAs α-γ (Figure 3.8) were linearized downstream of the BSMV coding sequence to function as template DNA for *in vitro* transcription by T7 RNA polymerase to prepare infectious RNA. Figure 3.10 shows the

electrophoretic analysis of BSMVΔCP plasmid linearization. The digested plasmids can be seen as a single band compared to the double bands that circular plasmids present in the gel. Between 600 and 1000 ng linear plasmid was used as template for RNA transcription. A fraction of the RNA was analysed on a 1% agarose gel (Figure 3.11) to determine the RNA yield of the *in vitro* transcription reaction. High concentrations of RNA were visible as smears of approximately 1.3 kb in the gel.
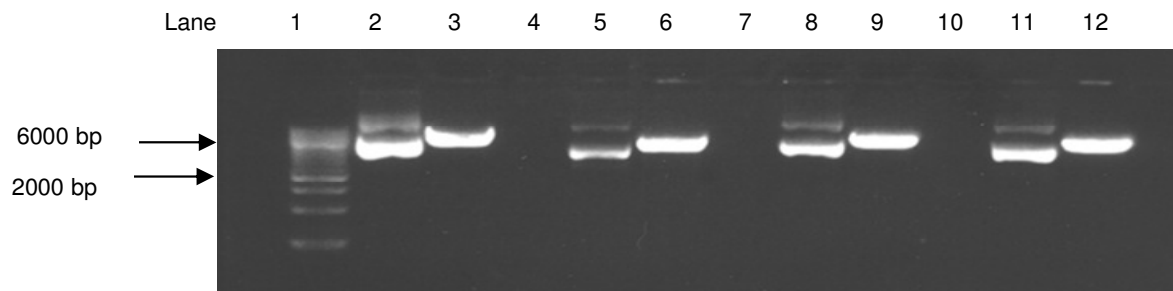

Figure 3.10 Electrophoretic analysis of BSMVΔCP plasmids linearization. Each of the BSMVΔCP component plasmids, pBSMVα, pBSMVβ, pBSMVγGFP, and pBSMVγPds_as were linearized with an appropriate restriction enzyme downstream of the viral coding sequence. A sample of undigested DNA of each plasmid was loaded to the left of the digested sample. Lane 2, 5, 8 and 11 reveal double bands, characteristic of circular DNA, of plasmids pBSMVα, pBSMVβ, pBSMVγGFP and pBSMVγPds_as respectively. Single bands, characteristic of linear template DNA, for each restricted plasmids are visible in Lane 3, 6, 9 and 12. Lane 1 shows the O' RangeRuler™ 500 bp DNA ladder (Fermentas).
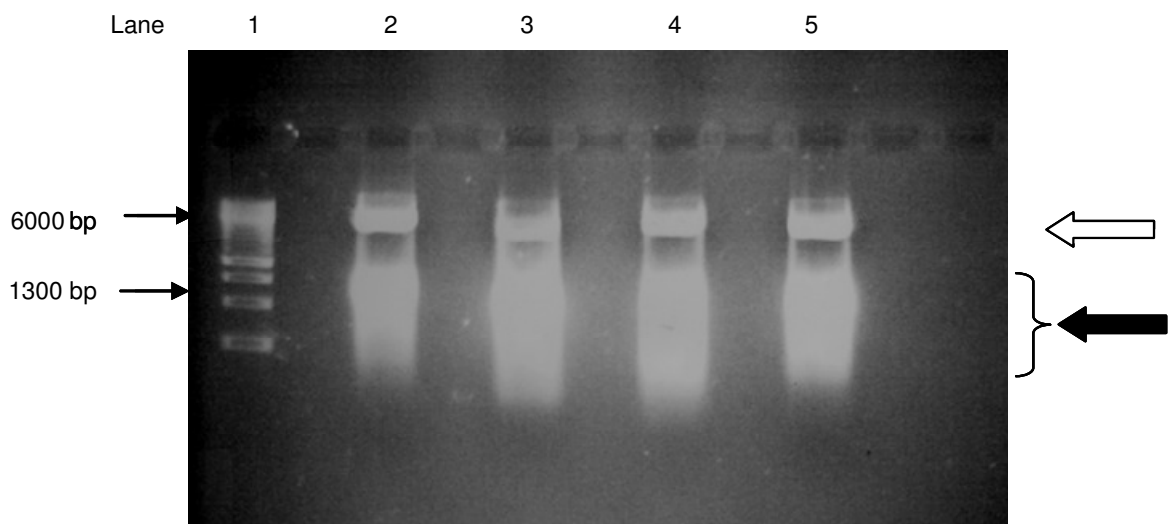

Figure 3.11 Agarose gel electrophoretic analysis of *in vitro* T7 transcription of BSMVΔCP RNAs. Lane 1: O' RangeRuler™ 500 bp molecular weight marker (Fermentas), lanes 2-5: 1 μl of RNA transcribed from pBSMVα, pBSMVβΔCP, pBSMVγGFP and pBSMVγPds_as, respectively. The brace and black arrow indicate the position of the 1.3 kb full-length RNA products while the thin white arrow indicates linearized DNA plasmids templates of approximately 6 kb.

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3.3.2 Symptoms of BSMVΔCP infection

The cotyledon and first true leaf of pearl millet lines ICML12, ICMB96222, and wheat cv. Tugela seedlings were infected with two specific BMSVΔCP constructs, a control construct namely BSMVΔCP:GFP (consisting of equal volumes of BSMVα, BSMVβΔCP and BSMVγGFP RNA) and the silencing construct BSMVΔCP:Pds (consisting of equal volumes of BSMVα, BSMVβΔCP and BSMVγPds_as RNA). Plants were monitored throughout the trial for any mosaic, chlorosis or necrotic viral infection symptoms or the occurrence of photobleaching, the phenotype associated with *Pds* silencing.

Extensive white streaking was observed in the BSMVΔCP:Pds infected wheat cv. Tugela seedlings from approximately 10 days post-inoculation (dpi) as seen in Figure 3.12, A. The streaking first appeared in the third leaf and thereafter in leaf 4 as well. The white areas in the leaves resembled tissue of the norflurazon treated wheat seedlings (Figure 9.3, C) except that the streaking did not extend over the entire lamina surface. Streaking patterns were not observed in any of the BSMVΔCP:GFP infected wheat cv. Tugela seedlings.
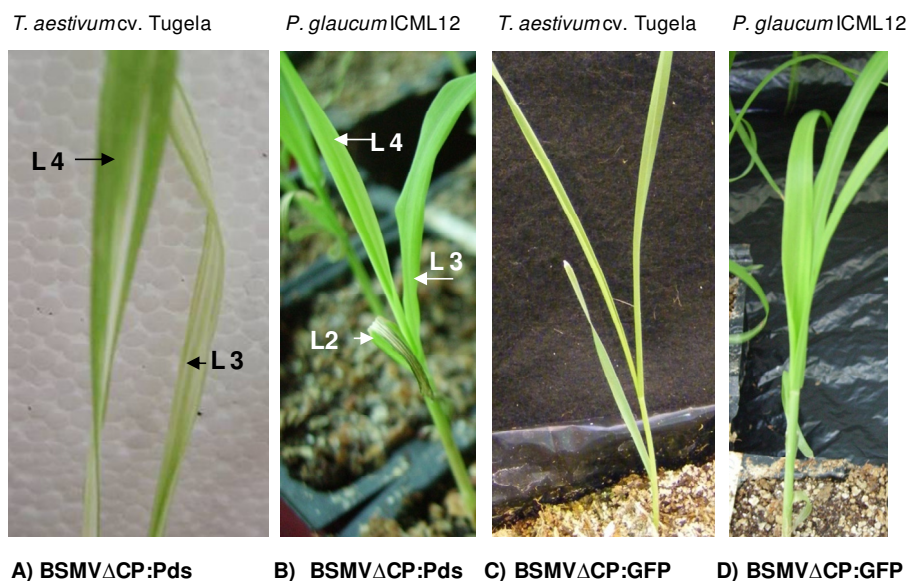

Figure 3.12 Silencing of *Pds* in wheat cv. Tugela by BSMVΔCP. Wheat and pearl millet seedlings were infected with *in vitro* transcribed RNAs of the BSMV VIGS vector BSMVΔCP:Pds and BSMVΔCP:GFP as a control for viral symptoms. A) Photobleaching was observed in wheat cv. Tugela in leaf 3 (L3) and leaf 4 (L4) from 10 dpi and persisted until 17 dpi when leaves were harvested. B) The second leaf (L2) where the pearl millet ICML12 seedlings were inoculated with BSMVΔCP:Pds underwent senescence. Leaf 3 (L3) and leaf 4 (L4) of the seedling showed no symptoms of photobleaching or viral infection symptoms. C and D) Both wheat and pearl millet seedlings infected with BSMVΔCP:GFP remained healthy and did not display any viral symptoms.

The pearl millet seedlings of both lines ICML12 and ICMB96222 infected with BSMVΔCP:Pds did not display any visible symptoms after infection (Figure 3.12, B). These seedlings were phenotypically indistinguishable from the uninfected control plants. The subset of seedlings infected with BSMVΔCP:GFP (Figure 3.12, D) did not display any streaking or other symptoms and remained asymptomatic throughout the experimental trial.

GFP fluorescence was analysed in BSMVΔCP:GFP infected wheat cv. Tugela seedlings at 48 hours after infection and again at 7 dpi. Red autofluorescence caused by chlorophyll could be detected in both BSMVΔCP:GFP and control BSMVΔCP:Pds infected seedlings but no visual GFP fluorescence was observed.

3.3.3 Molecular analysis of BSMVΔCP infection

A reverse transcription PCR (RT-PCR) specific to all three BSMV RNAs was devised to determine whether or not the BSMVΔCP virus was present in systemically infected leaves of the asymptomatic infected pearl millet lines. The symptomatic wheat cv. Tugela plants (Figure 3.12, A) were used as positive controls of viral infection. RNA was isolated from the third and fourth leaves of infected and control wheat and pearl millet plants (Figure 3.13) and used as template in a cDNA synthesis reaction. Integrity of the cDNA was verified using PCR primers specific to the actin housekeeping gene (Figure 3.14). The actin primers were designed to amplify a region of the pearl millet and wheat actin gene spanning an intron. Genomic DNA amplification with this actin primer pair will thus yield a 280 bp product whilst a 170 bp product is expected when cDNA is used as template (Crampton 2006).

The presence of the three viral components was analysed by PCR with primer pairs specific to each of the viral RNAs. The expected PCR product sizes were 668 bp for the BSMVα specific primer pair, 764 bp for the BSMVβ specific product and 664 bp for the BSMVγ product. All three viral RNAs; RNAα, RNAβ and RNAγ were amplified in the wheat cv. Tugela plants infected with BSMVΔCP:Pds but none in wheat uninfected control plants. No viral RNAs were detected in either the BSMVΔCP:Pds infected or uninfected control pearl millet lines. Results were the same for both of the pearl millet lines tested (Figure 3.15).
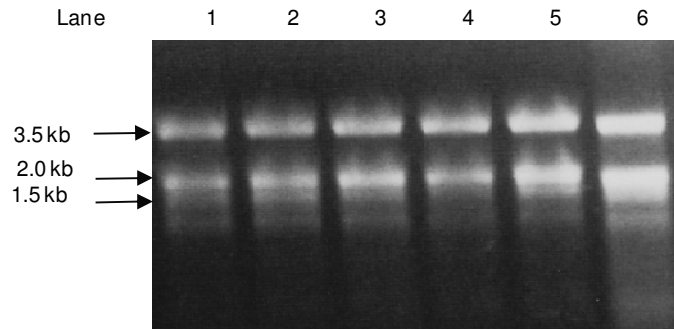
Figure 3.13 Formaldehyde agarose gel electrophoresis of total RNA isolated from wheat and pearl millet plants used in the BSMVΔCP trial. Lane 1: wheat infected with BSMVΔCP:Pds; lane 2: uninfected wheat; lane 3: ICML12 infected with BSMVΔCP:Pds; lane 4: uninfected ICML12 plants; lane 5: ICMB96222 infected with BSMVΔCP:Pds; lane 6: uninfected ICMB96222. Ribosomal RNA is present as distinctly sized bands on the gel.
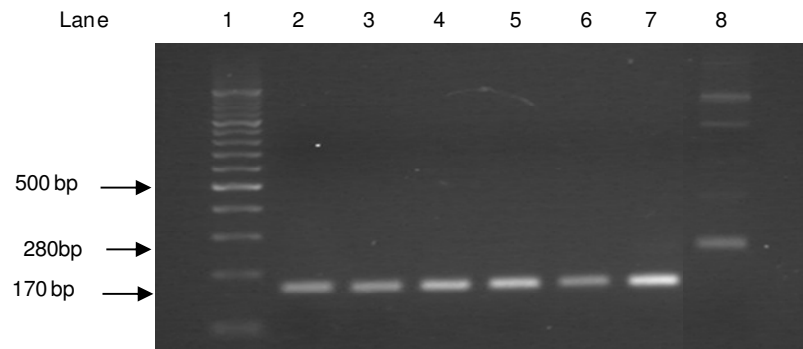

Figure 3.14 Reverse transcription of an actin gene fragment from RNA isolated from BSMVΔCP infected wheat and pearl millet leaves. cDNA samples were used to amplify a 170 bp actin product from cDNA of: lane 2: wheat BSMVΔCP:Pds; lane 3: uninfected wheat cDNA; lane 4: ICML12 BSMVΔCP:Pds; lane 5: uninfected ICML12; lane 6: ICMB96222 BSMVΔCP:Pds; lane 7: uninfected ICMB96222; lane 8: 280 bp actin fragment amplified from ICML12 genomic DNA. Lane 1: Gene Ruler[TM] 100 bp Plus DNA Ladder (Fermentas)
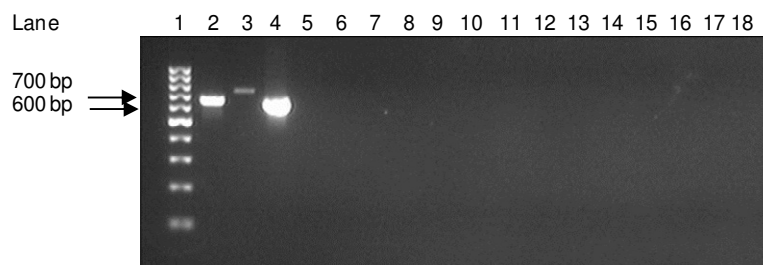
Figure 3.15 Agarose gel electrophoretic analysis of BSMV specific RT-PCR reveals pearl millet does not host the BSMVΔCP VIGS vector. Amplification of the 668 bp, 764 bp and 664 bp RNAα, RNAβ and RNAγ specific PCR products respectively from cDNA of lane 2-4: wheat BSMVΔCP:Pds; lane 5-7, uninfected wheat; lane 7-9: ICML12 BSMVΔCP:Pds; lane 10-12: uninfected ICML12; lane 13-15: ICMB96222 BSMVΔCP:Pds; lane 16-18: uninfected ICMB96222. Lane 1: Gene Ruler[TM] 100 bp DNA molecular weight marker (Fermentas).

Chapter 4 BMV results

4.1 Generating a BMV RNA 3 based vector

The H-BMV VIGS vector (Ding et al. 2006) consists of BMV RNA 1 cloned in a plasmid called pF1-11, BMV RNA 2 cloned in a plasmid called pF2-2 and BMV RNA 3 in a plasmid called pB3m. An alternative version of RNA 3 fused with a green fluorescent protein gene is cloned in a plasmid called pF13m-GFP. RNA 1 and RNA 2 originate from a strain of BMV isolated from tall fescue grass while RNA 3 is from the Russian strain of the virus (Ding et al. 2006). All plasmids consist of a pGEM®-T Easy vector backbone where the BMV genomic cDNAs are cloned under the control of a T3 promoter.

A fragment of the pearl millet *Pds* gene was amplified from ppmPds (Figure 3.3) using extension primers that incorporated *Nco*I and *Avr*II restriction enzyme recognition sites at the 5' and 3' ends of the PCR product. This amplicon was subsequently cloned into the pTZ57R/T cloning vector (Appendix Figure 2). PCR amplification using M13 primers verified cloning of the 300 bp fragment into the cloning vector (Figure 4.1).
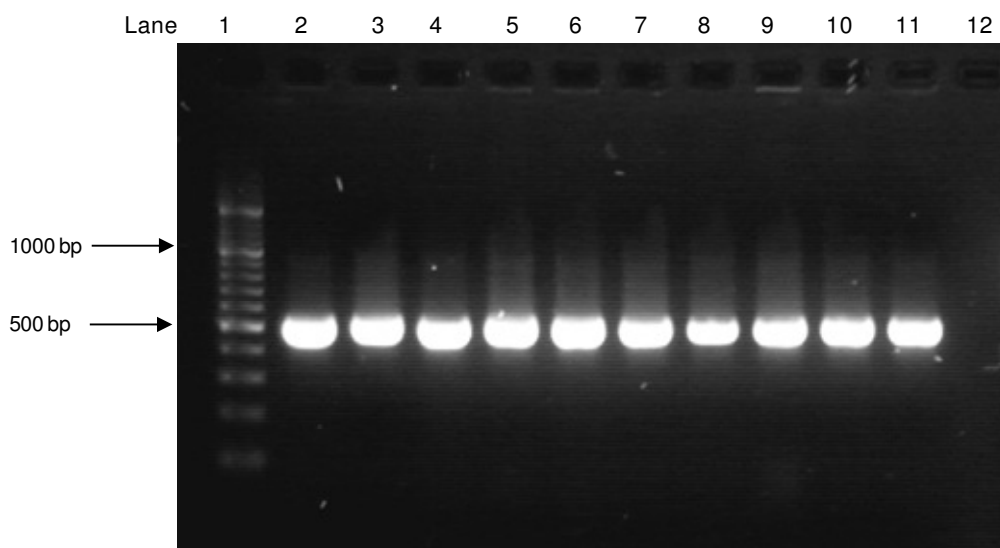

Figure 4.1 Agarose gel electrophoretic analysis of PCR amplification with M13 primers to confirm cloning of a pearl millet gene fragment into the pTZ57R/T cloning vector. Lane 1: 100 bp DNA ladder (New England Biolabs); lane 2-11: 500 bp amplification products from ten colonies screened with the M13 primer pair; lane 12: negative no template control.

The fragment was excised from the cloning vector using *Nco*I and *Avr*II restriction enzymes to generate a fragment with these enzyme overhangs which was subsequently cloned into the pB3m plasmid to generate plasmid pB3mPds300 (Figure 4.2).
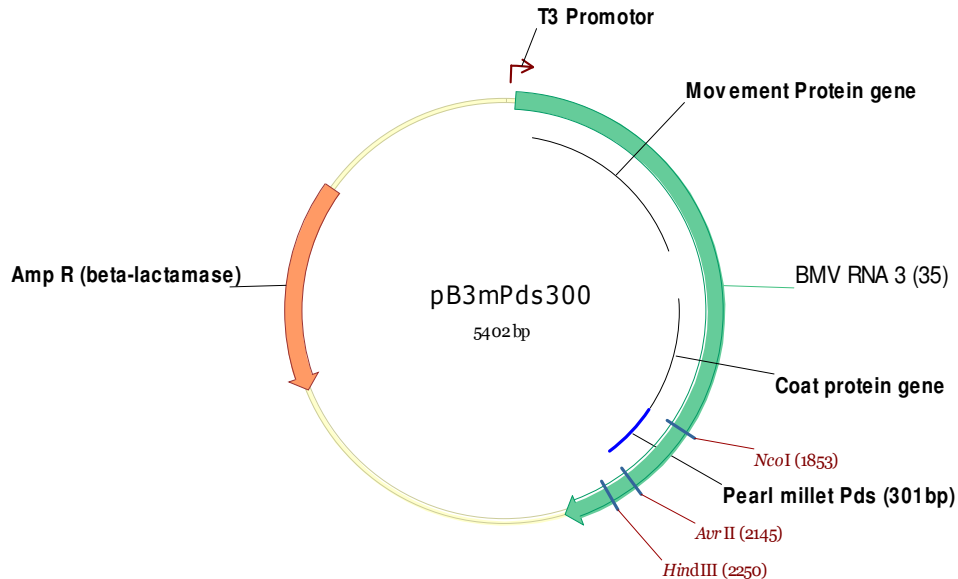

Figure 4.2 Recombinant BMV RNA 3 plasmid; pB3mPds300 with selected restriction sited indicated. A 301 bp fragment of the pearl millet *Pds* gene sequence is cloned in an antisense orientation into the *Nco*I/*Avr*II restriction enzyme recognition sites, immediately downstream of the coat protein gene. The total vector size is 5402 bp.
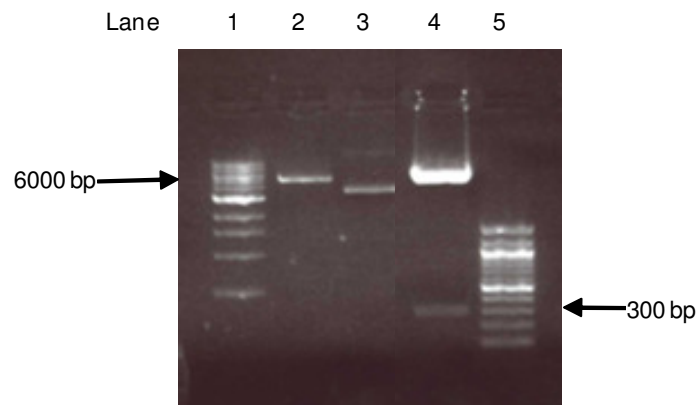
Figure 4.3 Restriction enzyme digestion of pB3mPds300 to confirm presence of the 301 bp pearl millet *Pds* gene insert. Lane 1: 1 kb DNA ladder (New England Biolabs); lane 2: pB3m digested with *Nco*I and *Avr*II; lane 3, circular pB3mPds300; lane 4: pB3mPds300 digested with *Nco*I and *Avr*II; lane 5: 100 bp DNA ladder (New England Biolabs).

Insertion of the *Pds* fragment in pB3mPds300 was confirmed with restriction enzyme digestion (Figure 4.3) and sequencing. The fragment of the pearl millet *Pds* gene cloned in the pB3mPds300 vector is 91% identical to a fragment of the maize *Pds* gene (GenBank

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accession ZMU37285), 85 % identical to a fragment of the barley *Pds* gene (GenBank accession AY062039) and 76% identical to the *N. benthamiana Pds* gene sequence (GenBank accession EU165355) over 284 nucleotides. The alignment is shown in Figure 4.4. Restriction enzyme digestion and sequencing revealed that the insert was incorporated into the plasmid and orientated correctly. pB3mPds300 was used in combination with the cloned copies of the other two BMV genomic RNAs, pF1-11 and pF2-2 to prepare the BMV:Pds construct for *in vitro* transcription of infectious RNA. pB3mGFP was used instead of pB3mPds300 in the BMV:GFP construct and unmodified pB3m in the case of the wild type BMV construct.
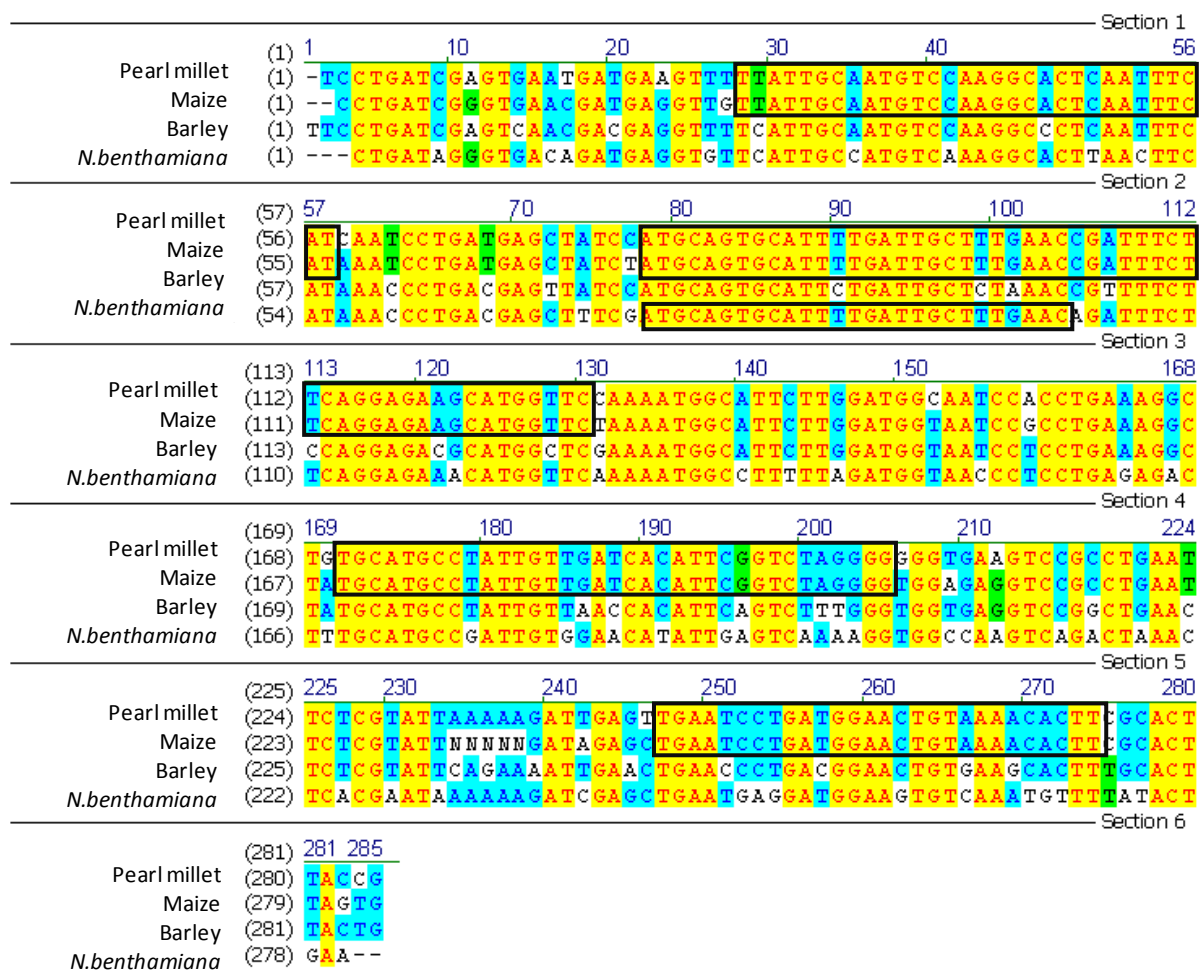
Figure 4.4 Nucleotide sequence alignment of the partial pearl millet, maize, barley and *Nicotiana bentamiana Pds* gene sequences. The fragment of the pearl millet *Pds* gene cloned in the pB3mPds300 vector shares 91%, 85 % and 76% nucleotide identity to fragments of the maize, barley and *N. benthamiana Pds* genes respectively. Non-similar residues are shown in black, conserved residues are highlighted in blue whereas identical residues are in red typeface, highlighted in yellow. Black boxes indicate stretches with 23 nucleotides or more similarity.

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4.2 Norflurazon treatment in barley

Norflurazon treatment was conducted on barley seedlings as described for pearl millet and wheat in section 3.2 of chapter 3 to obtain a phenotypic reference of *Pds* inhibition in barley. Barley seedlings displayed severe photobleaching. Symptoms were visible from 4 dpi, initiating in the leaf base and extending towards the apex (Figure 4.5). Homogeneous photobleaching occurred in all leaves that emerged after the treatment was applied and it was therefore apparent that norflurazon induced photobleaching symptoms in barley seedlings similar to those observed in pearl millet and wheat (Figure 3.9). Plants became weak and eventually died after prolonged norflurazon treatment.

Figure 4.5 Norflurazon induced photobleaching in barley seedlings. L3 of a 17 day old barley seedling displays whitening by 5 days after treatment with norflurazon. Photobleaching started from the leaf base (left) and progressed towards the leaf apex (right) of all leaves.

4.3 BMV direct RNA infection based VIGS experiments

4.3.1 BMV RNA vector linearization

Genomic organisation of the H-BMV RNA3 based VIGS vector is shown in Figure 4.6. BMV VIGS plasmids were linearized downstream of the BMV coding sequence to function as template DNA for *in vitro* transcription using T3 RNA polymerase. The first construct; BMV:Pds consisted of equal quantities of F1-11, F2-2 and B3mPds300 RNA. The second and third constructs BMV:GFP and BMV wild type (BMV:WT), differed only in containing an equal quantity of B3mGFP RNA or B3m instead of B3mPds300. Figure 4.7 shows the electrophoretic analysis of BMV constructs linearization where the digested plasmids can be seen as a single band in the agarose gel. 1000 ng linear plasmid DNA was used as template for the T3 polymerase transcription. A fraction of the RNA was analysed on a 1% agarose gel to determine the RNA yield of the *in vitro* transcription reaction. High concentrations of RNA were visible as smears of approximately 1.3 kb in the gel (Figure 4.8).

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A BMV F1-1

1a

B BMV F2-2

2a

T3 promoter
NcoI
AvrII

C BMV B3m:PDS

3a   CP   PDS

D BMV B3m:GFP

3a   CP   GFP
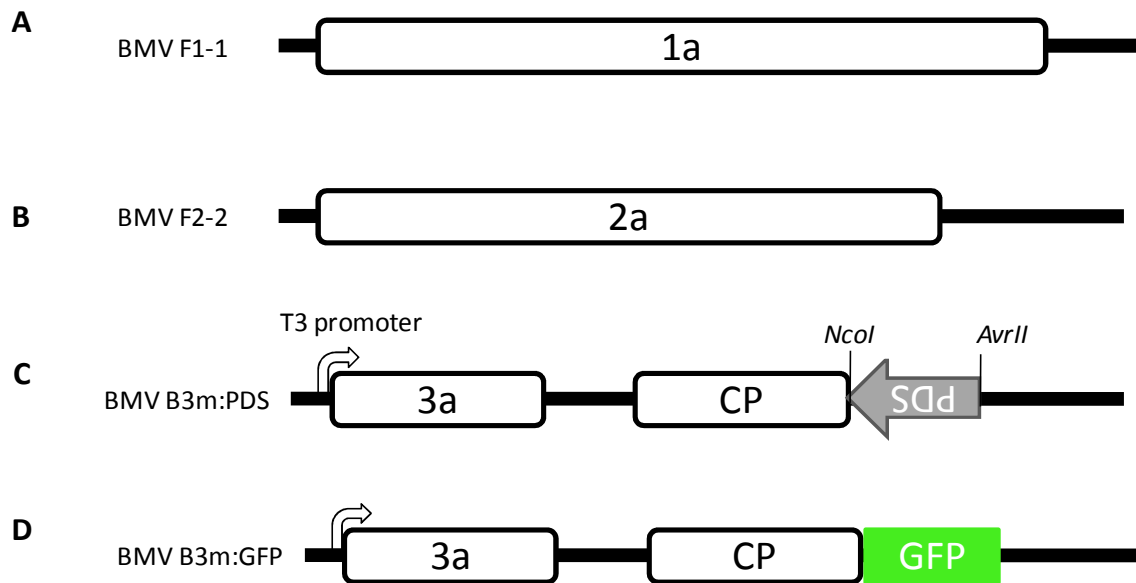
Figure 4.6 Genomic organisation of the H-BMV RNA3 based VIGS vectors. A and B) cDNA clones of F-BMV RNA 1 and RNA 2 respectively. C) A 301 bp fragment of pearl millet *Pds* inserted in an antisense orientation between the *Nco*I and *Avr*II restriction enzyme recognition sites of a cDNA copy of R-BMV RNA 3. D) BMV B3m:GFP contains the GFP gene (green box) downstream of the CP protein. Clear boxes represent ORF's.

Lane   1   2   3   4   5   6   7   8   9   10   11   12

6000 bp →

Figure 4.7 Electrophoretic analyses of linearized BMV plasmids. Lane 1: GeneRuler[Tm] 1 kb DNA ladder (Fermentas); lane 2-6: each of the BMV plasmids: pF1-1, pF2-2, pB3mPds300, pB3mGFP and pB3m before linearization; lane 8-12: vector plasmids visible as single bands after digestion with an appropriate restriction enzyme respectively.

Figure 4.8 Electrophoretic analysis of *in vitro* T3 BMV RNA transcription. Lane 1: GeneRuler[Tm] 1 kb Plus DNA ladder (Fermentas), lane 2-6: 1 μl of RNA transcribed from pF1-11, pF2-2, pB3mPds30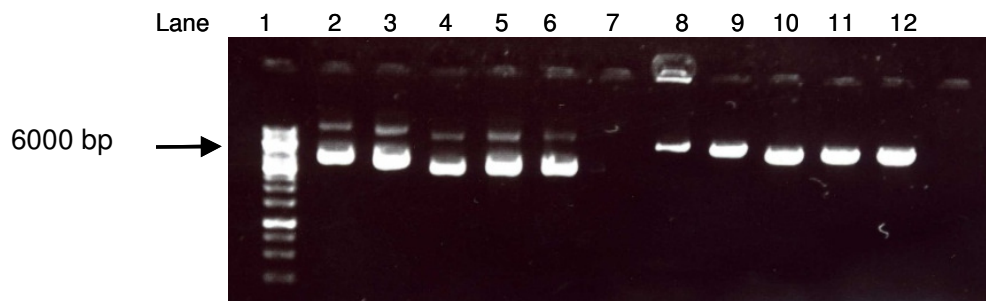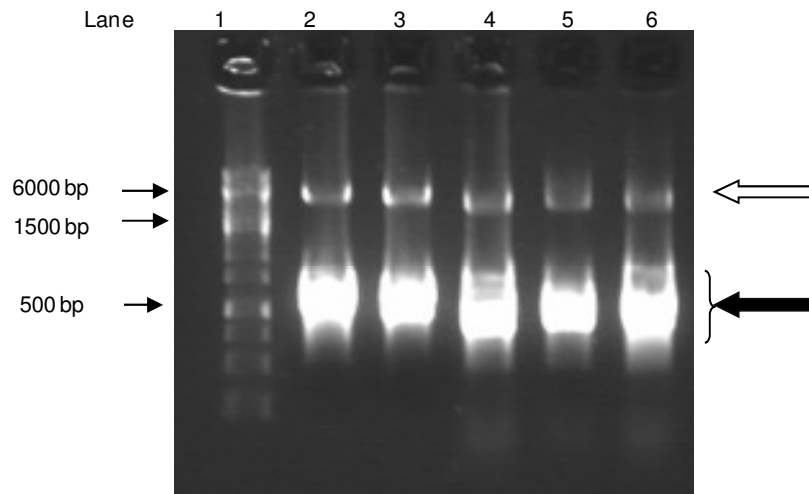0, pB3mGFP and pB3m respectively. The brace and black arrow indicate the position of the full-length RNA products whilst the thin white arrow indicates linearized DNA plasmids templates of approximately 6 kb.

4.3.2 Symptoms of direct RNA BMV infection

All three RNA components of each of the constructs, BMV:WT, BMV:GFP and BMV:Pds were mixed with an inoculation buffer in a 1:1:1 ratio and used to infect seedlings. Barley, a natural host for BMV (McKinney 1944) and *N. benthamiana*, which can be infected with BMV under laboratory conditions (Ford et al. 1970), were used as positive controls. Two pearl millet lines; ICML12 and ICMB96222, were infected. One set of plants was inoculated with the inoculation buffer only, as a negative control. The plants were analysed phenotypically from 7-28 dpi.

Barley cv. Morex plants infected with BMV:Pds, BMV:GFP and BMV:WT had systemic chlorotic streak symptoms which appeared from 12 dpi and increased in the newly emerging leaves (Figure 4.9, A-C). Control plants remained asymptomatic throughout the trial. (Figure 4.9, D). Necrosis was more prevalent in the BMV:WT infected plants (Figure 4.9, C) compared to either BMV:Pds or BMV:GFP (Figure 4.9, A and B). No significant visual distinction could be made between BMV:Pds and BMV:GFP infected barley.

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A) BMV:Pds   B) BMV:GFP   C) BMV:WT   D) Control

Figure 4.9 Phenotypic symptoms of barley infected with BMV VIGS vector RNA. Chlorotic mosaic patches and/or stripes were observed in the first emerging leaf (L3) after infection of barley seedlings with RNA from the A) BMV:Pds, B) BMV:GFP, C) BMV:WT vectors. The symptoms spread to most of the systemically infected leaves. Control barley seedlings infected with buffer only, D, did not display similar symptoms. Pictures were taken of L3 at 17 dpi and are representative of at least six samples.

A) BMV:Pds   B) BMV:GFP   C) BMV:WT   D) Control

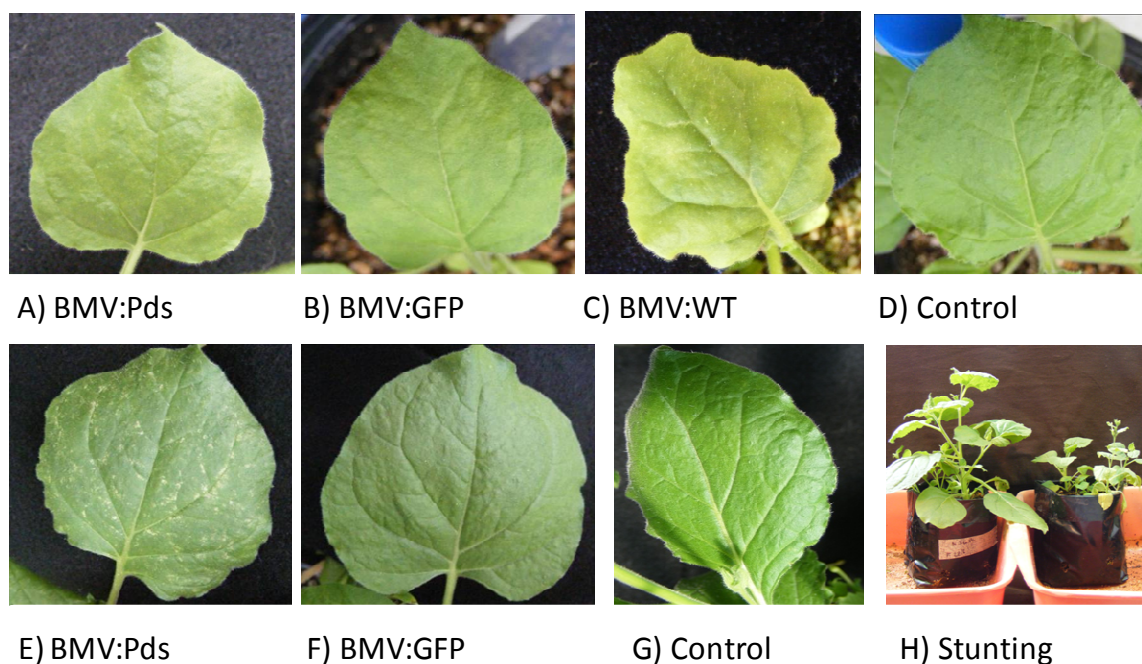E) BMV:Pds   F) BMV:GFP   G) Control   H) Stunting

Figure 4.10 Phenotypic symptoms of *N. benthamiana* infected with BMV VIGS vector RNA. Very faint, almost indistinguishable mosaics were observed in the first emerging leaf after infection of *N. benthamiana* seedlings infected with RNA from the A) BMV:Pds, B) BMV:GFP, C) BMV:WT vectors. Control *N. benthamiana* seedlings infected with buffer only, D, did not display similar symptoms. Pictures A-D were taken of L3 at 13 dpi and are representative of at least six samples. By 25 dpi the mosaics were more visible in *N. benthamiana* infected with BMV:Pds, E, compared to BMV:GFP, F. Control plants, G, remained healthy. H) BMV:WT infected plants (right) were stunted in comparison to control plants (left). Pictures were taken at 25 dpi.

BMV:Pds and BMV:GFP infected *N. benthamiana* plants displayed very faint mosaic symptoms by 13 dpi (Figure 4.10) in some, but not all of the leaves. The mosaic pattern intensified in the lower systemically infected leaves of BMV:Pds infected seedlings by 25 dpi. Small white patches could be observed in the proximity of the laminar venation (Figure 4.10, E). The plants infected with BMV:WT showed slight chlorosis by 13 dpi (Figure 4.10,

C) and were stunted and paler in colour compared to control plants throughout the trial (Figure 4.10, H). The control set of *N. benthamiana* plants remained asymptomatic throughout the experiment (Figure 4.10, D and G).

Both pearl millet lines, ICMB96222 and ICML12, infected with all three constructs BMV:Pds, BMV:GFP and the wild type BMV construct, as well as the control millet plants, remained asymptomatic throughout the trial (Figure 4.11). Slight striping occurred in some ICMB96222 plants (Figure 4.11, E-H), but this phenotype was observed in the same ratio amongst control seedlings and seems to be characteristic of the breeding line.

GFP fluorescence was analysed in BMV:GFP infected barley cv. Morex and *N. benthamiana* at 48 hours after infection and again at 7 dpi. Red autofluorescence caused by chlorophyll could be detected in both BMV:GFP and control BMV:Pds infected seedlings but no visual GFP fluorescence could be observed.

Therefore, phenotypic analysis of the infections revealed that the direct infection with BMV RNA induced visual symptoms in barley cv. Morex and *N. benthamiana* but not pearl millet. No distinction could be made between the symptoms in BMV:Pds, BMV:GFP infected barley cv. Morex seedlings, but in *N. benthamina* BMV:Pds infected plants showed pronounced mosaic symptoms, compared to BMV:GFP or BMV:WT plants, by 25 dpi.

A) BMV:Pds   B) BMV:GFP   C) BMV:WT   D) Control

E) BMV:Pds   F) BMV:GFP   G) BMV:WT   H) Control
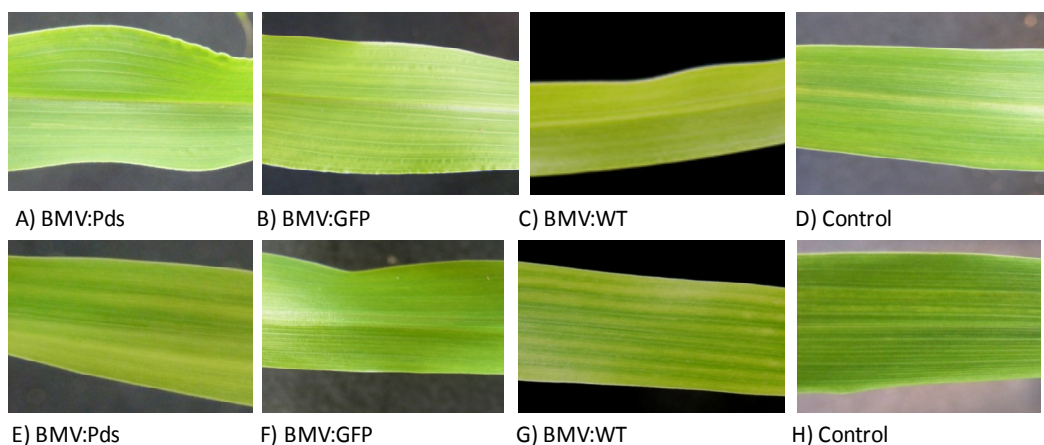
Figure 4.11 Phenotypic symptoms of pearl millet infected with BMV VIGS vector RNA. A-D) None of the ICML12 seedlings infected with RNA from the A) BMV:Pds, B) BMV GFP, C) BMV:WT constructs displayed any significant photobleaching or chlorotic symptoms. Control ICML12 seedlings infected with buffer only, D, remained healthy throughout the trial. E-H) ICMB96222 seedlings inoculated with infected RNA from the E) BMV:Pds, F) BMV GFP, G) BMV:WT vectors did not display any significant photobleaching or chlorotic symptoms and the control ICMB96222 seedlings, H, remained healthy throughout the trial. Pictures were taken of L3 at 16 dpi and are representative of at least six samples.

4.3.3 Molecular analysis of direct RNA BMV infection

The presence of viral RNAs in systemically infected leaves was determined through reverse transcription polymerase chain reaction (RT-PCR). RT-PCR primers designed to specifically amplify a part of each of the three BMV RNAs from cDNA were used for this purpose. To establish such a diagnostic tool; the RT-PCR was first performed on the known hosts of BMV, barley and *N. benthamiana*. The BMV RNA 1, RNA 2 and RNA 3 specific primers were designed to amplify 685 bp, 707 bp and 784 bp products respectively. RNA was isolated from barley and *N. benthamiana* infected with the BMV:Pds and BMV:GFP constructs as well as from control plants at 17 dpi. The RNA was subsequently converted to cDNA using random primers. The third leaf of two or more of the seedlings was used for RNA isolations in each treatment. A 170 bp fragment of the actin gene, in *N. benthamiana* and pearl millet or a 471 bp fragment of the small ribosomal subunit gene in barley, were amplified to confirm cDNA integrity of all the samples (Figure 4.12). Figure 4.13 shows the result of the diagnostic RT-PCR. All three RNAs were present in the barley and *N. benthamiana*, BMV:Pds and BMV:GFP infected plants. No amplification occurred in the control plants infected with buffer only.

The presence of BMV in the two pearl millet lines was subsequently analysed using these primer sets. None of the three BMV RNA's were detected in cDNA of either of the two pearl millet lines infected with RNA of wild type BMV (Figure 4.14) or the BMV:GFP and BMV:Pds constructs (Figure 4.15).
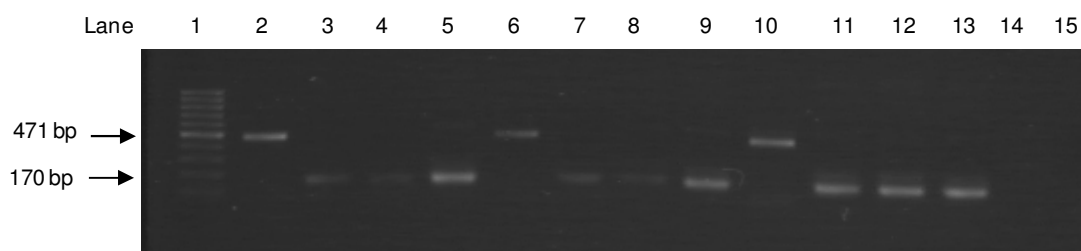
Figure 4.12 Agarose gel electrophoretic analysis of actin or small ribosomal subunit (sRs) housekeeping gene amplification verifies cDNA integrity. A 170 bp actin product was amplified from *N. benthamiana* and pearl millet, whilst a 471 bp sRs product was amplified from barley. Lane 1: GeneRuler[Tm] 100 bp DNA ladder (Fermentas); lane 2: uninfected barley; lane 3: uninfected *N. benthamiana*; lane 4: uninfected ICML12; lane 5: uninfected ICMB96222; lane 6: BMV:GFP barley; lane 7: BMV:GFP *N. benthamiana*; lane 8: BMV:GFP ICML12; lane 9: BMV:GFP ICMB96222; lane 10: BMV:Pds barley; lane 11: BMV:Pds *N. benthamiana*; lane 12: BMV:Pds ICML12; lane 13: BMV:Pds ICMB96222; lane 14: sRs no template control; lane 15, actin no template control.

Figure 4.13 Agarose gel electrophoretic analysis of BMV specific RT-PCR confirms that barley and *N. benthamiana* host BMV VIGS vectors. A, B and C: Lane 1: GeneRuler^{Tm} 100 bp DNA ladder (Fermentas). A) Amplification of the 685 bp BMV RNA 1 specific products from: lane 2: barley inoculated with buffer only; lane 3: barley inoculated with BMV:GFP; lane 4: barley inoculated with BMV:Pds; lane 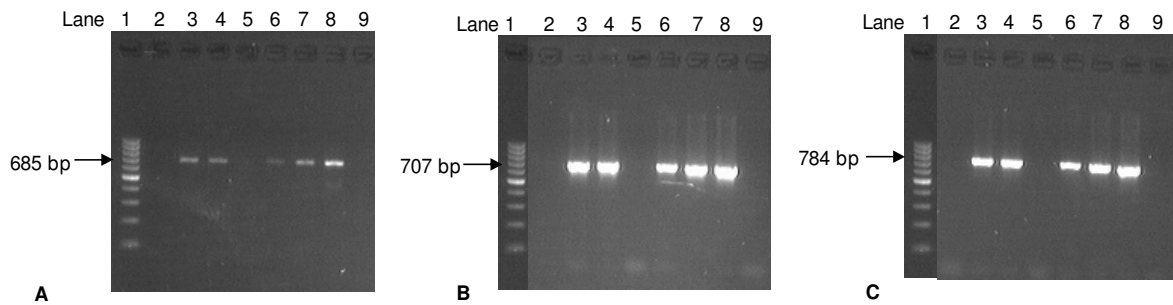5: *N. benthamiana* inoculated with buffer only; lane 6: *N. benthamiana* inoculated with BMV:GFP; lane 7: *N. benthamiana* inoculated with BMV:Pds; lane 8: positive pF1-1 plasmid control; lane 9: negative no template control. B) Amplification of the 707 bp BMV RNA 2 specific products from: lane 2: barley inoculated with buffer only; lane 3: barley inoculated with BMV:GFP; lane 4: barley inoculated with BMV:Pds; lane 5: *N. benthamiana* inoculated with buffer only; lane 6: *N. benthamiana* inoculated with BMV:GFP; lane 7: *N. benthamiana* inoculated with BMV:Pds; lane 8: positive pF2-2 plasmid control; lane 9: negative no template control. C) Amplification of the 784 bp BMV RNA 3 specific products from: lane 2: barley inoculated with buffer only; lane 3: barley inoculated with BMV:GFP; lane 4: barley inoculated with BMV:Pds; lane 5: *N. benthamiana* inoculated with buffer only; lane 6: *N. benthamiana* inoculated with BMV:GFP; lane 7: *N. benthamiana* inoculated with BMV:Pds; lane 8: positive pB3m plasmid control; lane 9: negative no template control.

Figure 4.14 Agarose gel electrophoretic analysis of BMV specific RT-PCR confirms that pearl millet does not host the wild type BMV vector. A-C) Lane 1: GeneRuler^{Tm} 100 bp DNA ladder (Fermentas); A) Amplification of the 685 bp RNA 1 specific product from cDNA of; lane 2: uninfected barley; lane 3: barley infected with BMV:WT; lane 4: uninfected ICML12; lane 5: ICML12 infected with BMV:WT; lane 6: uninfected ICMB96222, lane 7: ICMB96222 infected with BMV:WT; lane 8: no template control. B) Amplification of the 707 bp RNA 2 specific product from cDNA in the same order as in A. C) Amplification of the 784 bp RNA 3 specific product from cDNA in the same order as in A and B.
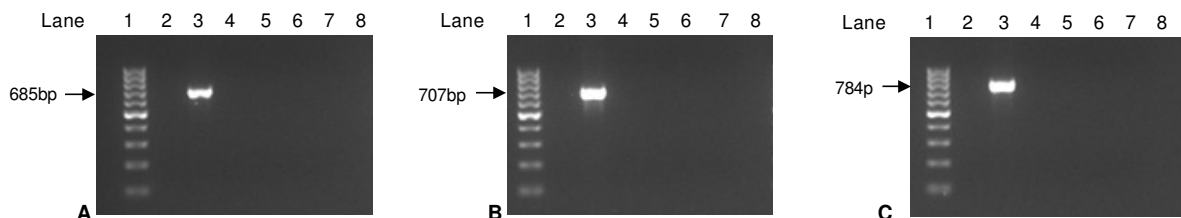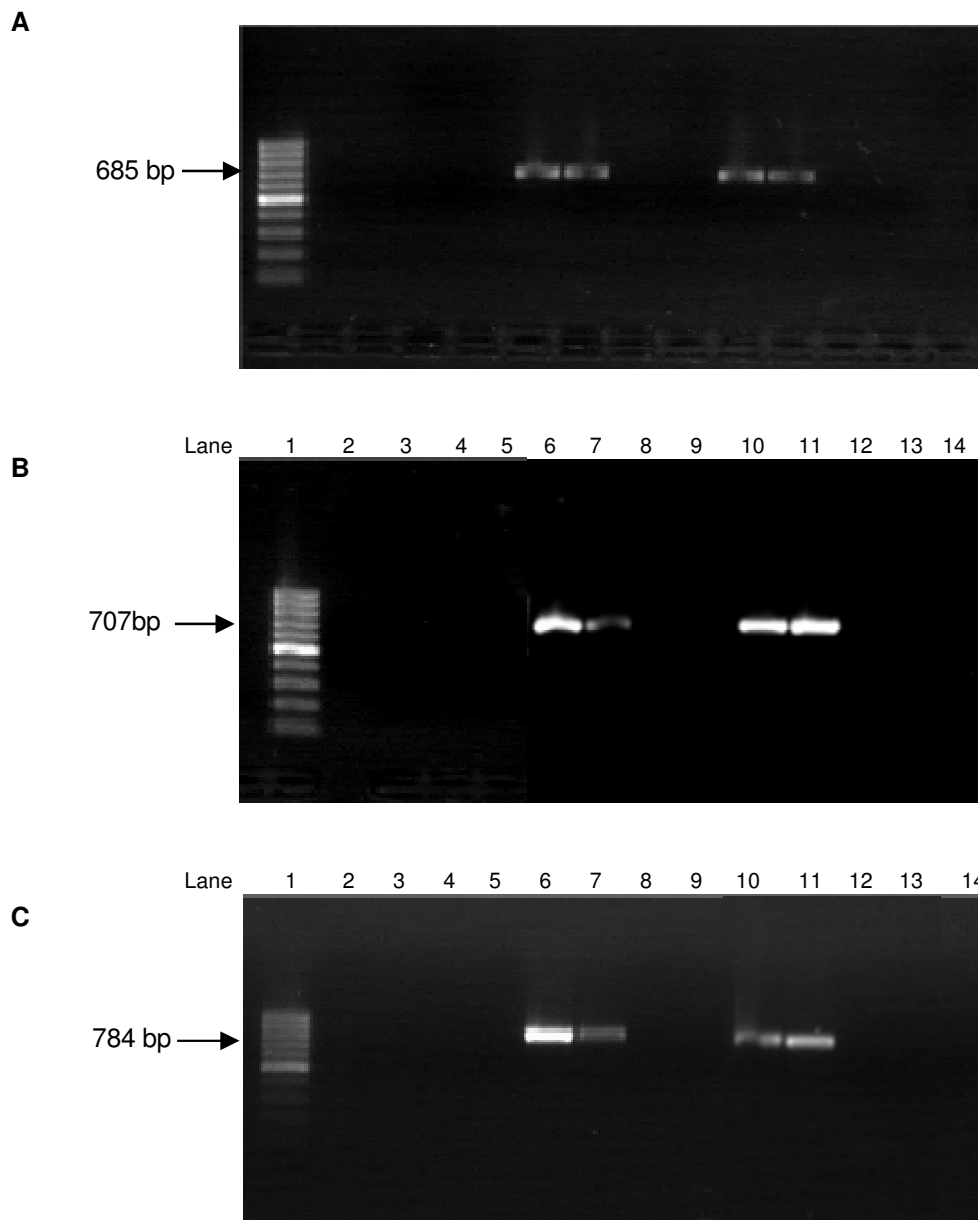
Figure 4.15 Agarose gel electrophoretic analysis of BMV specific RT-PCR confirms that pearl millet does not host BMV:Pds or BMV:GFP VIGS vector constructs. A-C) Lane 1: GeneRuler^{Tm} 100 bp DNA ladder (Fermentas); A) Amplification of the 685 bp RNA 1 specific products from cDNA of lane 2: uninfected barley; lane 3: uninfected *N. benthamiana*; lane 4: uninfected ICML12; lane 5: uninfected ICMB96222; lane 6: BMV:Pds infected barley; lane 7: BMV:Pds infected *N. benthamiana*; lane 8: BMV:Pds infected ICML12; lane 9: BMV:Pds infected ICMB96222; lane 10: BMV:GFP infected barley; lane 11: BMV:GFP infected *N. benthamiana*; lane 12: BMV:GFP infected ICML12; lane 13: BMV:GFP infected ICMB96222; lane 14: no template control. B) Amplification of the 707 bp RNA 2 specific products from cDNA, samples in the same order as A. C) Amplification of the 784 bp RNA 3 specific products from cDNA, samples in the same order as A and B.

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4.4 Indirect sap BMV infection VIGS experiments

A crude sap extract prepared from the symptomatic wild type BMV infected barley plants was used to infect a second set of barley cv. Morex and pearl millet ICML12 and ICMB96222 seedlings.

4.4.1 Symptoms of indirect sap BMV infection

Barley cv. Morex seedlings infected with the BMV:WT sap started to show mosaic streaking in L3 and L4 from 8 dpi (Figure 4.16). The symptoms increased in intensity and spread to all newly emerging leaves. By 18 dpi some of the leaves displayed necrosis (Figure 4.16, C).

A) BMV:WT   B) Control

C) BMV:WT 18 dpi   D) Control 18 dpi

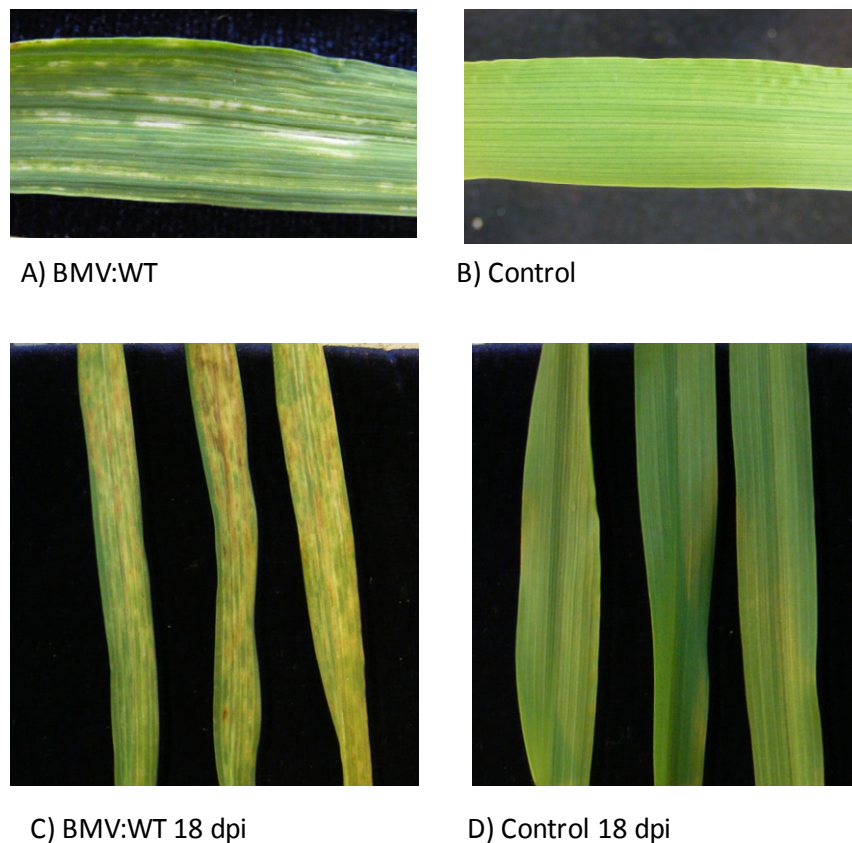**Figure 4.16** Phenotypic symptoms of barley infected with a wild type BMV (BMV WT) infected barley crude sap extract. A) Distinct white streaking in L3 of barley seedling at 8 dpi. B) Control plants, infected with phosphate buffer, did not display any symptoms. C) White streaking increased in the barley seedlings and by 18 dpi necrosis could be observed in L3 of all replicates, whilst all control plants remained healthy (D).

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Both pearl millet lines, ICMB96222 and ICML12, infected with the wild type BMV crude sap extract as well as the control millet plants, infected with phosphate buffer, remained asymptomatic throughout the trial (Figure 4.17).

Thus, as was seen with direct RNA infection, the crude sap extract induced viral infection symptoms in barley but not in pearl millet. The BMV vector used induced white streaking and necrosis in barley.

Two selected maize breeding lines X and Y, were also infected with the same crude sap extract that contains BMV:WT. No symptoms were observed in any of the infected maize seedlings (Figure 4.18).

A) BMV:WT sap   B) Control

C) BMV:WT sap   D) Control

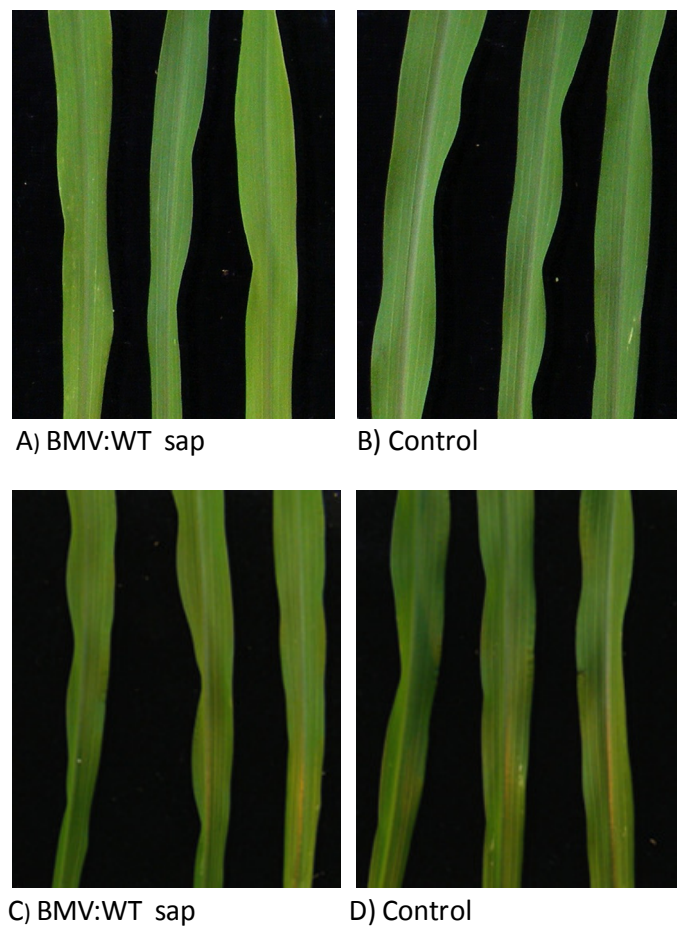**Figure 4.17** Phenotypic symptoms of pearl millet infected with a wild type BMV infected barley crude sap extract. A) L3 of a ICML12 seedlings infected with sap from BMV:WT infected barley cv. Morex looks similar to leaves of control ICML12 seedlings (B). D) L3 of ICMB96222 remained asymptomatic as did the control D). Pictures were taken of L3 at 18 dpi and are representative of at least 10 samples.
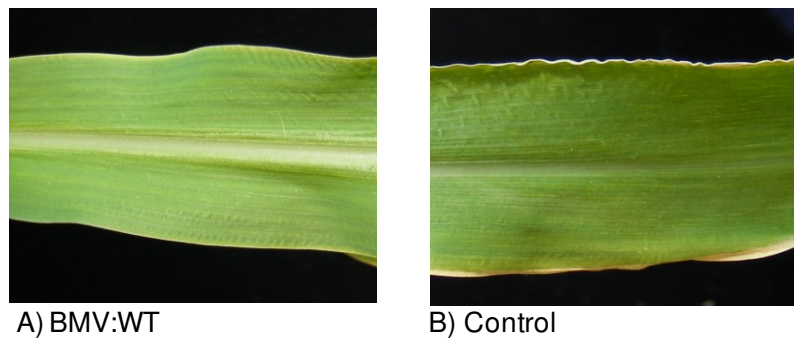
A) BMV:WT
B) Control

Figure 4.18 Phenotypic symptoms of maize infected with a wild type BMV-infected barley crude sap extract. A) L3 of maize breeding line X seedlings inoculated with BMV:WT infected barley sap resembles control maize seedlings in B. Pictures were taken of L3 or L4 at 25 dpi and are representative of at least six samples.

4.4.2 Molecular analysis of indirect sap BMV infection

No BMV specific products could be amplified from pearl millet ICML12 or ICMB96222 seedlings infected with a crude extract made from BMV:WT infected barley cv. Morex, whereas such products could be amplified from the barley cv. Morex seedlings infected with the same sap, used as positive controls for infection (Figure 4.19, A).
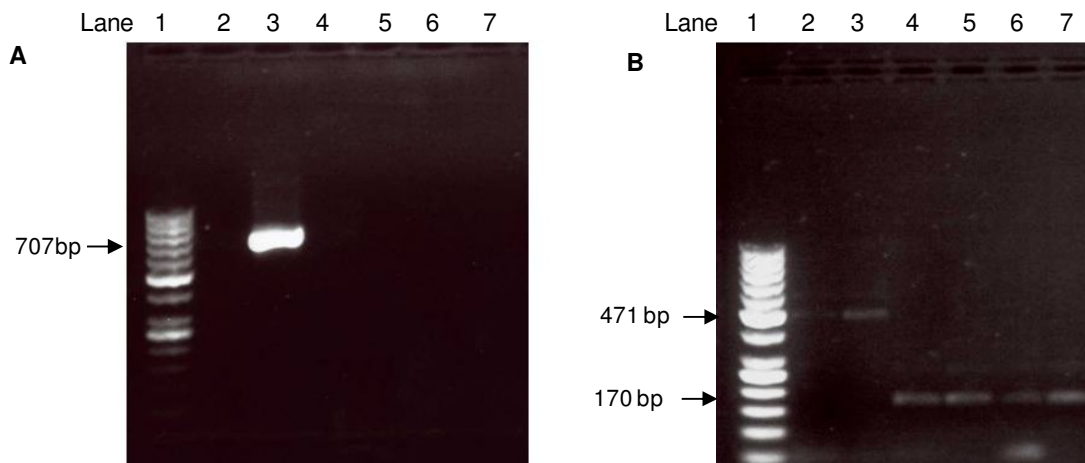
Figure 4.19 Agarose gel electrophoretic analysis of BMV specific RT-PCR confirms that asymptomatic pearl millet infected with a crude BMV sap extract do not host BMV. A and B) Lane 1: GeneRuler[Tm] 50 bp DNA ladder (Fermentas). A) Amplification of the 707 bp BMV RNA 2 specific products from cDNA of: lane 2, uninfected barley; lane 3: barley BMV:WT; lane 4: uninfected ICML12; lane 5: ICML12 BMV:WT; lane 6: uninfected ICMB96222; lane 7: ICMB96222 BMV:WT; B) Reverse transcription and PCR of a sRs or actin gene fragment from RNA isolated from barley and pearl millet leaves in the same order as A to confirm that the absence of amplification is not as a result of cDNA quality.

Although no BMV symptoms were observed (Figure 4.18), all three BMV specific products were amplified from the third and fourth leaves of BMV:WT infected maize breeding line X and Y (Figure 4.20).
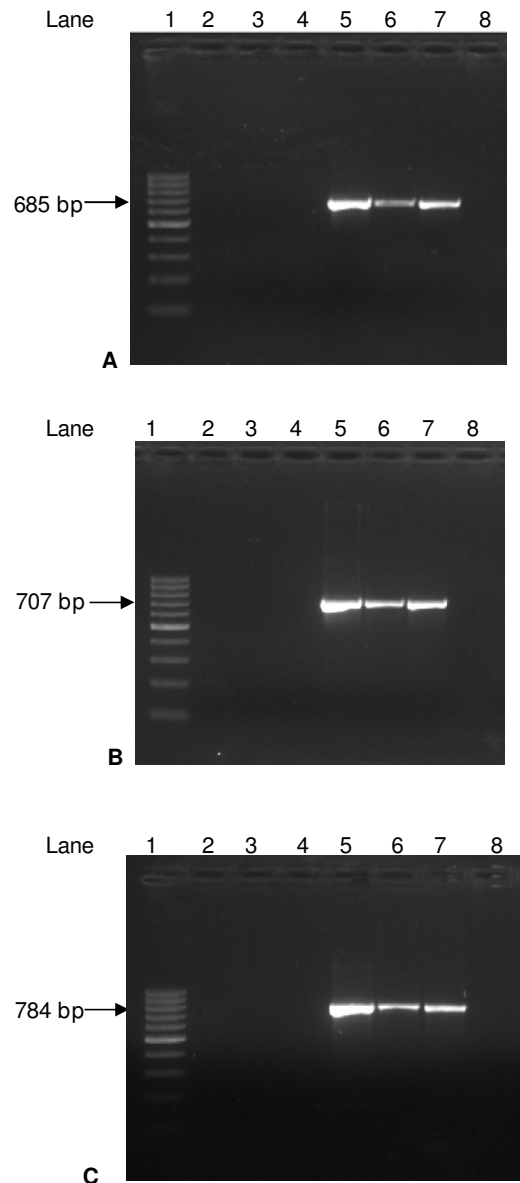
Figure 4.20 Agarose gel electrophoretic analysis of BMV specific RT-PCR in two selected maize breeding lines. Amplification of BMV specific products confirms that barley and maize seedlings host BMV when infected with a crude BMV:WT sap extract. A-C) Lane 1: GeneRuler^{Tm} 100 bp DNA ladder (Fermentas). A) Amplification of the 685 bp RNA 1 specific products from cDNA of lane 2: uninfected barley cv. Morex; lane 3: uninfected maize breeding line X; lane 4: uninfected maize breeding line Y; lane 5: BMV:WT infected barley cv. Morex, lane 6: BMV:WT infected maize breeding line X; lane 7: BMV:WT infected maize breeding line Y; lane 8: no template negative control. B) Amplification of the 707 bp RNA 2 specific products from cDNA, samples in the same order as A. C) Amplification of the 784 bp RNA 3 specific products from cDNA, samples in the same order as A and B.

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Chapter 5 Discussion

5.1 Discussion

The discovery and development of virus-induced gene silencing (VIGS), a powerful reverse genetics tool, has contributed significantly toward advances in functional genomics in many plant species (Purkayastha and Dasgupta, 2009). The technique has been widely applied in dicotyledonous species and a lot of research is currently focussed on the development of VIGS in monocotyledonous species. To date, four virus vectors; BSMV, BMV, CymMV (reviewed in Scofield and Nelson, 2009) and RTBV (Purkayastha et al. 2010) have successfully been used to conduct VIGS in monocotyledonous plants.

Phytoene desaturase has regularly been used as a visual reporter gene in VIGS experiments due to the easily discernable silencing phenotype, referred to as photobleaching, and was chosen as a reporter gene in this study. Conserved regions or a consensus of gene sequences in closely related species can be used to design primers if the nucleotide sequence of the gene is not available. This approach was used to isolate the barley *Pds* gene (Holzberg et al. 2002). The *Pds* nucleotide sequence for selected monocotyledonous species were aligned and compared to design primers that would amplify a fragment of the expressed pearl millet *Pds* gene. According to taxonomic studies, close relatives of pearl millet include foxtail millet, maize and sorghum as seen in Figure 1.1 (Devos and Gale, 1997). The maize *Pds* nucleotide sequence was used to design primers that successfully amplified a 900 bp pearl millet *Pds* fragment. Silencing is initiated by the virus if the nucleotide sequence of the insert is homologous to the exonic parts of the host target gene and therefore cDNA, instead of genomic DNA, was used to amplify the pearl millet silencing insert. The amplicon was cloned into the vector ppmPds (Figure 3.3) and nucleotide sequencing confirmed its identity.

A 365 bp or 301 bp fragment of this pearl millet *Pds* fragment was cloned in an antisense orientation into the BSMV or BMV vectors, respectively. The pearl millet *Pds* insert in the BSMV vector (GenBank accession number HS410757) was 87% identical to a part of the wheat *Pds* gene over 365 nucleotides (Figure 3.5) while the shorter *Pds* fragment in the BMV vector shared 91%, 85% and 76% sequence identity over 284 nucleotides with the *Pds* nucleotide sequence of maize, barley and *N. benthamiana*, respectively (Figure 4.4). The occurrence of PTGS is determined by the presence of short stretches of 21-23 nucleotides

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identical to the target gene, which can induce PTGS in plants, rather than by the average percentage homology shared between the target transcript and the insert (Thomas et al. 2001). The lower limit of insert size with 100% homology required to initiate VIGS of the GFP transgene in *N. benthamiana* has experimentally been determined to be 23 nt. In their study, Thomas et al. indicated that GFP silencing occurred when specific 23 nt or longer fragments were used as inducers and no silencing were observed with any fragments smaller than 23 nt (Thomas et al. 2001). Viruses initiate PTGS in plants when dsRNA, formed during viral replication, are processed by the DCL enzyme complex to produce siRNAs typically between 21 and 23 nucleotides long in plants (Hamilton and Baulcombe, 1999, Benedito et al. 2004, Shao et al. 2008). These siRNAs are used by the host plant to target mRNA degradation by cleaving homologous targets opposite the centre of the siRNA (Bernstein et al. 2001, Benedito et al. 2004). The maize *Pds* gene fragments share four sections of more than 23 nucleotides identical to the pearl millet *Pds* sequence, whereas only one such section occurs in wheat and *N. benthamiana* (indicated by black boxes in Figure 3.7 and Figure 4.4) which motivated the use of the pearl millet construct in these species. The pearl millet *Pds* inserts used in this study have sufficient sequence similarity to induce PTGS of *Pds*, and thus photobleaching, in pearl millet, maize, wheat, and *N. benthamiana*, but not in barley where the longest stretch of successive identical nucleotides is 17 nt long (Figure 4.4).

In VIGS, it is important to be able to distinguish any streaking or chlorosis as a result of viral symptoms from gene silencing resulting from PTGS (Holzberg et al. 2002, Robertson 2004). For this purpose two measurements can be implemented. Firstly; the expected *Pds* silencing symptoms can be induced chemically with norflurazon, an inhibitor of the Pds enzyme, as reference of the chemically-induced *Pds* silencing phenotype (Holzberg et al. 2002). Secondly; wild type viral constructs can to be included in all experimental trials to identify natural virus induced symptoms in the plant species or variety under investigation. In this study norflurazon-induced *Pds* silencing were conducted in all the plant species. The chemically-induced photobleaching appeared between four and six days after treatment in all the plant species used in this study. Symptoms initiated from the leaf base, extended towards the apex and saturated the entire lamina of the wheat, barley and pearl millet cultivars that were assessed. *Pds* has been found to be a poor visual marker gene in *Crassulacean* acid metabolism plants with slow growth rates, such as orchids (Lu et al. 2007). However, the norflurazon treatment executed here and comparative *Pds* silencing results in other monocotyledonous plants indicates that *Pds* would be an appropriate choice as visual reporter

of silencing in pearl millet, should a suitable viral vector be identified. Control viral constructs were included in all experiments to distinguish any potential symptoms caused by the BSMV and BMV VIGS vectors from *Pds* silencing symptoms. Either the wild type virus in the case of BMV and/or a construct that contained a full-length GFP sequence for monitoring purposes in the case of BSMV and BMV were used as such controls. Wild type BSMV infections were not conducted because no significant differences in symptoms caused by the wild type BSMV and BSMV:GFP insert control vector have been reported previously (Holzberg et al. 2002).

BSMV VIGS was first successfully conducted in barley (Holzberg et al. 2002) and wheat (Scofield et al. 2005) and has since been used to functionally characterise several genes such as genes associated with powdery mildew resistance in barley (Hein et al. 2005) and improved aphid resistance in wheat (van Eck et al. 2010) and to functionally confirm that the correct gene was isolated in the map based cloning of the wheat *Lr21* leaf rust R-gene (Cloutier et al. 2007). The CP, encoded by βa of BSMV RNA β, is disposable for systemic infection (Petty and Jackson, 1990, Lawrence and Jackson, 2001) and deleting this gene from the BSMV vector reportedly enhanced both viral symptoms and RNA silencing in barley (Holzberg et al. 2002). Scofield et al. (2005) found that this BSMV CP deletion vector brought about increased necrosis in barley and did not show any advantages to increase RNA silencing in wheat, and the wild type BSMV vector was used for VIGS in wheat (Scofield et al. 2005). In this study pearl millet was infected with the coat protein deletion mutant (BSMVΔCP) using wheat as positive control. Wheat cv. Tugela seedlings infected with the BSMVΔCP:Pds construct developed white streaking on leaf three and four from 10 days after infection. The white areas of these seedlings were comparable to norflurazon treated wheat, except that the symptoms did not penetrate the entire laminar surface as it did with the chemical Pds inhibition. Streaking was not observed in the wheat cv. Tugela seedlings infected with the BSMVΔCP:GFP control construct. These results indicate that the streaking observed in the BSMVΔCP:Pds infected wheat cv. Tugela was photobleaching likely to have been caused by *Pds* silencing and not by viral infection. Norflurazon-induced photobleaching symptoms have been reported to penetrate the entire laminar surface in barley, whereas BSMV mediated *Pds*-silencing occurred in a mosaic pattern or stripes in the majority of barley leaf blades (Holzberg et al. 2002). The areas of *Pds* silencing in barley resembled the norflurazon-induced photobleaching of leaves and not the necrosis or desiccation typical of BSMV infection (Holzberg et al. 2002). Similar results were observed

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where BSMVΔCP:Pds infected wheat cv. Tugela seedlings were devoid of typical BSMV symptoms, supporting that these symptoms (Figure 3.12, A) were the result of *Pds*-silencing and not of viral infection. The development of a BSMVΔCP VIGS construct containing a pearl millet *Pds* gene fragment was therefore successful and the experimental procedures and environmental conditions were conducive for VIGS to occur. Furthermore, virus replication and movement of the BSMVΔCP:Pds and BSVMΔCP:GFP constructs in wheat hosts were established by RT-PCR amplification of virus specific products in systemically infected wheat cv. Tugela leaves. Pearl millet seedlings of two different breeding lines ICML12 and ICMB96222 were infected with the BSMVΔCP:Pds and BSMVΔCP:GFP constructs. No phenotypic distinction could be made between these infected seedlings and uninfected control seedlings, nor could any symptoms similar to norflurazon treatment be observed. BSMV-targeted RT-PCR amplification confirmed that BSMV did not systemically infect these pearl millet seedlings. The BSMV vector tested is therefore suitable to conduct gene silencing in wheat, as previously reported, but not in the two pearl millet lines evaluated here.

A BMV VIGS vector has been developed and applied in barley, rice and maize plants (Ding et al. 2006). BMV has since been used to conduct functional characterisation of genes involved in corn/corn smut interactions (van der Linde et al. 2011). As discussed earlier, homology between the pearl millet *Pds* nucleotide sequence in the BMV:Pds construct and the barley *Pds* nucleotide sequence was insufficient to trigger PTGS and therefore photobleaching was not expected to occur in barley infected with the pearl millet BMV:Pds construct. Consequently, the dicotyledonous host *N. benthamiana,* recommended by the developers of the BMV VIGS vector, was included as positive control host in the BMV VIGS experiments. Mosaic and striping symptoms were observed in barley cv. Morex seedlings infected with either the BMV:Pds or the BMV:GFP constructs and no significant distinction could be made between these seedlings (Figure 4.9, A and B). These results indicate that the symptoms in barley cv. Morex infected with BMV:Pds were caused by the virus and are not indicative of *Pds* silencing, as expected. Mosaic patches of white tissue were observed in some, but not all, *N. benthamiana* seedlings infected with the BMV:Pds construct while all BMV:GFP infected seedlings remained asymptomatic. The inconsistency of photobleaching in *N. benthamiana* is likely to be the result of poor level of similarity (only a single stretch of more than 23 nucleotides) between the pearl millet *Pds* sequence in the BMV:Pds construct and the *N. benthamiana Pds* gene. It has also been reported that not all regions within a target gene are equally suited to initiate silencing (Reynolds et al. 2004) which could further

account for inconsistent *Pds* silencing in *N. benthamiana* with the pearl millet construct. Systemic infection of the BMV:Pds, BMV:GFP and a wild type BMV construct in both these known hosts were confirmed with RT-PCR. The wild type BMV construct was also shown to replicate in and infect two maize accessions. The BMV VIGS vectors were thus successful to infect and move systemically in barley cv. Morex, *N. benthamiana* and maize. The experimental procedures and environmental conditions therefore allowed BMV infection to take place, but molecular quantification of targeted transcripts remains to be done to conclude that VIGS of the targeted gene occurred. Pearl millet seedlings infected with the BMV constructs, BMV:GFP or BMV:Pds, remained asymptomatic and no norflurazon-like *Pds* inhibition was observed in these seedlings. RT-PCR confirmed that none of the BMV RNAs moved systemically in pearl millet. These results indicate that BMV can be used as VIGS vector in other monocotyledonous plants and *N. benthamiana*, but not in pearl millet.

The specific BMV VIGS vector used in this study, H-BMV, has been reported to induce mosaic symptoms, necrosis and stunting in rice. A chimeric BMV vector, C-BMV, which causes much milder symptoms and is therefore more suitable for VIGS, has been developed (Ding et al. 2006). Only if the H-BMV vector was successful to infect pearl millet but caused symptoms that could obscure the silencing phenotype, then one would consider testing the milder C-BMV vector. Interestingly, the H-BMV vector appeared to be more virulent in comparison to the BMV:Pds and BMV:GFP constructs in barley (Figure 4.9). The only significant difference between these viral constructs is the size thereof seeing that no other alterations were made to the viral genomes of the different vectors. Sasaki et al. (2003) have found that the total RNA levels of GFP tagged BMV were significantly lower than that of wild type BMV in *Chenopodium quinoa* protoplasts. A possible explanation for the increased virulence of wild type BMV compared to the BMV:Pds or BMV:GFP could be that wild type BMV replicates better or faster compared to the larger recombinant Pds or GFP vectors. However, the viral load in infected plants was not determined in this study to evaluate this theory.

Two methods of BMV delivery were compared in this study. An infective mixture of viral RNA transcripts was applied directly onto plants by means of physical inoculation or, alternatively, a crude sap extract made from an already infected host species was used as a source of viral inoculum. Direct infection, using RNA transcripts rather than a crude sap extract, is the recommended method of infection to conduct VIGS, since there is a lower chance for the viral vector to lose the insert during replication in the first host plant (Ding et

al. 2006). Large inserts have found to be unstable and are easily lost from recombinant viruses (Pogue et al. 2002, Avesani et al. 2007). There was no significant difference in the symptoms of barley or *N. benthamiana* infected with RNA or a crude sap extract and the two methods of infection tested yielded similar negative results in pearl millet.

This tendency of viruses to lose non-viral sequences inserted into their genomes (Pogue et al. 2002) could account for the absence of GFP fluorescence observed in either BSMV:GFP or BMV:GPF infected wheat cv. Tugela and barley cv. Morex seedlings respectively. This is a typical outcome in barley where only a few cultivars such as Black Hulless have shown GFP fluorescence in leaf 3 (I. Hein, unpublished). Protoplasts-based expression studies have shown BSMV constructs to lose large inserts such as GFP and GUS (Lawrence and Jackson, 2001). Published reports indicate that large inserts such as GFP are unstable and easily lost from the recombinant virus and optimal insert sizes have been defined for several of the established VIGS vectors (Avesani et al. 2007, Bruun-Rasmussen et al. 2007, Cakir and Tör, 2010). In BMV the maximum stable insert sizes have been determined to be approximately 400 bp (Ding et al. 2006) whereas inserts larger than 500 bp are lost or partially lost in BSMV VIGS (Cakir and Tör, 2010). *Pds* inserts used in either the BMV or BSMV vectors fell within the proposed size ranges of 250 to 350 bp in BMV (X.S. Ding, personal communication) or in BSMV (I. Hein, personal communication). A Western blot with GFP antibodies could provide a more clear indication of GFP expression in plants inoculated with the GFP expressing VIGS constructs.

The two pearl millet lines tested in this study, ICML12 and ICMB96222 were selected based on their known rust susceptibility/resistance for downstream gene characterisation studies. These two lines were developed in independent breeding programs and represent two diverse pearl millet genotypes. ICML12 was selected from bulk germplasm collected in Mali (Singh et al. 1990) whereas ICMB96222 was bred using a rust resistance source from the USA. (C.T. Hash, personal communication). In this study, no evidence of systemic BSMV or BMV movement could be found in these pearl millet breeding lines. This may be due to the fact that pearl millet does not serve as a host for these viruses. There may be many reasons for this, but two possibilities are that pearl millet either lacks a host factor necessary for susceptibility to BSMV and BMV or contains a viral resistance gene or genes which sufficiently prevent viral infection and the onset of RNA silencing. Research of the genetic elements of virus resistance in pearl millet will be valuable in the development of a virus-induced gene silencing system in this crop. De Jong and Ahlquist (1995) identified genetic

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determinants in all three BMV genomic RNAs of a specific BMV strain which are involved in expanding the host range of this virus strain to include cowpea as host. It would be valuable to evaluate the possibility of altering the genome of either of the existing monocotyledonous VIGS vectors to include pearl millet in its host range. Gene silencing phenotypes have been reported to vary in different cultivars of the same plant species (Holzberg et al. 2002, Chen et al. 2004), and it might still be possible that there is a pearl millet genotype that is susceptible to BSMV or BMV.

In conclusion, two viruses BSMV and BMV were evaluated as possible VIGS vectors for pearl millet in this study. The presence of viral RNA in inoculated plants was analysed using RT-PCR targeting viral genomic RNA. Viral RNA could not be detected in pearl millet seedlings inoculated with either BSMV or BMV which corresponded with the asymptomatic phenotypes observed in both pearl millet breeding lines infected with either the BSMV or the BMV *Pds* silencing constructs. These results indicate that neither of the viruses is hosted by pearl millet. Phenotypic *Pds* silencing and confirmation of systemic movement of the VIGS vectors were obtained in other monocotyledonous hosts using these same constructs.

5.2 Recommendations for future work

A VIGS system in pearl millet could potentially be developed by using one of the several other viruses that are documented to infect pearl millet. Some wild type viruses that infect pearl millet have been identified (Table 3) but the development of a suitable VIGS vector is a long and drawn out process. The virus has to be isolated, cloned and suitable sites to facilitate the cloning of foreign inserts have to be identified and engineered. Scofield and Nelson (2009) recently reviewed viruses, which have already been cloned, that could potentially be developed as VIGS vectors in grasses. Pearl millet is a natural host for two of these viruses, namely maize streak virus (MSV) (Mesfin et al. 1992) and wheat streak mosaic virus (WSMV) (Seifers et al. 1996). MSV, a member of *Geminiviridae* virus family, is the causal agent of maize streak disease, (Bosque-Pérez 2000). Geminiviruses are characterised by a single-stranded circular DNA genome and Gemini-vectors have been developed to conduct VIGS in *Arabidopsis, N. benthamiana* and cassava (Vanitharani et al. 2005). Many MSV strains with different degrees of virulence have been identified in maize and grass species in South Africa (Martin et al. 2001). The identification and isolation of specific viral strains that infect pearl millet with mild symptoms but with a sufficient titre to yield PTGS

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would be an important advance in developing a MSV based VIGS system in pearl millet. WSMV has been reported to infect pearl millet in Kansas in the USA (Seifers et al. 1996). A full length cDNA clone that can be used for *in vitro* transcription of this monopartite member of the *Potyvirideae* family has been developed (Choi et al. 1999) and could be used to evaluate the potential of WSMV as a pearl millet VIGS vector.

Alternatively, other methods for the functional characterisation of pearl millet genes have to be developed. The development of pearl millet TILLING is currently underway. A mutant population of the pearl millet IP144499 breeding line has been generated which can be used to analyse the phenotype of specific candidate gene mutant plants (C.T Hash, personal communication). One has to consider that numerous independent mutants will have to be evaluated since TILLING can mutate hundreds or even thousands of genes per plant.

A DNA based viral gene silencing vector has recently been developed in barley using BSMV (Meng et al. 2009). The DNA, as opposed to RNA, based VIGS vector is delivered by means of microprojectile bombardment. BSMV subunits, amplified from cDNA, were inserted into expression vectors. These vectors are able to express the BSMV genome components upon infection in the host. The expression of genes involved in powdery mildew resistance was successfully suppressed by the biolistic introduction of BSMV VIGS vector plasmids DNA into barley seedlings (Hu et al. 2009, Meng et al. 2009). Seven day old barley seedlings were bombarded with gold particles coated with a plasmid DNA of BSMV α, BSMV β, and modified BSMVγ containing the target gene fragment. Bombarded plants were subsequently infected with the powdery mildew pathogen to analyse resistance (Hu et al. 2009, Meng et al. 2009). Meng et al. (2009) reported that the biolistic-based delivery system is easier to handle and economical compared to direct RNA infection. DNA constructs are more stable than *in vitro* transcribed RNA, which increases the probability of attaining gene silencing in the host plants. DNA based VIGS would be a welcome alternative method of viral delivery if it was found that a crude sap extract from a known host works better to infect pearl millet with a VIGS vector than direct RNA inoculation. In this study neither direct RNA infection nor the sap extract were successful to introduce BMV into pearl millet, indicating that RNA stability is not the reason for the lack of infection.

Another possibility would be to silence pearl millet candidate gene homologues in maize, since these species are closely related (Devos and Gale, 1997). The pearl millet *Pds* gene fragment used in this study is 91% identical to a fragment of maize *Pds* gene and a high

percentage of similarity is also expected for other genes, especially genes with conserved functions across the species. Pearl millet gene equivalents could thus potentially be functionally characterised in maize. BMV mediated VIGS has been developed in the maize cv. Va35 where maize *Pds* silencing was observed in systemically infected leaves by 10 dpi with a H-BMV vector bearing a 86 bp maize *Pds* gene fragment (Ding et al. 2006). No distinct light yellow or white striping occurred in maize cv. Va35 infected with wild type H-BMV. Similar results were observed in this study where maize breeding lines X and Y seedlings infected with wild type H-BMV remained asymptomatic throughout the trial (Figure 4.18). All three BMV RNAs could be amplified using RT-PCR from these infected seedlings indicating that the H-BMV vector can spread systemically in the absence of any symptoms (Figure 4.20). This observation motivates the development of the H-BMV vector to be used for functional characterisation of pearl millet orthologous sequences in maize. A limitation of this approach is that it would not be suitable to characterise genes that are likely to differ between pearl millet and maize such as *R* genes that target pearl millet specific pathogens.

5.3 Concluding remarks

It has been postulated that both BSMV and BMV, which have a wide host range and have been used to conduct gene silencing in commercially important grass species, are likely to be effective VIGS vectors in other crop species (Scofield and Nelson, 2009). These two viruses were evaluated to conduct gene silencing in two pearl millet breeding lines using the phenotypic marker gene *Pds*. It was found that neither of the viruses could infect, nor induce gene silencing in pearl millet. The vectors analysed in this study were shown to systemically infect positive control monocotyledonous species and therefore the hypothesis that the existing monocotyledonous VIGS vectors, BSMV and BMV, can be transferred to conduct gene silencing in pearl millet can be discarded under the conditions tested in this study.

With the advances made in sequencing technology and the constant addition of more and more publicly available nucleotide sequence data the need for a reliable, reproducible and affordable method to functionally characterise genes in pearl millet is exceedingly urgent. Although this study did not identify a suitable VIGS vector, it does present some advances made towards the identification of a gene silencing system in pearl millet.

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Appendix
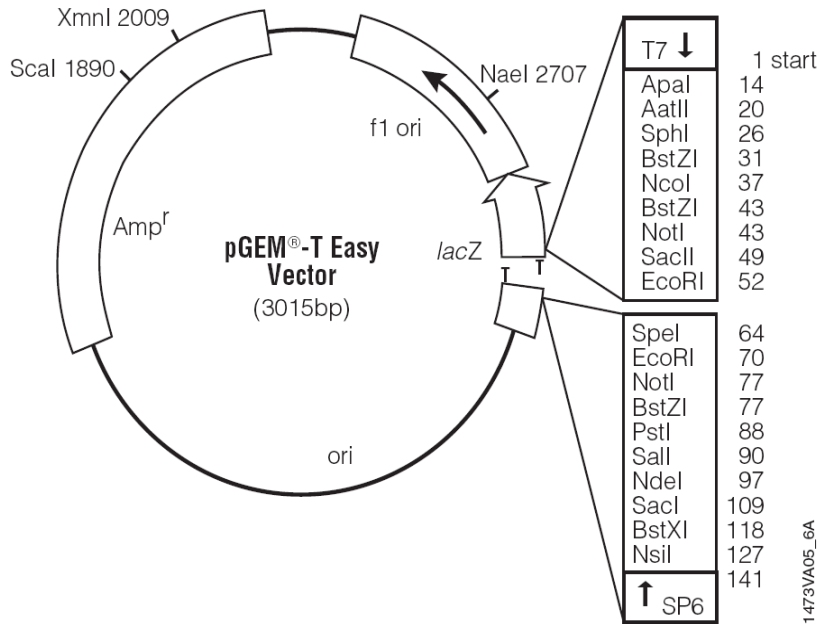
Appendix Figure 1 The pGEM®-T Easy vector (Promega) with restriction sites and relative positions is shown. Image from http://www.promega.com/tbs/tm042/tm042.pdf
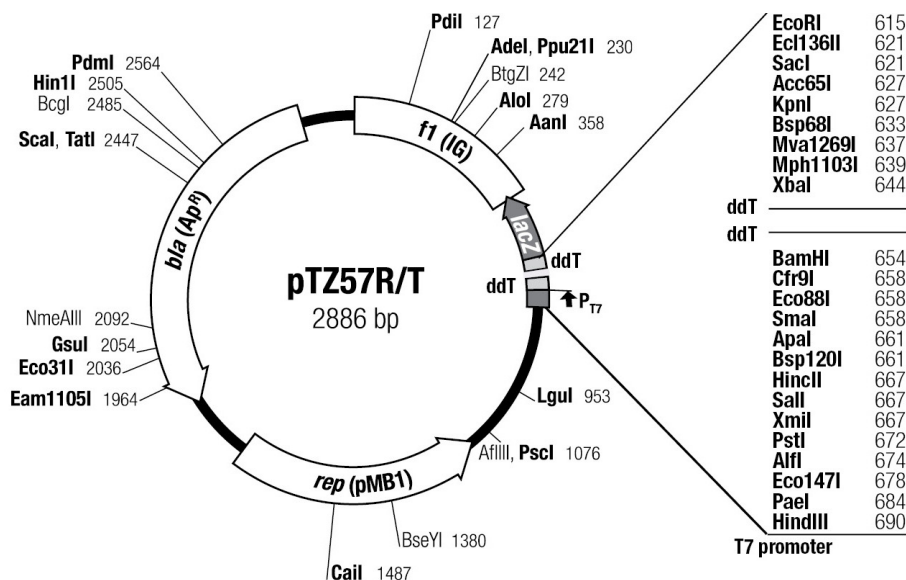
Appendix Figure 2 The InsTAclone™ PCR Cloning Vector with restriction sites and relative positions is shown. Image from http://www.fermentas.com/templates/files/tiny_mce/coa_pdf/coa_k1213.pdf

Appendix Table 1 Oligonucleotide primer sequences used in polymerase chain reactions.

Target gene	Forward primer name	Sequence (5'-3')	Reverse primer name	Sequence (5'-3')	Product size (bp)	Reference
Actin	Actin F	ACCGAAGCCCCTCTTAACCC	Actin R	GTATGGGTGACACCATCACC	170	Crampton (2006)
BMV RNA1	BMV1F	GACACGGAGCCATGAACATGAAGG	BMV1R	TCCTTCTCACGCAGTTGCTA	685	
BMV RNA2	BMV2F	GAGTGCTCAGCGAAGAGTTG	BMV2R	AGGCTCTGAGTCTTCGTTGT	707	
BMV RNA3	BMV3F	CACCAGTTCTGCGTTGGAAG	BMV3R	ACAGATGACGCGGATACAGA	784	
BSMV RNAα	BSMVαF	GATGTCACCGCAGCAATGTA	BSMVαR	TCCTTCTCACGCAGTTGCTA	668	
BSMV RNAβ	BSMVβF	AGTTCCTGGACCTGAGTTGA	BSMVβR	AGGCTCTGAGTCTTCGTTGT	764	
BSMV RNAγ	BSMVγF	CCAACGGAGGTTATGTGATG	BSMVγR	ATTCCAAGTACCGGCAAGT	664	
Pearl millet *Pds*	F2	AGCTGCTTGGAAGGATGAAG	R2	TTTGGGTCATAGTATTCCTTGC	908	
Pearl millet *Pds* AvrII/NcoI extension primers	AvrIIPdsF	GGCCTAGGTTCCTGATCGAGTGAATGA	NcoIPdsR	AACCATGGCGGTAAGTGCGAAGTGTT	301	
Pearl millet *Pds* NotI/PacI extension primers	300PdsF	aaGCGGCCGCATGGTCTAACGGTTTCAG	664PdsR	gcTTAATTAATGCCGCACAAACATAAGC	385	
Small ribosomal subunit	HvsRsF	ATGGCTTCGTCGGCTACC	HvsRsR	AAGGCGATGAAGCTGACG	471	Sasanum and Miyashita (1998)
Universal M13 vector insert	M13 +	GGTTTTCCCAGTCACGAC	M13-	AGCGGATAACAATTTCACAC	*	
Universal SP6/T7 primers	SP6	TACGATTTAGGTGACACT	T7	GTAATACGACTCACTATA	*	

* = product size depends on size of insert in cloning vector

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