An attempt to standardize VIGS based knockdown tool for floral Organ Identity Genes in *Coccinia grandis*

A thesis submitted to
Indian Institute of Science Education and Research Pune
in partial fulfilment of the requirements for the
BS-MS Dual Degree Programme

by

Shailendra Kushwaha
20101049

Under the guidance of

Dr. Anjan K. Banerjee

Associate Professor, Biology Department

Indian Institute of Science Education and Research, Pune

TABLE OF CONTENTS

TITLE **PAGE**

CERTIFICATE……………………………………………………………………….3
DECLARATION……………………………………………………………………...4
ABSTRACT…………………………………………………………………………..5
LIST OF FIGURES…………………………………………………………………..6
LIST OF TABLES……………………………………………………………………7
ACKNOWLEDGEMENT……………………………………………………………8

Chapter 1. INTRODUCTION…………………………………………………….…9
1.1 Background…………………………………………………………….…9
1.2 Virus Induced Gene Silencing (VIGS)……………………………………12
1.3 Hypothesis…………………………………………………………………..15
1.4 Objectives……………………………………………………………….....15
1.5 Approach……………………………………………………………………16
Chapter 2. MATERIALS AND METHODS……………………………………......18
2.1 Plant Material………………………………………………………..…18
2.2 Isolation of Partial *C. grandis PDS*……………………………………..…18
2.3 Plasmid Construction of *PDS* and *CgPI* silencing constructs………....18
2.4 Methodology of VIGS...…….20
2.5 RNA Isolation and RT-PCR analysis……………………………………...22
Chapter 3. RESULTS AND DISCUSSION…………………………………………..23
3.1 Transient GUS expression achieved in *C. grandis* by agroinfiltration………………………………………………………...23
3.2 pTRV1+ pTRV2-*NtPDS* VIGS vectors silenced *PDS* gene in *Nicotiana benthamiana*…………………………………….....24
3.3 Virus Induced Gene Silencing (VIGS) using pTRV1+ pTRV2-*NtPDS* in *C. grandis* resulted in yellowing of leaves…………..24
3.4 Cloning of *CgPDS A* and *CgPDS B* in pTRV2…………………………..26
3.5 Cloning of OIG fragments in pTRV2 for performing VIGS……………..30
Chapter 4.SUMMARY………………………………………………………………34
Chapter 4. REFERENCES…………………………………………………………35

भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान, पुणे
INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH (IISER) PUNE
(An Autonomous Institution of Ministry of Human Resource Development, Govt. of India)
Dr. Homi Bhabha Road, Pune - 411 008.

Certificate

This is to certify that this dissertation entitled "**An attempt to standardize VIGS based knockdown tool for floral Organ Identity Genes in *Coccinia grandis***" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents original research carried out by **Shailendra Kushwaha** at IISER Pune under the supervision of **Dr. Anjan K. Banerjee**, Associate Professor, Biology Department, IISER-Pune during the academic year 2014-2015.

Signature of supervisor

(Dr. Anjan K. Banerjee)

Date: 25 March 2015

Associate Professor
Indian Institute of Science
Education & Research (IISER)
PUNE - 411008

Declaration

I hereby declare that the matter embodied in the report entitled "**An attempt to standardize VIGS based knockdown tool for floral Organ Identity Genes in *Coccinia grandis***" are the results of the investigations carried out by me at the Biology Department of IISER-Pune, under the supervision of **Dr. Anjan K. Banerjee** and the same has not been submitted elsewhere for any other degree.

Signature of Student

Date: 25 March 2015

(Shailendra Kushwaha)

Abstract

Various forms of sexual systems are found among angiosperms and evolution of dioecy is one of the most extreme changes in the course of plant development. *Coccinia grandis*, a dioecious species belonging to Cucurbitaceae family was characterized by our lab as a good model system to study factors involved in sex expression in dioecious flowering plants. To elucidate the function of Organ Identity Genes (OIGs) or any other candidate genes in sex expression, we need a genetic transformation system for *C. grandis* which is currently unavailable. In this study a frequently used transient silencing technique named Virus Induced Gene Silencing (VIGS) was standardized for *C. grandis.* Viral vectors derived from Tobacco Rattle Virus (TRV), a bipartite, positive-strand RNA virus were used to silence phytoene desaturase (*PDS*) whose disruption shows a photobleaching phenotype. At the beginning of the experiments, the available vector containing *Nicotiana tabacum PDS* (*NtPDS)* was used for silencing which showed a very low silencing efficiency. To improve on this, a 224 base pair (bp) native *C. grandis* PDS sequence was isolated and cloned into the MCS region of TRV vector. Photobleaching phenotype was seen as yellowing of leaves starting 2 weeks post infiltration. Molecular analysis using semi-quantitative RT-PCR showed lower levels of *PDS* in infected plants compared to that in uninfected plants. Hence, VIGS technique could be used as a fast and an efficient gene knockdown tool in *C. grandis*. Concurrently, three fragments of *C. grandis PISTILLATA (CgPI)*, a B class gene were chosen for knockdown studies. These fragments were amplified from RNA extracted from the flowers and cloned individually into the TRV vector and infiltrated into the plants. Further work is under progress to study the effects of *CgPI* silencing.

LIST OF FIGURES

FIGURE **PAGE**

1. Flower structure of *C. grandis*…………………………………………………….......10

2. Model for evolution of dioecy………………………………………………………..11

3. Map of TRV based VIGS vectors……………………………….........................13

4. Mechanism of VIGS…………………………………………………………………...14

5. Overview of VIGS protocol in *C. grandis*……………………………………………22

6. GUS Assay carried out for leaf tissues infiltrated with *Agrobacterium*………......23

7. PDS silencing in agroinfiltrated *N. benthamiana* plants……………….................24

8. *C. grandis* plants treated with pTRV2-*NtPDS*………………………………..….25

9. VIGS effects on *PDS* levels in a mature *C. grandis* plant………………………..26

10. Confirmation of *CgPDS A* and *CgPDS B* cloned in pTRV2……………………...27

11. pTRV2-*CgPDS A* and pTRV2- *CgPDS B* silenced C. grandis plants………....28

12. Semi-quantitative analysis of *PDS*-silencing……………………………….....29

13. Pictorial representation of full length *CgPI*……………………………………..30

14. Confirmation of *CgPI A* and *CgPI B* and *CgPI C* cloned in pTRV2……….….31

15. TRV movement checked in pTRV2- *CgPI B* treated plants…………………....32

16. *CgPI C* silencing affected pollen development………………………….........33

LIST OF TABLES

TABLE **PAGE**

1. Description of all the primers used in this study…………………………………..19

2. Details of VIGS experiments……………………………………………………………27

Acknowledgements

I would like to thank my research advisor Dr. Anjan K. Banerjee for his constant support and critical suggestions on how to carry out my project in an intelligent way. He taught me how to always keep the big picture in mind and keep working towards it. Without his guidance and support my project would not have been possible.

Further, I would like to thank Ravi Devani, the PhD student with whom I used to discuss my day to day progress regarding the project work. His knowledge about the field and experience with the various techniques was very useful to me.

Next, I would like to thank all the members of PMB lab at IISER Pune. They were always very helpful and a cheerful lot of people.

Last but not the least I would like to thank my parents who have supported me at all paths of my life and my sister who has asked me so many questions about the project.

1. Introduction

1.1) Background

Among flower bearing plants referred to as angiosperms a majority are hermaphrodites i.e. produce bisexual flowers. About 6% of angiosperms produce male and female flowers on different individuals (Charlesworth, 2002; Renner et al., 1995), these are called dioecious plants. Various other sexual systems having combinations of unisexual and hermaphrodite flowers exist in nature. Dioecy has evolved independently and multiple times (Ainsworth & Wollaston, 1998; Guttman and Chalesworth, 1998). The mechanism of sex determination has been studied in dioecious plant species like *Silene latifolia* (Scutt et. al, 1997), *Rumex acitosa* (Ainsworth, 2000; Parker, 1995) and *Poplar* (Yin et al., 2008), each having different systems for sex determination involving sex chromosomes. Also in some dioecious plants, sex chromosomes are absent and sex is determined by a Mendelian segregation system. For example in squirting cucumber (*Ecballium elaterium*) sex determination is by segregation at one locus, and at multiple loci in annual mercury (*Mercurialis annua*).

Coccinia grandis is a dioecious species belonging to Cucurbitaceae family. *Coccinia* genus includes 27 species all of which are dioecious (Holstein and Renner, 2011). It has heteromorphic chromosomes and the chromosome composition in males is 22+XY and in females is 22+XX (Kumar et al., 1952). Y chromosome is conspicuously large and involved in sexual dimorphism (Kumar et al., 1952; Chakravorti, 1947; Bhaduri and Bose, 1947), but the genes involved in sex determination are still unknown. In addition to two sexual forms - male and female, an additional gynomonoecious form (GyM) was identified having hermaphrodite (GyM-H) and female flowers (GyM-F) on the same individual with chromosome composition 22+XX, same as that of a female plant (Ghadge et al., 2014). The presence of this form provides more dimensions to the study of sex determination in *C. grandis*.

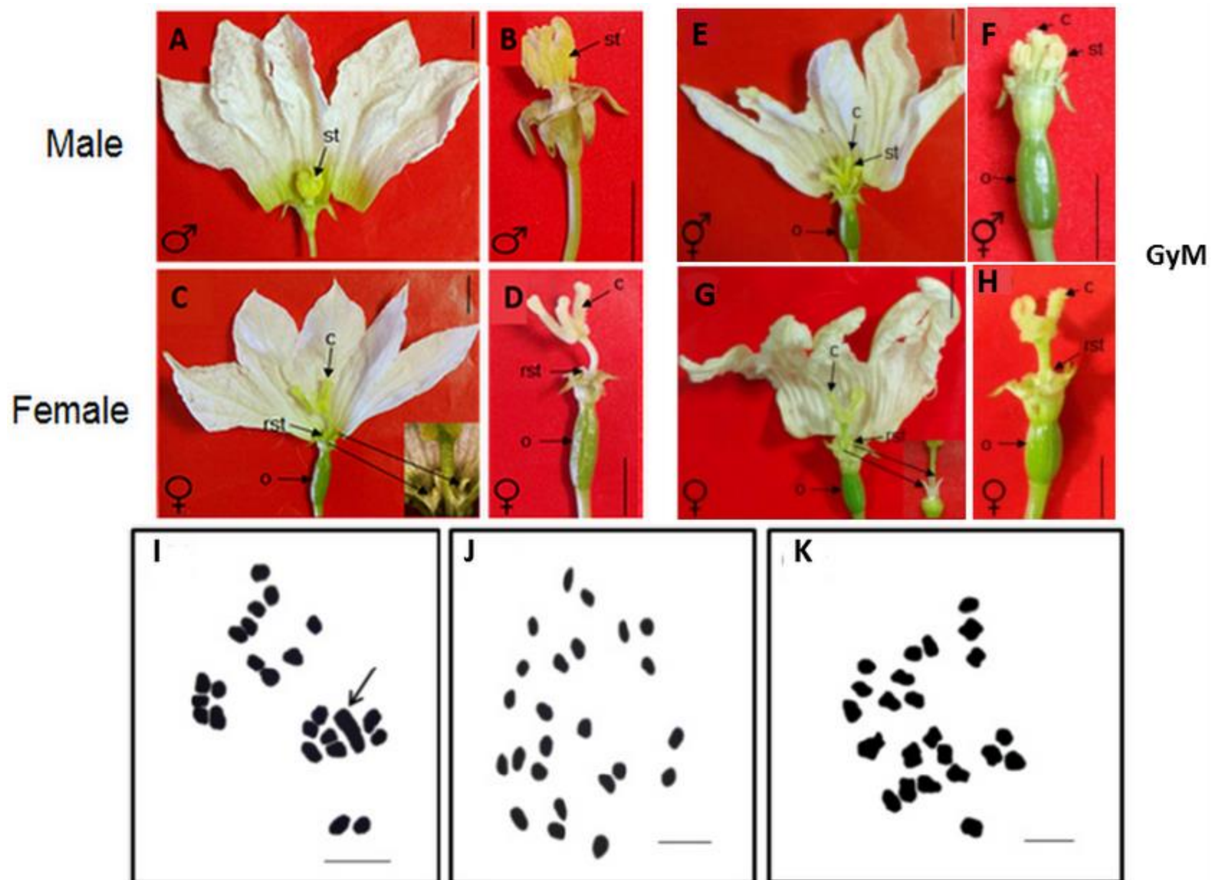


Fig. 1. Flower structure of *C. grandis* male A, B; female C, D; and gynomonoecius plant's hermaphrodite E,F; female G,H. Chromosome composition at Metaphase of male I, female J and gynomonoecious K, (adapted from Ghadge et al. 2014).

Organ Identity genes (OIGs) involved in development and identity of floral organs surely affects sex of the flower and might have a role to play in sex determination in dioecious species. Earlier in our lab, expression analysis of *Coccinia grandis PISTILATA* (*CgPI*), a B class OIG and *AGAMOUS* (*CgAG*) a C class OIG was carried out in early, middle and late staged flower buds of male and female flowers. The study found that *CgPI* expression is considerably high in male flower in all the early, middle and late stages of development in comparison to females, while there was a difference in expression of *CgAG* in early stages only, similar differences were found in stamen specific expression of *CgPI* and *CgAG* (Ghadge et al., 2014).

This differential expression could be a primary reason causing sex determination or a secondary outcome of organ degeneration. In *Coccinia grandis,* the male flowers completely lack carpels and hence OIGs might be involved in sex determination as organ initiation rather than organ maturation is regulated here. In contrast

unisexuality is achieved in female flowers by organ suppression since there is presence of rudimentary stamens and OIGs might not be directly involved in sex determination.

In the evolution of dioecy, the initial step in sexual dimorphism is mutations causing 'female supressing' functions in Y chromosome (M->m), next step is a female sterility mutation (Su^{f} -> Su^{Female}) (Lewis, 1942; Ross, 1978). There should be tight linkage between them to prevent appearance of neuters and hermaphrodites via recombination. In a hermaphrodite population, the number of plants with male sterility mutations (female plants) will increase if comparatively its seed production is equal or more. This will result in a gynodioecious population (Fig. 2). Male bias in hermaphrodite flowers will increase, eventually leading to female sterility and male individuals (Charlesworth and Beatrice, 2013).

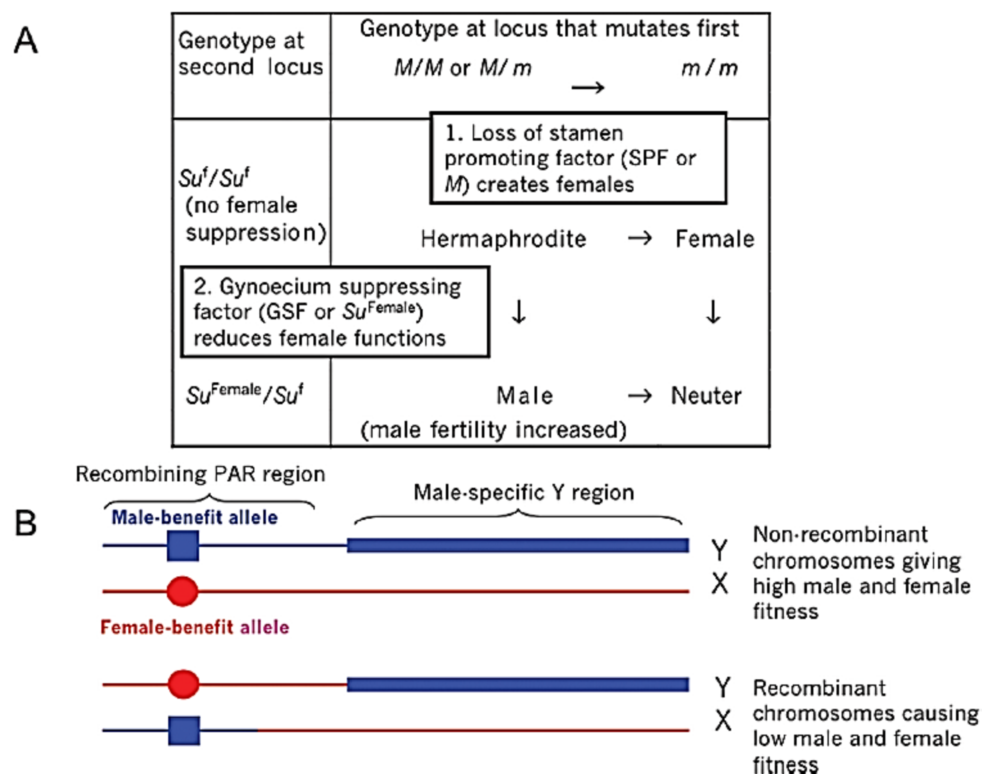


Fig. 2. Evolution of dioecy by segregation of two mutations leading to selection for reduced recombination. (A) In a population, male-female sterility mutations are not fixed because they cause sterility of each-other. (B) In the recombining region close to the Male determining region of Y, polymorphisms can be established by a sexually antagonistic mutation that improves male fitness but impedes female fitness. (Reproduced from Charlesworth and Beatrice, 2013)

To test if any of the B or C class genes acts as a factor in sex determination/ involved in sexual transitions, functional studies of these OIGs need to be carried out. Transformation techniques are currently unavailable for this species and various attempts at it previously had yielded no results. Thus, a transient silencing technique named Virus Induced Gene Silencing (VIGS) was adapted and standardized in *C. grandis*.

1.2) Virus Induced Gene Silencing (VIGS)

Virus Induced Gene Silencing (VIGS) is frequently used in various species to downregulate the target plant genes .We aimed to standardize VIGS in *C. grandis* in order to use it as a reverse genetics approach for studying role of candidate OIGs, *CgPI* and *CgAG* in sex determination in this species.

VIGS is one of the techniques which uses Post Transcriptional Gene Silencing (PTGS) to degrade sequence specific double-stranded RNAs (dsRNAs). The observation of recovery from a viral infection first suggested that PTGS was adopted by the plants as a defence mechanism against viruses (Ratcliff et al. 1997 ; Lindbo et al., 1993; Soosaar et al., 2005). This led to the idea that recombinant viruses carrying the plant genes can be used for knockdown of endogenous plant genes. This transient silencing technique termed as Virus Induced Gene Silencing (VIGS) has been widely used in many plants to study the functions of various genes. Several plant viruses such as tobacco rattle virus (TRV), potato virus-X (PVX), apple latent spherical virus (ASLV), tobacco mosaic virus (TMV), barley stripe mosaic virus (BSMV), tomato golden mosaic virus (TGMV) have been modified to carry out VIGS. But Tobacco rattle Virus (TRV) is most widely used due to its advantages like broader host range, moderate to low viral symptoms and hence better characterization of the silencing phenotype. Also, TRV infects meristem tissue which is useful in studies related to floral modification as is in my case. By literature survey it was found that TRV has not been used to carry out VIGS in Cucubitaceae before and another virus named Apple Latent Spherical Virus (ALSV) was shown to work in cucumber (Igarashi et al., 2009). But due to my requirement of studying downregulation effects on floral phenotypes, TRV which infects meristem tissue was chosen to be used as VIGS vector.

Viral vectors developed from Tobacco Rattle Virus (TRV), a bipartite virus having positive strand RNA with two genomes TRV1 and TRV2 were used here. TRV1 contains viral RNA dependent RNA polymerase (RdRp), Movement Protein (MP), a 16kDa Cysteine rich protein (16k) and a self-cleaving ribozyme (Rz). TRV2 contains the viral Coat protein (CP) and a Multiple Cloning Site (MCS), (Hayward et al., 2011) (Fig. 3). The endogenous gene sequence to be silenced was cloned into the MCS region of pTRV2 vector and the cassette was transformed into *Agrobacterium* which in turn was used to transfer this vector into the plant using agroinfiltration. *Agrobacterium* harbouring pTRV2 vector with cloned gene to be silenced has to be infiltrated along with pTRV1 for the silencing to work since the original virus has two genomes, each having different function to cause viral infection. Plant defence machinery against this modified virus targets and reduces the endogenous RNA transcript levels along with the viral transcripts leading to silencing of the desired gene.

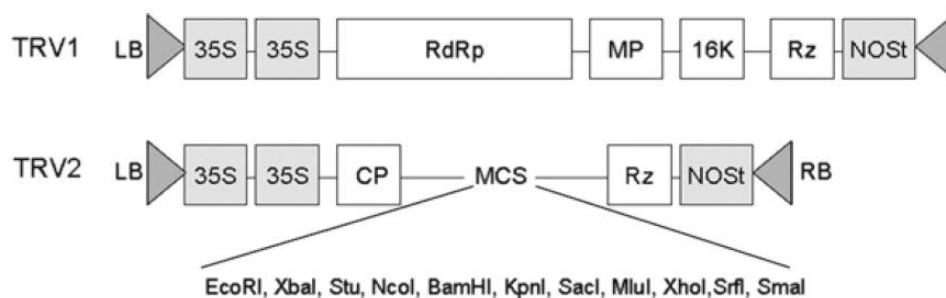


Fig. 3. Map of TRV based VIGS vectors. The target sequence is inserted between duplicated 35S promoters and Nopaline synthase terminator (NOSt) within pTRV2-MCS. TRV1 contains viral RNA dependent RNA polymerase (RdRp), Movement Protein (MP), a 16kDa Cysteine rich protein (16k) and a self-cleaving ribozyme (Rz). TRV2 contains the viral Coat protein (CP) and a Multiple Cloning Site (MCS). LB and RB are left and right borders of the TDNA. (Reproduced from- Hayward *et al*., 2011)

1.2.1) Mechanism of Virus Induced Gene Silencing (VIGS)

When virus attacks, the plant specifically targets the viral genome using RNAi mediated defense. If a viral vector carrying the genes from the plant is used, the defense mechanism of the plant will target the corresponding plant's mRNA and the respective gene will be silenced. For this, *Agrobacterium tumefaciens* is used which transfers its Ti plasmid carrying the plant gene fragment targeted for silencing integrated with the viral sequence. The virus spreads, and with it the silencing. When virus replicates, double stranded RNA (dsRNA) corresponding to the target gene are produced. The ribonuclease DICER cleaves these dsRNAs into siRNAs of 21 to 24 nucleotides in length. These siRNAs guide the RNA-induced silencing complex (RISC) to degrade the target mRNA (Becker and Lange, 1995).

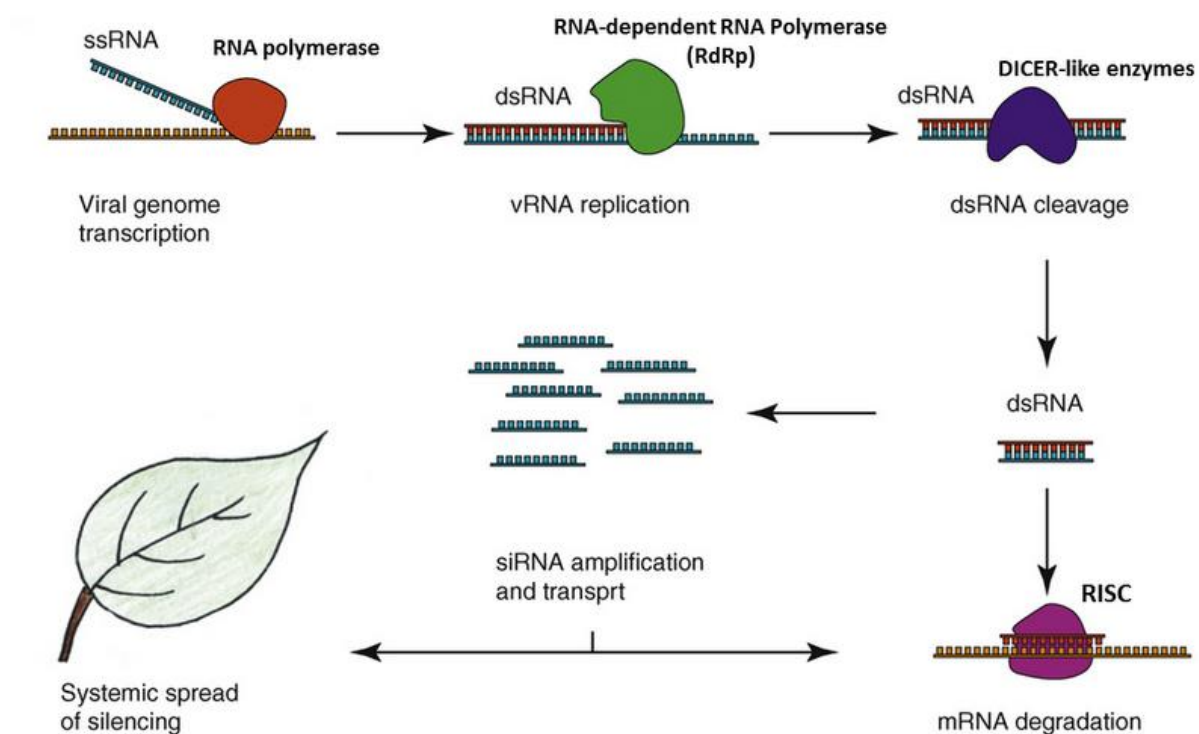


Fig. 4. Mechanism of VIGS. Upon infection, the *Agrobacterium* transfers Ti Plasmid containing Viral sequences along with plant gene into the plant genome. The sequence is transcribed using plant's RNA polymerase (red). RNA-dependent RNA Polymerase (green) produces dsRNA from the ssRNA viral transcript. DICER-like enzymes (blue) then recognizes dsRNA and cleave them into short interfering RNAs (siRNA). siRNAs are subsequently recognized by RISC (purple) which converts them into ssRNAs which are further used as template degradation of target gene. siRNAs acts as mobile signalling

molecules which are amplified and spread to distant parts of the plant leading to silencing phenotype at the whole plant level. (Adapted from Becker & Lange 1995).

VIGS has been earlier used in other dioecious species like spinach (Sather et al., 2010) and *Thalictrum diocum* (Larue et al., 2013) to study the function of OIGs in sex determination. In spinach, it has been shown that sexual dimorphism occurs through regulation of B-class genes. Sather *et al.* used *pWSRi* (plasmid Wayne State RNAi) vector constructed from Beet Curly Top Virus (BCTV) for silencing of OIGs. The constructs were transferred to plants via biolistic bombardment using coated tungsten particles. In *Thalictrum* similar to my study TRV vectors were used for silencing of *Thalictrum PISTILLATA (ThPI).* Sexual conversion of stamens into carpel was observed which suggested that homeosis is a viable model for dioecy in this species.

1.3) Hypothesis

- Assuming that *C. grandis* will carry out PTGS of endogeneous gene transcripts (which is cloned into the pTRV2 vector), upon infection with pTRV2 vector carrying *PDS* sequence, a photobleaching phenotype of leaves should be seen as whitening or yellowing of leaves.

- If silencing of *C. grandis PISTILLATA / AGAMOUS* causes sexual modifications along with expected homeotic transformations in flowers, then their possible role in sex determination could be deciphered.

1.4) Objectives

1. Transient expression of 35S-GUS marker in cotyledons and leaves of *Coccinia grandis* by agroinfiltration.

2. To confirm that the VIGS vectors pTRV1, pTRV2-*NtPDS* and pTRV2-*AtPDS* work efficiently in *Nicotiana benthamiana*

3. To check whether above mentioned TRV based VIGS vectors would function in *Coccinia grandis*.

4. To isolate and clone phytoene desaturase (*PDS)* from *Coccinia grandis* into pTRV2 vector to improve photobleaching in *C. grandis*.

5. Cloning of different fragments of Organ Identity Genes, *C. grandis PISTILLATA (PI)* and *AGAMOUS (AG)* in pTRV2 for conducting VIGS.

1.5) Approach

1. Transient expression of 35S-GUS marker in cotyledons and leaves of *Coccinia grandis* by agroinfiltration.

- First and foremost it was checked whether *Agrobacterium* would transfer its Ti plasmid in *C. grandis* which is a prerequisite for VIGS to work. *Agrobacterium* harbouring pBI121 vector having GUS under 35S promoter was used to agroinfiltrate *C. grandis* leaves and later GUS assay of infected leaves was carried out.

2. To confirm that the VIGS vectors pTRV1, pTRV2-*NtPDS* and pTRV2-*AtPDS* work efficiently in *Nicotiana benthamiana*.

- To standardize VIGS, the *C. grandis* plants belonging to different ages were agroinfiltrated using the VIGS protocol (with certain modifications) designed for *Nicotiana benthamiana* (Senthil-kumar and Mysore, 2014). pTRV1 and pTRV2-*NtPDS* vectors were used for this purpose which were ordered from The Arabidopsis Resource Centre (TAIR) and confirmed to work in *Nicotiana benthamiana.*

3. To check whether above mentioned TRV based VIGS vectors work in *Coccinia grandis*.

- pTRV2- *NtPDS* was used to carry out VIGS in *C. grandis*. It is logical that a *PDS* sequence of *Nicotiana benthamiana* (a distant species) would not yield good silencing efficiency. For better silencing efficiency, *PDS* sequence of *C. grandis* should be used for silencing.

4. To isolate and clone phytoene desaturase (*PDS)* from *Coccinia grandis* into pTRV2 vector to improve photobleaching in *C. grandis*.

- Degenerate primers were designed using closely related species and using them a partial *CgPDS* fragment was isolated. This fragment was further cloned into the TRV vector and used for carrying out VIGS experiment.

- Molecular analysis of silencing by checking endogenous *PDS* levels using Semiquantitative Reverse Transcription PCR (RT-PCR) from infected plant's RNA was the next step.

5. Cloning of different fragments of Organ Identity Genes, *C. grandis PISTILLATA (PI)* and *AGAMOUS (AG)* in pTRV2 for conducting VIGS.

- Once established, constructs of *CgPI* containing different regions of the genes were prepared and used to carry out knockdown studies. However, cloning of *CgAG* fragments is still in progress.

2) Materials and Methods

2.1) Plant Material

C. grandis plants were seed grown in pots and kept in greenhouse at long day (16h day, 8 h night) conditions. Plants aged from 2 weeks to 5 months were used for the study. Temperature kept was 20°C-22°C. For molecular analysis tissues were collected in liquid nitrogen and stored in -80°C freezer for future use.

2.2) Isolation of partial *C. grandis* PDS

Due to unavailability of *C. grandis* genome, *PDS* sequence of *Cucumis sativus* and *Cucumis melo*, close relatives of *C. grandis* were used to design primers which were used to amplify a 224 base pair partial *C. grandis PDS* from RNA extracted from *C. grandis* leaves. Several attempts were made to obtain full length *CgPDS* by 5' and 3' RACE but were unsuccessful.

2.3) Plasmid Construction of *PDS* and *CgPI* silencing constructs

pTRV1 and pTRV2 vectors are described in (Liu et al., 2002). pTRV2-*AtPDS* vector (Burch-smith et al., 2006) ordered from TAIR was cut using the restriction enzymes *EcoRI-XbaI* to remove the *AtPDS* insert. *EcoRI* and *XbaI* restriction sites were added to the primers designed for amplification of sequences to be cloned. cDNA from wild type *C. grandis* was used to amplify *CgPDS A*, *CgPDS B*, *CgPI A, CgPI B and CgPI C.* The PCR amplification was performed using *PfuTurbo* DNA polymerase. For the cloning of above mentioned PCR products, digestion of these PCR products using *EcoRI* and *XbaI* restriction enzymes was carried out. The digested product was ligated into *EcoRI-XbaI* cut pTRV2 vector using T4 DNA Ligase (NEB) using manufacturer's protocol.

Table 1. Description of all the primers used in this study.

Purpose	Primer Name	Sequence 5'-3'	Product size (bp)
Cloning *CgPDS A* in pTRV2	CgPDSA F	TGTGAATTCCATCTCCTTCAATTACATTC	224
	CgPDSA R	GATTCTAGATATTAACCCCGATGAACTTTCT	
Cloning *CgPDS B* in pTRV2	CgPDSB F	TGTGAATTCCTGTATCCTTGAATTAAGTC	154
	CgPDSB R	GTCTCTAGAATGCATTTTGATTGCTTTGAA	
Cloning *CgPI A* in pTRV2	CgPI A F	CCTGAATTCCTTCTACACCTTTGATTGACATCTTG	219
	CgPI A R	CCTTCTAGAGTGAGGCAGCCATTAACTCCTTG	
Cloning *CgPI B* in pTRV2	CgPI B F	CCTGAATTCATAGTGTGAGAGAAATGGATAATG	251
	CgPI B R	CCTTCTAGACATAACATAACACATAACCCAAAG	
Cloning *CgPI C* in pTRV2	CgPI C F	ATGCTCGAATTCAGAGGGAAGATTGAAATAAAG	630
	CgPI C R	ATGCCTTCTAGAATTAATTCTTTCTTGTAGATTTG	
Check TRV movement	TRV CP F	CTGGGTTACTAGCGGCACTGAATA	409
	TRV CP R	TCCACCAAACTTAATCCCGAATAC	
PDS cDNA synthesis	Sq PDS R	CCTTCCATAGAAGCTAAATAC	

2.4) Methodology of VIGS (adapted from Senthil-kumar and Mysore, 2014)

2.4.1) Overview

Agrobacterium tumefaciens having pTRV1 and pTRV2-GOI (Gene of Interest) were grown separately overnight and transferred to 10 ml media for secondary culture. The culture was grown to an appropriate optical density (OD). Acetosyringone was used to induce *Agrobacterium* Virulence (Vir) genes essential for delivery of T-DNA carrying virus into the plant genome. *Agrobacterium* strains carrying pTRV1 and pTRV2-GOI were mixed in 1:1 ratio and infiltrated into 3-4 leaves of the plant using a needleless syringe. Silencing was seen 2-3 weeks post infiltration. Maintenance of VIGS for longer periods was achieved by giving booster dosages. A schematic overview of the procedure is shown in Fig. 5.

2.4.2) Reagents Used

LB medium: LB powder is added to distilled water according to supplier's specifications and autoclaved. Stored at room temperature.

Acetosyringone: 200mM stock prepared by adding 39.24mg acetosyringone powder to 1ml DMSO. Stored at -20°C.

***Agrobacterium* induction buffer**: 10mM MES solution prepared by adding 0.195g in 100ml water. 200µl of acetosyringone (from 200mM stock) is added. pH adjusted to 5.5. ~10ml is required for 1 construct. Prepared freshly for each experiment.

Infiltration buffer: 5mM MES solution prepared by adding 0.0976g in 100ml. pH adjusted to 5.5.

2.4.3) Detailed Procedure

1. Single *Agrobacterium* colonies of both pTRV1 and pTRV2-GOI (GOI- Gene of Interest) were inoculated separately in 2 ml LB liquid medium containing rifampicin (10 µg/ml) and kanamycin (50 µg/ml). Incubated in 28 °C shaker at 200-250 r.p.m. for 10-14 h.

2. 0.5 ml of this primary culture was subcultured into 10 ml LB liquid medium containing rifampicin (10 µg/ml) and kanamycin (50 µg/ml). Incubated in 28 °C shaker at 200-250 r.p.m. for 5-6 h until OD_{600} (Optical density measured at 600 nm wavelength) of 0.5-0.6 is reached.

3. Cultures were centrifuged at 3,500 g for 5 min at room temperature.

4. Supernatant was discarded and 10 ml Agrobacterium induction buffer was used to resuspend the cells. Cultures were incubated at room temperature for 3 h in a shaker at ~50 r.p.m..

5. Cultures were centrifuged at 3,500g for 5 min and cells were resuspended independently in ~5-7 ml induction buffer depending on the final OD required. OD_{600} ranging from 0.4 - 0.7 was used in this study.

6. pTRV1 and pTRV2 cultures were mixed in 1:1 (vol/vol) ratio. Infiltration by needleless syringe (Syringe inoculation) after pricking a small dot in the leaves using the needle was the method used to infiltrate the pTRV1+pTRV2-GOI culture into the leaves.

7. Plants were maintained at 20-22 °C (optimal temperature for VIGS) in a greenhouse at long day conditions (16 h light 8 h dark).

8. Silencing phenotypes were checked starting from 10 days post infiltration. Phenotypes sometimes appeared after 2-3 weeks of inoculation.

9. For molecular validation of gene silencing, tissues were collected in liquid nitrogen and stored in -80 °C for future use.

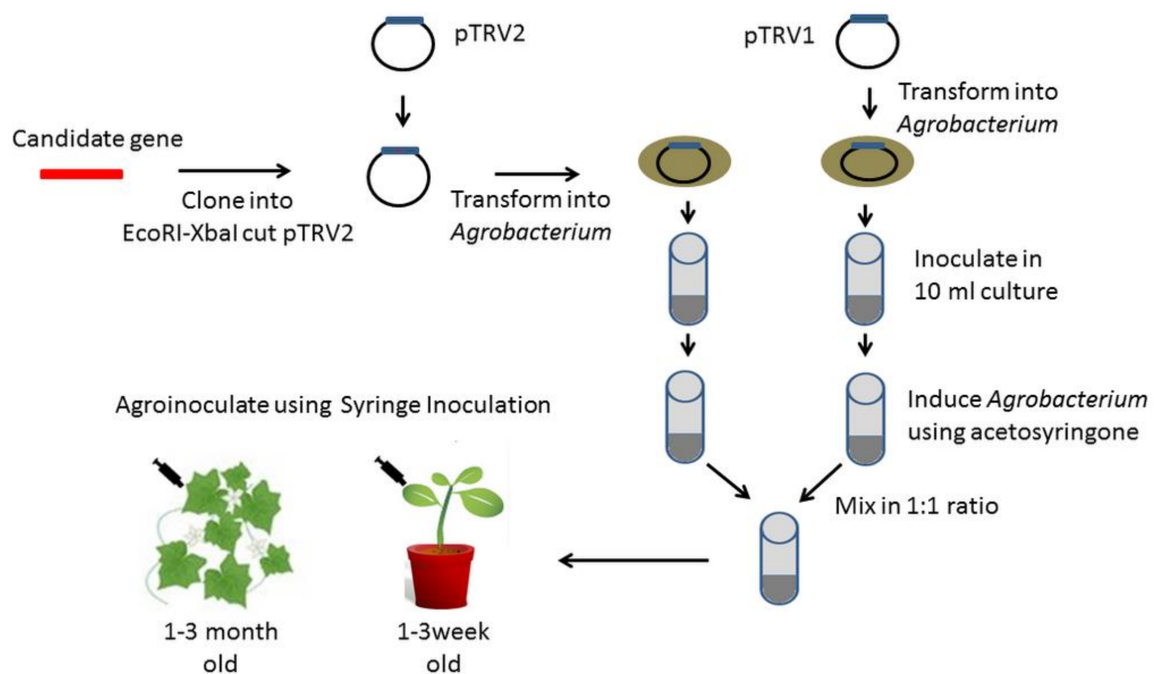


Fig. 5. Overview of VIGS protocol in *C. grandis*.

2.5) RNA Isolation and RT-PCR analysis

Total RNA was isolated from the pooled tissue or a single leaf depending on the requirement of the analysis. RNA isolation was done using TRIzol RNA isolation protocol followed by RNase-free DNase treatment (TURBO DNA-free kit, Ambion). First strand c-DNA was synthesized using 1 µg or 2 µg RNA depending on the concentration of all samples, using reverse primers for PDS, TRV Coat protein (TRV CP) and Actin using SuperScript reverse transcriptase III (Invitrogen) according to supplier's protocol. Cycling conditions were 1h at 55°C and 15 min at 70°C. For RT-PCR of *PDS*, primers used were outside the region that was cloned into pTRV2 vector.

2.6) Microscopy

Leica S8APO light microscope was used for taking magnified images of stamens.

2.6) Semiquantitative analysis using histograms

Histograms depicting semiquantitative analysis were made using ImageJ software.

3. Results and Discussion

3.1) Transient GUS expression achieved in *C. grandis* by agroinfiltration

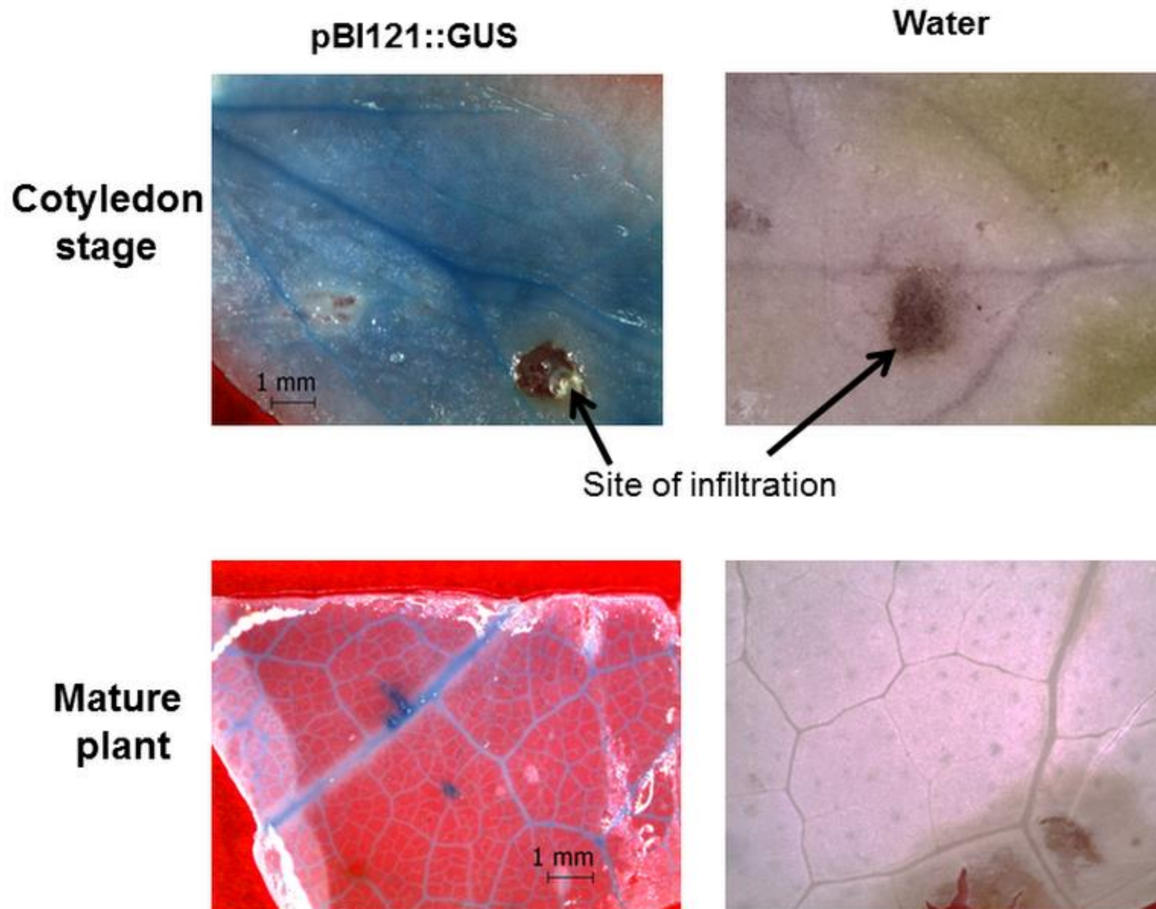


Fig. 6. GUS Assay carried out for leaf tissues infiltrated with *Agrobacterium* having pBI121 vector with GUS under 35S promoter. Blue staining shows transient GUS expression in infiltrated leaves. GUS staining was done 3 days post-infiltration.

Successful GUS staining shows that *Agrobacterium* can be used to transfer VIGS silencing construct into the plant. Hence, agroinfiltration method of transferring target gene clubbed with viral genome could be used for initiating Virus Induced Gene Silencing (VIGS) in *C. grandis*. Next we checked whether the ordered vectors pTRV2-*NtPDS* and pTRV2-*AtPDS* and pTRV1 work in *N. benthamiana* (species in which these vectors have been used to carry out VIGS before).

3.2) pTRV1+ pTRV2-*NtPDS* VIGS vectors silenced *PDS* gene in *Nicotiana benthamiana*

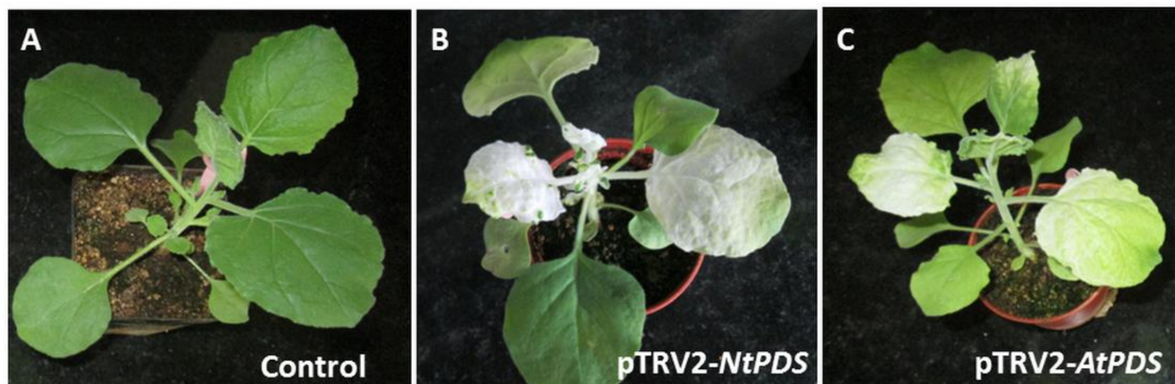
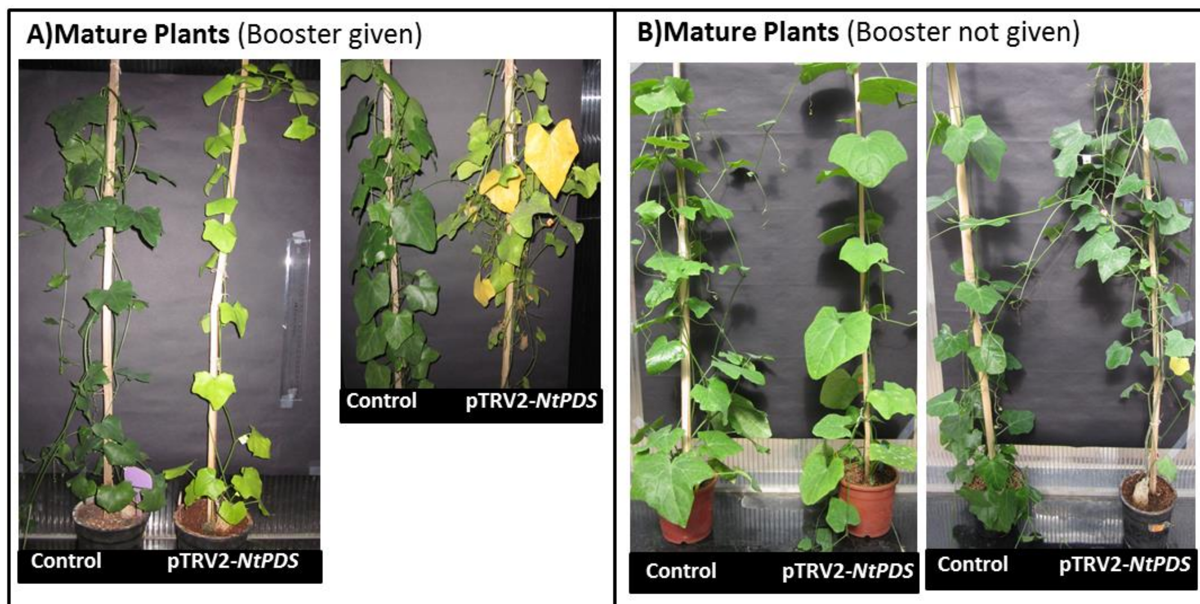


Fig. 7. *PDS* silencing seen as photobleaching phenotype in agroinfiltrated *N. benthamiana* plants. A. Wild–type plant. B. Plant agroinfiltrated with constructs pTRV1 + pTRV2-*NtPDS*. C. Plant agroinfiltrated with constructs pTRV1 + pTRV2-*AtPDS. PDS* silencing seen as photobleaching phenotype.

VIGS vectors were confirmed to be functional in *N. benthamiana*. pTRV2-*NtPDS* treated *N. benthamiana* plants started showing photobleaching phenotype after around 10 days post-infiltration. pTRV2-*AtPDS* treated plants also showed similar phenotype. pTRV2-*NtPDS* was then used for infiltration in *C. grandis* using the same protocol used for *N. benthamiana* plants

3.3) Virus Induced Gene Silencing (VIGS) using pTRV1+ pTRV2-*NtPDS* in *C. grandis* resulted in yellowing of leaves



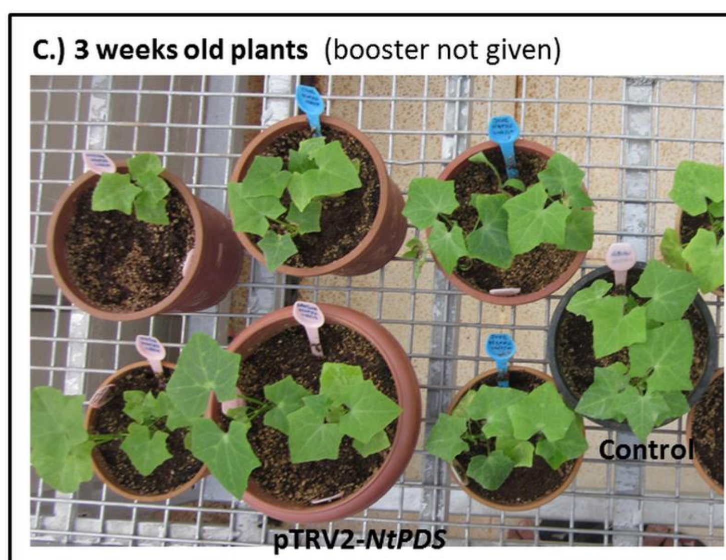


Fig. 8. In *C. grandis* plants treated with pTRV2-*NtPDS*, photobleaching phenotype was seen at very low frequency in both mature plants (2-3 months old) (B) as well as in 3 week old plants (C). When booster dosage was given to the treated mature plants 3- 4 weeks post first infiltration, better silencing was achieved (A).

pTRV2- *NtPDS* silenced *C. grandis* plants did not show good silencing even after 3 weeks post infiltration. When booster dosage was given to the mature plants 3-4 weeks after first infiltration, a better silencing phenotype as a yellowing of leaves was observed in some plants. Although, not all the plants which were given booster dosage showed this phenotype. To verify that the silencing phenotype was indeed due to silencing of *PDS* and that the virus was spreading the silencing, total RNA

was isolated from infiltrated and a non-infiltrated leaf from a single plant. Using Reverse Transcription PCR (RT-PCR) levels of *PDS* and TRV Coat Protein (TRV CP) as a proxy of viral movement were checked in Infiltrated and Non-Infiltrated leaves of *C. grandis*. *Actin* was used as a control.(Fig.6). Primers to check *PDS* levels were outside the fragment that was used for silencing.

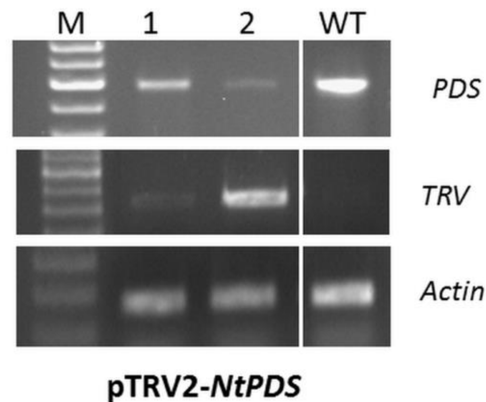


Fig. 9. VIGS effects on *PDS* levels in a mature *C. grandis* plant in which a booster dose was given 3 weeks post first treatment. Lane 1-Infiltrated leaf, Lane 2- Leaf nearby to Infiltrated leaf. *PDS* levels in both the leaves of infiltrated plants were lower than that of the non-treated plant. M-Ladder.

RT-PCR results showed that the *PDS* levels in both Infiltrated and Non-Infiltrated leaves were lower than that of un-treated plant. Also, TRV CP was detected in non-infiltrated leaf which confirmed viral movement. *PDS* levels were lower in Infiltrated leaf than in Non-Infiltrated leaf. This result showed that VIGS was working in *C. grandis.* But the silencing efficiency was low and a booster dosage was needed for better results. Hence, to get a better silencing efficiency *PDS* sequence from *C. grandis* was isolated and cloned into pTRV2 vector and used for conducting VIGS experiments.

3.4) Cloning of *CgPDS A* and *CgPDS B* into pTRV2 and performing VIGS

Due to unavailability of *C. grandis* genome, degenerate primers were prepared from *PDS* regions of *Cucumis sativus* and *Cucumis melo,* two closely related species to *C. grandis*. Using these degenerate primers a 224 bp *PDS* region was amplified from *C. grandis* cDNA. However we were unable to amplify longer sequences. This partial

C. grandis sequence was cloned into the pGEMT vector and sequence confirmed. Two fragments, one 224 bp and another 154 bp sequence were amplified and cloned into pTRV2 vector and used for VIGS experiments.

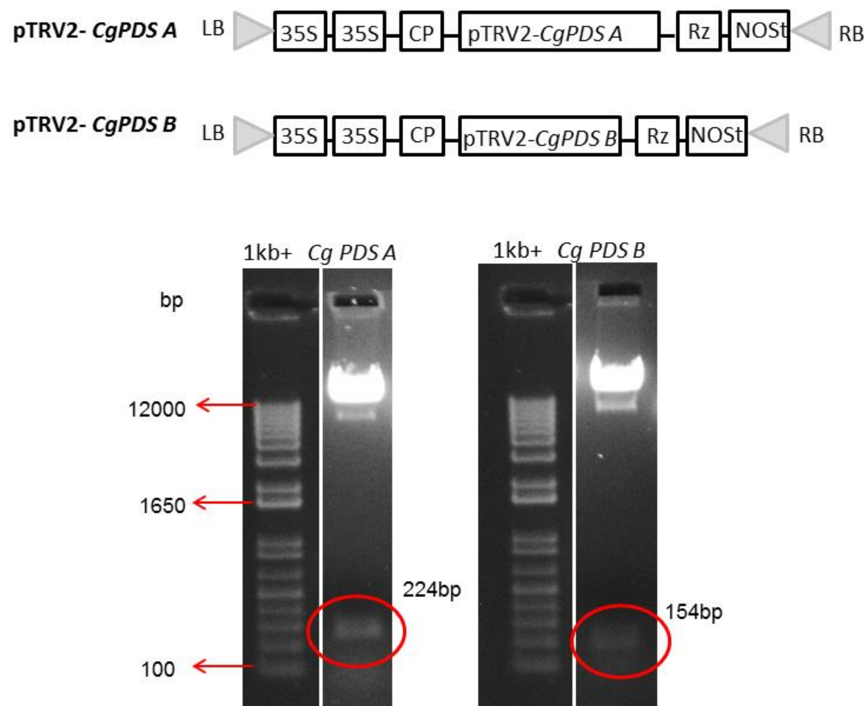


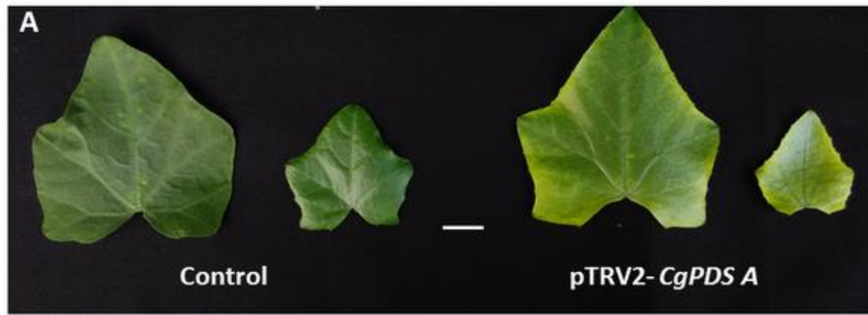
Fig. 10. Confirmation of *CgPDS A* and *CgPDS B* cloned in pTRV2 by restriction digestion using *EcoRI* and *XbaI.*

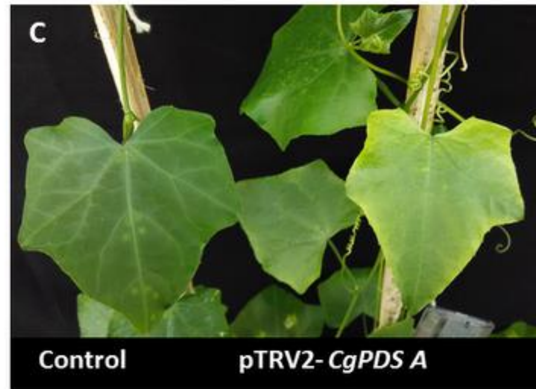
VIGS was carried out using pTRV2-*CgPDS A* and pTRV2- *CgPDS B* in a total of 29 plants of which 10 plants showed observable silencing to varying degrees. The following table summarizes the results.

Table 2. Details of VIGS experiments.

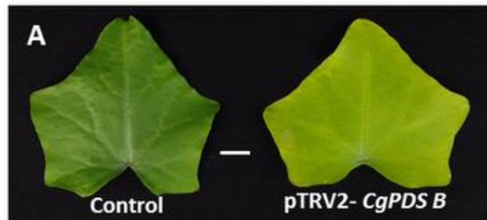
Construct	**Age of Plant**	**No. of plants Infiltrated**	**Plants showing photo bleaching phenotype**
pTRV2-*CgPDS A*	2-3 week old	10	4
	1-4 month old	4	2
pTRV2-*CgPDS B*	2-3 week old	11	2
	1-4 month old	4	2

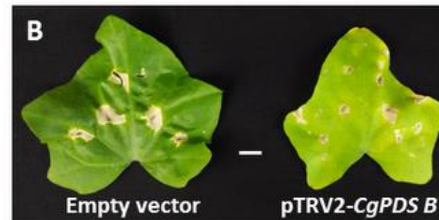
(1)





(2)





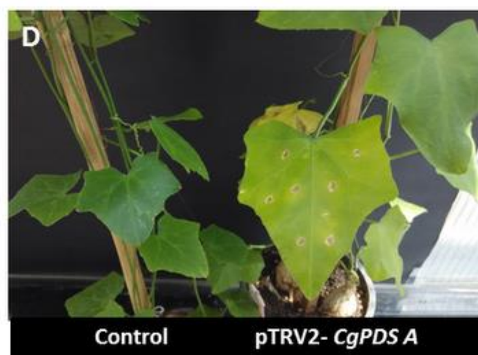
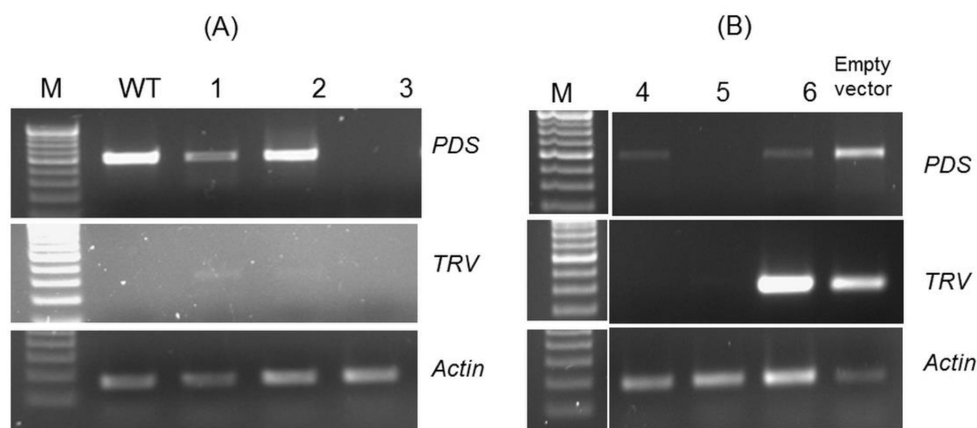


Fig. 11. pTRV2-*CgPDS A* and pTRV2- *CgPDS B* silenced *C. grandis* plants. Silencing phenotype seen as yellowing of leaves in (1) Plants aged 2-3 weeks when infiltrated and (2) Plants aged 1-4 months when infiltrated.1.A). Comparison of upper two leaves of a wild type control and treated plant. 1.B) differences at whole plant level and (1.C) zoomed in image of (1.B). 2. A comparion between a wild type control leaf and a leaf from a treated plant. 2.B comparison between a pTRV2-Empty vector treated leaf with pTRV2-*CgPDS B* treated leaf. (Scale-1cm)

To confirm that silencing is due to downregulation of *PDS* levels, RNA was isolated from pooled tissue of individual plants and semiquantitative RT-PCR to check the levels of *PDS* and TRV CP was carried out. Primers used to check *PDS* levels were outside the fragment that was used for silencing to prevent amplification from viral transcripts.



pTRV2-*CgPDS A* and pTRV2-*CgPDS B*

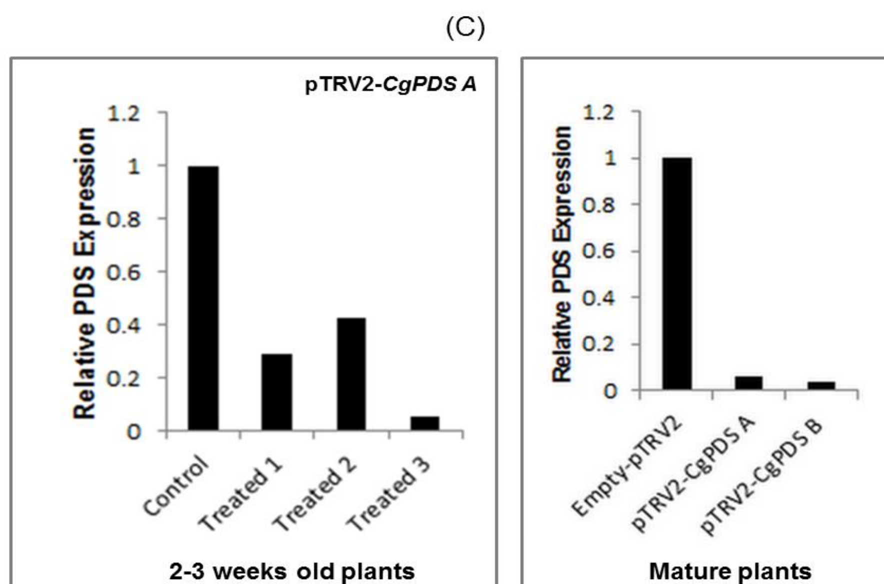


Fig. 12. Semi-quantitative analysis of *PDS*-silencing in (A) 2-3 week old plants when treated, (B) several month old plant when treated. (A) Lanes 1, 2, 3 show *PDS* and TRV levels in three biological replicates treated with pTRV2-*CgPDS A*. (B) Lane 4 – pTRV2- *CgPDS A* treated mature plant. Lane 5 and 6- pTRV2-*CgPDS B* treated mature plant, Lane 7- *PDS* and TRV CP levels in plant treated with pTRV2-empty vector. (C) Relative *PDS* expression represented as histograms of both (A) and (B) showing differences in *PDS* levels between WT and treated plants (using ImageJ 1.48v software).

From the semiquantitative analysis, out of 6 plants checked 3 of them showed clear downregulation in *PDS* levels. (Fig. 8 (A) Lane 1 and (B) Lane 4 and 6). TRV was also detected in these samples. From the silencing phenotype observed and RT-PCR analysis it could be concluded that VIGS protocol works in *C. grandis.* However the levels of *PDS* will also be confirmed by using a more sensitive Real Time qRT-PCR technique.

3.5) Cloning of OIG fragments in pTRV2 and performing VIGS

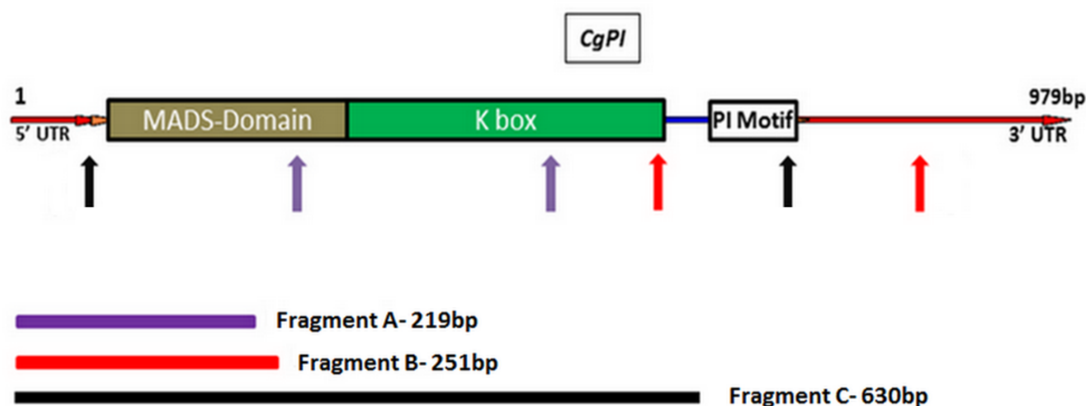


Fig. 13. Pictorial representation of full length *CgPI* (979bp) with conserved regions. Fragment A, B and C refers to three different fragments from *CgPI* amplified and cloned into pTRV2 for performing VIGS.

To carry out VIGS of *C. grandis PISTILLATA* (*CgPI*), a B class Organ Identity Gene three fragments of *CgPI* ; *CgPI A* (219 bp), *CgPI B* (251 bp) and *CgPI C* (630 bp)

were amplified from cDNA of wild type flower and cloned into pTRV2 vector. Fragment A and B were sequences unique to the *CgPI* whereas fragment C included all the conserved regions. The fragments were designed this way in order to look for all the phenotypes that may appear by silencing targeted at unique and conserved regions.

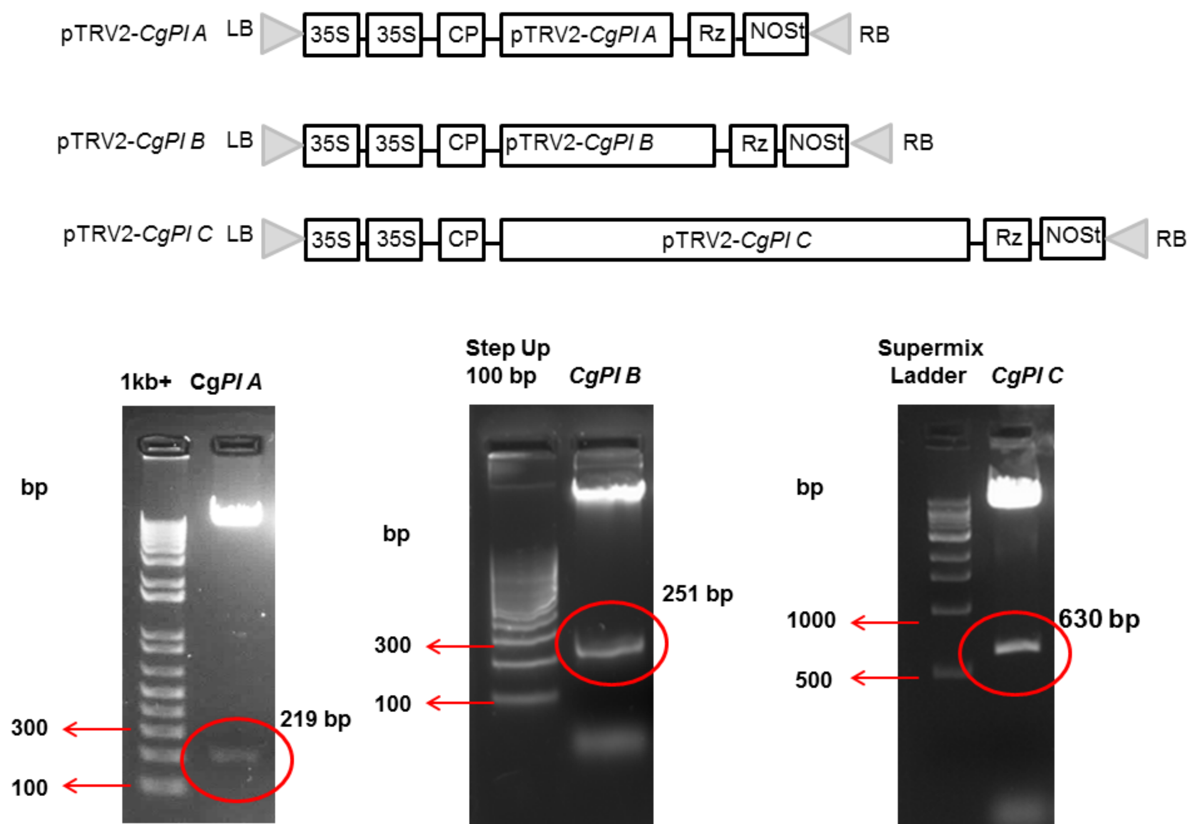


Fig.14. Confirmation of *CgPI A* and *CgPI B* and *CgPI C* cloned in pTRV2 by restriction digestion using *EcoRI* and *XbaI.*

A total of 20 plants were infiltrated using these three fragments of *CgPI* cloned into pTRV2. Unfortunately, none of the plants were flowering. One of the reasons might be lower temperature of the greenhouse (20°C- 22°C) where VIGS was performed and where plants were grown. However, TRV movement was confirmed in the pTRV2-*CgPI B* treated plants. TRV was detected in the very young leaves distant to the infiltrated leaf. This result showed that virus movement was taking place and the construct designed was working in *C. grandis* (Fig. 15).

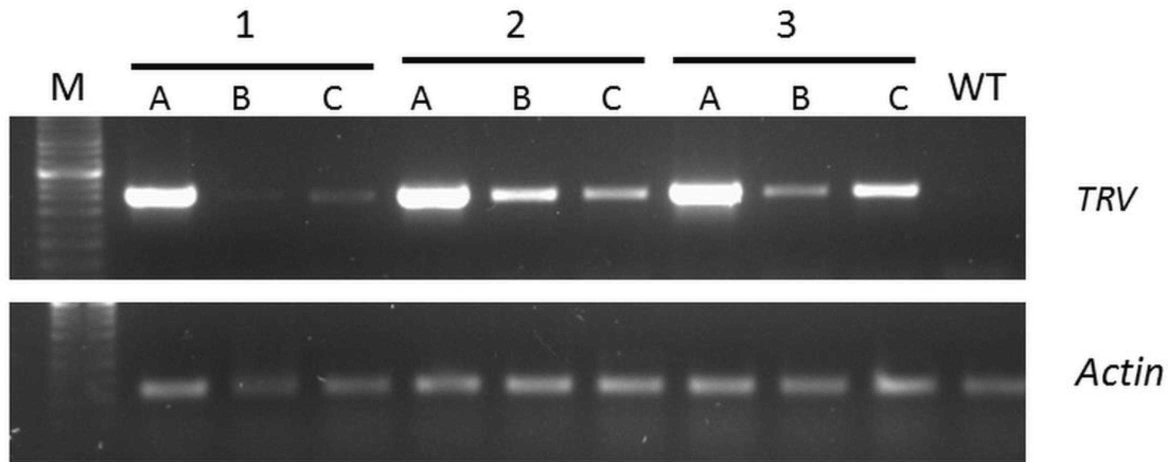


Fig.15 TRV movement was checked in three plants 1, 2 and 3 treated with pTRV2- *CgPI B* in A- infiltrated leaf, B- leaf next to infiltrated leaf and C- Distant leaf.

The low temperature was set because VIGS was shown to work better in lower temperature in other species. However, flowering in wild type *C. grandis* was observed to be most prominent during rainy season. Some of the plants were kept outside the greenhouse to induce flowering conditions. In one of the mature plants (3 months old) kept outside the greenhouse, flower buds started to appear. Agroinfiltration using the construct pTRV2- *CgPI C* was carried out in this plant. The plant was kept in the greenhouse for a week in lower temperature and then was transferred outside for flowering to occur.

VIGS has to be established before floral initiation for viable phenotypes to occur. Hence, a booster dosage was given to the plant 3 weeks after first infiltration and again after keeping it one week in greenhouse, the plant was transferred outside. The flowers which appeared next were observed under microscope for any phenotypic changes.

Fig.16 *CgPI C* silencing affected pollen development in one male stamen. A. Stamen with normal pollen development in an untreated plant. B1. A part of stamen showing hindered pollen growth. B2. Remaining part of the same stamen showing normal pollen development.

The phenotype looks like a chimeric silencing since only some part of the stamen had no pollen development. In *Arabidopsis* (a hermaphrodite), *PISTILLATA* knockdown caused stamens to retain their identity but pollen development was completely hindered (Wuest et al., 2012). In order to draw any conclusions regarding the function of *CgPI* in *C. grandis,* silencing phenotypes in more flowers and more individuals needs to be checked and molecular analysis by checking the levels *CgPI* needs to be carried out.

VIGS has proven to be a reliable technique for knockdown of genes in *C. grandis.* Only about one-third of the total number of plants treated with *PDS* silencing vector showed photobleaching phenotype, this could be because a short fragment of *CgPDS* was used for silencing. In the absence of any other transformation technique, VIGS protocol could be used as a great tool to do functional genomics in *C. grandis.* Along with deciphering the functions of *CgPI*, and *CgAG* in sex determination, functions of any other genes could be studied using this tool. With the availability of GyM plant this technique adds many more dimensions to the sex expression studies in this species.

Summary

1. Transient GUS expression was achieved in *C. grandis* by infiltration using *Agrobacterium* having pBI121 vector with GUS gene under 35S promoter. This indicates that *Agrobacterium* can be used to perform VIGS in *C. grandis.*

2. pTRV2-*NtPDS* vector used for VIGS in *C. grandis* showed very low silencing efficiency. Even after a booster dosage only some proportion of the plants showed good photobleaching phenotype.

3. To improve the silencing efficiency, a 224 bp partial *C. grandis PDS* was isolated from *C. grandis* cDNA using degenerate primers. Two fragments were prepared using this sequence and cloned into pTRV2 vector.

4. About one-third of the plants treated with these vectors resulted in a good photobleaching phenotype, where the bigger fragment (pTRV2-*CgPDS A*) seemed to work better.

5. Semi-quantitative RT-PCR analysis confirmed that photobleaching was due to lowering of *PDS* levels in these treated plants.

6. The next step was to check the effects of *PISTILLATA* silencing in *C. grandis* flowers. For this, three fragments of *CgPI* were amplified and cloned into pTRV2 vector to perform VIGS.

7. Several plants were treated with these *CgPI* silencing constructs. In one of the male flowers treated with pTRV2-*CgPI C* disruption of pollen development on the stamen was observed.

8. Further work is in progress.

References

Ainsworth, C. (2000). Boys and Girls Come Out to Play: The Molecular Biology of Dioecious Plants. Ann. Bot. *86*, 211–221.

Ainsworth, C., and Wollaston, V.B.- (1998). Sex Determination in Plants. *38*.

Becker, A., and Lange, M. (1995). VIGS – genomics goes functional. 1–4.

Bhaduri, P. N., & Bose, P. C. (1947). Cyto-genetical investigations in some common cucurbits, with special reference to fragmentation of chromosomes as physical basis of speciation. J Genet, 48(2), 237-256.

Burch-smith, T.M., Schiff, M., and Liu, Y. (2006). Efficient Virus-Induced Gene Silencing in Arabidopsis 1. *142*, 21–27.

Chakravorti, A. K. (1948, March). Cytology ofCoccinia Indica W. & A. with reference to the behaviour of its sex-chromosomes. In Proceedings of the Indian Academy of Sciences-Section B (Vol. 27, No. 3, pp. 74-86). Springer India.

Charlesworth, D. (2002). Plant sex determination and sex chromosomes. 94–101.

Charlesworth, D., and Beatrice, M. (2013). sex chromosome evolution induces changes in DNA In Posidonia oceanica cadmium methylation and chromatin patterning. *64*, 405–420.

Ghadge, A.G., Karmakar, K., Devani, R.S., Banerjee, J., Mohanasundaram, B., Sinha, R.K., Sinha, S., and Banerjee, A.K. (2014). Flower development , pollen fertility and sex expression analyses of three sexual phenotypes of Coccinia grandis. 1–15.

Holstein, N., and Renner, S.S. (2011). A dated phylogeny and collection records reveal repeated biome shifts in the African genus Coccinia (Cucurbitaceae). BMC Evol. Biol. *11*, 28.

Guttman, D. S., & Charlesworth, D. (1998). An X-linked gene with a degenerate Y-linked homologue in a dioecious plant. Nature, 393(6682), 263-266.

Igarashi, A., Yamagata, K., Sugai, T., Takahashi, Y., and Sugawara, E. (2009). Apple latent spherical virus vectors for reliable and effective virus-induced gene silencing among a broad range of plants including tobacco , tomato , Arabidopsis thaliana , cucurbits , and legumes. Virology *386*, 407–416.

Kumar, L. S. S., & Viseveshwaraiah, S. (1952). Sex mechanism in Coccinia indica Wight and Arn.

Larue, N.C., Sullivan, A.M., and Stilio, V.S. Di (2013). Functional recapitulation of transitions in sexual systems by homeosis during the evolution of dioecy in Thalictrum. *4*, 1–10.

Lindbo, J.A., Silva-rosales, L., Proebsting, W.M., and Dougherty, W.G. (1993). Induction of a Highly Specific Antiviral State in Transgenic Plants : Implications for Regulation of Gene Expression and Virus Resistance. *5*, 1749–1759.

Liu, Y., Schiff, M., and Marathe, R. (2002). Tobacco Rar1 , EDS1 and NPR1 / NIM1 like genes are required for N -mediated resistance to tobacco mosaic virus. *30*.

Parker, J. (1995). Male and Female Flowers of the Dioecious Plant Sorrel Show Different Patterns of MADS Box Gene Expression. *7*, 1583–1598.

Ratcliff, F., Harrison, B. D., & Baulcombe, D. C. (1997). A similarity between viral defense and gene silencing in plants. Science, 276(5318), 1558-1560.

Renner, S.S., Ricklefs, R.E., American, S., May, N., Renner, S.S., and Ricklefs, E. (2011). DIOECY AND ITS CORRELATES IN THE FLOWERING PLANTS groups. *82*, 596–606.

Sather, D.N., Jovanovic, M., and Golenberg, E.M. (2010). Functional analysis of B and C class floral organ genes in spinach demonstrates their role in sexual dimorphism.

Scutt, C. P., Li, Y., Robertson, S. E., Willis, M. E., & Gilmartin, P. M. (1997). Sex Determination in Dioecious Silene latifolia (Effects of the Y Chromosome and the Parasitic Smut Fungus (Ustilago violacea) on Gene Expression during Flower Development). Plant Physiology, 114(3), 969-979.

Senthil-kumar, M., and Mysore, K.S. (2014). Tobacco rattle virus – based virus-induced gene silencing in Nicotiana benthamiana. *9*, 1549–1562.

Soosaar, J.L.M., Burch-smith, T.M., and Dinesh-kumar, S.P. (2005). MECHANISMS OF PLANT RESISTANCE TO VIRUSES. *3*, 789–798.

Wuest, S.E., Maoileidigh, D.S.O., Rae, L., Kwasniewska, K., Raganelli, A., Hanczaryk, K., Lohan, A.J., Loftus, B., Graciet, E., and Wellmer, F. (2012). Molecular basis for the speci fi cation of fl oral organs by.

Yin, T., Difazio, S.P., Gunter, L.E., Zhang, X., Sewell, M.M., Woolbright, S.A., Allan, G.J., Kelleher, C.T., Douglas, C.J., Wang, M., et al. (2008). Genome structure and emerging evidence of an incipient sex chromosome in Populus. 422–430.