Cysteine protease-cystatin combination as an alternate strategy to barnase-barstar system for pollination control in plants

Thesis submitted to the University of Hyderabad for the degree of

DOCTOR OF PHILOSOPHY

By

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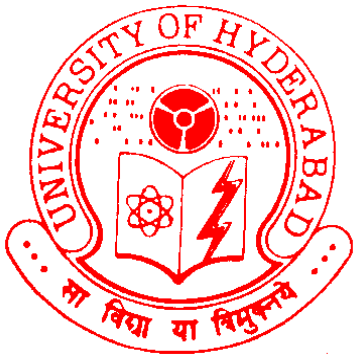

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DECLARATION

I, Pawan Shukla, hereby declare that this thesis entitled **"Cysteine protease-cystatin combination as an alternate strategy to barnase-barstar system for pollination control in plants"** submitted by me under the guidance and supervision of **Professor P.B. Kirti, Department of Plant Sciences, School of Life Sciences, University of Hyderabad** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled "**Cysteine protease-cystatin combination as an alternate strategy to barnase-barstar system for pollination control in plants**" is a bonafide work done by **Mr. Pawan Shukla**, a research scholar for Ph.D. programme in Plant Sciences, Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision. This thesis has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

Prof. P. B. Kirti
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Head
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Dedicated to My Parents

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Pawan Shukla

Abbreviations

µg	Microgram
µM	Micro Molar
µCi	Micro Curie
BAP	6-Benzylaminopurine
bp	Base pair
cDNA	Complementary DNA
CIAP	Calf Intestine Alkaline Phosphatase
CTAB	Cetyl/ Hexadecyltrimethyl Ammonium Bromide
Da	Dalton
DDW	Double distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide
dsDNA	Double stranded DNA
EDTA	Ethylene Diamine Tetra Acetic Acid
EtBr	Ethidium Bromide
hr/hrs	hour/s
kDa	Kilo Dalton
NAA	Naphthalene Acetic Acid
ng	Nanogram
nm	Nanometre
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
pmol	Picomol
PMSF	Phenyl Methane Sulphonyl Fluoride
PVDF	Polyvinylidine Fluoride
PVPP	Polyvinyl Polypyrrolidone
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SSC	Sodium Chloride Sodium Citrate
ssDNA	Single stranded DNA
SSPE	Sodium Chloride Sodium Phosphate EDTA
TAE	Tris Acetic acid EDTA
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween
TE	Tris EDTA
TEMED	N, N, N, N-Tetra Methyl Ethylene Diamine
Tris	Tris (hydroxymethyl) amino methane
β-Merc	β-mercapto ethanol

Chapter 1

Introduction

Introduction

Food security has become a major global challenge as the population is growing day by day. According to a report, on an average of an additional 73 million people are added annually and by 2050, world population is predicted to increase by 34% (FAO; 2009). Most of the additional population lives in the developing countries and nearly 1.2 billion people live in state of absolute poverty (FAO; 1996). A large proportion of the populations in developing and under-developed countries suffer from deficiency of micronutrients of iron, zinc and vitamin A (Pinstrup-Anderson and Cohen 2000). Food security and malnutrition result in a serious public health problem and loss of human potential (Sharma et al., 2002).

During the period 1950 – 1980, there was a remarkable increase in total food grain production as a result of the green revolution (Myers et al; 1999). This increment in grain production was mainly due to the increase in area under cultivation, irrigation, better agronomic practices. This had allowed food supply to keep pace with population growth. In the last few decades, the area of land for agriculture practices is decreasing consistently because of urbanization and land degradation. Continuous population growth has raised the global alarm. Recently, global economic downturn exacerbated these problems with high domestic food prices, lower incomes and increasing unemployment resulting in over one billion undernourished people worldwide (FAO; 2009). This raises the expectation of millions of people in developing countries for the demand of food, feed, fiber and fuel economic advancement for making pace with global food supply. Thus, food production must increase by 70% to feed the larger population.

To achieve this goal, there is an urgent need to increase the yield per unit area as the best agricultural lands are already in use and expanding additional lower quality areas face both environmental and resource (e.g. water) availability constraints. Since the yields of major crops have already reached a plateau, most of the productivity will have to be achieved through better natural resources management and crop improvement (Sharma et al., 2002).

To increase productivity, genetic improvement of crop plants emerges as a key input. This again opens up the door (after green revolution) to address food insecurity concerns. Genetic engineering offers a precise tool to introduce a desired gene and its related trait(s) into the high yielding and locally adapted crop cultivars. Development of hybrids using a genetic engineering approach has existed for a very long period as an effective method to increase agricultural productivity.

Heterosis results in the phenotypic superior performance of a heterozygous hybrid population over both homozygous parents involved in the cross with respect to traits such as growth rate, reproductive success, stability and yield. Hybrid vigor has been observed in most of the crops, but use of the hybrids has been limited to those crops for which there is an economically viable and effective means of pollination control. Thus commercial production of hybrids is only feasible, if a reliable and effective pollination control system is available.

Pollination control systems are procedures in plant breeding or systems adopted by a plant breeder in order to obtain desired hybrid cultivars. These systems render the pollen of one parent line non-viable (male sterile or female line) to ensure pollination by the chosen parent line. The objective is to develop a system using plant genes for pollination control

in order to facilitate hybrid crop varieties that exhibit heterosis in specific combinations resulting in wide adaptation to the environment over the years with stability of performance. Manual emasculation of male flower or flower parts is the most widely used method to avoid self-pollination, but major crops generally have small, bisexual flowers making manual emasculation impractical, laborious and expensive process.

Other pollination control systems include the use of male sterility in plants. Male sterility is defined as the inability of a plant to produce viable pollen grains retaining female fertility unaltered. In 1763, Kolreuter observed and first time documented the anther abortion within species and a specific hybrid. This phenomenon was naturally found in maternally inherited cytoplasmic male sterile (CMS) plants (Hanson, 1991). It is one of the conventional pollination control methods for hybrid seed production for example prior to the epidemic of Southern corn leaf blight in 1970; male sterile T (Texas) cytoplasm maize system was used to produce approximately 85% of hybrid maize seed in the USA. Using a CMS system, hybrid seed could be produced by developing female lines that carry a CMS cytoplasm lacking restorer genes and by developing male lines that carry the appropriate fertility restorer genes. Hence, F_1 hybrid seed carry the cms cytoplasm but yield fertile plants because of the action of male parent contributed nuclear fertility restorer genes (Patrick et al; 1998). So far, several plant species have been reported to exhibit cytoplasmic male sterility systems (Kaul, 1988) However, such effective pollination control systems were not found in most other crops used in agriculture. Also, CMS based hybrid seed production received a "black eye" after the epidemic of *Bipolaris maydis* on T-cytoplasm carrying lines of maize. This epidemic is often cited as a classic example of genetic vulnerability of major crop plants. In addition to Southern corn blight (CMS-T),

cold susceptibility (CMS Ogura) and Sorghum Ergot infection in the unfertilized stigma have been reported in CMS (Havey, 2004). Many of the CMS systems do not comprise proper restorer genes. Therefore, CMS systems are not sufficient enough to fulfill the global demand of the growing population.

In the recent past, genetic engineering approach as a pollination control method for improvement of crops emerged as a key input in increasing productivity. As a result, this biotechnological approach has gained a lot of popularity for the development of genetically engineered male sterile plants for pollination control. Several approaches, for instance using cytotoxic genes, altering metabolic process, RNA silencing method, RNA editing as a tool, by heterologous expression of CMS associated DNA, via chloroplast engineering and by developing conditional male sterile system have been used for the development of transgenic male sterile plants. However, programmed cell death related genes for the development of male sterility have been less tried.

In the present investigation, we have used a cysteine protease gene from *Arachis diogoi* for inducing male sterility in transgenic tobacco plants. Many reports suggested the critical role of cysteine proteases in programmed (Solomon et al., 1999). Currently, several reports have been documented for generating transgenic male sterile plants, but most of them lack suitable restoration system. Proper restoration system is essential for those plants where seed is the economically important product. Therefore, we have also attempted to develop a suitable restoration system by using a cysteine protease inhibitor-cystatin plants as restorer line. Hence, the thesis work involves **"Cysteine protease-cystatin combination as an alternate strategy to barnase-barstar system for pollination control in plants."**

Chapter 2

Review of Literature

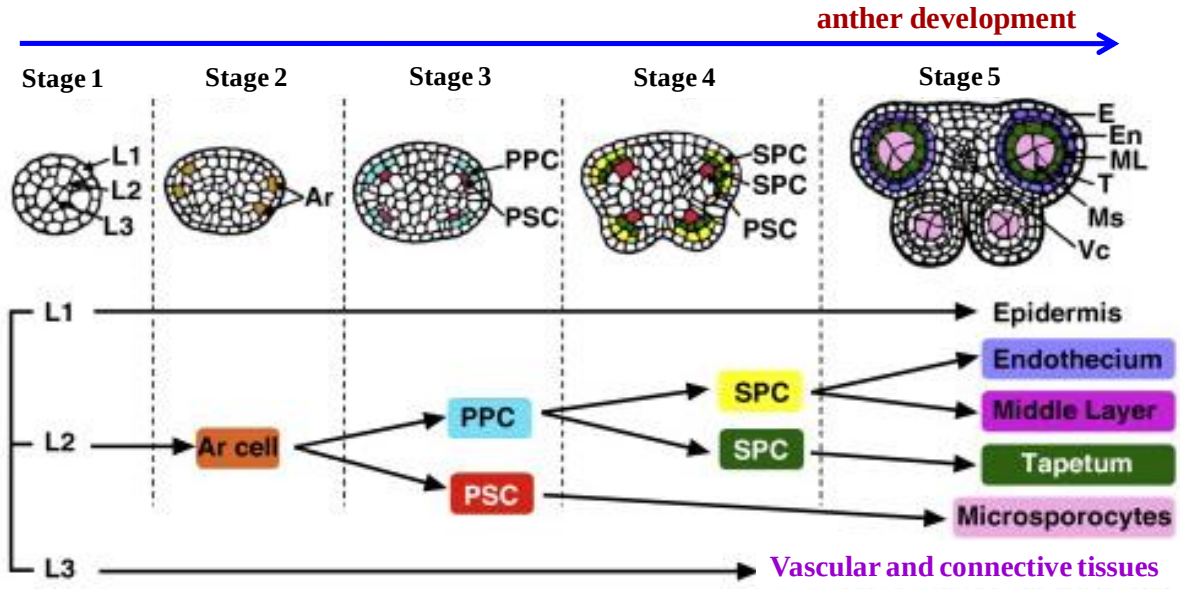
Review of Literature

From the agriculture perspective, the development of male sterile plants is one of the valuable tools in the hybrid seed industry by generating cross pollinated seeds without the need of labour intensive hand emasculation. In nature, male sterility is more prevalent than female sterility, probably because male gametophyte is less protected from environment than the female ovule and embryo sac. Male gametophyte development plays a vital role in plant fertility and crop production through the generation of male gametes. It is a very well-orchestrated and highly regulated process, in which anther plays a crucial role in male gametophyte development. Any disturbance in this process leads to male sterility.

Anther Development:

In Flowering plants, male reproductive processes require the formation of the male reproductive organ, stamen (Ma, 2005). Stamen comprises two morphologically distinct regions, the anther and the filament. Anther consists of multiple layers of specialized cell types including male meiotic cells and the filament provides support to the anther. In *Arabidopsis,* anther development is divided into 14 stages based on morphological and cellular features observed under the light microscope. At stage 1, the formation of the stamen (anther) primordium occurs through the division in the L1, L2 and L3 germ layers of floral meristem (Ma, 2005). These germ layers of floral meristem further divide and give rise to different anther tissues in the following stages. For example, epidermis and stomium are derived from the L1 layer; L2 layer gives rise to the archesporial cells, microspore mother cells (MMC), and endothecium and middle wall layers; L3 layer division results in the formation of connective tissue, vascular bundle and circular cell

cluster (Vinod, 2005). In addition to this, L2 and L3 layers contribute to the formation tapetum cell lineage (Vinod, 2005). By stage 5, anther morphogenesis is complete and it exhibits characteristic four anther lobes at each corner. In each lobe, there are four non-reproductive layers from surface to interior: the epidermis, the endothecium, the middle layer and the tapetum. These non-reproductive layers carry out special functions during pollen development and maturation. For example, the stomium and circular cell cluster are involved in anther dehiscence (Vinod, 2005). Tapetal cells play an important role in pollen maturation by providing amino acids, sugars and other nutrition to the male gametophytic cells.



(Chang et al., 2011, Current Opinion in Plant Biology 14:66-73)

Figure 1: Formation of *Arabidopsis* anther cell layers. The anther primordium only contains the L1, L2, and L3 layers at stage 1. At stage 2, some cells in the L2 layer become archesporial cells, which divide to produce the primary parietal cells (PPC, blue) and the primary sporogenous cells (PSC, red) at stage 3. Then the PPCs divide to form two layers of secondary parietal cells (SPC) at stage 4. Subsequently, the inner SPCs (green) form the tapetum (T, green), and the outer SPC (yellow) divide and differentiate into the middle layer (ML, dark pink) and the endothecium (En, purple). At the same time, the PSCs give rise to the microsporocytes (Ms, light pink) at stage 5.

In addition to this, tapetal cells also help in pollen wall formation and secrete β-1-3 glucanase, which is responsible for the dissolution of the cell walls of the tetrads, which results in the release of microspores from the pollen sacs. For this reason, the tapetal tissues become a major target for manipulating pollination control in plants. These non-reproductive tissues surround the MMCs. At stage 6, MMCs undergo meiosis and give rise to tetrads of four haploid microspores (Ma, 2005). Microspores divide mitotically and differentiate into multicellular male gametophytes or pollen grains that contain the sperm cells.

Manifestation of Male Sterility:

In nature, male sterility appears in plants in many different forms. It was broadly classified on the basis of phenotype and genotype (Kaul, 1988). Phenotypic male sterility can be structural, sporogenous and functional male sterility. Structural male sterility arises because of anomalies in male sex organs. Sporogenous male sterility displays abnormal development of microsporogenesis and functional male sterility exhibits the presence of inviable pollen, abnormal pollen maturation, inability of pollen grains to germinate on compatible stigma or it can be barrier other than incompatibility which prevents the pollen grains from reaching the ovule. On the basis of genotype, male sterility can be genic or genetic, cytoplasmic and cytoplasmic genetic. Cytoplasmic and cytoplasmic genetic male sterility is most commonly observed in nature.

Genetic Male Sterility (GMS):

GMS arises due to spontaneous mutations in one or more (dominant or recessive) nuclear genes. The gene inheritance follows a Mendelian inheritance pattern. In nature,

GMS has been identified in about 175 species. Most of them were due to spontaneous mutations in recessive nuclear genes and a few due to mutations in dominant genes (Chaudhury, 1993; Horner and Palmer, 1995).

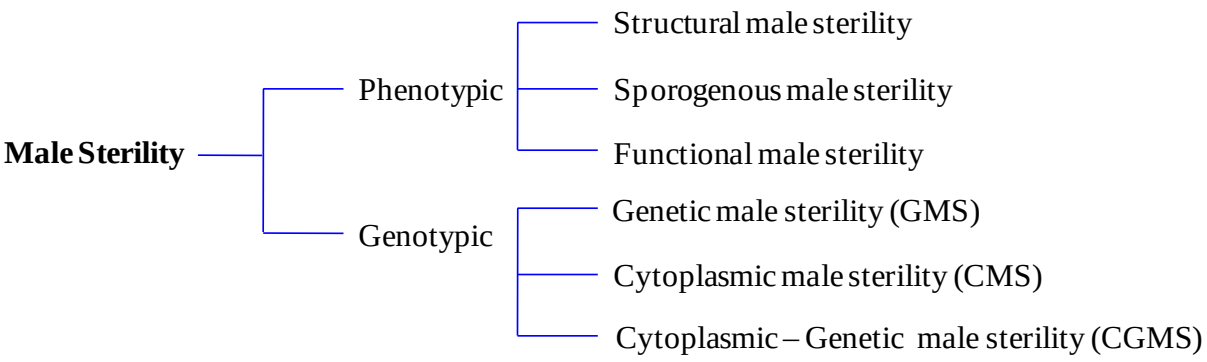


Figure 2: Classification of Male Sterility present in plant kingdom

Cytoplasmic Male Sterility (CMS):

It arises due to spontaneous mutation in cytoplasmic genes especially in mitochondrial genes. During microsporogenesis, anther cells are metabolically very active and are rich in mitochondria, which fulfill their energy demands. Any abnormalities in mitochondria lead to cytoplasmic male sterility. Sterile cytoplasm is designated as S, while the normal cytoplasm as N. It follows maternal inheritance (Hanson, 1991). This type of male sterility is not very common in the plant kingdom (Gutierres et al., 1997; Chase, 2007; Havey, 2004).

Cytoplasmic Genetic Male Sterility (CGMS):

CGMS involves interaction of both cytoplasmic and nuclear genes. The terms CMS and CGMS are often used interchangeably. It has been reported in more than 200 species

(Budar et al., 2007; Kaul, 1988; Mackenzie and McIntosh, 1999; Wise et al., 1999). CGMS has two types of cytoplasm, N (Normal) and S (Sterile) along with nuclear genes, restorers of fertility (Rf). The Rf genes are distinct from genetic male sterile genes and are required for restoration of male fertility in S cytoplasm induced sterility in plants. The Rf genes display expression in the presence of sterile cytoplasm only. Therefore, male sterile plants having S cytoplasm with rfrf genes, while fertile plants exhibit a combination of either N cytoplasm with rfrf genes or S cytoplasm with Rf- genes. It was one of the conventional methods for hybrid seed production. But, it has some limitations such as undesirable pleiotropic effects, imperfect fertility restoration.

Genetic Engineering for Male Sterility:

Conventional methods for commercial hybrid seed production have certain limitations and it cannot fulfill global demands of continuously growing population. Genetic Engineering offers a precise tool for the development of transgenic male sterile plants as an effective pollination control system. A suitable pollination control system is required for the production of hybrid seeds. In addition to this, it also acts as transgene biocontainment, which can minimize unwanted transgene movement via pollen dispersal. Several approaches have been employed for generating transgenic male sterile plants.

Dominant nuclear male sterility (Barnase and Barstar system):

In 1990, Mariani et al. developed the first genetically engineered male sterility system in crop plants. Two types of *RNAases* were used to manipulate this trait. One is *RNase T1* from *Aspergillus oryzae* and the other is Barnase from *Bacillus liquifaciens*. Both were fused with tapetum specific TA29 promoter separately and then introduced individually

into tobacco and Oilseed rape. About 10% of the TA29-*RNaseT1* transformants and 92% of TA29-*barnase* transformed were completely male sterile. Furthermore, studies revealed that the 5′ region of TA29 gene has driven the expression of *RNaseT1* and *barnase* genes specifically in tapetal cell lineage that caused selective and early cell death of tapetal cells that surround the pollen sacs, presumably by hydrolyzing the tapetal cells. This disrupted the pollen nourishment and induced male sterility.

Later, male fertility was restored by using Barstar, a Barnase specific RNAase inhibitor (Mariani et al., 1992). Barnase expressing male sterile plants were crossed with Barstar expressing fertile plants, which resulted in the coexpression of Barnase and Barstar genes in the anthers of F_1 progeny and the inactivation of Barnase by Barstar by making complex with Barnase leading to fertility restoration.

Male sterility by altering a metabolic process:

Carbohydrate supply to the developing microspores is essential and it is dependent on the hydrolysis of sucrose through cell wall bound invertase enzyme. Therefore, male sterility was achieved by impeding the carbohydrate supply through antisense suppression of invertase activity or through expressing invertase inhibitor in anthers using an invertase promoter P-Nin88 (Engelke et al., 2010; Hirsche et al., 2009). Latter, male fertility was restored by expressing yeast invertase under the same promoter (Engelke et al., 2010).

Similarly, glutamine plays an important role in nitrogen metabolism. Glutamine is synthesized from Glutamine synthetase (GS) by catalyzing the ATP dependent conversion of glutamate to glutamine utilizing ammonia as the substrate. GS is an octameric enzyme, found in two isoenzymatic forms, cytoplasmic GS1 and chloroplastic GS2. GS1 and GS2 are reported to be tightly regulated during male reproductive development (Cren and Hirel,

1999; Dubois et al., 1996; Becker et al., 1992). Using genetic engineering approach, Ribarits et al (2007) developed male sterile transgenic plants of *Arabidopsis* having mutated tobacco GS1 and GS2 driven by tapetum specific (TA29) and microspore specific promoter (NTM19) separately. Three constructs were prepared for inducing male sterility. In the first construct, N-terminal chloroplast targeting signal and 45 amino acid component of C-terminal activity domain of GS2 were deleted and fused with TA29 promoter (pTA29-ΔGS2-L construct). In the second and third constructs, two point-mutations at critical sites (N56A, R291L) were introduced in GS1 (GS1A56L291) and the mutants were fused with TA29 and NTM19 promoter. Tapetal expression of mutated GS1 or GS2 lead to the change in anther color from green to olive at about the first mitosis stage followed by drying up prior to dehiscence and this has released very small proportion of pollen grains. Microspore expression of the mutated GS1 in transgenic tobacco lead to 50% sterile pollen in T_0 transgenic, which has been conferred by the single copy insertion of the transgene and transgenic plants were able to produce seeds. Homozygous, 100% male sterile plants were produced by doubled haploids via microspore culture. These homozygous plants were used as female parents in a cross with a male fertile inbred line.

Callose is a plant polysaccharide. It is composed of β-1-3 glucan, which is deposited around the tetrad of microspores during meiosis. However, after the completion of meiosis and the beginning of microspore exine wall formation, the callose wall is broken down by the enzyme callase, a tapetally secreted β-1-3 glucanase, which degrades the callose and releases the microspores from the tetrad in a precisely timed manner. Hence, tight developmental regulation and timing of callase activity is necessary for proper microspore development. Worrall et al (1992) used a modified pathogenesis related glucanase (PR- β-

1-3 glucanase), which mimics the tapetal β-1-3 glucanase and expressed it in the tapetal cell lineage under A3 and A9 promoters. Transgenic tobacco plants expressing modified PR- β-1-3 glucanase resulted in premature dissolution of microsporocyte callose wall and this lead to moderate to complete male sterility.

Conditional male sterility:

Conditional male sterility refers to a situation in which plants are normally fertile, but when a particular condition is applied, it results in male sterility. Hawkes et al. (2011) described the use of inactive D- glufosinate as a male sterility agent on transgenic plant expressing a modified (F58K, M213S) form of the D-amino acid oxidase (DAAO) from *Rhodosporidium toruloides*. DAAO converts oxidized D-glufosinate to its 2-oxo derivative (2-oxo-4-methyl phosphinyl-butanoic acid), which is phytotoxic to the plants. For inducing male sterility, modified DAAO encoding gene was fused with TAP1 promoter from *Antirrhinum majus* and transformed into tobacco plants for generating the transgenic plants. When D-glufosinate was sprayed on these transgenic plants, it caused complete male sterility. This male sterility persisted for two or more weeks without exhibiting obvious phytotoxic symptoms or any measurable decline in female fertility.

Singh et al (2010) developed a novel three component tapetum specific and steroid inducible expression system for conditional male sterility. For this, *AtBECLIN1* gene from *Arabidopsis thaliana*, mutated TA29 promoter ($TA29_{TGTA}$), TATA binding protein mutant 3 (TBPm3), ligand binding domain (LBD) of glucocorticoid receptor (GR-LBD) from rat were used. *AtBECLIN1* gene is shown to be involved in a crosstalk between apoptosis and autophagy. Mutated TA29 promoter having TATA box with mutant TGTA does not recognize the native TATA binding protein, but the expression of mutated TGTA promoter

is restored to a higher level by coexpression of a mutant TBPm3, which recognizes TGTA. Three constructs were prepared. In the first construct, *AtBECLIN1* gene was placed under the control of mutated TA29 promoter ($TA29_{TGTA}$) as component one. In second construct, *AtBECLIN1* gene was expressed from mutated TA29 promoter ($TA29_{TGTA}$) along with the constitutive expression of TBPm3 as component two. In the second construct, component one was expressed along with constitutive expression of TBPm3 as component two. In the third construct, the component was same as in the component two, but the component two was modified with the TBPm3 mediated transcription fused with the ligand binding domain of the glucocorticoid receptor. The GR-LBD remains inactive in the absence of its cognate ligand or its agonist associated with a chaperone of Heat shock proteins (HSPs) in the cytoplasm and therefore, there would be no transcription from TBPm3 and no TBPm3 protein. However, the addition of its ligand (Glucocorticoid) or agonist (Dexamethasone) makes it active by dissociating from HSPs and then it translocates to the nucleus to mediate the transcriptional activation of TBPm3 gene. There was no sterility in transgenic plants expressing construct-I one. But in transgenic lines carrying the construct-II, non-viable pollen grains were observed and severe abnormality in microspore development, which resulted in male sterility. Dexamethasone treatment is required to induce male sterility in transgenic plants expressing the third construct.

Guerineau et al (2003) generated transgenic of *Arabidopsis* expressing a gene encoding temperature sensitive diphtheria toxin A-chain polypeptide driven by tapetum specific A9 promoter. Resultant transgenic plants were fully fertile at 26 $^{\circ}$C, but when the temperature was decreased to 18 $^{\circ}$C, male sterility was induced.

Kriete et al. (1996) developed an inducible male sterility system through the expression of bacterial gene *argE*. Gene *argE* encodes a deacetylase enzyme N-acetyl L-ornithine deacetylase, which removes the acetyl group from Non-phytotoxic compound, N-acetyl phosphinothricine (N-ac-pt) and produces a phytotoxic herbicide L-phosphinothricine (ppt, gluphosinate). The *argE* gene coding region was fused with the tapetum specific promoter (TA29) and transformed tobacco plants were generated. Foliar treatment of non-toxic compound N-ac-pt on these plant leads to early expression of L-phosphinothricine in tapetal cells resulting in male sterility, but female reproduction remain unaffected.

Male sterility via Post- transcriptional Gene Silencing:

Post-transcriptional gene silencing is defined as destroying or blocking of mRNA of a particular gene. In the recent past, it has gained a lot of importance as an approach towards inducing male sterility.

Jasmonic acid (JA) is a plant growth regulator that acts as a signal for developmentally induced alterations in gene expression (Wasternack and Parthier, 1997). It is involved in the control of developmental process in plants such as fruit ripening, root growth, tendril coiling, senescence and mechano-transduction (Creelman and Mullet, 1997). It also plays an important role in pollen maturation and anther dehiscence in *Arabidopsis* (Ishiguro et al., 2001; Sanders et al., 2000; McConn and Browse, 1996). Allene oxide synthase (AOS), a key enzyme in the JA biosynthetic pathway catalyzes the dehydration of 13-hydroxypenoxylinolenic acid to 12-13 epoxy linolenic acid (Creelman and Mullet 1997). Bae et al. (2010) used RNAi method to silence the activity of *OsAOS1* and *OsAOS2* with the promoter of anther specific genes *Osc4* and *Osg6b* separately. Four constructs, P-

Osc4::*OsAOS1*-RNAi (pSK230), P-*Osc4*:: *OsAOS2*-RNAi (pSK123), P-*Osg6b*:: *OsAOS*1-RNAi (pSK231) and P-*Osg6b*:: *OsAOS*1-RNAi (pSK124) were prepared and transformed into rice calli individually. Level of sterility in transformed plants with pSK230 was approximately 8% (3 of 37), with pSK231 approximately 28% (10 of 36) and with pSK123 23% (9 of 40). Complete sterility was found in 47% (9 of 19) of the plants from pSK124. It was concluded that *OsAOS2*-RNAi vector in combination with the *Osg6b* promoter is the most effective system for obtaining engineered genic male sterility in rice.

Woo et al. (2008) discovered a new chemically induced genic male sterility (GMS) gene ms-h in rice. GMS gene ms-h is a recessive gene and has pleiotropic effect on the chalky endosperm. Analysis of the ms-h gene through fine mapping and nucleotide sequencing displayed a single nucleotide substitution at the 3′splice junction of 14^{th} intron of UDP-glucose pyrophosphorylase1 (UGPase1, EC 2.7.7.9) gene, which causes aberrant splicing and leads to the expression of two mature transcripts with abnormal sizes. In vitro functional assay confirmed that both proteins encoded by the abnormal transcript had no UGPase activity. To establish a link between abnormal transcript and ms-h genic male sterility, role of UGPase activity with male sterility was demonstrated by suppressing the UGPase activity via RNAi construct having a 473 bp of the gene specific sequence of UGPase1 cDNA in wild plants and overexpressing the UGPase1 in mutant ms-h plant. Down-regulation of UGPase1 gene resulted in severely reduced transcript level in two male sterile lines, r23 and r29 with developmental retardation similar to the ms-h mutant phenotype. The overexpression of UGPase in ms-h mutants restored male fertility and transformants produced T_1 seeds that segregated into plants with normal and chalky

endosperms. This report also suggested that UGPase1 plays a key role in pollen developments as well as seed carbohydrate metabolism.

Nawaz-ul-Rehman et al. (2007) silenced the TA29 gene with hairpin RNAi construct, which resulted in about 10 out of 13 transgenic lines becoming male sterile.

Yui et al. (2003) tried to mimic the sugar beet CMS phenotype by antisense inhibition of mitochondrial pyruvate dehydrogenase (PDH)-E1α subunit gene. Pyruvate dehydrogenase plays an important role in the TCA cycle. Two subunits of PDH, E1α1, E1α2 were identified through screening of a cDNA library of sugar beet flower buds and it was found that the PDHE1α1 is highly expressed in the tap root, whereas bvPDH_E1α2 in flower buds. GFP fusion with bvPDH_E1α1 revealed its mitochondrial targeting property and a 300 bp of bvPDH_E1α1 cDNA sequence (from +620 to +926) was used for silencing PDH activity in mitochondria. The construct was prepared by fusing TA29 promoter with 300bp of bvPDH_E1α1 in reverse orientation and transformed into tobacco plants. Seven independent transformants were obtained of which, three tranformants, designated as #1, #2, #3 respectively, showed significantly low capacity to produce viable pollen grains relative to the non-transgenic tobacco plants. Their percentage of producing viable pollen grains were 15.0 % (#1), 47.1% (#2) and 65.5% (#3) respectively and another four were able to produce viable pollen grains at more than 80% efficiency. It was premised that the inhibition of PDH activity in tapetum prevented the conversion of pyruvate into acetyl CoA, and it adversely affected the operation of the TCA cycle, which was unable to fulfill the requirements in tapetal cells, leading to pollen sterility.

Similarly Zhang et al. (2001) demonstrated the down regulation of 304 bp sucrose non-fermenting1-related (*SnRK*1) protein kinase sequence by fusing it with a wheat high

molecular weight glutenin subunit gene promoter (Glu-1D-1) in reverse orientation, which caused abnormal pollen development resulting in male sterility in transgenic barley. Glu-1D-1 is a seed specific promoter driving the gene expression in the endosperm. But in contrast to this, Zhang et al had reported an inevitable conclusion that Glu-1D-1 promoter-derived the antisense *SnRK1* gene expression in the abnormal pollen grains. It was argued that starch accumulation was inhibited in the pollen grains that resulted in the arrest of pollen development at the binucleate stage. It was also proposed that in *SnRK1*-antisense expressing transgenic plants, cells were unable to adjust their carbon status and utilized the imported sucrose because of the reduced level of SnRK1. In effect, pollen grains were starved in a fashion similar to that in yeast Snf1 mutants starving on a sucrose medium.

Heiser et al. (1997) reported the antisense repression of mitochondrial NADH binding subunit of complex I in transgenic potato leads to male sterility. Antisense construct was prepared by cloning the gene encoding 55 kDa NADH binding protein in reverse orientation with 35S CaMV promoter. Antisense construct was further transformed into potato. This has resulted in a 33% decrease in the level of mRNA transcripts for 55 kDa subunit compared to wild type level and amount of 55 kDa protein in mitochondrial extract has also gone down to about 50% in transformed plants. Vegetative growth and tuber formation in transgenic plants were normal, but pollen maturation was disturbed. It was argued that down regulation of NADH binding component of respiratory complex caused an insufficient mitochondrial respiratory chain.

Xu et al. (1995) observed that the down regulation of *Bcp*1 gene in transgenic *Arabidopsis* plants lead to the arrest of pollen development. The *Bcp*1 gene from *Brassica oleracia* was identified as an anther specific gene, which is developmentally regulated and

expressed temporally in diploid tapetal cells and haploid microspore during pollen maturation. To confirm the role of Bcp1 gene, two antisense constructs were prepared. In first construct (Bcp1 promoter antisense construct), 0.5 kb of a *Bcp*1cDNA was fused with 0.77 kb of Bcp1 promoter in reverse orientation. In the second construct (LAT52 promoter antisense construct), LAT52 promoter from tomato was fused with antisense of 0.5 kb of a *Bcp1*cDNA and resultant constructs were transformed into *Arabidopsis* separately. About 36% (16 out of 42) in Bcp1 promoter-antisense Bcp1 construct containing transgenic plants were completely male sterile. Reduction in male fertility appeared to be correlated with the dosage level of antisense transgene. It was observed that in T_2 generation, homozygous transgenic plants were completely sterile, while hemizygous having single copy of antisense transgene resulted in a leaky male sterile phenotype. Transformed plants having LAT52-antisense Bcp1 construct had 1:1 segregation of viable/aborted pollen grains, which conferred the gametophytic control of *Bcp*1 gene.

Flavonoids and male sterility:

Among the three major flower pigments (flavonoids, carotenoids and betalains), flavonoids are the most common and important flower pigments for color. Apart from their role in pigmentation, they are also important for plant reproduction and disease related mechanisms. There are reports that any disturbance in flavonoid synthesis leads to altered pigmentation and male sterility in the plants.

Stilbene synthase gene (*STS*), reported to enhance disease resistance, can also induce male sterility when its expression is driven by an anther specific promoter. Fischer et al. (1997) expressed a Stibene synthase (*STS*) gene, cloned from grape vine (VstI), under the control of 35S RNA promoter strengthened by the duplication of enhancer region.

Transgenic tobacco plants expressing *STS* driven by the tapetum specific promoter (TapI) of *Antirrehinum majus* caused alteration in flower pigmentation and male sterility. Similar observations were also made when STS was expressed using male cone specific promoter (PrMALE1) from *Pinus radiate* (radiate pine) (Hofig et al., 2006). However, biochemical basis of *STS* induced male sterility is not fully understood. It was argued that the introduction of *STS* competes with endogenous *CHS* for the substrates, 4-coumaroyl CoA and malonyl CoA, which are important for sporopollenin and fatty acid biosynthesis. It was also argued that there was a decrease in p-coumaroyl availability, which resulted in impaired sporopollenin production and pollen wall formation. This eventually resulted in male sterility.

van der Meer et al. (1992) demonstrated that down regulation of chalcone synthase (*CHS*) gene under the control of modified 35S CaMV promoter having one, two or eight copies of the chs anther box, a homologous sequence present in the flavonoid specific gene and active during the early stages of anther development (van Tunen et al., 1988), leads to the down regulation of pigmentation and arrest of male gametophyte development in anther of transgenic tobacco plant. However, it was not concluded that the presence of the anther box in 35S CaMV can direct the gene expression in the tapetal cells, but it was argued that the module that confers organ specificity to the *CHS* or CaMV promoter may act in concert with the anther box.

RNA editing as a tool for inducing male sterility:

A basic principle of molecular biology is that primary sequence of RNA faithfully reflects the primary sequence of DNA from which it is transcribed. However, the discovery of RNA editing has challenged this concept (Covello and Gray, 1989). RNA editing has

been defined as change(s) in the nucleotide sequence of RNA that causes it to differ in sequence from the DNA that encoded it. RNA editing involves changes in nucleotide or insertion of a nucleotide, which alternatively results in changed amino acid sequence of the polypeptide. It was first reported in the kinetoplasts (mitochondria) of trypanosomes by Benne et al. (1986). This process has been described later in many biological systems. RNA editing in plant mitochondria involves post-transcription nucleotide identity switches, in most cases from C to U, but sometimes from U to C (Schuster et al., 1990; Gualberto et al., 1989; 1990, Covello and Gray, 1989). In CMS plants, male sterility is associated with mitochondrial DNA rearrangement creating new chimeric open reading frames (ORFs), which leads to a mitochondrial dysfunction. As in case of cms-T maize, ORF (T-URF13) gets transcribed and translated into a chimeric polypeptide (Dewey et al., 1986). Some other examples are the chimeric gene PCF-S of Petunia (Young and Hanson, 1987), ORF-B and ORF224 of the "Polima" CMS in rapeseed (Handa et al., 1995). The transcripts of all these chimeric genes, with the exception T-URF13, are edited only in the sequence formed by RNA fragment belonging to the transcripts that are usually edited in vivo in the normal transcripts from the gene where they expressed. Thus the chimeric proteins PCF and ORF B have originated from edited transcripts, and involved in petunia and rape seed cms respectively (Araya et al., 1998).

It was reported that editing of an *ATP6* gene may restore the fertility of CMS rice (Iwabuchi et al., 1993). Iwabuch et al (1993) analyzed the mitochondrial genome of CMS cybrid derived by transferring a small portion of the cms-bo mitochondrial genome to the fertile Nippon Bare through cell fusion. It has been observed that the cybrid has two *ATP6* genes, one with a normal cytoplasmic gene and the other one *B-ATP6* gene was also

identical to normal *ATP6*, but with 3′ flanking sequence having different starting at 49 bases downstream of the stop codon. In addition to this, the presence of RF-1 gene influences the processing and editing of the B-ATP6 RNA, (in the absence of RF-1, *B-ATP6* RNA remains unprocessed) and the translation of the ORF resulted in an altered phenotype related to CMS. But in the presence of RF1, *B-ATP6* RNA is processed and edited normally that resulted in fertility restoration in rice.

Hernould et al. (1993) expressed an unedited *ATP9* in mitochondria, which resulted in male sterility in transgenic tobacco plants.

Male sterility via Hormone imbalance:

Plant hormones play a vital role in different stages of development. Hormone imbalance at any particular stage would have adverse effects on the physiology of plants. Hormone imbalance approach was also attempted to disturb male gametophyte development for inducing male sterility in plants.

Involvement of GA in male gametophyte development was studied by using *gai* gene. Al-Ahmad and Gressel (2005) expressed *gai* gene driven by its own promoter, which also resulted in male sterility in transformed tobacco plants and male fertility was restored by exogenous application of kinetin.

In another report, rol B was kept under the control of meiosis specific *Arabidopsis* gene DMC1 (disrupted meiotic cDNA 1) promoter and petunia gene FBP7 (floral binding protein 7) specific promoter (Cecchetti et al., 2004). In P-DMC1::rol B containing transgenic plants, expression occurred earlier in male than in female development organs, which resulted in delay in anther dehiscence with respect to normal timing of pistil development and this leads to developmental uncoupling preventing self-pollination,

whereas transgenic plants containing P-FB7:: rol B resulted in a concomitant delay of both anther dehiscence and pistil development without affecting the self-pollination of plants.

Huang et al. (2003) studied the role of cytokinin and gibberellin in male gametophyte development by expressing *CKX1* (cytokinin oxidase 1) gene and gibberellic acid insensitive (*gai*) gene under the tissue specific promoter in maize. *CKX*, an enzyme, involved in the oxidative cytokinin degradation and GA insensitive (gai), a semi-dominant gene, negatively regulate GA response (Koorneef et al., 1985; Peng et al., 1997). *CKX1* constructs were developed by fusing the *CKX1* gene with maize anther specific promoter (P-Ztap) and pollen specific promoter (P-*Zmg13*) and transformed into maize individually. About 90% transformed plants of P-Ztap:: *CKX1* construct were male sterile with rudimentary terminal structure lacking recognizable male florets or spikelets compared to the ordinary develop tassel. However, leaky expression was also detected in young leaves. It was explained as either lack of promoter specificity or close proximity of the endogenous promoter to the 35CaMV enhancers.

Transgenic plants expressing the pZmg13:: *CKX1* construct exhibited 50% pollen abortion as expected in gametophyte expression of single insertion of the transgene. In addition to this, exogenous application of kinetin and thidiazuron (TDZ) partially restored the male development of sterile transgenic maize plants.

The *gai* gene was also driven by TA29 promoter and transformed into tobacco plants which resulted collapsed pollen sac during anther development leading to male sterility in about 81% transformed plants. However, leaky expression was not detected in floral tissue and pistils like in *CKX1* expressing plants. Fertility was restored in male sterile transgenic plants by the exogenous application of kinetin.

Spena et al. (1987) and Schmulling et al. (1988) observed the effect of independent and synergistic activity of rol A, B and C loci from *Agrobacterium rhizogenes* on plant development, but their main focus was on studying the synergistic action of the different rol loci in determining root induction in tobacco calli and kalanchoe leaves, and hairy root syndrome in transgenic tobacco plants. However, overexpression of rol B induced male sterility was less focused. Later on, the same group (Spena et al., 1992) demonstrated the role of rol B in male gametophyte development, in this study, rol B gene was expressed under the control of anther specific promoter of *tap*1, which resulted in alteration in anther development and whole flower growth. They have observed an increase in free IAA content and a decrease in gibberellins activity present in the anthers. It was argued that phenotypic alterations were a consequence of the increase content and activity of the auxin in anther.

Chloroplast engineering and male sterility:

In recent years, genetic transformation of plastid genome has become popular in the field of Biotechnology. This technology offers several advantages, which include high level expression of transgene, expression of bacterial genes without codon optimization, expression of multigene operons and maternal inheritance of the transgene. Its maternal inheritance feature has allowed us to induce male sterility via plastid transformation, which also showed the cms trait i.e. production of a uniform population of male sterile plants by simple cross pollination. In contrast to this, the pollen sterility via nuclear transformation cannot be true breeding as the male sterile plants must be propagated through pollination with wild type.

Ruiz and Daniell (2005) demonstrated for the first time a promising approach for inducing cytoplasmic male sterility via plastid engineering. In this approach, *phaA* gene from *Acinetobacter* sp. (accession no. L37761) that codes for β-ketothiolase was expressed in plastids through plastid transformation. It was found that the transgenic lines showed hyper-expression of β-ketothiolase in leaves, flower and anther, which caused abnormal thickening of the outer cell wall, enlarged endothecium and vaculation affecting pollen grain development and resulted in irregular shaped or collapsed male sterile phenotype. Reversible male sterile phenotype was observed under continuous illumination, which allows Acetyl CoA carboxylase (ACCase) to use available acetyl CoA that restores the normal fatty acid synthesis and less production of PHB via β-ketothiolase, which resulted in restoration of male fertility.

Heterologous expression:

It was a well-established fact that CMS is associated with mitochondrial dysfunction. This is accompanied by the expression of novel mitochondrial sequences that contain open reading frames. However, the molecular mechanism of male sterility was not completely understood till the genetic engineering approach was used to provide definite evidence that expression of abnormal mitochondrial genes could interrupt pollen development.

Nizampatnam et al. (2009) developed transgenic tobacco plant expressing, a pet1-CMS associated mitochondrial gene of sunflower, *orfH522* which was targeted to mitochondria and driven by TA29 promoter. About 35% of transformed tobacco plants were completely sterile and, premature DNA fragmentation and programmed cell death were observed at meiosis stage in the anthers of sterile plants. Later male fertility was restored by intron

hairpin and transitive RNAi mediated gene silencing of *orfH522* transcript (Nizampatnam and Dinesh Kumar, 2011)

Yamamoto et al. (2008) investigated the translational product of male sterile I-12 CMS cytoplasm derived from beets by combining with the normal fertile cytoplasm. In this study, it was observed that *orf129* was uniquely transcribed in I-12 CMS mitochondria, which they found to be a unique 12 kDa polypeptide protein in I-12 cms mitochondria. It was also observed that mitochondrial open reading frame (*orf129*) was uniquely transcribed in I-12 CMS and was large enough to encode a novel 12 kDa polypeptide. ORF 129 uniquely was present in the matrix and is loosely associated with the inner mitochondrial membrane. To investigate the functional role of *orf129*, its sequence was fused with mitochondrial targeting pre-sequence and placed under the control of *Arabidopsis* apetala 3 promoter. The resultant transgenic plants expressing *orf129* were male sterile.

Kim et al. (2007) investigated the functional role of the orf456 protein in plant mitochondria by expressing *orf456* fused with coxIV presequence in *Arabidopsis thaliana*. This has resulted in approximately 45% of the transgenic plants exhibiting the male sterility phenotype and no seed set in T_1 generation. This has been shown to be associated with the defects in the exine layer and vacuolated pollen phenotypes. In contrast to this, Stockmeyer et al (2007) developed transgenic *Arabidopsis* expressing CMS associated mitochondrial o*rf107* driven by different flower and anther specific promoters, but there was no effect of orf107 protein on male fertility of plant. Wintz et al. (1995) expressed CMS associated urf-S sequence (25 kDa) in mitochondria of petunia and tobacco using two different mitochondrial targeting sequences, but the fertility of transgenic plants was

not affected. He et al. (1996) introduced a cms associated mitochondrial sequence from common bean, ORF239, into the tobacco nuclear genome. Four constructs were developed with *orf239* with or without mitochondrial peptide driven by pollen specific promoter P-LAT52 and strong constitutive promoter P-SP separately. All four constructs were transformed in tobacco individually, which resulted in male fertile, semi- sterile and sterile transformed plants. This was explained to demonstrate that *orf239* can cause male sterility even without a mitochondrial signal peptide.

ORF138 (PCF), a mitochondrial encoded protein responsible for ogura cytoplasmic male sterility (cms) in radish (*Raphanus sativus*) and *Brassica* sp., was introduced into yeast and was transiently and stably expressed in plant cells. Its expression in yeast did not prevent the growth of yeast cells on fermentable or non-fermentable media, but it modified the cytological appearance of mitochondria in both yeast and plant cells. However, expression as a nuclear gene and mitochondrial targeting of *orf138* did not induce male sterility in transgenic *Arabidopsis* plants. It was argued on the basis of improper submitochondrial location of nuclearly expressed *orf138*.

Restoration of Male Fertility:

Several investigators have reported the development of transgenic male sterile plants, but very few have reported the development of appropriate restorer lines. Good fertility restoration mechanism is essential, if seed is the economic part of the plant and the crop is predominantly self- fertilized one. Restoration has been achieved using a Barnase-Barstar system (Mariani et al. 1990, 1992) at the protein level, gene silencing approach (Nizampatnam and Dinesh Kumar, 2011; Hird et al., 2000; Zabaleta et al., 1996) at RNA level and Site specific recombination system (CRE-LOX) (Bayer and Hess, 2005; Cao et

al., 2010) at DNA level. It is important for good restoration that restorer line must be capable of nullifying the function of transgene in induced male sterility.

Present study:

Present investigation involves the "**Study of the cysteine protease and its inhibitor-cystatin for developing a functional male sterility and fertility restoration system for pollination control in plants as an alternative strategy."**

Hence, the research work has been divided into two parts:

Part A:

Development of male sterile transgenic tobacco using a pathogen induced cysteine protease from wild peanut, *Arachis diogoi*.

Part B:

Restoration of male fertility using TA29-cystatin transgenic as a restorer line

Part B involves two activities-

I. Development of TA29-cystatin Transgenic Tobacco

II. Restoration of Fertility

Cysteine protease:

Plant proteolysis is a metabolic process. It involves different types of proteases mainly cysteine-, serine-, aspartic- and metallo-proteases (van der Hoorn, 2008). Plant genomes encode about 800 proteases and more than 140 correspond to cysteine proteases (CysProt) that are grouped in 15 families in 5 clans (Rawlings et al., 2010). Cysteine protease, belonging to C1A subfamily, was first identified and isolated from the latex of unripe papaya (*Carica papaya* L.) where it acts as an anti-herbivory agent against insects. A large

number of genes encoding C1A CysProt (papain like cysteine proteinase) have been identified in diverse plant species. Later, its homologous enzyme was discovered in viruses, Archae, bacteria, Protozoa, fungi, animals and plants. C1A proteases are present in all plant species analyzed. *Arabidopsis* plant genome alone has been reported to have 32 real and putative papain-like cysteine proteases (Simpson, 2001).

In general, papain-like cysteine proteases are synthesized as inactive precursors of the pre-pro-enzyme, which prevent inappropriate proteolysis. It consists of hydrophobic 10-26 amino acid (aa) pre-sequence serving as a signal peptide for co-translational translocation of the nascent peptide into the lumen of endoplasmic reticulum (ER). The 38-150 aa pro-domain is involved in blocking the catalytic site of enzyme and is important for proper folding and targeting. The pro-region contains the consensus motif GxNxFxD, which seems to be essential for the correct processing of the protease precursors and the non-contiguous ERFNIN signature (Ex3Rx3Fx3Nx3I/Vx3N) of unknown function (Grudkowska and Zagdanska, 2004; Martinez and Diaz, 2008). The pro-region is important for protease activity to ensure that mature enzyme is formed at the right place and/or at the right time (Vernet et al., 1995; Demidyuk Ilya et al., 2010). The mature enzyme is typically monomeric, 210-260 aa in length, and folds into two domains separated by the active site cleft. Cys25, His159 and Asp175 (papain numbering) form a "catalytic triad". In acidic pH, pro-region of enzyme gets cleaved and mature enzyme becomes active for proteolysis.

Involvement of cysteine protease in programmed cell death:

Cysteine proteases are reported to be ubiquitously involved in programmed cell death (PCD) in multicellular organisms. It is a well-characterized process in animals, where

cysteine proteases emerge as key regulators of animal programmed cell death. In plants, many reports suggest a critical regulatory role of cysteine protease in PCD (Pennell and Lamb, 1997; Trobacher et al., 2006). A number of genes encoding papain-like cysteine proteases have also been identified from senescing organs including leaves (Gepstein et al., 2003; Ueda et al., 2000), flowers (Eason et al., 2005), legume nodules (Kardailsky and Brewin, 1996) and germinating seeds (Ling et al., 2003). A papain-like cysteine protease, MsCyp15A, from *Medicago sativa* is reported to be important for the progression of leaf senescence. Antisense suppression of MsCyp15A in transgenic plants of *Medicago truncatula* resulted in delayed senescence (Sheokand et al., 2005).

SEN11 and SEN102, papain-like cysteine proteases have been shown to be expressed during flower senescence in *Hemerocallis* spp. (daylily) (Guerrero et al., 1998) and a similar gene *PRT5* is expressed during tepal senescence in *Sandersonia aurantiaca* (Eason et al., 2002). Broccoli (*Brassica oleracea*) *BoCP5* antisense suppression resulted in delayed floret senescence (Eason et al., 2005). The expression of eggplant (*Solanum melongena*) SmCP is associated with programmed cell death during leaf senescence, fruit senescence, xylogenesis and anther senescence (Xu and Chye, 1999; Xu et al., 2003).

During barley seed germination, 27 cysteine proteases among 42 proteases were found to be involved in protein degradation. Recently, a complete transcriptome analysis of barley grain germination in two tissue fractions (starchy endosperm/aleurone and embryo/scutellum) further revealed the induction of several cysteine protease genes during germination, most of them being mediated by gibberellins (Sreenivasulu et al., 2008). Moreover, several cathepsin L-like cysteine proteases from barley were found to be differentially expressed in seed tissues and involved in mobilization of hordeins, the main

storage proteins from barley (Davy et al., 2000; Davy et al., 1998; Martinez et al., 2009). Similarly, the orthologous genes encoding C1A proteases from wheat participate in bulk protein degradation of wheat endosperm during seed germination and subsequent seedling growth (Shi and Xu, 2009). Programmed cell death in the castor bean endosperm is associated with the accumulation and release of a cysteine endopeptidase from ricinosomes (Schmid et al., 1999), early stages of seed development in *Brassica napus*, a seed coat-specific cysteine protease is associated with programmed cell death of the inner integument (Wan et al., 2002).

In addition to this, cysteine proteases also play an important role in plant pathogen/pest interactions (McLellan et al., 2009; Shindo and Van der Hoorn, 2008; van der Hoorn, 2008). Several reports indicated that plants use cysteine proteases to protect themselves against different pests. Papain is a component of papaya latex and is involved in the defense of the papaya tree against different Lepidopteran caterpillars (Konno et al., 2004). In response to the herbivorous armyworm, *Spodoptera frugiperda*, Maize cysteine protease Mir1 gets induced (Pechan et al., 2000) and its proteolytic activity damages the insect's peritrophic matrix, impairing nutrient utilization (Pechan et al., 2002).

The expression of cysteine proteases also gets up-regulated during abiotic stresses like salt and drought treatment, extreme temperature, nutrient deprivation and oxidative stress induced PCD (Jenkins et al., 1997; Ning et al., 2002; Solomon et al., 1999). Rd19a and RD21a encoding two papain-like cysteine proteases were found to be up-regulated in *Arabidopsis* in response to salt and drought treatment (Koizumi et al., 1993). Similarly, *Pisum sativum* PSCYP15A, a homolog of Rd19a, is also up-regulated in response to salt and dehydration (Jones and Mullet, 1995). A tomato papain-like cysteine protease, C14 is

accumulated in fruits in response to both heat and cold treatments (Schaffer and Fischer, 1988; Schaffer and Fischer, 1990) and in senescing leaves of nutrient-deprived plants (Drake 1996). A festuca cysteine protease gene is up-regulated at temperatures of 44^{o}C (Zhang et al., 2005). Upon H_2O_2 induced oxidative stress, Suspension cultures of soybean showed increased activity of numerous proteases. Ectopic expression of an endogenous cysteine protease inhibitor reduced proteolysis and repressed the H_2O_2 triggered PCD (Solomon et al., 1999)

Some of the cysteine proteases get activated during developmental, PCD for example xylogenesis and reproduction also.

Cystatin:

Cystatins are the proteinaceous inhibitors of cysteine proteases. These are small peptides of 12-16 kDa. The first cystatin was first identified in egg white as chicken egg white cystatin (Colella et al., 1989). Later, they were found in all species so far analyzed. Cystatin superfamily of mammals was categorized into three families (Benchabane et al., 2010; Turk and Bode, 1991). Family 1, also known as stefin, includes cystatin of the molecular size of ~11 kDa, which lacks disulphide bridge and glycosylation sites. Generally, they are expressed intracellularly. Family 2 cystatins are 13-14 kDa in size having four conserved cysteine residues forming two disulphide bonds at the C-terminal portion of the protein, which stabilize the core protein backbone. Some members of the family also have glycosylation sites. Family 3 (Kininogens) comprise the multicystatin of molecular size 88-114 kDa. Repeats of stefin-like domain in kininogens are likely the results of gene duplication events. Plant cystatins are mostly 12-16 kDa in size without disulphide bridges and glycosylation sites (Martinez et al., 2012). However, there are

cystatins of ~23 kDa having a carboxy-terminal extension essential for the inhibition of a second family of cysteine protease, the C13 legumain peptidases (Martinez et al., 2009; Margis-Pinheiro et al., 2008; Martinez and Diaz, 2008 Martinez et al., 2007;). Recently multidomain cystatins of molecular size 85-87 kDa have also been reported in potato and tomato (Madureira et al., 2006; Nissen et al., 2009). Inhibitory cystatins form tight, reversible, equimolar complexes with cysteine proteases acting as pseudo-substrates to penetrate the active site of the target enzymes and block access to protein substrate.

They have inhibitory motif Gln-X-Val-X-Gly (where X is any amino acid) in the central region of the polypeptide chain and a Pro-Trp (or Leu-Trp) dipeptide motif in the C-terminal region and a conserved Gly residue in the N-terminal region (Benchabane et al., 2010). They have also plant specific signatures ([LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]- [HYFQ]-N) located in a α-helix (Martinez and Diaz, 2008). More than 200 plant members were reported to be cystatins. Oryzacystatin is the first well characterized cystatin of plant origin, which consists of a five stranded antiparallel β-sheet wrapped around a central α-helix, similar to the tertiary fold of well characterized animal cystatins such as chicken egg white cystatin and human stefin A (Abe et al., 1987; Nagata et al., 2000).

Interaction of cysteine protease and cystatin:

Cysteine protease and cystatin interactions are crucial for many cellular processes. Solomon et al. (1999) have reported that the coordinate expression of cysteine protease and cystatin regulates plant PCD. Several approaches were used to understand these interactions. For instance, physical *in vitro* interactions were determined through resolution of crystal protein complexes. The crystal structures of many plant cysteine proteases have

been resolved (e.g. castor oil CysEP, Than et al. 2004, barley EPB2, (Bethune et al., 2006). For cystatin, nuclear magnetic resonance (NMR) structure of oryzacystatin-1from rice is available (Nagata et al., 2000) and recently, the crystal structure of potato multicystatin 2 (PMC2) was resolved (Nissen et al., 2009). However, very few complex structures are available in the protein data bank (PDB) such as the human stefin- B-papain, stefin A,-cathepsin H and stefin B-cathepsin B, tarocystatin–papain complexes (Chu et al., 2011; Jenko et al., 2003; Renko et al., 2010; Stubbs et al., 1990). The crystal structure of tarocystatin–papain complex has suggested that human and plant cystatins have a similar binding mode to inhibit the cysteine protease activity. Since crystallization of protein complexes is difficult, *in silico* docking techniques have become a useful tool to study the protein interactions. In a recent study, homology based docking approach was applied for Cysteine protease and their inhibitor structures (Tastan Bishop and Kroon, 2011). Other approaches include inhibitory protease activity assay in which, recombinant proteins purified from bacteria or yeast are used commonly. The inhibition constant values are used to determine the specificity and capacity of inhibition of each protease by cystatin and therefore, indicate a physical interaction between both proteins (Tajima et al., 2011; Yamada et al., 1998). Martinez et al. (2009) have shown that almost all cystatin found in barley are able to inhibit C1A cysteine protease *in vitro* and they co-localize in the endoplasmic reticulum and Golgi bodies *in vivo.*

Chapter 3

Material and Methods

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Plant material:

Tobacco (*Nicotiana tabacum* cv. Samsun) plants grown under greenhouse conditions were used for plant transformation.

Plasmid DNA vectors:

pTZ57R (MBI Fermentas, Germany)

This vector is used in the cloning of PCR amplified fragments of cysteine protease, the tapetum specific promoter (P-TA29), T-NOS terminator and cystatin genes used in our study. It also has a bacterial selectable marker gene, β-lactamase and therefore can be selected on ampicillin.

pCAMBIA2300 (CAMBIA, Australia)

pCAMBIA2300 is a binary vector used for preparing TA29-cysteine protease construct. This binary vector has *nptII* gene encoding for kanamycin resistance for selection on bacteria and as a plant selectable marker respectively. This vector was modified by replacing its *nptII* cassette with basta cassette as a plant selection marker and used for preparing TA29-cystatin construct.

Bacterial Strains:

The bacterial strain Escherichia coli DH5α was used in the maintenance of the above plasmid clones. The *Agrobacterium tumefaciens* strains LBA4404 were used for transformation procedures of tobacco plants.

Preparation of competent cells of *Escherichia coli*:

Single colony of *E. coli* (DH5-α, Bangalore GENEI, India) culture was inoculated in 25 ml of LB (Luria Broth, Himedia, India) and was incubated overnight with constant shaking at 37 ºC. From the overnight culture, 1.0 ml was taken and re-inoculated in a fresh batch of 50 ml of LB and incubated again until the OD reached 0.5 at 600 nm. The cells were incubated on ice for 15 minutes and pelleted by centrifugation in a cooling centrifuge (Eppendorf®, Germany) at 4 ºC and 5000 rpm for 5 minutes. The supernatant was removed and the pellet was suspended in 40 ml of ice cold 0.1 M $CaCl_2$ solution and was stored on ice for 20 minutes. The cells were centrifuged at 4 ºC and 5000 rpm for 5 minutes, and the pellet was resuspended in ice cold 3 ml of 0.1 M $CaCl_2$ and 15% (v/v) sterile glycerol. These competent cells were stored at –70 ºC in aliquots of 0.2 ml of the competent cells. These aliquots can be used immediately for transformation after keeping the cells on ice for 30 minutes.

Transformation of *E. coli* competent cells:

E. coli competent cells (100 µl) were taken out from –70ºC freezer and 50 to 100 ng of the plasmid containing the desired gene was added to the competent cells and the cells were incubated on ice for 10 to 30 minutes. The cells were subjected to heat shock at 42 ºC for 90 seconds and then immediately chilled on ice for one minute. The volume was made up 1.0 ml by adding 800 µl of the sterile LB medium and tubes were incubated on a rotary shaker at 37 ºC for 1 hrs. Aliquots (100, 200 µl) of transformed cells were plated on LA medium (Luria Agar, Himedia, India) containing appropriate selection antibiotics. The plates were incubated in a bacteriological incubator overnight at 37 ºC for the appearance

of colonies. Once the colonies were visible, the plates were stored at 4 ºC in a refrigerator. Plasmid DNA was isolated from the colonies to confirm transformation. For long-term storage of the transformed cells, liquid cultures of the cells were stored at –70 ºC after adding sterile 50% glycerol or 0.075% of DMSO.

Plasmid DNA isolation from *E. coli* (mini preparation) (Sambrook et al. 1989):

Transformed *E. coli* colonies were inoculated in 5.0 ml of LB medium with appropriate antibiotics on a rotary shaker at 37ºC and 200 rpm for 12–16 hrs. These overnight grown cultures were harvested by centrifuged at 12000 rpm for 60 seconds at 4 ºC. Supernatant was removed and the pellet was resuspended in 100 µl sterile ice cold Solution I [25 mM Tris HCl (pH 8.0), 10 mM EDTA [Ethylene Diamine Tetra Acetic Acid (pH 8.0)], 50 mM Glucose, stored at 4 ºC] using a vortex mixer. The cells were lysed by adding 200 µl of Solution II [0.2 N NaOH, 1% SDS, freshly prepared and stored at RT]. The contents were mixed thoroughly by inversion till the solution became clear. The tubes were then incubated on ice for 5 minutes. After the lysate got chilled, 350 µl of Solution III [3.0 M potassium acetate (pH 4.8), autoclaved and stored at RT] was added, mixed thoroughly and incubated on ice for a further 5 minutes. The contents of the tubes were centrifuged at 4ºC and 12000 rpm for 10 minutes in a cooling centrifuge. The supernatant was collected into a fresh 1.5 ml micro-tube. From a stock of 10 mg ml^{-1} of RNase, 2–3 µl was added to the supernatant and incubated at 37ºC for 1 hr. The supernatant was treated with phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) successively and was centrifuged after each treatment at RT and 12000 rpm for 15 minutes to separate the aqueous phase from the organic layer. The upper aqueous layer was collected without disturbing the middle protein layer. The DNA was precipitated with an

equal volume of isopropyl alcohol. The mixture was allowed to stand for 10 minutes at room temperature and the DNA was pelleted by centrifuging at 4 ºC and 12000 rpm for 10 minutes. The supernatant was removed and the pellet was rinsed with 1.0 ml of 70% ethanol. The pellet was air dried and dissolved in 30 to 50 µl TE buffer [10 mM Tris HCl and 1.0 mM EDTA (pH 8.0)]. The isolated plasmid DNA was stored at –20ºC. Plasmid isolations were also carried out using a kit (Plasmid miniprep kit, Sigma Aldrich, USA) following the manufacturer's instructions.

Preparation of competent cells of *Agrobacterium*:

A single colony of parental *Agrobacterium* culture was inoculated in LB medium with appropriate antibiotics and was incubated in a bacteriological shaker at 28 ºC and 200 rpm for 24 hrs. When the OD reached 0.5–0.8 at 260 nm, the culture was placed on ice. The bacterial culture was centrifuged at 4 ºC and 5000 rpm for 5 minutes. The supernatant was discarded and 0.5 ml of 20 mM ice cold $CaCl_2$ solution and 15% (v/v) sterile glycerol was added under sterile conditions. These competent cells were stored at –70 ºC.

Transformation of *Agrobacterium* cells (Holsters et al. 1978):

To 100 µl of competent cells, 50 to 100 ng of the binary vector with the desired gene construct(s) was added. The cells were incubated in liquid nitrogen for 1–2 minutes and immediately transferring them to 37 ºC in water bath for 5 minutes. After heat shock, 0.5 ml of LB medium was added and incubated in a rotary shaker at 28 ºC and 200 rpm for 4–5 hrs. The cells were then centrifuged for 60 seconds and the pellet was resuspended in 0.1 ml of LB medium. The cells were then spread on LA plates containing appropriate antibiotics and incubated in at 28 ºC for 48–72 hrs for the appearance of transformed

colonies. The plates were stored at 4 ºC in a refrigerator, where the cells stayed viable for two months. Plasmid DNA was isolated from the colonies to confirm the transformation. For long-term storage of the transformed cells, liquid cultures of the cells were stored at –70 ºC after adding sterile 50% glycerol.

Plasmid isolation from *Agrobacterium*:

After transformation, a single colony of *Agrobacterium* cells was inoculated in 10 ml of LB medium containing appropriate antibiotics and incubated overnight on a rotary shaker at 28 ºC. The overnight grown culture harvested by centrifugation at 1000 rpm for 1 minute. The pellet was resuspended in 0.2 ml of ice-cold Solution I [4.0 mg l^{-1} lysosyme, 50 mM Glucose, 10 mM EDTA, 25 mM Tris HCl (pH 8.0)] and incubated at RT for 10 minutes. The cells were lysed by adding 0.2 ml of freshly prepared Solution II [1% SDS, 0.2 N NaOH]. The contents were mixed thoroughly by inversion and incubated for 5 minutes at RT. To this mixture, 30 µl of phenol equilibrated with 2 volumes of Solution II was added and mixed by vortexing till the suspension became viscous. To this viscous lysate, 150 µl of 3.0 M sodium acetate (pH 4.8) was added and was mixed well. The tubes were incubated at –70 ºC for 15 minutes and then centrifuged at 5000 rpm for 3 minutes. The supernatant was collected and ice cold 100% ethanol was added to fill the tube. The contents were mixed thoroughly by inversion and were stored at –70 ºC for 15 minutes. After incubation, the tubes were centrifuged at 5000 rpm for 3 minutes and the supernatant was discarded. The pellet was suspended in 0.5 ml of 0.3 M sodium acetate (pH 7.0) and the DNA was precipitated by adding ice-cold 95% ethanol. The DNA in sodium acetate and ethanol were mixed well by inversion and stored at –70 ºC for 15 minutes. After incubation, the tubes were centrifuged at 5000 rpm for 3 minutes. The supernatant was

decanted and the pellet was rinsed with 1.0 ml of 70% ice-cold ethanol. The supernatant was discarded and the pellet was air-dried. The dried DNA pellet was suspended in 50 µl TE buffer and was stored at –20 ºC.

Agarose gel preparation and electrophoresis:

DNA fragments were resolved in 0.8% agarose gel. Agarose gel was prepared by melting 0.8 g agarose (SeaKem LE AGAROSE) in 100 ml of 1x TAE buffer [50x TAE: 2.0 M Tris Cl, 1.0 M Acetate, and 100 mM EDTA (pH 8.0)]. The melted agarose was cooled to 50 ºC and 2 µl of ethidium bromide (EtBr) from a stock of 10 mg ml^{-1} was added. This was poured into a gel-casting tray with a comb placed properly in the grooves provided. After the gel got polymerized, it was kept inside the electrophoresis tank containing 1x TAE buffer so as to cover the gel. The DNA was mixed with 6x loading dye [0.15% bromophenol blue, 0.15% xylene cyanol, 5.0 mM EDTA, 40% sucrose] to a concentration of 1x and loaded in the wells created by the comb. The gel was electrophoresed at 60 V for 1 h or till the dye front covered almost 3 to 4 cm length of the gel. A molecular weight marker [λ DNA digested with E*co*RI/*Hin*dIII or *Hin*dIII alone (MBI Fermentas)] was loaded along with the samples for reference.

Gel elution:

For gel elution, the DNA fragments were electrophoresed on 0.8% agarose gel stained with ethidium bromide (EtBr). The fragment of interest cut out along with the gel slice was weighed and taken in a micro-tube. GenElute™ Gel Extraction Kit (Sigma Aldrich, USA) was used for extracting DNA from agarose gel following manufacturer‘s instructions. The DNA was eluted in 20 µl of warm sterile water (65 ºC). A small amount (1.0 µl) of the

eluted DNA was electrophoresed on agarose gel to check the concentration and quality before proceeding further. The gel eluted DNA was stored at –20 ºC**.**

Restriction digestion and Ligation:

Restriction digestion of the plasmid carrying the insert fragment and the binary vector plasmid was performed using *Hin*dIII for cloning the insert at the *Hin*dIII site of the vector. The restriction digestion reaction was set up as follows:

Plasmid	:	0.5 µg
10x Restriction buffer	:	2.0 µl (1x final concentration)
Restriction enzyme (Hind III)	:	0.2 µl (1 unit per µg of DNA)
DDW to make up to	:	20 µl

The reaction mixture was mixed well and was incubated at 37 ºC in a water bath for 90 minutes. The linearized vector and the insert fragments were gel eluted as detailed out earlier. Dephosphorylation of the 5'- termini of the linearized vector was done to avoid self-ligation using calf intestinal alkaline phosphatase (CIAP, MBI Fermentas Germany) treatment. The reaction mixture was prepared as follows:

Linearized DNA	:	10–40 µl (0.5 µg)
10x reaction buffer	:	5.0 µl (1x final concentration)
Deionized water to	:	49 µl
Alkaline phosphatase	:	1.0 µl (1 unit)

The reaction mixture was mixed well and the tube was incubated at 37 ºC in a water bath for 30 minutes. After incubation time, the reaction was stopped by heating it at 85 ºC for 15 minutes or the DNA was precipitated with ethanol. *Hin*dIII digested vector of pUC18 was ligated at 16 ºC for 12 hrs in a ligation bath (Multi Temp III, Amersham

Pharmacia Biotech, USA) at the *Hin*dIII site of the vector. The ligation mixture was prepared as given below to a final volume of 20 µl.

5x ligase reaction buffer	:	4.0 µl (1x final concentration)
Vector DNA	:	30 pmol
Insert DNA	:	90 pmol (3:1 fragment to vector)
Sterile DDW	:	20 µl
T4 DNA Ligase	:	1.0 unit (GIBCO BRL)

The mixture was mixed gently and centrifuged briefly to bring the contents at the bottom of the tube and was incubated at room temperature (RT) for 5 minutes. This reaction mixture after incubation period was used for transforming 100 µl of *E. coli* competent cells. For each transformation experiment, 2 to 3 µl of this reaction mixture was used. The ligation mixture was stored at –20 ºC. Plasmid DNA was isolated from the transformed *E. coli* culture and the presence of the gene was confirmed by *Hin*dIII digestion. The digested plasmid DNA was electrophoresed on 0.8% agarose gel stained with ethidium bromide and was visualized under a UV-Transilluminator (Hoefer, USA). The plasmid harboring the desired gene was used for transforming *Agrobacterium* cells.

DNA extraction from leaf tissues (miniprep):

CTAB method (Murray and Thompson 1980; Doyle and Doyle 1990)

DNA isolation was done from the second leaf from the shoot tip of young plants. The leaves were freshly collected or frozen in liquid nitrogen and stored at –70 ºC. The leaf tissue (100–500 mg) was ground using mortar and pestle in liquid nitrogen to a fine powder along with a pinch of PVPP (Polyvinyl Polypyrrolidone). Warm (65 ºC) CTAB (Cetyl/ Hexadecyltrimethyl Ammonium Bromide) extraction buffer (1.0 ml) [2% CTAB,

100 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl and 2% β- mercapto ethanol (β-merc)] solution was taken in 2.0 ml micro tubes and 200 mg of the powder was transferred to the tube, and mixed well to suspend the powder uniformly by repeated inversion of the tubes. The tubes were incubated at 65 ºC for 1 hr with intermittent mixing. After incubation, 0.5 ml of Chloroform: Isoamyl alcohol (24:1) mixture was added and mixed thoroughly by inversion. The two phases were separated by centrifugation at 12000 rpm for 15 minutes. The upper aqueous layer was taken in a fresh 2.0 ml tube without disturbing the middle and lower layers. The nucleic acid content was precipitated from the aqueous phase by mixing well by inversion after adding an equal volume of isopropyl alcohol and incubating the tubes at –20 °C for a minimum of 30 minutes. After the cold treatment, the tubes were centrifuged at 4 °C and 12000 rpm for 15 minutes to sediment the nucleic acids. The solution was decanted completely and 1.0 ml of 75% ethanol was added, and incubated for 5 minutes at RT. The tubes were centrifuged at 12000 rpm 3–5 minutes and ethanol was decanted. The pellet was air-dried and a required volume of TE [10 mM Tris HCl and 1.0 mM EDTA (pH 8.0)] solution was added. For further purification, the pellet was dissolved in 500µl of TE buffer.

Purification of genomic DNA:

The genomic DNA isolated from the tissue was purified from contaminating RNA and proteins in the purification procedure. This was necessary if the DNA was to be used for molecular analysis. To the nucleic acids, dissolved in TE buffer, 2–3 µl of RNase A from a stock of 10 mg ml^{-1} solution was added and the tubes were incubated at 37 °C in a water bath for 1–2 hrs. An equal volume (here 500 µl) of phenol: chloroform: isoamyl alcohol (25:24:1) mixture was added and mixed carefully by inversion. When the two phases got

mixed properly, the tubes were centrifuged at 12000 rpm for 15 minutes. The upper aqueous layer was transferred to a fresh tube without disturbing the middle protein and lower phenol mixture layers. An equal volume of chloroform: isoamyl alcohol (24:1) mixture was added, mixed thoroughly and centrifugation was repeated. Two volumes of 100% ethanol were added to the upper aqueous layer. The content was mixed well by inverting the tubes 5 to 6 times and was incubated at –20 °C for a minimum of 30 minutes. After incubation, the tubes were centrifuged at 12000 rpm for 10 minutes. The solution was decanted and the pellet was rinsed with 75% ethanol as explained earlier. The final pellet was air dried and suspended in a minimum volume (20 to 50 µl according to the pellet size) of deionized water or TE buffer. The isolated DNA was stored either at 4 °C (short term) or –20 °C (long term).

DNA Precipitation:

To the aqueous phase, 0.225 ml of 100% ethanol was added and mixed by inversion 6–8 times and was stored at RT for 5 minutes. Precipitated DNA was sedimented by centrifugation at 5000 rpm for 4 minutes, and resulting supernatant was discarded.

Quantification of DNA:

An aliquot (5.0 µl) of the solubilized DNA was mixed with 1.0 ml of TE buffer (pH 8.0) and the concentration was measured A_{260} and A_{280} of the resulting solution. The DNA content was calculated assuming that one A_{260} unit is equal to 50 µg of double stranded DNA/ml.

RNA isolation:

Total RNA was isolated from the plant organs using the TRI reagent (Sigma-Aldrich, USA) or TRIZOL (Invitrogen, Germany) as per the manufacturer‘s instruction.

PCR (Polymerase Chain Reaction):

Inserts were isolated by PCR with the primers specific to each of the component with the respective DNA. A reaction volume of 50 µl set up comprising 100 ng of genomic DNA, 1x PCR buffer, 1.5 mM MgCl2, 200 µM of each dNTP, 15 pmol each of both forward and reverse primers and 2.5 U of recombinant Taq DNA polymerase (Sigma Aldrich, USA) was taken. The cycling conditions were with an initial denaturation at 94 °C for 4 min followed by 32 cycles of 94 °C for 1 min, 57–60 °C for 1 min, 72 °C for 2 min and a final extension of 10 min at 72 °C. Lid temperature was set at 105 °C before the reaction was started. Amplified DNA fragments were electrophoresed on 0.8% agarose gel stained with EtBr. Gels were photographed under UV-light in a Gel-Documentation System (LTF Labortechnik, Germany).

Digestion of the genomic DNA:

For Southern analysis, 10–20 µg of genomic DNA was taken for digestion using restriction enzymes. The restriction enzymes, *Hind*III and *Eco*RI have been the most frequently used enzymes in this study for Southern analyses, and 20–30 units of the enzyme were used along with 1x reaction buffer. Usually, the restriction digestion was set up in a reaction volume of 40 µl.

Southern Blotting:

Digested DNA samples were electrophoresed on 0.8% neutral agarose gel at 30 V as explained earlier. The gel was allowed to run overnight and monitored by visualization and gel documentation. Before starting the gel treatments for blotting, the EtBr was removed by gently agitating the gel in sterile double distilled water for 30 minutes. The gel was treated with depurination solution (freshly prepared 0.125 M HCl) for 7 to 10 minutes on a gel rocker and then immersed in denaturation buffer [1.5 M NaCl, 0.5 M NaOH] for 30 minutes with gentle agitation. After denaturation, the gel was the buffer was submerged in neutralization buffer [1.0 M Tris-Cl, 1.5 M NaCl (pH 7.5)] and incubated for 30 minutes with gentle agitation. The DNA was transferred onto Hybond-N^+ nylon membrane (Amersham Biosciences, UK) overnight by the capillary method with 20X SSC (1.5 M NaCl, 0.15 M Sodium citrate, pH 7.0) as a transfer buffer. Capillary blotting technique, first described by Southern (1975), is the most widely used technique for transferring separated nucleic acid fragments from an agarose gel to a solid support. A sheet of positively charged nylon membrane was cut to an appropriate size and appropriately wetted with the transfer buffer. A tray was half filled with transfer buffer [20x SSC/SSPE (pH 7.5)] and a platform was prepared that was covered with a wick of 3MM Whatman sheet saturated in transfer buffer. The treated gel was gently kept on the wick platform to avoid any air bubble getting trapped between the gel and wick. The membrane was positioned on top of the gel and three sheets of 3MM paper cut to size and saturated in transfer buffer was placed on top of the membrane. A stack of absorbent crude filter papers cut to size (at least 5 cm high) was placed on the top of 3MM paper. Finally, a glass plate and a weight (500 g) were kept on top of the paper stack and incubated overnight to allow

proper transfer. The DNA transfer on the membrane was fixed using a UV-cross linker by exposing the membrane to 120 milli joules of ultraviolet radiation. Membrane was prehybridized at 65 °C for 3–4 hrs in phosphate buffer (0.5 M phosphate buffer, pH 7.2, 7% (w/v) SDS, 10 mM EDTA and 1% BSA) and hybridized for 16 h with α-32P dATP radiolabelled DNA using Prime-a-gene labeling system of Promega, USA. After hybridization, the membranes were washed twice with 2x SSC, 0.1% SDS at 65 °C for 10 min followed by 1x SSC, 0.1% SDS and 0.1x SSC and 0.1% SDS for 5 min each respectively. The membranes were exposed at –70 °C and autoradiographed.

Total Protein Extraction:

For total protein extraction, Leaf tissue was ground in liquid nitrogen using mortar and pestle adding a pinch of PVPP and the fine powder was collected in pre-chilled micro-tubes. Into the tubes kept on ice, 1.0 ml of chilled extraction buffer [50 mM Tris-Cl and 1.0 mM PMSF (Phenyl Methane Sulphonyl Fluoride)] was added, mixed thoroughly and were made to stand at 4 ºC for half an hour with intermittent shaking. The tubes were centrifuged at 4 ºC and 15000 rpm for 20 minutes and the supernatant containing the protein was collected in a fresh tube. All the steps were performed at 4 ºC. Isolated protein samples were immediately used or stored at 4 ºC. It stayed without degradation for one week.

Quantification of the leaf-extracted total protein:

Bradford (1976) method for protein quantification:

Bradford dye concentrate was prepared by dissolving 100 mg Coomassie Brilliant blue G250 in 50 ml of 95% ethanol. To this, 100 ml concentrated phosphoric acid was added

and the volume was made up to 200 ml with H_2O. The Bradford dye concentrate was stable for 6 months at 4 ºC. Samples of BSA were prepared with 10, 20, 30, 40, 50, 75 and 100 μg/100 μl of BSA (fraction V) in the same buffer solution in which the protein samples were extracted. The Bradford dye concentrate was diluted to 5x with double distilled water and 1.0 ml was added to each sample. The red dye turned blue after binding to the protein. The samples were allowed to develop color for at least 5 minutes (not longer than 30 minutes). Absorbance at 595 nm was read, and a linear standard curve was prepared to calculate the concentration of protein.

Polyacrylamide Gel Electrophoresis (PAGE):

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with acrylamide: N, N'-bis acrylamide (30:1) was carried out according to the standard protocol (Laemmli, 1970). A **12% (standardized for Cysteine protease protein)** 7.5 cm gel was prepared with 1 cm stacking gel. Electrode buffer was prepared by dissolving 10 g SDS, 30.3 g Tris and 144.1 g glycine in 800 ml H_2O [25 mM Tris and 192 mM glycine with 1% SDS]. The volume is adjusted to 1 liter with H_2O and stored at RT. Protein samples are prepared by boiling in a water bath for 3–5 minutes after mixing 50 μg of each sample with one-tenth volume of the sample buffer [0.125 M Tris-HCI (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue]. Electrophoresis is carried out at 90 V in an electrophoresis and electro-transfer unit (Hoefer mini VE, Amersham Pharmacia Biotech, USA) till the dye came out of the gel into the electrode buffer.

Coomassie Blue staining of the gel:

Coomassie Brilliant Blue R250 staining detection limit is 300 to 1000 ng protein and is used in the experiments. The gel with electrophoretically separated proteins is incubated for staining in Coomassie solution (0.025% Coomassie Brilliant blue-R250 in 45% methanol and 10% acetic acid) for 30 minutes. Destaining was done with a destaining solution [45% methanol and 10% acetic acid] to remove background staining. The destaining solution is replaced every 10–15 minutes with a fresh solution, until the protein bands are visible.

Western Blotting and Immuno-staining:

After electrophoresis, the protein samples are electro-blotted on to Polyvinylidine fluoride (PVDF)/ nitrocellulose membrane (Pall Gellmann Corporation USA) using Trans-Blot apparatus (electrophoresis and electro-transfer unit, Hoefer mini VE, Amersham Pharmacia Biotech, USA) according to the manufacturer‘s instruction using Towbin buffer (Towbin et al. 1979). The gel is first equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine and 20% methanol) and the membrane is kept above the gel towards the positive end of apparatus and is stacked in between Whatman filter papers taking care not to trap any air bubbles. Transfer is conducted for 4 hrs at 25V. The transfer of protein to the membrane was checked by reversible Ponceau S staining (100 mg Ponceau S in 5% acetic acid). The stain was removed by 3-4 washes with TBST [10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween-20 (v/v)]. Blocking of the membrane is carried out using 3% BSA (w/v) in TBS for 1 hr at RT followed by 5 washes with TBS for 10 minutes each. The blot was then incubated overnight with the primary antibody diluted in TBS containing 3%

BSA at 4 ºC. This is again followed by five thorough washes, each of 10 minutes in TBS. Then, the blot is incubated in secondary antibody-Goat anti-rabbit IgG (Bangalore GENEi, India) ALP conjugate. The bands are visualized after staining with BCIP/NBT (Bangalore GENEi, India), which is a substrate for ALP conjugate. Proteases are ubiquitous and difficult to separate from proteins. Hence, protease inhibitor, PMSF is used throughout the procedure for successful isolation of total protein from the plants. Protein solutions are stored in aliquots at 2–8 ºC or at –70 ºC after addition of 20% glycerol and shock freezing in liquid nitrogen. During experiments, proteins are always maintained on ice. Vigorous pipetting and vortexing are avoided. β-mercaptoethanol is avoided in the extraction buffer as it can form disulfide bridges with thiol groups of proteins leading to aggregation and/ or inactivation. Molecular weight of amino acids ranges between 75 and 204 Da. For calculations, the average molecular weight of amino acid (110 Daltons) is considered. Ponceau stain is used only in the initial experiments; later this step is avoided in the western blot for checking the transfer, as the proteins are visible even without using the stain while drying the blot.

RT-PCR:

Total RNA is also isolated from the leaves without any stress or pathogen treatment using TRI-reagent (Sigma-Aldrich, USA). For RT-PCR, the first strand of the cDNA is synthesized by reverse transcription with an oligo-(dT) primer and M-MLV reverse transcriptase enzyme (Sigma-Aldrich, USA). The program for the RT-PCR is followed as per the instruction previously.

Biological Software used for detection of deducing the amino acid sequence:

Nucleotide and deduced amino acid sequence comparisons are made using the BLAST (Basic Local Alignment Search Tool) programs such as BLASTN (Zhang et al., 2000) and BLASTX (Altschul et al., 1997) respectively, on the non-redundant database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Expasy tool is used to deduce the amino acid sequence. Clustal W was used for multiple sequence alignments.

Transformation of Tobacco leaf discs (Horsch et al. 1985):

Tobacco leaves were surface sterilized by rinsing in 400 ml of water containing 5% of commercial bleaching powder and 20 drops of Tween 20. The leaves were rinsed for 5 minutes in distilled water to remove the bleach and the surfactant. The midribs of the leaves were cut out and the lamina was cut into uniform squares using a sterile blade. The leaf pieces were rinsed for 5 minutes in sterile distilled water 5 times followed by the treatment with $HgCl_2$ (0.01%) for 5 minutes. Then, they were washed with sterile water five times for 5 minutes each. The leaf pieces were carefully placed on sterile tissue paper and the wound the leaf edges and were cut out carefully. They were carefully kept in plates containing pre-solidified MS medium with 2 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. The leaf discs were co-cultivated with *Agrobacterium* for a period of 3 days and then they were transferred to regeneration medium containing the antibiotic cefatoxime to control bacterial overgrowth.

In one to two weeks the leaf disks start expanding and callusing. At that stage, the leaf pieces were subcultured to the bottles containing regeneration medium i.e. MS medium

with 2 mg l^{-1} BAP and 0.1 mg l^{-1} NAA. Each piece was cut into four and the pieces were pressed into the medium in order to ensure a good contact between the plant tissue and the medium. Shoots started appearing about 3 weeks after inoculation and they generally grew from the edge of the disks or from internal area wounded by the forceps. When the shoots were at least 5-mm long, before they were excised avoiding any callus tissue and the shoots were carefully placed in the rooting medium i.e. MS medium. Cefotaxime was kept in the medium to avoid *Agrobacterium* growth. The rooted plantlets were then taken out from the medium and the agar was washed from the root system under running tap and the plantlets were transferred to a mixture of sterile soil and vermiculite (1: 3). To harden the *in vitro* plantlets, which need to develop a cuticle to control water losses, each plantlet was covered with a polythene bag.

Development of transgenic tobacco plants:

Cysteine Protease gene (size 1083bp, accession no. EU310415) was isolated from wild species of peanut, *Arachis diogoi* after *Phaeoisariopsis personata* pathogen challenge (Kumar and Kirti, 2011) and cloned in pTZ57R vector (MBI Fermentas, Germany) at *Xho*I and *Apa*I restriction sites. A plant gene promoter viz., tapetum specific promoter, TA29 from tobacco (Koltunow et al. 1990) was used for driving cysteine protease expression. TA29 promoter from genomic DNA and T-NOS terminator from plasmid DNA of the binary vector were amplified separately and cloned in the pTZ57R vector. Finally, the TA29 promoter, cysteine protease and T-NOS terminator were excised with specific enzymes as mentioned in Table 1 and cloned in the binary vector pCAMBIA2300 in the sense orientation. The confirmed recombinant clones were mobilized into *Agrobacterium tumifaciens* strain LBA4404 (pAL4404) using freeze-thaw method (Chen et al. 1994).

Transformed tobacco plants were generated using standard leaf disc transformation procedure. The regenerated putative transgenic plants were acclimatized at 28 $^{\circ}$C in growth room and then transferred in the green house for further molecular analysis.

Scanning electron microscopy (SEM):

Pollen grains from both the transgenic as well as non-transgenic control plant anthers of flowering bud stage 7 were placed on adhesive coated aluminium stubs and coated with gold using a sputter coater (Quorum–model Q150RES). For SEM observations, pollen grains were photographed by using 10 KV in a Phillips ESEM, model XL30 electron microscope.

Pollen fertility analysis:

Pollen grains from three flowers of each transgenic line were tested for viability assay using acetocarmine staining method. Pollen germination assay was done on 10% sucrose and 3 mgl^{-1} boric acid media using same flower, >500 pollen grains were randomly selected for observation from each line. Based on viability test and germination assay, plants were classified as fertile, sterile and semi-sterile.

Histological analysis:

Anthers from early stages of development were fixed in glutaraladehyde, dehydrated in ethanol series and embedded in paraffin wax. Sections of 10µm were cut through microtomy (LeicaRM 2125). For microscopic studies, tissue was stained with toludine blue and Bright field images of an anther cross section were taken with an Olympus UCTR30-2 microscope.

Progeny Analysis:

The male sterile plants were backcrossed with untransformed fertile control plants to obtain seeds for T_1 plants. For genetic segregation analysis of *neomycin phosphotransferase* II (*npt*II) plants, seeds obtained from T_0 were germinated on media containing 125mgl^{-1} kanamycin for the selection of T_1 transgenic plants. After 15 days, germinated seedlings with dark green leaves were scored as positive (carrying transgene) and non-germinated or bleached seedlings as negative. The ratio of positive and negative seedling was taken for statistical analysis to predict the number of insertion of the transgene.

Protein extraction and two-dimensional electrophoresis (2-DE):

Flower bud of 3.0 mm to 1.0 cm size were collected and immediately kept in liquid nitrogen. Samples were stored in –80 $^{\circ}$C freezer. Anther protein extraction was done using a phenol extraction method (Sengupta et al., 2011; Saravanan and Rose, 2004). Proteins were resuspended in 100 µL of the rehydration solution [8M (w/v) urea, 2M (w/v) thiourea, 4% (w/v) CHAPS, 30 mM DTT, 0.8% (v/v) IPG buffer pH range 4–7 (GE, Healthcare)] and the protein concentration was determined by Bradford method. Proteins were resolved by two-dimensional electrophoresis (2-DE) as describe by Sengupta et al. (2011). It involves active rehydration of protein (450 µg) on immobilized pH gradient (IPG) strips (11 cm, 4–7 pH linear gradient; Amersham, GE) for 12 hrs at 50 V. Isoelectric focusing (IEF) was done in Ettan IPGphor II (GE Healthcare). After IEF, the strips were equilibrated twice for 30 min with gentle rocking at room temperature in equilibration buffers (6M urea, 50 mM Tris-HCl buffer (pH 8.8), 30% (w/v) glycerol, 2% (w/v) SDS).

First equilibrium was performed in a solution containing equilibrium buffer with 2% DTT and second with 2.5% (w/v) iodoacetamide instead of DTT. Second dimension separation of proteins was done through SDS-PAGE (12% vertical polyacrylamide slab gels). The gels were stained with modified colloidal coomassie blue, scanned densitometric scanner (GE, Healthcare) and analyzed (normalization, spot matching, expression analyses, and statistics) using Image Master 2-D Platinum version 7 image analysis software (GE, Healthcare). The Spots showing statistically significant ($P < 0.05$) increase was selected and manually picked for digestion and identification. In gel trypsin digestion and MALDI-TOF/TOF mass spectrometer matrix- assisted laser desorption/ionization time of flight mass spectrometric (MALDI-TOF MS) analysis was conducted with a MALDI- TOF/TOF mass spectrometer (Bruker Autoflex III smartbeam, Bruker Daltonics, Germany) according to the method described by Shevchenko et al. (1996) with slight modifications. The data were analyzed by similarity search for mass value against sequence information from NCBInr and Swiss Prot database using a MASCOT program (http://www.matrixscience.com).

Methodology for *in-silico* analysis of cysteine protease and cystatin interaction

Data set:

Cysteine protease (FJ716616.2) and cystatin (EU723567.1) encoding genes were identified from *Arachis diogoi* and their nucleotide sequences and amino acid sequences were deposited in NCBI. We have considered these deposited amino acid sequences for the modeling of complex cysteine protease with the inhibitor cystatin. Templates were identified that contained a cysteine protease in complex with inhibitor irrespective of the

source of occurrences of these proteins (Tastan Bishop and Kroon, 2011). Complex cysteine protease with inhibitor templates were identified by key word search in PDB and by BlastP (http://www.ebi.ac.uk/blast) (Altschul et al., 1990). Eight crystal structures (table 1) were identified and all the known 3-D structural data of biological information were retrieved from PDB (Dutta et al., 2008) (www.rcsb.org/.). Pairwise sequence alignment was done by Emboss package (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) employing needleman-wunsch algorithm. BLOSUM62 matrix, an open gap penalty of 10 and an extension penalty of 0.5 were the parameters employed for pairwise sequence alignment.

Structure prediction and validation:

3D models of Complex protein of Cysteine protease with cystatin was modeled using the modeller software well-known computer program for comparative protein structure modeling. MODELLER 9v8 (Eswar et al., 2008) implements comparative protein structures modeling by satisfaction of spatial restraints (Sali and Blundell, 1993). Complex AdCysPro and cystatin of target sequence was modeled with the all available eight crystal complex structure of cysteine protease with an inhibitor. For each template-target pair, approximately 10 models were constructed and the accurate model was validated based on the PROCHECK, Profile-3D and the RMSD values. PROCHECK is a suite of program that offers a detailed analysis on the stereochemistry of a protein structure (Laskowski et al., 1993) where the structure was validated by Ramachandran plot (Ramachandran et al., 1963). RMSD check with respect to Cα atoms was performed by submitting the modeled protein complexes and the template structure to InsightII. The complex modeled structures were minimized with AMBER 3.0 (Case et al., 2005) with 100 steps of steepest descent

and 1500 steps of conjugate gradient optimization. The minimized complex models were further evaluated by calculating energy scores DOPE Z score (Shen and Sali, 2006) and reliability score Ga341 score (Melo et al., 2002). DOPE (Discrete Optimized Protein Energy) is a statistical potential used for the evaluation of homology in protein structure prediction. DOPE is based on an improved reference state that corresponds to noninteracting atoms in a homogeneous sphere with the radius dependent on a sample native structure; it thus accounts for the finite and spherical shape of the native structures. Lower the normalize z-DOPE score better the model is. GA341 score derived from statistical potentials (Melo et al., 2002). A model is predicted to be reliable when the model score is higher than a pre-specified cutoff (0.7) or near to one. Based on the above, the best fit model with lowest energy suitable one was selected for cysteine protease-cystatin interaction studies. Complex cysteine protease and cystatin interactions were observed using discovery studios 3.5 (http://accelrys.com/products/discovery-studio/visualization-download.php). Also, complex models were checked for presences of any errors, models was submitted to ProSA server (https://prosa.services.came.sbg.ac.at/prosa.php) validate the structure based on the score and energy plot (Wiederstein and Sippl, 2007). Z score value of all the known PDB (X-ray, NMR) structure was plotted and checked whether Z score of the query model falls within the range or not. If the z score falls out of the specified range, then it infers the structural errors.

Chapter 4
Development of male sterile transgenic tobacco expressing a pathogen induced cysteine protease from wild peanut, *Arachis diogoi*

Results:

1. Cloning and characterization of Arachis Cysteine protease (AdCysPro):

Cysteine protease was identified as one of the genes that have been differentially expressed by a wild peanut *Arachis diogoi* challenged with the germinating conidia of the late leaf spot pathogen, *Phaeoisariopsis personata* (Rajesh Kumar and Kirti; 2011). *Arachis* cysteine protease is composed of 1083 bp (**Fig. 4.1.1**) long nucleotide sequence, which codes for a protein of 360 amino acids (**Fig. 4.1.2**). Comparison with other cysteine proteases (**Fig. 4.1.3**) revealed that (i) AdCysPro is a pre-pro-protein; with (ii) a highly hydrophobic region (amino acid 2–18) putatively that functions in vacuole-targeting; (iii) Gln-145, Cys-151, His-293 and Asn-320 amino acid residues in the cysteine proteinase active center; (iv) the highly conserved Gly-Cys-Asn-Gly-Gly motif corresponded to amino acids 197–201; and (v) the cysteine residues involved in disulphide-bridge formation are Cys-147/Cys-198, Cys-182/Cys-231 and Cys-287/Cys-341. It also has a consensus motif GxNxFxD and the non-contiguous ERFNIN signature (Ex3Rx3Fx3Nx3I/Vx3N) in pro region (**Fig. 4.1.4**). It shows significant similarity with Glycine, Rd19a like cysteine protease (75%), Brinjal SmCP Cysteine protease (72.78%), *Arabidopsis*, Rd19, (67.78%), and tobacco (67.5%).

2. Preparation of TA29-cysteine protease construct:

Cysteine protease gene was amplified and cloned in pTZ57R vector (**Fig.4.2.2**). Clones were confirmed through restriction digestion and sequencing. Tapetum specific promoter (P-TA29, 870 bp) was used to drive the expression of cysteine protease gene. TA29 promoter is a well characterized, dominant, non-leaky and tapetum specific promoter for

expressing genes for male sterility. It was amplified from tobacco genomic DNA using TA29 promoter specific forward and reverse primers and cloned in pTZ57R vector (**Fig.4.2.1**). Clones were confirmed through restriction digestion with *Kpn*I and *Xho*I enzymes and sequencing. Similarly, T-NOS terminator from plasmid DNA of the binary vector were amplified separately and cloned in the pTZ57R vector and cloned were confirmed through restriction digestion (**Fig.4.2.3**).

Figure 4.1.1 Nucleotide sequence of *Arachis diogoi* cysteine protease (FJ716616.2)

```
ATGGCTCGCCTCTCCCTCCTCCTCCTCCTCGTCGCCGCCGTCGCAACCGCCGTCGACGACCAAGCCG
ATCCCTTGATCCGTCAAGTAACCGATGGAGACCATCACATGCTCAACGCCGAGCACCACTTCACAACCTT
CAAGACTAAGTTCGGAAAGTCTTATGCCACTCAAGAAGAGCACGATTACCGCTTTGGCGTCTTCCGGGCG
AACCTGAGGAGGGCGAAGCTGCACGCGAAGCTGGATCCGTCGGCGGAGCACGGTGTCACGAAATTCTCCG
ATCTGACGCCAGAGGAGTTCAAGAGGCAGTACCTTGGGTTGAAGCCGCTGCGGCTTCCGTCGACCGCTAA
CAAGGCTCCAATCCTGCCGACGAGCGATCTTCCAGAGAATTTCGATTGGCGTGACAAGGGTGCTGTTACT
CCAGTCAAGAACCAGGGCTCTTGTGGTTCATGTTGGGCGTTTAGCACGACTGGGGCTTTGGAAGGCGCTC
ATTATCTGTCCACCGGAGAGCTTGTCAGCCTTAGTGAGCAACAGCTTGTAGACTGTGACCATGTGTGTGA
TCCAGAAGAATATGGCGCATGTGACGCAGGCTGCAATGGTGGGTTGATGAACAATGCTTTCGACTACATA
CTCCAGGCTGGAGGAGTACAGACAGAGAAGGACTATCCTTACAGTGGAAGAGACGAGACCTGCAAATTCG
ACAAGAGCAAGGTCGCAGCTACAGTTGCGAATTTCAGTGTGGTTTCCCTTGATGAAGACCAAATTGCTGC
AAATCTAGTGAAGCATGGCCCTCTTGCAGTTGGTATCAATGCAATATTCATGCAGACATACATAGGGGGA
GTCTCATGCCCCTACATCTGCGGCAAGAATCTGGATCATGGCGTGCTCCTAGTGGGTTATGGCGCGGCCG
GATATGCTCCCATTCGCTTCAAGGACAAGCCATTCTGGATCATCAAGAATTCATGGGGAGAGAGCTGGGG
AGAGGATGGATACTACAAGATCTGCAGGGGTAAGAATGTGTGTGGAGTGGATTCCATGGTCTCAAGTGTA
GTAGCTACTACATTTACATCTAGCAACAATTAA
```

Figure 4.1.2 Deduced Amino acid sequence of cysteine protease (360 aa):

```
MARLSLLLLLLVAAVATAVDDQADPLIRQVTDGDHHMLNAEHHFTTFKTKFGKSYATQEEHDYRFGVFQANLR
RAKLHAKLDPSAEHGVTKFSDLTPEEFKRQYLGLKPLRLPSTANKAPILPTSDLPENFDWRDKGAVTPVKNQG
SCGSCWAFSTTGALEGAHYLSTGELVSLSEQQLVDCDHVYDPEEYGACDAGCNGGLMNNAFDYILQAGGVQTE
KDYPYSGRDETCKFDKSKVAATVANFSVVSLDEDQIAANLVKHGPLAVGINAIFMQTYIGGVSCPYICGKNLD
HGVLLVGYGAAGYAPIRFKDKPFWIIKNSWGESWGEDGYYKICRGKNVCGVDSMVSSVVATTFTSSNN
```

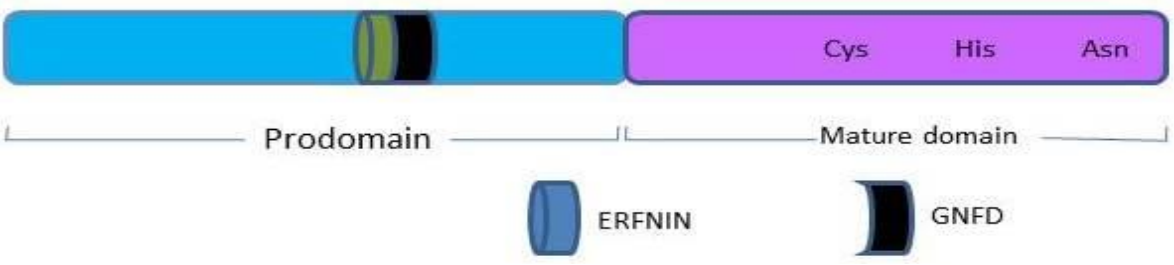
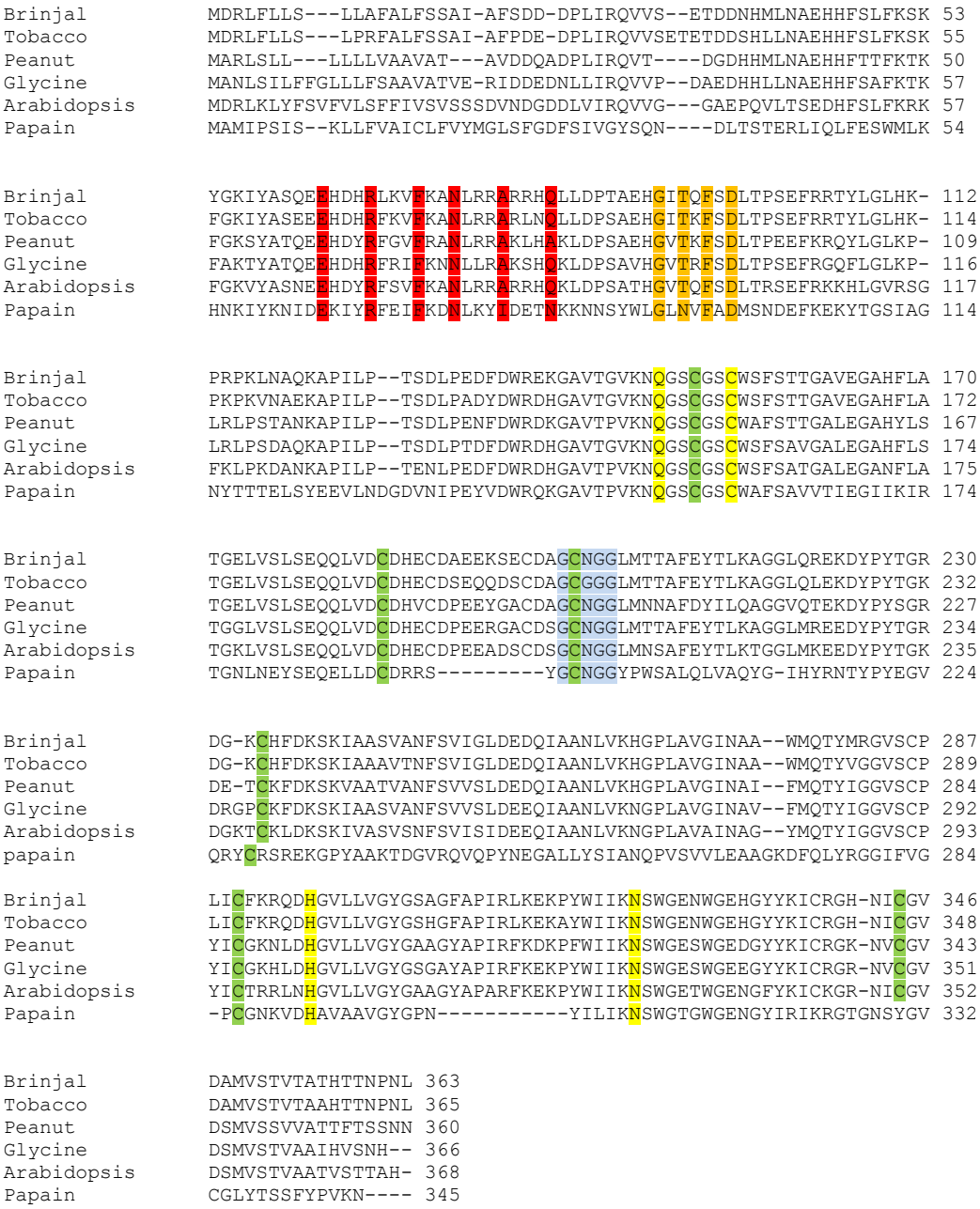
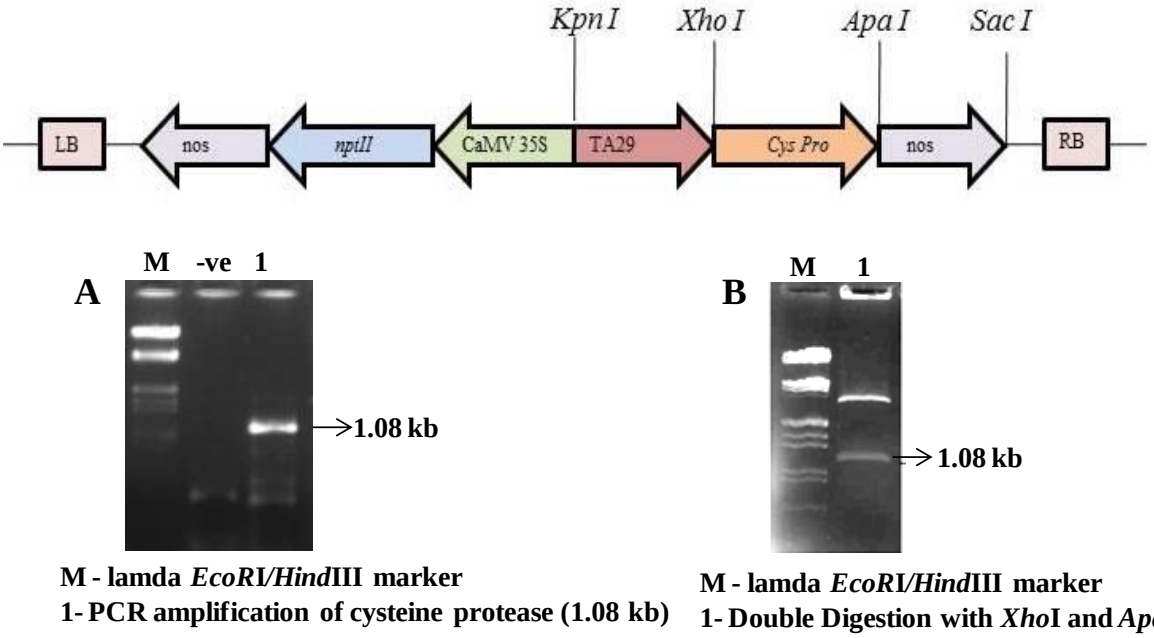


Figure 4.1.3 Schematic diagram of cysteine protease

Fig 4.1.4 Comparison of *Arachis diogoi* cysteine protease with other plant cysteine proteases



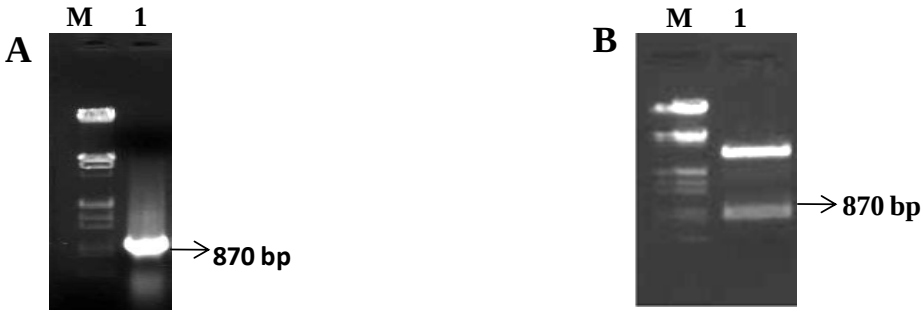
Subsequently, cysteine protease, TA29 promoter and T-NOS were transcriptionally fused and cloned in binary vector pCAMBIA2300 (**Table 3, Fig. 4.2.3**). Final construct was then mobilized into *Agrobacterium tumefaciens* strain LBA4404 using freeze-thaw method as described in the section Material and Methods. The presence of recombinant pCAMBIA2300 in bacteria was confirmed and subsequently used for plant transformation



M - lamda *EcoRI/Hind*III marker
1- PCR amplification of cysteine protease (1.08 kb)

M - lamda *EcoRI/Hind*III marker
1- Double Digestion with *Xho*I and *Apa*I

Figure 4.2.1 Cloning of Cysteine protease (1.08 kb) in pTZ57R (**A**, **B**)



M - lamda *EcoRI/Hind* III marker
1- PCR amplification of TA29 promoter (870 bp)

M - lamda *EcoRI/Hind* III marker
1- Double Digestion with *Kpn*I and *Xho*I

Figure 4.2.2 Cloning of TA29 promoter (870 bp) in pTZ57R (**A**, **B**)

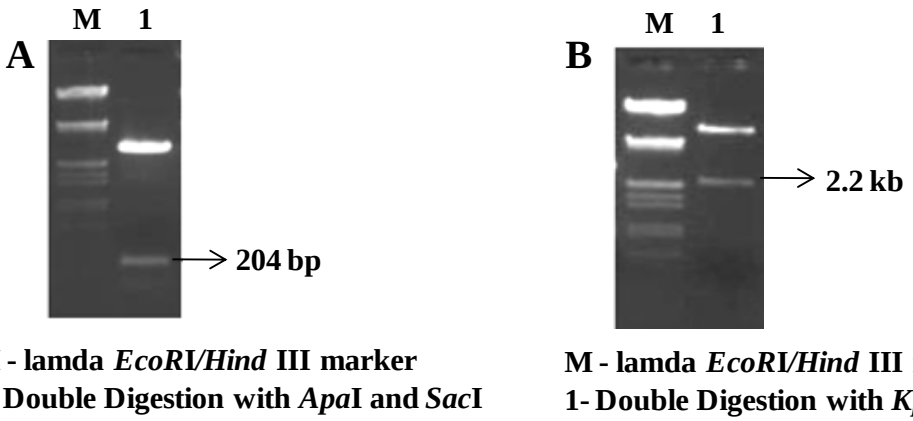


Figure 4.2.1 Preparation of TA29-cysteine protease construct. **A**. Cloning of T-NOS terminator in pTZ57R vector, **B**. A transcriptional fusion of TA29 promoter, cysteine protease and NOS-terminator for preparation of cysteine protease cassette and cloned in binary vector pCAMBIA2300.

3. Tobacco transformation:

Tobacco transformation was done using the standard tobacco transformation protocol using leaf discs (Horsch et al. 1985). After transformation, the explants were kept on MS medium supplemented with 2 mgl^{-1} BAP and 0.1 mgl^{-1} NAA and 250 mgl^{-1} cefotaxime and 125mgl^{-1} kanamycin in the media. Shoots were obtained about a month after initiation of the transformation experiments. Rooting occurred when the shoots were transferred to the growth regulator free half strength MS medium (rooting media), and root formation occurred in about two weeks (**Fig. 4.2.2**). Rooted plantlets were hardened in a mixture of sterile vermiculite and soil (3:1).

4. Screening of putative transgenic plants:

In different transformation experiments 15 independent putative transgenic plants were generated. Total DNA was isolated from putative transgenic plants including non-transformed control plant. Presence of the transgene was confirmed by PCR amplification

using the forward and reverse primers for the marker gene *nptII*. PCR amplified 700 bp fragment of *nptII* gene was observed in all transgenic plants and was absent in non-transgenic control plants.

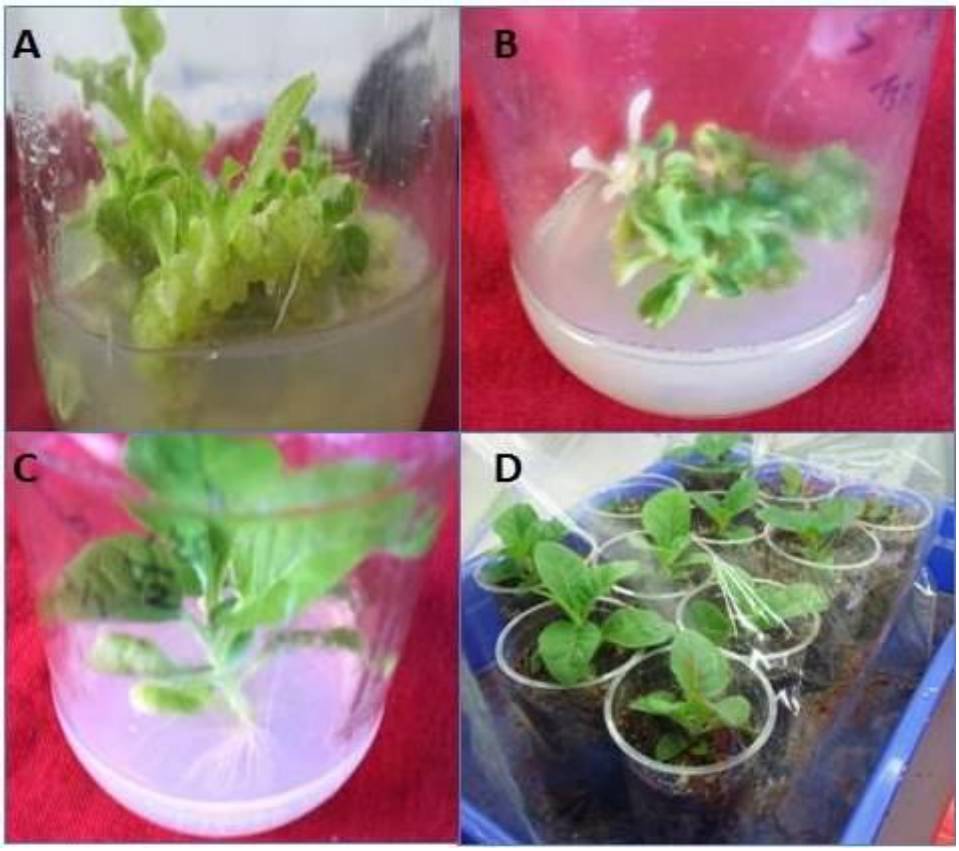


Figure 4.3.1 Different stages of tobacco regeneration after transformation. (A) Shoot Induction from callus on shoot inducing medium (MS + BAP 2.0 mg l^{-1} + NAA 0.1 mg l^{-1}), (B) Shoot Elongation on medium (MS + BAP at 1 mg l^{-1}+ NAA 0.1 mg l^{-1}), (C) Rooted plantlet and (D) Hardening of plants in the glass house.

In addition to this, for further confirmation of transgene integration, total genomic DNA was isolated and digested with *Hin*dIII enzyme, which did not have a site in *nptII* gene. Hence, Southern hybridization was performed using *nptII* as a probe, which indicates the number of sites of integrations, which correspond to the number of bands obtained in the autoradiograph. Different copy numbers were observed in the transgenic plants ranging

from single copy to multiple copies. There were four plants having single copy integration in the genome and were designated as T1, T2, T4, T6.

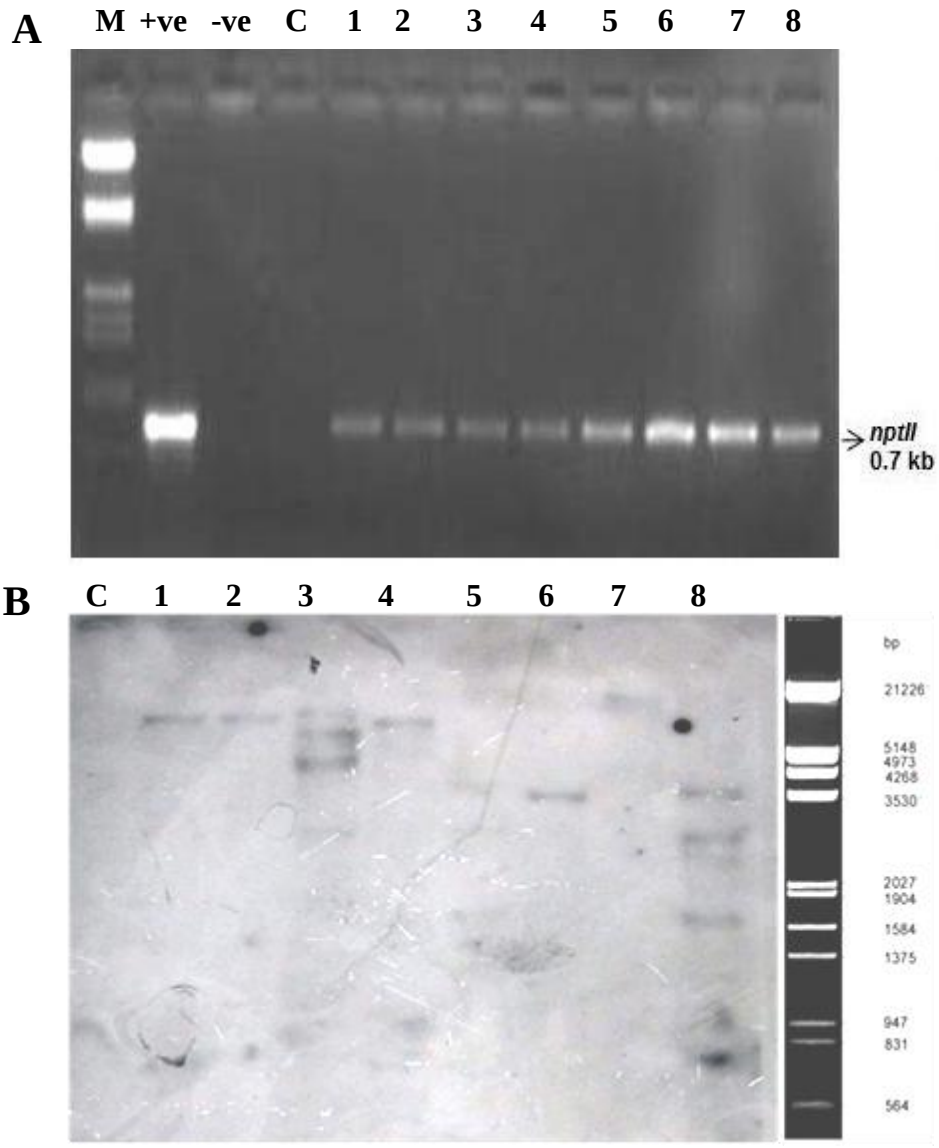


Figure 4.4.1 Molecular analysis of T_0 TA29-Cysteine protease transgenic tobacco plants.

- **A.** PCR confirmation of TA29-Cysteine protease transgenic plants using *nptII* forward and reverse primer that would amplify 0.7 kb fragment.
- **B.** Southern analysis of T_0 transgenic plants. Genomic DNA of non-transformed control plant (C) and T_0 transgenic plants were digested with *Hin*dIII and restriction fragments electrophoresed, blotted and hybridized with [$^{\alpha\text{-}32}$P] –labeled *npt*II gene as a probe

5. Tapetal expression of cysteine protease:

During anthesis, flower morphology and their pollen production were analyzed **(Fig. 4.5.1)**. It was found that out of 15 plants, three plants were completely male (Transgenic plant numbered T1, T2 and T6) and nine plants were identified as semi- sterile (transgenic plant no. T4, T5, T7, T8, T9, T11, T12, T13, T15). Most prominent changes that occurred in the transgenic male sterile plant flowers include stamen filament and petal size. Stamen filament was reduced by a factor of 1.5 and petal size was decreased by a factor of 1.3 as compared to that of non-transformed control plants. Similar morphology was also observed in subsequent generation of male sterile plants. Male sterile anthers were small, shrunken, green colored, which dried up earlier compared to those in fertile flowers.

Pollen viability test was done with acetocarmine staining (**Fig. 4.5.2)**. It revealed that fertile pollen grains have stained positively with dark uniform coloration, while sterile plants pollen grains did not get stained or stained very lightly.

Furthermore, SEM observations also showed that the pollen of male sterile line were shrunken, deformed and with exine sculpturing showing smooth, spherical to elliptical, irregular shaped grooves, as a fossiculate orientation when compared to the normal control pollen having regulated perforated orientation over the exine (**Fig. 4.5.2)**.

The germination frequency of pollen grains was observed on sucrose-boric acid medium (**Fig. 4.5.3)**. Pollen grains from untransformed control fertile plants exhibited 60-70% of germination, while semi-sterile plants showed a decrease in pollen germination ability and completely sterile plants failed to germinate even after 24 hrs also (**Table 4.1**).

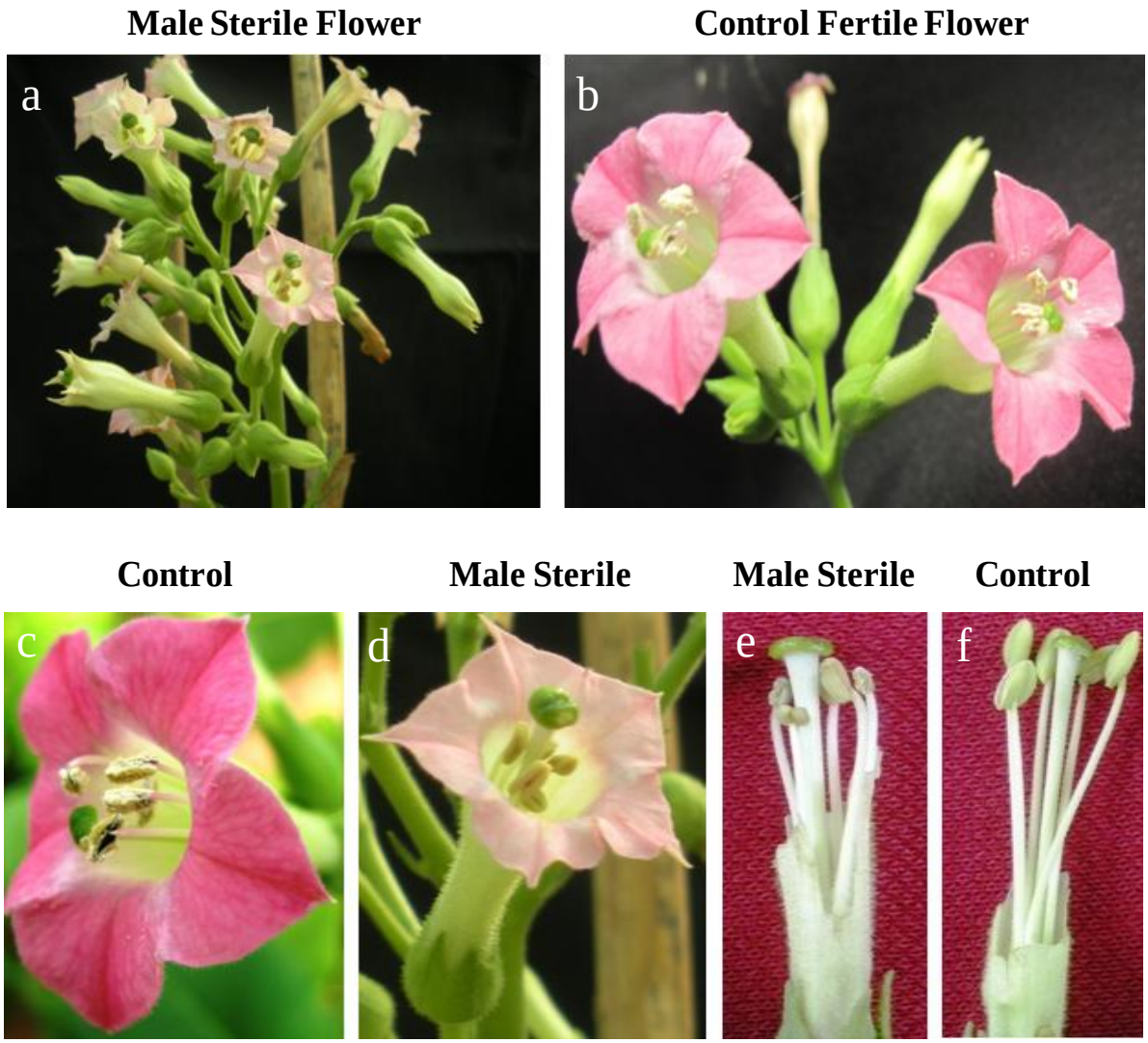


Figure 4.5.1 Comparison of Flower morphology of male sterile T2 transgenic line and fully opened non-transformed control plant. Fully opened male sterile flower (a, c). Fully opened non-transformed control flower (b, d). Stamen length has been reduced in male sterile transgenic line T2 (e) compared to the non-transformed control plant (f).

Control **Sterile**

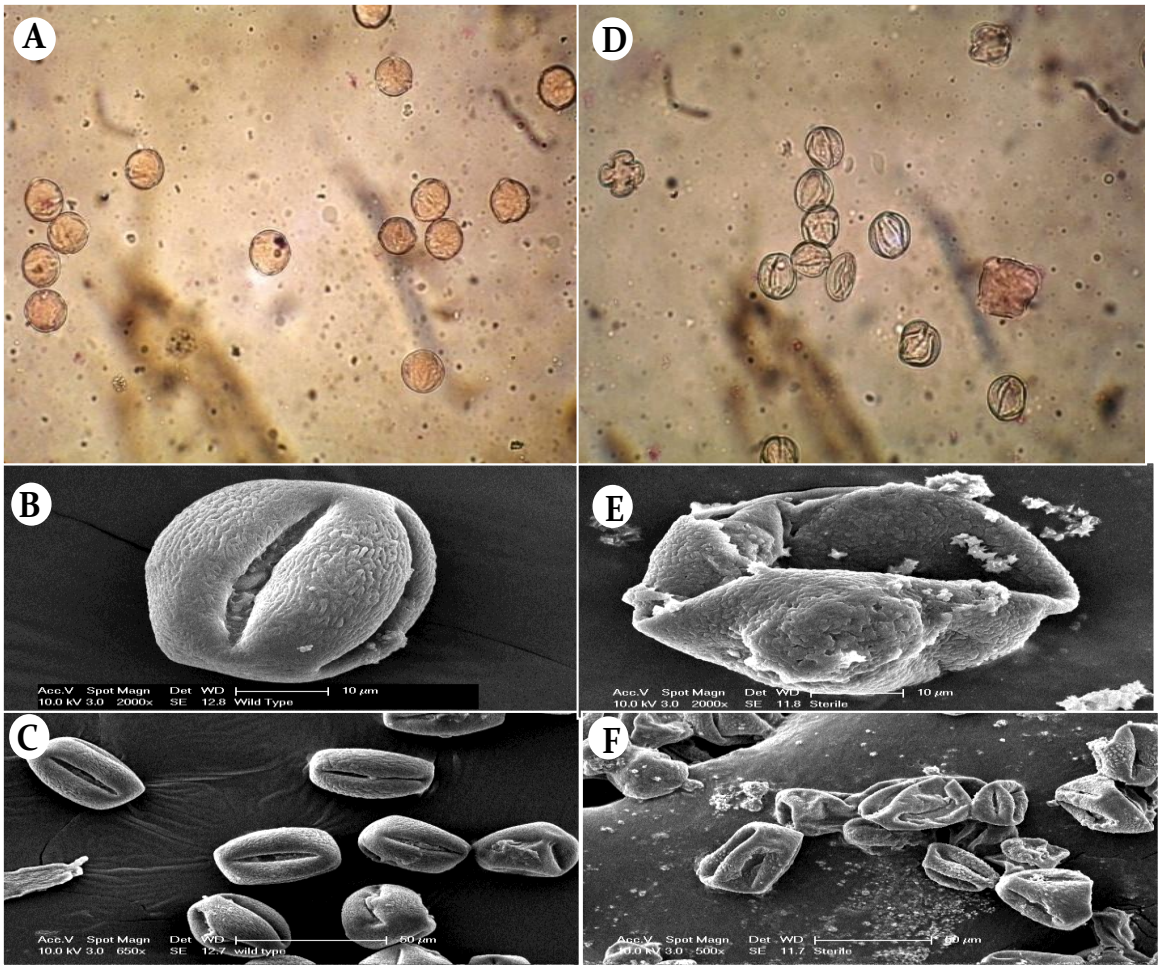


Figure 4.5.2 Pollen characteristic of male sterile transformed and untransformed control plant. **A, B, C** - Untransformed control plant pollen, **D, E, F** - Sterile Pollen

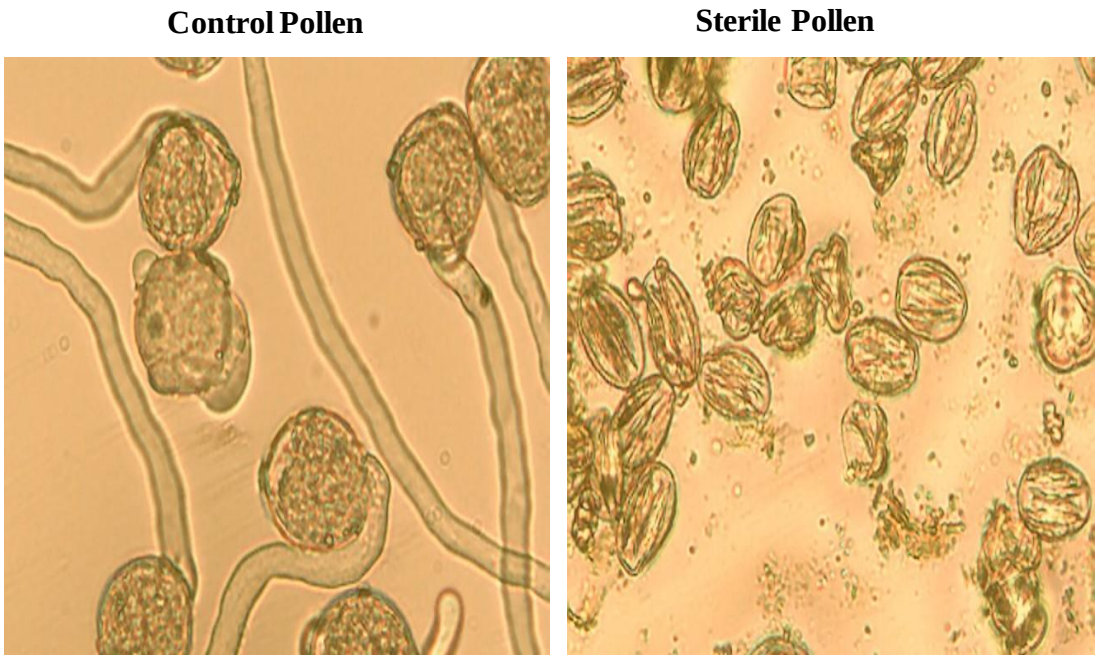


Figure 4.5.3 *In Vitro* pollen germination of untransformed control plant and sterile T2 transgenic plants. Pollen grains were germinated on sucrose-boric acid media and >500 pollen grains were observed

6. Fertility analysis:

Fertility in the transgenic plants was also determined by observing the capsule formation efficiency after selfing or backcrossing with pollen from non-transformed plants. Male sterile flowers were unable to form capsule even after selfing. However, manual pollination of male sterile flowers with the pollen grains from untransformed control plants resulted in a normal capsule formation.

7. Histological study of the anther:

Histological study was performed by taking the transverse section of the developing anther from the male sterile and non-transformed control plants (**Fig. 4.7.1**). Study of anther sections showed that fertile plants anther sacs were filled with normal round shaped

pollen grains while male sterile anther sacs were having very less and deformed pollen grains. Also, the tapetal layer in the anther has become ablates and appeared to be unsuitable for supporting the gametophytic tissues.

Table 4.1 Evaluation of pollen viability and *In Vitro* pollen germination rate in cysteine protease transgenic plants

Transgenic line	Pollen viability (%)	Germination Frequency (%)
T-1	0	0
T-2	0	0
T-3	95	62
T-4	17	5
T-5	56	37
T-6	0	0
T-7	13	9
T-8	67	33
T-9	42	28
T-10	86	57
T-11	11	7
T-12	23	13
T-13	28	17
T-14	93	72
T-15	39	24

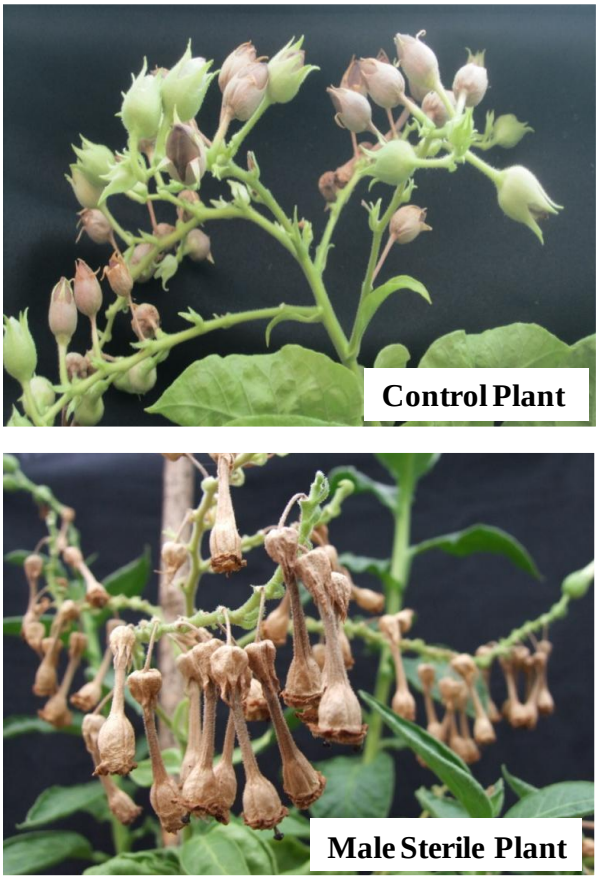


Figure 4.6.1 Capsule failed to form upon self-pollination in Sterile T2 line while normal capsule formed in untransformed control plant.

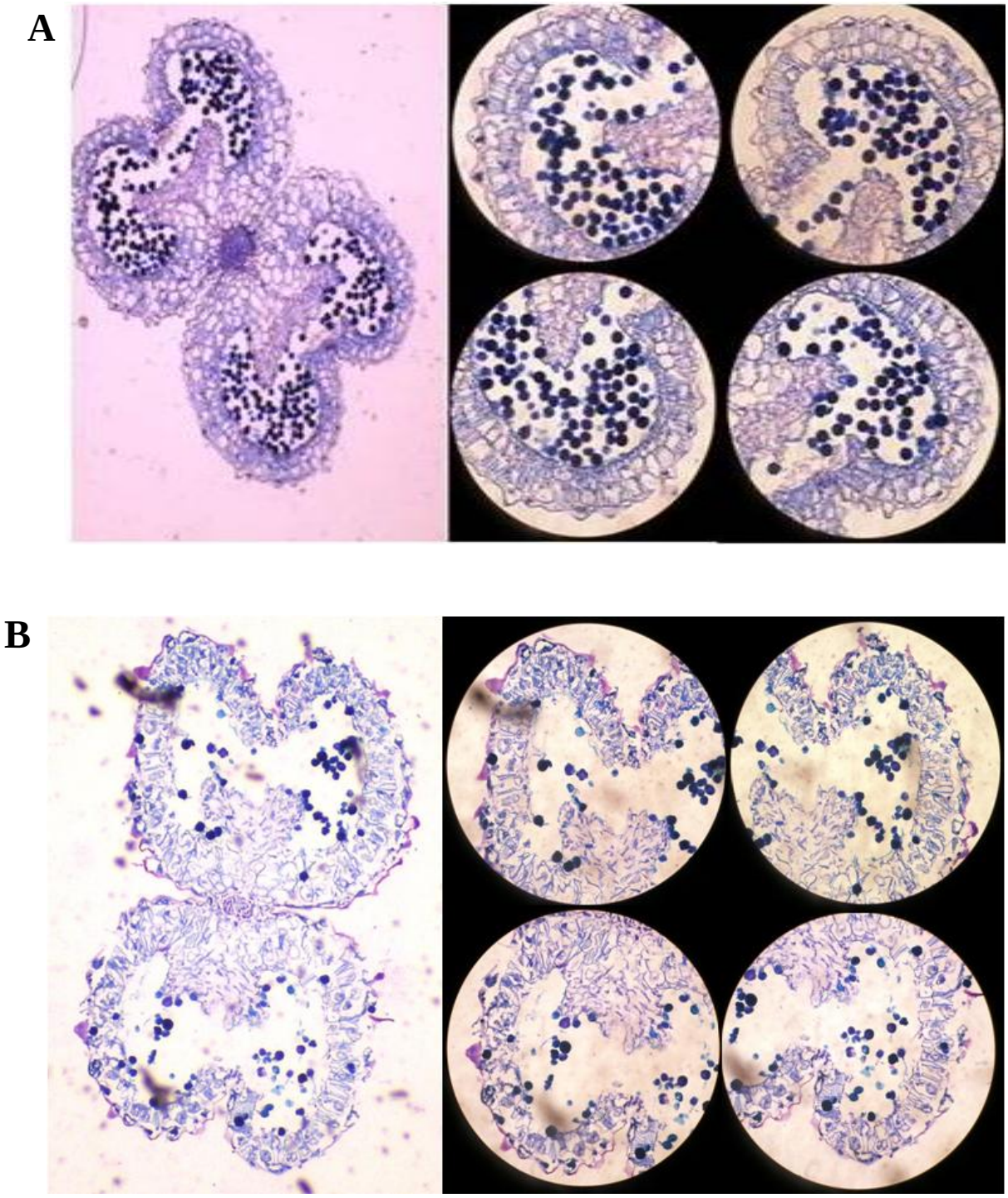


Figure 4.7.1 Transverse section of anther of untransformed control plant (**A**) and transgenic male sterile line (**B**)

8. Segregation analysis:

Segregation analysis was performed with the three male sterile (Transgenic plants designated as T-1, T-2 and T-6) transgenic plants pollinated with the pollen grains of untransformed control fertile plant. The selection medium contained 125 mg/l-1 kanamycin in the seed germination medium, which completely arrests the germination of seed from plants that do not express the *nptII* gene. Hence, the segregation into plants with the transgene and nulls can be observed. Seed germination of T_1 transgenic of male sterile plants showed 1:1 segregation ratio (**Table 4.2**) with respect to kanamycin resistance.

Table 4.2 Segregation analysis of male sterile T1 seeds by germinating on selection medium containing 125mg/l kanamycin

Male sterile T_0 plants	**No. of seeds germinated on selection medium**	**χ^2**	**Probability**
T-1	117/226	0.283	0.5947
T-2	68/132	0.12	0.7290
T-6	82/170	0.211	0.6459

9. Expression Analysis of cysteine protease in anther:

Total RNA was isolated from anthers of all the plants and semi-quantitative PCR analysis using cysteine protease specific primer was performed (**Fig. 4.9.1**). It was observed that complete male sterile plants showed high expression of the cysteine protease transcripts as compared to the semi-sterile plants and there was no expression of cysteine protease transcripts in the anther tissues of the untransformed control plant.

Western blot analysis with total protein from anther was performed to ascertain the production of transgene encoded protein, the cysteine protease (**Fig. 4.9.2**).

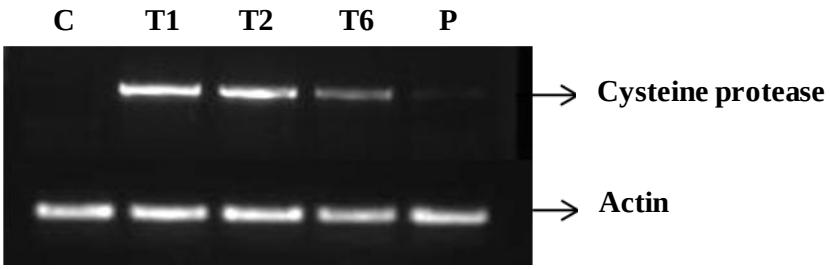


Figure 4.9.1 Semi-quantitative RT-PCR analysis of cysteine protease expression in the anther of untransformed control (C) and male sterile transgenic plants. cDNA was synthesized from total RNA from anther of control and male sterile transgenic plants and amplified with Cysteine protease specific forward and reverse primer. Actin served as an internal control.

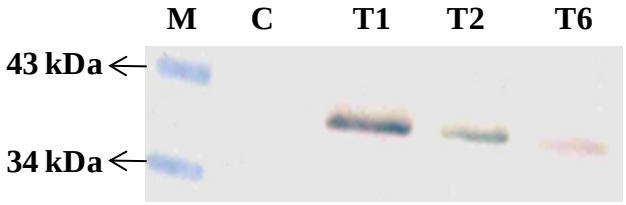


Figure 4.9.2 Western Blot analysis of transgene protein. Cysteine protease expression analysis in the anther of male sterile lines (T1, T2, T6) and untransformed control plant.

Complete male sterile plants exhibited increased in expression of cysteine protease and no signal were detected in the untransformed control plant. The expression of the transgene encoded protein is restricted to only anther and not detected in other tissues of complete male sterile plants.

10. Proteomic Analysis of cysteine protease induced male sterile transgenic tobacco:

To determine the molecular mechanism of cysteine protease induced male sterility in transgenic plants, the proteome of anthers from transgenic male sterile plants and untransformed control plant were analyzed by 2-dimensional electrophoresis followed by MALDI-TOF-TOF analysis (**Fig. 4.10.1**). Gel analyses using Image Master Platinum software suggested that more than 230 protein spots were reproducible and all gels showed

similar distribution patterns in 2D images (**Fig. 4.10.2**). Further analysis revealed that 56 matched spots showed significantly up-regulated (≥1. 5) (**Fig. 4.10.3**). Furthermore, we have identified two important proteins that are novel proteins up-regulated in the cysteine protease expressing transgenic tobacco plants, a patatin homologs and a Dicer like protein-4 through MALDI-TOF analyses.

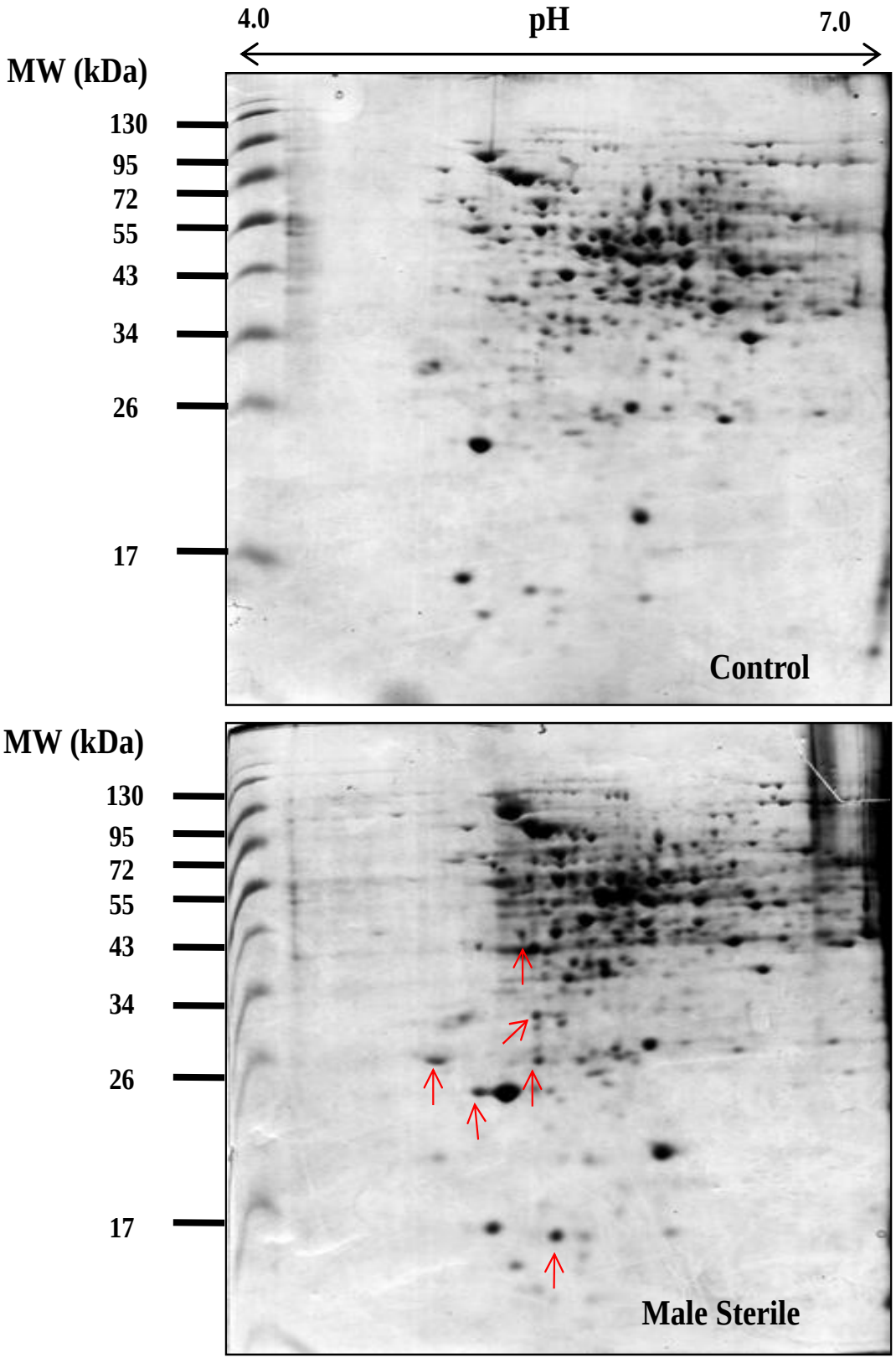


Figure 4.10.1 Colloidal Coomassie stained 2D gels of protein Extract from anther.

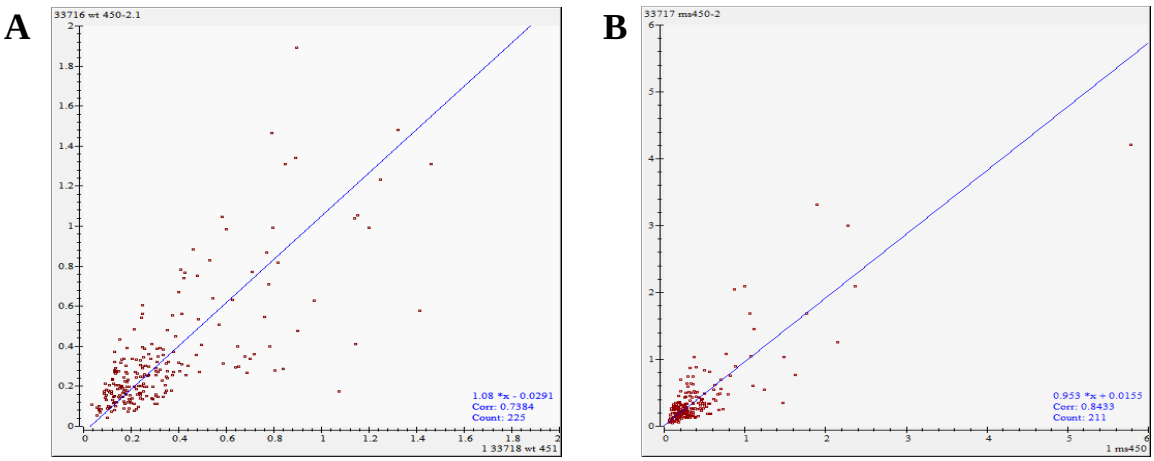


Figure 4.10.2 Scatter Plot of protein spots in 2D gels of anther from untransformed control (A) and Male sterile (B)

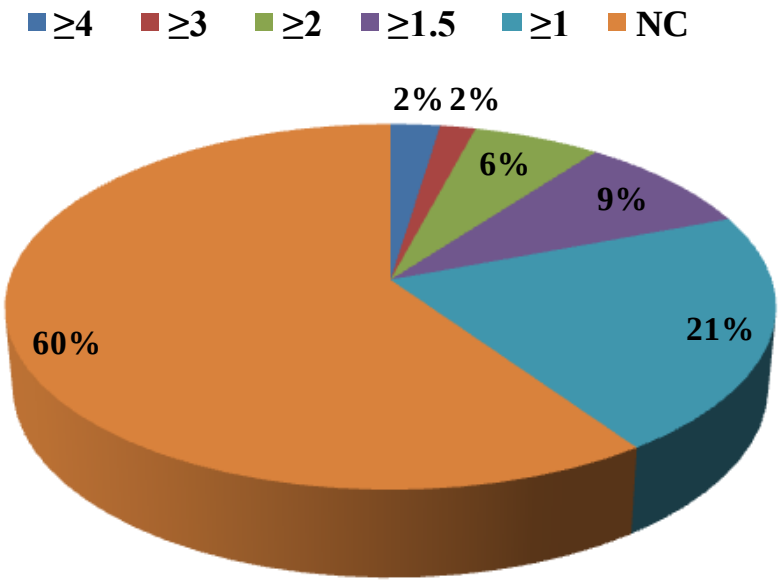


Figure 4.10.3 Protein Expression Profile Pattern: Fold change

Discussion:

Genetic engineering has become a successful and rapid approach for developing male sterility in many economically important crops. Most of these approaches were based on early destruction of tapetal cell lineages, which provide nourishment to the growing pollen grains. In general, tapetal cells undergo programmed cell death after nourishment during male gametophyte development. The timing of tapetal cell death is important for the maturation and release of pollen grains from pollen sacs. Any interference with the tapetal cell developments result in sterility of the pollen grains due to improper development tor arrest of pollen release.

In the present study, we have investigated the potentiality of a pathogen induced cysteine protease (AdCysPro) from *Arachis diogoi* for inducing male sterility in tobacco plants. AdCysP shows significant similarity with a cysteine protease (SmCP) of *Solanum melongena*. SmCP has been reported to be involved in developmentally regulated programmed cell death including leaf senescence, fruit senescence, xylogenesis and anther senescence (Xu and Chye, 1999; Xu et al., 2003). This suggests that AdCysPro may also have a role in programmed cell death and possibly it could be manipulated for inducing male sterility in crop plants. Early destruction of tapetal cell lineage in anther has been a widely used strategy for this purpose (Mariani et al. 1990, Nizampatnam et al. 2009). A tapetum specific promoter, TA29 from tobacco was manipulated to drive the expression of cysteine protease. The TA29 promoter has been shown to express during the early stages of tapetum development (Koltunow et al., 1990) and was efficiently used to drive the expression of genes involved in tapetum cell death (Mariani et al. 1990; Kriete et al., 1996; Nizampatnam et al., 2009).

In the present study, the TA29-cysteine protease construct was developed in binary vector pCAMBIA2300 and transformed into tobacco using the Agrobacterium strain LBA4404 to obtain transgenic plants. Stable transgene integration in the PCR positive plants was confirmed by Southern hybridization analysis. The confirmed transgenic plants were observed for flower morphology and their pollen production characteristics. Three plants have shown distinct flower morphology with a reduction in the length of stamen filament, which correlated with the arrest of microsporogenesis (Mariani et al. 1990). These three complete male sterile plants were found to have single copy integration of the T-DNA. However, single copy integration did not correlate with the level of male sterility of the plants as one of the plants with single copy integration plants did not exhibit complete male sterility. Furthermore, transcript analysis showed that male sterile plants displayed high expression of cysteine protease transcripts compare to partial sterile plants. A similar observation was also noticed by Kim et al. (2007)

SEM study and histological analysis further confirmed the male sterility as described earlier. Female fertility did not get affected as capsule formation was normal, when male sterile plants were pollinated with the pollen from untransformed control plant. This indicates that the tapetal expression of cysteine protease affected only male gametophyte development and did not meddle with the female gametophyte development.

These observations indicate that cysteine protease expression in tapetum resulted in improper microsporogenesis, which resulted in male sterility. RT-PCR and western blot analysis results showed the proper expression of the transgene and the cysteine protease expression could be correlated to male sterility of the transgenic plants.

In addition to this, we have observed the differential expression of the transgene encoded proteins in the transgenic plants and the untransformed control plant through proteomic analysis and we have identified patatin-like protein-2 homolog and the dicer-like protein-4, which were differentially up-regulated in male sterile anther proteome. Patatin like proteins act as lipid acyl hydrolases and hydrolyze phospholipids and galactolipids (Scherer et al., 2010). Induction of Patatin-like protein genes (NtPat1-3) from tobacco have been reported during HR in response to tobacco mosaic virus infection prior to JA accumulation (Dhondt et al., 2000). A pathogen induced patatin-like protein (PLP2) from *Arabidopsis* promoted cell death and negatively affected resistance against *Botrytis cinerea* and *Pseudomonas syringae*. In contrast to this, it also conferred resistance against cucumber mosaic virus (CMV), which is an obligate pathogen (La Camera et al., 2009). Patatin like protein expression was also reported in the anthers of potato, sweet pepper flowers and tobacco petal development (Drews et al., 1992; Vancanneyt et al., 1989). Recently, Rajesh Kumar et al. (2011) have reported the up-regulation of NtPAT3 in AdRSZ21 induces cell death of leaves. Dicer-like protein 4 (DCL4) was reported to act in the biogenesis of Trans-acting small interfering RNAs (ta-siRNAs) (Dunoyer et al., 2005). *Arabidopsis* DCL4 is reported to be involved in silencing of endogenous gene-nuclear-localized RNA binding protein (FCA). Because of this, DCL4 promoted the expression of endogenous genes by contributing to the "tidying up" of 3′ end formation at genes where, for some reason, effective termination and polyadenylation has not occurred (Liu et al., 2012). It was reported that transcriptome analysis of NaDCL4 silenced plant during plant-herbivore interaction (*Nicotiana attenuate-Manduca sexta* interaction) exhibited silencing of many genes including patatin-like protein encoding (Bozorov et al., 2012).

The expression of patatin-like protein and dicer-like protein in cysteine protease induce male sterile anther proteome suggested that all these proteins might act in a common pathway of programmed cell death. This might require further investigation.

Our results are analogous to BoCysP1 (Konagaya et al., 2008), where Brassica cysteine protease (BoCysP1) has been shown to be involved in PCD of the inner integument (Wan et al. 2002). Overexpression of BoCysP gene also has led to induce male sterility in *Arabidopsis* driven by A9 and A3 promoter individually. In contrast to this, silencing of OsCP1 (Lee et al., 2004) and NtCP56 (Xiao-mei et al. 2009) also result in the sterility of the pollen grains. It can be explained as there is an increase in protease activity during anther development (DeGuzman and Riggs, 2000). This suggests that the disturbance of the protease cascade prior to the tapetal cell PCD could lead to the male sterility. Detailed investigation is required to establish the link between AdCysPro and PCD.

In conclusion, using AdCysP as a plant gene for inducing male sterile crop plants would be a good approach in modern biotechnology. To some extent, it will address environmental and consumer safety related concern in using GM crops.

Table 4.3: Sequences of the oligoes/primers used in the present study

Name of the Primer	**Primer Sequence (5'-3')**
TA29 F	GGCGGTACCTTTTTGGTTAGCGAATGC
TA29 R	CGGGCTCGAGTTTTAGCTAATTTCTTTAAG
Cysteine protease F	GCCTCGAGATGGCTCGCCTCTC
Cysteine protease R	TCTAGATTAATTGTTGCTAGATGTAAATG
T-NOS F	TAGGGCCCTAATTCGGGGGATC
T-NOS R	CGG AGCTCGTCG ATCGACAAGC
Actin F	TGGCATCACACTTTCTACAA
Actin R	CAACGGAATCTCTCAGCTCC

Chapter 5

Restoration of male fertility in cysteine protease expressing male sterile transgenic plants using TA29-cystatin transgenic as a restorer line

I. Development of TA29-cystatin Transgenic Tobacco as a restorer line

Results:

1. Identification of *Arachis* cystatin:

Cystatin (297 bp) was identified from *Arachis diogoi* (**Fig. 5.I.1.1**). It was found that it encodes a small peptide of 98 amino acids (**Fig. 5.I.1.2**) and it shows significant homology with other plant cystatins.

Figure 5.I.1.1 Nucleotide sequence of *Arachis diogoi* cystatin (EU723567.1, 297 bp)

ATGGCAGCAGTGGGTGCACCTCGCGAAGTAGCCGGAAACGAGAACAGCCTCGAGA
TCGATAGTCTTGCTCGCTTTGCTGTCGATGAACACAACAAGAAACAGAATGGCCT
TCTCGAGTTTAAGAGGGTTATAAGTGCTAAGCAGCAAGTTGTTGCTGGGACTTTG
CACCACATCACTTTGGAGGCAGCAAGTGGTGATAGTAAGAATGTTTATGAAGCCA
AGGTGTGGGAAAAGCCATGGATGAACTTCAAGGAGGTTCAGGAGTTCAAGCTTGC
TGGTGATGGCTCCAATGCTTAA

Figure 5.I.1.2 Amino Acid Sequence of cystatin (98 aa)

MAAVGAPREVAGNENSLEIDSLARFAVDEHNKKQNGLLEFKRVISAKQQVVAGTL
HHITLEAASGDSKNVYEAKVWEKPWMNFKEVQEFKLAGDGSNA

Figure 5.I.1.3 Multiple Sequence Alignment of cystatin

```
Arachis         MAAVGAPREVAGNENSLEIDSLARFAVDEHNKKQNGLLEFKRVISAKQQVVAGTLHHITL 60
Glycine         MAALGGNRDVAGSQNSLEIDGLARFAVEEHNKKQNALLEFEKVVSAKQQVVSGTLYTITL 60
Solanum         MATLGGIREAGGSENSLEINDLARFAVDEHNKKQNALLEFGKVVNVKEQVVAGTMYYITL 60
Hevea           MAKLGGVKEVEGSANSVEINSLARYAVDDYNQKQNALLEFKKVVNAKQQVVAGTIYYITL 60

Arachis         EAASGDSKNVYEAKVWEKPWMNFKEVQEFKLAGDG---SNA 98
Glycine         EAKDGGQKKVYEAKVWEKAWLNFKEVQEFKLVGDA---PA- 97
Solanum         EATEGGKKKAYEAKVWVKPWQNFKQVEDFKLIGDA---ATA 98
Hevea           EVIDGGQKKVYEAKVWEKPWLNFKEVQEFKLIGDAPSDSTA 101
```

Comparison of *Arachis* cystatin with other plant cystatins suggested that this cystatin also has inhibitory motif Gln-X-Val-X-Gly (where X is any amino acid) in the central

region of the polypeptide chain and a Pro-Trp (or Leu-Trp) dipeptide motif in the C-terminal region and a conserved Gly residue in the N-terminal region (**Fig. 5.I.1.3**).

2. Preparation of TA29-cystatin construct:

Cystatin gene was amplified using gene specific primers (**Table 5.I.2.1**) and cloned in pTZ57R vector. Defensin apoplastic signal sequence was transcriptionally fused with cystatin gene through PCR and cloned in pTZ57R. Confirmed clone was digested with appropriate restriction enzymes and subsequently cloned in modified pCAMBIA2300 vector having *bar* gene as plant selection marker under the control of the constitutive 35S promoter along with TA29 promoter driving the expression of the chimeric cystatin gene with the T-NOS terminator in the expression cassette for cystatin.

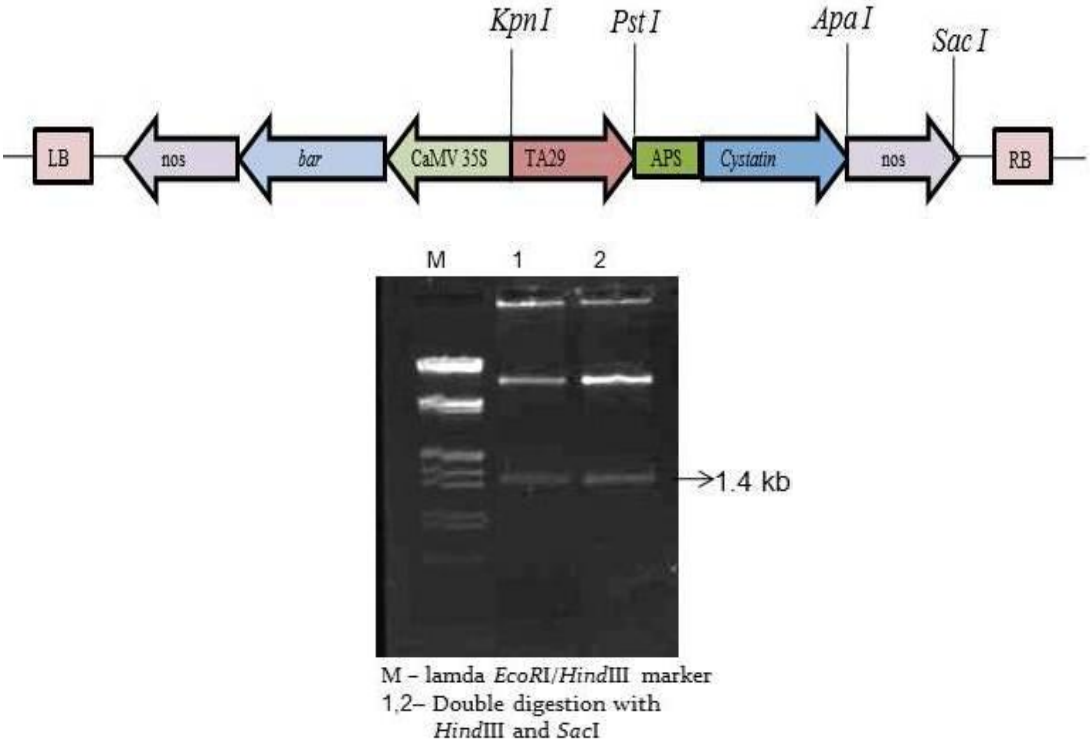


Figure 5.I.2.1 Preparation of TA29-cystatin construct. Transcriptional fusion of TA29 promoter, cysteine protease and NOS-terminator for preparation of cysteine protease cassette and cloned in modified binary vector pCAMBIA2300.

Clones were confirmed through restriction digestion (**Fig. 5.I.2.1**). Modified pCAMBIA2300 was prepared by removing *nptII* cassette through *Xho*I restriction digestion and replaced by bar cassette, which was amplified from pEGAD binary vector and cloned in pTZ57R. Recombinant modified pCAMBIA2300 was mobilized into *Agrobacterium tumefaciens* strain LBA4404 using freeze-thaw method as describe in material and methods. Confirmed bacterial strain having recombinant binary vector was used for plant transformation.

3. Tobacco transformation and Screening of putative transgenic:

Tobacco transformation was carried out using the standard tobacco transformation protocol (Horshe et al. 1985). Putative transformed shoots were selected on 250 mg/l cefotaxime and 1.0 mg/l phosphinothricine.

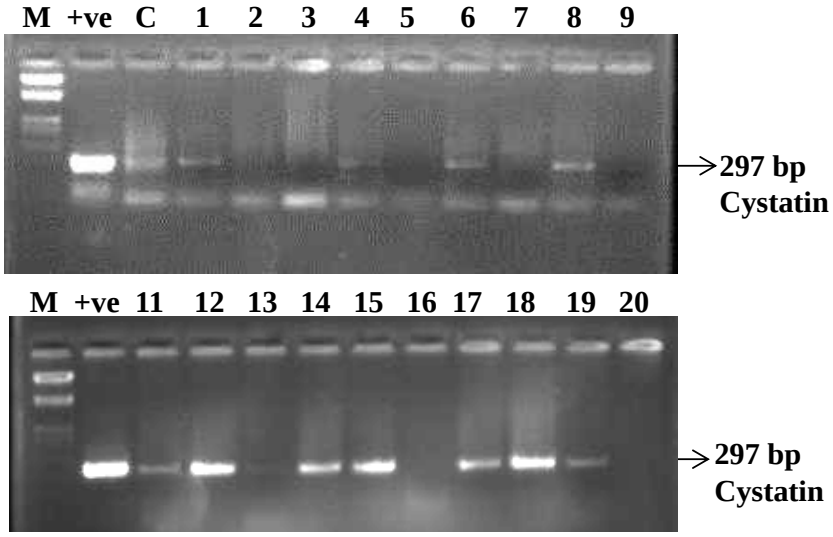


Figure 5.I.3.1 PCR confirmation of TA29-Cystatin transgenic plants using cystatin forward and reverse primers that would amplify the 297 bp fragment.

The presence of the transgene in the putative tobacco transgenic plants was confirmed by PCR amplification using forward and reverse primers of cystatin gene. PCR amplified 297 bp fragment of cystatin gene was observed in transgenic plants and was absent in non-transgenic control plants (**Fig. 5.I.3.1**).

4. Expression analysis of cystatin in anthers:

Total RNA was isolated from the anthers of PCR positive transgenic plants and semi-quantitative PCR analysis using cystatin specific primers was performed (**Fig. 5.I.4.1**). It was observed that the transgenic plants exhibited specific cystatin transcripts in anthers and three plants (#15, #17, and #18) showed high expression in anther. These high expression lines were selected as restorer lines. Transgenic plants displayed a similar phenotype as untransformed control plants without any noticeable changes in morphology

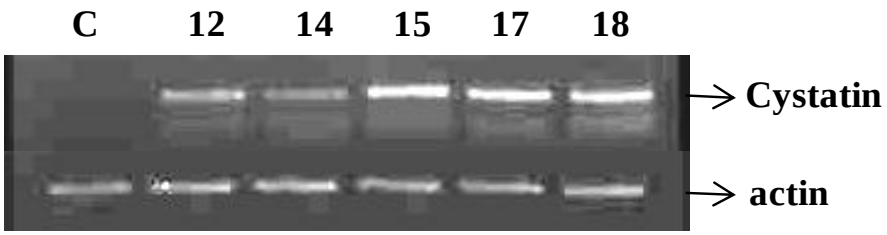


Figure 5.I.4.1 Semi-quantitative RT-PCR analysis of cystatin expression in the anther of untransformed control (C) and transgenic plants. cDNA was synthesized from total RNA from the anthers of control and male sterile transgenic plants and amplified with cystatin specific forward and reverse primers. The amplification of actin served as an internal control.

II. Restoration of Male Fertility in Transgenic Plants expressing Cysteine Protease

1. *In-silico* analysis of cysteine protease and cystatin interaction:

Cysteine protease and cystatin interaction have been well studied in other systems like human cathepsin B with cystatin A (*H. sapiens*) and chagasin (*T. cruzi*), Falcipain-2 (*P. falciparum*) with chagasin, Papain (*C. papaya*) with chagasin, cystatin B (*H. sapiens*) and tarocystatin (*C. esculenta*). These studies revealed the conserve inhibitory interaction of cystatin with cysteine protease. Each functional cystatin domain composes of three conserved motifs for interacting with target cysteine proteases: (1) the first major binding loop (L1) with Q-X-V-X-G; (2) the second binding loop (L2) with a conserved aromatic residue, W or H; and (3) the N-terminal trunk with a conserved G (Chu et al., 2011).

Arachis cystatin was also found to have the conserved motif, which forms the glutamine-valine-glycine (Q-X-V-X-G) loop at the N terminal side and a hairpin loop formed by proline-tryptophan (PW) residues. *Arachis* cysteine protease (360 aa) considered in this study was aligned with other cysteine proteases shown to have all the conserved residues like, the motifs EHDYRFGVFRANLRRA (Ex3Rx3Fx3Nx3I/Vx3N) and GVTKFSD (GxNxFxD), which are necessary for the correct processing of the protease for the generation of the mature protein. This N-terminal region was found to have secretory signal peptide and the peptide cleavage site for the generation of the mature protein during its transfer to the target site. This N-terminal region was not considered for modeling in this analysis. This propeptide region at the N terminal region was found to

have no matching alignment with available crystal structure data of cysteine proteases in the protein data bank.

Even though there is increased amount of information on its multifunctional properties of cysteine proteases, the complexity still remains on their diverse functional processing roles, which form the subject of further study. The complexity of its enzyme activity can be brought down by in-detailed comparative analysis of protein inhibitor complex studies. Complex protein modeling was undertaken as the protein-protein docking study was observed to be cumbersome and it is difficult to get the accurate model as it is a highly challenging task. Simple approach suggested by Bishop and Kroon (2011) enabled the protein- protein interaction studies simpler. Complex Protein modeling of the cysteine proteases with their inhibitors were modeled and studied as suggested by Bishop and Kroon (2011). The eight selected template crystal structures showed to have the same cysteine protease from papain, while the inhibitor was found to be from different sources as has been mentioned in Table 5.II.1. The different inhibitors were aligned with *Arachis* cystatin and the percentage identity and similarity were mentioned in **Table 5.II.1.1**. It was seen that tarocystatin was found to be nearly perfectly aligned with *Arachis* cystatin compared to other inhibitors as tarocystatin was from the plant source C. esculenta, while the other inhibitors are from different sources, *H. sapiens* (3K9M,1STF, 1NB5), T. cruzi (3CBJ, 2NQD, 2OUL 3E1Z). All the crystal structures were considered for building the complex model for *Arachis* cysteine proteases using Modeler 9v8. Out of 10 models that were constructed using one complex template crystal structure, only one accurate model was obtained, which was selected based on the parameters (PROCHECK and RMSD) that are mentioned in Methods and Materials. The accurate models for each of the eight

template structures that were selected are given in Table 1. From Table 1, it can be observed that the model generated using the 3IMA complex crystal structure was the best fit model. Target *Arachis* cystatin showed highest sequence identity of 56% with the inhibitor (Tarocystatin). Ramachandran plot of the complex built model showed only 1.1% of residues in the disallowed region and 87.5% of residues in the allowed region, which infers that the model was good and can be considered for the further studies. The model was also found to be more reliable with Ga341 score of 1.0 and zDOPE score of -0.178; lower DOPE score infers a more stable structure. The best fit model generated by 3IMA was found to be energetically more stable compared to other models generated. According to the pro-A server, the z-score of this complex structure falls within the range of the specified z-score (**Fig. 5.II.1.2**) thereby inferring the absence of structural errors in the model generated in the present study.

The best fit model was considered to identify the residues involved in inhibitor and cysteine protease interactions before proceeding for restoration. Cystatin interacts with cysteine proteases with N-terminal region making two interactions in the loop region and one in the β- sheet. Three other interactions were seen in the first β- hairpin loop from the N-terminal side. A total of eight interactions were seen between cystatin and cysteine proteases. Residues Gly 75, Trp196, Asp166, Cys72, Asn73, Gly20 of the cysteine proteases were found to be involved in the interaction with cystatin (**Fig. 5.II.1.1**, Chu et al., 2011). A side chain amine group of Glutamine 49 of cystatin was found to be important, which is interacting with the backbone carbonyl oxygen of both Cys72 and Asn73 of the cysteine proteases. While the side chain of Asp 166 of cysteine protease forms hydrogen bond with side chain of arginine 8 of cystatin and side chain of Trp 196 in

interacting with the backbone carbonyl oxygen of valine 51 of cystatin. Other interaction involved are between the backbone nitrogen and backbone oxygen, which involve the hydrophobic residues val4, Val51, Ala52, Gly5 of cystatin and Gly75, 20 of cysteine proteases forming a hydrophobic cluster in the interface area. The interacting residues between the cystatin and cysteine proteases can be seen in **Fig. 5.II.1.1**.

Even though a lot of information is available on the multifunctional properties of cysteine protease, the complexity still focuses on their diverse functional processing roles, which form the subject of further study. The complexity of its enzyme activity can be brought down by an in depth comparative analysis of protein inhibitor complex studies.

This complex modeling study will thus help us understand the inhibitory action of cystatin on cysteine protease at the molecular level in the present study.

Table 5.II.1.1: comparison of different models generated for cysteine protease-cystatin complex based on sequence identity, RMSD value, hydrogen bond between the complexes, Ramchandran plot score, GA341 Score and Z-Score.

Protein ID	Inhibitor From is source	Percent identity with cysteine proteases	Percent identity with cystatin	RMSD	Total no of H-H bonds Between the complex	Ramchandran plot score (%)	GA341 Score	zDOPE Score
3IMA	Tarocystatin (C. esculenta)	37.5%	56%	0.688	8	87.5	1.00	-0.178
1NB5	Cystatin-A (H. sapiens)	40.1%	35%	1.005	10	84.7	1.00	0.424
3K9M	Cystatin-A (T. cruzi)	27.6%	35%	1.772	6	81.4	1.00	0.690
1STF	Cystatin-B (H. sapiens)	37.5%	29%	2.938	6	85.2	1.00	0.256
3CBJ	Chagasin (T. cruzi)	27.6%	34%	2.609	4	84.2	1.00	0.850
2NQD	Chagasin (T. cruzi)	40.1%	34%	2.937	6	80.1	0.395	1.153
2OUL	Chagasin (T. cruzi)	31.6%	34%	1.628	19	86.10	1.00	0.406
3EIZ	Chagasin (T. cruzi)	37.5%	34%	2.899	2	84.9	0.40	0.337

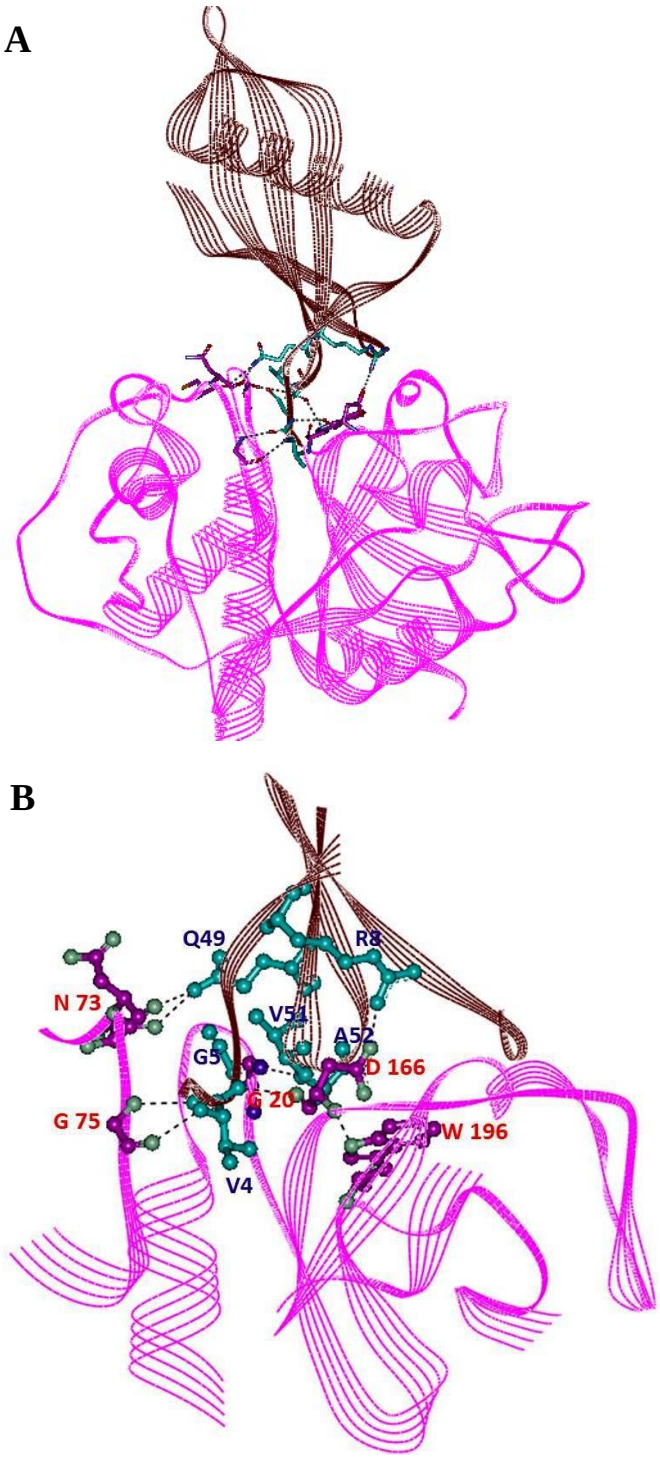


Figure 5.II.1.1 Structure of the cysteine protease (AdCysPro)-Cystatin complex. A. View along the active site cleft. B. Interacting residue of the cysteine protease-cystatin complex. Cysteine protease is shown in pink and cystatin in brown color. Interacting amino acids in respective proteins have been numbered.

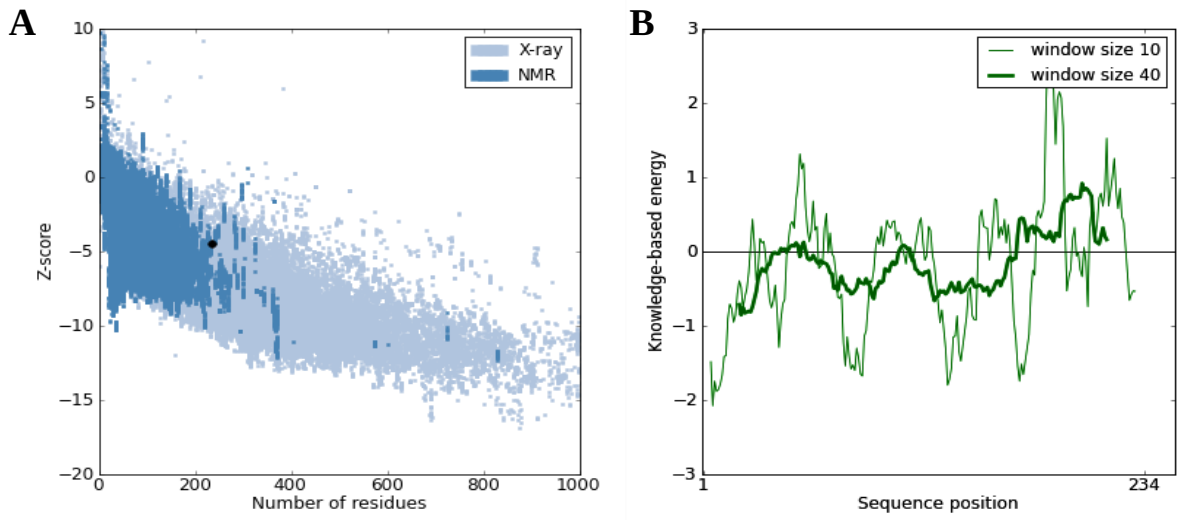


Figure 5.II.1.2 Validation of Cysteine protease-cystatin complex models based on Pro-SA evaluation Z-Score plot (A) and energy plot (B)

2. Development of putative restored progeny through crossing:

Two cysteine protease induced male sterile plants having a single copy insert were pollinated with the pollen from the high expression heterozygous transgenic plants of cystatin to check the possibility fertility restoration on the cysteine protease expressing transgenic plants (Restorer action) individually and seeds were collected for F_1 generation analysis (**Fig. 5.B.2.1**).Seeds were germinated on half strength MS media with Kan-125 mgl^{-1} and phosphinothricine (BASTA) -1.0 mgl^{-1}. Genetic analysis for putative restored plants was performed (**Fig. 5.B.2.2**) which suggested that the genotypes having both cysteine protease and cystatin transgenes would survive on the double selection medium containing both kanamycin and phosphinothricine and all other, genotypes would not survive the double selection.

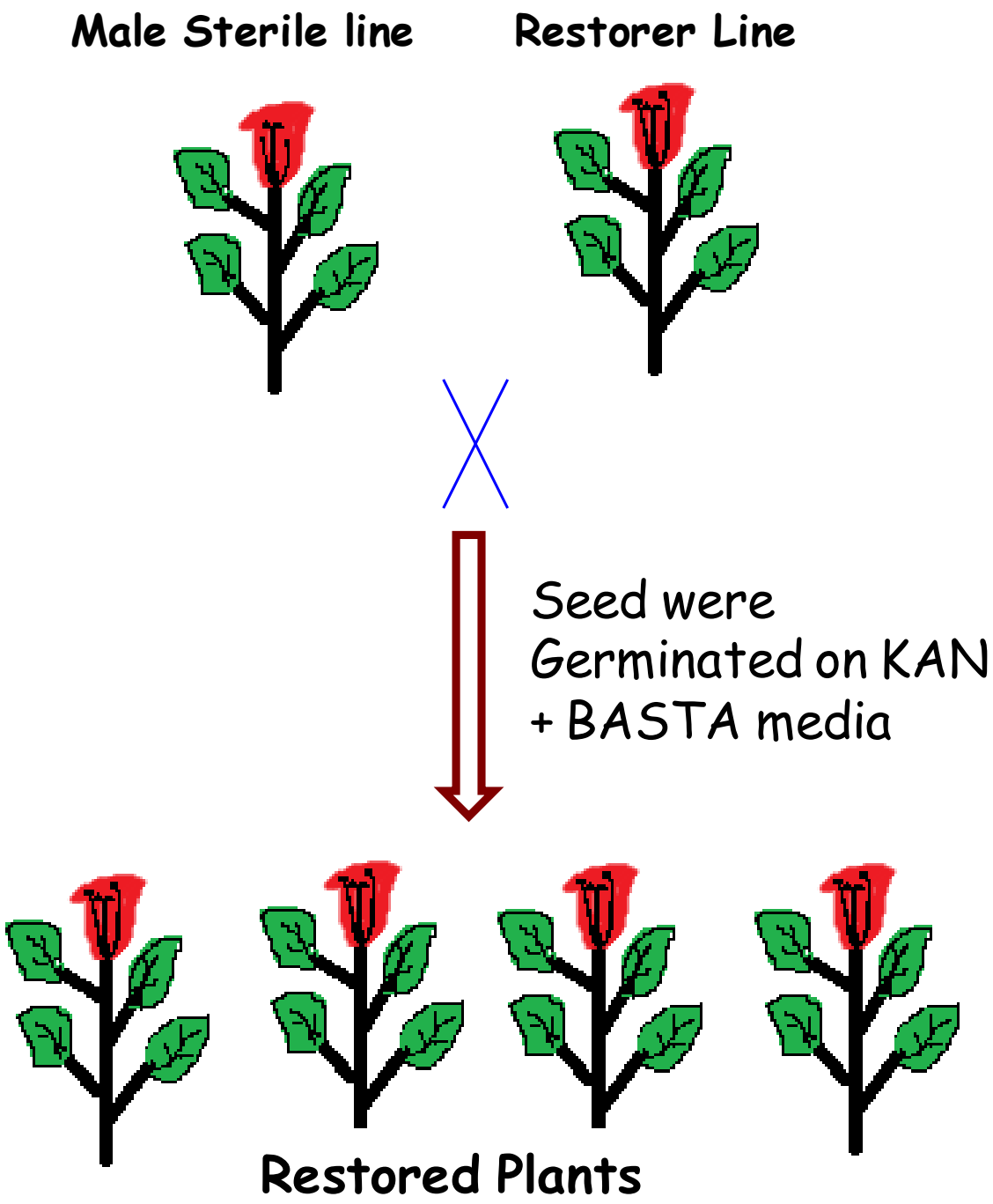


Figure 5.B.2.1 Schematic diagram for the development of restored plants. Cysteine protease induced male sterile plants were crossed with restorer line having cystatin transgene and resultant seed were germinated on half MS with Kan-125 mgl^{-1} and phosphinothricine (BASTA) -1.0 mgl^{-1}. The plants that survived the double selection are purported to be carrying the two transgenes with the respective selection marker genes and thus, are able to survive. Seedlings carrying one or neither of the marker genes would not survive the double selection.

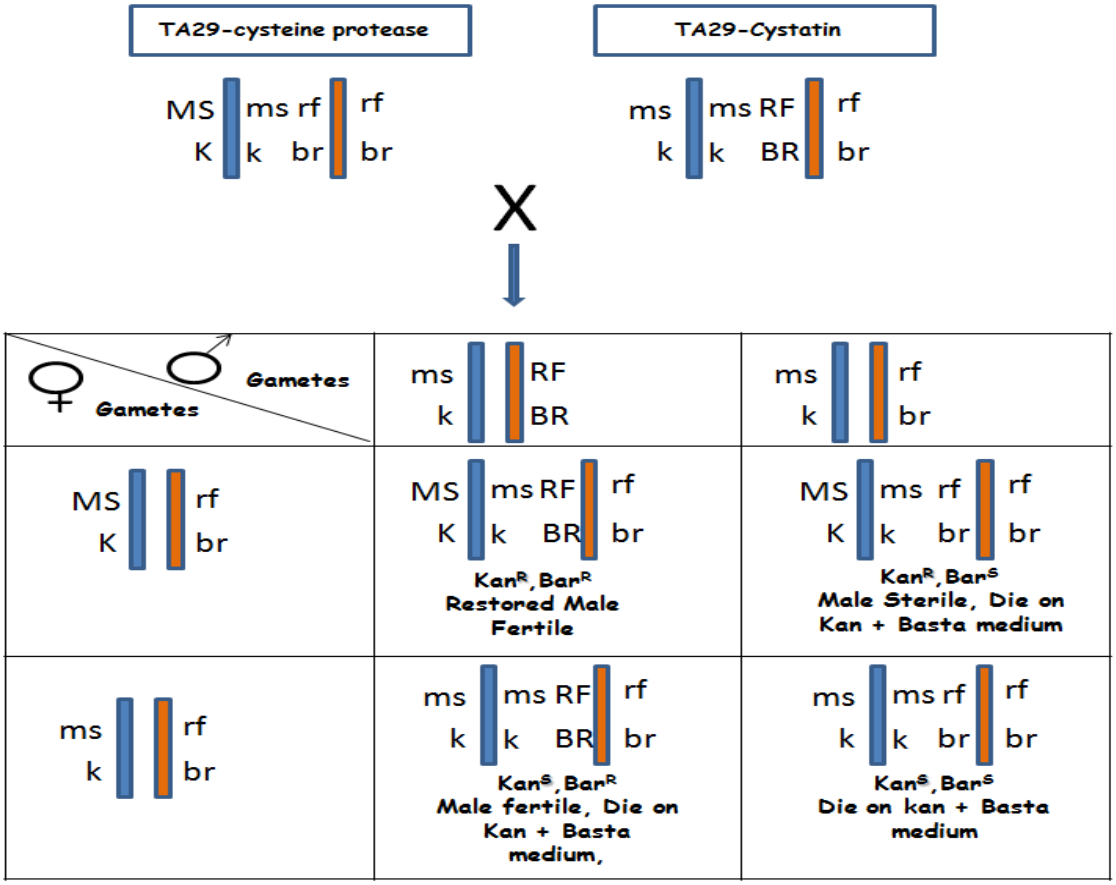


Figure 5.B.2.2 Genetic Analysis of putative restored line. Genotypic representation of male sterile plants MS and Restorer plants RF, Kanamycin Resistant K, Basta Resistant BR and ms, k, rf, br represent the hemizygous chromosomal loci, which lacks TA29-cysteine protease, *nptII*, TA29-cystatin and bar genes respectively.

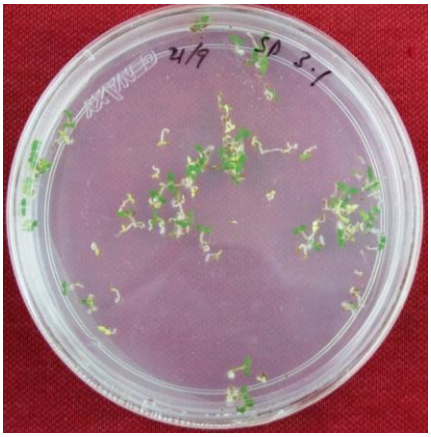


Figure 5.B.2.3 Seed germination on half MS media with Kan-125 mgl^{-1} and phosphinothricine (BASTA) - 1.0 mgl^{-1}.

3. Screening of putative restored plants:

After the seed germination on half strength MS double selection medium with Kan-125 mgl^{-1} and phosphinothricine (BASTA) -1.0 mgl^{-1}, green seedlings were selected and transferred to the green house for further analyses to confirm the possibility of restoration. Total DNA was isolated from putative restored plants including untransformed control plant. Presence of the cysteine protease and cystatin transgenes was confirmed through PCR amplification using the forward and reverse primer for both the genes respectively (**Fig. 5.B.3.1**). PCR amplified 1.08 kb fragment of cysteine protease gene was observed in putative restored plants and was absent in non-transgenic control plants. Similarly, the 297 bp fragment of cystatin gene was also noticed in putative restored plants.

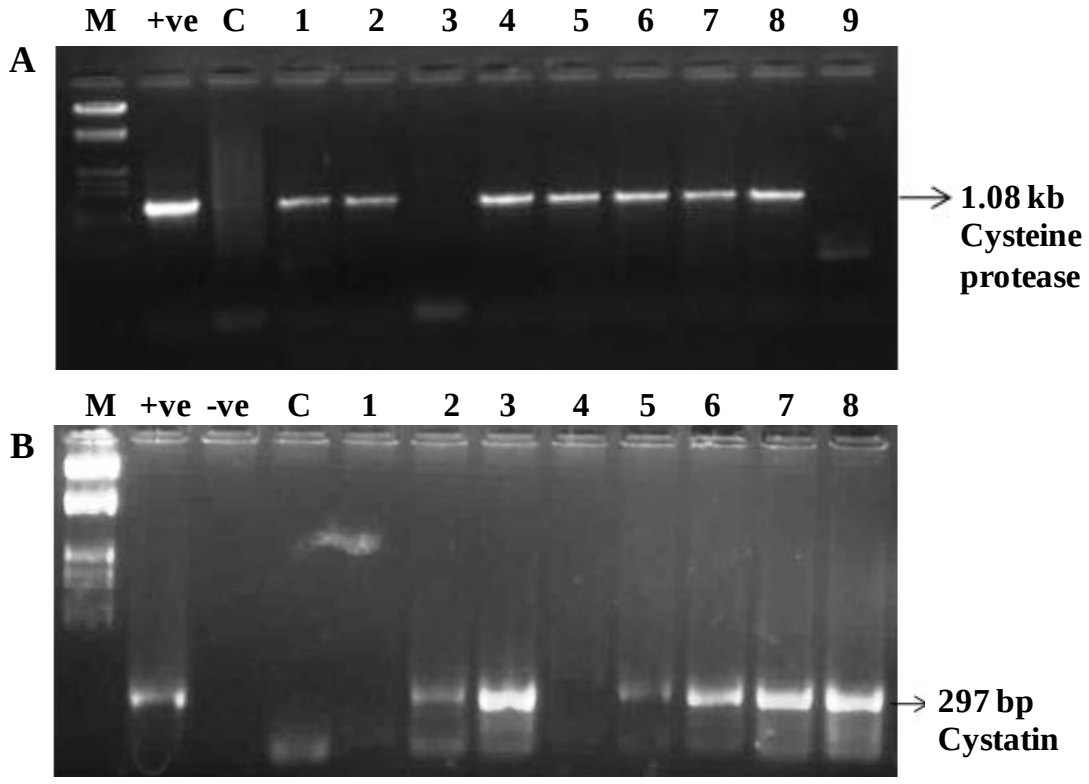


Figure 5.B.3.1 Screening of putative restored plants using PCR. **A.** PCR amplification of cysteine protease gene using forward and reverse primer that would amplify 1.08 kb fragment. **B.** PCR confirmation of cystatin fragment of 297 bp using forward and reverse primer of cystatin gene.

4. Evaluation of flower morphology and pollen characteristics of restored plants:

During anthesis, flower morphology and pollen characteristics of PCR confirmed fertility restored plants were observed (**Fig. 5.B.4.1**). Based on flower morphology and pollen characteristics, it was noticed that out of seven plants, four plants display flower morphology similar to the control plants, while three plants exhibit male sterile phenotype similar to cysteine protease induce male sterility feature. Stamen length also became normal in restored plants similar to the control plants. Restored four plants were also assayed for pollen production characteristic.

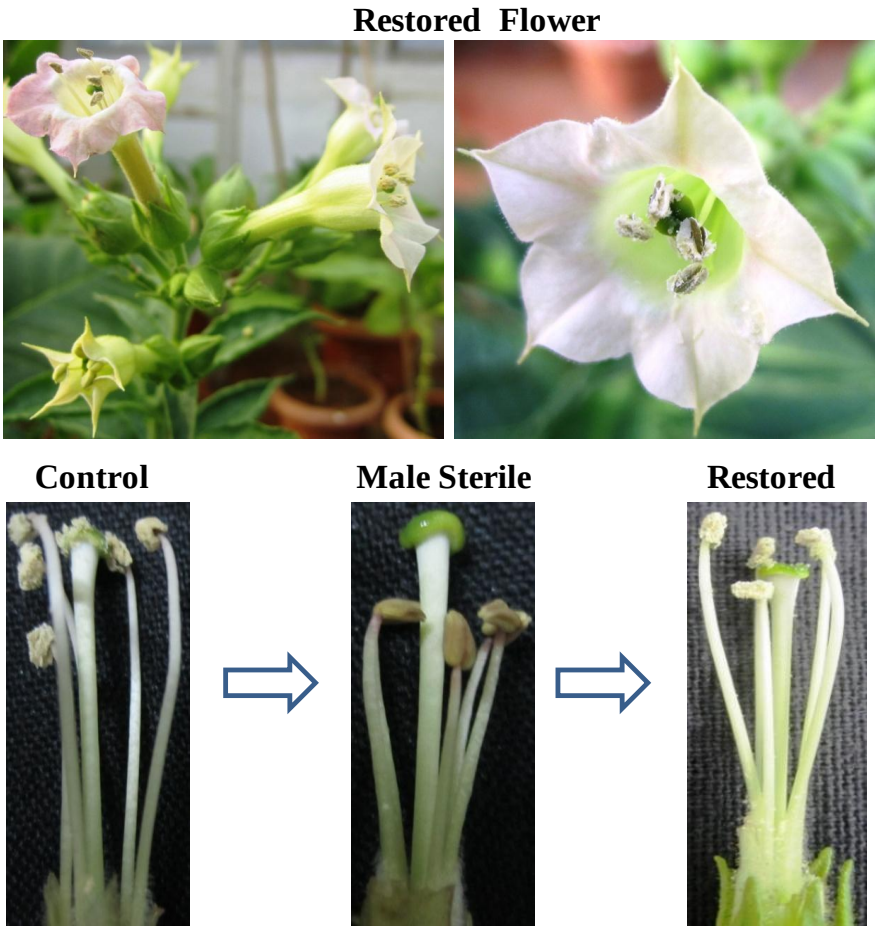


Figure 5.B.4.1 Flower morphology of restored plant. Flower of restored plant was similar to the flower of control plant. Stamen length became normal in restored plant compare to sterile plant.

Pollen viability was tested with acetocarmine staining (**Fig. 5.B.4.2)**. It revealed that anthers of the restored plants had fertile pollen grains which stained positively, while sterile plants pollen grain did not get stained or stained very lightly. Pollen morphology was also examined by scanning electron micrography (SEM), which showed the presence of viable pollen grains with spherical, regular shape similar to normal pollen grains of control plants.

Furthermore, pollen germination assay was performed in sucrose-boric acid medium and germination of pollen grain was noticed. Based on viability and germination, fertility evaluation was done and it was found that restored plants were able to restore fertility up to 70%.

Finally, seed set was observed on restored plants. Completely normal capsule formation was observed in the fertility restored plants upon self-pollination, which was similar to control plant. Seed set was also similar to control plant.

5. Transcript analysis of cysteine protease and cystatin in restored plant:

Total RNA was isolated from the anthers of restored plants and semi-quantitative duplex RT-PCR was performed using gene specific primer for cysteine protease and cystatin (**Fig. 5.B.5.1**). It was observed that restored plant showed expression of both the transcript and expression of any transcript was not detected control plant.

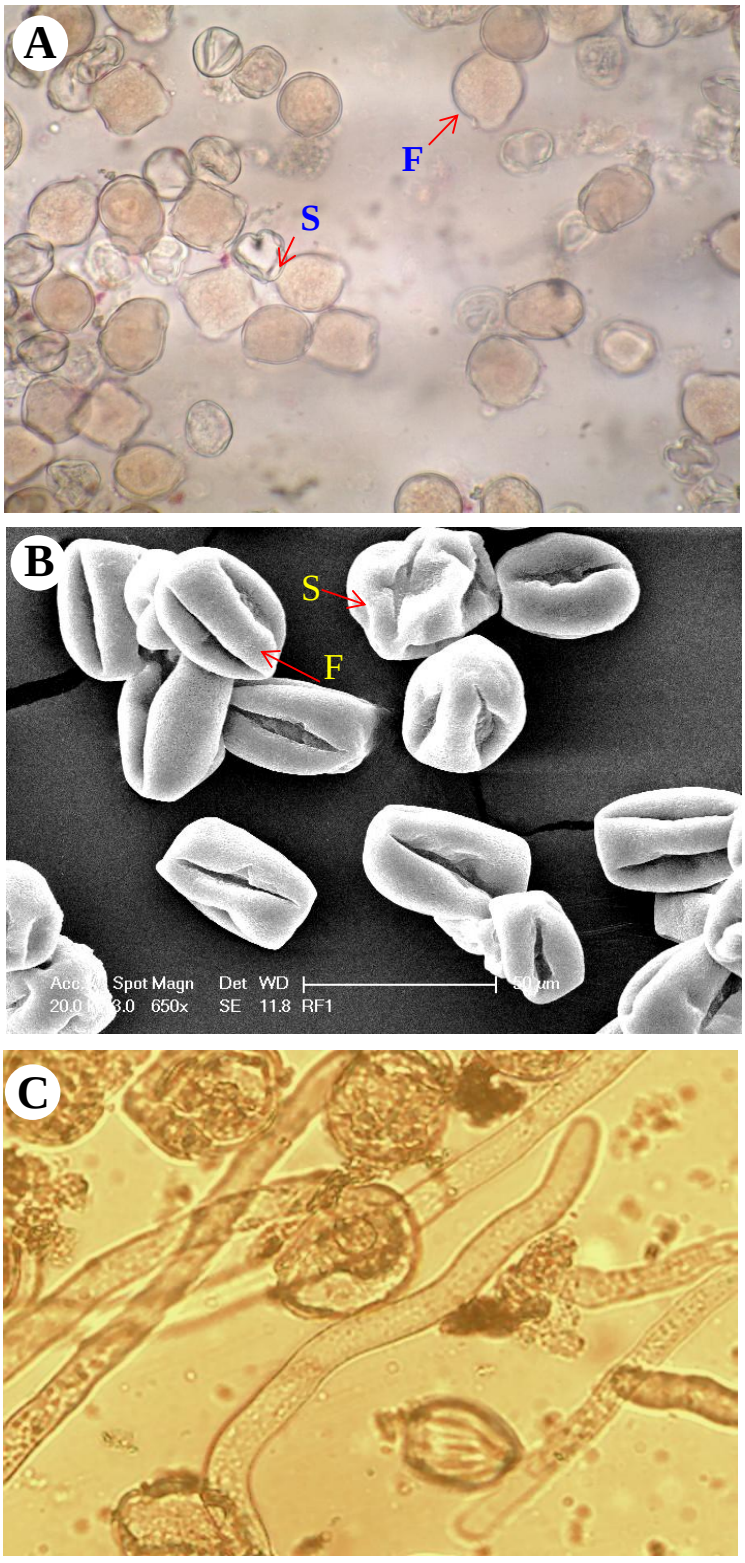


Figure 5.B.4.2 Pollen morphology of restored plant. **A -** Acetocarmine staining of pollen grains. **B -** Scanning electron micrograph (SEM) of pollen grains. **C -** Pollen germination assay on sucrose-boric acid media.

Figure 5.B.4.3 Comparison of capsule formation of restored plant, male sterile plant and control plant. Capsule formation in restored plant was similar to control plant.

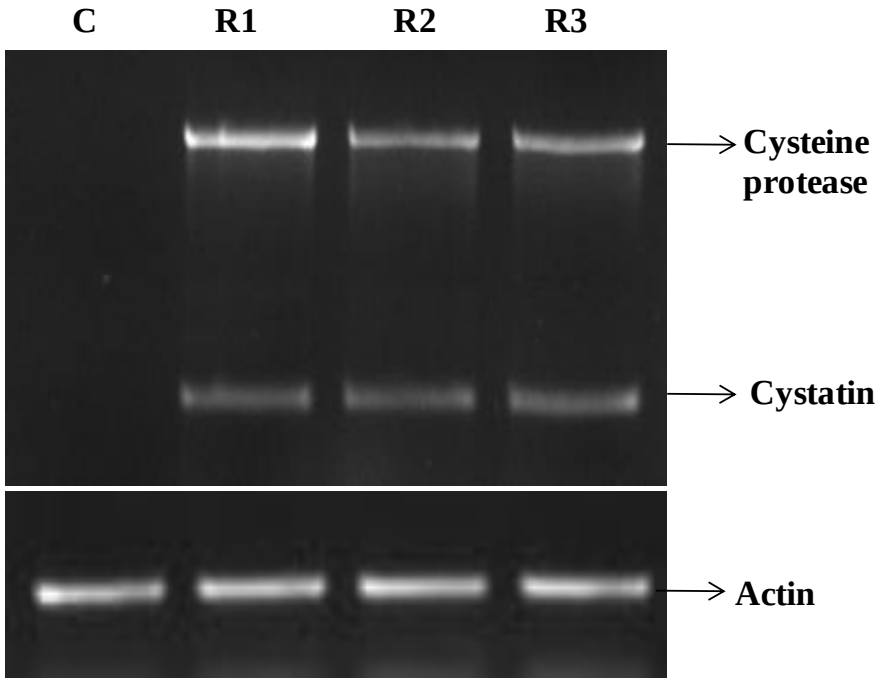


Figure 5.B.5.1 Semi-quantitative RT-PCR (duplex RT-PCR) analysis of cysteine protease and cystatin expression in the anther of untransformed control (C) and restored plants. cDNA was synthesized from total RNA from anther of control and restored plants and amplified with cysteine protease and cystatin specific forward and reverse primer respectively. Actin served as an internal control.

Discussion:

We have earlier reported that the tapetal expression of cysteine protease led to male sterility of the transgenic tobacco plants. It was hypothesized that male fertility could be achieved by the tapetal expression of cystatin in cysteine protease induced male sterile plants. Therefore cystatin construct was prepared and transformed tobacco plants were generated. Transcript analysis was performed on PCR confirmed plants and three high expression lines were selected for exploring the possibility of restoring fertility on the male sterile plants expressing the cysteine protease in the tapetal tissues. Several reports suggested that cystatin can inhibit cysteine protease activity efficiently. We have selected *Arachis* cystatin for restoration as it has been reported that any phytocystatin of 12-14 kDa has the ability to inhibit papain-like cysteine protease activity in vitro and their *in vivo* interaction has also been reported (Martinez et al., 2009). We have also performed *in silico* interaction of cysteine protease and cystatin of *Arachis*, which further revealed that occurrence of interaction between them with proper bond formation between appropriate amino acid residues. For a high-level of expression of cystatin, we have used TA29 promoter. It is a strong promoter and specially expressed in the tapetal cell layer in the early stages of microsporogenesis in the anther. Same promoter has also been used for cysteine protease induced male sterility. The present study was, therefore, undertaken to confirm the possible interaction between the toxic protein and its inhibitor by co-expressing both the genes at same developmental stages for proper inhibition of cysteine protease in a strategy similar strategy similar to that of Mariani et al., 1990, 1992), who used a cytotoxic protein, barnase whose action can be inhibited by naturally occurring inhibitor, barstar from the bacterium, *Bacillus amyloliquefaciens*.

It has been reported that cysteine protease interacts with cystatin at the Golgi and endoplasmic reticulum (Martinez et al., 2009). To ensure proper inhibition of cysteine protease even beyond its inhibitory interaction with cystatin at the Golgi and the endoplasmic reticulum stage, we transcriptionally fused defensin apoplastic signal (Vijayan's unpublished work) with cystatin. We have used the high expression hemizygous plants of T_0 cystatin transgenic plants for pollinating the cysteine protease expressing male sterile plants. After pollination, seeds were collected and screening of putative restored plants was performed on half strength MS medium with Kan-125 mgl^{-1} and phosphinothricine (BASTA) -1.0 mgl^{-1}. Genetic analysis of F_1 progeny suggested that only plants expressing the kanamycin resistance gene associated with the cysteine protease and the basta resistance gene associated with cystatin would survive double selection on kanamycin-phosphinothricin medium. However, we have observed some male sterile plants in the progeny of the cross, which might be because of the use of a lower dose of phosphinothricin in the double selection medium. As hypothesized, plants expressing both the cysteine protease and cystatin in the tapetal layer are fertile to a great extent because of the occurrence of the expression of both the genes in the crossed plants (the cross has been done between a hemizygous plants expressing cysteine protease and cystatin). Flower morphology, pollen characteristics of restored plants was similar to control non-transformed plant. Restored plants produced viable and functional pollen grains. Interestingly shorter stamen length was not found in restored plant as seen in male sterile plants. Anther development was normal and indistinguishable from that of the non-transformed control plants. Similarly, capsule formation and seed set were also normal as in control plants. Duplex RT-PCR also displayed the co-expression of cysteine protease

and cystatin in the anthers of fertility restored plant only and not in the control plants. It has been shown earlier also that co-expression of Barstar and Barnase resulted in restoration of fertility (Mariani et al., 1992).

These investigations indicated that cystatin was able to inhibit cysteine protease activity in the anthers and efficiently suppressed the male sterile phenotypes. Since our main purpose was to restore male fertility in cysteine protease induced male sterile transgenic lines using apoplastic targeted cystatin as a restorer line, we have mainly focused on restored plant analysis only. Comparative study of cytosolic and secretory cystatin efficiency of restoring male fertility might require further investigation. Our results are analogous to barnase-barstar system developed by Mariani et al. (1990, 1992). Since our system is of genes controlling the expression of the genes, whose proteins are of universal occurrence in all plants, we presume that it is better than the barnase-barstar system in terms of ethical and environmental issues and more acceptable in engineering pollination control in crop plants.

In conclusion, the present work has established that the male sterility induced by cysteine protease could be restored using cystatin expressing restorer lines. Since both genes used in this study were plant origin therefore Exploiting plant gene/s could be a novel tool for the development of genetically engineered male sterile plants, whose fertility can be restored.

Table 5.I.2.1: Sequences of the oligos /primers used in the present study

Name of the Primer	**Primer Sequence (5'-3')**
Cystatin F	ACCTCGAGATGGCAGCAGTGGGTG
Cystatin R	TAGGGCCCTTAAGCATTGGAGCCATCAC
Defensin Apoplastic Signal	ATGGAGAAGAAATCACTAGCTGGCTTGTGCTTCCTCTTC CTCGTTCTCTTTGTTGCACAAGAAATTGTGGTGACTGAA GCC
Apoplastic signal F	CTGCAG ATGGAGAAGA AATCACTAGC
Actin F	TGGCATCACACTTTCTACAA
Actin R	CAACGGAATCTCTCAGCTCC

Summary and Conclusion

Summary and Conclusion

Usable male sterility systems have immense potential in developing hybrid varieties in crop plants and are also used as a biological safety method to prevent horizontal transgene flow. Barnase-barstar system developed earlier was the first approach to engineer male sterility in plants in this direction. Since the genes involved in the system are of prokaryotic origin, we have made attempts to engineer male sterility and fertility restoration using a plant pathogen induced gene, cysteine protease, for inducing male sterility. This gene was identified in the wild peanut, *Arachis diogoi* in its interaction with the pathogen *Phaeoisariopsis personata* that causes late leaf spot in peanut. The role of cysteine protease in plant PCD has been well documented. Therefore, we have made an attempt to devise a strategy of engineering male sterility in crop plants by expressing this gene under the strong tapetum specific promoter, TA29 for early degeneration of tapetal cell lineages.

TA29 promoter and cysteine protease encoding genes were cloned in pTZ57R vector. TA29 promoter and cysteine protease were translationally fused together along with T-NOS terminator from pCAMBIA2300 followed by cloning in plant expression binary vector pCAMBIA2300. This construct was further transformed into *Agrobacterium* strain LBA4404 (pAL4404) by the freeze-thaw method. Finally, tobacco transformation was done with the cysteine protease construct. Putatively transformed plants were obtained and were hardened under the culture room conditions, and were maintained at 28 ºC till maturity in the glass house. Putative transgenic plants were confirmed through PCR and Southern blot. Phenotypic analysis was done to confirm their level of male sterility. Flower size, anther filament length have been measured. It has been found that out of 15 plants

three plants were completely male sterile and nine have shown partial sterility. Most prominent changes that appeared in male sterile plants were a noticeable reduction in the length of flower size and stamen filament. Flower size has decreased by a factor of 1.3 and stamen filament size was reduced by a factor of 1.5. In addition to this, acetocarmine staining for pollen grains also showed that male sterile pollen failed to take intense stain that is characteristic of fertile pollen and these pollen grains lacked cytoplasm and were deformed in shape while fertile pollen were with regular round shape properly developed pollen wall structure. Pollen morphology was observed using a Scanning Electron Microscope in which empty and aberrant pollen grains were clearly visible. Sterile pollen did not show any signs of germination in pollen germination medium at 28 ºC while fertile pollen grains did germinate well after four hours of incubation. Histological study also showed that fertile plants have anther sacs filled with pollen grains, while male sterile plants anther sacs exhibited fewer and deformed pollen grains. Furthermore, RT-PCR analysis and western blot analysis also confirmed the cysteine protease expression was confined only to the anthers of the male sterile plants and there was no expression in fertile control plants.

To restore male fertility, we have developed a construct using cysteine protease inhibitor called cystatin, which was also isolated from wild peanut and we expressed this gene in tapetal cell layer like the cysteine protease. Putative transgenic plants expressing cystatin were raised and confirmed through PCR. Three high expression plants of cystatin transgenic plants were identified selected for the crossing with the transgenic male sterile plants to observe the possible fertility restoration.

We have also modeled the interaction of the cysteine protease with cystatin isolated from *Arachis diogoi* using bioinformatics approach and observed that there is possible intermolecular interaction between the toxic and the inhibitory proteins. Cysteine protease induced male sterile lines having a single copy insert were pollinated with pollen grains of high expression cystatin plants individually and seeds were collected F_1 generation analysis. Seeds were germinated on half strength MS medium having Kanamycin -125 mgl^{-1} and phosphinothricine (BASTA) -1.0 mgl^{-1}. Our genetic analysis suggested that only restored plants would survive on Kanamycin + phosphinothricine supplemented medium. Putative restored plants were confirmed through PCR amplification cysteine protease and cystatin gene using their respective primers. During anthesis, flower morphology and pollen characteristic of PCR confirmed restored plants were observed. It was found that four plants exhibited normal feature similar to the fertile control plants. Stamen length became normal in restored plants. Acetocarmine staining and SEM analysis showed the presence of viable pollen grains in restored plants, which was further confirmed through *in vitro* pollen germination assay. Based on viability and germination, fertility evaluation was done and it was found that the expression of cystatin in the tapetum was able to restore fertility from up to 70% in the cysteine protease expressing male sterile transgenic plants. These restored plants displayed normal capsule formation upon self-pollination. Semi-quantitative duplex RT-PCR analysis with total anther RNA has been performed, which showed the expression of cysteine protease and cystatin transcripts in the anthers while these transcripts were not detected in the anthers of the control plants.

It was concluded that cysteine protease-cystatin combination can be used as a pollination control system for the development of hybrid varieties. Exploiting plant gene/s

could be a novel tool for the development of genetically engineered male sterile plants and their restorer plants as they may face less serious problems in the respect of regulatory issues in commercial deployment compared to the already commercially deployed barnase-barstar system, which uses genes of bacterial origin.

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