Induction of male sterility and disease resistance with the expression of harpin$_{Pss}$ in transgenic tobacco

Thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that Mr. Debashish Dey has carried out the research work embodied in the present thesis under the supervision and guidance of Prof. Appa Rao Podile for a full period prescribed under the Ph.D. ordinances of this University. We recommend his thesis ***"Induction of male sterility and disease resistance with the expression of harpin$_{Pss}$ in transgenic tobacco"*** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

This is to declare that the work embodied in this thesis entitled ***"Induction of male sterility and disease resistance with the expression of harpin$_{Pss}$ in transgenic tobacco"*** has been carried out by Debashish Dey under the supervision of Prof. Appa Rao Podile. This has not been submitted for any degree or diploma in any other University earlier.

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dedicated
to my
beloved
mother

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ABBREVIATIONS

µg	: microgram
µM	: micromolar
ALP	: alkaline phosphatase
BAP	: 6-benzyl amino purine
BCIP	: 5-bromo-4-chloro-3-indolyl phosphate
Bp	: base pair
° C	: degree centigrade/degree Celsius
CaMV	: cauliflower mosaic virus
cDNA	: complementary DNA
CMS	: cytoplasmic male sterility
CTAB	: cetyltrimethylammoniumbromide
C-terminal	: carboxy terminal
DEPC	: diethylpyrocarbonate
DNA	: deoxy ribonucleic acid
dNTPs	: deoxy nucleotide triphosphates
EDTA	: ethylene diamine tetra acetic acid
g	: gram
GUS	: β-glucuronidase
h	: hour(s)
HR	: hypersensitive response
Hrp	: harpin
hrpZ	: gene encoding harpin
IgG	: immunoglobulin G
IPTG	: isopropyl β-D-thiogalactoside
kb	: kilobase pair
kDa	: kilodalton
L	: litre
LB	: Luria-Bertani
LS	: leader sequence
M	: molar
mg	: milligram
min	: minute
ml	: milliliter
mM	: millimolar
MS	: Murashige and Skoog
MTT	: 3-(4,5 Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NBT	: nitroblue tetrozolium
N-terminal	: amino terminal
OD	: optical density
PAGE	: polyacrylamide gel electrophoresis
PCR	: polymerase chain reaction
PMSF	: phenylmethylsulfonylfluoride

PR proteins	: pathogenesis-related proteins
Pss	: *Pseudomonas syringae* pv. *syringae*
Psph	: *Pseudomonas syringae* pv. *phaseolicola*
PVP	: polyvinylpyrrolidone
RNA	: ribonucleic acid
RNase	: ribonuclease
rpm	: revolutions per minute
RT-PCR	: reverse transcriptase-polymerase chain reaction
SDS	: sodium dodecyl sulphate
SDW	: single distilled water
sec	: seconds
SEM	: scanning electron microscopy
SP	: signal peptide
TE	: Tris-EDTA
Tris	: tris-(Hydroxymethyl) aminoethane
T3SS	: type three secretion system
V	: volts
X-gal	: 5-bromo-4-chloro-3-indolyl β-D- galactoside

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Introduction

1. 1 Impact of plant diseases on crop yield

Plant diseases, caused by several phytopathogenic bacteria and fungi, result in loss of crop productivity threatening food security. Apart from the application of novel agrochemicals and the conventional breeding for resistant cultivars, there have been attempts to use transgenic technology as an alternative approach to protect plants from microbial diseases. Various reports demonstrate enhanced resistance against pathogens in plants through introduction of one or more genes.

Disease resistance mechanisms in plants against pathogens are primarily of two classes: 1) constitutive, and 2) induced upon exposure to a pathogen. Plants are able to recognize microbial invaders through specific surface determinants, collectively called pathogen-associated molecular patterns (PAMPs) and to react through defense signaling cascades (Zipfel *et. al*., 2005). The interaction of pathogens with plants leads to a disruption in cellular homeostasis, often leading to cell death, in both compatible and incompatible relationships.

1. 2 Hypersensitive response (HR) in plants

Hypersensitive response (HR), the most distinguished hallmark of resistance, is characterized by a rapid, localized plant cell death at the site of infection (Park, 2005). The HR is generally recognized by the presence of brown, dead cells at the infection site. Phytopathogenic bacteria harbor *hrp* gene cluster that controls pathogenicity in susceptible plants and the ability to elicit an HR in non-host or resistant cultivars of host plants (Guo *et. al*., 2009). Some *hrp* genes encode elements of type III secretion system (T3SS) by which effector proteins are exported into the host cytoplasm, which then interacts with plant intracellular proteins, and activate defense response. HR involves two phases - Phase I involves ion fluxes with efflux of hydroxide and potassium and influx of calcium and hydrogen ions into the cell. Phase II involves oxidative burst by producing reactive oxygen species (ROS), superoxide anions, hydrogen peroxide, hydroxyl radicals and nitrous oxide. These compounds affect cellular membrane function, in part by inducing lipid peroxidation and by causing lipid damage. The alteration of ion components in the cell, and the breakdown of cellular components in the presence of ROS, results in the death of affected cells and the formation of local lesions.

1. 3. 1 Harpin, a proteinaceous bacterial elicitor HR in plants

Harpins constitute a group of effector proteins exported by T3SS of phytopathogenic gram negative bacteria like *Erwinia*, *Pseudomonas* and *Ralstonia* spp. (Galan and Collmer, 1999). Harpin is acidic, hydrophilic in nature, rich in glycine, lacking cysteine, heat stable with no reported enzymatic activity. Harpins induce pathogen resistance by activating HR (He *et. al*., 1993 and 1994). However, when infiltrated into non-host plants, harpins trigger disease resistance-associated responses, such as transcript accumulation of pathogenesis-related (PR) genes and systemic acquired resistance (Fig. 1.1) (He *et. al*., 1993; Strobel *et. al*., 1996; Dong *et. al*., 1999; Galan and Collmer, 1999). *hrpZ* gene from *P. syringae* pv. *syringae* 61 encodes a 34.7 kDa extracellular protein, $harpin_{Pss}$, that elicits HR in tobacco and other non-host plants. Infiltration of $harpin_{Pss}$ imto tobacco leaves exogenously induced HR within 12 h. Following pathogen recognition, the earliest reactions are the regulation of specific ion channels and the formation of reactive oxygen intermediates. $Harpin_{Pss}$ induces a homolog of gp91-phox, which is a component of NADPH oxidase causing an oxidative burst (Desikan *et. al.*, 1998), or induce mitogen-activated protein (MAP) kinase in cultured cells of *Arabidopsis thaliana* (Desikan *et. al.*, 1993). Harpins were reported to inactivate mitochondria in *Arabidopsis* suspension cells (Krause *et. al*., 2004).

The physiological binding site for harpin action is not very clear. Immunolocalization studies revealed a Ca^{2+}-dependent association of *P. syringae* pv. *syringae* harpin with tobacco cell walls (Hoyos *et. al*., 1996) but other reports indicating harpin-induced K^+/H^+ exchange at the plant plasma membrane and subsequent plasma membrane depolarization (Hoyos *et. al*., 1996; Pike *et. al*., 1998) open debate on the concept of a cell wall binding site mediating such responses. Harpins may also interact with membranes directly and trigger plant defense responses in an ionophore-like manner (Lee *et. al*., 2001).

Harpin enhances plant growth, crop yield and quality, protect plants against a broad array of viral, fungal and bacterial diseases, including some for which no effective treatment is currently available, and also enhance resistance to attack by insects. Harpins are active across wide array of crops, which makes it a suitable candidate for engineering plant disease resistance.

Figure 1.1: The binding of harpin to a putative plant receptor results in disturbance of ion exchange across the plasma membrane followed by generation of reactive oxygen species and signal amplification. The various signal transduction events lead to the activation of salicylic acid pathway, jasmonate pathway and their responsive genes. Harpin also activates gene expression involved in plant growth.

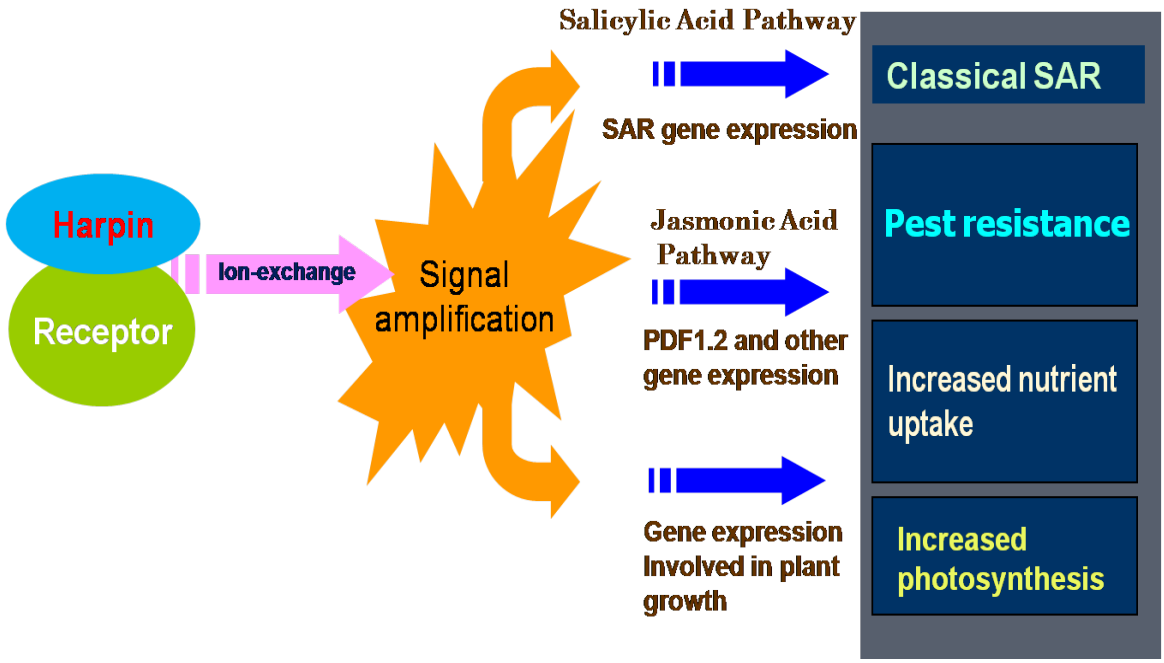


Figure 1.1: Harpin-induced biochemical changes and activation of signal transduction pathways

(http://ag.arizona.edu/crops/diseases/papers/dischemistry.html).

1. 3. 2 Harpin transgenics developed for disease resistance

Constitutive expression of harpin controlled by 35S promoter resulted in no morphological changes in leaves, stems and flower and seed fertility was normal in absence of pathogen. However, the plants were observed to be resistant and HR-like local lesions were generated after pathogen attack (Takakura *et. al*., 2004; Tampakaki *et. al*., 2000). Similar report of constitutive expression of harpin gene (*hrpG*) from *Xanthomonas oryzae* pv. *oryzae* and (*hrpN*) from *Erwinia amylovora* in transgenic tobacco and pear conferred resistance to *Ralstonia solanacearum* (Peng *et. al.*, 2004) and to *Erwinia amylovora* (Malnoy *et. al*., 2005), respectively. The transgenic cotton plants constitutively expressing harpin gene (hpa_{Xoo}) from *X. oryzae* pv. *oryzae* conferred resistance to *Verticillium dahliae* (Miao *et. al*., 2010). Expression of harpin under PAL1 promoter resulted in low basal level expression in absence of pathogen but HR like local lesions were observed in presence of fungal pathogen, *E. cichoracearum* (Takakura *et. al.*, 2004). High-level expression of extracellularly secretable form of harpin induced hypersensitive cell death *in planta* that was accompanied by induction of a HR-specific gene transcript, *hsr203J* prior to the onset of necrotic symptoms (Tampakaki *et. al.*, 2000). The transgenic *Arabidopsis* plants expressing *hrpN* gene from *Erwinia amylovora* driven by Nos and Gst1 promoter conferred resistance to *Peronospora parasitica* (Bauer *et. al*., 1999). When the same *hrpN* was targeted to apoplast by fusion with a signal peptide gene and driven by the same Gst1 promoter, it resulted in development of citrus plants resistant to *Xanthomonas axonopodis* (Mendes *et. al*., 2009). The transgenic tobacco plants expressing *hrpN* gene from *Erwinia pyrifoliae* downstream to Cc1 promoter were resistant to *Botrytis cinerea* (Sohn *et. al*., 2007). The expression of *hrpZ* gene from *P. syringae* pv. *syringae* downstream to TA29 promoter resulted in generation of partial male sterile plants due to expression of harpin gene in the tapetal layer (Madhuri, 2006).

The elicitor (harpin) induced HR-mediated cell death can be exploited in a transgenic approach to confer various traits to plants useful in crop improvement *e.g.*, induce male sterility by expressing harpin in the tapetal layer and to generate disease resistant plants by expressing harpin downstream to pathogen-inducible promoters.

1. 4. 1 Microsporogenesis

In flowering plants, male reproductive development begins in the sporophytic generation with the initiation and formation of the male reproductive organ, stamen in the flower. The development of stamen involves the formation of an anther that has multiple specialized cell types and that houses male meiotic cells and a filament that supports the anther. The process of microgametogenesis occurs within the developing pollen, yet it depends on sporophytic functions provided by the surrounding anther tissues.

The anther development is divided into 12 different stages (Fig. 1.2; Ma, 2005). At stage 1, divisions in the L1, L2, and L3 layers of the floral meristem result in the formation of the stamen (anther) primordium. From stages 2 to 5, anticlinal cell division in L1 expands the surface area of the anther to form the epidermis. At the same time, cells in the L3 layer divide and differentiate to form the connective and vascular tissues. Periclinal and anticlinal cell divisions are responsible for the formation of internal cell layers in the four anther lobes. Specifically, four clusters of archesporial cells are formed at stage 2 from periclinal division of L2 cells. The archesporial cells then divide at stage 3 to form both the primary parietal layer and the sporogenous cells; the primary parietal layer is just beneath the epidermal layer and surrounds the sporogenous cells. Cell division in the primary parietal layer then forms two secondary parietal layers. The outer secondary parietal layer further divides and differentiates at stage 4 into an endothecium layer and a middle layer. During this stage, cells of the inner secondary parietal layer divide and develop into the tapetum layer.

Meiosis of microspore mother cells (MMCs) results in the formation of microspores. Following meiosis, the development of pollen grains from the microspores involves cell divisions and cell differentiation to form a vegetative cell and two sperm cells. Early in pollen development, the microspore becomes vacuolated after release from the tetrad; the formation of a large centralized vacuole is accompanied by the migration of the microspore nucleus to one side of the cell. The first mitosis in pollen development then occurs and is asymmetric, producing a large vegetative cell and a small generative cell. The vegetative cell contains a dispersed nucleus and most of the cytoplasm from the microspore, whereas the generative cell has highly condensed chromatin and very little cytoplasm.

The total flower developmental process was also categorized in to 12 different stages by Goldberg (1988) that serve as reference points for the expression of specific

Figure 1.2: The 12 different stages of *Arabidopsis thaliana* anther development are shown (Ma, 2005). The numbers indicate stages.

Ar, archesporial; E, epidermis; En, endothecium; L1, L2, L3, Layer 1, 2, 3; ML, middle layer; Ms, microsporocytes; Msp, microspore; PG, pollen grain; PPC, primary parietal cell; PSC, primary sporogenous cell; Sm, septum; SPC, secondary parietal cell; St, stomium; T, tapetum; Tds, tetrads.

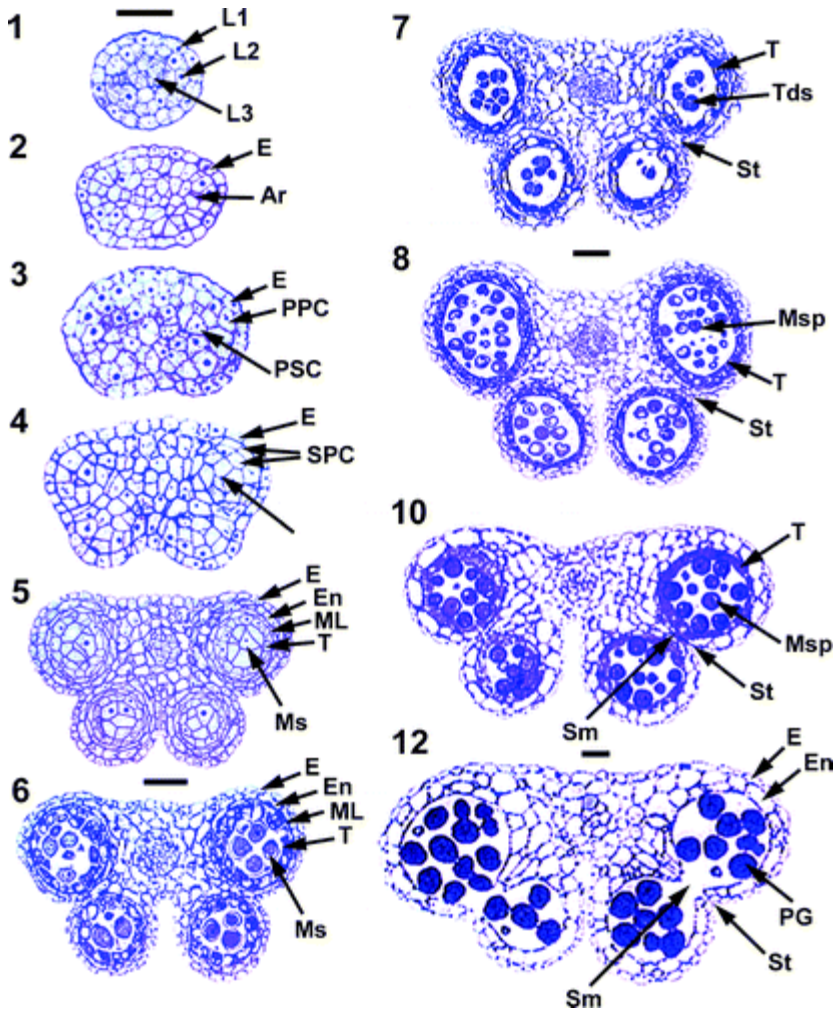


Figure 1.2: Different stages of anther development.

genes in different floral organs (Fig. 1.3). The 12 stages of flower were divided based on morphological markers (Table 1.1; Koltunow *et. al*., 1990) of flower bud and anther. The flower bud (calyx and corolla) increases in weight and length from stage 1 to 12 (Fig. 1.3, top panel) whereas the anthers increase in weight only till stage 5, then remain constant until stage 11 followed by decrease in weight after anther dehiscence (Fig. 1.3, bottom panel).

1. 4. 2 The tapetum is a specialized anther tissue

Microsporogenesis in angiosperms takes place within the anther. Microspores are surrounded by a layer of cells, the tapetum, which degenerates during the later stages of pollen development with cytological features characteristic of programmed cell death (PCD). The tapetal cells are usually bigger and normally have 2 nuclei per cell (polyploid). It helps in pollen wall formation, transportation of nutrients to inner side of anther, synthesis of callase enzyme for separation of microspore tetrads. The roles of tapetum being (i) supply of metabolic substrates and nutrients required for the growth and development of spores and pollen, (ii) provision of the sporopollenin precursors for the formation of exine following the dissolution of the callose wall and (iii) deposition of a coating on the pollen surface which is known to play a role in self-incompatibility systems and pollen dispersal (Shukla *et. al.*, 1998).

The tapetum acts as a nurse tissue during sporal development. During the early differentiation of the sporogenous tissue, the tapetal cells become meristematic, multinucleate and metabolically hyperactive. Tapetal cells undertake active synthesis of nucleic acids and rich reserve of metabolites (Mascarenhas, 1989). Further, when the spores continue to develop and differentiate, the tapetum enters gradually into a phase of decline and senescence. The lysis products of tapetum are also utilized by the developing spores and pollen grains. Therefore, functioning of tapetum is vital for the normal development of the pollen grains and any interference in the functioning of tapetum results in the development of male sterility.

Figure 1.3: The entire tobacco flower development process (from 8-mm bud to opening) was divided into 12 different stages (Top panel) on the basis of size and morphological criteria (Table 1.2). The flower buds of 8-mm size with the tetrads present within the anther were designated as stage 1. Stage 12 was designated as fully opened flower with dehisced anthers. The bottom panel shows anther development from stage 1 to stage 12.

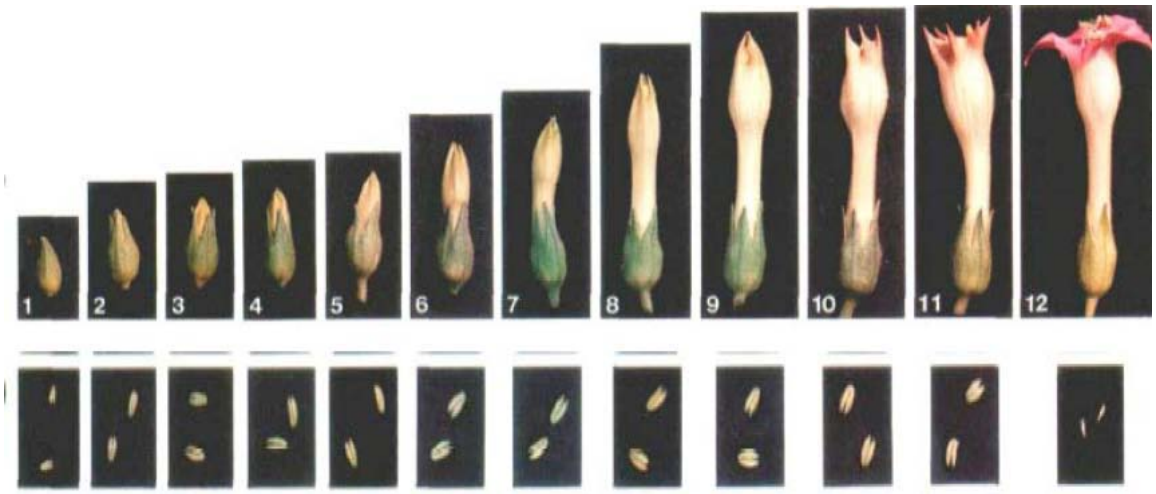


Figure 1.3: Different stages of tobacco flower development (Koltunow *et al*., 1990).

Table 1. 1: The abbreviations used in the table are shown below -

A, archesporial cells; C, connective; E, epidermis; En, endothecium; MMC, microspore mother cells; Msp, microspores; P, parietal layer; PG, pollen grains; S, stomium; Sp, sporogenous cells; T, tapetum; TDS, tetrads; V, vascular tissue.

Flower			Anther	
Stage	Bud Length[a]	Morphological Markers[b]	Tissues Present[c]	Major Events and Morphological Markers[d]
−7	0.75	Petal and stamen primordia present; carpels forming; calyx almost closed.	E, V, A, C	Rounded primordium; tissue differentiation begun.
−6	1.5	Calyx closed; carpels not fused.	E, V, Sp, P, C	Intense mitotic activity in four corners; invagination of inner side.
−5	3	Carpels fused; stamen filaments elongated; petals equal in length with anthers.	E, En, T, V, MMC, C	Wall layers including endothecium and tapetum being formed; connective established.
−4	4	Anthers yellowish; petals enclose anthers; stigma forming.	E, En, T, V, MMC, C	Tapetum and pollen sacs distinct; inner and outer tapetum morphologically different; middle layer crushed.
−3	5	Style elongating; stamen filament extension continues; anthers below stigma.	E, En, T, V, MMC, C	Meiosis begins; callose deposition between microspore mother cells evident.
−2	6	Style clearly elongated; ovary expanded; anthers below stigma.	E, En, T, V, MMC, C, S	Meiosis in progress; tapetum large and multinucleate; stomium differentiation begins; thick callose walls between microspore mother cells.
−1	7	Petals approaching top of sepals.	E, En, T, V, MMC, C, S	Meiosis in progress; continued stomium differentiation.
1	8	Anthers and pistil fully differentiated and green.	E, En, T, V, TDS, C, S	Meiosis complete; microspores in tetrad; stomium differentiated; all sporophytic tissues formed.
2	11	Calyx opens slightly at top of bud.	E, En, T, V, Msp, C, S	Microspores separate.
3	14	Corolla emerges from calyx.	E, En, T, V, Msp, C, S	Tapetum shrunken; secondary thickening in outer wall layers; pollen grains begin to form.
4	16	Sepals completely separated at top of calyx.	E, En, T, V, Msp, C, S	Cells adjacent to stomium degenerated; tapetum degenerating.
5	20	Corolla tube bulge just inside calyx.	E, En, T, V, Msp, C, S	Secondary thickening in outer wall layers intensified.
6	22	Corolla tube bulge at tip of calyx.	E, En, T, V, Msp, C, S	Remnants of tapetum present; microspore nucleus dividing.
7	28	Corolla tube bulge above calyx; petals closed.	E, En, V, Msp, C, S	Degradation of connective tissue in stomium region.
8	39	Corolla elongating; petals green and slightly open.	E, En, V, Msp, C, S	Disruption of connective tissue separating pollen sac.
9	43	Corolla tube bulge enlarging; petal tips becoming pink.	E, En, V, Msp, C, S	Continued connective degradation.
10	45	Corolla limb beginning to open; petal tips pink.	E, En, V, Msp, S	Connective tissue almost fully degraded; pollen binucleate.
11	47	Corolla limb halfway open; stigma and anthers visible.	E, En, V, Msp, S	Anthers bilocular; connective absent; locules filled with mature pollen grains.
12	46	Flower open; anthers dehisced; corolla limb fully expanded and deep pink.	E, En, V, PG	Anthers dehisce along stomium; pollen released.

Table 1. 1: Different stages of tobacco anther development (Koltunow *et. al.*, 1990).

1. 4. 3 Male Sterility

Male sterility is defined as the failure of plants to produce functional anthers, pollen, or male gametes. It is very difficult to make large number of crosses required for hybrid seed production in case of self-pollinated crops in field conditions. The use of male sterile plants has solved this limitation. The phenotypic characteristics of male sterility vary from the complete absence of male organs, the abortion of pollen at any stage of development, the failure to develop normal sporogenous tissues, the absence of stamen dehiscence or the inability of the mature pollen to germinate on the stigma of the flower. The generation of mostly nuclear encoded male sterility is of agricultural importance for the production of hybrids to improve crop yield.

1. 4. 4 Development of male sterile plant for production of hybrid seeds

Due to hybrid vigor or heterosis, the heterozygous hybrid progeny outperforms both homozygous parents. Since the effects of heterosis are observed only in the F1 generation, hybrid seeds always need to be produced by the breeder. Most crops show hybrid vigor, but commercial production of hybrids is feasible only if some kind of pollination control system is available which is both reliable and cost-effective. Pollination control refers to the practices employed to prevent sib- or self-pollination of the female parent, thereby ensuring hybridization between the male and female parent. Many methods can be employed to control self pollination of the female line such as mechanical removal (emasculation) of anthers or male flowers, application of male-specific gametocides, or use of genetic cytoplasmic or nuclear-encoded male sterility. Emasculation of the female line by hand increases costs and labor expenses of seed production. Maize has separate male and female flowers, and the male flowers are located at the top of the plant in the tassel. Pollination control by physical emasculation is therefore, feasible and widely practiced in countries where cheap labor is available. Most of the commercial crops have small bisexual flowers making emasculation difficult and hybrids seeds can be produced with incorporation of some kind of male sterility in these crops.

In case of cytoplasmic male sterility (CMS), the trait is controlled through mitochondrial genome and is transmitted maternally and is successful in case of crops like rice, sorghum and sunflower. However, CMS can be used only if (1) CMS mutants are

available in a given crop; (2) (nuclear) restorer genes are available to restore the fertility of the CMS lines when the seed is the harvested product; and (3) the CMS mutation is not associated with a yield penalty. With the advancements in biotechnology, it has opened many possibilities to generate male sterile plants in any crop plant to generate hybrid seeds such as nuclear-encoded male sterility has been engineered in important crops such as corn and oil seed rape (*Brassica napus*) (Mariani *et. al.*, 1990).

1. 4. 4. 1 Cytoplasmic Male Sterility (CMS)

CMS is observed in both non-transformed and domestic plants wherein the plants are unable to shed viable pollen. This form of male sterility is very useful for hybrid seed industry as a means to generate cross-pollinated seed without the need for labor-intensive hand emasculations and as a strategy for preventing pollen escape in transgenic crops. Different morphological processes including premature dissolution of the callose surrounding the tetrad and premature autolysis of the tapetum layer are involved in CMS associated abortion of the meiocytes result in male sterility. In most cases of CMS, pollen sterility is associated with abnormal mitochondrial function in the sporophytic (maternal) tissue and it represents an excellent model to study the interaction between nuclear and cytoplasmic factors, because fertility restoration relies on nuclear genes that suppress cytoplasmic dysfunction. A variety of mitochondrial DNA rearrangements such as low frequency, illegitimate recombination, or nonhomologous end joining activity within the mitochondrial genome is responsible for the CMS trait (Sandhu *et. al*., 2006).

CMS, the trait resulting in the formation of non-functional microspores or pollen grains, is commonly used by plant breeders for hybrid seeds production. Numerous studies aimed at the explanation of both biological and molecular mechanisms leading to the pollen abortion have been carried out in the past thirty years. Among the cytological events accompanying CMS, the most pronounced one concerns the tapetum - tissue surrounding differentiating microspore mother cells (MMCs) - and involves its abnormal vacuolization, fusion of cells into multinuclear syncytia, and disturbances in the time of the programmed tapetum death. Development of MMCs, depending upon the species, is arrested either during meiosis or in post-meiotic phase, and is usually related to the failure in the deposition of the microspore (pollen) wall. Ultrastructural and morphometric analysis

clearly showed that mitochondria in both tapetum and MMCs are seriously affected in CMS plants, which is reflected in changes of their number, size and structure.

1. 4. 4. 2 Nuclear Male Sterility

Many nuclear genes involved in pollen development have been identified as mutants leading to pollen abortion and male sterility. This nuclear or genic male sterility is useful for hybrid seed production but it has limitations due to the need to maintain female parent lines as heterozygotes and the segregation of fertile and sterile plants each generation (Esser *et. al*., 2006). Nuclear male sterility in plants includes both spontaneous and engineered sterility. Spontaneous mutations leading to nuclear male sterility commonly occur in plants with high frequency. Such mutations can easily be induced by chemical mutagens or ionizing radiation. In many crops, nuclear male sterility does not permit effective production of population with 100% male sterile plants which limits its use in hybrid seed production (Williams, 1995).

1. 4. 5 Development of male sterility through genetic engineering

The advancements in plant biotechnology have added new possibilities to obtain male-sterile plants, thereby, broadening the potential to produce hybrid seeds for different crops. Cytoplasmic male sterility has not yet been achieved but nuclear-encoded male sterility has been successfully engineered in important crops such as corn and oil seed rape (*Brassica napus*). The developmentally regulated promoters were used to drive the expression of cytotoxin genes or the genes involved in normal functioning of anther or pollens, thereby, generating male sterility. Many strategies to produce male-sterile plants have been reported which could be categorized into six different approaches (Table 1. 2).

1. 4. 5. 1 Male sterility induced by genes encoding cytotoxic enzymes

The first report of successfully engineered male sterility was reported in tobacco and oil seed rape was by Mariani *et. al.* (1990). Two genes encoding ribonucleases; a natural gene called *barnase*, from the bacterium *Bacillus amyloliquefaciens* (Hartley, 1988; 1989) and a chemically synthesized *RNase-T1* gene from the fungus *Aspergillus oryzae* (Quaas *et. al*., 1988) were expressed specifically in the tapetum layer under TA29 promoter. The

Table 1.2: Dominant transgenes used to induce male sterility in plants by genetic engineering.

Abbreviations: p, promoter; c, constitutive, t-s, tapetum-specific, psp-s, petal/stamen primordial-specific; p-s, pollen-specific; DTA, diphtheria toxin A-chain; CHS, chalcone synthase, N. A., not analyzed.

S. No	Approach	Transgene	Recipient plant	Effect on female fertility	References
1.	Male sterility by gene encoding cytotoxic enzymes	t-s pTA29/*barnase Rnase* t-s pTA29/T1 *Rnase* t-s pA9/*barnase Rnase* t-s pA3, pA9/PR vacuolar β−1, 3-glucanase	Tobacco, oilseed rape, maize,mustard. Tobacco, oilseed rape Tobacco, mustard Tobacco	None None None None	Mariani *et al., 1990; 1992; 1993* *Jagannath et al., 2001* *Mariani et al., 1990 Paul et al., 1992 Jagannath et al., 2001* *Worral et al., 1992*
2.	Male sterility by gene encoding cytotoxic Proteins	t-s pTA29/DTA Slg-s S-locus glycoprotein promoter/DTA psp-s pAP3/DTA t-s pA9/DTA c p35S/rolC t-s pTA29/RIP cDNA	Tobacco Tobacco Arabidopsis Arabidopsis Arabidopsis Tobacco, Potato, Arabidopsis Tobacco	N.A Yes None None Reduced None	Koltunow *et al., 1990* Thorness *et al., 1991; 1993* Day *et al., 1994; 1995* Guerineau *et al., 2003* Schmulling *et al., 1988; 1993* Cho *et al., 2001*
3.	Male sterility by regulating genes involve in pollen development.	t-s pOsg6B/β-1,3- glucanase	Tobacco	None	Tsuchiya et al., 1995
4.	Chemically induced male sterility	t-s pTA29/P450SU1 t-s pTA29/*argE*	Tobacco Tobacco	None None	O'Keefe *et al., 1994* Kriete et al, 1996
5.	Male sterility by RNA interference	*RAFTIN* c pubiquitin/OsGEN-L	Wheat Rice	None None	Wang *et al., 2003* Moritohl et al., 2005
6.	Male sterility by antisense technology	c p35S: anther expression box or pCHS/antisense CHS cDNA pNin88 /antisense Nin88	Petunia Tobacco	None None	van der Meer *et al., 1992* Goetz et al., 2001

expression of RNase specifically in the tapetum layer led to degradation of all the cellular RNA, precocious degeneration of the tapetal cells, the arrest of microspore development, and hence the development of male sterility. The transformants were similar to the untransformed non-transformed plants with respect to growth rate, height, morphology of vegetative and floral organ systems, time of flowering, flower coloration pattern and female fertility except for the male sterile trait.

Another report of male sterility exploiting degeneration of tapetum was reported where the A6 and A9 promoters were used to express *barnase* in transgenic plants (Hird *et. al*., 1993; Paul *et. al.*, 1992). The *barnase* gene was introduced into the Indian oilseed mustard germplasm - *Brassica juncea*, for the production of improved hybrid seeds by Prof. Deepak Pental's group (Jagannath *et. al*., 2001, 2002). Two tapetum-specific promoters - TA29 (Koltunow *et. al*., 1990) and A9 (Paul *et. al.*, 1992) were used to drive the expression of *barnase* gene and constitutive promoter (CaMV 35S or its double-enhancer variant) to express marker gene (bar gene) to confer herbicide resistance in these plants. The transformation frequency was extremely low with the transgenics showing abnormalities in vegetative morphology, poor female fertility and low seed germination frequencies which might be due to the leaky expression of *barnase* gene from 35S promoter which is a strong promoter acting bi-directionally. Further, the use of a spacer element between the *barnase* gene (downstream to TA29 promoter) and nearby 35S promoter (driving bar gene expression) eliminated unwanted leaky expression and tissue specific expression of barnase gene resulting in desirable male sterile plants with no abnormalities (Jagannath *et. al*., 2001).

In another report, partial and complete male sterile plants were generated by expressing β-1, 3-glucanase in the tapetal layer prior to the appearance of callose activity in the locule, leading to the premature dissolution of the callose walls surrounding the microsporogenous cells (Worral *et. al*., 1992).

1. 4. 5. 2 Male sterility induced by genes encoding cytotoxic proteins

The diphtheria toxin A chain polypeptide (DTA) gene was expressed downstream to tapetum-specific TA29 promoter resulting in dominant male sterile plants (Koltunow *et. al*., 1990). Further, when the S-locus glycoprotein gene promoter of *Brassica* was fused to the

DTA gene, self sterile tobacco (Thorness *et. al*., 1991) and *Arabidopsis* (Thorness *et. al*., 1993) plants were generated due to expression of the gene in both pistil and anthers. When the same DTA gene was fused with the petal/stamen primordial-specific AP3 promoter, it resulted in complete degeneration of petals and stamens in flowers of transgenic *Arabidopsis* (Day *et. al*., 1994; 1995). A temperature dependent male sterile system was developed with a temperature-sensitive polypeptide (DTA) when it was expressed downstream to A9 promoter. The resulting plants were male sterile and female fertile at 18°C whereas completely sterile at 26°C (Guerineau *et. al*., 2003).

The ribosome inactivating protein (RIP) gene from *Dianthus sinensis* is a cytotoxin gene which was used to induce male sterility in tobacco plants (Cho *et. al*., 2001) by expressing the transgene under the TA29 promoter. The anther sections of these transgenic plants revealed the selective and complete destruction of the tapetal layer in the anther resulting in the generation of complete male sterile plants.

1. 4. 5. 3 Male sterility induced by regulating the genes involved in pollen development

The cDNA for endo-β-1, 3-glucanase, a pathogenesis related (PR) protein gene from soybean was used for induction of male sterility. The glucanase gene was expressed downstream to rice tapetum specific promoter (*Osg6B* promoter) (Tsuchiya *et. al*., 1995). The transgenic lines showed premature dissolution of the callose wall in pollen tetrads resulting in decrease in pollen viability, thereby, development of male sterility.

1. 4. 5. 4 Male sterility induced by chemical means

The *argE* gene isolated from *Escherichia coli* was expressed downstream to TA29 promoter for tapetum-specific expression in transgenic tobacco (Kriete *et. al*., 1996). In the tapetal layer, the *argE* gene product acts as an enzyme (ArgE) deacetylating the non-toxic herbicide N-acetyl phosphoinothricin into an active toxic herbicide resulting in the death of tapetal cells. When the transgenics were treated with the herbicide, the flowers contained empty anthers resulting in development of male sterile plants whereas the untreated flowers remained completely fertile, similar to non-transformed plants.

O'Keefe *et. al*., (1994) reported generation of male sterility by combining the use of biotechnology and chemical application. Transgenic tobacco plants expressing a

cytochrome $P450_{SU1}$ gene downstream to TA29 promoter accumulated the gene product specifically in the tapetal layer. In the chloroplast of the tapetal cells, the cytochrome $P450_{SU1}$ gets converted from its normally non-herbicidal form to highly phytotoxic form. When the plants were treated with sulfonyl urea compound R7402, the protein targeted to the chloroplast converts it into a 500-times more toxic form, resulting in non-viable pollen whereas the untreated plants were fertile. However, generation of male sterility in this system was limited only in appropriate flower developmental stages during application of the compound which could be due to the rapid metabolism of R7402 compound in the plant cells.

1. 4. 5. 5 Male sterility induced by RNA interference

RAFTIN, a structural protein is essential for normal pollen development (Wang *et. al*., 2003). RAFTIN appears to be essential for the late phase of pollen development in rice. The expression of the gene encoding for RAFTIN is specific only to the anther tissue. Therefore, RNA interference (RNAi)-mediated suppression of RAFTIN expression in the anther tissue resulted in generation of male sterile plant in rice.

Male sterility was also observed while studying the function of OsGEN-like (OsGEN-L) gene from rice (*Oryza sativa* L.) which is a member of the RAD2/XPG nuclease family (Moritohl *et. al*., 2005). The transgenic OsGEN-L-RNAi rice plants in which OsGEN-L expression was silenced displayed low fertility, and some of them were male sterile. These transgenic plants lacked mature pollen due to a defect in early microspore development. This indicates that the OsGEN-L is involved in the DNA metabolism required for the early microspore development in rice.

1. 4. 5. 6 Male sterility induced by antisense RNA technology

In *Petunia hybrida* the flavonoid pigments are very much essential for the normal development of the pollen. To silence the *chs* gene (Chalcone synthase involved in the flavonoid biosynthetic pathway), through antisense approach, the gene was expressed downstream to a modified CaMV 35S promoter in transgenic petunia. The resulting transgenics developed white anthers due to inhibition of pigment synthesis leading to generation of male sterility (van der Meer *et. al*., 1992).

In another antisense approach, the extracellular invertase gene, which is essential for phloem unloading *via* an apoplastic pathway, was targeted. The repression of invertase isoenzyme Nin88, under control of the corresponding promoter, resulted in male sterile transgenics in tobacco due to tissue specific abolition of invertase activity during early stages of pollen development. This report illustrated the significant role of various enzymes like extracellular invertase in pollen development (Goetz *et. al*., 2001).

1. 5. 1 Plant disease resistance

One of the major interests in plant biology is the production of crops with increased and durable resistance to a spectrum of diseases. Conventional breeding approaches and the widespread use of pesticides were the major means of achieving durable resistance until recently. Disease is rare in nature because plants carry different layers of defense – from structural barriers and pre-formed antimicrobials to offering different mechanisms of resistance to pathogens that encompass non-host, race–specific and race non-specific resistance. Plants possess both preformed and inducible mechanisms to resist pathogen invasion. Extant morphological barriers, secondary metabolites (phytoanticipins), and antimicrobial proteins must be avoided or overcome for pathogens to be able to invade a plant. Once contact has been established, elicitors produced and released by the pathogen induce further defenses, comprising the reinforcement of cell walls, the production of phytoalexins, and the synthesis of defense-related proteins.

In both compatible and incompatible plant-pathogen interactions, hundreds of genes are up- and down regulated. In many cases, differences between susceptibility and resistance are associated with differences in the timing and magnitude of these changes rather than with the expression of different sets of genes. Whether or not a plant turns out to be susceptible or resistant is likely determined by the speed and magnitude with which these mechanisms are activated and expressed and by their effectiveness against individual pathogens with different modes of attack (Loon *et. al.*, 2006). Upon infection by various types of pathogens, defense-related genes are coordinately activated and may be expressed in both infected and noninfected tissues concomitant with the development of SAR. All *PR-1* genes in plants appear to be inducible by SA, and endogenous production or exogenous application of SA has been shown to be both necessary and sufficient to elicit

the induced state (Vernooij *et. al.*, 1994). Active oxygen species (AOS) are involved in SAR and, indeed, tissue damage invariably leads to the production of AOS and synthesis of SA. Progressive damage will amplify SA production even further, and inevitably lead to induction of defense-related genes and SAR.

With the present understanding of plant disease resistance, two new avenues are being pursued; non-transgenic strategies that use marker-assisted breeding and transgenic approaches. Several resistant cultivars have been developed in crop plants to combat diseases by conventional methods. However, the success of these methods is limited due to fertilization barriers leading to adoption of modern molecular tools such as introduction of transgenes. Many of the initial transgenic approaches to reduce severity of diseases caused by fungal pathogens aimed at the constitutive production of one component of the plants' own defense *e.g.*, PR proteins. Constitutive expression of chitinase and/or β-1, 3 glucanase genes results in increased disease resistance (Broglie *et. al.*, 1991; Zhu *et. al.*, 1994; Lin *et. al.*, 1995). However, the approach was not successful in developing fungal disease resistant plants. Lack of detailed fundamental knowledge of host pathogen interactions is the main limitation to development of disease resistant transgenics.

Expression of *Xa*-21 (R gene) under constitutive promoter in transgenic rice conferred durable resistance against bacterial blight pathogen, *Xanthomonas campestris* pv. *oryzae* (Wang *et. al.*, 1996). However, similar strategy to confer resistance against fungal pathogens has not been successful. When the resistance is brought about by one or few in-bred disease resistance genes per crop species, as in case of cultivation of huge areas of genetically identical crops, pathogen can easily overcome such disease resistance genes. An alternate strategy for engineering broad-spectrum resistance, based on coordinate expression of an *R* gene and a corresponding *Avr* transgene, controlled by a pathogen-inducible promoter has been proposed (McDowell *et. al.*, 2003) which enables the induction of resistance against multiple pathogens without pyramiding the *R* genes. There is no loss of durability of *R* genes as the *Avr* gene is not under selective pressure for mutation.

1. 5. 2 Pathogen inducible promoters for generation of transgenics

The constitutive expression of the transgenes might confer increased disease resistance but the plants might show reduced size, altered morphology or disease symptoms in the absence of pathogens (Fitzgerald *et. al.*, 2004). Expression of the transgene only when and where it is needed at the sites of infection is possible with the use of pathogen-inducible promoters. The detrimental effects on plant growth and development, owing to unwanted transgene expression in disease-free conditions, can be eliminated using pathogen-inducible promoters. An ideal pathogen-inducible promoter should be activated rapidly in response to a wide range of pathogens conferring broad-spectrum resistance and also must be inactive under disease free conditions to ensure that there are no unwanted defense responses triggered by leaky expression of the transgene (McDowell *et. al.*, 2003).

Osmotin is a small, basic, pathogenesis-related protein (PR-5), which has *in vitro* and *in vivo* antifungal activity. There are more than 10 inducers that activate the osmotin gene in various plant tissues. Transgenic 'Royal Gala' apple (*Malus domestica* Borkh.) plants expressing a modified cecropin gene *MB39* joined to a secretory coding sequence from barley α-amylase, and placed under the control of tobacco osmotin promoter exhibited 2.5- to 3.3-fold more resistance to *Erwinia amylovora* (Ingersoll *et. al*., 2001).

Phenylalanine ammonia lyase (PAL, 1.4 kb) promoter is pathogen and wound-responsive but also active during normal development of xylem and flower (Bevan *et. al.*, 1989). HSR203J (1.4 kb) promoter drives rapid, high-level, localized and HR specific activation of candidate genes in response to pathogen (Pontier *et. al.*, 1994). The induction of HSR203J promoter occurs during incompatible interactions with bacterial and viral pathogens and also with inducers of HR such as harpin, elicitin and PopA1 proteins (Pontier *et. al.*, 1994 and 1998; Takahashi, 2004). HSR203J promoter confers pathogen-inducible cryptogein production without damaging transgenic plants (Keller *et. al*., 1999).

1. 6 Hypothesis and objectives of the study

A transgenic approach, to exploit the harpin-mediated cell death to induce male sterility by expressing harpin in tapetal layer, resulted in development of partial male sterile transgenic tobacco (Madhuri, 2006). The tapetum-specific expression of harpin in intracellular form leads to accumulation of harpin in the tapetal cells. Since the harpin receptor is on the cell wall (Tampakaki *et. al.*, 2000), harpin was unable to access its receptor to cause HR-mediated death of tapetal cells. However, towards later stages of anther development, the tapetum undergoes natural process of degeneration releasing the harpin molecules which binds to the pollen cell wall and subsequent cell death resulting in development of partial male sterile lines.

The present study was undertaken to improve the male sterility with harpin developed by Madhuri (2006). Suitable constructs were devised for signal peptide-mediated release of harpin into the apoplast for a 'better' interaction with the putative receptor in the cell walls leading to HR-mediated cell death (Fig. 1.4). This approach will allow us to test the possibility of inducing complete male sterility and disease resistance by expression of harpin under tightly regulated promoters using a transgenic approach. The present thesis reports generation of tobacco transgenics with harpin gene in intracellular and secretable form and characterization of the transgenics with the following objectives:

A) Generate suitable constructs to target harpin to the apoplast making use of signal sequence of the PR1a gene to express either in tapetal tissue or in presence of pathogen

B) Develop and compare the efficiency of apoplastic trageting of harpin to the intracellular expression to induce male sterility in tobacco

C) Develop disease-resistant transgenic tobacco expressing harpin in a pathogen–inducible system

Figure 1.4: Two different strategies of harpin expression (intracellularly or secretable) were devised using appropriate promoters. In intracellular form, upon expression, harpin gets accumulated inside the cells and the wounds created by pathogens contribute to leakage of harpin to the apoplast whereas in the secretable form of harpin is directly translocated to the apoplast. In the apoplast, harpin is expected to bind to its receptor present in the cell wall resulting in the signal transduction events leading to HR-mediated cell death.

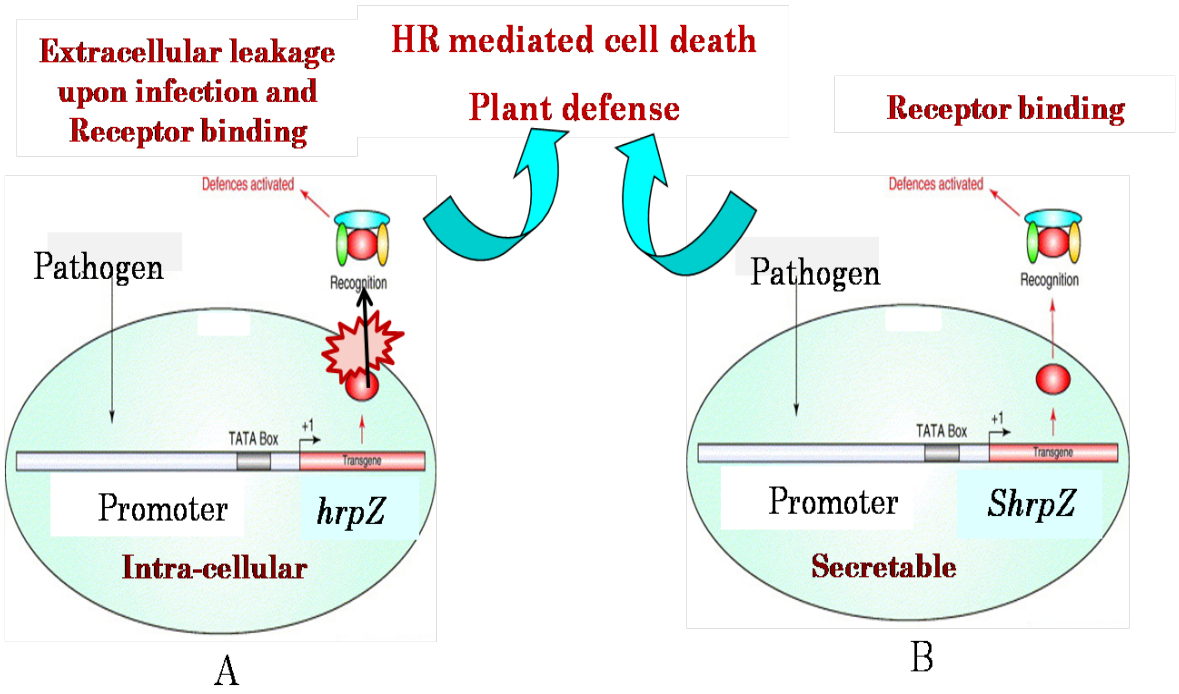


Figure 1.4: Strategies of harpin expression in transgenics.

Materials and Methods

2. 1. 1 Bacterial cultures/ Tobacco cultivar

Escherichia coli DH5α

Agrobacterium tumefaciens strain EHA105

Nicotiana tabacum cv. xanthi

2. 1. 2 Media used

• Luria Bertani (LB) medium – To 900 ml of water, 10 g tryptone, 10 g NaCl, 5 g Yeast Extract and 15 g Agar was added and the pH was adjusted to 7.2 and volume was made up to 1 litre. LB medium was used to grow *Escherichia coli* and *Agrobacterium* cultures with appropriate antibiotics.

• Murashige and Skoog (MS) medium for culturing and transformation of tobacco:

Germination medium- 2.2 g/L MS salts, 3% sucrose, 0.8% agar, pH 5.6-5.8.

Cocultivation medium- 4.4 g/L MS salts, 3% sucrose, 0.8% agar, pH 5.6-5.8.

Regeneration (shoot inducing) medium- 4.4 g/L MS salts, 3% sucrose, 1 mg/l 6-BAP, 100 mg/l timentin, 25 mg/l hygromycin, 0.7 % agar, pH 5.6-5.8.

Rooting medium- 2.2 g/L MS salts, 3% sucrose, 100 mg/l timentin, 25 mg/l hygromycin, 0.7 % agar, pH 5.6-5.8.

2. 1. 3 Chemicals

X-Gluc was procured from Duchefa. Isopropyl-β-D-thiogalactoside (IPTG), X-Gal, phenol solution Tris equilibrated, GeneElute HP Plasmid miniprep kit, Agarose and plant growth regulators were purchased from Sigma. All other chemicals, antibiotics and routine media components for bacterial and plant tissue culture, including readymade MS

salts, were of analytical grade and obtained from HiMedia laboratories (India) unless otherwise stated.

2. 1. 4 Phytohormones

6-benzylaminopurine (6-BAP): 1 mg/l.

2. 1. 5 Antibiotics

- kanamycin, rifampicin -50 µg/ml.
- timentin (GlaxoSmithKline) -150 µg/ml.
- hygromycin (Calbiochem, Germany) -25 µg/ml.

2. 1. 6 Kits and enzymes

Plasmid isolation kit and Taq DNA polymerase were from Simga-Aldrich Ltd while Gel extraction kit and DNeasy Plant mini kit for plant genomic DNA isolation were from Qiagen. pGEM-T cloning kit was procured from Promega for T/A-cloning of PCR products. Easy-A™ High fidelity PCR cloning enzyme from Stratagene. *Pfu DNA* polymerase, T4 DNA Ligase and all restriction enzymes from MBI Fermentas (Germany) and were used as per the manufacturers' instructions. Recombinant DNase I and blueprint 1st strand cDNA Synthesis Kit was procured from TaKaRa. The Gene Images™ AlkPhos Direct™ labeling kit with CDP-*Star*™ chemiluminescent detection reagents were procured from Amersham Biosciences.

2. 2 Plasmids

The brief details of the vectors used and the constructs generated in this study are given in Table 2.1.

2. 3 Primers used for PCR

The primers used in this study (Table 2.2) were procured either from MWG biotech Pvt. Ltd. or from Sigma-Aldrich Pvt. Ltd. The cycling conditions used for PCR amplification of the promoters, *hrpZ* and *ShrpZ* sequences are given in Table 2.3.

plasmid (designation)	**purpose**	**Source**
pCAMBIA1300 and pCAMBIA2301	cloning and plant transformation	Prof. Kirti, P. B., University of Hyderabad, India
pCAMBIA1303	cloning and plant transformation	Prof. Sharma, R. P., University of Hyderabad, India
pYEUT- *hrpZ*	Source of *hrpZ*	Prof. Feng,T.-Y., Institute of Academia Sinica, Taiwan
pGEMT-*ShrpZ*	Source of *ShrpZ*	This study
pRT101-PAL	Source of tomato PAL promoter	Prof. Rajam, M.V., University of Delhi.
pRT100	Source of 35S promoter	Our collection.
pCAMBIA2301-TA29-*hrpZ-nos* (TH)	Source of TA29 promoter	Our collection.
pCAMBIA2301-TA29-*ShrpZ-nos* (TSH)	plant transformation	this study
pCAMBIA1300-PAL-*ShrpZ-nos* (PSH)	plant transformation	this study
pCAMBIA1303-HSR -*ShrpZ -nos* (HSH)	plant transformation	this study
pCAMBIA1300-35S -*ShrpZ-nos* (CSH)	plant transformation	this study
pCAMBIA1300-35S -*hrpZ -nos* (CH)	plant transformation	this study
pCAMBIA1300-OSM -*ShrpZ -nos* (OSH)	plant transformation	this study

Table 2.1: Brief details of plasmids used and/or generated in the present study.

Sl No.	Name	Sequence	Restriction sites
1	HrpZ FP1	5'-ACA TGG AGC TCA TGC AGA GTC TCA G-3'	*Sac*I
2	HrpZ RP1	5'- ACG AGC TCT CAG GCT GCA GCC TGA TT-3'	*Sac*I
3	HrpZ RP2	5'-CGC GGA TCC TCA GGC TGC AGC CTG ATT GCG-3'	*Bam*HI
4	Ch Hrp FP	5'- AAC TCT CAA CAA GTC AGT CTT AAC AGC-3'	None
5	Ch SP RP	5'- GTT AAG ACT GAC TTG TTG AGA GTT TTG G-3'	None
6	SP FP1	5'-CAT GCC ATG GGA TTT GTT CTC TTT TC-3'	*Nco*I
7	SP FP2	5'-CAT GCA TAT GGG ATT TGT TCT C-3'	*Nde*I
8	SP RP1	5'-CAT GGA GCT CTT GTT GAG AGT TTT GGG C -3'	*Sac*I
9	TA29 FP	5'-ACG CGT CGA CTT TTT GGT TAG CGA ATG C 3'	*Sal*I
10	TA29 RP	5'- CAT GCC ATG GCT ACC ATG GTA GCT AAT TCC – 3'	*Nco*I
11	OSM FP	5'-TGA CAC TGC AGC CAT ATT TGG ACC TTT TTC CG-3'	*Nco*I(*Pst*I)
12	OSM RP	5'-GCG CAT GCC ATG GTT GGA TAT AGT GAC AAA TTG-3'	*Nco*I
13	HSR FP	5'-CGA TGG CTG CAG CAA TAT AAT ACA TTT TCA AAT TAA ATT AG-3'	*Nco*I(*Pst*I)
14	HSR RP	5'-ACA TAT GTTTGG TTT GGA TGT GCG TGG C -3'	*Nde*I
15	Nos FP	5'-CAT CTA GAG CTC GCA AAT CAC CAG TC-3'	*Sac*I
16	Nos RP	5'-AAG CTT GAG CTC CTG CAG GTC ACT G-3'	*Sac*I-*Pst*I
17	NptII FP	5'-GAG AGG CTA TTC GGC TAT GAC T -3'	None
18	NptII RP	5'-GAT ACC GTA AAC CAC GAG GAA G-3'	None
19	Hyg FP	5'-CCC CCG TTA CTC TAT ACT TTT TCG G -3'	None
20	Hyg RP	5'-CTA GCT GTC TAG GCC AGC CGT AGA T -3'	None
21	HSR203 FP	5'-TGT ACT ACA CTG TCT ACA CGC -3'	None
22	HSR203 RP	5'-GAT AAA AGC TAT GTC CCA CTC C-3'	None
23	PR1 FP	5'-GAT GCC CAT AAC ACA GCT CG -3'	None
24	PR1 RP	5'-TTT ACA GAT CCA GTT CTT CAG AGG -3'	None
25	PR2 FP	5'-CTG CCC TTG TAC TTG TTG GG-3'	None
26	PR2 RP	5'-TCC AGG TTT CTT TGG AGT TCC -3'	None
27	PR3 FP	5'-GGT TCT ATT GTA ACG AGT GAC -3'	None
28	PR3 RP	5'-TTC TAT GTA ACG AAG CCT AGC -3'	None
29	HIN1 FP	5'-TCT CAT GTT TCC TTC TCC GG-3'	None
30	HIN1 RP	5'-CAA AGT AAC CTA GCA ATC CTC TAC C -3'	None

Table 2.2: Details of the primers used in this study.

	SP	*hrpZ*	*ShrpZ*	Promoters		
				TA29	OSM	HSR
Step 1	94°C - 3 min	94°C- 3 min	94°C - 3 min	94°C - 3 min	94°C - 3 min	94°C - 3 min
Step 2	94°C - 40 sec	94°C - 40 sec	94°C - 40 sec	94°C - 40 sec	94°C - 40 sec	94°C - 40 sec
Step 3	55°C - 40 sec	58°C - 40 sec	58°C – 40 sec	58°C - 40 sec	60°C - 40 sec	55°C - 40 sec
Step 4	72°C – 40 sec	72°C - 1 min	72°C - 1 min 15 sec	72°C - 1 min	72°C - 1 min	72°C - 1 min
Step 5	Go to step '2' 30 more times					
Step 6	72°C - 5 min				72°C - 10 min	72°C - 10 min

Table 2. 3: Cycling conditions for PCR amplification of *hrpZ*, *ShrpZ*, SP and different promoters (TA29, OSM and HSR).

2. 4. 1 Genes

The *hrpZ* (1.02 kb) gene was available in the lab as pYEUT-*hrpZ*. The gene was amplified using plasmid DNA (pYEUT-*hrpZ*) as template with the gene specific primers HrpZ-FP1 and HrpZ-RP1 (Table 2.2) using *Pfu* DNA polymerase.

The signal peptide (*SP*) portion (90 bp) of Pathogenesis Related (*PR1a*) gene was PCR-amplified from genomic DNA of tobacco non-transformed plant cv. *xanthi* with signal peptide region specific primers SP-FP1 & SP-RP1 using *Pfu* DNA polymerase. The amplicon (*SP* including primer sequences =135 bp) was gel purified using Qiagen Gel Clean up kit and cloned in pGEM-T cloning vector according to the protocol given by the manufacturer (Promega) at 4 ˚C for overnight. The plasmids isolated from white colonies were analyzed and sequenced.

The *SP* and *hrpZ* genes were fused *in-frame* through PCR-based approach to generate fusion gene (*ShrpZ*, 1.2 kb) (Fig. 2.1) which was used for generation of constructs. The signal *SP* and *hrpZ* were amplified separately with chimeric gene overlapping primers- Ch Hrp-FP and Ch SP-RP (Table 2.2) in first PCR of 30 cycles and gel purified using Sigma Gel clean up kit. Different concentrations of these two PCR amplicons were used for annealing together in second PCR of 20 cycles without adding any primers. In third PCR of 30 cycles, a small aliquot (4-6 µl) from the second PCR was used as template with SP-FP1 and HrpZ-RP1. All these three PCRs were done at an annealing temperature of 58˚C using *Pfu* DNA polymerase. The *SP* and *hrpZ* gene fusion product (*ShrpZ*, 1.2 kb) was gel purified and re-amplified using Easy-A^{TM} High-Fidelity PCR cloning enzyme (Stratagene) which has the property of proof reading as well as non template dependent 3'-A addition property to PCR products. The reaction products were gel purified and cloned in pGEM-T cloning vector for T/A-cloning of the fusion gene (*ShrpZ*). The plasmids from white colonies were analyzed and positive clones were sequenced to confirm the faithfulness of fusion and the sequence of nucleotides.

Figure 2.1: The *SP* and *hrpZ* genes were amplified individually using chimeric primers in first round PCR. The amplicons were gel purified and equal concentrations of *SP* and *hrpZ* were annealed together in second round PCR. The reaction product (2 µl) of second round PCR was used as a template for third round PCR to amplify the entire fusion gene (*ShrpZ*) using SP-FP and HrpZ-RP.

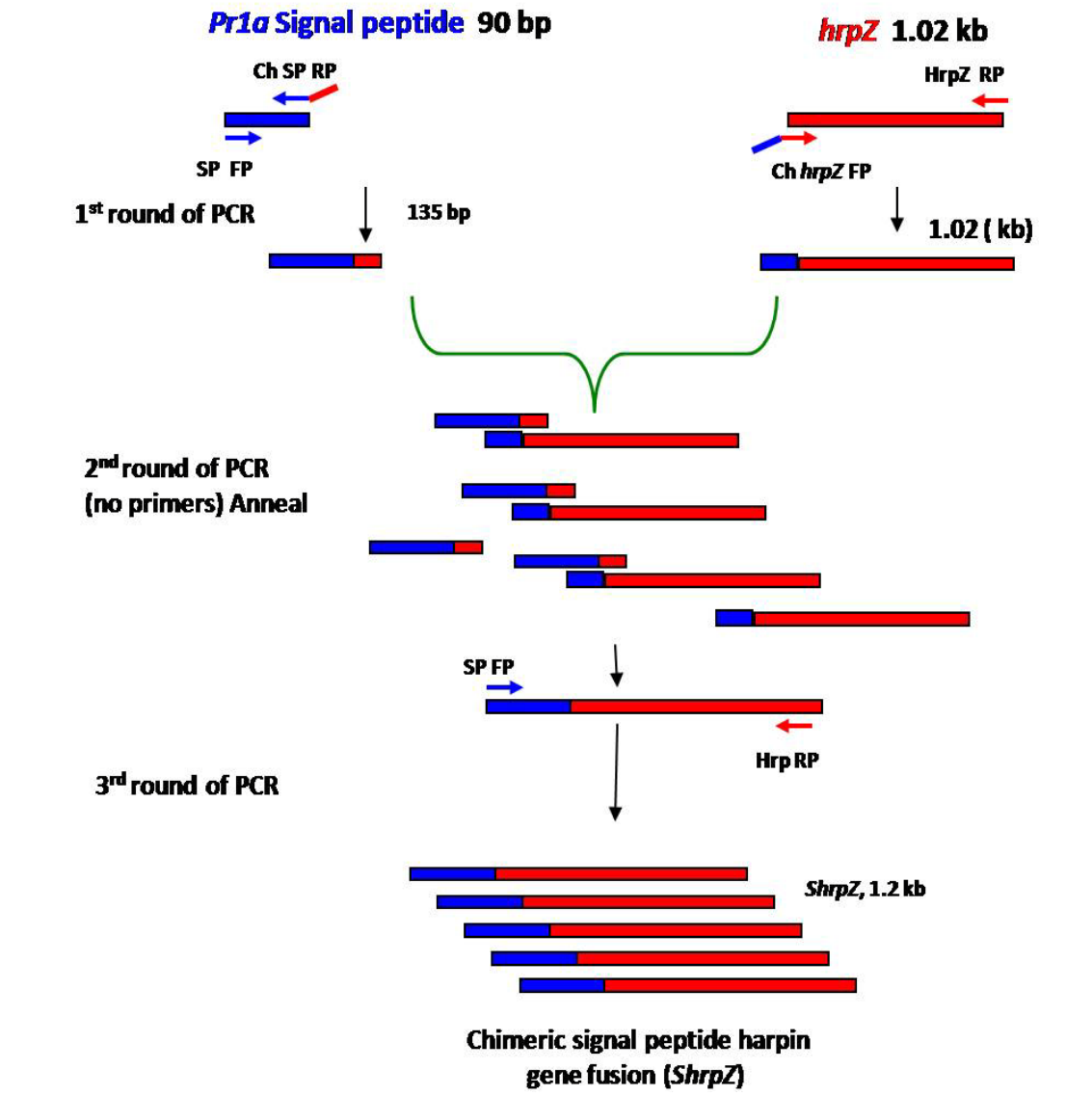


Figure 2.1: PCR-based gene fusion of signal peptide (SP, 90 bp; blue colour) and harpin (*hrpZ* gene, 1.02 kb; red colour) genes to generate fusion gene (*ShrpZ*, 1.2 kb).

2. 4. 2 Promoters used in the generation of transgenics

2. 4. 2.1 Developmentally regulated promoter (TA29) - The tapetum-specific promoter (TA29, 870 bp) was available in our lab as pCAMBIA1300-TA29-*hrpZ-nos* (TH) construct. This construct was used to generate the pCAMBIA2301-TA29-*ShrpZ-nos* (TSH) construct.

2. 4. 2. 2 Constitutive promoter (35S) - The 35S promoter (500 bp) was available as 35S promoter-MCS-*nos* cassette in pRT100 (Our collection).

2. 4. 2. 3 Pathogen inducible promoters (PIP) –

a) **Osmotin promoter (OSM)** - The pathogen inducible promoter - osmotin (OSM, Accession no. - S68111) from tobacco is of 2 kb including a suppressor element at the 5'- region of the promoter. The 1 kb region of the promoter (from +1 to - 1051 bp) was PCR-amplified from the genomic DNA of tobacco *cv. xanthi* (cycling conditions – Table 2.3) and used in generation of the construct.

b) **Tomato PAL promoter (PAL)** - The tomato PAL promoter (232 bp, Accession no.- M83314) was a gift from Prof. M.V. Rajam, University of Delhi in the form of PAL-MCS-*nos* cassette in pRT101.

c) **Tobacco HSR promoter (HSR) -** The HSR203J promoter (1.3 kb) sequence was available in the NCBI database (Accession no-X77136). This promoter also contains a suppressor element at the 5'- region. Therefore, only 660 bp region of the promoter (from +1 to - 660 bp) was PCR amplified (cycling conditions – Table 2.3) from genomic DNA of tobacco *cv. xanthi* using HSR FP and HSR RP (Table 2.2). The amplicon was gel purified, T/A-cloned in pGEM-T cloning vector as per manufacturer's (Promega) instruction and confirmed cloning of amplicon by double digestion.

2.5 Handling of DNA

All the molecular biology protocols including plasmid and genomic DNA isolation, restriction digestion, agarose gel electrophoresis, ligation, competent cell preparation and transformation were done following Sambrook *et. al.* (1989).

2. 6 Generation of promoter-*ShrpZ-nos* and promoter-*hrpZ-nos* constructs

The following DNA manipulations were carried out to transcriptionally fuse the candidate genes (*hrpZ* and *ShrpZ*) under the control of tapetum-specific promoter (TA29), constitutive promoter (CaMV 35S) and pathogen-inducible promoters (HSR, PAL, and OSM). The details of the constructs generated in the different pCAMBIA binary vectors are given in Table 2. 4.

2. 6. 1 Generation of pCAMBIA2301- TA29-*ShrpZ-nos* (TSH) construct

The *hrpZ* (1.02 kb) gene was released from pCAMBIA1300-TA29-*hrpZ-nos* (TH) construct as *Nco*I/*Sac*I fragment and the *ShrpZ* (1.2 kb) fusion gene was cloned into the TH backbone generating the TSH cassette in pCAMBIA1300 vector. The entire TA29-*ShrpZ-nos* cassette was released as *Pst*I/*Eco*RI fragment from pCAMBIA1300 vector and sub-cloned into pCAMBIA2301 generating pCAMBIA2301:TA29-*ShrpZ-nos*, designated as TSH construct (Fig 2.2; Table 2. 1, 2. 4).

2. 6. 2 Generation of pCAMBIA1300-35S-*ShrpZ-nos* (CSH) construct

The *ShrpZ* (1.2 kb) fusion gene was PCR amplified (cycling parameters, Table 2. 3) as a single band with *Pfu* polymerase with the primers (SP FP1 and HrpZ RP2; Table 2. 2) using pGEMT-*ShrpZ* as a template. The gene was cloned as *Nco*I/*Bam*HI fragment in to pRT100. The entire 35S-*ShrpZ-nos* cassette was released as *Pst*I fragment and cloned into *Pst*I site of pCAMBIA1300 generating pCAMBIA1300-35S-*ShrpZ*-nos (CSH) construct (Fig.2.3; Table 2. 1, 2. 4).

Constructs	Promoter (size in kb)	Gene (size in kb)	*nos* terminator (size in kb)	Cassettes (size in kb)	Binary Vector	Total construct size (in kb)
TSH	**TA29 (0.87)**	***ShrpZ* (1.2)**	***nos* (0.2)**	**TA29-*ShrpZ-nos* (2.27)**	**pCAMBIA2301 (11.63)**	**~13.9**
PSH	**PAL (0.6)**			**PAL-*ShrpZ-nos* (2.0)**	**pCAMBIA1300 (8.96)**	**~11.0**
HSH	**HSR (0.7)**			**HSR-*ShrpZ-nos* (2.1)**	**pCAMBIA1303 (12.36)**	**~14.5**
CSH	**35S (0.5)**			**35S-*ShrpZ-nos* (1.9)**	**pCAMBIA1300 (8.96)**	**~10.9**
OSH	**OSM (1.0)**			**OSM- *ShrpZ-nos* (2.4)**	**pCAMBIA1300 (8.96)**	**~11.4**
CH	**35S (0.5)**	***hrpZ* (1.02)**		**35S-*hrpZ*-nos (1.72)**	**pCAMBIA1300 (8.96)**	**~10.7**

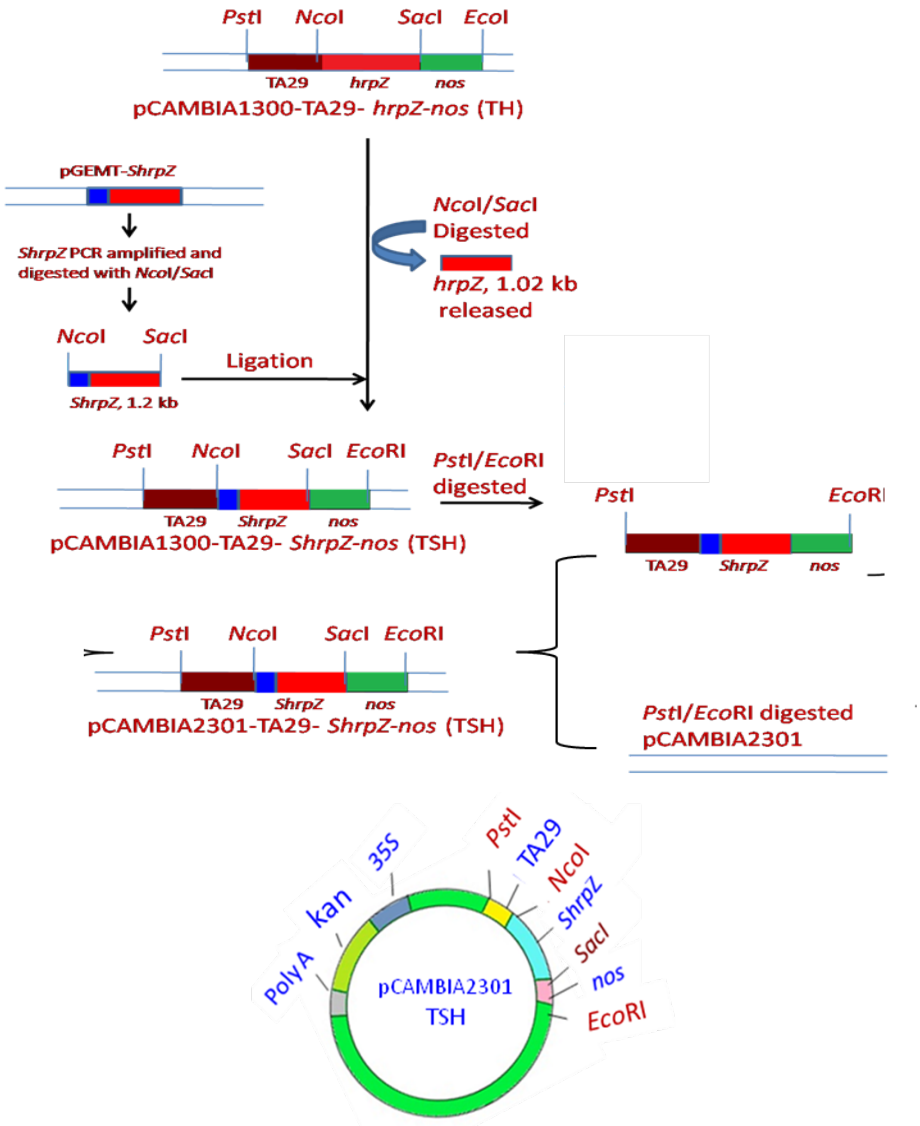
Table 2. 4: Details of different constructs generated in the pCAMBIA2301, pCAMBIA1300 and pCAMBIA1303.

Figure 2.2: **The map and sequence of TSH construct in pCAMBIA2301 (11.63 kb)**

(a) Schematic diagram showing the construction and map of pCAMBIA2301:TA29-*ShrpZ-nos* (TSH) construct (13.9 kb). The *ShrpZ* gene was PCR amplified from pGEMT-*ShrpZ*, digested with *Nco*I/*Sac*I and substituted at *hrpZ* position in pCAMBIA1300-TA29-*hrpZ-nos* (TH) construct resulting in generation of TA29-*ShrpZ-nos* (TSH) cassette in pCAMBIA1300. The TSH cassette was digested out as *Pst*I/*Eco*RI fragment and cloned into pCAMBIA2301 generating pCAMBIA2301-TA29-*ShrpZ-nos* (TSH) construct.

(b) Sequence of TA29-*ShrpZ*-nos cassette (2.27 kb). The sequence of *ShrpZ* gene is underlined.

(a)



(b)

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TGACTTGTCACTTTATGAAAGATATTCAAACATGATTTTTTATGTACTAATATATACATCCTACTCGAATTAAAGCGACATAGGCTCGAAGTATGCACATTTAGCAATGTAAATTAAATCAGTTTTTGAAT
CAAGCTAAAAGAGACTTGCATAAGGTGGGTGGCTGGACTAGAATAAACATCTTCTCTAGCACAGCTTCATAATGTAATTTCCATAACTGAAATCAGGGTGAGACAAAATTTTGGTACTTTTTCCTCACA
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TGACGAATTC

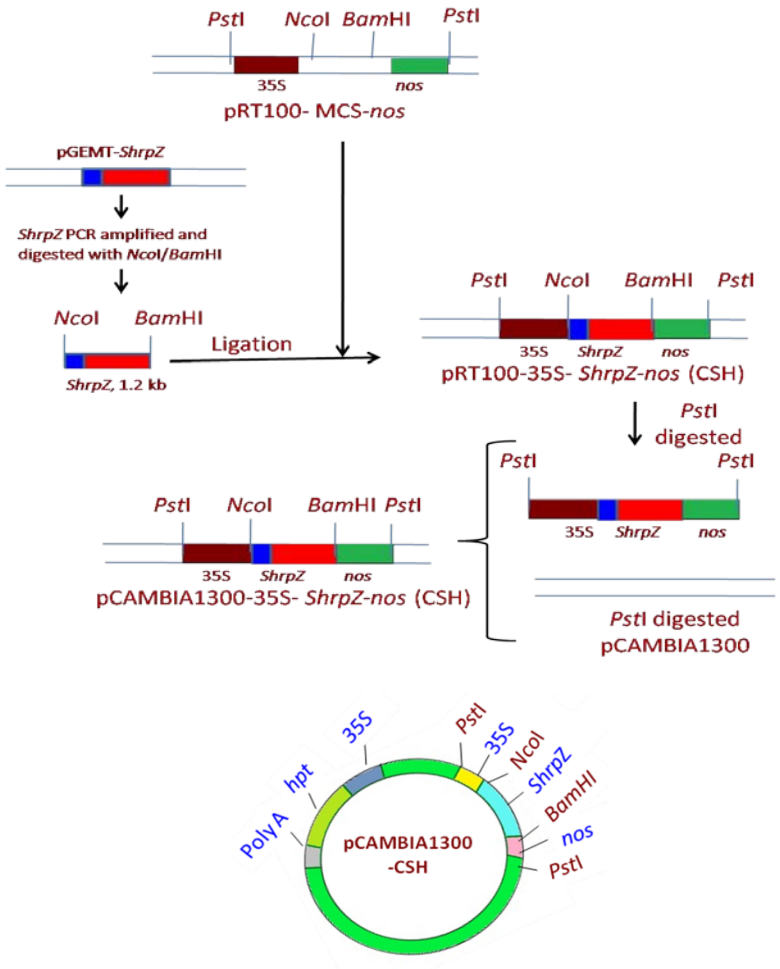
Figure 2.2: **TSH construct in pCAMBIA2301 vector (11.63 kb).**

Figure 2.3: **The map and sequence of CSH construct in pCAMBIA1300 (9 kb)**

(a) Schematic diagram showing the construction and map of pCAMBIA1300: 35S-*ShrpZ-nos* (CSH) construct (~10.9 kb). The *ShrpZ* gene was PCR amplified from pGEMT-*ShrpZ*, digested with *Nco*I/*Bam*HI and cloned in pRT101 downstream to 35S promoter resulting in generation of 35S-*ShrpZ-nos* (CSH) cassette in pCAMBIA1300 vector. The CSH cassette was digested out as *Pst*I fragment and cloned into pCAMBIA1300 vector generating pCAMBIA1300-35S-*ShrpZ-nos* (CSH) construct.

(b) Sequence of 35S-*ShrpZ-nos* cassette (1.9 kb). The sequence of *ShrpZ* gene is underlined.

(a)



(b)

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Figure 2.3: **CSH construct in pCAMBIA1300 vector (9 kb).**

2. 6. 3 Generation of pCAMBIA1300-PAL-*ShrpZ-nos* (PSH) construct

The *ShrpZ* (1.2 kb) gene released from CSH construct as *Xho*I/*Bam*HI fragment was cloned in to pRT101 harbouring tomato PAL promoter in place of 35S promoter. The entire PAL-*ShrpZ-nos* cassette was released as *Pst*I fragment and cloned into *Pst*I site of pCAMBIA1300 generating pCAMBIA1300-PAL- *ShrpZ* -nos (PSH) construct (Fig.2.4; Table 2. 1, 2. 4).

2. 6. 4 Generation of pCAMBIA1300-35S-*hrpZ-nos* (CH) construct

The *hrpZ* (1.02 kb) gene was PCR amplified and cloned in to pRT100 as *Sac*I/*Sac*I fragment. The entire 35S-*hrpZ*-nos cassette was released as *Pst*I fragment and cloned into *Pst*I site of pCAMBIA1300 generating pCAMBIA1300-35S-*hrpZ*-nos (CH) construct (Fig.2.5; Table 2. 1, 2. 4).

2. 6. 5 Generation of pCAMBIA1300-OSM-*ShrpZ-nos* (OSH) construct

The *ShrpZ* (1.02 kb) gene was PCR amplified as *Nco*I/*Sac*I product with SP FP1 and HrpZ RP1 using Easy-A high fidelity PCR cloning enzyme (Stratagene) from pGEMT-*ShrpZ* and T/A-cloned in pGEM-T vector. The osmotin promoter (OSM) was PCR amplified as *Nco*I(*Pst*I)/*Nco*I fragment using TrOsm FP and Osm RP (Table 2.2) and cloned upstream of *ShrpZ* gene. The *nos* terminator was PCR amplified as *Sac*I/(*Pst*I)*Sac*I fragment and cloned downstream to *ShrpZ* gene. The orientation of OSM promoter and *nos* terminator was determined through PCR and the entire OSM-*ShrpZ*-nos cassette was released as *Pst*I fragment and cloned into *Pst*I site of pCAMBIA1300 generating pCAMBIA1300-OSM-*ShrpZ*-nos (OSH) construct (Fig.2.6; Table 2. 1, 2. 4).

2. 6. 6 Generation of pCAMBIA1303-HSR-*ShrpZ-nos* (HSH) construct

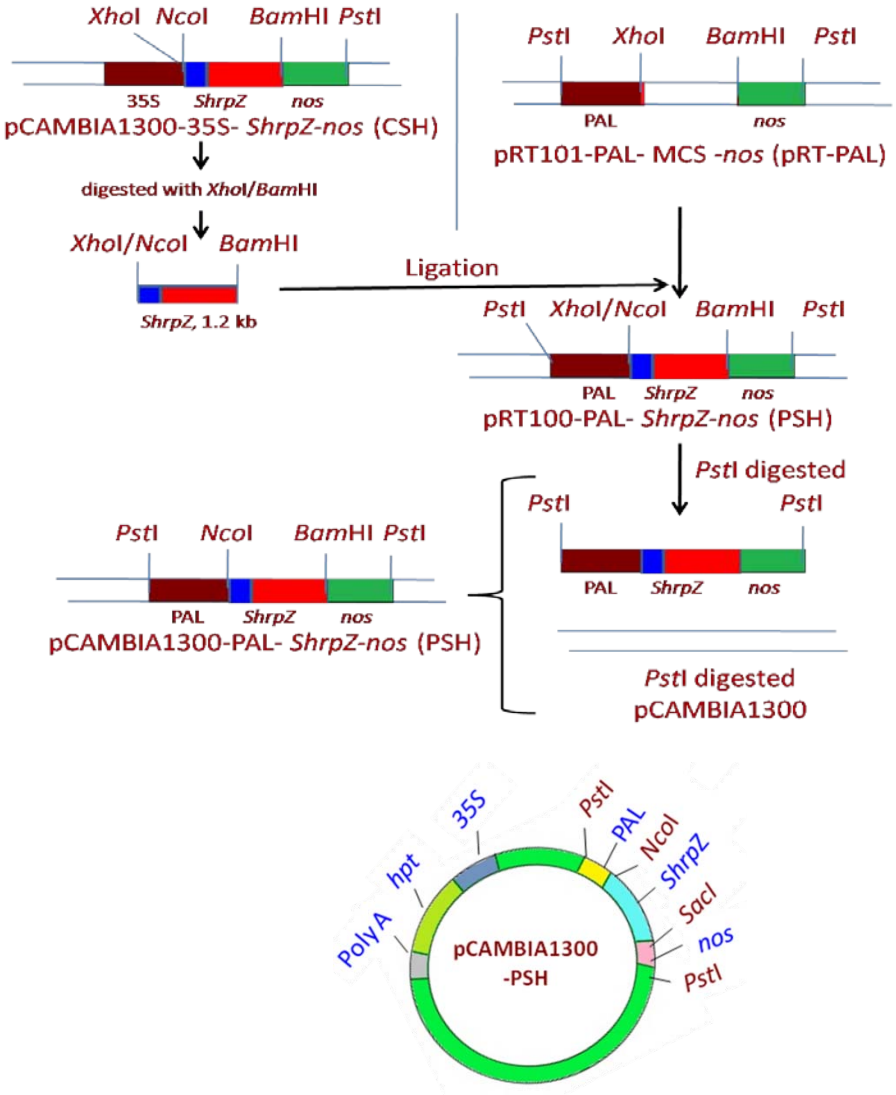
The *ShrpZ* (1.02 kb) gene was PCR amplified as *Nde*I/*Sac*I product with SP FP2 and HrpZ RP1 using Easy-A high fidelity PCR cloning enzyme (Stratagene) from pGEMT-*ShrpZ* and T/A-cloned in pGEM-T. The HSR promoter (HSR) was PCR amplified as *Nco*I(*Pst*I)/*Nde*I fragment using HSRF2 FP and HSR(Nde) RP (Table 2.2)

Figure 2.4: **The map and sequence of PSH construct in pCAMBIA1300 (9 kb)**

(a) Schematic diagram showing the construction and map of pCAMBIA1300: PAL-*ShrpZ-nos* (PSH) construct (~11 kb). The *ShrpZ* gene was digested out from CSH construct as *Xho*I/*Bam*HI fragment and cloned in pRT101 downstream to PAL promoter resulting in generation of PAL-*ShrpZ-nos* (PSH) cassette. The PSH cassette was digested out as *Pst*I fragment from pRT101 vector and cloned into pCAMBIA1300 generating pCAMBIA1300-PAL-*ShrpZ-nos* (PSH) construct.

(b) Sequence of PAL-*ShrpZ-nos* cassette (2 kb). The *ShrpZ* gene is underlined.

(a)



(b)

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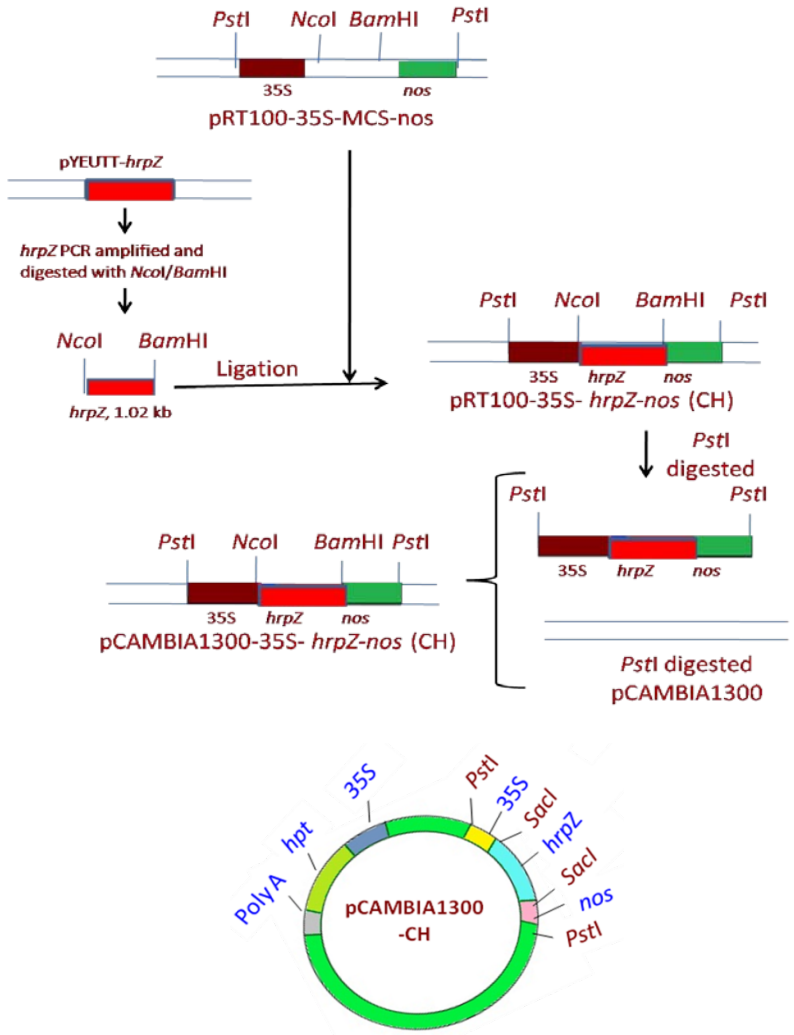
Figure 2.4: **PSH construct in pCAMBIA1300 vector (9 kb).**

Figure 2.5: **The map and sequence of CH construct in pCAMBIA1300 (9 kb)**

(a) Schematic diagram showing the construction and map of pCAMBIA1300: 35S-*hrpZ-nos* (CH) construct (~10.7 kb). The *hrpZ* gene was PCR amplified from pYEUT-*hrpZ* as *Nco*I/*Bam*HI fragment and cloned into pRT101 downstream to 35S promoter resulting in generation of 35S-*hrpZ-nos* (CH) cassette in pRT100 vector. The CH cassette was digested out as *Pst*I fragment and cloned into pCAMBIA1300 generating pCAMBIA1300-35S-*hrpZ-nos* (CH) construct.

(b) Sequence of 35S-*hrpZ-nos* cassette (1.7 kb). The sequence of *hrpZ* gene is underlined.

(a)



(b)

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GTTCCGTTCGGCACTCGACATCATTGGCCAGCAACTGGGTAATCAGCAGAGTGACGCTGGCAGTCTGGCAGGGACGGGTGGAGGTCTGGGCACTCCGAGCAGTTTTTCCAACAACTCGTCCGTGATGGGTGA
TCCGCTGATCGACGCCAATACCGGTCCCGGTGACAGCGGCAATACCCGTGGTGAAGCGGGGCAACTGATCGGCGAGCTTATCGACCGTGGCCTGCAATCGGTATTGGCCGGTGGTGGACTGGGCACACCCGT
AAACACCCCGCAGACCGGTACGTCGGCGAATGGCGGACAGTCCGCTCAGGATCTTGATCAGTTGCTGGGCGGCTTGCTGCTCAAGGGCCTGGAGGCAACGCTCAAGGATGCCGGGCAAACAGGCACCGACG
TGCAGTCGAGCGCTGCGCAAATCGCCACCTTGCTGGTCAGTACGCTGCTGCAAGGCACCCGCAATCAGGCTGCAGCCTGAGGATCCTCTAGAGTCCGCAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCT
ATTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATT
TCTAATTCCTAAAACCAAAATCCAGTGACCTGCAGGCATGCAAGCTT

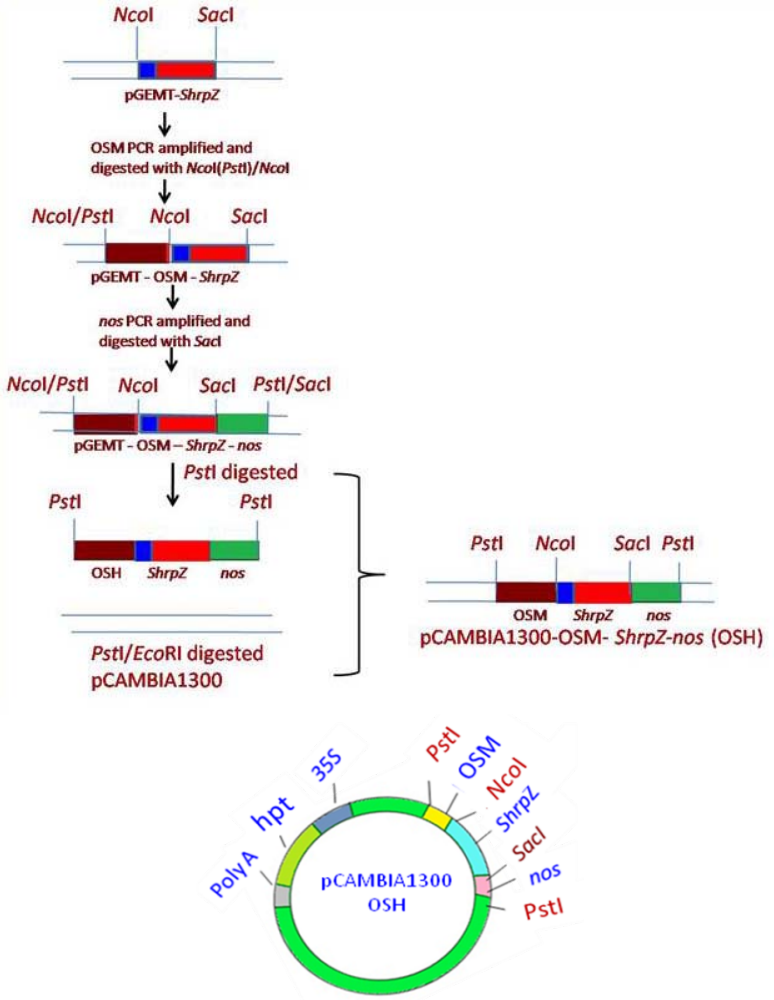
Figure 2.5: CH construct in pCAMBIA1300 vector (9 kb).

Figure 2.6: **The map and sequence of OSH construct in pCAMBIA1300 (9 kb)**

(a) Schematic diagram showing the construction and map of pCAMBIA1300: OSM-*ShrpZ-nos* (OSH) construct (11.4 kb). The *ShrpZ* gene was PCR amplified from pYEUT-*ShrpZ* as *Nco*I/*Bam*HI fragment and cloned into pRT101 downstream to 35S promoter resulting in generation of OSM-*ShrpZ-nos* (OSH) cassette in pRT100. The OSH cassette was digested out as *Pst*I fragment and cloned into pCAMBIA1300 generating pCAMBIA1300-OSM-*ShrpZ-nos* (OSH) construct.

(b) Sequence of OSM-*ShrpZ-nos* cassette (2.4 kb). The sequence of *hrpZ* gene is underlined.

(a)



(b)

CTGCAG**CCATATTTGG**ACCTTTTTCGTAAATTTTATGTAGATTTAGAAAAAAGCAACAACCTATAAGGGGTTGGTCTTTAAATATTGTCTTCATTTTTTAATGTAC
TTAAAGAATGAGCTCTGGACCTATATAGTTCTTCAGAGATTTTTCTATTGGATCGCTAGAATTTATGTTATATTTATTCTACTTTTATTGTTAAGTGTTCACAAATTT
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GGAAAAATTTATGATCGGTGCAAACTCCATAAAAAATTTCGGAAGTACAAAATGTGGAGTTCAAACTGATAAACAAACTCTAATAAATTTCTTATAATTTTTTTAT
ATTTTTGTGACGAATATTATTGTTTGAGTTTTATTTTCACATTAAAAACTAAATATTGAATAGCTTTAAAATGATGGCTATCTGCCAAAAAGTGGCTATCTGTCAAT
TTCTTGCGAATTAAAAAATGGTATAGATAAAAGAAAGCAAGAAATTGACTAAAAGAGATATTGTTACAAGTGTCACGTTACAGAGATTATAGGTCAGCGTTATTA
CCAAATAAATTGACTTCTATATTCATAAAATAATTAATTATTAGGCGGCTCTTATGTTTAAGCGCCGCCTCCATCTTTGCCAAAGCATCCTTGAGATATATCCGTTT
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ATTTGTTCTCTTTTCACAATTGCCTTCATTTCTTCTTGTCTCTACACTTCTCTTATTCCTAGTAATATCCCACTCTTGCCGTGCCCAAAACTCTCAACAAGTCAGTCTT
AACAGCAGCTCGCTGCAAACCCCGGCAATGGCCCTTGTCCTGGTACGTCCTGAAGCCGAGACGACTGGCAGTACGTCGAGCAAGGCGCTTCAGGAAGTTGTCGT
GAAGCTGGCCGAGGAACTGATGCGCAATGGTCAACTCGACGACAGCTCGCCATTGGGAAAACTGTTGGCCAAGTCGATGGCCGCAGATGGCAAGGCGGGCGGC
GGTATTGAGGATGTCATCGCTGCGCTGGACAAGCTGATCCATGAAAAGCTCGGTGACAACTTCGGCGCGTCTGCGGACAGCGCCTCGGGTACCGGACAGCAGGA
CCTGATGACTCAGGTGCTCAATGGCCTGGCCAAGTCGATGCTCGATGATCTTCTGACCAAGCAGGATGGCGGGACAAGCTTCTCCGAAGACGATATGCCGATGCT
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TATCTCTCTCTATTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTA
GTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTGACCTGCAG

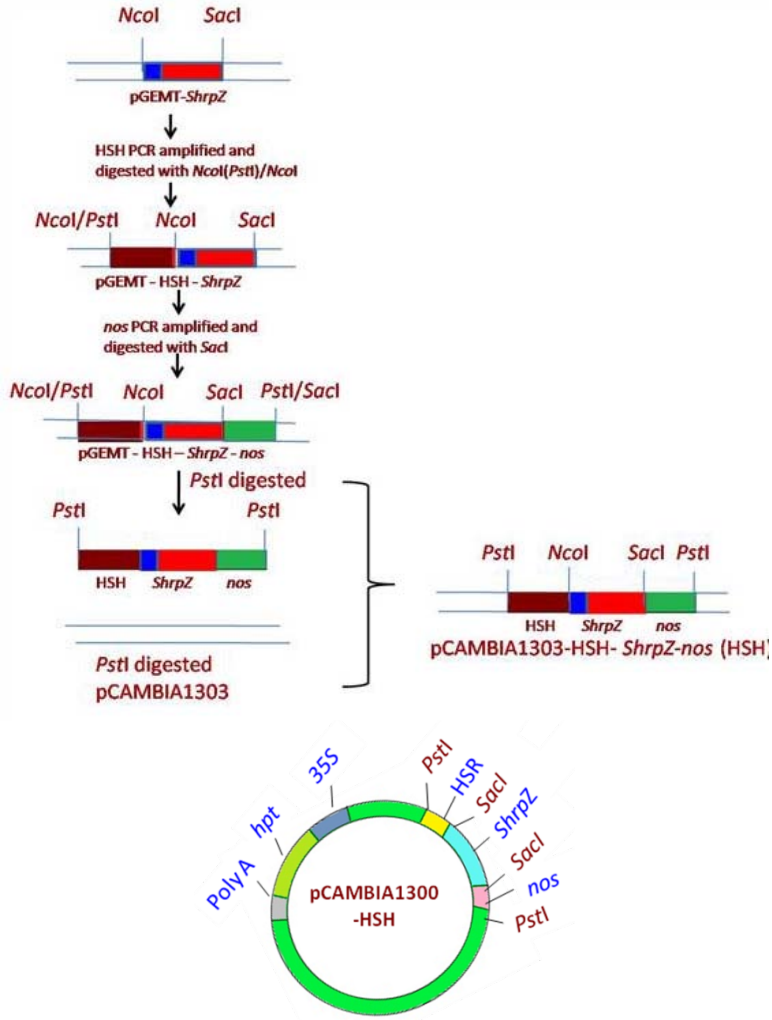
Figure 2.6: **OSH construct in pCAMBIA1300 vector (9 kb).**

Figure 2.7: **The map and sequence of HSH construct in pCAMBIA1303 (12.4 kb)**

(a) Schematic diagram showing the construction and map of pCAMBIA1303: HSR-*ShrpZ-nos* (HSH) construct (14.5 kb). The *ShrpZ* gene was PCR amplified from pYEUT-*ShrpZ* as *Nco*I/*Bam*HI fragment and cloned into pRT101 downstream to 35S promoter resulting in generation of HSR-*ShrpZ-nos* (CH) cassette in pRT100. The HSH cassette was digested out as *Pst*I fragment and cloned into pCAMBIA1303 generating pCAMBIA1303-HSR-*ShrpZ-nos* (HSH) construct.

(b) Sequence of HSR-*ShrpZ-nos* cassette (2.1 kb). The sequence of *ShrpZ* gene is underlined.

(a)



(b)

CTGCAGCAATATAATACATTTTCAAATTAAATTAGTATCAAATAATTGTATTTTTGCTTTTTTAATAATTTATACGCATGAATTTCATAATCCAGCATATTATGCTAGAACTTTTCGTGTTTCAACTAAAATA
ATGACTATTTTTCAATGTTACAAACACTGACTAATTTTTGATTGCAGTCCGAAAACTATCTAGTCTATGCTATTTTCACTTTTCTAAACTCCCTGCCACTGTATGCTTTCATTGGATTAACCTTTAACCACAC
AAATATTTTAAAGAGTAATGTTTGACAGCGTAATTTGAAACATCTACTATGCCTCTGTATATAATATCTAATGTTTGTTCGTAGACCAATATTCTAATTCCTCTCTTGTAGACTAAACGGGGCTGTAACTA
ACTAACCACCATAGTTATCTAAATTAGTGACCCTAGCGACCATTGATAATTTGATACTGATCATTGACTTCCACCAAATCTACTTTCTAAATGTGGACTGACTCATTATGAATTTGTGAGGAAAATACTTT
CCTAAAATCTACTTTCTAAATGTGGACTGACTCATTATGAATTTGTGAGGAAAATACTTTCCTAATGCTAGTGCTCTTCCCATTATCTAAACTCCAAAATTTTGTAAAATTCTTTGAACCTTCCTTTAAACTA
CCACAAATTTTCTTATCCTTTCCTATCTCACCATTATAAATAGCCACGCACACGCAAACCAAACATATGGGATTTGTTCTCTTTTCACAATTGCCTTCATTTCTTCTTGTCTCTACACTTCTCTTATTCCTAGT
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GTATTGAGGATGTCATCGCTGCGCTGGACAAGCTGATCCATGAAAAGCTCGGTGACAACTTCGGCGCGTCTGCGGACAGCGCCTCGGGTACCGGACAGCAGGACCTGATGACTCAGGTGCTCAATGG
CCTGGCCAAGTCGATGCTCGATGATCTTCTGACCAAGCAGGATGGCGGGACAAGCTTCTCCGAAGACGATATGCCGATGCTGAACAAGATCGCGCAGTTCATGGATGACAATCCCGCACAGTTTCCCA
AGCCGGACTCGGGCTCCTGGGTGAACGAACTCAAGGAAGACAACTTCCTTGATGGCGACGAAACGGCTGCGTTCCGTTCGGCACTCGACATCATTGGCCAGCAACTGGGTAATCAGCAGAGTGACGC
TGGCAGTCTGGCAGGGACGGGTGGAGGTCTGGGCACTCCGAGCAGTTTTTCCAACAACTCGTCCGTGATGGGTGATCCGCTGATCGACGCCAATACCGGTCCCGGTGACAGCGGCAATACCCGTGGT
GAAGCGGGGCAACTGATCGGCGAGCTTATCGACCGTGGCCTGCAATCGGTATTGGCCGGTGGTGGACTGGGCACACCCGTAAACACCCCGCAGACCGGTACGTCGGCGAATGGCGGACAGTCCGCT
CAGGATCTTGATCAGTTGCTGGGCGGCTTGCTGCTCAAGGGCCTGGAGGCAACGCTCAAGGATGCCGGGCAAACAGGCACCGACGTGCAGTCGAGCGCTGCGCAAATCGCCACCTTGCTGGTCAGTA
CGCTGCTGCAAGGCACCCGCAATCAGGCTGCAGCCTGAGAGCTCGCAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTATTTTCTCCAGAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAG
GGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGTGACCTGCAG

Figure 2.7: **HSH construct in pCAMBIA1300 vector (9 kb).**

and cloned upstream of *ShrpZ* gene. The *nos* terminator was PCR amplified as *Sac*I/(*Pst*I)*Sac*I fragment and cloned downstream to *ShrpZ* gene. The orientation of HSR promoter and *nos* terminator was determined through PCR and the entire HSR-*ShrpZ*-nos cassette was released as *Pst*I fragment and cloned into *Pst*I site of pCAMBIA1303 generating pCAMBIA1303-HSR-*ShrpZ*-nos (HSH) construct (Fig.2.7; Table 2. 1, 2. 4).

2. 7 Transformation

2. 7. 1 *E. coli* transformation

The ultracompetent cells of *E. coli* DH5α were prepared as described by Sambrook *et. al.,* (1989) and used for all cloning work related to generation of the required constructs and selected on LB plates containing suitable antibiotics.

2. 7. 2 *Agrobacterium* transformation

Agrobacterium tumefaciens EHA105 was transformed with the plasmids TSH, PSH, CSH, CH, OSH and HSH by the freeze-thaw method (Hofgen *et al*., 1988). Single colony of *Agrobacterium* was inoculated in 5 ml of LB medium supplemented with rifampicin (50 µg/ml) and cultured at 28°C overnight. Next day, 1 ml of culture was inoculated into 50 ml of LB medium with rifampicin (50 µg/ml). After 3-4 h of incubation at 28°C, the cells were centrifuged at 3000 g for 10 min at 4°C. The pellet was washed once in 10 ml pre-cooled 20 mM CaCl2 and dissolved in 2 ml of 20 mM CaCl2. Aliquots of 100 µl were used directly for transformation or frozen in liquid nitrogen and stored at –70°C. Stored cells were thawed on ice prior to transformation. Competent agrobacterial cells were mixed with 0.5-1.0 µg of plasmid DNA. The cells were incubated successively for 5 min on liquid nitrogen, 5 min at 37°C and 5 min on ice. 1 ml of LB medium was added to the transformed cells and allowed to grow for 4 h at 28°C. Aliquots of 100 µl were spread on LB plates supplemented with rifampicin (50 µg/ml) and kanamycin (50 µg/ml) and cultured at 28°C for 48 h. A few colonies were inoculated for plasmid isolation from *Agrobacterium* cells and confirmation through PCR.

2. 7. 2. 1 Plasmid isolation from *Agrobacterium* (Hooykas and Mozo, 1994):

The agrobacterial cultures were grown in 5 ml of LB broth overnight for 36-48 h at 28°C with 50 µg/ml each of kanamycin and rifampicin. The cultures were centrifuged at 1500 g for 10 min at 4°C. The supernatant was discarded and the pellets were kept on ice until ready to be resuspended. The pellets were resuspended in 200 µl of Solution I (50 mM glucose, 10 mM EDTA and 25 mM Tris-Cl, pH 8.0 with 5 mg/ml freshly added lysozyme) by vortexing and incubated for 10 min at 25°C. Fresh Solution II (1 % SDS, 0.2 N NaOH) was added (400 µl) and tubes inverted four times and incubated for 10 min at 25°C. Sixty µl of fresh alkaline phenol (2 volumes of 0.2 N NaOH and 1 volume of Tris-saturated phenol mixed fresh before using) was added and mixed by inverting about sixteen times. Immediately, 300 µl of 3 M sodium acetate, pH 5.0, was added, mixed by inverting about twenty times and incubated at -20°C for 20 min. The samples were centrifuged for 5 min at 25°C using a microfuge that has slow acceleration and deceleration options and the supernatant was collected. Equal volumes of Tris-equilibrated phenol were added and DNA was extracted by inverting about 20 times. The samples were centrifuged at 1500 g for 5 min at 25°C. The upper phase was pipetted off and two volumes of ice cold 95 % ethanol were added. The samples were mixed by inverting about four times and centrifuged at 1500 g for 10 min at 25°C. The pellet was washed with 500 µl of ice cold 70 % ethanol and centrifuged for 2 min at 1500 g. The pellet was dried for 10 min in a vacuum desiccator and resuspended gently in 40 µl of 1x TE.

2. 7. 2. 2 PCR analysis of *Agrobacterium* transformants:

The plasmids were isolated from *A. tumefaciens* transformed with constructs TSH, PSH, CSH, CH, OSH and HSH as described in section 2. 8. 2. 1; and used as templates for PCR (cycling parameters, Table 2. 3) to confirm the transformation of *Agrobacterium*. Glycerol stocks were prepared from the confirmed clones by mixing 800 µl of *Agrobacterium* culture and 200 µl of autoclaved glycerol (100%) and stored at -80°C.

2. 7. 3 *Agrobacterium*-mediated leaf disc transformation of tobacco

Nicotiana tabacum cv. *xanthi* leaf discs were transformed with *A. tumefaciens* EHA105, harboring the constructs TSH, PSH, CSH, CH, OSH and HSH as described by Horsch *et. al*. (1985) and Rogers *et. al*. (1988).

2. 7. 3. 1 Surface sterilization of tobacco leaves:

Well grown healthy plants maintained in green house were selected for choosing 6-7 medium-sized leaves. The leaves were washed thoroughly under tap water followed by sterilization under laminar hood. The leaves were kept in an autoclaved beaker (250 ml) and rinsed once with autoclaved water followed by surface sterilization with 70% ethanol for 1 min. The leaves were washed with water once and then mercuric chloride (0.1%) treatment was given for 5 min. The leaves were properly washed with sufficient water for three times to remove traces of mercuric chloride and used for *Agrobacterium*-mediated transformation.

2. 7. 3. 2 Preparation of explants:

The leaves were kept in a petridish and the tip, base and edges from the leaves were excised out using sterile, thin, scalpel blades and fine forceps, making sure that minimal damage is caused to the tissues to generate explants of about 5-10 mm^2.

2. 7. 3. 3 Cocultivation

A. tumefaciens EHA105 (harbouring TSH/PSH/CSH/OSH/HSH/CH) were grown overnight at 28°C in LB with rifampicin-50 µg/ml and kanamycin-50 µg/ml until the OD_{660} reaches to 0.6-0.8. The agrobacterial cells were pelleted at 3000 g for 10 min followed by washing once with ½-strength MS medium once. The pellet was dissolved in

½-strength MS medium. Leaf disc explants (section 2.8.3.2) were transferred into the *Agrobacterium* suspension and ensured that they were fully submerged for at least 5 min. The explants were then briefly blot-dried on sterile filter paper and transferred to cocultivation medium, about 30-40 discs per petri dish. The plates were sealed with parafilm and incubated in growth chamber at 25°C for 48 h under dark.

2. 7. 3. 4 Selection and regeneration of transformants:

After coculture, the explants were transferred to tissue culture bottles containing MS medium supplemented with BAP (1 mg/l), timentin (150 mg/l) and selection agent [hygromycin (25 mg/l) or kanamycin (100 mg/l)] to select the transformed shoots and to stop the growth of *Agrobacterium* after coculture. The control and non-transformed explants turned brown and appeared necrotic after selection on selection medium. The selected regions developed shoot buds and shoots after one month of culturing in the same medium. Once the shoots elongated to 2-3 cm, the entire explants with buds and shoots were sub-cultured onto ½ strength MS medium without any growth regulators and supplemented with timentin (100 mg/l) for root induction.

2. 7. 3. 5 Transfer to green house:

The rooted plants were taken out of tissue culture bottles and agar was washed off from the roots very carefully not to cause any damage to any part of the plant. The plants were potted into small plastic cups containing red soil: sand: manure: vermiculite (2:1:1:1) and kept in tissue culture room. The cups were placed on petriplates containing water and plants were covered with plastic bags to retain the humidity. After one week of hardening in tissue culture room, the plants were transferred to earthen pots in green house.

2. 8 Analysis of primary transgenics through PCR:

Genomic DNA was isolated from the leaves of the putative transgenic (male sterile and disease resistant) tobacco plants by the CTAB method (Rogers and Bendich, 1994). Medium sized tobacco leaves (5 g) were ground to fine powder in a mortar and pestle using liquid nitrogen. The powder was transferred to 50 ml falcon tube containing pre heated genomic DNA extraction buffer (15 ml) [100 mM Tris-HCl (pH 8.0); 1.4 mM NaCl; 2 0 mM EDTA; 0 . 1% β-mercaptoethanol and 2% CTAB]. The mixture was incubated at 65°C for 1-2 h with intermittent mixing by inverting followed by addition of chloroform: isoamyl alcohol (24: 1) mixture (8 ml) and slowly mixed by inverting for 15 min. The mixture was centrifuged for 5 min at 10,000 g at 4°C and the upper aqueous phase was transferred to new tube. An equal volume of ice-cold isopropanol was added and stored at -20°C. The DNA was collected by spooling through a glass rod and washed with 70% ethanol twice followed by air drying of the DNA pellet. The DNA was dissolved in 200 µl of 10 mM Tris (pH-8). The RNA was removed from the DNA by treating with 2 µl of RNase (10 mg/l) and incubation at 37°C for 30 min. The DNA was precipitated by addition of $1/10^{th}$ volume of sodium acetate (3M, pH 6.8) and 2 volumes of 96% ethanol. The mixture was centrifuged at maximum speed for 10 min at 4°C followed by washing the DNA pellet with 70% ethanol and air drying. The DNA was re-suspended in 200 µl of 10 mM Tris (pH-8). The genomic DNA was used as a template for PCR amplification of genes (*hrpZ* and *ShrpZ*), promoters (HSR, OSM, PAL and 35S) and marker genes (hygromycin and kanamycin).

2. 9 Protein isolation

2. 9. 1 Protein isolation from male sterile transgenics

Twenty TSH anthers were collected from buds of male sterile plants at the appropriate developmental stage (Stages 3-6), when the TA29 promoter is most active (Table 1. 2, Fig. 1. 4; Koltunow *et al*., 1990). The anthers were collected from time to

time, frozen in liquid nitrogen and stored at -80°C. The anthers were homogenized into a fine powder in a 1.5 ml eppendorf tube using a micropestle. To the powder, 1 ml of TRIzol reagent (Sigma) per 100 mg of tissue was added and incubated for 5 min at 25°C. Then 200 µl of chloroform was added per 1 ml of TRIzol reagent, vigorously mixed for 15 min and incubated for 3 min at 25°C. The samples were centrifuged at 12,000 g for 15 min at 4°C and three different layers were visible – a lower phenol-chloroform phase, an interphase and a colorless upper phase. The upper phase was removed and 300 µl of 100% ethanol was added (Per 1 ml of TRIzol used) to the mixture of interphase and lower organic phase. The samples were mixed well and incubated for 3 min at 25°C. The samples were centrifuged at 2,000 g for 15 min at 4°C, the supernatants were collected and 1.5 ml of isopropanol was added (Per 1 ml of TRIzol used). The samples were incubated at 25°C for 10 min followed by centrifugation at 12,000 g for 10 min at 4°C to collect the protein pellet. To the protein pellet, 2 ml of 0.3 M guanidine hydrochloride solution in 95 % ethanol (Per 1 ml of TRIzol used) was added and incubated for 20 min at 25°C followed by centrifugation at 7,000 g for 5 min at 4°C. After the final wash, protein pellet was vortexed in 2 ml of ethanol and left in ethanol for 20 min at 25°C followed by centrifugation at 7,500 g for 5 min at 4°C. The protein pellet was vacuum dried for 5-10 min and dissolved by pipette mixing in a solution containing 50 mM phosphate buffer (pH 7.0) having 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM β- mercaptoethanol, 0.1 % Triton X-100, 0.1 % sarcosyl, 0.6 % Polyvinyl polypyrrolidene (PVP) and 25 µg/ml Phenylmethyl-sulphonylfluoride (PMSF). The samples were incubated at 50°C to dissolve the proteins and the insoluble fractions were removed by centrifugation at 10,000 g for 10 min at 4 °C and the supernatant was transferred to a fresh tube. The concentration of protein was determined by Lowry's method (1951) and used for polyacrylamide gel electrophoresis (PAGE) and western blotting.

2. 9. 2 Protein isolation from putative disease resistant transgenics

The proteins from pathogen challenged leaves of putative transgenics were isolated following the protocol of Isaacson *et. al.* (2006). The leaves (1 g) were ground to a fine powder in a mortar and pestle and 15 ml of TCA extraction buffer was added [0.7

M sucrose, 0.1 M KCl, 0.5 M Tris-HCl, pH 7.5, 50 mM EDTA, 2% β- mercaptoethanol and 1mM PMSF]. The samples were mixed and stored at -20°C overnight. The samples were centrifuged at 5,000 g for 30 min at 4°C. Carefully the supernatant was removed and 10 ml of ice cold acetone was added to the pellet and centrifuged for 10 min at 5,000 g at 4°C. The supernatant was pipette out and discarded. This step was repeated twice and the pellet was air dried. The pellet was resuspended in 50 mM Tris-HCl (pH 6.8). The concentration of protein was determined using Lowry's method (1951) and used for PAGE and western blotting.

2. 10 SDS-PAGE

The protein samples (anther proteins from putatuve male sterile lines or leaf proteins from putative disease resistant plants) were separated by SDS-PAGE on vertical slab gels according to Laemmli (1970). The stacking gel contained 4.5 % polyacrylamide in 0.125 M Tris-HCl, pH-6.8 and the resolving gel contained 12% polyacrylamide in 0.375 M Tris-HCl, pH 8.8. Electrode buffer contained 0.025 M Tris-HCl, 0.192 M glycine and 0.1% (w/v) SDS of pH 8.5. The samples were boiled at 100°C for 5 min in sample buffer [1% SDS (w/v) and 12% glycerol (v/v), in 0.063 M Tris-HCl, pH 6.8] and electrophoresis was carried out at 50V in stacking gel and at 100V in resolving gel.

2. 11 Western blotting

The total soluble proteins isolated from leaf/anther of putative transgenics were separated on SDS-PAGE and transferred on a nitrocellulose membrane (Millipore, U. S. A.) using semidry transfer apparatus at 10V for 20 min. The membrane was blocked with 5% non fat milk in TBS buffer (0.3 % Tris, 0.8 % NaCl) for 1 h. The membrane was washed twice with TBST (0.3 % Tris, 0.8 % NaCl and 0.1% Tween) for 5 min and then once with TBS for 5 min. The membrane was incubated with primary antibody (1: 200 in TBS buffer with 3% non fat milk) raised against $harpin_{Pss}$ at 4°C for 5-6 h. The membrane was washed twice with TBST for 5 min and then once with TBS for 5 min. Then the membrane was incubated with secondary antibody (goat anti-rabbit IgG ALP conjugate,

1: 2000 in TBS buffer with 3% non fat milk) for 2-3 h. The membrane was washed four times with TBST buffer and the blot was development by addition of NBT-BCIP as a substrate for alkaline phosphatase (Sambrook *et. al*., 1989).

2. 12 Pollen viability tests – MTT assay

Pollen from freshly opened flowers was collected and assessed for viability by the 3-(4,5 Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT) assay (Slater *et. al*., 1963). More than 300 pollen grains were counted and an average count of ten fields was taken. The pollen grains were placed on a slide in a 5-10 µl droplet of 1 % solution of MTT or thiazolyl blue tetrazolium bromide or methyl thiazole tetrazolium in 5 % sucrose and mixed well, allowed to dry, and the procedure repeated thrice at 25°C. Finally, a small drop of glycerine was added to the stained dry sample before observing under the microscope. The pollen grains were counted on a haemocytometer. Dark violet-purple-brown colour indicated the presence of dehydrogenases, signifying that the pollen grains are viable.

2. 13 Scanning electron microscopy (SEM)

For SEM studies, anthers and pollens were spread on stubs coated with sticky tape to secure the specimen on to the stubs and coated with gold on a Sputter Coater (JEOL FC 1100) with a gold film thickness of 150A° and observed under scanning electron microscope.

Processing samples for microscopy

Flower buds were collected from TH, TSH and non-transformed plants and classified in different stages based on their size (Mariani *et. al.*,1990). The anthers were carefully excised and immediately fixed at room temperature in a solution of 4% formaldehyde in PBS buffer pH 7.4 for three hours followed by three washings (15 min each) in PBS buffer pH 7.4. The anthers were further washed twice with methanol (10 min

each) and stored at -80°C. After 12 h the anthers were dehydrated with a series of increased concentration of ethanol in water: 70%, 90% for 3 h and 100% for 1 h at room temperature. The specimens were then treated with xylene till the tissue becomes transparent. Infiltration in paraffin wax was carried out three times for 1 h each and kept at 4°C overnight.

Sectioning and observation

Thin sections of (4-6 µm) were cut from the polymerized blocks in a microtome (Leica). Sections were stained with 1% toluidine blue O and observed on a Leica confocal microscope (TCS-SP2-AOBS) under bright field and photographed using Olympus DC10 digital camera.

2. 14 Immunohistochemical localization of harpin

Thick sections (10-15 µm) of the floral buds, used for immunohistochemistry, were washed twice (5 min each) in PBS buffer pH 7.4, incubated with blocking solution (PBS containing 0.5% (w/v) BSA) for 1 h at room temperature. Slides were rinsed with PBS for 5 minutes and incubated with harpin primary antibodies for 12 h at 4°C in a humid chamber. Followed by rinsing with PBST (PBS and 0.25% (w/v) tween 20) for 5 min each and incubated with FITC conjugated goat anti- rabbit IgG antibodies for 2 h at 25°C and rinsed with PBST thrice (for 5 min each) and overlaid with (1:1) PBS and glycerol and examined under bright field in a Leica confocal microscope and photographed using Olympus DC10 digital camera.

2. 15 Capsule formation

Fruit capsules were collected from the PCR-positive TSH transgenics and non-transformed tobacco plants and the average capsule weight was determined (in grams), with at least twenty counts per plant.

2. 16 Effect on female fertility of TH transgenics

To confirm whether the female fertility of TSH transgenics remained unaffected due to transgene expression, and that the sterility was restricted to the anthers only, the flowers of the TSH transgenics were emasculated, cross pollinated with the pollen grains of non-transformed tobacco flowers and bagged. The bag was removed after capsules started to form.

2. 17 Southern blotting

Genomic DNA was isolated from non-transformed and transgenic plants (TSH lines) and a total of 20 µg of DNA was digested with *Eco*RI overnight at 37°C and was run on agarose gel (0.8%) for 3 h at 80V. The gel was subjected to depurination in depurination solution for 15 min followed by soaking in alkaline transfer buffer for 30 min. The DNA fragments were vacuum blotted to positively charged nylon membrane using vacuum blot apparatus (Amersham Biosciences) in alkaline transfer buffer for 2 h at 60 mm of Hg pressure. After transfer the membrane was neutralized in neutralization buffer-II for 10 min followed by UV-fixation in UV-transilluminator for 3 min and was stored at 4°C wrapped in whatmann filter paper. Southern blotting was done using the Gene Images^{TM} AlkPhos Direct^{TM} labeling kit (Amersham Biosciences) with CDP-*Star*™ chemiluminescent detection reagents which utilize the probe-bound alkaline phosphatase to catalyze the decomposition of a stabilized dioxetane substrate. The probe preparation, pre-hybridization, hybridization and stringency washes were done following manufacturer's protocol. The membrane was treated with detection reagent in a dark room. Upon appearance of fluorescence, the membrane was exposed to x-ray film for 30 sec to 3 min followed by development.

2.18 Fungal bioassay of disease resistant transgenics for resistance to *Fusarium oxysporum*

The resistance of transgenic tobacco plants (CH, PSH, OSH and HSH) and non-transformed plants to *Fusarium oxysporum* infection was studied *via* detached leaf bioassay. Tobacco leaves were collected 6 weeks after the adaptation of the regenerated transgenic plants in soil and put in plastic trays on wet filter paper. After the inoculation the trays were covered with transparent polyethylene cover in order to sustain high humidity. The fungal strain of *F. oxysporum* f. sp. *solani* was cultured on potato dextrose agar for 7 days at 25°C. On each side of midrib of leaf, two small plugs (5 mm) of agar with mycelium facing the leaf surface were placed. The leaves were incubated at 24°C in 16 h light/ 8 h dark photoperiod. The symptoms were evaluated 7 days after the inoculation.

2.19 RT-PCR

A total of 15 anthers of stage 3 from male sterile lines were selected and stored in -80°C. These anthers were used for RT-PCR analysis to detect the expression of harpin gene. The pathogen challenged leaves from non-transformed and different disease resistant transgenics (CH, PSH, OSH and HSH) were taken for RT-PCR analysis to detect the harpin gene expression and the transcript levels of different PR genes.

The anther or leaf samples were ground to a fine powder using liquid nitrogen in a diethylpyrocarbonate (DEPC) -treated mortar and pestle. The powder was transferred to 2 ml eppendorf tube and 1 ml TRIzol was added per 100 mg of sample. The samples were mixed and incubated for 10 min at room temperature followed by centrifugation at 15,000 g for 15 min to remove the debris. The supernatant was transferred to new eppendorf and 500 µl of chloroform was added, mixed vigorously and allowed to stand for 15 min. The samples were spun at 20,000 g at 4°C for 15 min. The upper phase was transferred to new eppendorf and 500 µl of isopropanol was added, mixed and stored at -20°C. The samples were centrifuged at 20,000 g for 15 min at 4°C. The pellet was washed with 70% ethanol for 10 min at -20°C and centrifuged at 20,000 g for 15 min at

4°C. The pellet was air dried and 40 ul of RNase free water was added. The samples were incubated at 50°C for 15 min to dissolve the RNA. The RNA was given recombinant DNase I (RNase free, TaKaRa) treatment for 30 min at 37°C followed by heat inactivation of DNase I at 80°C for 2 min.

The total RNA was converted into cDNA using BluePrint 1st Strand cDNA Synthesis Kit (TaKaRa) following manufacturer's protocol. The cDNA was used as template in a PCR to amplify the harpin gene and different defense response genes (*PR1*, *PR2*, *PR3*, *HSR203J* and *HIN1*). The RNA (5 µg) was mixed with 1 µl of oligo dT primers (50 µm) and 1 µl of dNTP mix (10 mM each) and the reaction volume was made up to 10 µl. The mixture was incubated at 65°C for 5 min followed by cooling on ice for 5 min. To the above template RNA primer mix (10 µl), 4 µl of 5X BluePrint 1st strand buffer, 0.5 µl of recombinant RNase inhibitor (40 U/µl) and 1 µl of BluePrint RTase was added and the volume was made up to 20 µl with RNase free water. The reaction contents were mixed and incubated at 42°C for 60 min. The enzyme was inactivated by incubation at 95°C for 5 min, followed by cooling on ice and stored at -80°C. The cDNA synthesized from RNA from male sterile anthers was used for PCR amplification of harpin gene. In case of disease resistant transgenics, the cDNA synthesized from RNA from challenged leaf was used to detect the harpin gene expression and the transcript levels of different defense response genes (*PR1*, *PR2*, *PR3*, *HSR203J* and *HIN1*).

Results

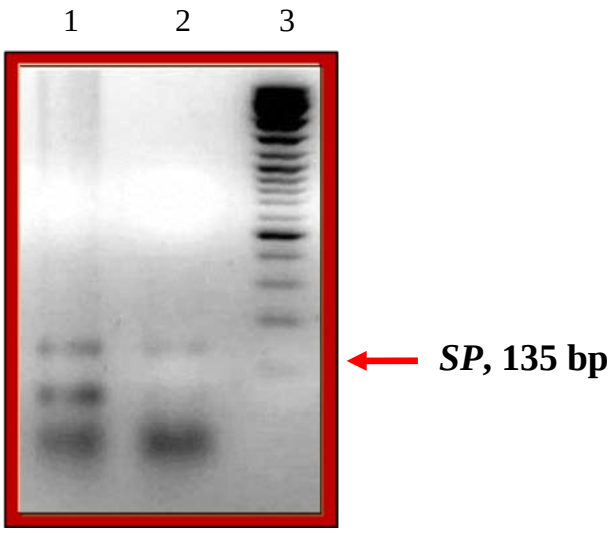
3. 1 PCR amplification of genes (*SP, hrpZ* and *ShrpZ*) and promoters (HSR and osmotin).

To induce male sterility and/or disease resistance in tobacco, with expression of harpin gene using suitable promoters, the required genes and promoters were amplified. The harpin gene (*hrpZ*) was amplified from pYEUT-*hrpZ* which was available in our lab. To target harpin protein to the apoplast, for its interaction with the receptor in the cell wall, a secretable form of harpin gene (*ShrpZ*) was generated by fusing *hrpZ* with a signal peptide (*SP*) sequence of Pathogenesis Related (*PR1a*) gene. The *SP* was PCR amplified (Fig. 3.1a) (SP FP1 and SP RP1, Table 2.2) from genomic DNA of tobacco while the *hrpZ* was PCR amplified from pYEUT-*hrpZ* (Fig. 3.1b) (HrpZ FP1 and HrpZ RP1, Table 2.2) using gene specific primers with desired restriction sites. The *SP* (135 bp) was fused with *hrpZ* (1.02 kb) through PCR-based approach to generate the fusion gene (*ShrpZ*, 1.2 kb) using chimeric primers (Ch SP RP and Ch HrpZ FP, Table 2.2). In-frame fusion of the two genes was confirmed with sequencing (Fig. 3.2). During fusion of two genes, the first four codons of harpin protein were omitted and fifth amino acid residue was replaced with valine. The fused protein consists of 376 residues. The peptide expected after removal of the signal peptide (343 residues) includes six amino acids (QNSQQV), which substitute for the first four residues of harpin (MQSL) (Fig. 3.2d).

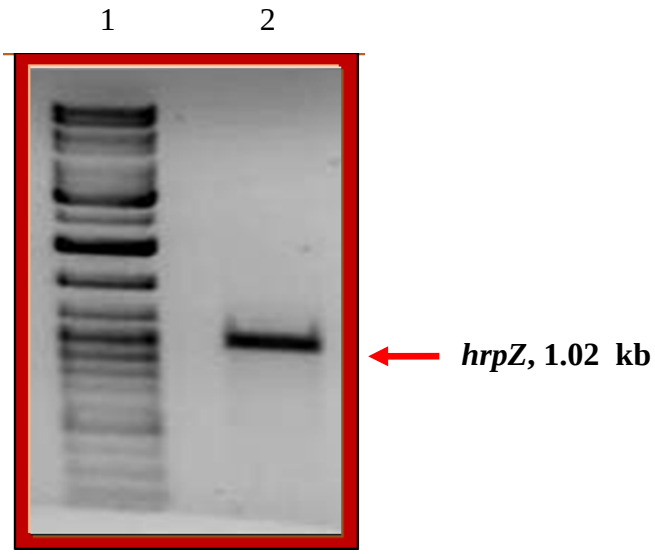
The TA29 promoter, active only in the tapetum layer, was originally obtained from Prof. DeepaK Pental, University of Delhi and was used for generation of male sterile lines. The promoter was available in the lab as pCAMBIA1300-TA29-*hrpZ-nos* designated as (TH) construct.

The hypersensitive-related (HSR) and osmotin promoters were reported as pathogen inducible promoters from tobacco but contain silencer regions at 5’ end. Primers were designed to amplify the HSR and osmotin promoters without the 5’ silencer regions. The HSR promoter (650 bp) and osmotin promoter (OSM, 1 kb) were amplified with (HSR FP and HSR RP) and (OSM FP and OSM RP) respectively (Table 2.2) from genomic DNA of tobacco (Fig. 3.3) and sequenced. For constitutive expression of harpin, the 35S promoter was used in this study and the promoter was available in the lab as pRT100 having 35S-MCS-*nos* cassette. The tomato PAL promoter, also a pathogen inducible promoter, was obtained from Prof. M.V. Rajam, University of Delhi in the form

Figure 3.1: a) The signal peptide (*SP*) portion of the Pathogenesis-Related (PR1a) gene was PCR amplified (Lanes 1 and 2) from genomic DNA of tobacco and was run in 3% agarose gel. The 135 bp band was cloned and sequenced. b) The harpin gene was PCR amplified from pYEUT-*hrpZ* (Lane 2). The molecular weight marker used is DNA ladder mix (100 bp – 10 kb).



a) Signal peptide (SP)



b) Harpin gene (*hrpZ*)

Figure 3.1: PCR amplification of *PR1a* signal peptide and harpin gene.

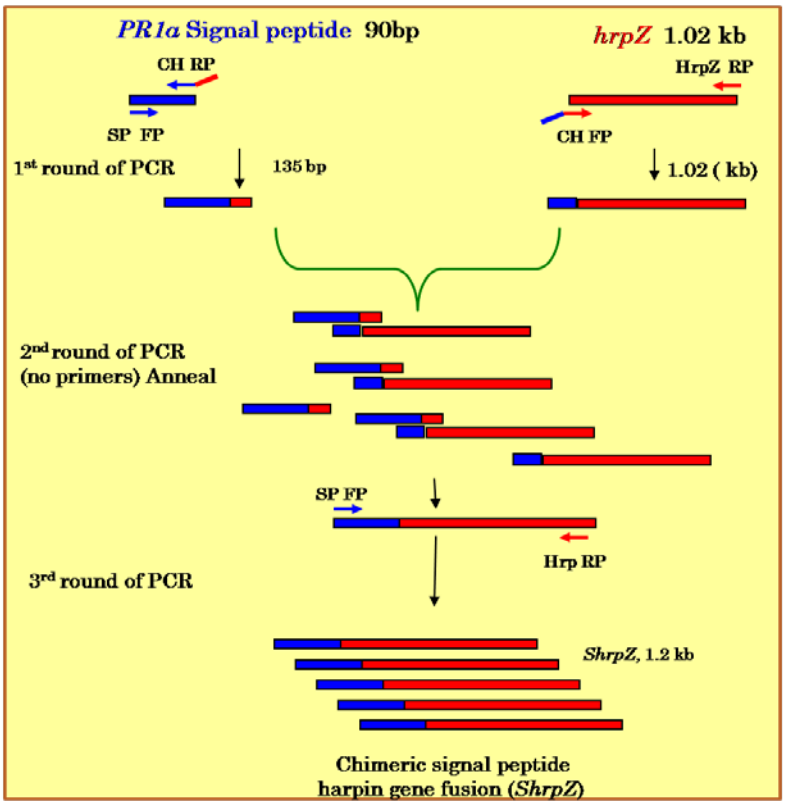
Figure 3.2: PCR-based generation of generate fusion gene (*ShrpZ*).

a) Three step PCR-based gene fusion of *SP* (135 bp) and *hrpZ* (1.02 kb) generating fusion gene (*ShrpZ*, 1.2 kb). The *SP* and *hrpZ* genes were amplified separately using chimeric primers having overlapping sequences in the first round of PCR. In the second round of PCR, the chimeric products of *SP* and *hrpZ* were allowed to anneal together. In third round of PCR, the entire fusion gene was PCR amplified by using SP FP and HrpZ RP.

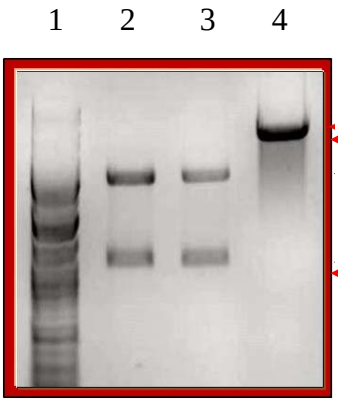
b) Cloning confirmation of fusion gene in pGEM-T vector by single digestion with *Sal*I (Lanes 2 and 3) and double digestion with *Nco*I & *Sac*I releasing the insert (*ShrpZ*, 1.2 kb; Lane 4). The molecular weight marker used is DNA ladder mix (100 bp – 10 kb; Lane 1).

c) Sequence of fusion gene (*ShrpZ*) as per the sequencing result showing the successful in-frame fusion of *SP* sequences (in blue) with that of *hrpZ* sequences (red, underlined).

d) Amino acid sequence of the complete fusion gene (*ShrpZ*) including SP sequences (in blue) and *hrpZ* sequences (red, underlined).



a)



pGEMT-*ShrpZ*, 4.2 kb

ShrpZ , 1.2 kb

b)

ATGGGATTTGTTCTCTTTTCACAATTGCCTTCATTTCTTC
TTGTCTCTACACTTCTCTTATTCCTAGTAATATCCCACTCT
TGCCGTGCCCAAAACTCTCAACAAGTCAGTCTTAACAGC
AGCTCGCTGCAAACCCCGGCAATGGCCCTTGTCCTGGT
ACGTCCTGAAGCCGAGACGACTGGCAGTACGTCGAGC
AAGGCGCTTCAGGAAGTTGTCGTGAAGCTGGCCGAGG
AACTGATGCGCAATGGTCAACTCGACGACAGCTCGCCA
TTGGGAAAACTGTTGGCCAAGTCGATGGCCGCAGATG
GCAAGGCGGGCGGCGGTATTGAGGATGTCATCGC

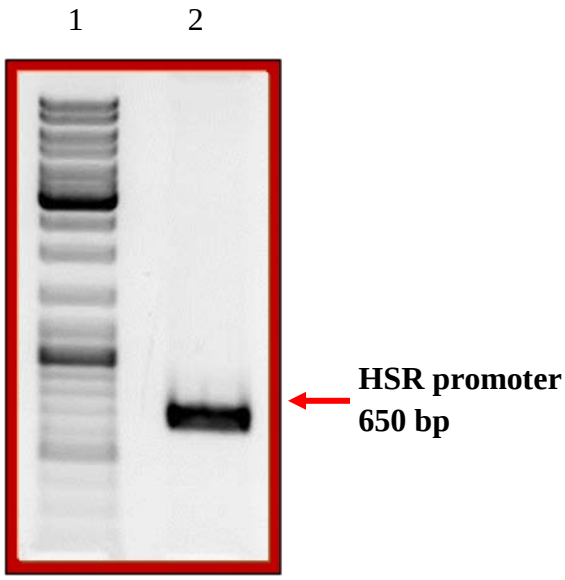
c)

MGFVLFSQLPSFLLVSTLLLFLVISHSCRAQNSQQSLNSSSLQTPAMALVLVRPEAETTGSTSSKALQEVVVKLAEELMR
NGQLDDSSPLGKLLAKSMAADGKAGGGIEDVIAALDKLIHEKLGDNFGASADSASGTGQQDLMTQVLNGLAKSML
DDLLTKQDGGTSFSEDDMPMLNKIAQFMDDNPAQFPKPDSGSWVNELKEDNFLDGDETAAFRSALDIIGQQLGN
QQSDAGSLAGTGGGLGTPSSFSNNSSVMGDPLIDANTGPGDSGNTRGEAGQLIGELIDRGLQSVLAGGGLGTPVN
TPQTGTSANGGQSAQDLDQLLGGLLLKGLEATLKDAGQTGTDVQSSAAQIATLLVSTLLQGTRNQAAA

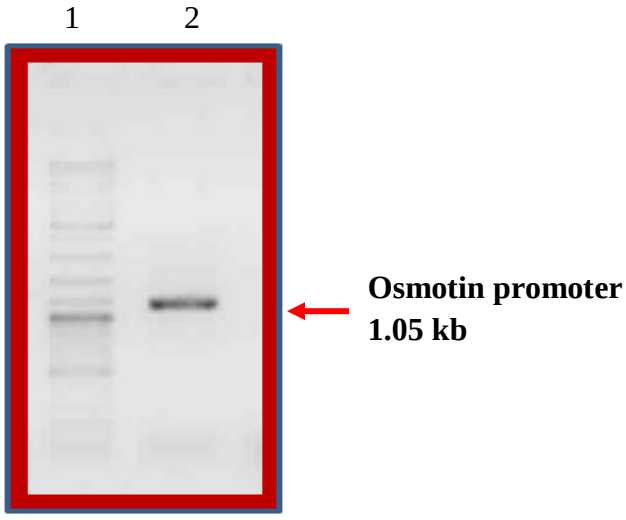
d)

Figure 3.2: PCR-based gene fusion of *SP* and *hrpZ* genes to generate fusion gene (*ShrpZ*).

Figure 3.3: PCR-based amplification of a) HSR promoter (650 bp, lane 2) using HSR FP and HSR RP primers and b) osmotin promoter (1 kb, Lane 2) using OSM FP and OSM RP from genomic DNA of tobacco. Both of these promoters were reported to contain 5' silencer. The HSR and osmotin promoters lacking silencer regions were PCR amplified and used in this study. The molecular weight marker used is DNA ladder mix (100 bp – 10 kb, lane 1).



a) HSR promoter



b) Osmotin promoter

Figure 3.3: PCR amplification of HSR and osmotin promoters.

of pRT101 having PAL-MCS-*nos* cassette.

All these above promoters were used for generation of binary constructs for expression of harpin either intracellularly or extracellularly towards generation of male sterile and disease resistance transgenics in tobacco.

3. 2 Generation of promoter-*hrpZ-nos* and promoter-*ShrpZ-nos* binary constructs

To develop male sterile tobacco, TSH (T=TA29; S=Signal peptide; H=harpin) construct was generated by placing the fusion gene (*ShrpZ*) downstream to TA29 promoter. To generate disease resistant tobacco, the *ShrpZ* gene was placed downstream to different pathogen inducible promoters (HSR, OSM and PAL) and obtained three binary constructs – HSH, OSH and PSH (the first letter denotes the promoter). The *ShrpZ* or *hrpZ* genes were placed downstream to 35S promoter for generation of CSH and CH constructs (C=for constitutive expression). The CH construct was intended for intracellular expression of harpin gene whereas the other constructs are meant for a secretable form of harpin to target $harpin_{Pss}$ to apoplast.

3. 2. 1 Generation of pCAMBIA2301-TA29-*ShrpZ-nos* (TSH)

The pCAMBIA2301-TA29-*ShrpZ-nos* construct was confirmed by restriction digestion and PCR. Single digestion of TSH construct with *Eco*RI resulted in 13.9 kb single band (Fig.3.4a). The double digestion of TSH construct with *Pst*I resulted in release of TSH cassette (2.27 kb) from pCAMBIA2301 (11.63 kb). The *ShrpZ* gene (1.2 kb) was PCR amplified using TSH construct as a template.

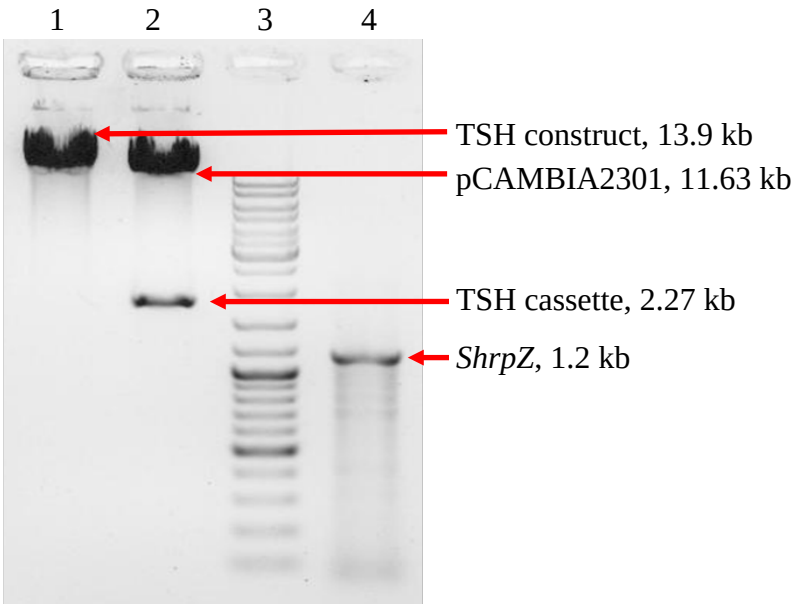
3. 2. 2 Generation of pCAMBIA1300-35S-*ShrpZ-nos* (CSH)

The pCAMBIA1300-35S-*ShrpZ-nos* (CSH) construct was confirmed both by restriction digestion and PCR. Single digestion of CSH construct with *Eco*RI resulted in single band of 10.9 kb (Fig. 3.4b). The double digestion of CSH construct with *Pst*I released the CSH cassette of 1.9 kb from the vector backbone. The *ShrpZ* gene was PCR amplified using CSH construct as a template.

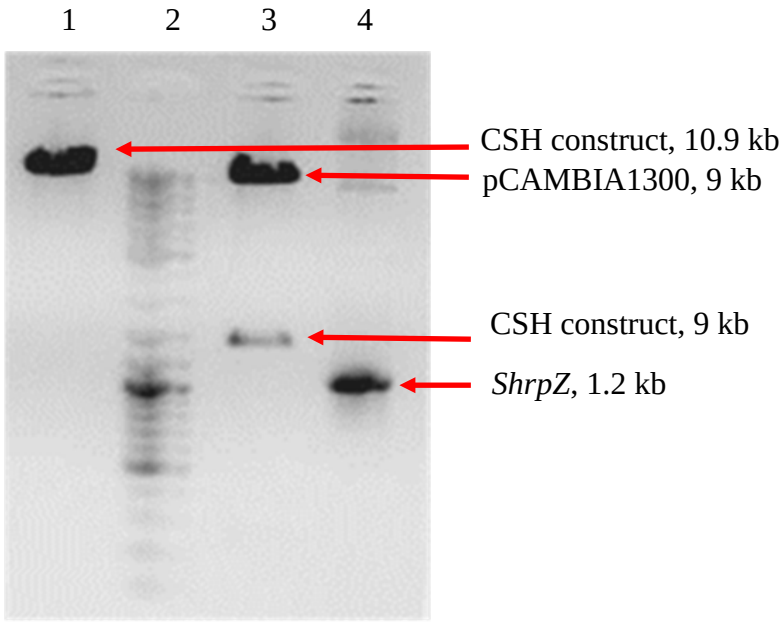
Figure 3.4: Cloning confirmation of TSH and CSH constructs. The *ShrpZ* gene was cloned downstream to TA29 and 35S promoters generating (a) TSH and (b) CSH constructs in pCAMBIA2301 and pCAMBIA1300. These two constructs were confirmed by digestion and by PCR.

a) TSH construct confirmation – Single digestion of TSH construct (Lane 1) with *Eco*RI results in generation of 13.9 kb band. Double digestion of TSH construct with *Pst*I and *Eco*RI releases the TSH cassette (2.27 kb) from the pCAMBIA2301 (Lane 3). The *ShrpZ* gene was PCR amplified using the TSH construct as template (Lane 4). The amplicons were compared with DNA ladder mix (100 bp – 10 kb, Lane 2).

b) CSH construct confirmation - Single digestion of CSH construct (Lane 1) with *Eco*RI results in generation of 10.9 kb band. Double digestion of CSH construct with *Pst*I releases the CSH cassette (2.27 kb) from the pCAMBIA1300 (Lane 2). The *ShrpZ* gene was PCR amplified using the CSH construct as template (Lane 4). The amplicons were compared with DNA ladder mix (100 bp - 10 kb, Lane 3).



a) Cloning confirmation of pCAMBIA2301-TA29-*ShrpZ-nos* (TSH)



b) Cloning confirmation of pCAMBIA1300-35S-*ShrpZ-nos* (CSH)

Figure 3.4: Cloning confirmation of TSH and CSH constructs.

3. 2. 3 Generation of pCAMBIA1300-PAL-*ShrpZ-nos* (PSH)

The pCAMBIA1300-PAL-*ShrpZ-nos* (PSH) construct was confirmed both by restriction digestion and PCR. Single digestion of PSH construct with *Eco*RI resulted in single band of 11 kb (Fig.3.5a). The double digestion of PSH construct with *Pst*I released the PSH cassette of 2 kb from the vector backbone. The *ShrpZ* gene was PCR amplified using PSH construct as a template.

3. 2. 4 Generation of pCAMBIA1300-35S-*hrpZ-nos* (CH)

The pCAMBIA1300-35S-*hrpZ-nos* (CH) construct was confirmed both by restriction digestion and PCR. Single digestion of CH construct with *Eco*RI resulted in single band of 10.7 kb (Fig. 3.5b). The double digestion of CH construct with *Pst*I released the CH cassette of 1.72 kb from the vector backbone. The *ShrpZ* gene was PCR amplified using CH construct as a template.

3. 2. 5 Generation of pCAMBIA1300-OSM-*ShrpZ-nos* (OSH)

The pCAMBIA1300-OSM-*ShrpZ-nos* (OSH) construct was confirmed both by restriction digestion and PCR. Single digestion of OSH construct with *Eco*RI resulted in single band of 11.4 kb (Fig. 3.6a). The double digestion of OSH construct with *Pst*I released the OSH cassette of 2.4 kb from the vector backbone. The *ShrpZ* gene was PCR amplified using OSH construct as a template.

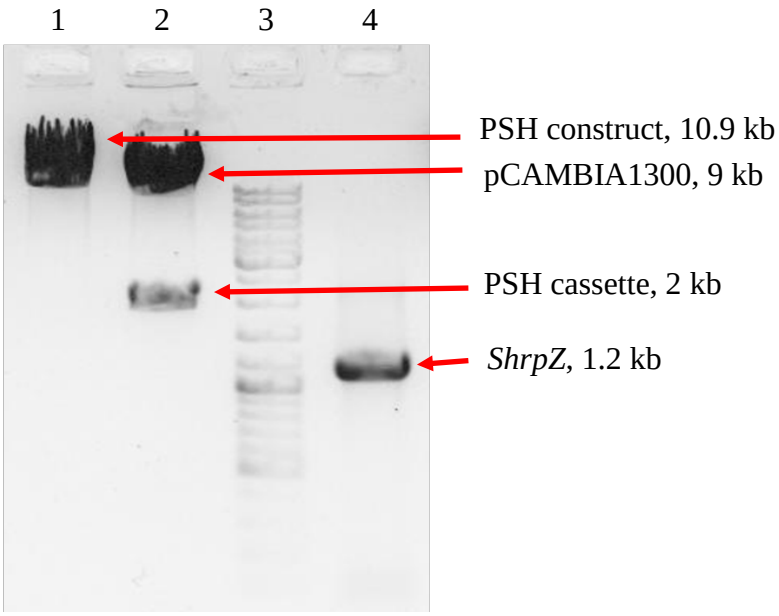
3. 2. 6 Generation of pCAMBIA1303-HSR-*ShrpZ-nos* (HSH)

The pCAMBIA1303-HSR-*ShrpZ-nos* (HSH) construct was confirmed by restriction digestion and PCR. Single digestion of HSH construct with *Eco*RI resulted in single band of 14.5 kb band (Fig. 3.6b). The double digestion of the HSH construct with *Pst*I resulted in release of HSH cassette (2.1 kb) from the vector. The *ShrpZ* gene was PCR amplified using HSH construct as a template.

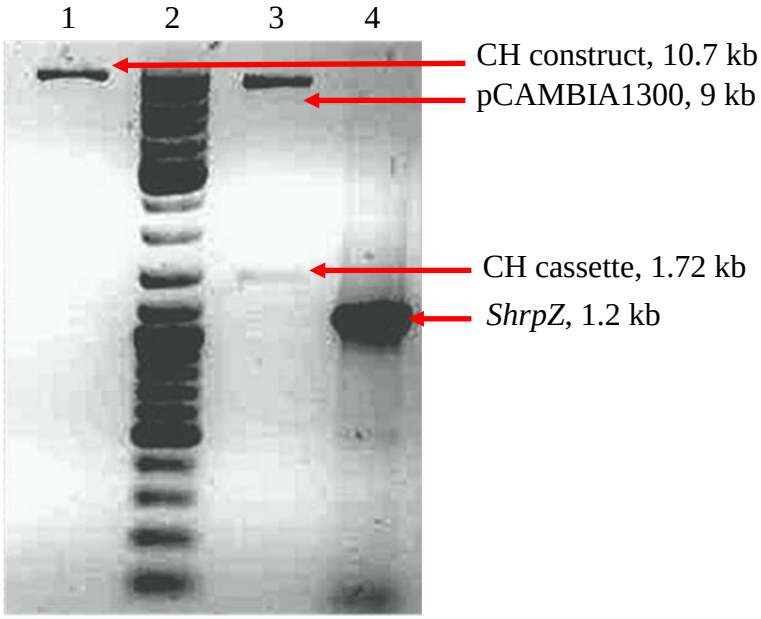
Figure 3.5: Cloning confirmation of PSH and CH constructs. The *ShrpZ* gene was cloned downstream to PAL promoter whereas hrpZ gene was cloned downstream to 35S promoter generating (a) PSH and (b) CH constructs in pCAMBIA1300. These two constructs were confirmed by digestion and by PCR.

a) PSH construct confirmation – Single digestion of PSH construct (Lane 1) with *Eco*RI results in generation of 11 kb band. Double digestion of PSH construct with *Pst*I releases the PSH cassette (2 kb) from the pCAMBIA1300 (Lane 2). The *ShrpZ* gene was PCR amplified using the PSH construct as template (Lane 4). The amplicons were compared with DNA ladder mix (100 bp – 10 kb, Lane 3).

b) CH construct confirmation – Single digestion of CH construct (Lane 1) with *Eco*RI results in generation of 10.7 kb band. Double digestion of CH construct with *Pst*I releases the CH cassette (2.27 kb) from the pCAMBIA1300 (Lane 3). The *hrpZ* gene was PCR amplified using the CH construct as template (Lane 4). The amplicons were compared with DNA ladder mix (100 bp – 10 kb, Lane 2).



a) Cloning confirmation of pCAMBIA1300-PAL-*ShrpZ-nos* (PSH)



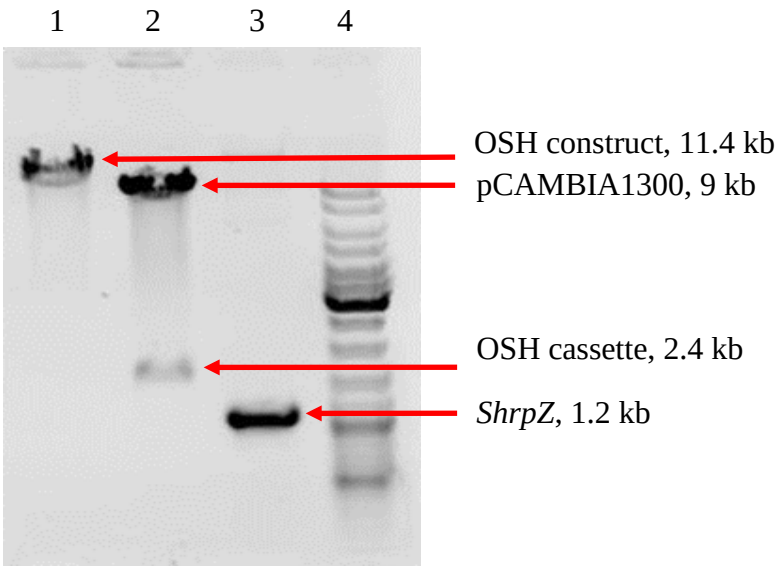
b) Cloning confirmation of pCAMBIA1300-35S-*hrpZ-nos* (CH)

Figure 3.5: Cloning confirmation of PSH and CH constructs.

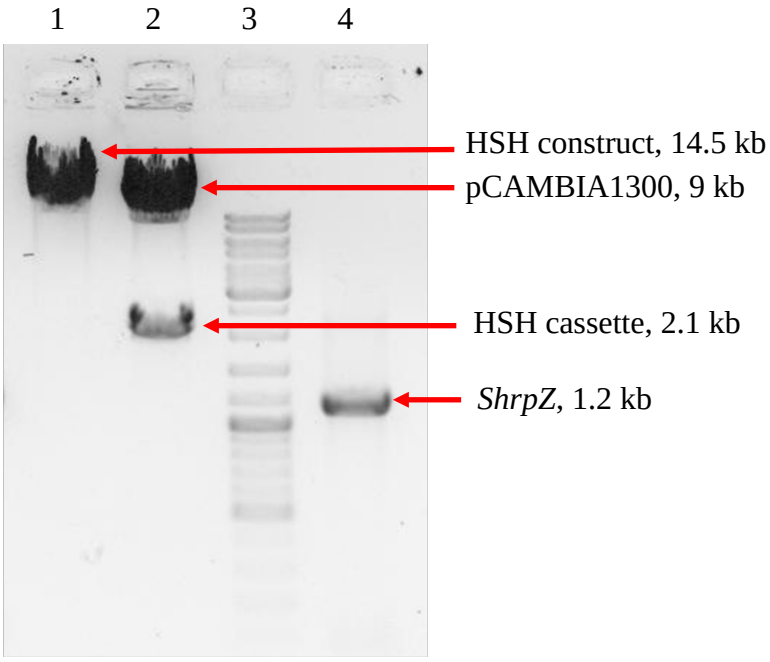
Figure 3.6: Cloning confirmation of OSH and HSH constructs. The *ShrpZ* gene was cloned downstream to HSR promoter generating (a) OSH and (b) HSH constructs in pCAMBIA1300 and pCAMBIA1303 respectively (d). The construct was confirmed by digestion and by PCR.

a) OSH construct confirmation - Single digestion of OSH construct (Lane 1) with *Eco*RI results in generation of 11.4 kb band. Double digestion of OSH construct with *Pst*I releases the OSH cassette (2.4 kb) from the pCAMBIA1300 (Lane 2). The *ShrpZ* gene was PCR amplified using the OSH construct as template (Lane 3). The amplicons were compared with DNA ladder mix (100 bp - 10 kb, Lane 4).

b) HSH construct confirmation - Single digestion of HSH construct (Lane 1) with *Eco*RI results in generation of 14.5 kb band. Double digestion of HSH construct with *Pst*I releases the HSH cassette (2.1 kb) from the pCAMBIA1303 (Lane 2). The *ShrpZ* gene was PCR amplified using the HSH construct as template (Lane 4). The amplicons were compared with DNA ladder mix (100 bp - 10 kb, Lane 3).



a) Cloning confirmation of pCAMBIA1300-OSM-*ShrpZ-nos* (OSH)



b) Cloning confirmation of pCAMBIA1303-HSR-*ShrpZ-nos* (HSH)

Figure 3.6: Cloning confirmation of OSH and HSH constructs.

3. 3 *Agrobacterium* transformation and confirmation

The supervirulent *Agrobacterium tumefaciens* EHA105 was used for tobacco transformation to generate male sterile and disease resistant transgenic plants. The competent agrobacterial cells were independently transformed through freeze thaw method with all the constructs generated in pCAMBIA1300, pCAMBIA1303 and pCAMBIA2301 *viz.*, TSH, PSH, CH, CSH, OSH and HSH (Tables 2.1, 3.1).

Plasmids were isolated from the agrobacterial cultures transformed with TSH, PSH, CH, CSH, OSH and HSH. These plasmids were used as template in PCR to amplify the *hrpZ* gene from CH construct whereas fusion gene (*ShrpZ)* in case of rest of the constructs (Fig. 3.7) to confirm the uptake of plasmids by *Agrobacterium.*

3. 4 *Agrobacterium*-mediated leaf disc transformation of tobacco

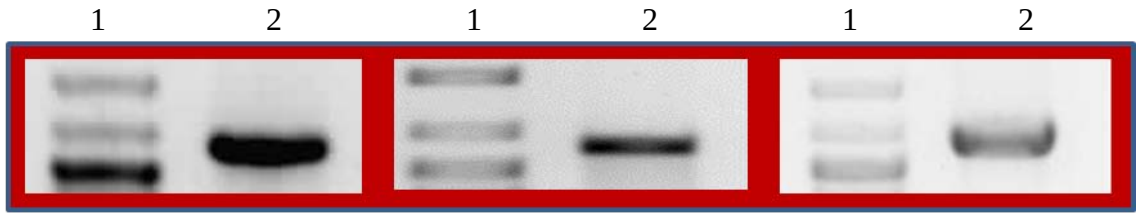
The healthy leaves of 5-6 cm length were selected from green house grown plants of *Nicotiana tabacum* cv. *Xanthi* for *Agrobacterium*-mediated leaf disc transformation. After surface sterilization, the leaves were cut into small leaf disks and used as a source of explants. The tobacco leaf discs were infected separately with *A. tumefaciens* EHA105 harbouring the TSH, PSH, CH, CSH, OSH and HSH constructs for 10 min and blotted in filter paper to remove excess bacteria and cocultivated on cocultivation medium (30-40 disks per petri dish, Fig. 3.8a) at 28°C in dark for 2 days. After cocultivation, all the explants were transferred to the shoot induction medium (Fig. 3.8b), and weekly subcultured to fresh shooting medium. At the end of 3 weeks, callus formation was observed from the margins of all the leaf disk explants. Once the shoots were 2-4 mm in length, they were either excised from the surrounding shoot buds or transferred to rooting medium or the shoots with small shoot buds were allowed to grow in a bunch until rooting. Rooting was observed after about 3 weeks (Fig. 3.8c) on half strength MS medium. The plantlets with roots were hardened in culture room followed by transfer to the green house (Fig. 3.8d, e). Putative tobacco transgenics were designated according to the plasmid construct followed by a serial number (Table 3.1).

Table 3.1: The table shows the details of all the constructs generated in this study. The construct CH with 35S promoter would express harpin constitutively within the cell (intracellular form) whereas all the other constructs would express fusion gene targeting harpin to the apoplast. In case of TSH construct, kanamycin is plant selectable marker whereas hygromycin is the marker gene in rest of the constructs.

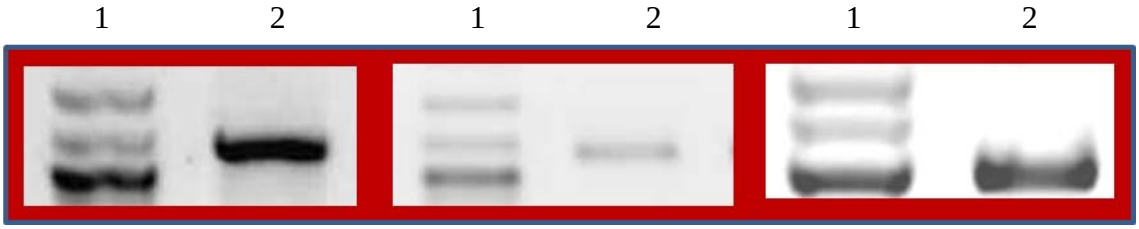
Promoters			Genes	Termina tor	Cassettes	Binary construct	Marker gene	Desig-nation
Name	Origin	Size						
35S	CaMV	500 bp	*hrpZ* (1 kb)	*nos* (200 bp)	35S-*hrpZ*-nos (1.72)	pCAMBIA 1300-35S-*hrpZ-nos*	*hpt*	CH ~10.7
			ShrpZ (1.2 kb)		35S-*ShrpZ-nos* (1.9)	pCAMBIA 1300-35S-*ShrpZ-nos*	*hpt*	CSH ~10.9
TA29	Tobacco	870 bp	*ShrpZ* (1.2 kb)		TA29-*ShrpZ-nos* (2.27)	pCAMBIA 2301-TA29-*ShrpZ-nos*	*nptII*	TSH ~13.9
PAL	Tomato	232 bp			PAL-*ShrpZ-nos* (2.0)	pCAMBIA 1300-PAL-*ShrpZ-nos*	*hpt*	PSH ~11.0
Osmotin	Tobacco	1.0 kb			OSM- *ShrpZ-nos* (2.4)	pCAMBIA 1300-OSM-*ShrpZ-nos*	*hpt*	OSH ~11.4
HSR203J	Tobacco	700 bp			HSR-*ShrpZ-nos* (2.1)	pCAMBIA 1303-HSR-*ShrpZ-nos*	*hpt*	HSH ~14.5

Table 3.1: The details of all the constructs generated in this study.

Figure. 3.7: The SP FP and Hrp RP were used to amplify the fusion gene (*ShrpZ*) from (a) TSH, (b) PSH, (c) OSH, (d) HSH, (e) CSH whereas Hrp FP and Hrp RP were used to amplify *hrpZ* gene from (f) CH plasmids isolated from agrobacterial cultures. In all the figures, lane 1 shows the DNA ladder mix (100 bp – 10 kb) and lane 2 shows the amplicon of *ShrpZ*/*hrpZ* from *Agrobacterium* transformed with TSH/PSH/OSH/HSH/CSH/CH.



a) TSH **b) PSH** **c) OSH**



d) HSH **e) CSH** **f) CH**

Figure. 3.7: **Confirmation of *Agrobacterium* transformation by PCR.**

Figure 3.8: Leaves from green house grown plants were selected and were cut into small leaf disks after surface sterilization. The leaf disks were cocultivated with *Agrobacterium tumefaciens* strain EHA105 harbouring the desired construct for 2 days followed by selection on selection medium. The transformed tissues formed calli whereas the untransformed tissues turned pale and necrotic. The shoots were induced to elongation followed by rooting. The plantlets were hardened in culture room followed by subsequent transfer to pots in green house.

(a) Leaves from green house grown plantlets of *Nicotiana tabacum* cv. xanthi were used as source of leaf disk explants.

(b) Selection cum shoot induction from leaf explants on selection medium.

(c) Initiation of rooting from elongated shoots.

(d) Transfer of plantlets to soil after hardening in culture room.

(e) Growth of putative transgenics to maturity under green house conditions.

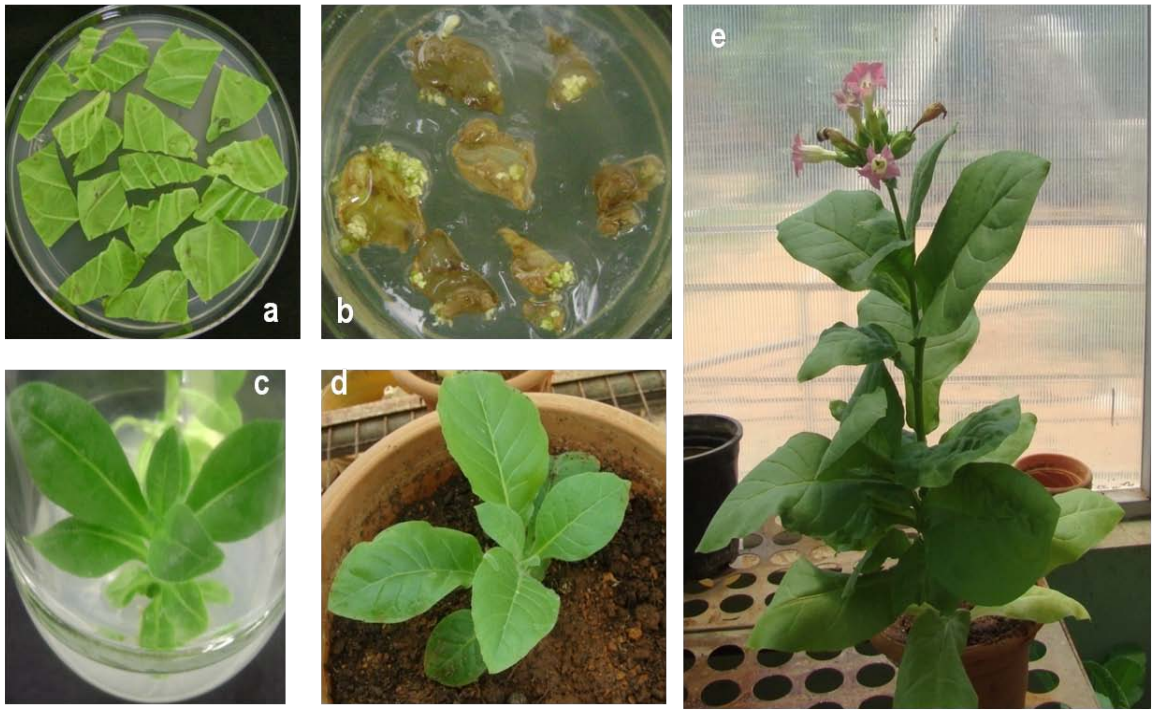


Figure 3.8: Leaf-disc transformation of tobacco.

3.5 Analysis of the primary transgenics

The plantlets obtained through tissue culture for male sterile and disease resistant lines were further analyzed for the confirmation of their transgenic nature and characterization of the transgenics.

3.5.1 Analysis of primary male sterile transgenics

3.5.1.1 PCR and western analysis

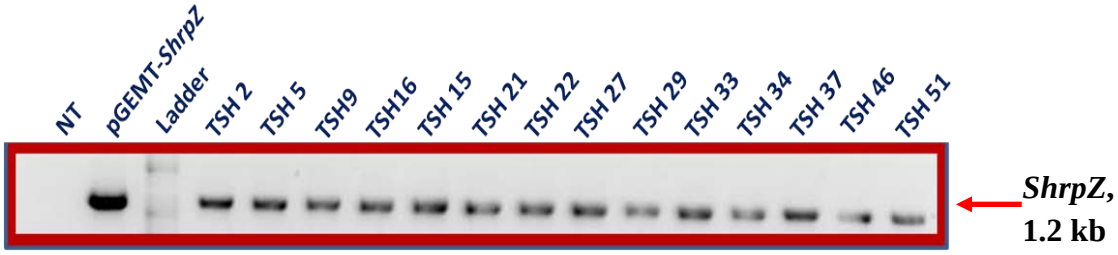
The male sterile lines generated by using TSH construct were first screened through PCR. A total of 53 independent transgenic lines were transferred to green house and screened through PCR, of which 42 plants were found positive for kanamycin marker gene (Fig. 3.9a) (NptII FP and NptII RP, Table 2.2). Of the 42 kanamycin positive plants, 27 plants were positive for fusion gene (Fig. 3.9b) (SP FP and H2 RP, Table 2.2). Out of 53 plants, 11 plants did not show amplification of kanamycin gene which indicates they might be escapes generated during kanamycin selection. For western blot analysis, proteins were isolated from stage 3 anthers of non-transformed and transgenic lines and were separated on PAGE gels followed by semi-dry transfer onto nitrocellulose membrane and western blotting with harpin antibody. All the 27 transgenics were positive for harpin (Data shown for 5 plants – TSH2, TSH16, TSH27, TSH33 and TSH34) (Fig. 3.9c). These PCR and western positive plants were selected for further morphological and histological analysis.

3.5.1.2 MTT assay to test pollen viability of TSH lines

To determine the percentage of pollen viability of partial and complete male sterile lines, the MTT assay was performed. The non-transformed type pollen grains reduce MTT to a purple colored complex and stain purple indicating the presence of dehydrogenases suggesting that they are viable (Fig. 3.10). When the pollens from the partial male sterile plants were tested for pollen viability using MTT assays, transgenic lines showed gradient reduction in pollen viability ranging from 20% to 70% as compared to 94% in non-transformed. The complete male sterile plant TSH34 showed no pollen grain formation and hence no anther dehiscence was observed.

Figure 3.9: PCR was done from genomic DNA of putative transgenics for the amplification of a) kanamycin (*NptII*) gene and b) fusion (*ShrpZ*) gene. c) The protein isolated from stage 3 anthers was used for western blotting of harpin$_{Pss}$ from non-transformed, complete and partial male sterile plants. The molecular weight marker used is DNA ladder mix (100 bp – 10 kb) for agarose gels.

a) PCR for kanamycin gene from putative TSH transgenics

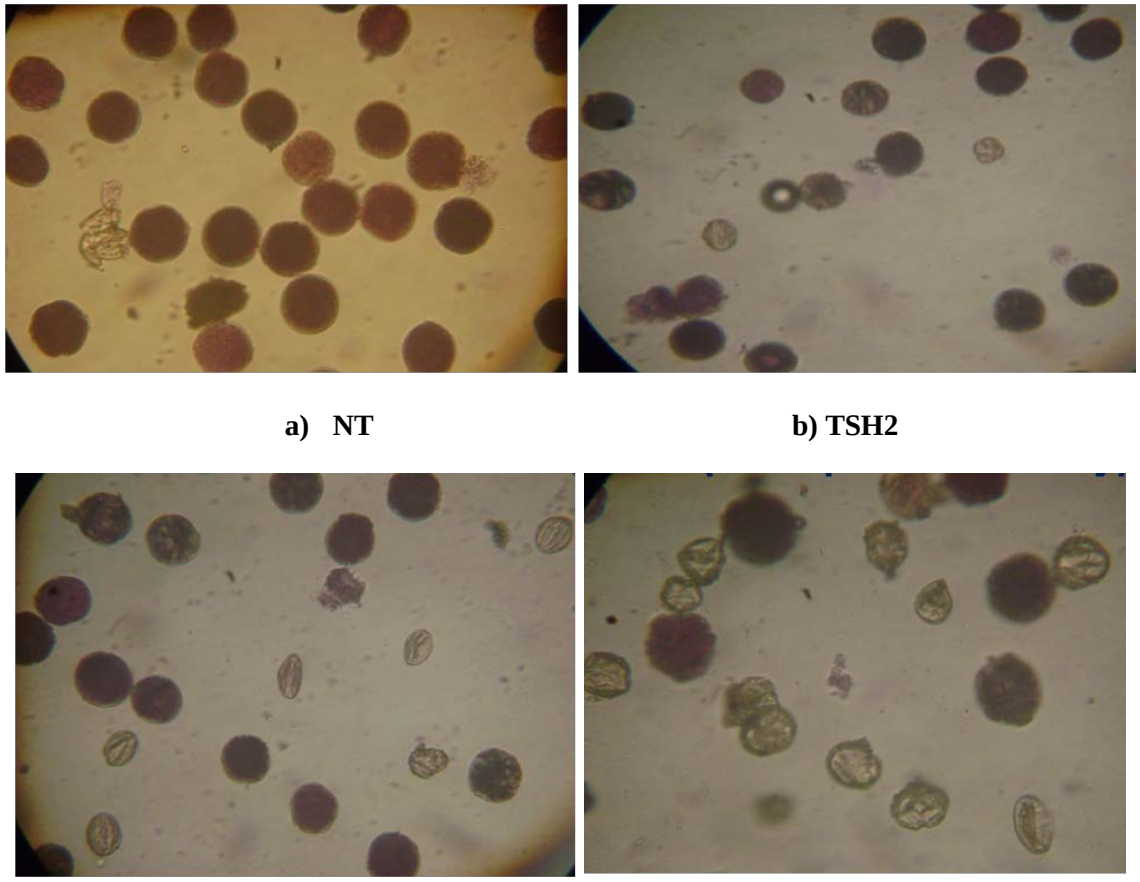


b) PCR for *ShrpZ* from putative transgenics

c) Western confirmation of the putative TSH transgenics

Figure 3.9: PCR and western confirmation of putative male sterile transgenics.

Figure 3.10: Samples of shed pollen of non-transformed (NT) plants and partial male sterile transgenics (TSH2, TSH27 and TSH16) following MTT staining assessed for pollen viability. Microscopic observations were made (100X) to count the proportion of viable pollen.



a) NT **b) TSH2**

c) TSH27 **d) TSH16**

Figure 3.10: MTT assay to assess the pollen viability of non-transformed and partial male sterile lines.

3.5.1.3 Morphological analysis of male sterile transgenics

Morphological observations, especially of anthers, play key role in screening the male sterile lines. Apart from sterility of anther or pollens they may show other morphological differences also. In morphological analysis of different TSH lines, out of 27 putative transgenics, variations were observed only in 2 lines - TSH16 and TSH34 whereas rest of the lines were similar to non-transformed plants (Fig. 3.11). The size of the leaf in TSH16 and 34, was much reduced as compared to non-transformed and the number of leaves also increased (Fig. 3.12). The leaves of these plants were thick where as other transgenic lines were similar to non-transformed plants.

We hypothesized that the male sterile lines of TSH should not develop pollen grains due to degeneration of tapetal layer. Therefore, examination of different lines for anther necrosis helps in screening the male sterile lines. TSH34 flowers showed aberration in anther development (Fig. 3.13). The anthers in TSH34 did not dehisce at all and no pollen formation was observed. On first day of flower opening, one or two anthers were observed to be necrotized and gradually with the progression of days, the other anthers also showed necrosis which could be due to harpin expression in the tapetal layer. Therefore, TSH34 line was completely male sterile whereas rest all other lines including TSH16 showed anther dehiscence and were referred to as partial male sterile lines. The complete male sterile plant (TSH34) showed reduced length of flower and stamen whereas in case of partial male sterile plants, only some flowers showed reduced length of stamens as compared to the non-transformed plants. Moreover, petals of complete male sterile flowers were of lighter shade with rough texture as compared to dark colour and soft texture of petals in non-transformed plants.

3.5.1.4 SEM studies of anthers and pollens of TSH lines

The degeneration of tapetal layer due to harpin induced HR-mediated cell death may generate deformities in the anther and pollen cell wall. The anthers and pollen from partial and complete male sterile lines were gold coated and observed in SEM. The necrotized anthers from TSH34 showed completely deformed morphology of the anther lobes as compared to anthers from partial and non-transformed plants (Fig. 3.14). The pollen grains from TSH2 and TSH16 were observed at different magnifications and only

Figure 3.11: The green house grown plants of non-transformed and putative transgenics of different TSH plants.

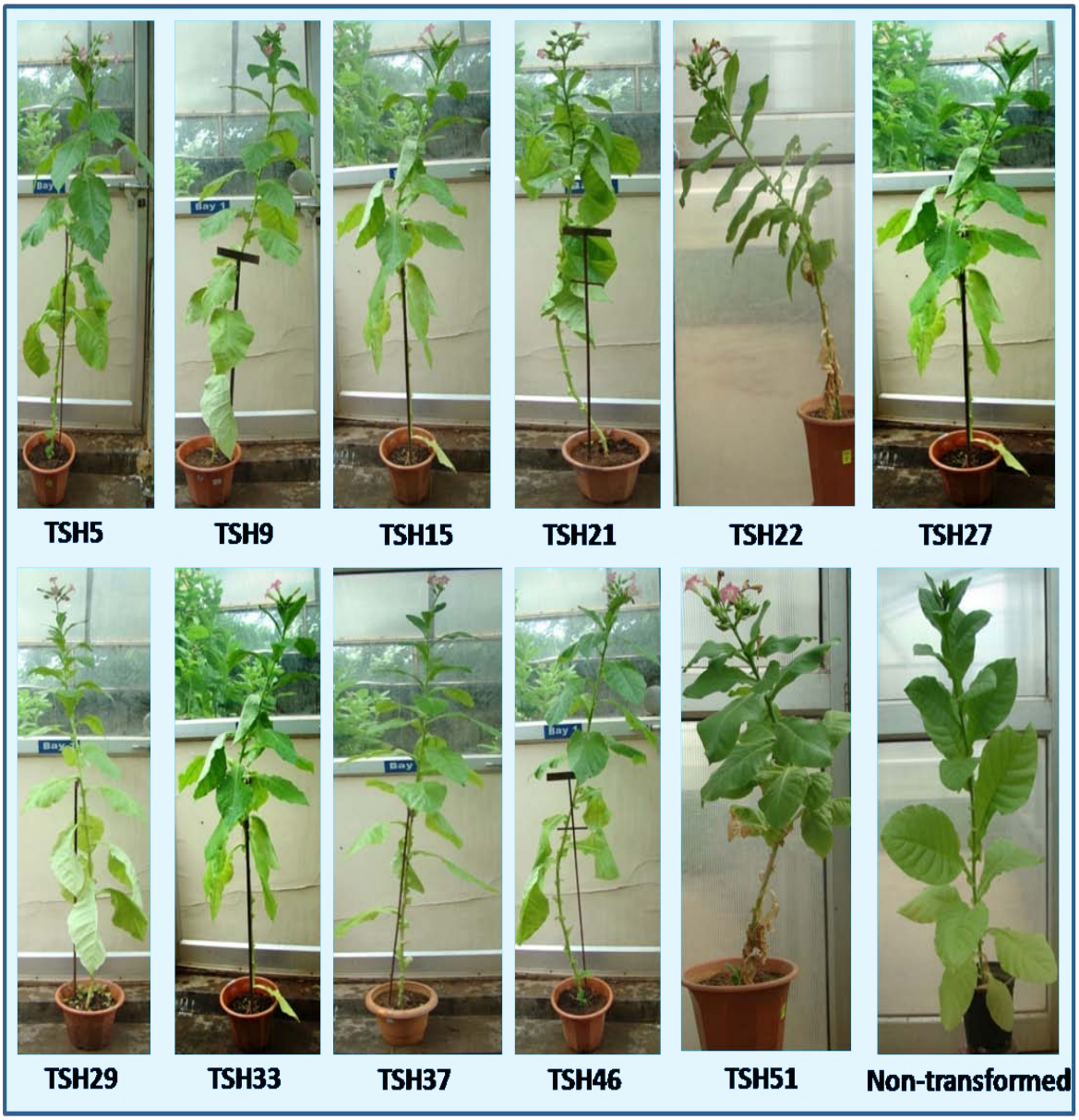


Figure 3.11: **Mature plants of non-transformed and putative transgenics of different TSH lines.**

Figure 3.12: Morphological observations on non-transformed (NT) and different TSH transgenic plants.

1) Top Row shows 90 days old plants of non-transformed, partial male sterile plants TSH2 and TSH16; and complete male sterile plant TSH 34 (left to right).

2) Middle row shows single leaf of the respective plant from the top row.

3) Bottom row shows open flower morphology of respective plant from the top row.

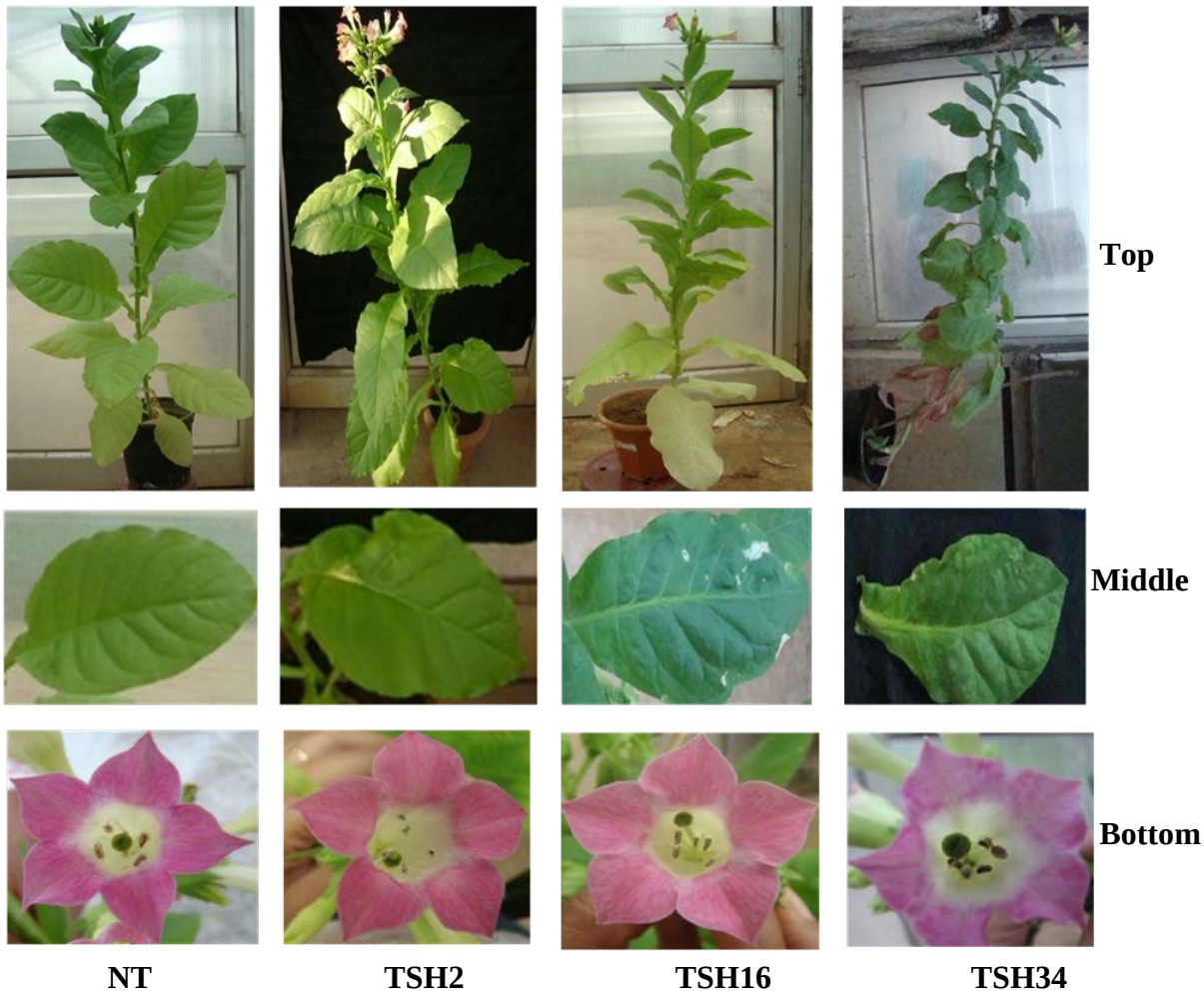


Figure 3.12: Morphological analysis of male sterile transgenics.

Figure 3.13: Morphological observation of flower from non-transformed plants and complete male sterile plant (TSH34).

1) Top row: left panel shows complete flower and right panel shows anther with filament from NT and TSH34 plants respectively.
2) Middle row: extreme left panel shows NT flower with viable anthers. The other three panels show gradual necrosis of anthers in the flowers from complete male sterile plant (TSH34) at different days (day 1-5) after flower opening.
3) Bottom row: vertical section of flowers from NT and TSH34 plants showing the morphology of anthers.

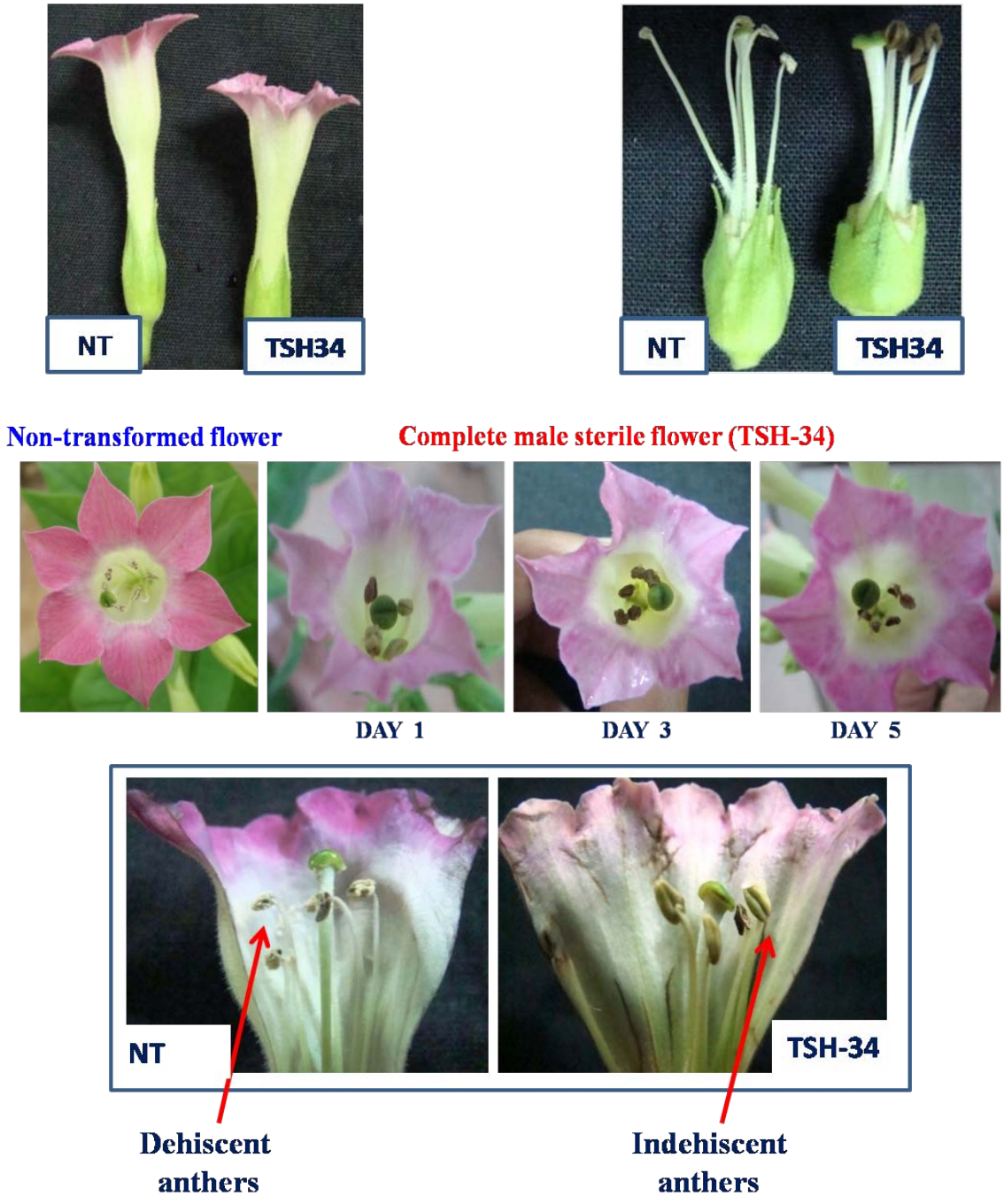


Figure 3.13: Observation of flower morphology of complete male sterile plant TSH34.

Figure 3.14: Scanning Electron Micrograph (SEM) of

a) anther from non-transformed (NT) plant.

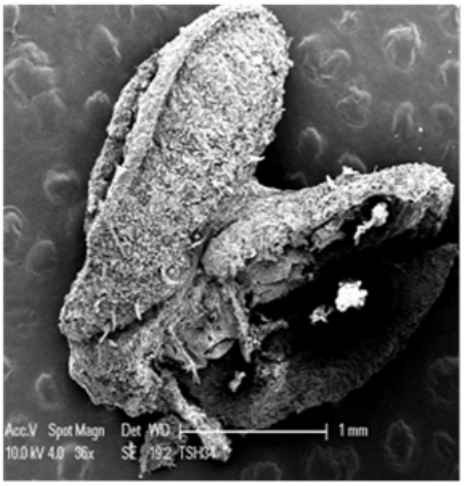
b) anther from complete male sterile plant (TSH34).

c) pollen grains from non-transformed and partial male sterile plants (TSH2 and TSH16) surface morphology studies.

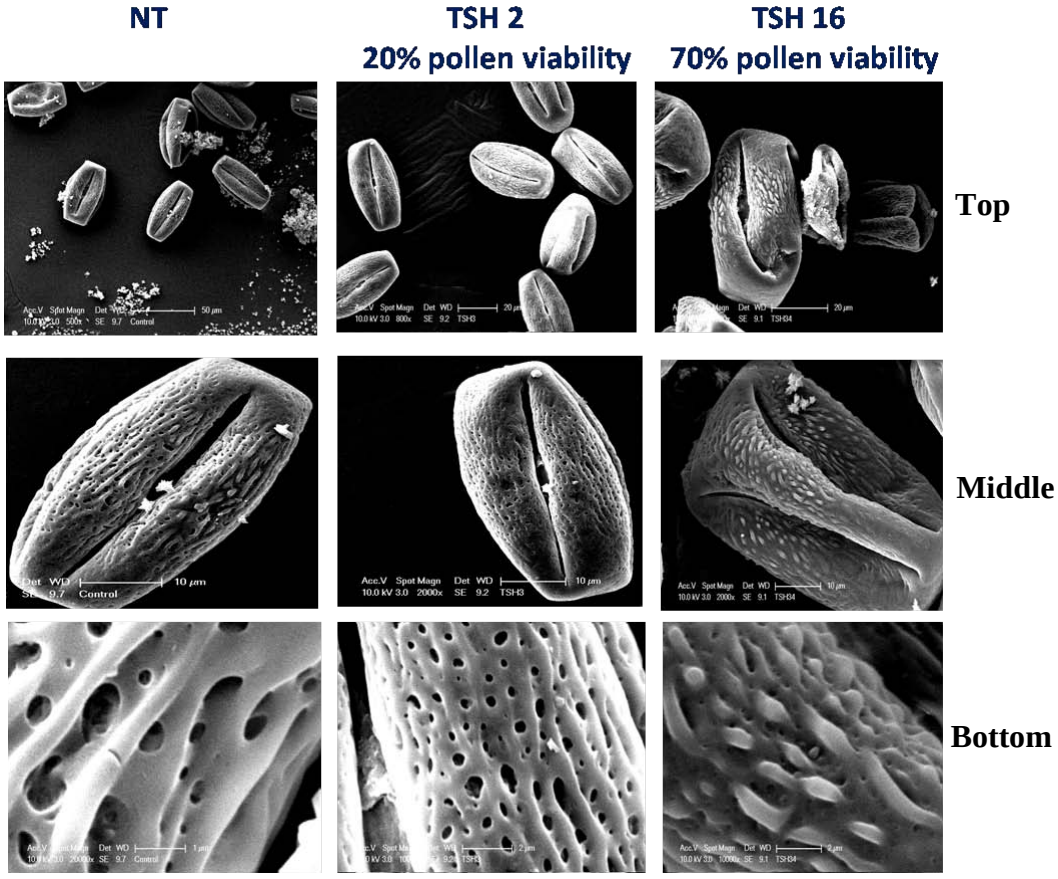
    1) Top row shows pollen grains at 200 µm magnification of NT TSH2 and TSH16 (from left to right).

    2) Middle row shows pollen grains at 10 µm magnification of the respective plants from top row.

    3) Bottom row shows pollen grains at 2 µm magnification of the respective plants from top row.



a) NT anther **b) TSH anther**



c) Wavy ornamentation of the pollen wall

Figure 3.14: SEM studies of anthers and pollens of TSH lines.

at 2 µ size, significant differences were observed in the mescolopodium of the pollen cell wall. In the partial male sterile lines, the outgrowth of the furrows and pores were reduced in comparison to the non-transformed which denotes the improper development of pollen cell wall and this can be attributed to harpin expression in the tapetum. As with the increase in male sterility percentage, the abnormalities in pollen cell wall also increased.

3.5.1.5 Histological analysis on anthers from male sterile lines

The TSH34 and TSH16 show deformities in the anther cell wall which could be due to harpin expression in the tapetal layer. The anthers from these lines were subjected to histological analysis using confocal microscopy for observing the deformities in the wall layer. The anthers from non-transformed plants at stage 2 showed healthy cells with pollen development and at stage 12, it showed complete dehiscence of the anthers (Fig. 3.15). Structural aberration was found in complete and partial male sterile lines in comparison to non-transformed. In TSH16 at stage 2, one of the anther lobes was deformed while the other lobe was completely normal with development of pollen grains. In TSH34, tissue specific expression of harpin in tapetum led to arrest of cell development and no pollen formation was observed. Hence, no dehiscence had taken place. Intact anther lobes were observed even at stage 12 of TSH34. Partial male sterile plants showed structural deformities during anther development process which resulted in different percentages of male sterility in comparison to non-transformed.

Immuno-localization of harpin in anthers from TSH34

To determine the localization of harpin$_{Pss}$ in the anther walls of transgenic tobacco plants, immuno-histochemical examination of anthers [TA29 active stages (Stage 6) and TA29 deprived stages (Stage 12)] were conducted using light microscopy. A strong signal was observed in the developing anther at stage 6 while very less signal was observed at stage 12 of complete male sterile plant (Fig. 3.16). No signal was detected in the non-transformed plants at these stages. This reveals the expression of harpin protein during active stages of TA29 during anther development leading to development of male sterility in tobacco plant.

Figure 3.15: The anther sections from stage 2 (upper panel) and stage 12 (lower panel) of non-transformed (NT), partial male sterile plant (TSH16) and complete male sterile plant (TSH34) were observed under light microscope.

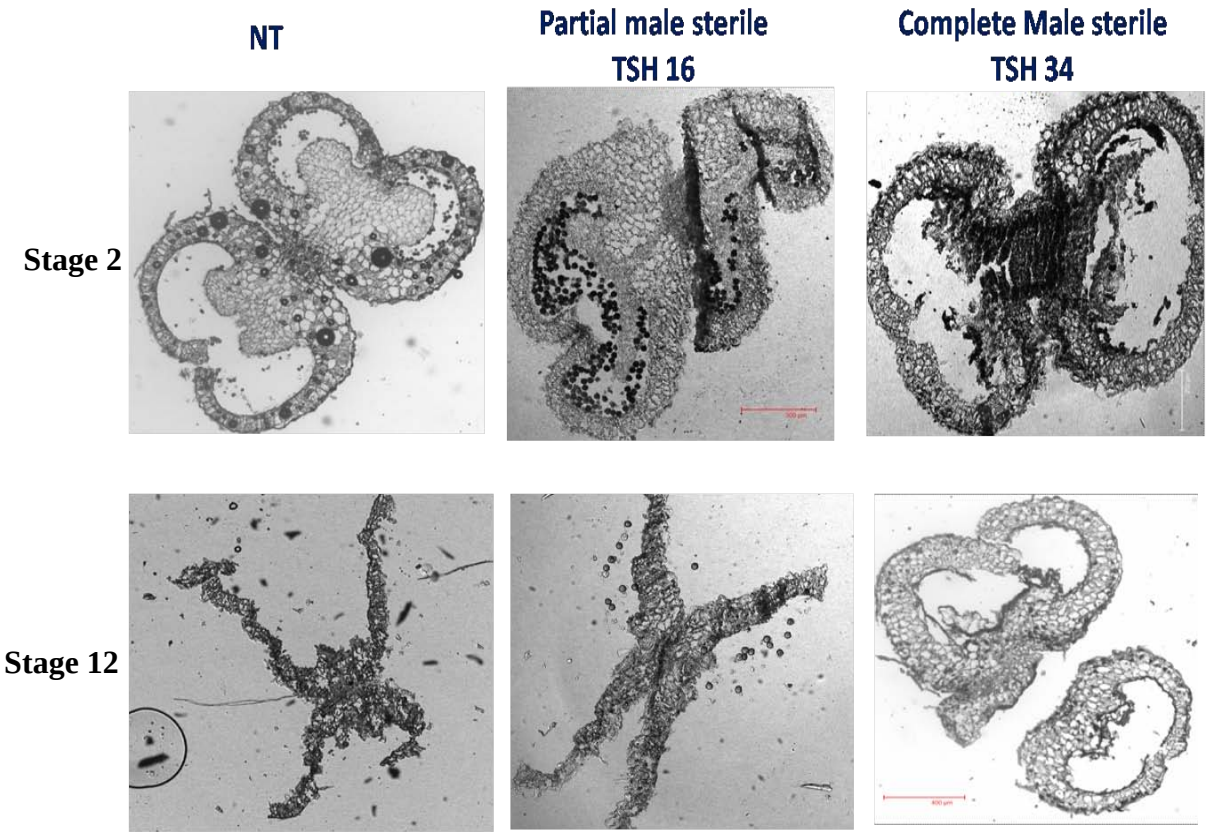


Figure 3.15: Histological studies with anthers from non-transformed and male sterile plants.

Figure 3.16 The anther sections from complete male sterile plant (TSH34) at stage 6 and 12 were prepared using FITC tagged secondary antibodies. Since TA29 is functional till stage 6, this stage was selected for the present study. The fluorescence signal was localized around the tapetal layers (indicated with arrows) of the TSH34 anthers at stage 6 (right top) and stage 12 (right bottom) which confirmed the tapetum specific expression of harpin whereas in non-transformed (NT) plants, no such signal was detected (left top and left bottom). Scale bars (–) indicate 400 µm.

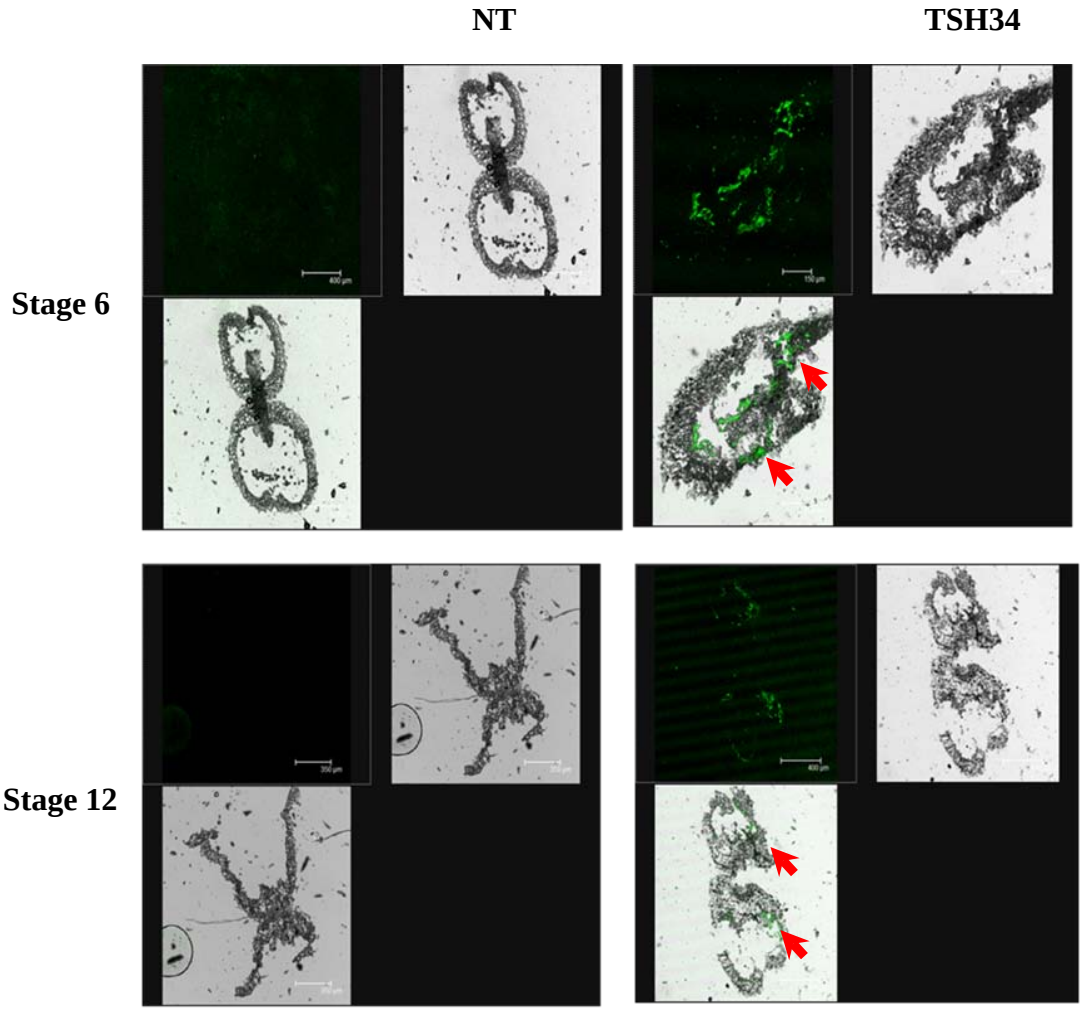


Figure 3.16: Immuno-localization for harpin in anthers at stage 6 and 12 from non-transformed and TSH34 plants.

3.5.1.6 Capsule formation

The capsule formation was observed in all the partial male sterile TSH plants. The average weight of 20 capsules was determined from these transgenic plants and compared with non-transformed plants (Fig. 3.17). There was significant reduction in the weight of capsules from partial TSH plants due to tapetum-specific expression of harpin gene and subsequent reduction in the formation of viable pollen grains.

3.5.1.7 Effect on female fertility of TSH transgenics

The female fertility of male sterile plants should essentially remain unaffected for its use in the crop breeding program and in hybrid seeds production. When the flowers from complete and partially male sterile lines were fertilized with pollen from non-transformed plants, normal capsule formation was observed indicating that the female fertility of male sterile lines was not affected (Fig. 3.18).

3.5.1.8 Southern blotting for TSH lines

To confirm the integration of transgene and also to assess the copy number of the putative male sterile transgenics, southern blotting was carried out using *ShrpZ* gene as probe. The genomic DNA was isolated from non-transformed, complete and partial male sterile plants (TSH2, TSH5, TSH6, TSH9, TSH10, TSH11, TSH27, TSH33 and TSH34). The DNA was digested overnight with *Eco*RI separated on agarose gel. The DNA was vacuum blotted to nylon N^+ membrane after depurination and denaturation. The southern blotting was done using the Gene Images™ AlkPhos Direct™ labeling kit (Amersham Biosciences) with CDP-*Star*™ chemiluminescent detection reagents. Southern analysis of different TSH lines showed two copy integrations in TSH34 where as single copy integrations in other lines (Fig. 3.19). The two copy integration in TSH34 might result in increased harpin expression in the tapetal layer leading to complete sterility as compared to single copy integration in partially male sterile lines.

Figure 3.17: Bar graph showing the comparison of capsule weights of the PCR-positive TSH transgenics with that of non-transformed plants (NT). Vertical bars denote the standard error.

Figure 3.18: Capsules obtained upon cross pollination of flowers from partial (TSH2) and complete (TSH34) male sterile plants with viable pollen from non-transformed (NT) plants.

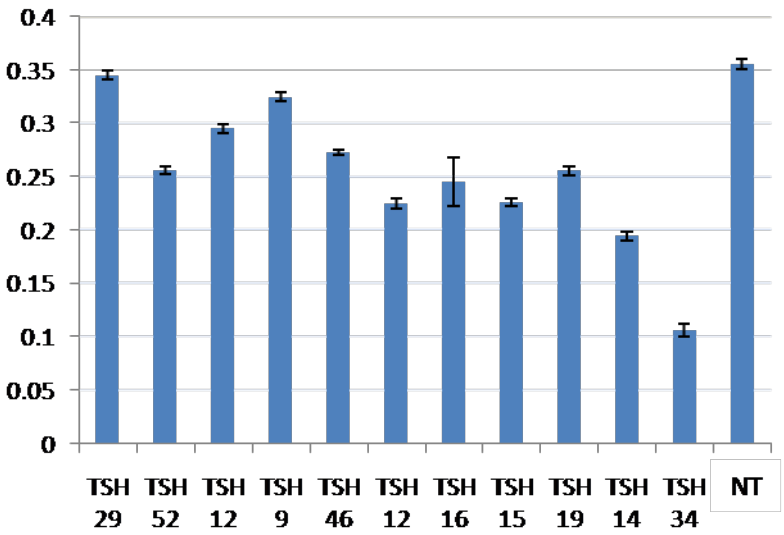


Figure 3.17: Bar graph showing the average mass of seeds per 20 capsules in the non-transformed and different TSH transgenics.

Figure 3.18: Effect on female fertility of partial and complete male sterile TSH transgenics.

Figure 3.19: The southern blotting of different TSH transgenic lines shows double copy integration in complete male sterile line TSH34 while single copy integrations in other partial male sterile lines. *ShrpZ* gene was used as probe which was synthesized and southern blotting was performed as per manufacturer's protocol (Amersham biosciences).

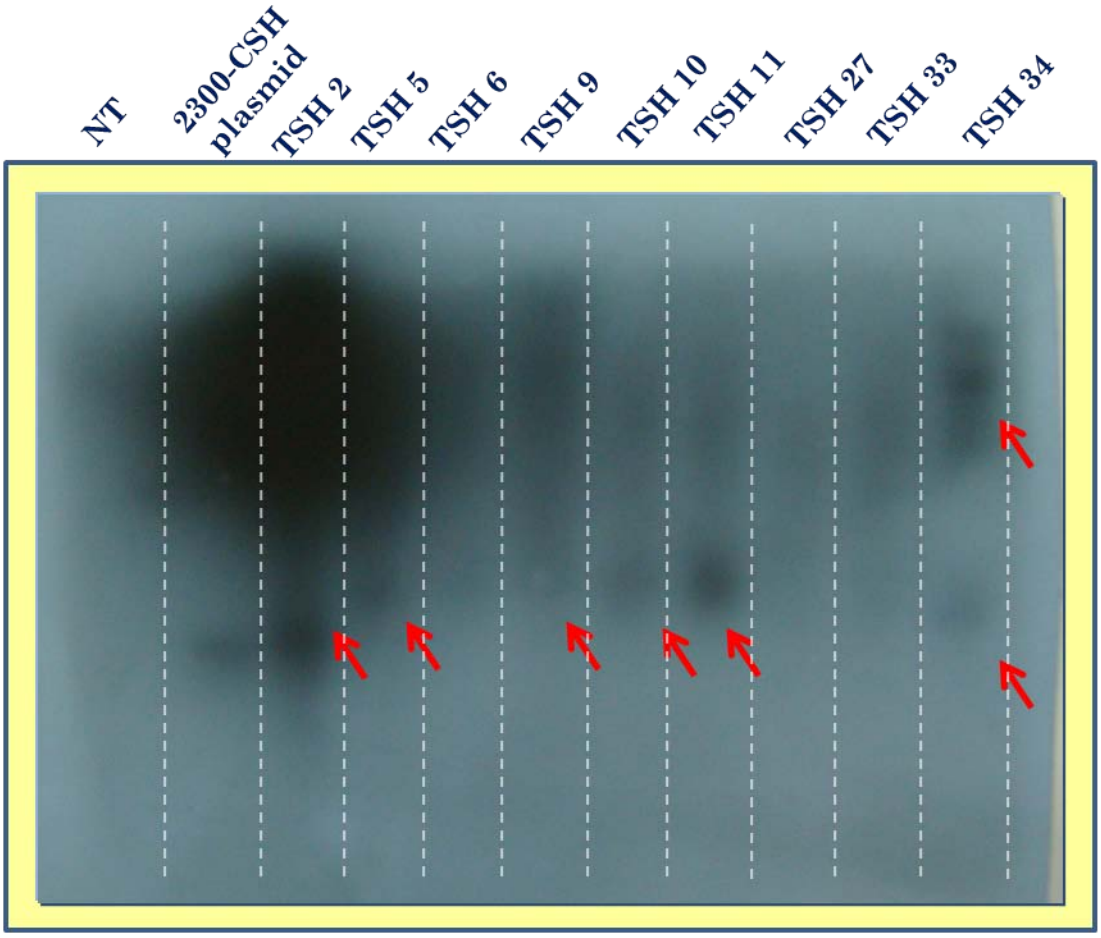


Figure 3.19: Southern blotting for TSH lines.

3.5.1.9 RT-PCR

The transgene (*ShrpZ*) expression in the tapetal tissue and the comparative levels of expression in different lines was studied by isolating RNA followed by RT-PCR analysis. To confirm the harpin gene expression in the tapetal layer, a total of 15 anthers (stage 3) from flowers of male sterile lines were selected and stored in -80°C. Since the tapetal layer is functional until stage 6, RT-PCR analysis was performed with stage 3 anthers. The total RNA was isolated from the anther samples using trizol method. Total RNA (5 µg) was converted into cDNA using cDNA synthesis kit (TaKaRa) following manufacturer's protocol. The cDNA was used as template in a PCR to amplify the fusion gene (*ShrpZ*). The harpin gene expression was observed in all male sterile lines (Fig. 3.20). However, the expression level of *ShrpZ* was highest in TSH34, the complete male sterile plant followed by the partial male sterile plant TSH16 and other plants. No amplification of fusion gene was observed from the non-transformed plants.

Figure 3.20: The first strand cDNA from RNA isolated from anthers of TSH transgenics was synthesized as per manufacturers protocol (TaKaRa) and used as template for amplification of *ShrpZ* (1.2 kb). The cDNA isolated from anthers of non-transformed (NT) plants was used as control.

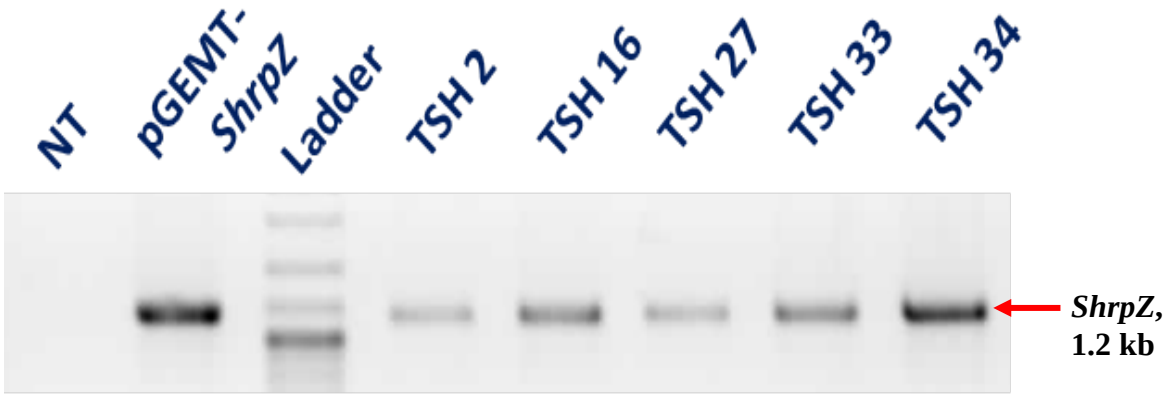


Figure 3.20: RT-PCR analysis of different TSH transgenic lines.

3. 5. 2 Analysis of primary disease resistant transgenics

During the initial stages of transformation, we faced a major challenge in recovery of transformed explants. Normal tissue culture procedure involves cutting of shoot buds during subculture. This led to wound-induced expression of harpin and subsequent necrosis of the explants (Fig. 3.21). In order to avoid wound-induced expression, the shoots were rather separated from the shoot buds and allowed to grow in a bunch until rooting. Then the bottles were broken and the whole plant was transferred to the green house. In this way, the required transgenics for all constructs were generated.

3. 5. 2. 1 PCR and western confirmation

The different disease resistant lines generated for each construct were screened first through PCR followed by western analysis for confirmation of their transgenic nature. For western analysis of all these lines, the leaves challenged with *Fusarium oxysporum* f.sp. *solani* spores were selected for protein isolation. A total of 15 putative transgenics for PSH construct were transferred to the green house and all of them were PCR positive for *ShrpZ* and *hpt* gene (Fig. 3.22). The plant leaves challenged with fungal spores were used for protein isolation and all the plants were western positive.

Transgenics for CH construct were also raised similarly as PSH and a total of 8 transgenics were transferred to green house (Fig. 3.23). All of them were confirmed to be *hrpZ* and *hpt* gene positive through PCR and all these eight plants showed harpin signal in western analysis.

A total of five putative transgenic plants were transferred to green house for OSH construct and all of them were screened to be positive for *ShrpZ* and *hpt* gene (Fig. 3.24). The leaves challenged with pathogen were used for protein isolation and all the plants were found to be western positive. A total of 7 transgenics for HSH construct were transferred to green house and all of them were PCR positive for *ShrpZ* and *hpt* gene and all the plants were found to be western positive (Fig. 3.25). However, we could not generate any transgenics for CSH construct (Fig. 3.26). This result confirms that constitutive expression of fusion gene (*ShrpZ*) results in continuous production and secretion of harpin to the apoplast leading to subsequent HR-mediated cell death in all the transformed tissues leading to necrosis of explants on selection medium.

Figure 3.21: During regeneration of PSH transgenics, (a) the shoots were cut and separated from bunch of shoot buds and sub-cultured to fresh medium which turned necrotic. b) The shoots were allowed to grow in a bunch until rooting and the bottles were broken and plants were transferred to pots.

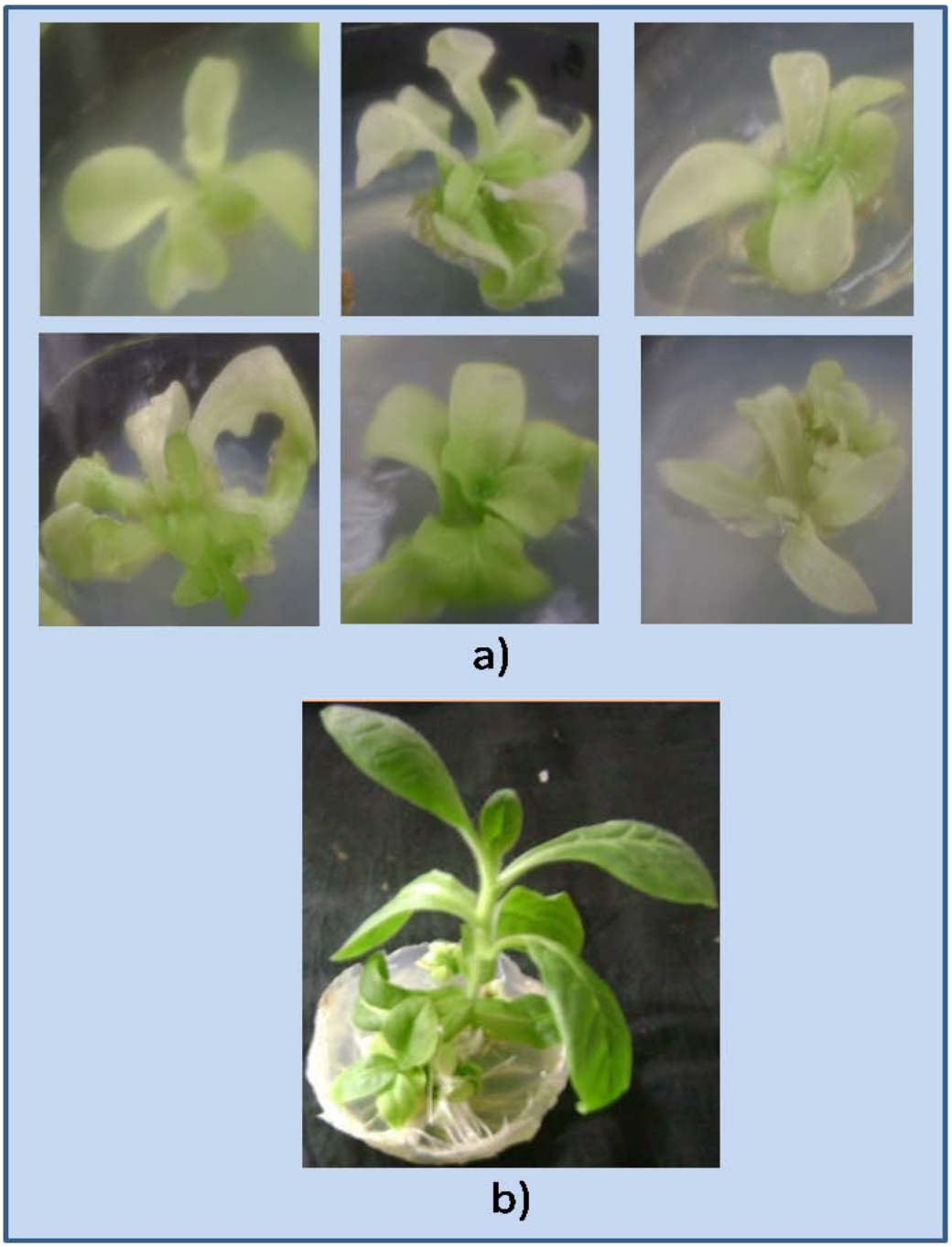
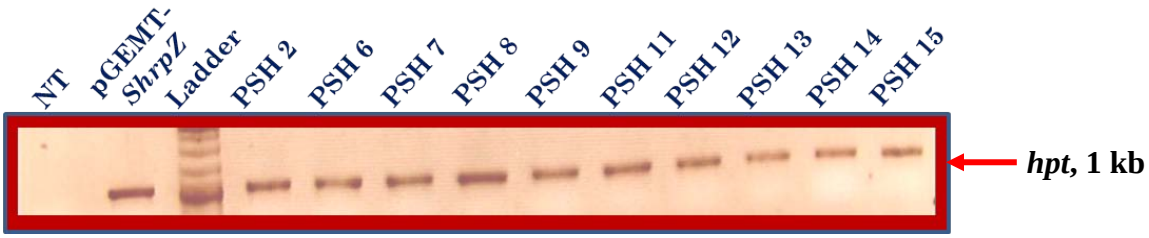


Figure 3.21: Generation of putative disease resistant transgenics of PSH.

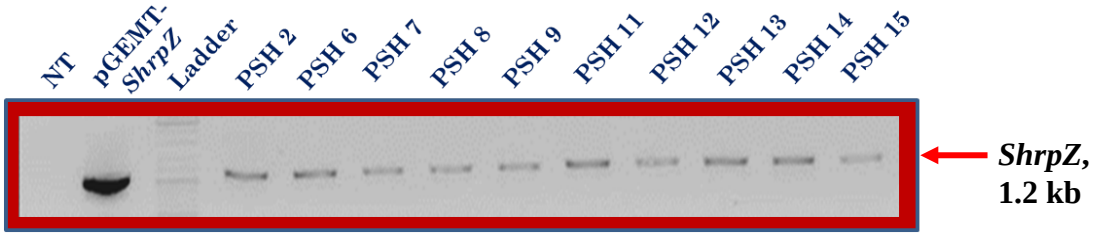
Figure 3.22: PCR was done from genomic DNA of a) non-transformed and putative PSH transgenics for the amplification of b) *hpt* gene (1 kb) and c) *ShrpZ* gene (1.2 kb). d) The protein isolated from leaves challenged with *Fusarium* was used for western blotting of $harpin_{Pss}$ from non-transformed and putative PSH transgenic plants. The molecular weight marker used is DNA ladder mix (100 bp – 10 kb) for agarose gels.

NT **PSH**

a) Putative transgenic for PSH construct.



b) PCR amplification of hygromycin from PSH transgenics.



c) PCR amplification of *ShrpZ* from PSH transgenics.

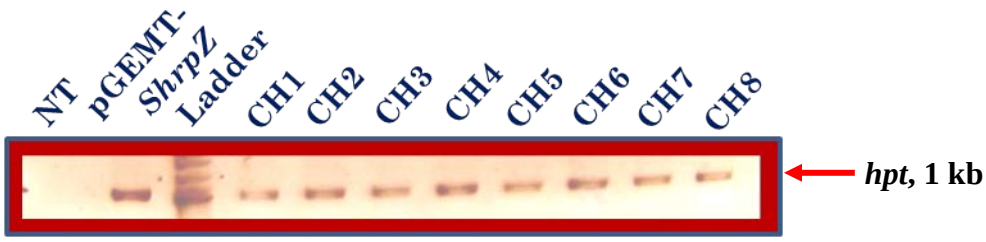
d) Western blotting for harpin$_{Pss}$ from PSH transgenics.

Figure 3.22: PCR and western confirmation of PSH transgenics.

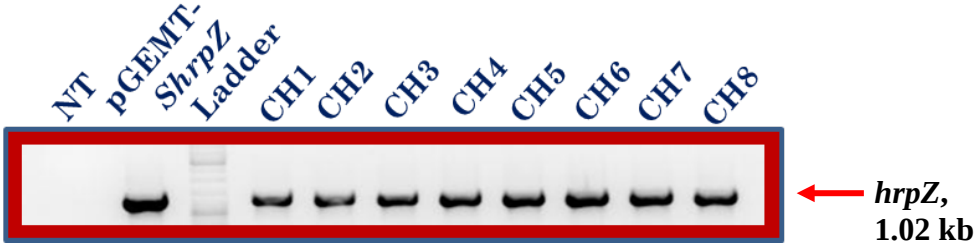
Figure 3.23: PCR was done from genomic DNA of a) non-transformed and putative CH transgenics for the amplification of b) *hpt* gene (1 kb) and c) *hrpZ* gene (1.02 kb). d) The protein isolated from leaves challenged with *Fusarium* was used for western blotting of $harpin_{Pss}$ from non-transformed and putative CH transgenic plants. The molecular weight marker used is DNA ladder mix (100 bp – 10 kb) for agarose gels.

NT **CH**

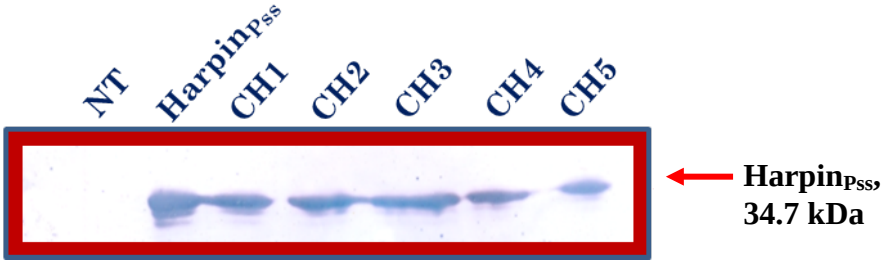
a) Putative transgenic for CH construct.



b) PCR amplification of hygromycin from CH transgenics.



c) PCR amplification of *ShrpZ* from CH transgenics.



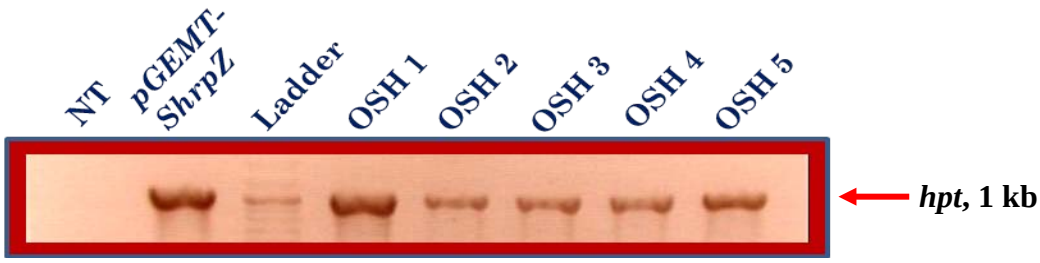
d) Western blotting for harpin$_{Pss}$ from CH transgenics.

Figure 3.23: PCR and western confirmation of CH transgenics.

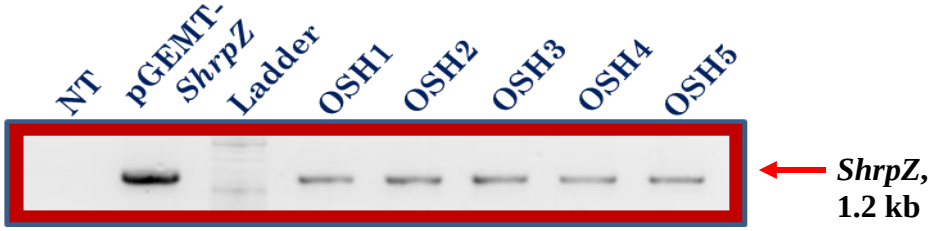
Figure 3.24: PCR was done from genomic DNA of a) non-transformed and putative OSH transgenics for the amplification of b) *hpt* gene (1 kb) and c) *ShrpZ* gene (1.2 kb). d) The protein isolated from leaves challenged with *Fusarium* was used for western blotting of $harpin_{Pss}$ from non-transformed and putative OSH transgenic plants. The molecular weight marker used is DNA ladder mix (100 bp – 10 kb) for agarose gels.

NT **OSH**

a) Putative transgenic for OSH construct.



a) PCR amplification of hygromycin from OSH transgenics.



b) PCR amplification of *ShrpZ* from OSH transgenics.

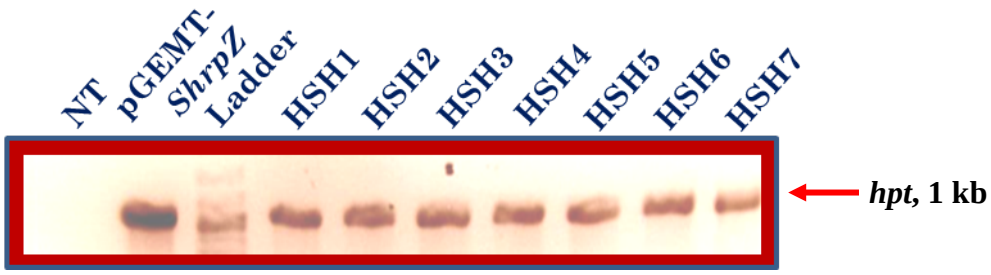
c) Western blotting for harpin$_{Pss}$ from OSH transgenics.

Figure 3.24: PCR and western confirmation of OSH transgenics.

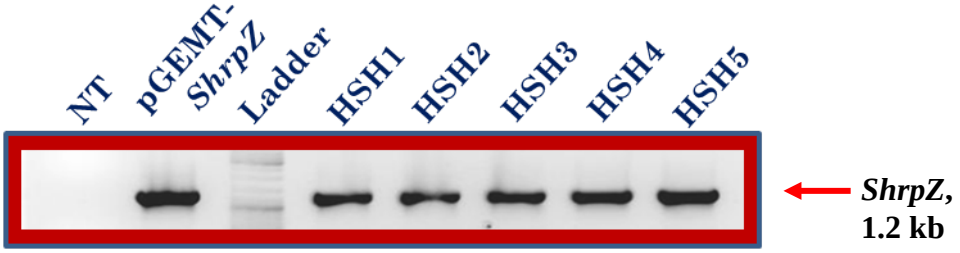
Figure 3.25: PCR was done from genomic DNA of a) non-transformed and putative HSH transgenics for the amplification of b) *hpt* gene (1 kb) and c) *ShrpZ* gene (1.2 kb). d) The protein isolated from leaves challenged with *Fusarium* was used for western blotting of $harpin_{Pss}$ from non-transformed and putative HSH transgenic plants. The molecular weight marker used is DNA ladder mix (100 bp – 10 kb) for agarose gels.

NT **HSH**

a) Putative transgenic for HSH construct.



b) PCR amplification of hygromycin from HSH transgenics.



c) PCR amplification of *ShrpZ* from HSH transgenics.

d) Western blotting for harpin$_{Pss}$ from HSH transgenics.

Figure 3.25: PCR and western confirmation of HSH transgenics.

Figure 3.26: Selection of explants on hygromycin medium one week after transformation with *Agrobacterium* harbouring CSH construct. Upon selection, the explants turned necrotic on selection medium after two weeks (a- d).

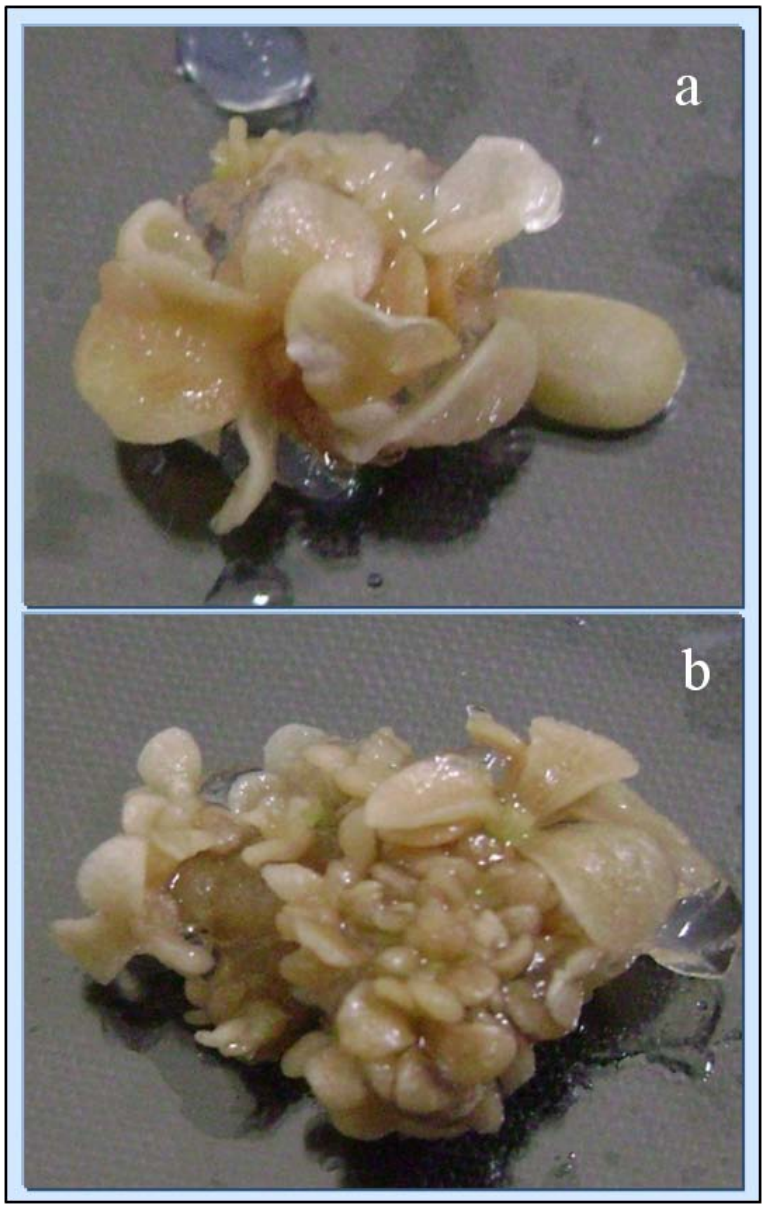


Figure 3.26: Selection of CSH transgenics on hygromycin medium.

3. 5. 2. 2 Fungal bioassay

To confirm the disease resistant nature of different PSH, CH, OSH and HSH transgenics, PCR and western confirmed transgenics were selected for fungal bioassay with *Fusarium oxysporum* f.sp. *solani* using detached leaf method. The transgenic lines challenged with the fungus showed minimal disease symptoms as compared to the non-transformed plants at the end of 7 days. The non-transformed plant leaf showed disease symptoms at 2nd day of challenge inoculation with the fungus. In case of PSH transgenics, PSH13 and 15 showed browning of leaves surrounding the site of inoculation after 4 days whereas PSH9 and 11 showed minor symptoms at the end of 7 days (Fig. 3.27a).

Late appearance of disease symptoms was observed in case of CH transgenics as compared to the non-transformed plants (Fig. 3.27b). CH5 followed by CH2 showed minimal disease symptoms.

Similar results as in other transgenics were also observed in case of OSH (Fig. 3.27c) and HSH lines (Fig. 3.27d). OSH4 followed by OSH2 showed minimal disease symptoms. In case of HSH, all the lines conferred better resistance compared to the non-transformed plants and the disease severity was also observed to be less in HSH transgenics.

By comparing the disease severity in harpin expressing transgenics with different promoters, the HSH transgenics appear to have higher degree of resistance to *Fusarium oxysporum* (Fig. 3.27).

Figure 3.27: The leaves from non-transformed and different harpin transgenics (PSH, OSH, HSH and CH) were challenged against *Fusarium oxysporum* f.sp. *solani* and observed after 7 days. All the HSH lines showed minimal disease symptoms as compared to non-transformed. PSH9 and PSH11, in case of PSH lines; CH5 followed by CH2, in case of CH lines; OSH4 and OSH2, in case of OSH lines showed delayed onset of disease symptoms.

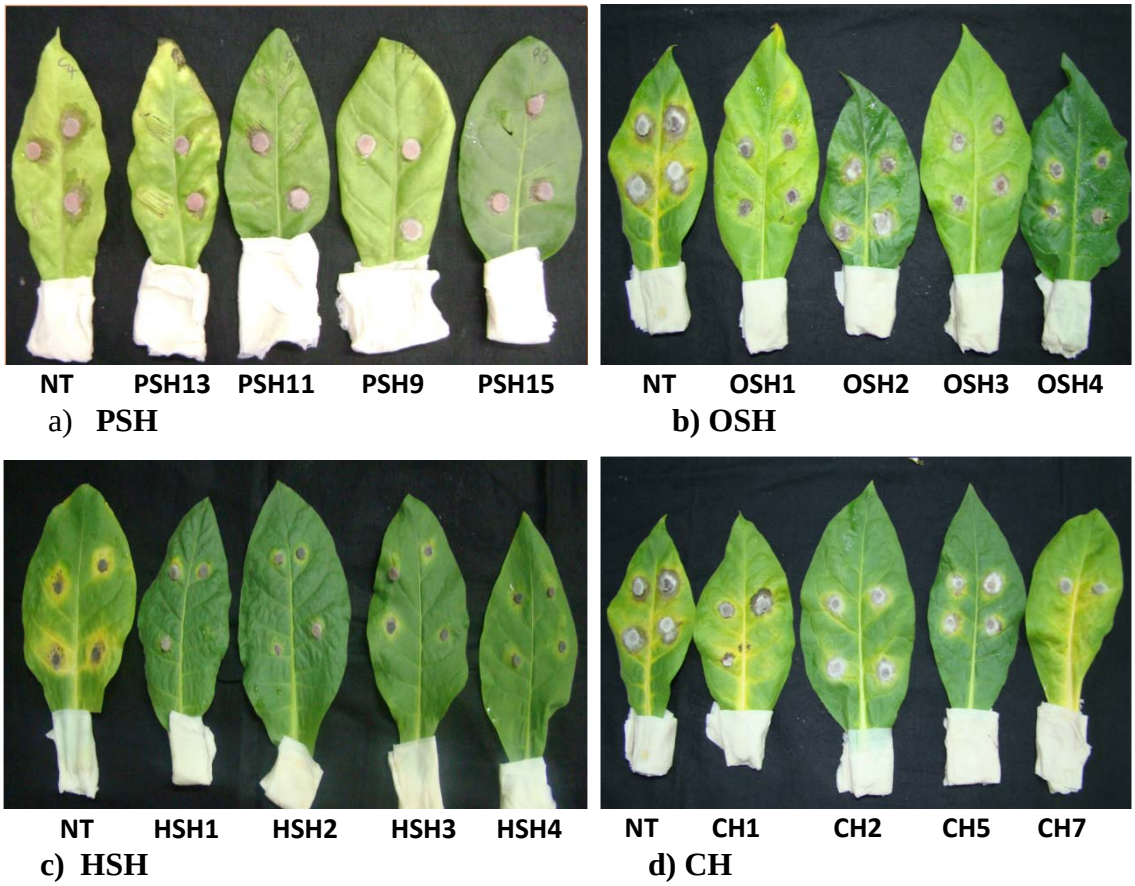


Figure 3.27: Fungal bioassay of harpin transgenics (PSH, OSH, HSH and CH) lines against *Fusarium*.

3. 5. 2. 3 RT-PCR analysis

To confirm the expression of harpin and fusion gene in different disease resistant lines and to analyze the harpin-induced expression of different defense genes (PR1, PR2, PR3, HSR and HIN1) the RT-PCR analysis was undertaken.

The non-transformed and different disease resistant transgenics (CH, PSH, OSH and HSH) confirmed through PCR and western analyses were challenged with *Fusarium oxysporum* f.sp. *solani* and the leaves were collected from treated and untreated plants after 12 h and stored at -80°C for RT-PCR analysis to detect the harpin and fusion gene expression and the transcript levels of different defense genes.

The cDNA prepared from RNA isolated from transgenic leaves challenged with fungus was used as template in PCR to amplify the harpin and fusion gene (*ShrpZ*) (Fig. 3.28). The fusion gene expression was observed in all disease resistant transgenics of PSH, OSH and HSH and harpin gene expression in CH transgenics. However, the harpin expression was observed in both treated and untreated samples of CH lines and the expression levels of harpin in CH transgenic were higher than the levels of fusion gene expression in other three transgenics. This could be due to constitutive expression of harpin gene under 35S promoter in CH transgenics compared to pathogen-inducible expression of fusion gene in other three transgenics under HSR, OSM and PAL promoters. Among all these disease resistant lines, HSH transgenics showed higher levels of *ShrpZ* expression compared to other lines which might be responsible for minimal disease symptoms observed in HSH lines in fungal bioassay against *Fusarium*.

The amplification of different defense genes like PR1, PR2, PR3, HSR and HIN1 was observed from the different disease resistant transgenics upon challenge with *Fusarium* (Fig. 3.29). The levels of expression of these defense genes were higher in all disease resistant transgenics than in non-transformed plants. The increased level of expression of defense genes in different disease resistant transgenics contributes to the resistance observed in these transgenics to *Fusarium*.

Figure 3.28: Non-transformed (NT) and harpin transgenics of PSH, OSH, HSH and CH plants were challenged against *Fusarium* spores and leaves were collected after 12 h. The first strand cDNA from RNA isolated from challenged (+)(Lanes 2, 6, 8, 10 and 12) and mock (-)(1, 5, 7, 9 and 11) leaves of NT and a) PSH b) OSH c) HSH and d) CH transgenics was synthesized as per manufacturers protocol (TaKaRa) and used as template for amplification of *ShrpZ* and *hrpZ* genes. The molecular weight marker used was DNA ladder mix (100 bp – 10 kb, Lane 4).

Figure 3.29: Non-transformed (NT) and harpin transgenics of PSH, OSH, HSH and CH plants were challenged against *Fusarium* spores and leaves were collected after 12 h. The first strand cDNA from RNA isolated from challenged (+) (Lanes 3, 5, 7, 9 and 11) and mock (-) (Lanes 2, 4, 6, 8 and 10) leaves of NT and a) PSH b) OSH c) HSH and d) CH transgenics was synthesized as per manufacturers protocol (TaKaRa) and used as template for amplification of defense-response genes *viz. PR1*, *PR2*, *PR3*, *HSR* and *HIN1*. The molecular weight marker used was DNA ladder mix (100 bp – 10 kb, Lane 1).

Figure 3.28: First strand cDNA was synthesized from *Fusarium* challenged and mock leaves of non-transformed and different harpin transgenics (PSH, OSH, HSH and CH) and used as template for amplification of harpin gene.

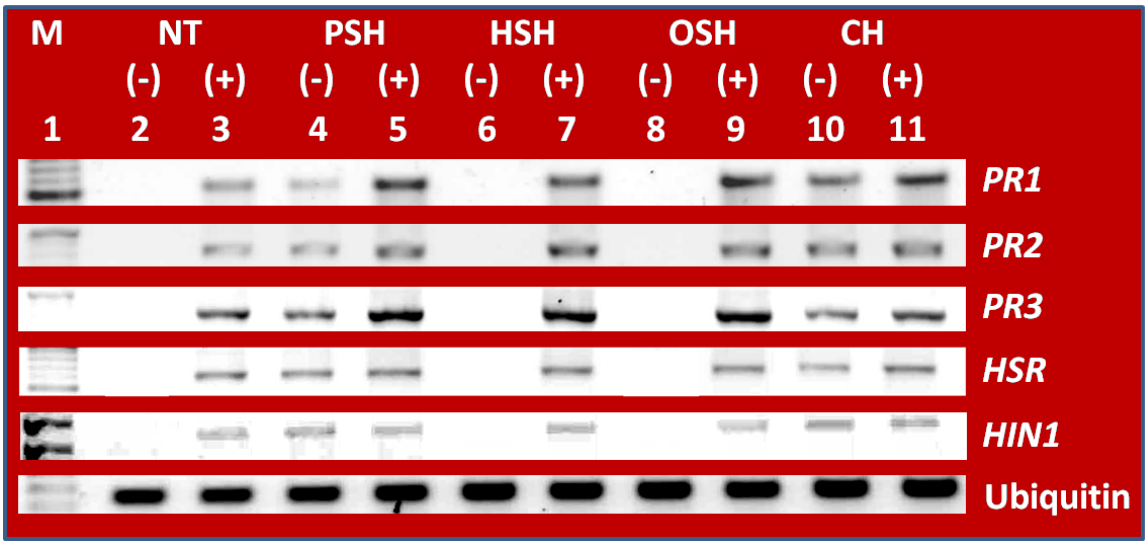


Figure 3.29: First strand cDNA was synthesized from *Fusarium* challenged and mock leaves of non-transformed (NT) and different harpin transgenics (PSH, OSH, HSH and CH); and used as template for amplification of different defense-response genes.

Discussion

4. 1 Harpin$_{Pss}$ mediated hypersensitive-cell death in plants

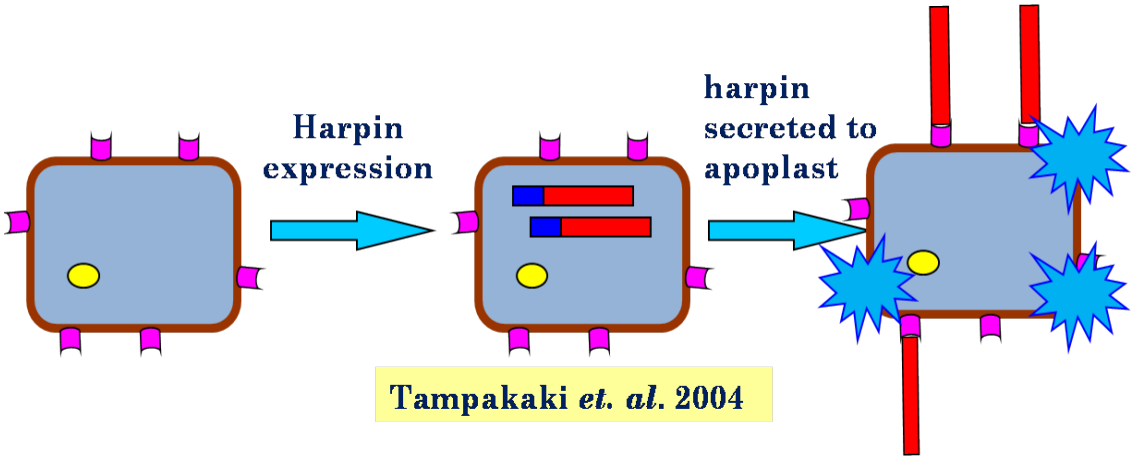
Harpin, a proteinaceous elicitor, mediates a plant defense reaction known as the hypersensitive response (HR). Harpin gene was initially isolated from *Erwinia amylovora* and later a few gram –ve phytopathogenic bacteria including *Pseudomonas syringae* pv. *syringae* (He *et. al*., 1993). Harpin$_{Pss}$ is a 34.7 kDa protein, encoded by *hrpZ* gene (1.02 kb). The infiltration of harpin protein into intercellular spaces of leaves of non-host plants triggers disease resistance–associated responses, such as HR, transcript accumulation of pathogenesis-related (*PR*) protein genes, and systemic acquired resistance (Baker *et. al.*, 1993; He *et. al.*, 1993; Gopalan *et. al.*, 1996; Strobel *et. al.*, 1996; Dong *et. al.*, 1999; Galan and Collmer, 1999) suggesting the target receptor for harpin could be in the apoplastic region. Further, the immunolocalization studies revealed a Ca^{2+}-dependent association of harpin$_{Pss}$ with tobacco cell walls (Hoyos *et. al.*, 1996), while harpin-induced K^+ /H^+ exchange at the plant plasma membrane and subsequent plasma membrane depolarization (Hoyos *et. al.*, 1996; Pike *et. al.*, 1998) generated ambiguity regarding the localization of harpin receptor in the cell. Harpins were also reported to interact with membranes directly and trigger plant defense responses in an ionophore-like manner by associating with the synthetic bilayer membranes and evoking cation currents of large unitary conductance (Lee *et. al*., 2001).

The expression of harpin gene under TA29 promoter resulted in development of partial male sterile lines (Madhuri, 2006). The expression of harpin gene downstream to 35S promoter resulted in no un-induced expression of harpin gene and no morphological changes were observed in leaves, stems and flower and seed fertility of transgenics. However HR-like local lesions generated after pathogen attack (Takakura *et. al.*, 2004; Tampakaki *et. al.*, 2000, Fig. 4.1). When the harpin gene was expressed downstream to PAL1 promoter, the transgenics showed resistance to pathogens and HR like lesions were only visible upon pathogen attack (Takakura *et. al*, 2004, Fig. 4.1). In both these cases, intracellular harpin accumulation didn't induce HR response in disease free conditions; however upon pathogen challenge, harpin leaked out of the wounds and accessed its receptor on the cell wall resulting in HR like lesion development at infection sites.

In the present study, we hypothesized that tagging of signal peptide to harpin gene (*ShrpZ*) and its expression downstream to developmentally regulated promoters such as

Figure 4.1: Proposed model of harpin$_{Pss}$ action. The harpin gene is expressed in two forms a) intracellular and b) secretable (using signal peptide) to induce both male sterility and disease resistance in tobacco.

a) Intracellular form of harpin



b) Secretable form of harpin.

Figure 4.1: Proposed model of harpin$_{Pss}$ action.

tapetum-specific TA29 promoter would result in generation of male sterile lines. Similarly the expression of *ShrpZ* driven by constitutive and/or pathogen inducible promoters would result in generation of disease resistant transgenics.

4. 2. 1 Generation of male sterile lines using TA29 promoter holds great promise

For generation of male sterile plants using genetic engineering, the uses of cytotxic genes are of great promise e.g., the use of barnase gene (Mariani *et. al*., 1990). However, the importance of the promoters plays very crucial role in driving the expression of such toxic genes. The expression of the transgene shouldn't confer any undesirable side effects in the introduced plant. The tapetum-specific expression of the transgene (cytotoxic) under TA29 (Koltunow *et. al.*, 1990; Mariani *et. al.*, 1990; Kriete *et. al.*, 1996; Cho *et. al.*, 2001) or A9 (Paul *et. al.*, 1992; Worrall *et. al.*, 1992) or *Osg6B* (Tsuchiya *et. al.*, 1994; 1995) promoters resulted in development of successful male sterile plants.

These promoters drive the expression of the transgene only in the desired tapetal layer resulting in selective degeneration or dysfunction of the tapetal layer. The tapetum plays vital role in the development of pollen mother cells and pollen by providing nourishment and substances that deposit as pollen exine besides acting as a protective anther wall layer (Echlin, 1971). The degeneration of tapetum, therefore, compromises the tapetal function and hampers the maturation of pollen mother cells (Shivanna *et. al*., 1997). The degeneration of tapetum doesn't interfere with any of the normal functions of other organs or tissues in the plant since developmentally tapetum is required towards the early stages of microsporogenesis and it undergoes degeneration naturally at the later stages. The TA29 promoter contains all the *cis* elements necessary to drive the expression of the transgene only in the tapetal layer during the active stages of tapetum.

4. 2. 2 The expression of signal peptide tagged harpin gene (*ShrpZ*) downstream to tapetum-specific TA29 promoter resulted in development of male sterility

Based on our earlier observations on partially male sterile TA29-*hrpZ* (TH) transgenics (Madhuri, 2006) we have revised our hypothesis that signal peptide mediated extracellular targeting of harpin protein might result in development of complete male

sterile plants. Suitable constructs were developed having TA29-*ShrpZ*-nos (TSH) cassette and generated transgenic tobacco plants through *Agrobacterium*-mediated transformation. The plants were screened through PCR for kanamycin and *ShrpZ*. Further confirmation was done through western blotting of proteins isolated from stage 3 anthers of the TSH transgenics, probed with harpin antibodies.

A total of 53 independent lines were transferred to the green house and only 42 plants were found to be *NptII* positive. The remaining 11 plants did not show amplification of *NptII* gene which suggests that these plants could be escapes during kanamycin selection. The 42 plants were further PCR screened for *ShrpZ* gene and 27 plants were positive for both kanamycin and fusion gene (*ShrpZ*). Out of 27 plants, only two plants TSH16 and TSH34 were morphologically distinct showing smaller leaf size and thick texture whereas the rest of the plants were more or less similar to non-transformed plants (Fig. 3.14).

The morphological observation of anthers of the 27 plants showed anther dehiscence in 26 plants including TSH16. However, TSH34 flowers revealed no dehiscence of anthers with gradual anther necrosis and no pollen grain formation. Therefore, TSH34 was referred to as complete male sterile whereas rests of the plants were referred to as partially male sterile. The length of the flower and stamen in TSH34 were reduced and the colour and texture (lighter and rough) of the flowers were different from that (dark and soft) of flowers from non-transformed plants (Fig. 3.15).

The scanning electron micrographs (SEM) of anthers from TSH34 revealed deformed morphology. Aberrations were visible in the pollen wall of partially male sterile lines (TSH2, TSH16 and other TSH lines) wherein the furrows and pores of the mescolopodium of the pollen cell wall were reduced in size. Previous report with *Arabidopsis* mutants showed differences between the non-transformed and mutant anthers only with respect to abnormal pollen wall development (Guan, *et. al*., 2008). The MTT assay demonstrated that the pollen viability of partial male sterile lines showed varying reduction (20-70%) in viability as compared to non-transformed plants (94%). Similar report of generation of partial and complete male sterile plants was achieved with RNAi-mediated silencing of *Os-Gen-L* resulting in defects in micropsores in rice

(Moritoh, *et. al*., 2005).

In an attempt to develop male sterility through specific disruption of tapetal layer by expression of barnase gene downstream to *apg* promoter, partial male sterile plants were generated despite demonstration of barnase expression and premature degeneration of the tapetum (Roberts *et. al.,* 1995). This is in contrast to the previous successful reports of development of male sterile plants with the tapetum-specific expression of barnase gene (Mariani *et. al*., 1990; Jagannath *et. al*., 2001). We now report development of one completely male sterile event (TSH34) and several partially male sterile events through the revised strategy.

The anthers from partially male sterile TSH16, at stage 2, showed development of pollen in one lobe of anther while the other lobe was morphologically deformed. Since there was pollen development in these partial lines therefore complete dehiscence of anthers was observed at stage 12. Contrary to this, there was no pollen formation in TSH34 in all the stages; no dehiscence of anthers was observed even at stage 12. The immunolocalization studies of anthers (Stage 6) for harpin using FITC tagged secondary antibodies resulted in localization of harpin signal in the anther wall layers which confirms the tapetum-specific expression of the harpin gene and concomitant HR-mediated cell death. Degeneration of the tapetal layer resulted in the development of complete male sterile event in TSH34.

The female fertility of complete and partial male sterile lines was not affected. Capsule formation was observed in all the male sterile lines when pollen from non-transformed plants was used to pollinate the flowers.

The expression of *ShrpZ* gene was confirmed through RT-PCR analysis using RNA isolated from the stage 3 anthers. The expression levels of *ShrpZ* gene were higher (almost double) in TSH34 as compared to other partial lines. The southern analysis of male sterile lines showed two copies of integration in TSH34, while rest of the partial lines had single copy integration. The high copy integration in TSH34 might have resulted in higher expression levels of harpin thereby generation of complete male sterility in TSH34.

Several reports correlated between copy numbers and expression levels in plants. Gendloff *et. al*., (1990) showed a significant correlation between transgene copy number and the expression level. However, an inverse correlation was reported between copy numbers and transgene expression by Allen *et. al.*, (1993) and Linn *et. al*., (1990). On the contrary, Shirstat *et. al*., (1989) and Van der krol *et. al*., (1990) demonstrated no significant correlation between transgene expression and copy numbers. McCabe *et. al.*, (1999) and Voelker *et. al*., (1996) reported that the increment of transgene expression is correlated directly to high copy numbers of transgenes. Plants with multiple copies of transgenes might contribute to higher expression of transgenes than plants with lower copies of transgenes.

The effect of genomic position and copy number towards transgene expression has been reported in numerous plants (Allen *et. al.,* 2000). Foreign DNA integrates in randon non-homologous sites; hence some of the transgenes may integrate in chromatin active areas or stiff chromatin transcription areas. Thus, transgenes that integrate in heterochromatic areas, such as centromere, tend to experience gene silencing, which resulted in low gene expression level (Mengiste and Paszkowski 1999). Also, due to the effect of "Expressivity", there could be variation in the expression level in different TSH transgenics even though all received a copy of transgene and could result in the generation of partial male sterile lines.

By comparing the present study of development of complete male sterile transgenic (TSH34) with our earlier results of generation of partial male sterile ones (TH plants; Madhuri, 2006), we conclude that secretable form of harpin (Fig. 4.1b) is quite promising as compared to intracellular form (Fig. 4.1a) towards generation of male sterile lines using the same TA29 promoter in transgenic tobacco. We need to improve the frequency at which the complete male sterility events could be improved.

4. 3. 1 Generation of disease resistant transgenics by expressing *hrpZ* / *ShrpZ* downstream to pathogen-inducible promoters holds great promise

The general approach of expressing the transgene under strong constitutive promoters results in constitutive expression of the gene all the time in all tissues of the crop which can result in detrimental effects on plant growth, development and yield. The

pathogen-inducible promoters might allow highly restricted expression of the transgene only at the infection sites resulting in development of crops with increased disease resistance (Gurr and Rushton, 2005b).

Different genetic engineering strategies have been used to obtain transgenic plants resistant to fungal diseases (Cornelissen *et. al.*, 1993). The initial approaches involved constitutive expression of one antifungal compound of the plants' own defense machinery, e.g. PR proteins or phytoalexins, thereby having the plant in a constant state of alert. The other approaches for development of fungal resistance relied on the activation of the whole array of defense responses by activating an HR (Honee, 1999). The HR coincides with a number of defense responses that occur around the infection site and distally in uninfected parts of the plant. These plants exhibited SAR against a broad spectrum of pathogens (Ryals *et. al*., 1994, 1996).

In case of race-specific resistance (e.g., R-gene mediated resistance), host responds with a hypersensitive reaction (HR) (Dangl *et. al.* 1996; Goodman and Novacky 1994; Lamb and Dixon 1997) limiting pathogen spread but durability is easily lost by one or a few mutations in the candidate genes. This can be overcome by growing alternate rows of resistant and normal cultivars reducing selection pressure (on '*Avr'* gene) in pathogen or by pyramiding several candidate genes, which is time consuming. Another alternative could be expressing '*Avr'* gene downstream to pathogen-inducible promoters thereby activating plant defense and keeping the plants alert before the actual pathogen invasion. Success has been achieved upon expressing molecules of pathogen origin in transgenic plants conferring resistance to the respective pathogen e.g. transgenic papaya resistant to papaya ringspot virus (Tennant *et. al.*, 2001).

Therefore, the expression of harpin gene (derived from a pathogen) downstream to pathogen-inducible promoters is expected to reduce the cost and burden of transgene expression on the plant and will result in generation of disease resistant transgenic plants.

4. 3. 2 Generation of harpin transgenics downstream to different promoters (35S, PAL, OSM and HSR)

The harpin gene was expressed in two different forms – intracellular form downstream to 35S promoter only and secretable form downstream to 35S, PAL, osmotin

and HSR promoters. A total of five different constructs were generated in pCAMBIA1300 and 1303. Putative transgenics were raised for all the five constructs through *Agrobacterium*-mediated leaf disk transformation of tobacco. Towards the initial phases, we faced difficulties in the regeneration of shoots. The routine tissue culture procedure involves unavoidable cutting of the explants during subculture resulting in wounding the explants. The wound-induced expression of harpin in shoot buds resulted in HR-cell death and necrosis of the buds compromising the regeneration process though the harpin gene was placed downstream to well studied pathogen inducible promoters. This could be due to the pathogen and wound signaling through the same *cis*-acting elements (Durrant *et. al*., 2000; Rushton, *et. al*., 2002). However, in the later phases, the shoots along with buds were allowed to grow on a bunch until rooting, and bottles were broken to take out the plant without causing damage to plants followed by transfer to green house. This procedure was followed for all the constructs to avoid loss of putative transgenics in the regeneration process.

Previous reports of constitutive expression of harpin protein in transgenic plants with or without signal peptide did not kill the plants or interfere with regeneration process (Rugang *et. al*., 1999). However, this response was due to insufficient expression of harpin protein in those transgenic plants and very low levels of harpin protein was accumulated. At a low concentration, harpin could only induce disease resistance, but not cell death and therefore, they did not face any limitation in the potato regeneration step. Mendes *et. al*. (2009) suggested that harpin (*hrpN*) might inhibit shoot regeneration and plantlet development during development of citrus transgenics and that could be the reason for low transformation efficiency and *in vitro* grafting survival of the seedlings. However, there are couple of other successful reports with harpin gene such as *Arabidopsis* plants expressing *hrpN* (Bauer *et. al*., 1999), tobacco plants expressing *hrpG* (Peng *et. al.,* 2004), pear plants expressing *hrpN* (Malnoy *et. al.,* 2005), tobacco plants expressing *hrpN* (Sohn *et. al*., 2007) and cotton plants expressing hpa_{Xoo} (Miao *et. al*., 2010).

A total of 15 different lines for PSH construct, 5 lines for OSH construct, 7 lines for HSH construct and 8 lines for CH construct were generated. All 35 plants were

positive for harpin and *hpt* gene in a PCR-based approach.

We could not regenerate transgenics for the CSH construct possibly due to high constitutive expression of harpin gene resulting in continuous production and secretion of harpin to the apoplast leading to subsequent HR-mediated cell death in all of the transformed cells (tissues) of the explants.

Except for the CH construct which intends constitutive and high level expression of harpin gene downstream to 35S promoter leading to intracellular accumulation of harpin protein irrespective of any HR induction, rest of the transgenic lines of PSH, OSH and HSH will presumably express the *ShrpZ* gene upon challenge infection with the pathogen since the *ShrpZ* gene was placed under pathogen-inducible promoters. Therefore, healthy and the *Fusarium* challenged leaves from the harpin transgenics were selected after 12 h for the protein isolation and western blotting. All the transgenic lines, for all the constructs, were western positive for harpin$_{Pss}$ (Fig. 3.20, Fig. 3.21, Fig. 3.22 and Fig. 3.23).

Harpin is reported to confer broad spectrum resistance against a wide array of pathogens such as bacterial, fungal (Dong *et. al.,* 1999; Peng, *et. al.,* 2003) and viral (Strobel *et. al*., 1996) pathogens as well as insects. Harpins such as harpin$_{Ea}$ and harpin$_{Xoo}$ are reported to be successful in controlling diseases such as *Fusarium* wilt and *Verticillium* wilt on cotton (Zhang *et. al*., 2004) and conferred resistance against *Phytophthora infestans* and *P. nicotianae* (Zhang *et. al*., 2004). To assess the disease resistance response of the putative harpin$_{Pss}$ transgenics, the PCR and western confirmed harpin transgenic plants were taken further for fungal bioassay with *Fusarium oxysporum* f. sp. *solani* using detached leaf bioassay. The non-transformed plants showed severe disease symptoms while the transgenic lines showed minimal damage. In case of PSH construct, PSH9 and PSH11 showed maximal disease resistance. In case of CH transgenics, CH5 showed best resistance compared to CH2 and CH1. In case of OSH transgenics, OSH4 followed by OSH2 showed minimal disease symptoms and disease severity as compared to the non-transformed plants. The HSH3 and HSH4 lines conferred the most resistance to *Fusarium* as compared to non-transformed plants.

The previous reports illustrated harpins ability to stimulate the accumulation of SA and ethylene (Zhang *et. al.,* 2004) and the expression of acidic and basic PR genes in

Arabidopsis spp. and tobacco (Dong *et. al.*, 1999; Peng *et. al*., 2003). The defense-response genes like *GST1, Chia5*, *PR-1a*, *PR-1b etc.* are activated by exogenous application of harpins to plants during the induction of pathogen resistance (Dong *et. al.*, 1999; Peng *et. al*., 2003, Strobel *et. al*., 1996).

The pathogen (*Fusarium*) challenged leaves from putative harpin transgenics were taken for RT-PCR analysis to study the expression of harpin gene and defense-response genes. Harpin expression was observed in all the harpin transgenics in response to pathogen challenge. The RT-PCR analysis of these transgenics for the expression of defense genes revealed pathogen-induced expression of *PR1, PR2, PR3, HSR* and *HIN1* genes. The expression of these defense-response genes in synergy with harpin gene might have contributed to the defense response of the transgenics towards *Fusarium*. The defense response of different harpin transgenics revealed that the HSH transgenics conferred the best resistance followed by PSH transgenics. This could be due to the very rapid, high level and localized activation of HSR promoter in response to pathogen infection thereby limiting the spread of the disease at the earliest (Pontier *et. al*., 1994 and 1998; Takahashi, 2004).

Previous reports of transgenics with harpins such as harpin$_{Ea}$ and harpin$_{Xoo}$ in both intracellular and secretable form downstream to 35S and other PIPs conferred resistance to several fungal and bacterial pathogens (Rugang *et.al.*1999, Peng *et. al.* 2004, Malnoy *et. al.* 2005, Sohn *et. al*. 2007, Mendes *et. al.* 2009). Both the constitutive or pathogen-induced expression of harpin protein reduced the lesion growth rate of fungus *Phytophthora infestans* in transgenic potato (Rugang *et. al*., 1999). In the present study, we also observed that both constitutive expression driven by 35S promoter and pathogen induced expression driven by PAL, OSM and HSR promoters conferred resistance to *Fusarium*. There was no difference in resistance between intracellular and secretable form of harpin expression in these harpin transgenics, as was reported in potato by Rugang *et. al*. (1999).

Minor difference in length of amino acid sequence of harpin (*hrpZ*) and fusion gene (*ShrpZ*) did not affect harpins function of inducing resistance in transgenic plants. This study shows the development of disease resistant transgenics in tobacco by expressing harpin in both intracellular and secretable forms under constitutive and

pathogen inducible promoters conferring fungal disease resistance. Similar studies of harpin transgenics could be raised in commercially and economically important crops such as cotton (Miao *et. al*., 2010) *etc.* towards reducing crop loss due to diseases.

Most of the transgenic approaches used constitutive promoters for expression of transgene. However, it has limitations in case of toxin genes whose constant over- or under-expression has deleterious or toxic effects on the plant (Zuo *et. al.*, 2000). These limitations can be overcome by use of tissue-specific, pathogen-specific or chemical-inducible promoters which enable very low level of expression of toxin genes only at the desired places when it is required. The best possible strategy will be choosing the required *cis*-regulatory elements to design synthetic promoters which can be used to drive pathogen-inducible expression of the toxin genes.

Summary

5. 1 Background

In a few specific interactions with pathogen-derived molecules, cell death is triggered in the interacting plant cells. Earlier studies in our lab adopted a transgenic approach to exploit the elicitor-mediated cell death to induce male sterility wherein harpin was expressed under tapetum specific TA29 promoter, resulting in partial male sterile lines. In an attempt to achieve complete male sterility using harpin, we hypothesized that the protein needs to be secreted to the apoplast. Expression of the signal peptide tagged protein downstream to tapetum-specific TA29 promoter might lead to the development of completely male sterile plants. Therefore, suitable constructs were devised to release harpin into the apoplast for a 'better' interaction with the putative receptor in cell walls. In this work, we have tested this hypothesis to induce both male sterility and disease resistance in tobacco.

$Harpin_{Pss}$, a 34.7 kDa extracellular protein which elicits hypersensitive response (HR) in plants (He *et. al*., 1993; Alfano *et. al*., 1996) is encoded by *hrpZ* gene, originally cloned from *Pseudomonas syringae* pv. *syringae* 61. Intracellular expression of $harpin_{Pss}$ doesn't lead to un-induced necrosis in transgenic plants and resulted in generation of partial male sterile lines upon expression in the tapetal layer (Madhuri, 2006). An attempt was made to develop an alternate inducible male sterility system by expressing signal peptide tagged harpin gene under the transcriptional control of tapetum-specific TA29 promoter. The second part of the study focused on expressing intracellular and secretable form of harpin gene downstream to constitutive (CaMV 35S) and pathogen inducible promoters (PAL, OSM and HSR) for generation of disease resistant transgenics.

5. 2 Development of male sterility by expressing *ShrpZ* under tapetum-specific TA29 promoter

The tapetum-specific TA29 promoter was available as TA29-*hrpZ*-nos (TH) construct in the lab. The *ShrpZ* gene was cloned downstream to the TA29 promoter generating the pCAMBIA2301-TA29-*ShrpZ-nos* (TSH) construct. *Agrobacterium tumefaciens* EHA105 was used for tobacco leaf disk transformation and generation of putative transgenics which were analyzed through PCR, western and morphological observations.

Out of 53 transgenics transferred to green house, only 27 lines were screened positive for both kanamycin and *ShrpZ* gene. Among these 27 lines, only two plants TSH34 and TSH16 showed morphological differences *i. e.*, leaves of small size and thick texture. The TSH34 flowers were lighter in shade and of rough texture in addition to reduced length of flower and stamen as compared to that of non-transformed plants. Significantly, the anthers in TSH34 were never observed to dehisce and no pollen formation was observed which could be linked to harpin induced damage to the tapetal layer. Therefore, TSH34 line was referred as completely male sterile whereas other lines including TSH16 showed anther dehiscence and were referred to as partial male sterile lines. The MTT assay of pollens from partial male sterile lines showed a gradient reduction in pollen viability ranging from 20% to 70% as compared to 94% in non-transformed plants.

The anthers from TSH34 and the pollen from other partial male sterile lines were observed under SEM. The anther lobes of TSH34 were completely deformed. The pollen from partial male sterile lines showed significant differences wherein the outgrowth of the furrows and pores of mescolopodium of pollen cell wall were reduced in comparison to the non-transformed plants denoting the improper development of pollen cell wall.

The histological observations of anthers (stage 2 and 12) from complete (TSH34) and partial male sterile lines (TSH2 and TSH16) showed structural aberrations in comparison to non-transformed plants. The non-transformed anther at stage 2 showed healthy cells with pollen development and at stage 12, it showed complete dehiscence of the anthers. In partial male sterile plant TSH16 at stage 2, one of the anther lobes was deformed while the other lobe was completely normal with development of pollen grains and therefore, anther dehiscence was observed at stage 12. In TSH34, tissue specific expression of harpin in tapetum led to arrest of cell development and resulted in no pollen formation. Therefore, no anther dehiscence was observed in TSH34 anthers at stage 12.

Immuno-histochemical examination of complete male sterile anthers from TSH34 showed strong signal at stage 6 (TA29 scanty stage) and very low signal at stage 12 (TA29 degenerated stages) using light microscopy. The localization signal was observed around the anther wall layers illustrating the harpin expression in the tapetal layer. No signal was detected in the non-transformed plants at these stages. This reveals the

constitutive expression of harpin protein during all the active stages of tapetum leading to development of complete male sterility in TSH34.

The female fertility of the complete and partial male sterile lines remained unaffected as normal capsule formation was observed in these plants when the pollen from non-transformed flowers was used to pollinate male sterile lines.

The southern analysis of TSH34 showed two copy integrations while the partial male sterile lines showed single copy integrations. The increased number of transgene integration in TSH34 may have resulted in complete male sterility.

The RT-PCR analysis of the complete and partial male sterile lines revealed harpin gene expression in all male sterile lines with the expression level of *ShrpZ* being the highest in TSH34, the complete male sterile plant followed by other partial male sterile plants. No amplification of *ShrpZ* was observed from the non-transformed plants.

5. 3 Generation of disease resistant transgenics by expressing *hrpZ* under constitutive (CaMV 35S) and *ShrpZ* under pathogen-inducible promoters (PAL, OSM and HSR)

While regeneration of disease resistant transgenics from transformed explants, it is requisite to cut and separate the shoots from the bunch of buds. This wounding of explants during tissue culture process induced harpin expression and subsequent necrosis of the explants. To avoid wounding, the shoots were allowed to grow in bunch until rooting and then transferred to green house and in this way the required number of transgenics for all the constructs were generated.

A total of 15 putative transgenics for PSH, 5 plants for OSH, 8 plants for CH and 7 plants for HSH were generated. All these plants were screened to be PCR positive for hygromycin marker gene. All the transgenics for CH were positive for harpin gene while rest of the transgenics (PSH, OSH and HSH) was positive for *ShrpZ*. The transgenic lines from each of the constructs were challenged with *Fusarium* spores and were used for protein isolation and western analysis. All the transgenic plants from each of the construct were found to be western positive.

The PCR and western positive plants were further taken for fungal bioassay with *Fusarium oxysporum* f. sp. *solani* using detached leaf method and were observed at the end of 7 days. PSH9 and PSH11 from PSH lines; CH5 and CH2 from CH lines; OSH4 and OSH2 from OSH lines and HSH3 and HSH4 from HSH lines showed greater resistance to the fungal pathogen. The HSH transgenics showed the best resistance to fungal infection followed by PSH ones.

The RT-PCR analysis of the cDNA prepared from RNA isolated from healthy and *Fusarium* challenged transgenic leaves (collected after 12 h) revealed harpin gene expression in CH transgenics while *ShrpZ* gene expression in PSH, OSH, and HSH transgenics. The expression of different defense genes like PR1, PR2, PR3, HSR and HIN1 were also observed concomitant with the harpin gene expression. The levels of expression of these defense genes were higher in pathogen inoculated samples than the healthy samples and non-transformed plants, which confirms the basis of resistance observed in different transgenics.

5. 3. 1 Conclusions on harpin transgenics for induction of male sterility

- Out of 27 transgenics, TSH34 was 100% male sterile while others were partially male sterile (20-70%).
- Non-transformed and male sterile plants showed morphological differences in terms of number, size and texture of leaves.
- The flowers from male sterile lines were smaller in size with reduced stamen length.
- The transgenic flowers were lighter in shade and the petals were rough in texture as compared to soft texture of non-transformed flowers.
- There was no dehiscence of anthers and no pollen formation in complete male sterile plant (TSH-34) while 20-70% male sterility was observed in partial male sterile plants accompanied by anther dehiscence.
- Female fertility remained unaffected in both complete and partial male sterile lines.
- Significant morphological differences were observed in the anther lobes of TSH34 and the wavy ornamentation of pollen cell wall was altered in partial male

sterile lines. There was gradient reduction in furrows length and pore size of pollen mescolopodium.

- Improper development of anther lobes was observed in partial and complete male sterile plants.
- The immuno-localization studies revealed the presence of harpin in tapetal layer of anthers in male sterile lines.
- The RT-PCR analysis confirmed fusion gene expression in all TSH transgenics and the level of expression of fusion gene was highest in the complete male sterile plant TSH34 followed by the partial male sterile transgenics.

5. 3. 2 Conclusions on harpin trasngenics for disease resistance

- Wounding of explants during tissue culture process induced harpin expression which interfered with the regeneration process.
- Transgenics of PSH, CH, OSH and HSH constructs were raised and confirmed through PCR and western analysis.
- Fungal bioassay of transgenics showed a delay in development of disease symptoms compared to the non-transformed plants.
- CSH transgenics could not be generated due to constitutive expression of harpin and its secretion to the apoplast.
- The RT-PCR analysis confirmed the expression of harpin gene in response to pathogen challenge in different transgenics. The harpin expression was accompanied by the expression of several defense-response genes (*PR1*, *PR2*, *PR3*, *HSR203J* and *HIN1*) in the transgenics and the expression levels of defense genes were higher in transgenics than non-transformed plants.

5. 4 Major findings of the present study

Male sterile transgenics:

- Complete male sterile transgenic line TSH34 was obtained using signal peptide-mediated targeting of harpin to the apoplast of the tapetal layer, while most of the TSH transgenics were partially male sterile.
- Distinct morphological and histological differences were observed between the non-transformed and transgenic (both partial and complete male sterile) plants.
- Immuno-localization and RT-PCR analysis revealed expression of harpin in all male sterile lines, highest being in TSH34.

Disease resistant transgenics:

- Transgenics expressing harpin downstream to 35S /PAL /HSR/OSM promoters showed considerable resistance to *F. oxysporum* f.sp. *solani.*
- RT-PCR analysis confirmed harpin gene expression in response to pathogens along with defense gene expression in all the transgenic lines.
- Comparison of disease development and severity among all the trangenics showed that HSR promoter appears to be most suitable and PAL promoter was the next best to express harpin in a pathogen-inducible manner.

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