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Tapetum-specific expression of harpin$_{Pss}$ causes male sterility in transgenic tobacco

B. MADHURI, S. RAUT, D. DEY, A. NAZNEEN, B. UMA and A.R. PODILE*

Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad-500046, India

Abstract

Harpin, an elicitor molecule of bacterial origin induces hypersensitive response (HR) in non-host plants. In an attempt to induce male sterility, harpin was tagged with a signal peptide and expressed downstream to tapetum-specific TA29 promoter resulting in extracellular secretion, subsequent degeneration of tapetum and development of male sterility in tobacco. Putative transgenics were analyzed by PCR amplification of transgene, semiquantitative RT-PCR analysis from total RNA extracts from anther tissue with transgene specific probe, Western blotting using polyclonal antibody raised against harpin, by transmission and scanning electron microscopy, and by confocal microscopy of anthers and pollen at various stages of development. Varying degrees of male sterility (30 - 100 %) was observed with plants showing complete and partial male sterility as well as several morphological variations were seen especially in leaves and flowers. Further, some of the transgenics showed un-induced of HR-like local lesions in the vegetative tissues. Harpin$_{Pss}$ got deposited on the pollen grains upon tapetal degeneration resulting in significant alterations in the morphology of pollen cell wall. However, megagametogenesis was not affected in complete and partial male sterile plants and female gametes were completely fertile. The complete male sterility was attributed to premature tapetal cell death due to sufficient extracellular harpin$_{Pss}$ accumulation whereas insufficient protein content might be the reason for partial male sterility. These findings indicate the possible use of cytotoxic harpin$_{Pss}$ for the development of male sterile plants.

Additional key words: exine, hypersensitive response, *Nicotiana tabacum*, signal peptide, TA29 promoter.

Introduction

Production of hybrid seeds requires a pollination control system to prevent unwanted self-pollination. Methods used to prevent self-pollination include mechanical removal of anthers or male flowers, application of male-specific gametocides, or use of genetic cytoplasmic or nuclear-encoded male sterility**.** Male sterility in plants could be engineered through controlled/specific genetic ablation of cells. Two genetic components are essential to develop an efficient male sterility system; a cytotoxic gene to remove target cells, and an appropriate promoter to control the spatial and temporal expression of the cytotoxic gene (Mariani *et al*. 1990, Kriete *et al*. 1996, Cho *et al*. 2001). Male-sterile mutation(s) or nuclear expression of cytotoxic proteins at specific stages of the anther development destroys tapetal cells, causes microspore abortion and prevents normal pollen development. Thus, tapetal cells are the target of choice to produce male sterile plants (Goetz *et al.* 2001).

Mariani *et al.* (1990) induced male sterility in tobacco and oilseed rape using two different ribonuclease genes under the control of a tapetum-specific TA29 promoter. Soybean pathogenesis related (PR) endo-β-1,3-glucanase was fused with the tapetum-specific *Osg6B* promoter resulting in premature dissolution of callose wall in pollen tetrads causing male sterility (Tsuchiya *et al.* 1995). Expression of cytotoxic ribosome inactivating

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Abbreviations: HR - hypersensitive response; LB medium - Luria-Bertani medium; RT-PCR - reverse transcriptase - polymerase chain reaction.

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* Corresponding author: fax: (+ 91) 40 23010120, e-mail: arpsl@uohyd.ernet.in

protein (RIP) from *Dianthus sinensis* downstream to TA29 promoter developed male sterile tobacco (Cho *et al*. 2001). Another system for the inducible male-sterility, based on the deacetylation of the non-toxic compound *N*-acetyl-L-phosphinothricin (*N*-ac-Pt), was developed by Kriete *et al.* (1996) expressing *argE* under TA29 promoter. An application of *N*-ac-Pt led to empty anthers and male-sterile plants. However, without the *N*-ac-Pt treatment, the plants were completely fertile. In another report, expression of *AtBI-1*, which suppresses Bax-induced cell death in the tapetum at the tetrad stage, inhibits tapetum degeneration, results in pollen abortion and male sterility (Kawanabe *et al*. 2006). In the present study, tapetum-specific expression of harpin was carried out to induce HR-mediated degeneration of tapetum and hence development of male sterility in tobacco.

Harpin is a bacterial proteinaceous elicitor that triggers hypersensitive reaction (HR) in non-host plants (Wei *et al.* 1992). The *hrpZ* gene from *Pseudomonas syringae* pv. *syringae* 61 encodes harpin, a 34.7 kDa extracellular protein (He *et al.* 1993) known to induce resistance against pathogens and pests in plants by activating the defense genes. Their ability to activate defense-responses prompted the transgenic expression of harpins to produce plants resistant to pathogens (Takakura *et al.* 2004). Such defense gene activation may not be the original action of the harpin, but possibly a reflection of its cytotoxic effect on cells. $Harpin_{Pss}$ is known to cause yeast cell death that shares conserved features of cell death pathway (among eukaryotes) with harpin-mediated hypersensitive cell death in plants (Podile *et al.* 2001). Therefore, transgenic expression of harpin for tapetal cell ablation could be useful to induce male sterility in plants. Expression of intracellular form of *hrpZ* under TA29 promoter resulted in 13 independent partial male sterile lines of transgenic tobacco which were morphologically normal with varying degrees of pollen viability (Madhuri 2006). Further, to achieve complete male sterility in tobacco, we designed an expression cassette to secrete $harpin_{Pss}$ into the intercellular spaces and demonstrate that the secretable form of $harpin_{Pss}$ induced absolute male sterility as well as partial lines with varying levels of male sterility.

Materials and methods

The binary constructs pCAMBIA1300-TA29-*hrpZ-nos* and pCAMBIA2301-TA29-*SP-hrpZ-nos* were used for *Agrobacterium*-mediated transformation of tobacco plants. The *SP* gene (90 bp) of tobacco *PR1a* fused in-frame with *hrpZ* gene (~1 kb) from *Pseudomonas syringae* pv. *syringae* was cloned between *Nco1-Sac1* restriction sites and expressed downstream to tapetum-specific TA29 promoter (870 bp). *Agrobacterium tumefaciens* strains LBA4404 and EHA105 harbouring the construct were grown at 28 °C in Luria-Bertani (LB) medium containing rifampicin (50 mg dm^{-3}) and kanamycin (50 mg dm^{-3}) until absorbance (A_{600}) reached to 0.6 - 0.8. The cells were pelleted and resuspended in MS medium to a final A_{600} of 0.2 and was used for co-cultivation of tobacco leaf disc explants.

Leaf discs from 2-month-old healthy tobacco (*Nicotiana tabacum* L. cv. *xanthi*) plants were used for trans-formation using the standard protocol (Horsch *et al*. 1985). Primary transformants were selected on medium supplemented with kanamycin (50 mg dm^{-3}). Regenerated shoots were subsequently rooted and transferred to soil. The transgenic plants were grown in greenhouse at day/night temperature of 30/22 ± 2 °C. Total genomic DNA was isolated from transformed and non-transformed tobacco plants using the cetyl-tri-methyl ammonium bromide (CTAB) method (Rogers and Bendich 1994) and used as template for PCR to check the presence of the transgene.

The expression of the *SP-hrpZ* gene in the tapetal layer, was studied using semiquantitative RT-PCR with cDNA prepared from total RNA isolated from anthers (stage 6) of non-transformed and transformed plants. The RNA was isolated using *TRIzol* (*Sigma*, St. Louis, USA). cDNA was synthesized using *BluePrint* 1st strand cDNA synthesis kit (*TaKaRa*, Tokyo, Japan) following manufacturer's protocol.

Total soluble protein was extracted using the *TRIzol* reagent from twenty anthers (stage 6) from transformed and non-transformed tobacco plants. The protein (70 µg) was separated on 12 % SDS-PAGE and blotted onto a nitrocellulose membrane (*Millipore*, New Bedford, USA). Primary antibody raised against $harpin_{Pss}$ (1: 5000 in Tris-buffered saline and *Tween 20* (TBST buffer) and goat anti-rabbit IgG ALP conjugate (1: 2000 in TBST buffer) was used for immunoblotting as described by Podile *et al.* (2001). The immunoblot was visualized by the alkaline phosphatase-catalysed color reaction using 5-bromo-4-chloro-3-indolyl phosphate - nitroblue tetrazolium (BCIP-NBT) as substrate.

For scanning electron microscopy (SEM), mature pollen grains were gold coated on a sputter coater (*JEOL FC 1100*, Tokyo, Japan) with a gold film thickness of 15 µm for direct observation under the scanning electron microscope *XL 30 ESEM*.

For light microscopy, the anthers (stages 3, 6 and 11; Koltunow *et al*. 1990) were fixed in a solution of 4 % formaldehyde in phosphate buffer saline (PBS; pH 7.4) for 3 h at 30 ± 2 °C and washed thrice (15 min each) in PBS. Further, the anthers were washed twice with methanol (10 min each) and stored at -80 °C. After 12 h, the anthers were dehydrated in a series of increasing concentration of ethanol (70 and 90 % for 3 h, and 100 % for 1 h) at 30 ± 2 °C. The anthers were then treated with xylene till the tissue becomes transparent, infiltrated in paraffin wax for 3 h, and kept at 4 °C overnight. Thin sections (4 - 6 µm) were cut from the polymerized blocks

on a microtome (*Leica*, Wetzlar, Germany) and stained with 1 % toluidine blue O.

For immuno-localization of harpin, the anther (stages 6 and 12) sections (10 - 15 µm) were washed twice (5 min each) in PBS, pH 7.4, and incubated with blocking solution [PBS containing 0.5 % (m/v) BSA] for 1 h at 30 ± 2 °C. Slides were rinsed with PBS for 5 min and incubated with $harpin_{Pss}$ primary antibody for 12 h at 4 °C in a humid chamber, followed by rinsing with PBST [PBS and 0.25 % (m/v) *Tween 20*] for three times (5 min each). Thereafter, slides were incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG antibody for 2 h at 25 °C, rinsed with PBST thrice (5 min each) followed by overlaying with (1:1) PBS and glycerol. The sections were examined on a *Leica TCS SPZ ABOS* confocal microscope under bright field and photographed using *Olympus* (Tokyo, Japan) *DC10* digital camera.

The viability of the pollen grains from transformed and non-transformed plants was assessed by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [1 % MTT in 5 % (m/v) sucrose] and also by *in vitro* pollen germination. Three hundred pollen grains were counted and an average count of ten fields was recorded.

To check the female fertility of male sterile transgenics, the stamens were emasculated and flowers were cross-pollinated with pollen from non-transformed plants and bagged.

Results

Generation of male sterile transgenics and molecular characterization: The non-secretable form of harpin was generated by fusing the TA29-*hrp Z-nos* (TH) construct into the binary vector pCAMBIA 1300, while the secretable form of harpin was generated by fusing the N-terminus of *hrpZ* with C-terminus of *SP* from *PR1a* generating *SP-hrpZ* gene which was cloned downstream to TA29 promoter. The TA29-*SP-hrpZ* (*TSH*) cassette was cloned in pCAMBIA2301 generating the TSH construct. Both the TH and TSH constructs were mobilized into *A. tumefaciens* and used for tobacco leaf disc transformation. All the 10 TH and 27 independent TSH transgenics were screened by PCR (Fig. 1). A 1 kb amplicon corresponding to the size of *hrpZ* and a 1.9 kb amplicon corresponding to TA29-*hrpZ* were generated, confirming the presence of the transgene in the TH transgenics. Amplicons of 2.0 kb and 1.2 kb, corresponding to *TSH* cassette and *SP-hrpZ,* respectively, were amplified in all TSH T_0 transformants, indicating the integration of *TSH* cassette in these transgenics. None of the TH and TSH transgenics were positive for *virG* amplication (data not shown). To confirm *hrpZ* expression in the tapetum, semi-quantitative RT-PCR was done for *SP-hrpZ* from cDNA made from total RNA isolated from anthers (stage 6) of complete and partial male sterile plants. RT-PCR analysis confirmed expression of 1.2 kb of *SP-hrpZ* in the anther tissues of all 27 TSH transgenics with the expression levels being highest in complete male sterile plant TSH24 (no pollen) followed by partial male sterile plants. Immunoblot analysis was done using total protein isolated from anthers (stage 6) from non-transformed and transformed plants and $harpin_{Pss}$ was detected using rabbit polyclonal antibodies raised against $harpin_{Pss}$. $Harpin_{Pss}$ was detectable in the anther tissues of complete and partial male sterile transformants (Fig. 1) but not in the non-transformed plants. The intensity of signal was high in TSH24 and the pattern of $harpin_{Pss}$ accumulation was similar to that of the transcript levels assessed in RT-PCR.

All these 27 transformants were categorized into complete (single transformant-TSH24) and partial (remaining 26 transformants) male sterile plants based on

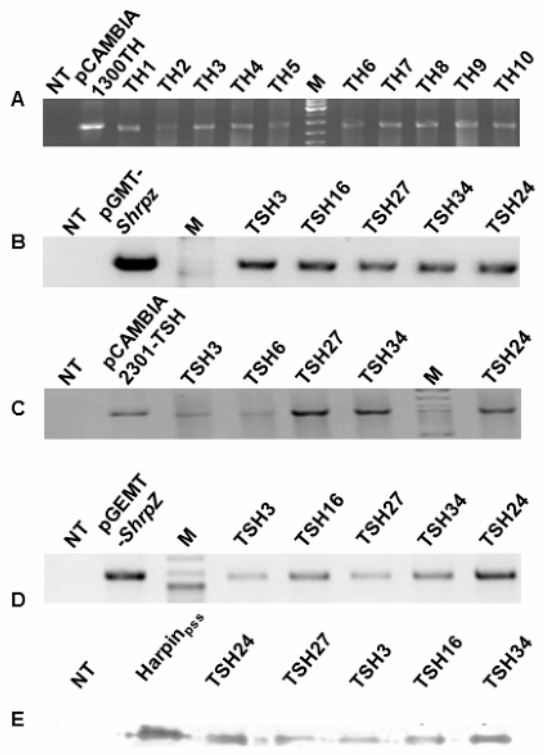

Fig. 1. Integration and expression of TH and TSH cassette in transgenic tobacco plants. Ten TH and five TSH transgenic lines (partially male sterile TSH3, TSH16, TSH27, TSH34 and completely male sterile TSH24) were compared with non-transformed (NT) plant. *A* - Amplified TH from genomic DNA of T_0 plants. *B,C* - PCR amplified *SP-hrpZ* and *TSH* from genomic DNA of T_0 plants. *D* - Semi-quantitative RT-PCR for the expression of *SP-hrpZ* gene from transgenic and NT plants *E* - Total soluble proteins (70 µg) extracted from anthers (stage 6) from transgenic and NT plants were subjected to Western blot analysis with anti-harpin serum. The alkaline phosphatase system was used for visualizing the immunoreactive protein. M represents DNA ladder mix.

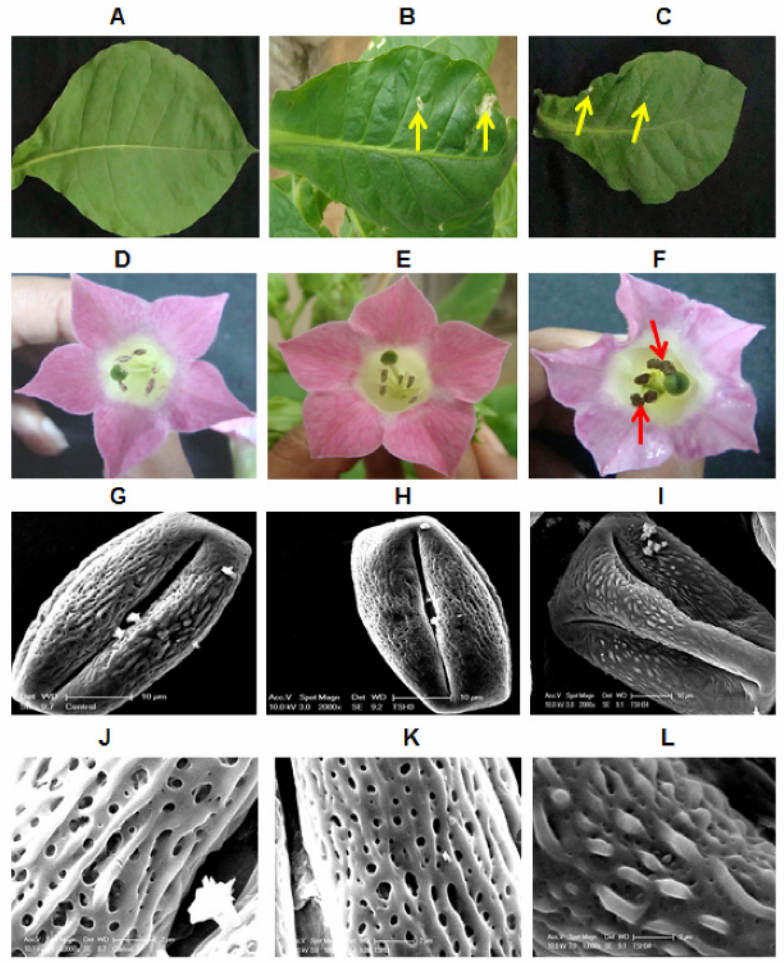

Fig. 2. Comparison of leaf and flower morphology, and pollen grains of non-transformed (NT), partial (TSH3) and complete male sterile (TSH24) transformants: *A,B,C* - leaves from NT, TSH3 and TSH24 plants, HR-like lesions were observed on TSH3 and TSH24 (*yellow arrows*); *D,E,F* - flowers from NT, TSH3 and TSH24 plants, necrotized anthers we observed (*red arrows*). *G* to *L* - ridges and furrows (*arrows*) on pollen grain surface under SEM of NT, TSH3 and TSH24, furrows are faint in TSH3 (*K*) or absent in TSH34 (*L*).

morphological observations. Transformants developed more than twice as many leaves than the non-transformed control plants, but the leaves were only about half the size of the control (Fig. 2). The leaves were wrinkled with thick texture. Uninduced localized HR-like lesions developed on leaves post 30 d transfer to the greenhouse. Complete male sterile plant (TSH24) showed abnormal floral development such as reduced flower length, thick-textured sepals and petals**,** large gynoecium and the anther filaments remained below stigma. Further, the anther locules collapsed and the anthers failed to dehisce. The cross-pollination of flowers from partial and complete male sterile plants with pollens from non-transformed plants resulted in normal capsule development and seed setting indicating that female fertility was unaffected. Flowers of the 27 TSH transgenic lines showed a delay in petal senescence up to 2 d as compared to non-transformed plants, with the effect being strongest in complete male sterile line (TSH 24).

Anomalous microsporogenesis of complete male sterile plant: The MTT assay was done to assess pollen viability**.** The TH transgenics showed varying degrees of fertility ranging from 24 - 51 %. Among the TSH transgenics, the anther development was completely impaired in complete male sterile plant (TSH24) with no pollen grain formation whereas pollens from partial male sterile plants showed varying levels of viability (30 - 80 %). The degree of male sterility in transgenics expressing harpin intracellularly or in secretable form, did not show major variation, except that one event with the secretable form was completely male sterile (TSH24). The spatial and temporal availability of harpin in the transgenic line was critical to induce male sterility rather than the intracellular or extracellular release of harpin.

Localization studies of harpin$_{Pss}$ in the anthers at stage 6 showed a strong signal in aborted tapetal cells of complete male sterile plant (TSH24) and the intensity of the signal remained the same till the last stage of anther

development (photomicrographs not shown). In case of partial male sterile plants, the signal was also observed in pollen grains. No signal was detected in non-transformants. In the complete male sterile plant (TSH24), the tapetal layer aborted before stage 3 and hence, no pollen grains formed. At stage 6, remnant of tapetum was present whereas by stage 11, connective tissue was completely degraded and the anther became bilocular without dehiscence (Fig. 3). Partial male sterile plants showed anther development mostly similar to non-transformed plants.

SEM studies of pollens from partial male sterile plants, revealed significant aberration of pollen grain and alteration in the exine pattern (Fig. 2) in transformants. Some of the pollen grains even collapsed. The pollen exine showed a reduced number of homogenously sized holes and furrows, and the protuberances were flattened (Fig. 2*G-L*) in contrast to the regular exine pattern of pollen in general.

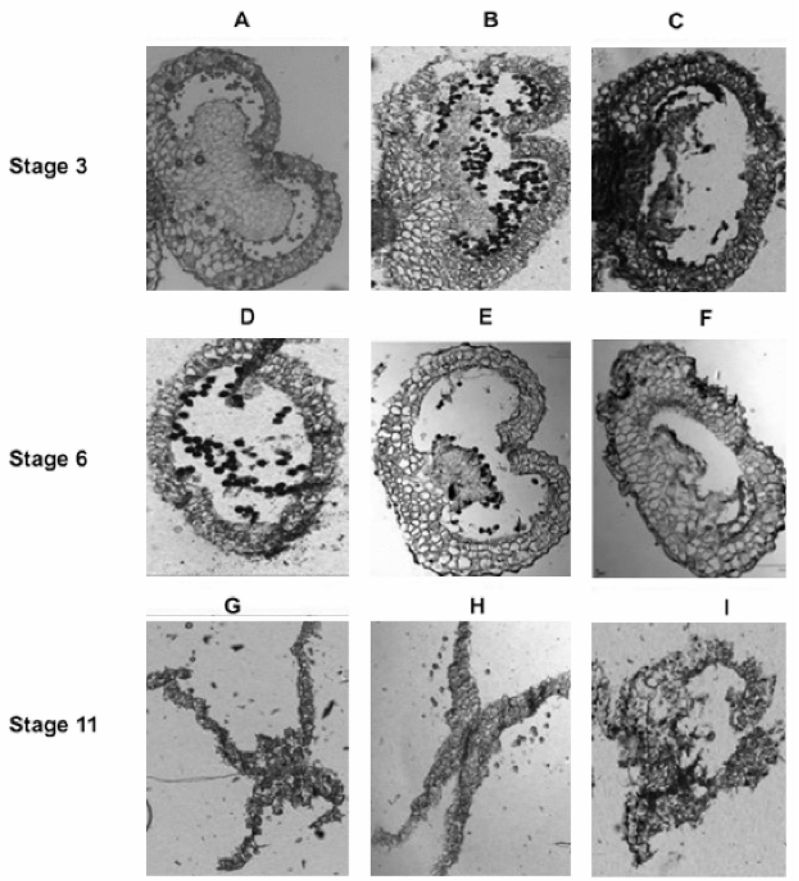

Fig. 3. Comparative study of anther development. Semi-thin sections from anther lobes of non-transformed (NT) (*A,D,G*), partial male sterile TSH3 (*B,E,H*) and completely male sterile TSH24 (*C,F,I*) were observed at three stages - stage 3 shrinking of tapetum starts, stage 6 remnants of tapetum present, stage 11 anther dehiscence along the stomium occurs. Pollen development proceeds normally in NT and partial male sterile plants. In TSH24, the early degeneration of tapetum and no pollen formation before stage 3 and connective tissue degradation started at stage 6. No signs of anther dehiscence at stage 11.

Discussion

Male sterile lines are of particular significance in agriculture because of their usefulness in hybrid seed production. Among the various strategies used for development of male sterile plants, the use of cytotoxic genes has been promising. There are several reports of generation of successful male sterile plants using TA29 promoter for the tapetum-specific expression of the cytotoxic transgenes (Koltunow *et al.* 1990, Mariani *et al.* 1990, Kriete *et al.* 1996, Cho *et al.* 2001). The harpin gene (*hrpZ*) which is an elicitor molecule of HR-mediated cell death in plants was used for developing male sterility in this report. Our previous attempt to generate male sterile transgenics by expressing harpin intracellularly downstream to TA29 promoter resulted in generation of 10 independent partial lines. The male fertility of these transgenics varied between 24 - 51 %

and none of them were completely male sterile. One of the possible reasons could be the putative harpin receptor which is hypothesized to be extracellular. Therefore, intracellular accumulation of harpin is non-toxic to the plant as evident with previous report of 35S-mediated constitutive expression of harpin intracellularly showing no morphological changes in leaves, stems and flower and normal seed fertility in absence of pathogen (Takakura *et al.* 2004, Tampakaki and Panopoulos 2000). However, the plants were resistant and HR-like local lesions were generated after pathogen attack. However, when the harpin gene was over-expressed in secretable form, HR-induced necrotic lesions were observed in tobacco plant leaves (Tampakaki and Panopoulos 2000). These reports confirm our hypothesis that the putative harpin receptor is in the outer lamina of plasma membrane or in the apoplast. We, therefore, generated the secretable form of harpin by fusing signal peptide of tobacco *PR1a* with *hrpZ* and expressed the transgene under TA29 promoter in the tapetal layer.

A total of 27 independent TSH transgenic lines were generated which were confirmed through PCR and Western blot. Based on the phenotype, these were divided into complete male sterile (single transformant-TSH24) and partial male sterile lines (remaining 26 transformants). Tapetum-specific expression of $harpin_{Pss}$ resulted in complete and partial sterility, which was proportional to $harpin_{Pss}$ accumulation in these transgenics (Fig. 1). The transformants with high transgene expression showed abnormalities in leaf size, number of leaves and HR-like localized lesions in leaf whereas transformants with low transgene expression were almost normal. Differences in floral size, length of filament and delay in petal senescence were also observed. Light microscopic studies revealed that the anther development and pollen grains of partial male sterile lines were similar to that of non-transformants (Fig. 2) but the reduced viability of pollen grains in such transformants could be due to defects in development of tapetum caused by $harpin_{Pss}$ expression (Fig. 3). In case of complete male sterile line, the anthers remained undehisced due to lack of pollen formation. Harpin was localized around the anther wall layers in complete male sterile plant where as no signal was detected in the non-transformants.

Vigorous growth and development of transgenic tobacco plants expressing $harpin_{Pss}$ was reported (Jang *et al*. 2006). However, there was no growth promotion observed in male sterile transgenics generated in this study (except increase in number of leaves that are smaller). HR-like local lesions were observed interfering in normal development and resulting in morphological abnormalities. These abnormalities could be either due to leaky expression of transgene from neighboring 35S promoter which is known to act bidirectionally (Jagannath *et al*. 2001) or due to gene disruptions upon insertion of transgenes, although unlikely in polyploidy tobacco.

The transgenic plants also showed a delay in anther dehiscence and petal senescence. Pollination reduces floral attraction and accelerates the senescence or abscission of floral organs (Jones 2008). Since there was no scope for self-pollination in complete male sterile line, the petal senescence was considerably delayed. Paxson-Sowders (2001) and Barrena *et al.* (2006) demonstrated the link between male sterility and pollen wall development particularly about exine pattern in *Arabidopsis* mutants. In this study, the transformants with highest reduction in pollen viability had most defective exine pattern formation and *vice-versa* supporting the link between the exine pattern and male sterility in plants. Callose synthesis during pollen wall formation has a vital function in building a properly sculpted exine, the integrity of which is essential for pollen viability. $Harpin_{Pss}$ expression might have interfered with the callose synthesis leading to alterations in pollen cell wall and reduction in pollen viability.

The complete male sterile line generated using $harpin_{Pss}$ showed indehiscent anthers contrary to the dehiscent anthers of male sterile lines generated using other cytotoxic genes like *barnase* and *RIP* (Mariani *et al*. 1990, Cho *et al*. 2001). The cytotoxic $harpin_{Pss}$ was expressed in a secretable form that realeased $harpin_{Pss}$ into the apoplast, while the *barnase* and *RIP* were expressed intracellularly. Beals and Goldberg (1997) showed that the anther dehiscence requires functional stomial cells. The apoplastic movement of harpin might contribute to impaired functioning of the stomial cells resulting in indehiscent anthers.

Our observations provide a strong basis to consider the use of $harpin_{Pss}$ to develop male sterile plants. Further work is required to generate high frequency of complete male sterile lines by using more copies of *hrpZ* in secretable form and also develop a functional fertility restorer system making use of genes encoding proteins that negate $harpin_{Pss}$ action.

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