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Biochemical and molecular dissection of thermo-sensitive genetic male sterility in diploid cotton (*Gossypium arboreum* L.)

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Abstract

Diploid cotton, due to its inherent problem of stamen brittleness, its found unsuitable for traditional method of hybrid seed production which involves hand emasculation followed by pollination. Due to shortfall in other methods *viz.,* Genetic Male Sterility (GMS), as well as, Cytoplasmic Genetic Male Sterility (CGMS), hybrid seed production in diploid cotton becomes costly and thereby, covers less area among the total cotton grown area. Thermo-sensitive genetic male sterility, which overcomes the drawbacks of both GMS and CGMS can be an effective tool in coming years for hybrid cotton research. Understanding fertility and sterility variations, their relation with biochemical changes in plant is important before its application in plant breeding. Hence, the available TGMS line, Ga TGMS-3 obtained at Cotton Research Centre, UAS, Dharwad was studied for callase activity and markers associated with TGMS. The line Ga TGMS-3 had fertile anthers and showed less callase enzyme activity at pre-meiosis stage, high enzyme activity at tetrad releasing microspore stage and no callase activity during other stages. The counterpart TGMS sterile anthers displayed little higher callase activity at pre-meiosis stage, high activity at tetrad stage, but poor activity at tetrad releasing microspore stage. During tetrad stage, TGMS sterile anthers showed high callase enzyme activity giving every chance for early release of poorly developed microspores as compared to fertile anthers. At tetrad releasing microspores stage during which fertile anthers had strong callase enzyme activity led to microspores being released normally and developed normal pollen grains as compared to sterile anthers. The present investigation revealed that NAU2176, NAU2096 and BNL1227 primers can be used as tightly linked markers for TGMS trait, as evident from their differential expression in fertile and sterile anthers.

Key words

EST-SSR markers, Diploid cotton, Genetic male sterility, q-PCR, Thermo-sensitive

Introduction

Male sterility is an effective means of producing hybrid cotton since it does not require labour intensive hand emasculation and pollination. In cotton, two types of male sterility systems viz., Cytoplasmic Genetic Male Sterility (CGMS) and Genetic Male Sterility (GMS) systems can be used for hybrid seed production. Though GMS is preferred than CGMS as it overcomes the drawbacks of cytoplasmic effect and problem of fertility restoration, but is less stable and requires more area as 50% of the population is fertile (Singh *et al.,* 2002). Thermo-sensitive genetic male sterility (TGMS) has been reported in diploid cotton by Khadi *et al.* (2003) at ARS Dharwad, which can overcome drawbacks of the above two systems. Among cultivated species, diploid cottons have wide adaptability and are relatively tolerant to biotic and abiotic stresses. Diploid cotton hybrids are high yielding with short to medium fibre (22.0 to 25.0 mm) and GOT around 35-40%. In-spite of these qualities, diploid hybrids covers an insignificant area due to non availability of

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suitable cost effective system for large scale production of quality F_1 seed. The two-line hybrid proved advantageous over three line hybrids and has been commercially exploited in case of rice (Pham *et al.,* 2004; Reddy *et al*., 2000). For successful utilization of this novel male sterility system, knowledge of breeding and fertility behaviour of TGMS line is essential. Inheritance of thermo sensitive genetic male sterility (TGMS) has been widely studied in China, Japan, IRRI and India in case of rice (Lee *et al.,* 2005). Genetic or biochemical information regarding TGMS in diploid cotton is meagre. Use of molecular marker(s) closely linked to TGMS trait could be a reliable approach for easy identification of trait and helps in accelerating further breeding procedures. For successful exploitation of this novel male sterility system in heterosis breeding, more TGMS lines need to be developed and characterized for their sterile and fertile phases, understand the critical stages and temperature for fertility alteration. It is therefore imperative to have an idea about the mechanism of male sterility in TGMS lines of diploid cotton. The practical potentiality of TGMS in hybrid seed production depends mainly on stability of sterility during seed production period. In spite of its practical importance in plant breeding, the causal mechanism of such male sterility system in diploid cotton has not been clearly understood, though several workers have reported the histological basis of male sterility in cotton (Borakakati and Virmani, 1996 and Reddy *et al*., 2000).

Pollen development involves number of events, commencing at the end of meiosis with the formation of tetrads of haploid microspores (Scott *et al.,* 2004). Individual microspores in each tetrad are surrounded by thick callose wall composed of β-1, 3-glucanase between primary cell wall and plasma membrane (Eschrich and Currier, 1964). At the end of meiosis, tetrads of haploid microspores are liberated into the locule (Steiglitz and Stern ,1973). The anther specific expression of β-1, 3-glucanase mRNA and protein is detectable during meiosis in many plant species, and these enzymes are thought to participate in tetrad dissolution during pollen development (Hird *et al.,* 1993; Bucciaglia and Smith, 1994). Abnormal dissolution of callose surrounding the tetrads causes male sterility in cytoplasmic male-sterile lines of petunia (Shamay and Rafael, 1971), and Sorghum (Warmke and Overman, 1972). An idea about the mechanism of male sterility in TGMS lines of diploid cotton, therefore, becomes essential to know whether similar mechanism applies for cotton or not. Present study aims at revealing the role of callase enzyme activity in TGMS lines to induce male sterility. At cellular level, mechanism of sterility induction is assumed to

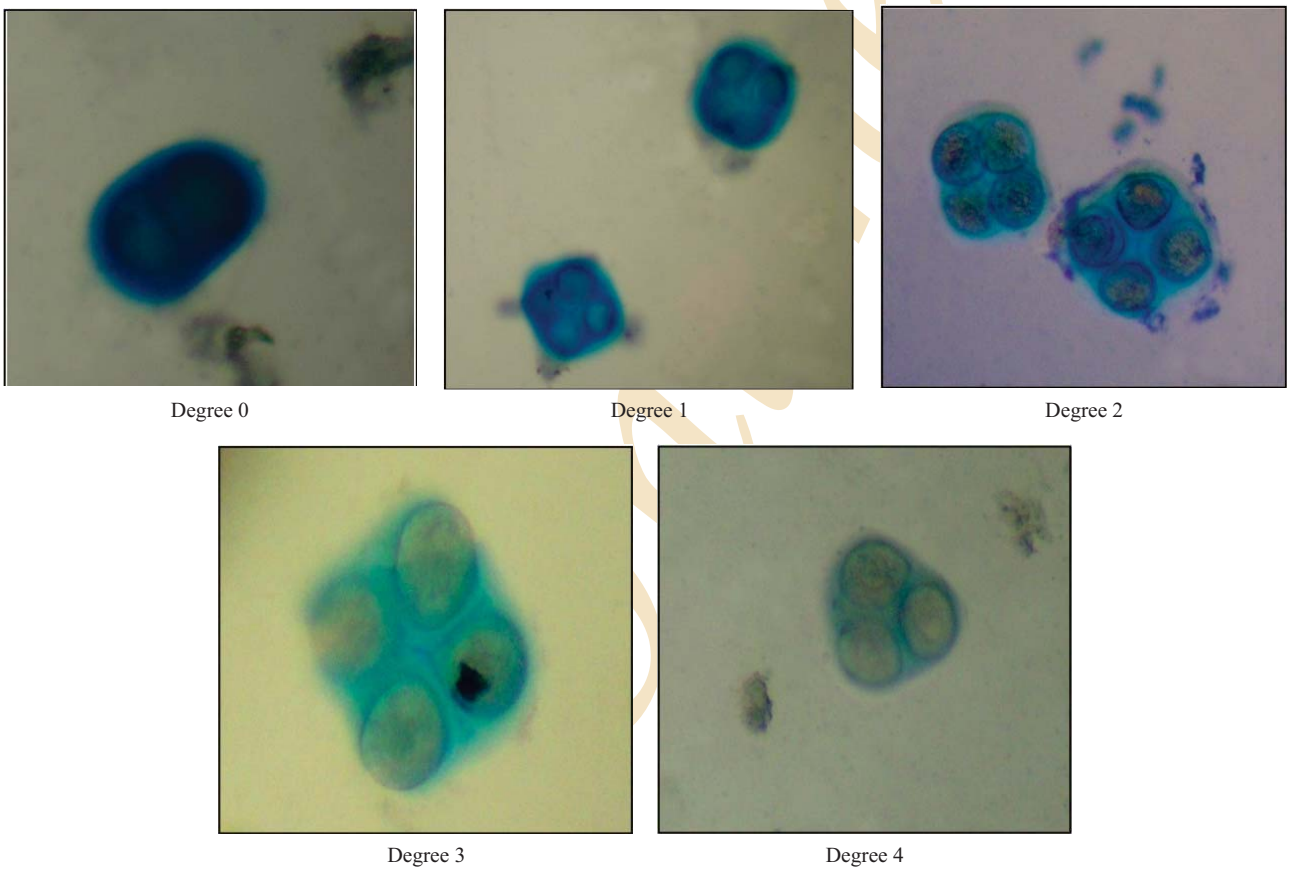

Fig. 1 : Callase activity at different developmental stages of anthers in Ga TGMS-3 line of diploid cotton

be common among the different male sterility systems. In this context, the present study was undertaken to biochemically characterize new TGMS line Ga TGMS-3 in diploid cotton. In addition, an attempt was made to identify EST-SSR markers associated with TGMS in Ga TGMS-3 as well.

Materials and Methods

Callase (Glucanase) activity in anthers : Anthers from male sterile and fertile flowers of Ga TGMS-3 line were powdered in liquid nitrogen, crushed in cold acetate NaCl buffer (0.08M acetate+0.02 M NaCl, pH 4.8) and solution was used for β-1, 3-glucanase extract by centrifuging at 10,000 rpm at 4°C for 30 min. Supernatant obtained was used as enzyme source. Substrate consisting of young tetrads from fertile anthers (Pronounced callose in their walls and bindings) was put in a well slide, and the enzyme preparation was added and the reaction mixture was incubated for about 18 hr at 38 ºC. After incubation, lacmoid (0.1% resorc in blue in absolute ethanol) was added to stop the reaction and to stain the undigested callose. The colour intensity of tetrad walls and bindings (indicating the extent of callose digestion) was determined under a microscope (Frankel *et al.,* 1969). This determination was based on a five degree scale ranging from dark blue (degree 0) to colorless (degree 4) as seen in Fig. 3.

Identification of molecular markers (EST-SSR) linked to male sterility : It was observed that during high temperature (> 18°C) anthers become sterile in diploid cotton (Khadi *et al.,* 2003). At this stage anthers were collected from genotype, Ga TGMS-3, in liquid nitrogen and kept in -80 ºC for further preservation. Fertile anthers of same plant was obtained for its fertile stage during low temperature (<18°C). Total RNA was extracted from both sterile and fertile anthers using spectrum total RNA isolation Kit (Sigma Aldrich) according to manual instructions. RNA extracted from anthers at sterile and fertile stages of the same plant was used for qRT-PCR. The cDNA was synthesized using DyNAmo™ cDNA Synthesis Kit according to the manual instructions. The qRT-PCR reactions were carried out using Corbett Q-PCR, according to manual instructions. PCR reactions (final volume of 10 ul) were performed using gene-specific primers (Table 1) obtained from cotton marker database (CMD) originally tagged for GMS trait and synthesized by Sigma Aldrich. Among these primers, NAU 3561 and NAU 2096 were closely flanking with ms_5 gene of chromosome 12 and NAU 2176 in chromosome 12, as well as, BNL 1227 in chromosome 26 linked with ms_{15} in tetraploid cotton (Chen *et al.,* 2009). The passive reference dye cyber green was used to normalize fluorescence across the plate. Reaction conditions were as follows: 50 ºC for 2 min, 94 ºC for 10 min, followed by 40 cycles of 94 ºC for 15 sec and 60 ºC for 1 min. Values were normalized to the expression of reference histone, differences in expression were identified using the Student's t-test.

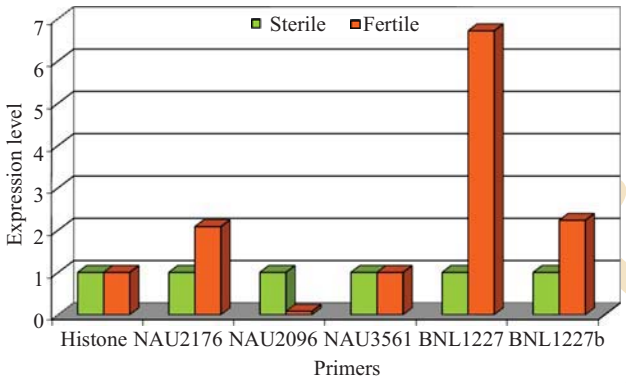

Fig. 2 : Expression EST based SSR marker linked to TGMS in Ga TGMS-3 line of diploid cotton

Results and Discussion

The results of callase activity at different developmental stages in fertile and sterile anthers of TGMS lines are presented in Table 2. A semi-quantitative assay, which is comparative assay was developed and used to measure the enzyme activity by lacmoid staining (Fig 1). The results indicated that callase enzyme activity at pre-meiotic

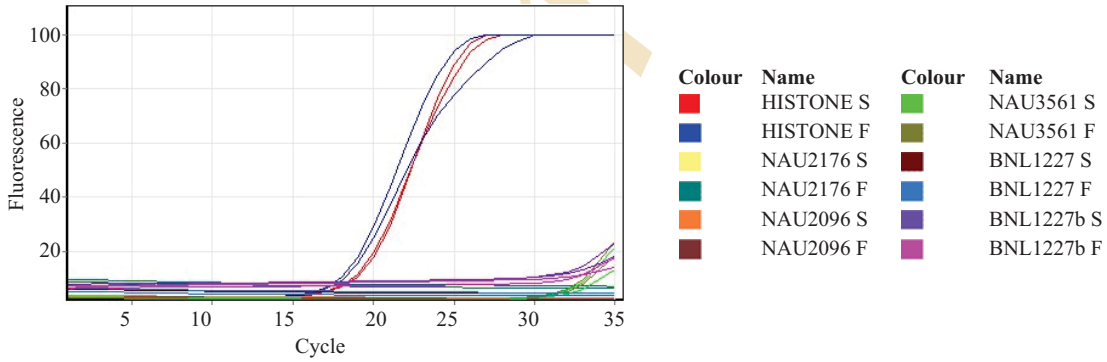

Fig. 3 : Amplification/Expression level of EST SSR primers along with histone in Ga TGMS-3 line in diploid cotton

Table 1 : List of EST-SSR marker used to study the expression of the TGMS trait in diploid cotton.

Primer name	F/R	Primer sequence
Histone	F	TCAAGACTGATTTGCGTTTCCA
	R	GCGCAAAGGTTGGTGTCTTC
NAU2176	F	TGGCACTTGCTAAACCATAA
	R	GGTTTGAGCAGAAGGGTATG
NAU2096	F	GAGAGATAAACGGAGGTGGA
	R	TTAGTCAAACCGAGCAGCTT
NAU3561	F	GAGCAGAATTGAAGGGAGAA
	R	CTACCCACAGGTTGAGCTTT
BNL1227	F	CATCAAGATCTATCTCTCTCTATACCG
	R	TTTACCCTCCGATCTCAACG
NAU127b	F	ATCATGGAACCTGGTTGTTC
	R	ATGAATTGCGGAGTCTAAGG

F- Forward R- Reverse

stage in fertile anther was low (degree 1) as compared to sterile anther (degree 2).

At tetrad stage, fertile anther had no callase activity (degree 0) indicating profound callose wall which inhibited callase activity. In sterile anthers of TGMS line, high callase enzyme activity (degree 3) was observed. During tetrad releasing microspore stage, the fertile anther showed higher callase enzyme activity (degree 4), whereas in sterile anthers callase enzyme activity was poor (degree 1), which indicated that the release of microspores was affected in sterile anthers as compared to fertile anthers. At old microspore stage, the fertile anthers, as well as, sterile anthers showed no callase activity (degree 0). At pollen grain stage, irrespective of sterile and fertile stages the callase enzyme activity was zero (degree 0).

Overall, seven TGMS lines showed moderate callase enzyme activity during pre-meiosis and tetrad stage (data not shown). At tetrad releasing microspore stage, enzyme activity was minimum. In fertile anthers, the enzyme activity was minimum at tetrad stage and maximum at tetrad releasing microspore stage indicating proper release of microspore. The results indicated that callase is active in both male fertile and sterile anthers of TGMS lines, but the timing of its activity was different. Relatively poor activity was indicated at pre-meiotic stages in both the male sterile and fertile anthers of TGMS lines. At tetrad stage higher callase activity was seen in sterile anthers of TGMS lines, whereas fertile anthers had no callase activity. During tetrad releasing microspore stage, the fertile anthers had strong callase activity (Degree 4) as compared to sterile anthers. At old microspore and pollen grain stage, irrespective of sterile and fertile anthers, all lines showed zero callase activity because at this stage there was no role of callose walls and hence, callase too.

Timing of callase activity plays an important role in the formation and degradation of cell walls during microsporogenesis. High callase activity is required for normal release of microspores from tetrads at tetrad releasing microspore stage. In fertile anthers, the enzyme activity was strong as compared to sterile lines. Hence, release of microspores was not affected in fertile anthers. The delayed release of microspores and their further growth is abnormal in TGMS lines resulting in male sterility (Govindraju *et al*., 2004). The shrunken microspores were eventually released at late microspore stage, but with loss of fertility. The results demonstrated that the timing of callose degradation is important and delay in release of microspores affects the subsequent development of pollen, resulting in male sterility (Frankel *et al.,* 1969 and Izar and Rafael, 1971). During tetrad stage, TGMS sterile anther had high callase enzyme activity, hence, there was every chance of early release of microspores as compared to fertile anthers with poor development of microspores. At tetrad releasing microspores stage, fertile anthers had strong callase enzyme activity, hence, microspores were released normally and developed into normal pollen grains as compared to sterile anthers (Frankel *et al.,* 1969).

Molecular insights of TGMS : The TGMS trait is due to post transcriptional changes, because the same plant behaves as sterile at high (>18 ºC) mean minimum temperature and as fertile at low temperature (<18 ºC). The genome being the same but differences arose during the post transcriptional level (Wang *et al.,* 1995). So, it was considered better to study gene expression using markers at cDNA level rather than at genomic level which can give clear-cut meaning for this situation. The mRNA isolated from two stages on the same TGMS line (Ga TGMS-3) was converted into cDNA. EST-Expressed Sequence Tags SSR- Simple Sequence Repeat markers (primer) linked to male sterility were used to understand the expression levels.

Primer NAU2176 was highly expressed (2.08 folds) during fertile stage, whereas primer NAU2096 was expressed poorly (0.079 folds). Primer NAU3561 did not

Table 2 : Callase enzyme activity in the sterile and fertile anthers of Ga TGMS-3 line of diploid cotton.

Anther type	Stages				
	Pre-meiosis	Tetrad stage	Tetrad releasing Microspore	Old microspores	Pollen grains
Fertile anthers	Degree1	Degree 0	Degree 4	Degree 0	Degree 0
Sterile anthers	Degree 2	Degree 3	Degree 1	Degree 0	Degree 0

Table 3 : Expression EST based SSR marker linked to TGMS in Ga TGMS-3 line of diploid cotton

Primer	cDNA from sterile anthers	cDNA from fertile anthers	Expression
Histone	1.00	1.0	No / Constitute
NAU2176	1.00	2.08	High
NAU2096	1.00	0.079	Low
NAU3561	1.00	1.00	No
BNL1227	1.00	6.71	High
BNL1227b	1.00	2.23	High

show any expression difference between the two different stages. BNL1227 primer had 6.71 folds more expression during fertile stage as compared to sterile stage indicated its stage specific expression. Primer BNL1227b also had 2.23 folds more expression during fertile stage when compared to its counterpart sterile stage. Hence, three out of five primers used showed differential expression (Fig.2, Table 3) during sterile and fertile stages of the same TGMS line. This was due to their linkage with ms_{15} and ms_5 GMS genes on chromosome 12 (linkage group V) in cotton (Chen *et al.,* 2009). BNL1227 primer showed 6.71 folds more expression in fertile cDNA over sterile cDNA as standard control. BNL1227b in fertile cDNA also had 2.23 folds more expression as compared to sterile cDNA. These two primers were highly linked to ms_6 GMS gene of cotton on chromosome 26 (Linkage group IV) (Chen *et al.*, 2009 and Xiaoding *et al.,* 2007). The histone primer used as control did not change their expression irrespective of fertile and sterile anthers, indicating their constitutive expression across the situations, as it is a housekeeping gene (Fig.3).

Stage and time of activity of enzyme callase or glucanase plays major role in TGMS in cotton. High callase activity in tetrad stage makes plant sterile where as high glucanase activity at tetrad releasing stage makes plant fertile which was majorly influenced by prevailing temperature. Expression of TGMS is limited to reproductive stage of the plant and breeder needs to wait for the genetic manipulation till plant flowers. Detection of linked maker to the TGMS traits makes the way ease by facilitating the identification of TGMS line at even vegetative stage also. This makes breeding cycle short and economical. In present study, it was found that NAU2176, NAU2096 and BNL 1227 markers were linked with fertile stage of plant, where as NAU 2096 marker with sterility in diploid cotton. These markers can be utilized for detection of TGMS at an early stage of plant growth and in crop improvement program of diploid cotton in an effective manner.

References

Borkakati, R. P. and S. S. Virmani: Inheritance of a thermosensitive genic male-sterile mutant of Indica rice. *Rice Genet. Newslett.,* **10,** 45-49 (1996)

Bucciaglia, P.A. and A.G. Smith: Cloning and characterization of Tag 1, a tobacco anther beta-1, 3-glucanase expressed during tetrad dissolution. *Plant Mol Biol.,* **24,**903–914 (1994)

Chen, D., Y. Ding, W. Guo and T. Zhang: Molecular mapping of genic male sterile genes ms 15, ms 5 and ms 6 in tetraploid cotton. *Plant Breed.,* **128,** 193-198. (2009)

Eschrich, W. and H.B. Currier: Aniline Blue Fluorochrome, *Stain Technology.,* **39**, 303(1964).

Frankel, S.I. and J. Nitsan: Timing of callase activity and Cytoplasmic male sterility in petunia. *Biochem. Genet.,* **3**, 451-455 (1969)

Govinda, Raju, N.S., B.M. Khadi, A.S. Nalini and I.S. Katageri: Histological studies in thermo sensitive genetic male sterile genotypes of cotton. Int. Symp. on Strategies for Sustainable Cotton Production - A Global Vision Crop Improvement, 23-25 November, 2004, Univ. Agric. Sci., Dharwad, Karnataka (India) (2004)

Hird, D.L., D. Worrall, R. Hodge, S. Smartt, W. Paul and R. Scott: The anther-specific protein encoded by the Brassica napus and Arabidopsis thaliana A6 gene displays similarity to beta-1,3-glucanases. *Plant J.,* **4**,1023-1033 (1993)

Izar, S. and F. Rafael: Mechanism of male sterility in petunia: the relationship between pH, callase activity in the anthers, and the breakdown of the microsporogenesis. *Theor. Appl. Genet.,* **41**,104–108 (1971)

Khadi, B.M., A.M. Badigannavar, V.N. Kulkarni and I.S. Katageri: Development of TGMS system in diploid cotton *(Gossypium arboreum* L.) *Indian J. Genet.,* **63**, 269-270 (2003)

Lee, D.S., L.J. Chen and S. Suh: Genetic characterization and fine mapping of a novel thermo-sensitive genic male – sterile gene tms6 in rice (*Oryza sativa* L.). *Theor. Appl. Genet.,* **111**, 1271-1277(2005)

Pham Cuong, V an, S. Murayama, Y.M. Ishimine, Y. Kawamistu, K. Motomura and E. Tsuzuki: Sterility of thermo-sensitive genic male sterile line, heterosis for grain yield and related characters in F1 Hybrid rice (*Oryza sativa*). *Plant Prod. Sci.,***7**, 22-29. 2004

Reddy, O.U.K., E.A. Siddiq, N.P. Sarma, J. Ali, A.J. Hussain, P. Nimmakayala, P. Ramasamy, S. Pammi and A.S. Reddy: Genetic analysis of temperature-sensitive male sterilty in rice. *Theor. Appl. Genet.,* **100**, 794–801(2000)

Scott, R.J., M. Spielman and H.G. Dickinson: Stamen structure and function. *Plant Cell.,***16** (Suppl), S46-S60 (2004)

Shamay, I. and and F. Rafael: Mechanism of male sterility in Petunia: The relationship between pH, callase activity in the anthers, and the breakdown of the microsporogenesis. *Theor Appl Genet.,* **41**, 104 (1971)

Singh, S.B., P. Singh and C.D. Mayee: Male sterility in cotton, CICR Tech. Bltn. No. 24, Central Institute for Cotton Research, Nagpur, India (2002)

Steiglitz, H. and Stern H: Regulation of β-1,3 glucanase activity in developing anthers of Lilium. *Dev Biol.,* **34**, 169-173 (1973)

Wang, B., J.Z. Wang, W. Wu, H.G. Zheng, Z.Y. Yang, W.E. Xu, J.P. Ray and H.T. Nguyen: Tagging and mapping the thermosensitive genic male sterile gene in rice (*Oryza sativa* L.) with molecular markers. *Theor. Appl. Genet.,* **91,** 1111-1114 (1995)

Warmke, H.E. and M.A. Overman: Cytoplasmic male sterility in sorghum. 1. Callose behavior in fertile and sterile anthers. *J. Hered*., **63**, 103–108 (1972)

Xiaoding, Ma, Chaozhu Xing, Liping Guo, Yangcang Gong, Hailin Wang, Yunlei Zhao and Jianyong Wu:, Analysis of differentially expressed genes in genic male sterility cotton (*Gossypium hirsutum* L.) using cDNA-AFLP. *J. Genet. Genom.,* **34**, 536-543. (2007)

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