The role of *Somatic embryogenesis receptor-like kinase* 1 in controlling pollen production of the *Gossypium* anther

Ya-li Shi · San-dui Guo · Rui Zhang · Zhi-gang Meng · Mao-zhi Ren

Abstract In flowering plants, male gametophytes are generated in anthers from microsporocytes. However, more evidence is needed to reveal the genetic mechanisms which regulate the differentiation and interaction of these highly specialized cells in anthers. Here we report the characterization of a series of male-sterile cotton (*Gossypium hirsutum*) mutants, including mutants with normal fertility, semi-sterility and complete sterility. These mutants are forms of transgenic cotton containing RNAi vectors with partial cDNA fragments of *GhSERK1*. The *GhSERK1* gene encodes a putative leucine-rich repeat receptor protein kinase (LRR-RLK), and generally has 11 domains. In previous research, we found plants containing *GhSERK1* produce an abundance of male reproductive tissue. In this paper, three RNAi constructs were designed separately to analyze its function in anther. After the three RNAi vectors were transformed into the cotton, transgenic plants with the specialized fragment exhibited normal fertility or the pollen energy decreased slightly, as ones with the homologous fragments exhibited various degrees of male sterility with different expression levels of *GhSERK1* mRNA. In conclusion, for the transgenic plants with conserved fragments, lower expression levels of *GhSERK1* mRNA were in transgenic plants, and a higher degree of male sterility was observed. Taking together, these findings demonstrate the *GhSERK1* gene has a role in the development of anthers, especially in the formation of pollen grains. Also, we infer there must be another homolog of *GhSERK1* in cotton, and both of *GhSERK1* and its homolog function redundantly as important control points in controlling anther pollen production.

Keywords Anther · *Somatic embryogenesis receptor-like kinase* 1 · *Gossypium hirsutum* · Homolog · RNAi

Abbreviations

LRR-RLK	Leucine-rich repeat receptor protein kinase
SERK	Somatic embryogenesis receptor-like kinases
EMS1	Excess microsporocytes1
EXS	Extra sporogenous cells
TPD1	Tapetum determinant1
BAM1/2	Barely any meristem1/2
RPK2	Receptor-like protein kinase1
SPP	Ser-Pro-Pro motif
TM domain	Transmembrane domain
Nt	Nucleotide

Ya-li Shi and San-dui Guo contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s11033-013-2875-x) contains supplementary material, which is available to authorized users.

Y. Shi · S. Guo (✉) · R. Zhang · Z. Meng · M. Ren
Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China
e-mail: gsdui@mail.caas.net.cn

Y. Shi
e-mail: shiyali2006@126.com

Introduction

In the life cycles of flowering plants, the haploid gametophyte and diploid sporophyte generation alternate. Male gametophytes develop in anthers. The anther consists of highly specialized reproductive and nonreproductive tissues [1]. The reproductive tissues with microsporocytes develop into pollen grains though microsporogenesis. The nonreproductive tissues in anthers are necessary for the development and release of normal pollen, including the

epidermis, endothecium, middle layer, and tapetum. The existence and disintegration of the tapetum layer is necessary for nutrients to be supplied to the microsporocytes and for the release of pollen [2, 3]. Therefore, the interaction between the microsporocytes and surrounding somatic cells in anthers is important to the formation of microspores and eventually pollen grains.

The developmental process of anthers consists of two sequential phases [1]. During the first phase, the differentiation of specialized cells is completed, and anther morphology is established. In detail, archesporial cells give rise to the microsporocytes, tapetum, middle layer and endothecium. Microsporocytes then undergo meiosis. In the second phase, microspores develop into pollen grains, and then the anther dehisces to release the pollen grains. To date, only a few genes have been identified which have roles in the regulation of anther cell division and the differentiation events. For example, the genes EXCESS MICROSPOROCYTES1 (EMS1)/EXTRA SPOROGENOUS CELLS (EXS) [4, 5] and SOMATIC EMBRYOGENESIS RECEPTORLIKE KINASES1/2 (SERK1/2) [6–8] play an important role in the differentiation of tapetal cells. The TAPETUM DETERMINANT1 (TPD1) gene product signals the differentiation of the tapetum [9]. BARELY ANY MERISTEM1/2 (BAM1/2) is required for the differentiation of anther somatic cell layers, including the endothecium, middle layer, and tapetum [10, 11]. Also, RECEPTORLIKE PROTEIN KINASE2 (RPK2) defines middle layer cells [12].

Somatic embryogenesis receptor-like kinases (SERKs) are a large family of receptor-like kinases with an extracellular domain, a transmembrane domain and a putative serine/threonine kinase domain. Until now, several SERK genes have been identified from many monocotyledonous and dicotyledonous plants. The predicted protein structure of the SERK proteins starts at the N terminus with a single peptide followed by a Leu zipper domain, five Leu-rich repeats (LRRs), a Pro-rich domain called the Ser-Pro-Pro motif, a single transmembrane domain, a Ser/Thr kinase and a C-terminal Leu-rich domain [7]. The kinase domain has the highest identity among the 11 domains of SERK. For example, for AtSERK1, AtSERK2, AtSERK3, AtSERK4, and AtSERK5, identity within the kinase domain is 85 to 95 % for all five sequences and all contain the core sequences characteristic of Ser/Thr kinases. Identity is about 66 % in the LRR region and 54 % in transmembrane domain. The greatest divergence, <45 %, is seen in the SPP and C-terminal domains [13]. A similar phenomenon is seen among the three SERKs in maize [15]. So there is homology in the kinase domains among SERKs, and specificity in the SPP and TM domains.

Until now, a number of reports have suggested a variety of roles for SERKs, such as in somatic embryogenesis [13–17], sporogenesis [7, 8], hormone response [18–20] and host defense response [21–23]. For example, Jan Salaj et al.

[14] found four sites of AtSERK1 expression in the embryogenic cultures: embryogenic callus, the basal parts of primary somatic embryos, the outer layers of cotyledons of primary somatic embryos and provascular and vascular strands of developing somatic embryos. The in vitro expression of AtSERK1::GUS coincides with embryogenic development, and it was expressed in single cells and small cell clusters. The results indicated that AtSERK1 maybe marks embryogenic competence. In the report by Santos et al. [23], using antisense RNA, transgenic lettuce with silenced SERK exhibit absence of detectable LsSERK gene transcripts. As the transgenic lines presented a reduction in their ability to form in vitro somatic embryonic structures, and showed enhanced susceptibility to the pathogenic fungus Sclerotinia sclerotiorum, when compared to control plants. The results support the idea that SERK genes might not only be involved in embryogenic competence, but also in a general mechanism of biotic and abiotic stress perception.

According to the previous annotation, the full-length cDNA of SERK1 in Gossypium hirsutum is 2,502 bp (GenBank Accession no. HQ621831). Expression analysis showed GhSERK1 mRNA was present in all organs of cotton plants, and at different developmental stages. However, its transcripts were most abundant in reproductive organs.

Our current work was designed to characterize the function of the GhSERK1 gene in the Gossypium anther. So we designed three RNAi constructs. One construct with the specialized fragment was aimed to produce single knockdown mutants of GhSERK1 itself. The others with two different homologous fragments were aimed to down-regulate the normal expression level of GhSERK1 and its homologous gene. After genes are transformed into the cotton, transgenic plants with the specialized fragment showed normal fertility or the pollen energy decreased slightly, and ones with the homologous fragments showed various degrees of male sterility with different expression levels of GhSERK1 mRNA. In conclusion, for the transgenic plants with conserved fragments, lower expression levels of GhSERK1 mRNA in transgenic plants resulted in a higher degree of male sterility. Taken together, these findings demonstrate GhSERK1 takes part in sporophytic development of anther. Also, we infer there is another homolog in cotton, and both of GhSERK1 and its homolog function redundantly as an important control point in controlling anther pollen production.

Materials and methods

Plant materials

Four Gossypium hirsutum lines were prepared in the laboratory: Sumian12 (Su12), P30A, P30B and Y18.

Sumian12 (Su12), P30B and Y18 are inbred lines. P30A is a cytoplasmic male sterile line, and it is cross-pollinated with its maintainer line P30B. Transgenic plants were generated from the T1 generation line of Sumian12. The cotton field used in these experiments was used for normal agricultural practices. No specific permits were required for the described field studies. The cotton field used in these experiments was owned by our lab. All the cotton seeds were preserved in our lab. The field studies did not involve endangered or protected species.

Southern hybridization

Genomic DNA samples were extracted using a modified CTAB method [24] from the four *G. hirsutum* lines. 50 µg of genomic DNA was separated on 1 % agarose gels after digestion with *Eco*RI and *Hin*dIII (Promega, USA). Then the DNA was transferred to Hybond N+ nylon membranes (GE Healthcare, USA) using a vacuum-transfer apparatus (Vacuum Blotter 785; Bio-Rad, USA). Next, it was fixed on the nylon membranes by UV crosslinking (CL-1000 UV Crosslinker; UVP, USA) for 1 min (0.1 J/cm^2). 1 µg of DNA fragments were amplified with specific primers (Table 1), and then used as a template DNA for probe labeling. Consequently, probe labeling, hybridization, and detection were performed based on the DIG High Prime Labeling and Detection Starter Kit II manual (Roche Applied Science, Germany). A Gene-ruler DNA ladder Mix (Fermentas, No 0331 Lithuania, Canada) was labeled and used as a size standard.

Generation of *GhSERK1* RNAi constructs

To produce knockdown mutants, we used an RNAi approach. RNAi constructs were generated using the Gateway system pK7GWIW (I) (Invitrogen, USA). The site-specific recombination sequences to be used in Gateway were introduced in the kinase regions of the G*hSERK1* coding sequence by PCR amplification. Three fragments were separately chosen to construct three RNAi vectors. The three fragments of *GhSERK1* cDNA were 184-nt (nt 102–285 in CDS), 343-nt (nt 1,207–1,549 in CDS), and 169-nt (nt 1,689–1,857 in CDS). These were amplified by PCR using the primers in Table 2. The PCR product was introduced into the pENTR/D-TOPO vector (Invitrogen, USA) and subsequently inserted into the plant transformation RNAi vector pK7GWIWG2 (I) by means of a lambda reconstruction (LR) recombination reaction (Invitrogen, USA). The construct with 184-nt was designed to down-regulate the expression level of *GhSERK1* itself, named NG514. The others with 343-nt and 169-nt were aimed to down-regulate the expression level of *GhSERK1* and its homolog, separately named NG1683 and NG7016.

Cotton transformation

The RNAi constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 and transformed into Sumian12 by the floral spraying method. In this method, MS liquid medium containing Agrobacterium was cultured until its OD$_{600}$ value was 0.8. Then the culture medium was centrifugated, and the pellet Agrobacterium was collected. The cell pellet was resuspended in 0.5 % Sucrose and 0.2 ml/L Silwet L-77 until its OD$_{600}$ value was 0.4. The suspension was used to spray the developing flowers. Seeds were harvested from the dipped plants and were then sown under normal agricultural management practices.

Screening on the transformed plants

The transformed plants were screened using kanamycin and the resistant plants were further confirmed by PCR and sequence analyses (Screening primers on Table 1). Genomic DNA samples were extracted from transgenic plants using a modified CTAB method [24]. Genomic DNA was used as a template for PCR. The PCR reaction mixture consisted of 1 µL of genomic DNA (25 ng), 1 µL each of FP1 and RP1 (10 µmol L^{-1}) primers, 1 µL of dNTPs

Table 1 Primers used in the study

Primer name	Primer sequence
Southern Blot	
GhSERK1-FP1	5′-GTGCCTTAGGTGGTTGTTGTTCC-3′
GhSERK1-RP1	5′-TGTCACCAAATCTTGAACCCTCC-3′
GhSERK-FP	5′-CAACACGGAAGAGAATTGCGTTG-3′
GhSERK-RP	5′-CATCATCATTTGCAAGACGAGC-3′
RNAi constructs	
SP-514(184)	5′-CTTAAGCAAGCCAAGCTGCGG-3′
RP-514	5′-ATTGCACAGCTAAGGACCAACTTG-3′
SP-1683(169)	5′-CTCCCCAATGGACCGGCC-3′
RP-1683	5′-ATGCAGATTGTCAGTTGAGTCCAC-3′
SP-7016(343)	5′-GCCAACACGGAAGAGAATTGCGTTG-3′
RP-7016	5′-CCATCATCATTTGCAAGACGAGC-3′
Screening primers	
pK7G-R:	5′-CCATAGGGGTTTAGATGCAACTG-3′
NG514-F	5′-CTGCAGAGTTGGGATCCTACC-3′
NG1683-F	5′-GAATGCTGGAAGGTGATGGG-3′
NG7016-F	5′-TTGCGTTGGGATCTGCTAGG-3′
Real-time PCR	
GS1-RTF	5′-GTTGGCCGAGAGATGGGATG-3′
GS1-RTR	5′-ATGCGGACTGGGCTTACAGG-3′
18F	5′-AGCCTTGCGACCATACTC-3′
18R	5′-CCATAAACGATGCCGACCAG-3′

Table 2 Compared the phenotype and the *GhSERK1* transcript between the wildtype and the transgenic plants

No. of plants	Phenotype of the anther	The percentage of active pollen	The relative expression of the GhSERK1 gene(compared with WT)
WT	Normally dehiscent, abundance of pollen	90–98 %	100 %
NG514-1, NG514-3, NG514-4, NG514-5, NG514-6	Normally dehiscent, abundance of pollen	80–89 %	10–20 %
NG514-2	Normally dehiscent, abundance of pollen	70–79 %	1–9 %
NG1683-7	Normally dehiscent, abundance of pollen	90–95 %	40 %
NG1683-6, NG1683-12	Normally dehiscent, less pollen	70–89 %	20–39 %
NG1683-1, NG1683-2, NG1683-3, NG1683-5, NG1683-8, NG1683-9, NG1683-10, NG1683-11, NG1683-13	Indehiscent, less pollen	30–69 %	5–19 %
NG1683-4	Indehiscent, without pollen	0	0
NG7016-15	Normally dehiscent, abundance of pollen	90–95 %	40–60 %
NG7016-1, NG7016-12,	Normally dehiscent, less pollen	70–89 %	20–39 %
NG7016-2, NG7016-3, NG7016-4, NG7016-4, NG7016-4, NG7016-5, NG7016-6, NG7016-7, NG7016-9, NG7016-10, NG7016-12, NG7016-17, NG7016-20,	Indehiscent, less pollen	30–69 %	5–19 %
NG7016-8, NG7016-11, NG7016-13, NG7016-18, NG7016-19, NG7016-14	Indehiscent, without pollen	0	<5 %

(2.5 mmol L^{-1} each), 5 μL of PCR reaction buffer, 0.25 μL of *ExTaq* DNA polymerase (5.0 units μL^{-1}) (TaKaRa) and 42.5 μL of distilled water. The PCR conditions were as follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, and a final extension at 72 °C for 5 min. The products were cloned into pGEM-T vectors (Promega, USA) and sequenced.

Phenotypic analysis and pollen stained

Phenotypic analysis of transformed lines was performed in the T1 generation. In all experiments, vector-transformed plants were analyzed together with wild-type plants as controls. Flowers were imaged using a digital camera (Canon, Japan) and a dissecting microscope (Leica, Germany). Pollen activity was evaluated using 2,3,5-triphenyl-2 h-tetrazolium chloride (TTC) staining, in TTC staining; pollen grains were soaked in 0.1 % TTC solution. Viable pollen stains red because the NADH/NADPH produced deoxidized TTC to TTF (which is red). These stained

pollen grains were observed under the microscope (ZEISS AXIOSKOP40, Germany).

Real-time PCR

The expression of the *GhSERK1* gene in transgenic cotton was further analyzed by real-time PCR. RNA extraction was performed from the leaves of the seedlings using the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) based on the instructions of the manufacturer. The RNA isolation protocol included an on-column DNaseI (Promega, USA) digestion to remove contaminating genomic DNA. RNA was reverse transcribed into cDNA using AMV reverse transcriptase (ToKoBo, Japan). Real-time PCR was performed using the SYBR Green kit (BioRad, USA) following the manufacturer's standard instructions. As a control, parallel amplification reactions for 18SrRNA were performed. The *GhSERK1* specific primers were GS1-RTF and GS1-RTR, and the 18SrRNA specific primers were 18F and 18R (Table 1). Each sample was run three times to confirm the results (primer sequences in Table 1). Real-time PCR

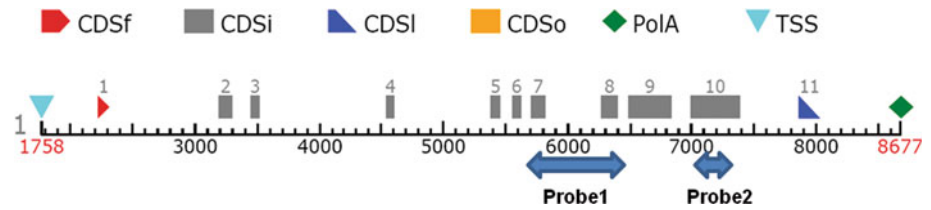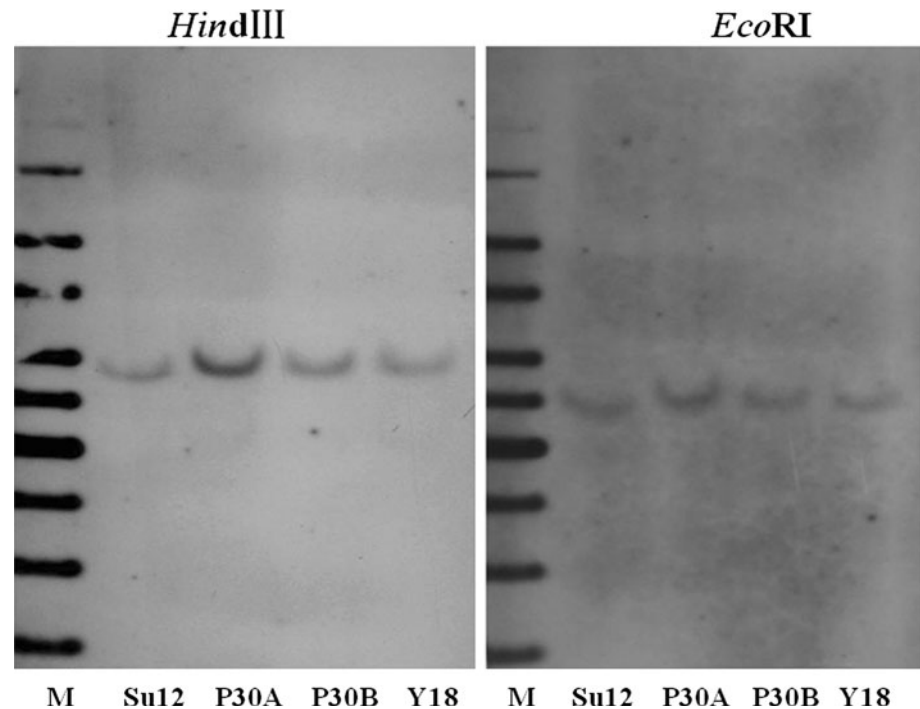**Fig. 1** Schematic map of the two probes for the southern blot in the *GhSERK1* gene

Fig. 2 Southern blot of *GhSERK1* with the probe 1 (the unique fragment). *Left*: *Hin*dIII; *Right*: *Eco*RI; *M* DNA marker

results were analyzed according to the method of $2^{-\Delta\Delta Ct}$ [25].

Results

G. hirsutum has one copy of *GhSERK1* gene, and contains at least two to three members of *SERK* genes

Southern-blot hybridization analysis was performed to investigate the genomic organization of *GhSERK1* and the members of the *SERK* family in *G. hirsutum*. All genomic DNA was extracted from *G. hirsutum* and digested with *Eco*RI and *Hin*dIII restriction enzymes. One probe was obtained from genomic DNA amplification with specific primers (*GhSERK1*-FP and *GhSERK1*-RP) corresponding to the SPP and transmembrane domains of *GhSERK1* (5,665–6,448 bp) (Fig. 1 and Supplementary material). It was named Probe1. The 834-bp-long probe was designed to analyze copies of the *GhSERK1* gene. The hybridization

on the genomic DNA digested with *Hin*dIII and *Eco*RI resulted in one band for the ten lines (Fig. 2).

The other probe was cloned from the cDNA corresponding to the kinase domain of *GhSERK1*, and primers were *GhSERK*-FP and *GhSERK*-RP (Table 1). It was named Probe2 (7,016–7,356 bp, Fig. 1). The 334-bp-long probe was used to analysis the members of the *SERK* family. Three hybridization products were found for genomic DNA. The presence of multiple copies of *SERK* genes in the *G. hirsutum* genome agrees with the findings of multiple members documented in genomes of other species (Fig. 3).

The cotton transformants containing RNAi constructs displayed various degrees of male sterility

To determine whether the *GhSERK1* gene took part in the regulation of anther development, transgenic cotton plants were generated separately after transformation with *Agrobacterium* containing the NG514, NG1683, and NG7016 constructs. Among the 29 kanamycin-resistant cotton

Fig. 3 Southern Bolt of *GhSERK1* with probe 2 (the homologous fragment). *Left*: Genomic DNAs on the left of the marker were digested with *Hind*III. The ones on the *right* of the marker were digested with *Eco*RI. *Right*: Genomic DNAs digested with *Eco*RI
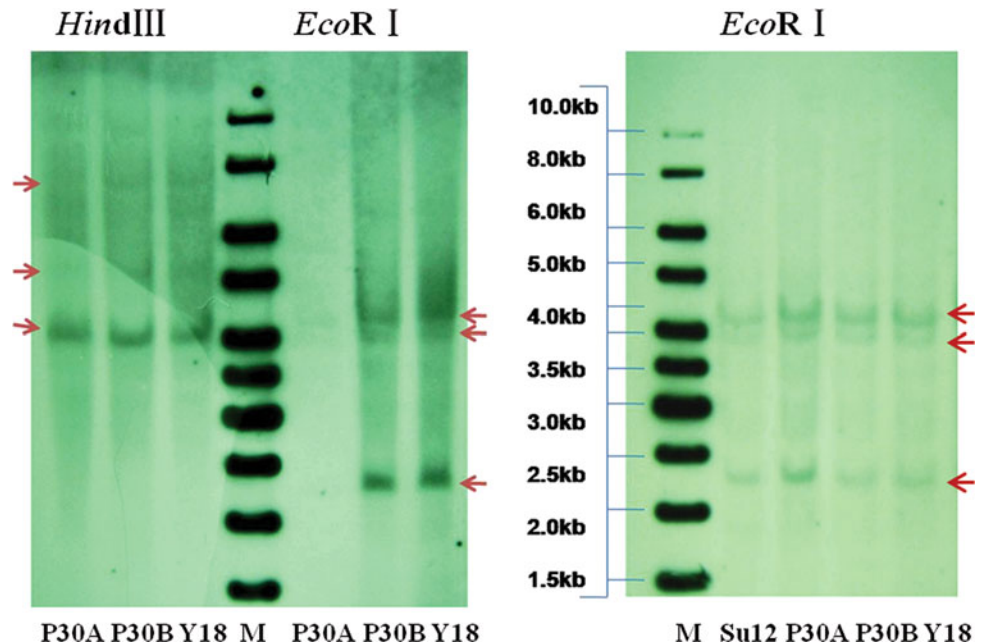
plants, six plants contained NG514, 13 contained NG1683, and 20 contained NG7016.

When grown to maturity in soil, all of the transgenic plants grew into normal plants and exhibited normal vegetative development. Meanwhile there were no differences between the transgenic plants containing NG514 and the wild type, and the pollen energy ranged from 70 to 89 % (Fig. 7). In other words, all of the plants with NG514-construct were fertile.

However, all but two plants of the transgenic lines containing NG1683 or NG7016 constructs exhibited various degrees of male sterility. Two plants named NG1683-7 and NG7016-15 were fully male-fertile (data not shown). There was no difference between them and the wild type. Compared with the wild type (Figs. 4a, 5b) another four plants displayed fewer pollen grains in the anther, though the stamens of them were normally dehiscent. In the anthers of 22 other plants (Figs. 4b, c, 5b) there were some indehiscent stamens with fewer pollen grains. We scored the 26 lines as semi-sterile plants. Seven transgenic plants containing NG1683 or NG7016 were fully male-sterile as there were no pollen grains in their anthers (Figs. 4d, 5c). Thus, they failed to form seeds by themselves. For all the transgenic plants, the pistil was normal. In detail, we compared the flowers of the wild type and the completely male-sterile line (Fig. 6); the flowers of these plants exhibited normal filaments and their anthers seemed full (Fig. 6b). But their anther walls were indehiscent and there were no pollen grains in their anthers (Fig. 6d).

To determine the activity of pollen grains, staining the pollen of the transformants and the wild type with TTC was performed (Fig. 7). The pollen energy of the wild type was above 90 % (Fig. 8a). It ranged from 80 to 89 % for the plants containing NG514 constructs and the two fully male-fertile lines (NG1683-7 and NG7016-15) (Fig. 8b). Among the 26 semi-sterile plants, the activity of pollen was 70–89 % (Fig. 8c) for the four of them; it was 30–69 % for another 22 of them (Fig. 8d, e).

Various degrees of male sterility were coupled with different expression levels of *GhSERK1* in transgenic plants

Real-Time PCR analysis was performed to check for expression of *GhSERK1* in transgenic lines, as well as in untransformed cotton plants (Fig. 9; Table 2). For the transgenic lines containing NG514, the expression of *GhSERK1* ranged from 10 to 20 % in the fully male fertile lines, and from 1 to 9 % in the lines with decreased pollen energy. For the transgenic lines containing NG1683 or NG7016, the expression level of *GhSERK1* in the fully male fertile lines was about 40 % compared with the wild type. As it was from 5 to 39 % in the semi-sterile plants, and it was below 5 % in the completely male sterile lines.

Discussion

There were at least two to three homologous with *GhSERK1* in *G. hirsutum*

In 1997, Schmidt et al. [6] found the first *SERK* gene in carrot (*Daucus carota*) and named it *DcSERK*. Consequently, many *SERK* genes have been cloned from other
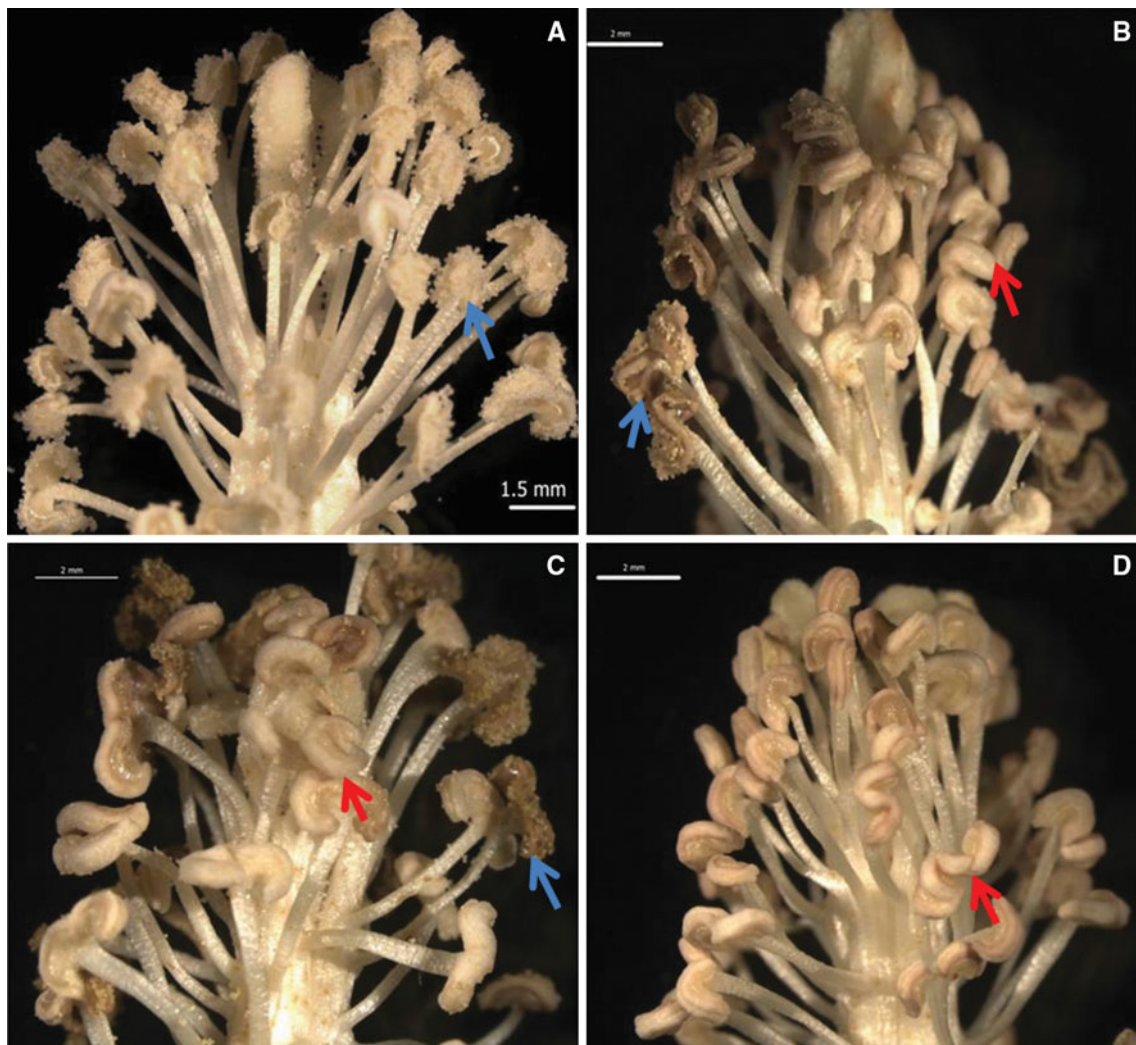
Fig. 4 Phenotypic analysis of male-sterile transformants carrying RNAi-vectors. **a** Flower morphology of non-transgenic cotton; **b, c** Semi-sterile transformants (NG1683 or NG7016); **b** The indehiscent stamen accounts for 30 %; **c** The indehiscent stamen accounts for 50 %; **d** A full male-sterile transformant. *Red arrow* sterile anther; *blue arrow* fertile anther. *Scale bar* A = 1.5 mm; B, C, D = 2 mm. (Color figure online)
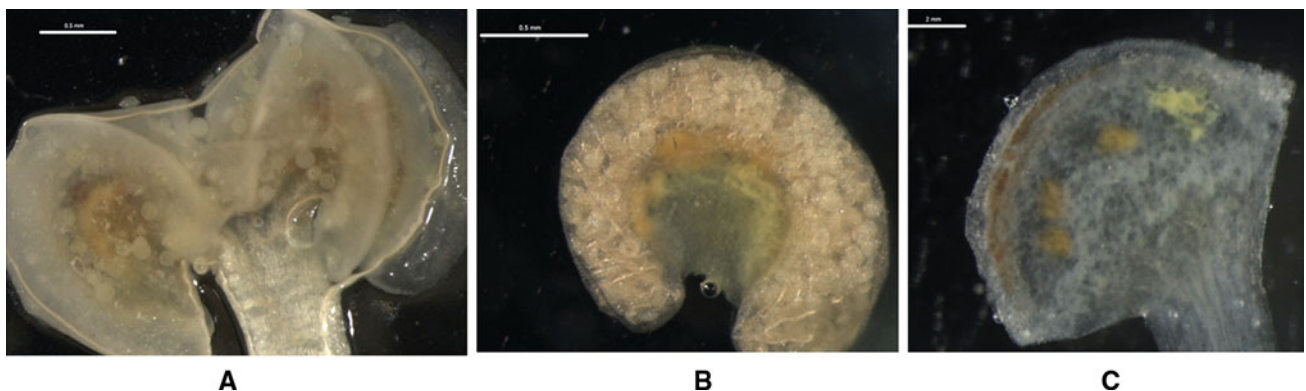

Fig. 5 Comparison of the anther of transgenic plants containing NG1683 or NG7016 and wildtype. **a** Anther of a non-transgenic cotton plant: dehiscent and full of pollen grains; **b** Indehiscent anther of a semi-sterile transformant carrying NG1683 or NG7016; **c** Stamen of a fully male-sterile transformant carrying NG1683 or NG7016: indehiscent and absence of pollen grains. *Scale bar* A, B = 0.5 mm; C = 2 mm
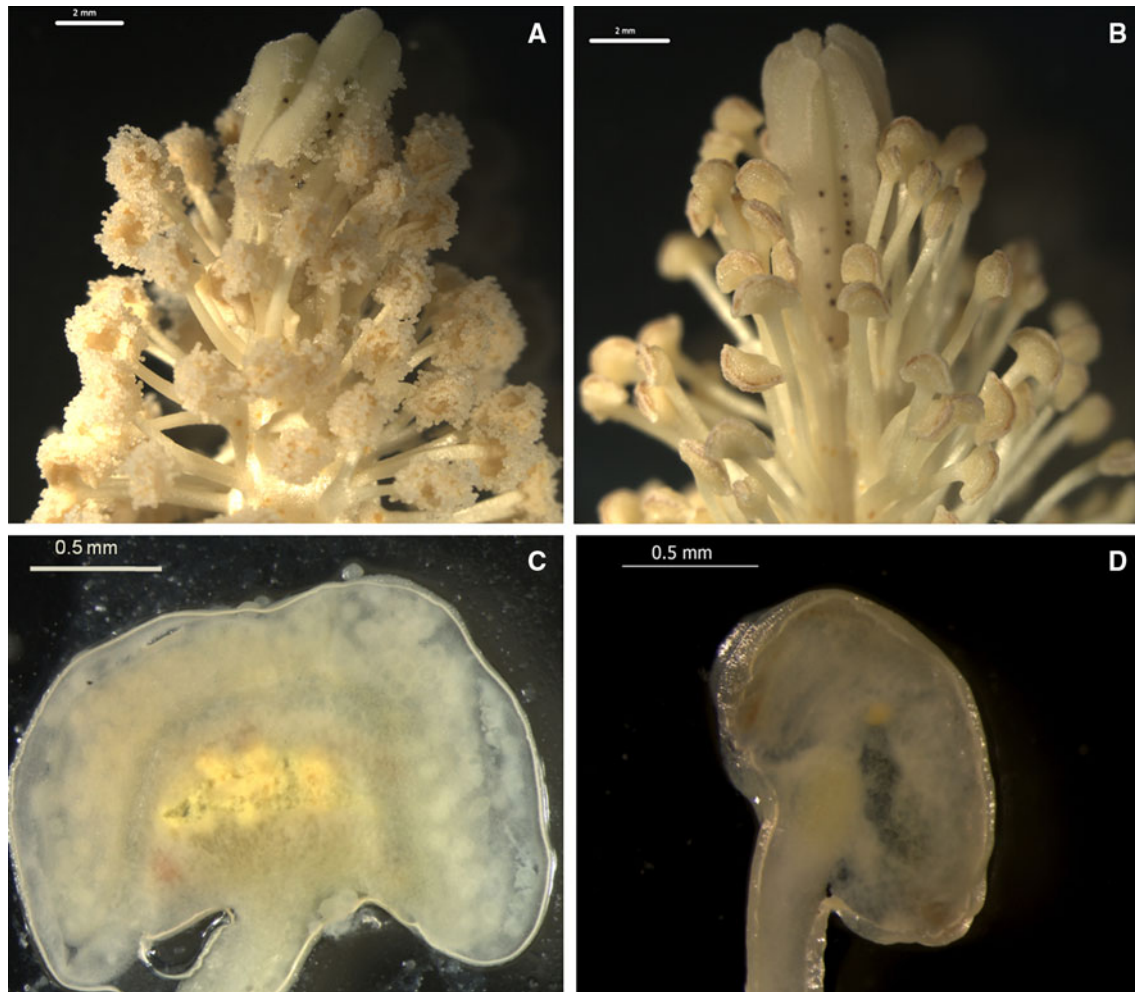
Fig. 6 Comparison of the flowers of the wild type and the completely male-sterile line. **a** the flower of the wild type; **b** the flower of the completely male-sterile line; **c** the anther of the wild type; **d** the anther of the completely male-sterile line. *Scale bar* A, B = 2 mm; C, D = 0.5 mm
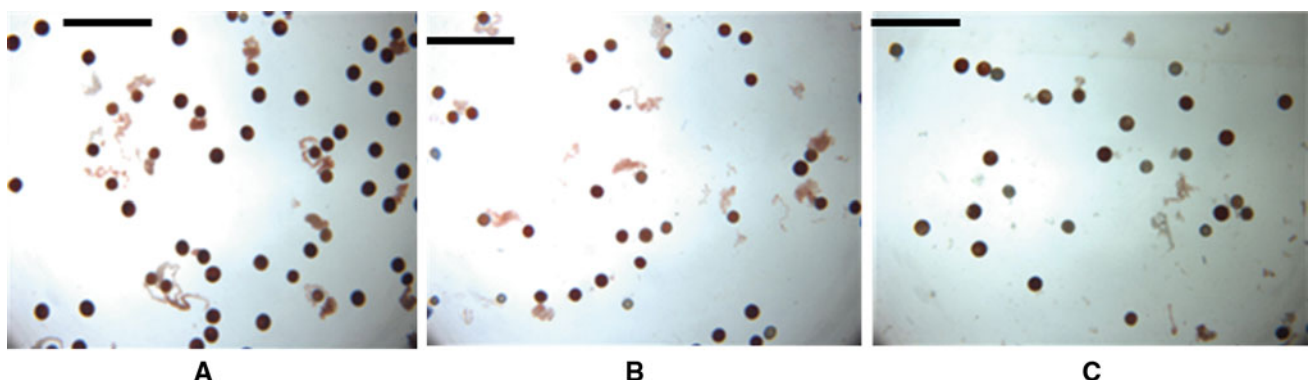

Fig. 7 Comparison of the pollen energy of the wild type (**a**) and transgenic plants carrying RNAi-514 (**b**, **c**) with TTC. **a** The active pollen of the wild type accounts for 95 %; **b**, **c** The active pollen of the transgenic plant carrying RNAi-514 accounts for 80 % (**b**) to 70 % (**c**). *Scale bar* = 0.5 mm

plants, such as *Arabidopsis thaliana* (*AtSERK*; [13]), *Zea mays* (*ZmSERK*; [15]), *Medicago truncatula* (*MtSERK*; [26]), *Oryza sativa* (*OsSERK* [21, 27], *OsBISERK* [28]), *Triticum aestivum* (*TaSERK*, [29]) and *Vitis vinifera* (*VvSERK*; [30]), *Rosa hybrid* cv. Linda (*RhSERK*; [31]). Also, two or more members have been isolated from
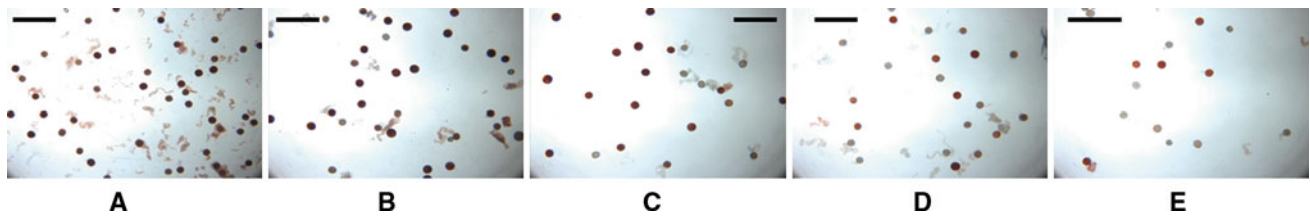
Fig. 8 Comparing the pollen energy of the wild type and transgenic plants carrying RNAi-1683 or RNAi-7016 with TTC. A: The active pollen of the wild type accounts for 95 %; **b** The active pollen of the fully fertile transformants accounts for 90 %. **c–e** The active pollen of the semi-sterile transformants accounts for 80 % (**c**), 60 % (**d**) and 35 % (**e**). *Scale bar* = 0.5 mm
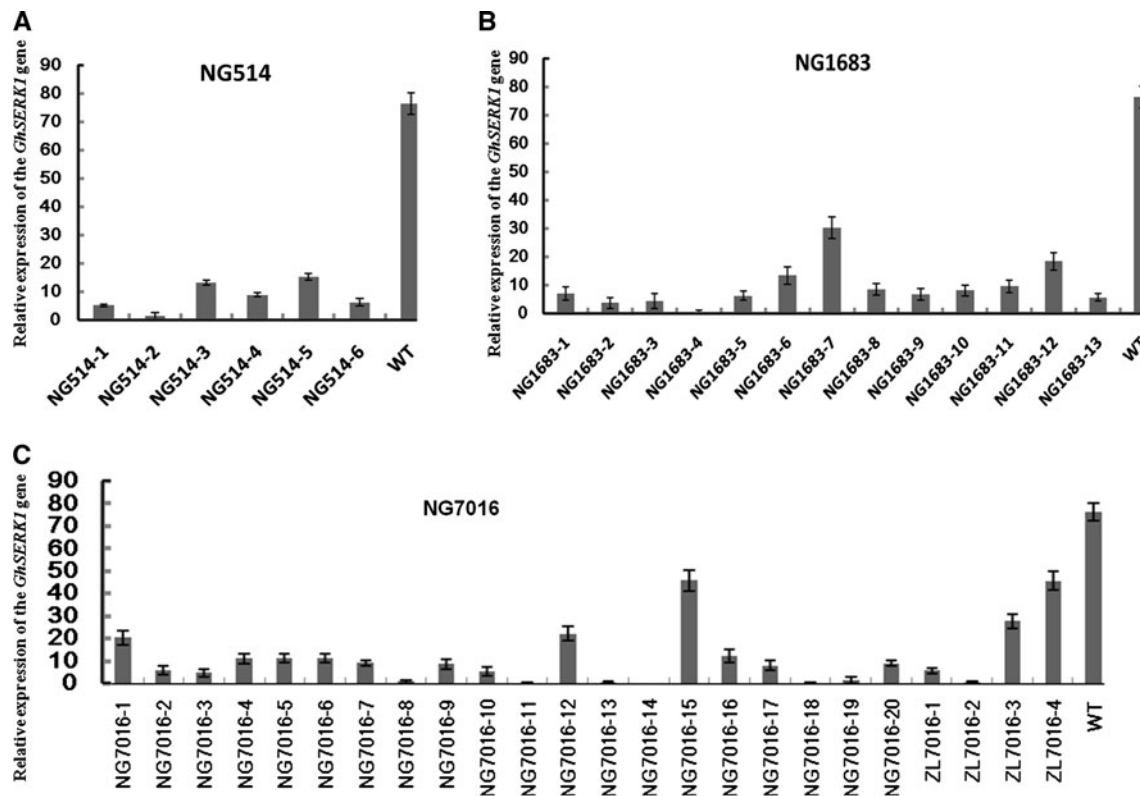

Fig. 9 The relative expression of the *GhSERK1* gene in the wildtype and transgenic plants. **a**, **b**, and **c** The relative expression of the *GhSERK1* gene in the wildtype and transgenic plants containing NG514, NG1683, and NG7016, respectively

specific plant species. For example, five *Arabidopsis SERKs* (*AtSERK1–5*) [13], three maize *SERKs* (*ZmSERK1–3*) [15], three wheat *SERKs* (*TaSERK1–3*) [29], three grapevine *SERKs* (*VvSERK1–3*) [30], and four rose *SERKs* (*RhSERK1–4*) [31]) have been identified. So, many studies have found SERKs were a subfamily of the LRR-RLKs family. Meanwhile the kinase domain has the highest identity among the 11 domains of SERK, as the greatest divergence is seen in the SPP and C-terminal domains (below 45 %) [13, 15].

In our studies, the *GhSERK1* gene was one copy in the genomic DNA of *G. hirsutum* proved by southern blot. Including that, we also wanted to know how many members of *SERK* exist in *G. hirsutum*. A probe was designed to locate in the kinase domain of GhSERK1 according to its

highest identity. Three hybridization products were found for genomic DNA of *G. hirsutum*. One of the three bands was *GhSERK1* gene, and the other bands were one or two *SERK* genes. There were at least two or three *SERK* genes in *G. hirsutum*.

The role of *GhSERK1* in the male reproduction of *G. hirsutum* and in the network of pollen development

The SERK1/2 gene in *Arabidopsis* was reported to be involved in anther development. In *Arabidopsis*, the expression level of *AtSERK1* mRNA was highest in closed flower buds before fertilization and in flowers with
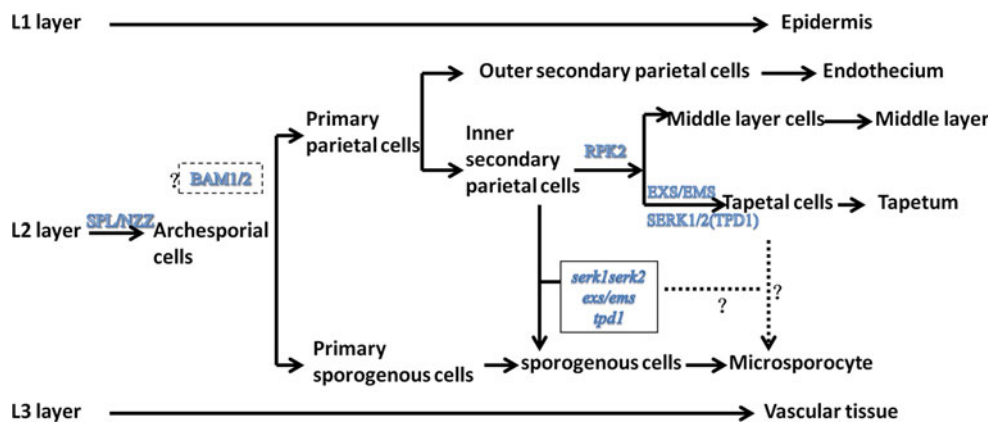
Fig. 10 Signal pathways including SPL/NZZ, EMS1/EXS, SERK1/2, TPD1, BAM1/2 and RPK2, and their possible integrations in regulating anther cell differentiation. SPL/NZZ: SPOROCYTELESS/NOZZLE; EMS1/EXS: EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS; SERK1/2: SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINAES1/2; TPD1: TAPETUM DETERMINANT1; BAM1/2: BARELY ANY MERISTEM; RPK2: RECEPTOR-LIKE PROTEIN KINASE 2

embryos of stage 1 through 7 after pollination. Quantitative analysis shows the abundance of *AtSERK1* mRNA in the flower buds was about ten times higher than in the leaf tissue [6]. The expression patterns of *AtSERK1* and *At-SERK2* in wild-type plants were similar based on the semiquantitative RT-PCR analysis. Also, the *serk1* and *serk2* single insertion mutants show no differences compared to the wild type, but *serk1serk2* double mutants were not able to produce seeds because the mutant anthers produced no pollen. Meanwhile, the AtSERK1 and AtSERK2 proteins can form homodimers or heterodimers in vivo. These results demonstrate *AtSERK1* and *AtSERK2* play an important but redundant role in sporophytic development by controlling male gametophyte production [7, 8]. Similarly, in a previous study [32], we found the transcripts of the *GhSERK1* gene were most abundant in reproductive organs, especially in anthers. *GhSERK1* mRNA was most abundant in closed flower buds before fertilization, especially in 14- and 18-day-old buds containing pollen. In the floral organ, the expression of the *GhSERK1* gene was higher in anthers and ovules than in the sepals and petals. Also, the anthers in a male-sterile cotton cultivar line (P30A) showed obvious abortion in the PMC meiosis stage, and the level of *GhSERK1* mRNA was sharply decreased in this stage compared with that of the male-fertile line. This phenomenon indicated the expression of the *GhSERK1* was related to the development of anthers. Here single knockdown mutants of *GhSERK1* showed male-fertility only with slightly decreased pollen energy. However, transgenic plants with homologous fragments RNAi vector showed various degrees of male sterility. The lower the expression levels were in *GhSERK1* mRNA in transgenic plants, the higher degree of male sterility they exhibited. So we infer *GhSERK1* is responsible for the male gametophyte development in *Gossypium*

anthers. Also, there is another homolog in cotton; the role of *GhSERK1* and its homolog in sporophytic development is probably redundant. It would be interesting to identify other SERK members related to anther cell differentiation in *G. hirsutum*. If found, this will verify the SERKs play an important role in the signal pathway of microsporogenesis in *G. hirsutum*.

Similar to SERK1/2, only a few genes have been known to be involved in early anther cell division and cell differentiation events, including SPOROCYTELESS/NOZZLE (SPL/NZZ) [33–35], EMS1/EXS [4, 5], TPD1 [9], RPK2 [12] and BAM1/2 [10, 11]. The SPL/NZZ protein is related to MADS box transcription factors and is involved in regulating anther cell differentiation. The *spl/nzz* mutant is not able to produce microsporocytes or anther walls. So, the SPL/NZZ gene is required for the differentiation of microsporocytes and anther walls, including the tapetum [33, 34]. Also, ectopic expression of *SPL/NZZ* not only affects flower development in the wild-type background but also results in the transformation of petal-like organs into stamen-like organs in flowers of *ap2-1*, a weak *ap2* mutant allele. The results indicate the "floral organ-building" gene *SPL/NZZ* is engaged in controlling stamen identity via interacting with genes required for stamen identity in *Arabidopsis* [35]. The *EMS1/EXS* gene encodes a putative leucine-rich repeat receptor protein kinase (LRR-RLK), and it mediates signals controlling the fate of reproductive cells and their contiguous somatic cells during early anther development. The anthers in the *ems1/exs* mutant lack tapetum, but produce more microsporocytes than the wild type. In fact, the *ems1* anthers produce excess microsporocytes at the expense of tapetal cells, since the number of microsporocytes in *ems1* mutant anthers is close to the sum of microsporocytes and tapetal cells in wild-type anthers. This indicates a trade-off exists between somatic and

reproductive cells [4, 5]. The *TPD1* gene is reported to play an important role on the differentiation of tapetal cells in *Arabidopsis* anthers. Morphological analysis of *tpd1*, the male-sterile mutant, revealed the precursors of tapetal cells differentiate and develop into microsporocytes instead of tapetum. As a result, extra microsporocytes were produced and tapetum was absent in developing *tpd1* anthers. The *tpd1* mutant was phenotypically similar to the *ems1/exs* mutant. The *RECEPTOR-LIKE PROTEIN KINASE2* (*RPK2*) gene belongs to the LRR-RLK family, and is required for differentiation of the middle layer and the tapetum in anthers. The middle layer is absent in the *rpk2* mutant anther, and the tapetum displays hypertrophy. So the disruption of RPK2 function affects the pollen maturation [12]. BARELY ANY MERISTEM1 (BAM1) and BAM2 are two novel LRR-RLKs which are involved in regulating early anther cell differentiation in *Arabidopsis* [10], [11], and [36]. The *bam1* and *bam2* single mutants have no detectable phenotypes. However, *bam1bam2* double mutants exhibit various developmental abnormalities, including reduced meristem size and aberrant leaves, as well as irregular male and female fertility [10]. Additional detailed morphological analysis of anther development reveals the *bam1bam2* double mutant anthers have no endothecium, middle layer, and tapetum [11]. Nevertheless, the double mutant anthers produce only microsporocytes. Both *BAM1* and *BAM2* genes in *Arabidopsis* are expressed in archesporial cells at as early as stage 2, while in later stages they are preferentially expressed in sporogenous cells and microsporocytes. The results demonstrate the *BAM1* and *BAM2* genes are related to the development of anthers. Also, some evidence strongly suggests these genes interact with each other. The phenotype of the mutant *tpd1* is nearly identical to the ones of the *ems1/exs* single mutant and *serk1serk2* double mutant, showing the absence of tapetum and the formation of excess microsporocytes [9]. Also, TPD1 is a novel secreted protein and interacts with EMS1/EXS in vitro and in vivo and the ectopic expression of *TPD1* in the *ems1* mutant affects the differentiation of tapetal cells and microsporocytes. The results indicate *TPD1* and *EMS1/EXS* probably function in the same genetic pathway [37]. However, the expression of SPL/NZZ in *bam1bam2* anthers is expanded to most of the L2-derived cells. So *BAM1/2* probably represses the expression of *SPL/NZZ* [11]. These results remind us to propose these questions: Do *these genes* function in the same signal pathway? (Fig. 10) How do the mechanisms of the interaction function? Further studies should focus on studying the crosstalk among these signaling networks.

References

1. Goldberg RB, Beals TP, Sanders PM (1993) Anther development: basic principles and practical applications. Plant Cell 5(10):1217–1229
2. Mariani C, Beuckeleer MD, Truettner J, Leemans J, Goldberg RB (1990) Induction of male sterility in plants by a chimaeric ribonuclease gene. Nature 347:737–741
3. Mariani C, Gossele V, Debeuckeleer M, Deblock M, Goldberg RB, Degreef W, Leemans J (1992) A chimaeric ribonuclease-inhibitor gene restores fertility to male sterile plants. Nature 357(6377):384–387
4. Canales C, Bhatt AM, Scott R, Dickinson H (2002) EXS, a putative LRR receptor kinase, regulates male germline cell number and tapetal identity and promotes seed development in *Arabidopsis*. Curr Biol 12(20):1718–1727
5. Zhao DZ, Wang GF, Speal B, Ma H (2002) The *EXCESS MICROSPOROCYTES1* gene encodes a putative leucine-rich repeat receptor protein kinase that controls somatic and reproductive cell fates in the *Arabidopsis* anther. Genes Dev 16(15):2021–2031
6. Schmidt ED, Guzzo F, Toonen MA, de Vries SC (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development 124(10):2049–2062
7. Albrecht C, Russinova E, Hecht V, Baaijens E, de Vries S (2005) The *Arabidopsis thaliana* SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES1 and 2 control male sporogenesis. Plant Cell 17(12):3337–3349
8. Colcombet J, Boisson-Dernier A, Ros-Palau R, Vera CE, Schroeder JI (2005) *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASES1 and 2 are essential for tapetum development and microspore maturation. Plant Cell 17(12):3350–3361
9. Yang SL, Xie LF, Mao HZ, Puah CS, Yang WC, Jiang L, Sundaresan V, Ye D (2003) Tapetum Determinant1 is required for cell specialization in the *Arabidopsis* anther. Plant Cell 15(12):2792–2804
10. DeYoung BJ, Bickle KL, Schrage KJ, Muskett P, Patel K, Clark SE (2006) The CLAVATA1-related BAM1, BAM2 and BAM3 receptor kinase-like proteins are required for meristem function in *Arabidopsis*. Plant J 45(1):1–16
11. Hord CL, Chen C, Deyoung BJ, Clark SE, Ma H (2006) The BAM1/BAM2 receptor-like kinases are important regulators of *Arabidopsis* early anther development. Plant Cell 18(7):1667–1680
12. Mizuno S, Osakabe Y, Maruyama K, Ito T, Osakabe K, Sato T, Shinozaki K, Yamaguchi-Shinozaki K (2007) Receptor-like protein kinase 2 (RPK2) is a novel factor controlling anther development in *Arabidopsis thaliana*. Plant J 50(5):751–766
13. Hecht V, Vielle-Calzada JP, Hartog MV, Ed Schmidt DL, Boutilier K, Grossniklaus U, de Vries SC (2001) The *Arabidopsis somatic embryogenesis receptor kinase* 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol 127(3):803–816
14. Salaj Jan, von Recklinghausen Iris R, Hecht Valerie, de Vries Sacco C, Schel Jan HN, van Lammeren André′ AM (2008) AtSERK1 expression precedes and coincides with early somatic embryogenesis in *Arabidopsis thaliana*. Plant Physiol Biochem 46:709–714
15. Baudino S, Hansen S, Brettschneider R, Hecht VF, Dresselhaus T, Lörz H, Dumas C, Rogowsky PM (2001) Molecular characterisation of two novel maize LRR receptor-like kinases, which belong to the *SERK* gene family. Planta 213(1):1–10

16. Shimada T, Hirabayashi T, Endo T, Fujii H, Kita M, Omura M (2005) Isolation and characterization of the *somatic embryogenesis receptor-like kinase* gene homologue (*CitSERK1*) from *Citrus unshiu Marc*. Sci Hortic 103(2):233–238

17. de Oliveira Santos M, Romano E, Yotoko KSC, Tinoco MLP, Dias BBA, Aragão FJL (2005) Characterisation of the cacao *somatic embryogenesis receptor-like kinase* (*SERK*) gene expressed during somatic embryogenesis. Plant Sci 168(3):723–729

18. Li JM, Chory J (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90(5):929–938

19. Noguchi T, Fujioka S, Choe S, Takatsuto S, Yoshida S, Yuan H, Feldmann KA, Tax FE (1999) Brassinosteroid-insen-sitive dwarf mutants of *Arabidopsis* accumulate brassinosteroids. Plant Physiol 121(3):743–752

20. He K, Gou X, Yuan T, Lin H, Asami T, Yoshida S, Russell SD, Li J (2007) BAK1 and BKK1 regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways. Curr Biol 17(13):1109–1115

21. Hu H, Xiong L, Yang Y (2005) Rice *SERK1* gene positively regulates somatic embryogenesis of cultured cell and host defense response against fungal infection. Planta 222(1):107–117

22. Kemmerling B, Schwedt A, Rodriguez P, Mazzotta S, Frank M, Qamar SA, Mengiste T, Betsuyaku S, Parker JE, Müssig C, Thomma BP, Albrecht C, de Vries SC, Hirt H, Nürnberger T (2007) The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. Curr Biol 17(13):1116–1122

23. Santos MO, Romano E, Vieira LS, Baldoni AB, Aragao FJ (2009) Suppression of *SERK* gene expression affects fungus tolerance and somatic embryogenesis in transgenic lettuce. Plant Biol 11(1):83–89

24. Khan IA, Awan FS, Ahmad A, Khan AA (2004) A modified mini-Prep method for economical and rapid extraction of genomic DNA in plants. Plant Mol Biol Report 22(1):89a–89e

25. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25(4):402–408

26. Nolan KE, Irwanto RR, Rose RJ (2003) Auxin up-regulates *MtSERK1* expression in both *Medicago truncatula* root-forming and embryogenic cultures. Plant Physiol 133(1):218–230

27. Ito Y, Takaya K, Kurata N (2005) Expression of *SERK* family *receptor-like protein kinase* gene in rice. Biochem Biophys Acta 1730(3):253–258

28. Song D, Li G, Song F, Zheng Z (2008) Molecular characterization and expression analysis of *OsBISERK1*, a gene encoding a leucine-rich repeat receptor-like kinase, during disease resistance responses in rice. Mol Biol Rep 35(2):275–283

29. Singla B, Tyagi AK, Khurana JP, Khurana P (2007) Analysis of expression profile of selected genes expressed during auxin-induced somatic embryogenesis in leaf base system of wheat (*Triticum aestivum*) and their possible interactions. Plant Mol Biol 65(5):677–692

30. Schellenbaum P, Jacques A, Maillot P, Bertsch C, Mazet F, Farine S, Walter B (2008) Characterization of *VvSERK1*, *VvSERK2*, *VvSERK3* and *VvL1L* genes and their expression during somatic embryogenesis of grapevine (*Vitis vinifera* L.). Plant Cell Rep 27(12):1799–1809

31. Zakizadeh H, Bjarne M, Stummann, Lütken H, Müller R (2010) Isolation and characterization of four *somatic embryogenesis receptor-like kinase* (*RhSERK*) genes from miniature potted rose (*Rosa hybrida* cv. *Linda*). Plant Cell Tiss Organ Cult 101(3):331–338

32. Shi YL, Zhang R, Wu XP, Meng ZG, Guo SD (2012) Cloning and characterization of a *Somatic Embryogenesis Receptor-Like Kinase* Gene in cotton (*Gossypium hirsutum*). Journal of Integrative Agriculture 11(6):898–909

33. Schiefthaler U, Balasubramanian S, Sieber P, Chevalier D, Wisman E, Schneitz K (1999) Molecular analysis of *NOZZLE*, a gene involved in pattern formation and early sporogenes is during sex organ development in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 96(20):11664–11669

34. Yang WC, Ye D, Xu J, Sundaresan V (1999) The *SPOROCYTELESS* gene of *Arabidopsis* is required for initiation of sporogenesis and encodes a novel nuclear protein. Genes Dev 13(16):2108–2117

35. Liu X, Huang J, Parameswaran S, Ito T, Seubert B, Auer M, Rymaszewski A, Jia G, Owen HA, Zhao D (2009) The *SPOROCYTELESS/NOZZLE* Gene Is Involved in Controlling Stamen Identity in *Arabidopsis*. Plant Physiol 151(3):1401–1411

36. Shiu SH, Bleecker AB (2001) Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci USA 98(19):10763–10768

37. Jia G, Liu X, Owen HA, Zhao D (2008) Signaling of cell fate determination by the TPD1 small protein and EMS1 receptor kinase. Proc Natl Acad Sci USA 105(6):2220–2225