communications biology

Article

https://doi.org/10.1038/s42003-024-06961-5

Loss-of-function in testis-specific serine/threonine protein kinase triggers male infertility in an invasive moth

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Genetic biocontrol technologies present promising and eco-friendly strategies for the management of pest and insect-transmitted diseases. Although considerable advancements achieve in gene drive applications targeting mosquitoes, endeavors to combat agricultural pests have been somewhat restricted. Here, we identify that the testis-specific serine/threonine kinases (*TSSKs*) family is uniquely expressed in the testes of *Cydia pomonella*, a prominent global invasive species. We further generated male moths with disrupted the expression of *TSSKs* and those with *TSSKs* disrupted using RNA interference and CRISPR/Cas9 genetic editing techniques, resulting in significant disruptions in spermiogenesis, decreased sperm motility, and hindered development of eggs. Further explorations into the underlying post-transcriptional regulatory mechanisms reveales the involvement of lnc117962 as a competing endogenous RNA (ceRNA) for miR-3960, thereby regulating *TSSKs*. Notably, orchard trials demonstrates that the release of male strains can effectively suppress population growth. Our findings indicate that targeting *TSSKs* could serve as a feasible avenue for managing *C. pomonella* populations, offering significant insights and potential strategies for controlling invasive pests through genetic sterile insect technique (gSIT) technology.

Arthropods, constituting approximately 1.1 to 1.2 million species, are widely distributed in various habitats, including soil and plant surfaces, and are known to parasitize both humans and animals[1]. Insects, the largest class among arthropods, have been extensively studied with a recorded count exceeding one million species[2]. According to the Food and Agriculture Organization of the United Nations (FAO), insect pests contribute to nearly 40% of annual global crop yield losses, resulting in over $70 billion losses primarily due to invasive species. The challenges posed by insects have heightened in recent years due to factors such as climate change, rapid globalization and urbanization, and the proliferation of invasive species[3,4].

Integrated Pest Management (IPM) is a methodology for pest control that emphasizes technical integration of various strategies to maintain pest populations below economically acceptable thresholds[5]. In light of the imperative for increased food production driven by population growth, the utilization of pesticides remains pivotal within the framework of IPM[6–8]. Nevertheless, challenges arose following the initiation of insecticide-based control measures, leading to detrimental consequences such as insect outbreaks, ecological disruption, and the persistence of pesticide residues[9]. The adverse effects were linked to the eradication of natural enemies and the development of insecticide resistance[10]. Hence, there is a critical need to explore sustainable and eco-friendly approaches to pest management in agricultural and forestry practices.

The sterile insect technique (SIT) has become a crucial method for controlling populations by producing and releasing sterile males targeting specific species[11–13]. Initially developed in the mid-1930s, SIT utilizes radiation to induce dominant lethal mutations, as seen in the successful eradication of *Cochliomyia hominivorax* in United States[11]. The Okanagan-Kootenay Insect Sterile Release (OK SIR) project is a prominent example of regional management for *Cydia pomonella*, covering extensive orchards and neighboring areas urban in British Columbia, Canada[14,15]. Traditional sterilization methods using DNA-damaging agents can reduce the fitness and mating competitiveness of released males[16]. Newer approaches include microbial-mediated of SIT variations like the Wolbachia-based incompatible insect technique (IIT)[17], genetically similar SIT-like systems such as

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release of insects carrying dominant lethal genes (RIDL)[18], and methods targeting female elimination like female-specific RIDL (fsRIDL)[19]. While these first-generation gene SIT technologies present a notable advancement, but each approach has its limitations. For instance, IIT requires that infected females cannot be released, posing practical challenges in field settings[20]. The use of tetracycline, an antibiotic in the microbiome, could impact the fitness of RIDL/fsRIDL males[16].

Recently, CRISPR based self-limiting approaches such as Inherited Female Elimination by Genetically Encoded Nucleases to Interrupt Alleles (Ifegenia)[21], and precision-guided SIT (pgSIT)[22–26] have emerged as potential game-changers for genetic biocontrol. These approaches leverage advancements in genetic manipulation to disrupt or suppress populations while minimizing adverse environmental impacts. To engineer these technologies in *C. pomonella*, we first need to identify molecular targets linked to female viability and male fertility. Numerous studies have been conducted to explore the genetic and molecular processes that govern spermatogenesis to identify potential targets for gene-specific treatments for infertility[27,28]. The testicular-specific serine/threonine protein kinases (*TSSKs*) are exclusively expressed after sperm cell meiosis, and their distinct patterns of expression across developmental stages and in various tissues suggest their involvement in the regulation of spermatogenesis[29,30]. For example, experiments delving into the suppression of *TSSK1* in *Drosophila melanogaster* and *Zeugodacus cucurbitae* have unveiled a significant decline in sperm viability and an adverse impact on male fertility, culminating in diminished egg hatching rates[31,32]. Similarly, the use of CRISPR/Cas9 technology to knockout the serine protease 2 (*ser2*) gene in *Bombyx mori* has resulted in the inability of sperm and eggs to fertilize, ultimately leading to infertility in subsequent generations[33]. Furthermore, the reduction of the *tektin* gene, which is associated with sperm flagella, has been found to cause a significant decrease in the hatching rate of offspring[31]. These findings underscore the pivotal role of testis-specific genes in male reproduction, albeit the precise regulatory mechanisms remain ambiguous.

Eukaryotic gene expression has traditionally been assessed through steady-state mRNA levels. However, this fails to consider translational activation variability or the stability of specific mRNA types. Consequently, post-transcriptional regulation plays a pivotal role in the overall coordination of gene expression[34]. It is important to highlight that the maturation of insect sperm involves extensive post-transcriptional regulation, whereby a significant number of transcripts are preserved for translation at a later stage[35]. In recent years, long non-coding RNAs (lncRNAs) have emerged as crucial factors in post-transcriptional regulation[36,37]. These lncRNAs play a significant role in regulating gene expression through various mechanisms, including chromatin modification, RNA decoying, transcriptional co-activation, ribonucleoprotein complex formation, and microRNA sequestration[38–40]. LncRNAs exhibit a multifaceted modus operandi involving various regulatory modalities, encompassing direct inhibition of gene expression by lncRNAs, as well as their role as competitive endogenous RNAs (ceRNAs), sequestering microRNAs (miRNAs) to modulate the expression of messenger RNAs (mRNAs)[37]. Notably, the knockdown of 128 testicle-specific lncRNAs in fruit flies demonstrated that silencing nine of these lncRNAs led to sterility or reduced fertility[41]. The *lnc94638* is crucial for spermatogenesis in *Z. cucurbitae* and significantly affects male fertility[42]. Therefore, further investigation of the post-transcriptional regulatory mechanisms involved in spermatogenesis is necessary to advance the development of male sterile technology.

The invasive agricultural pest of *C. pomonella*, has been listed among the world's 20 most resistant pests to insecticides by the insecticide resistance action committee (IRAC)[43]. To address resistance problem, the use of SIT as part of area-wide integrated pest management (AW-IPM) has been proposed as an environmentally friendly control technology[12]. Previous studies have shown the effectiveness of SIT in controlling *C. pomonella*[15]. However, the most commonly used radiation-based sterile technology (rSIT) technology is not aligned with the current trend of green agriculture development due to issues such as cobalt source waste, and radioactive safety hazards[33]. To explore the concept of genetic SIT for pest control, this study focused on investigating genes related to male reproduction. Through genome and testis transcriptome analysis, five *TSSKs* genes were identified. To understand the functional role of *TSSKs*, an RNA interference (RNAi) and CRISPR/Cas9-based loss-of-function approach was employed, comparing *C. pomonella* mutant and wild-type (wt) individuals. The study revealed that *TSSKs* are highly expressed in the testes of adult males. Surprisingly, disruption of the *TSSKs* genes in males resulted in sterility, reduced sperm motility, decreased sperm counts, and abnormal development of fertilized eggs. Furthermore, analysis of the lncRNA transcriptome identified as lnc17962 significantly expressed in testis. Notably, lnc17962 was found to competitively bind with miR-3960, regulating the expression of *TSSKs* and ultimately causing sterility in offspring. These findings emphasize the crucial role of *TSSKs* and lnc17962 in sperm function and suggest their potential as targets for the applications of gSIT technology in *C. pomonella* control.

Results

TSSKs are specifically expressed in male testis of *C. pomonella*

During the larval stage, the *C. pomonella*, like other Lepidopteran insects, possesses two separate testes. However, as the insect enters the prepupal stage, two separate testes gradually move closer to each other and fuse to form a single mature testis in the adult stage (Fig. 1A). To investigate the genes that are specifically expressed in the testes of adult males and their role in mating, RNA-seq was carried out on the testes of male *C. pomonella* at two developmental stages: the second day (presexual maturity, CpT2D) and the fifth day (postsexual maturity, CpT5D). The entire male body, excluding the testes, served as the control groups (CpT2DC and CpT5DC, respectively). Comparative analysis revealed differential expression of 13,095 differentially expressed genes (DEGs) between presexual maturity and control samples, with 1,507 DEGs uniquely expressed in CpT2D compared to CpT2DC. Similarly, 12,882 DEGs exhibited differential expression, including 1,641 DEGs exclusively expressed in CpT5D when compared to CpT5DC. Notably, 7 DEGs displayed unique differential expression solely in the CpT2D versus CpT5DC treatment comparison (Fig. 1B, S1A, S1B). Furthermore, GO enrichment analysis highlighted the crucial role played by the differential genes (Table S1, Fig. S1C, S1D). Among DEGs, the genes responsible for the testicle-specific serine/threonine protein kinase (*TSSK*), were significantly enriched in 7 BP and 4 MF processes, suggesting a potential role in regulating spermatogenesis (Table S1, Figs. S1C and 1D). Notably, the testes exhibited high expression of serine protease genes (Figure S2), tektin (Figure S3A), cyclin (Figure S3B), ubiquitin (Figure S3C), and tubulin (Figure S3D). These DEGs may serve as potential candidates for modulating the fecundity of male *C. pomonella*. Members of the *TSSK* gene family, including *TSSK1*, *TSSK1a*, *TSSK2*, *TSSK2a*, and *TSSK4*, were selected for further investigation to elucidate their role in spermatogenesis. Through phylogenetic analysis, it was observed that TSSK2 and TSSK4 formed a cluster with other lepidoptera species, while TSSK1, TSSK1a, and TSSK2a displayed a long evolutionary relationship with other Lepidoptera species (Fig. 1C; Table S2). Collinearity analysis further supports their presence in Lepidoptera (Fig. S4). Multiple sequence comparisons confirmed the conservation of TSSK residues in the S-TKc region, as well as the adenosine triphosphate (ATP) and substrate binding domains (Fig. 1D). Additionally, RT-qPCR analysis of *TSSKs* revealed a moderate level of expression during the pupal stages, which significantly increased upon reaching sexual maturity. Notably, *TSSKs* were exclusively expressed in the male testis, with *TSSK4* displaying the highest expression level during the developmental stages (Fig. 1F). These findings suggest that these testis-specifically expressed *TSSKs* play an important role in the male fertility of *C. pomonella*.

Loss function of *TSSKs* influence male fertility of *C. pomonella*

Following a 48 h period of injection, the efficiency of RNAi was observed to increase from 37.32% (Fig. S5) to 86.09% (Fig. 2A), suggesting that double injections method enhances the interference efficiency over time. Upon injecting adult subjects with dsTSSKs and conducting subsequent

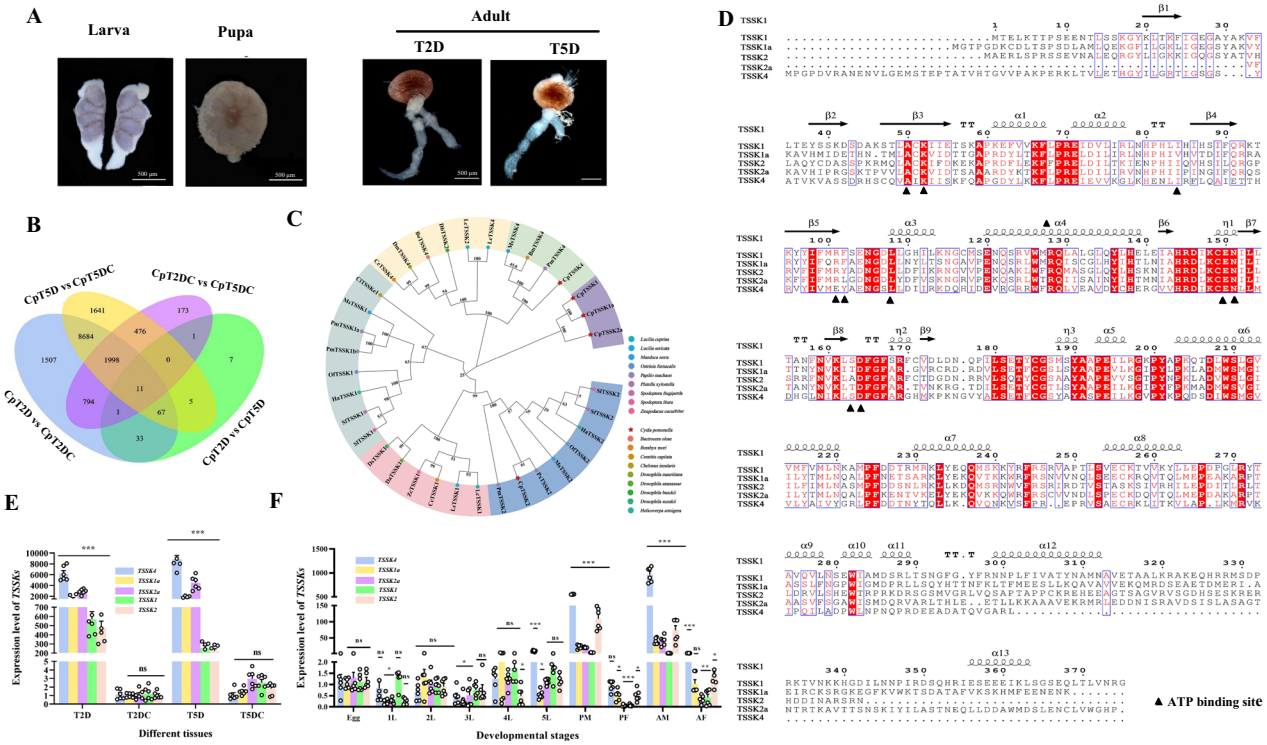

Fig. 1 | Identification of testis specific expression genes of *C. pomonella*. **A** Testis development dynamics; T2D: testes of male adult 2 days old after emergence; T5D: testes of male adult 5 days old after emergence. **B** The number of differentially expressed genes (DEGs) in the testes compared with the whole body of adult males excluding the testis. 2TDC: whole body of 2 days old adult males excluding the testis; 5TDC: whole body of 5 days old adult males excluding the testis. **C** Phylogenetic analysis of TSSK gene family of *C. pomonella* and other insects. **D** Multiple amino acid sequence alignment of TSSK family proteins. ATP binding sites are respectively represented with black triangles. **E** RT-qPCR verification of *TSSKs* expression in different tissues of *C. pomonella*. **F** RT-qPCR verification of *TSSKs* expression in development stages of *C. pomonella*. Data is mean ± SD. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns not significant).

observations after 24 h, it was found that the expression levels of *TSSK1*, *TSSK1a*, *TSSK2*, *TSSK2a*, and *TSSK4* were significantly reduced by 56.13% ($p = 0.001$), 86.09% ($p < 0.001$), 42.67% ($p = 0.038$), 72.08% ($p < 0.001$), and 75.62% ($p < 0.001$), respectively, compared to control treatment with dsGFP (Fig. 2A). Although the number of eggs produced by the females mated with dsTSSKs treated males remained unchanged (Fig. 2B), the hatching rate of these eggs was significantly lower compared to dsGFP treatment (Fig. 2C). The sterility rates of *TSSK1*, *TSSK1a*, *TSSK2*, *TSSK2a*, and *TSSK4* knockdown lines were found to be 90.61%, 83.93%, 100%, 100%, and 100%, respectively (Fig. 2C). The unhatched eggs exhibited progressive desiccation during development, with no evidence of reaching the blackhead stage (Fig. 2D). To ascertain whether the underdeveloped eggs were a result of lack of fertilization in the female, a spermatophore examination was conducted, ruling out this possibility (Fig. S6). Sperm viability was then assessed in both the dsGFP and dsTSSKs treatment groups, revealing a higher number of dead eupyrene and apyrene sperm bundles in the dsTSSKs treatment group compared to the dsGFP group (Fig. 2E). In males, the number of spermatozoa was reduced by 28.61% (dsTSSK1, $p = 0.0014$), 63.03% (dsTSSK1a, $p < 0.001$), 39.00% (dsTSSK2, $p = 0.0\,030$), 46.77% (dsTSSK2a, $p < 0.001$), and 30.38% (dsTSSK4, $p = 0.0024$) in the dsTSSKs treatment groups compared to the control (Fig. 2F).

In addition, we employed the CRISPR/Cas9 gene editing system to induce mutation in the TSSK genes (Fig. 2G), which led to deletions at the desired location (Fig. 2H, I). The mutation rate of the TSSK genes varied from 9.38% to 12.46% (Table S4), and these genetic modifications did not have any impact on the insect's developmental period (Table S5). When TSSKs$^{-/-}$ males were mated with wt females, the egg-laying levels were similar to the results of RNAi (Fig. 2J). However, a significant decrease in hatchability, consistent with the results of RNAi approach, was observed (Fig. 2K). The findings from the dsTSSK2a and TSSK2a-KO studies were not consistent, potentially owing to the fact that depletion of nucleotide bases in multiples of three in TSSKs$^{-/-}$ males did not result in protein frameshift. Additionally, the limited effect of the absent amino acids on functionality suggests that there was no significant compromise to the operational efficacy of TSSK2a. Most embryos derived from TSSKs$^{-/-}$ males failed to develop properly, even after seven days following ovulation. Consequently, TSSKs$^{-/-}$ males exhibit normal sexual behavior but are ultimately sterile.

Lnc117962 is specifically expressed in male testis of *C. pomonella*

The male *C. pomonella*' internal reproductive system encompasses the testis, accessory gland, seminal vesicles, and vas deferens (Fig. 3A). The testis holds significant importance as it is the primary organ responsible for spermatozoa production and sustenance, ensuring their nourishment and protection during development. Consequently, our focus was on conducting lncRNA transcriptome sequencing specifically on testis tissues. By analyzing the Venn diagram, it was found that 6361 lncRNAs were specifically expressed in the testis (CpT1D vs CpT1DC). Furthermore, the testis exhibited specific expression of 7390 lncRNAs in CpT3D compared to CpT3DC, with 4430 lncRNAs being expressed in both CpT1D vs CpT1DC and CpT3D vs CpT3DC (Fig. 3B). By employing a log2fold change > 5.00 as the screening parameter, twelve lncRNAs exhibiting differential expression were identified. Their tissue-specific expression patterns across various developmental stages were validated using RT-qPCR. The RT-qPCR results demonstrated that all 12 lncRNAs exhibited high expression levels in the testis (Fig. 3C) while displaying lower expression levels in the vas deferens, accessory glands, and seminal vesicles (Fig. 3D–F). Notably, lnc117962 showed the highest expression level in the testes, with subsequent decreased expression as sperm matured and shifted (Fig. 3G). Furthermore,

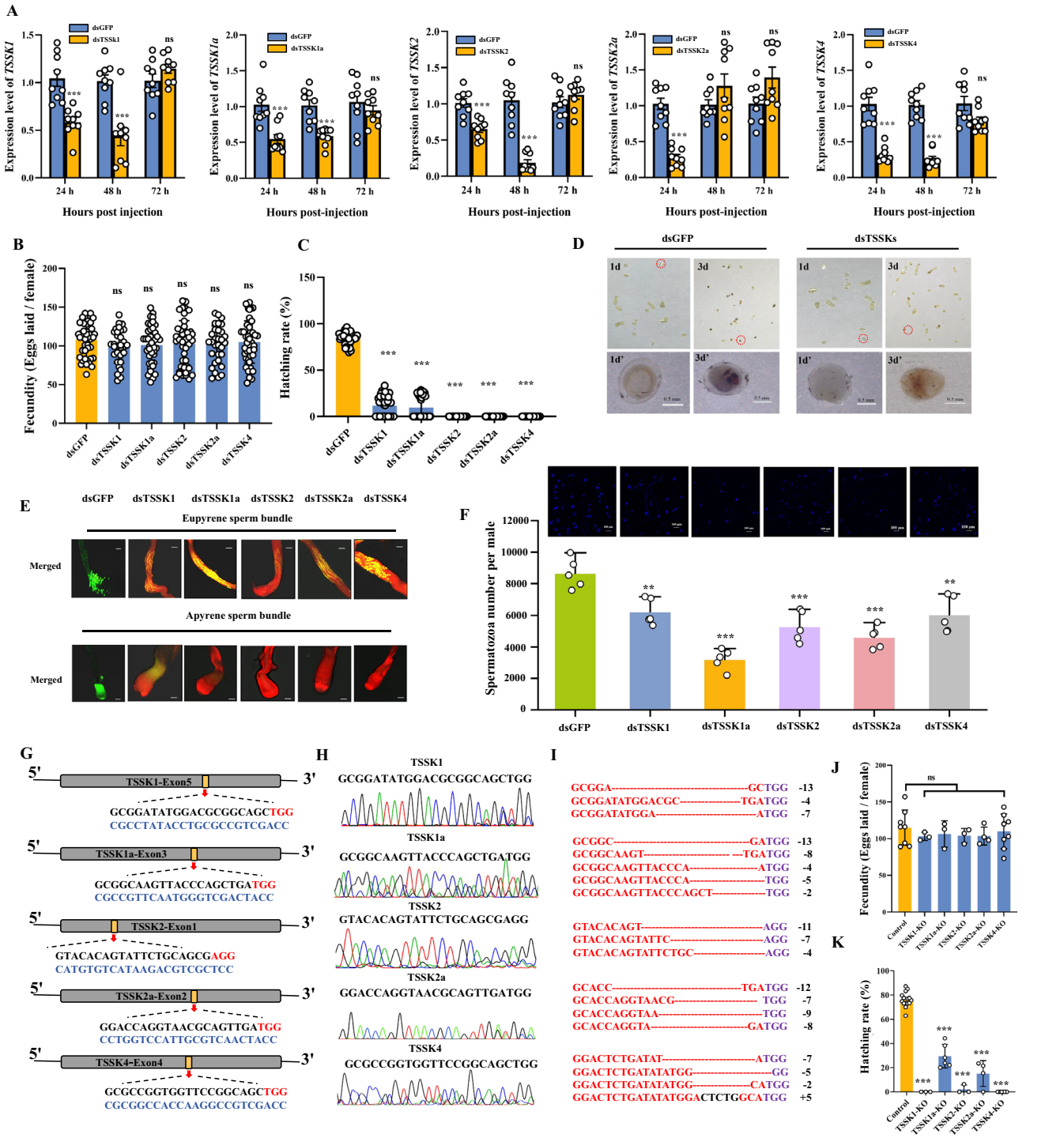

Fig. 2 | *TSSKs* in males limit fecundity and fertility. A The expression level of *TSSKs* after RNAi knockdown 24 h, 48 h, and 72 h. **B** Analysis of female fecundity after RNAi knockdown of *TSSKs*. **C** Analysis of fertility after RNAi knockdown of *TSSKs*. ($n = 45$ for dsGFP; $n = 33$ for dsTSSK1; $n = 45$ for dsTSSK1a; $n = 45$ for dsTSSK2; $n = 32$ for dsTSSK2a; $n = 45$ for dsTSSK4). **D** The effect of TSSKs knockdown on the developmental dynamics of eggs in *C. pomonella* is being investigated. **E** The live and dead eupyrene/apyrene sperm bundle per male were observed after injection dsTSSKs. Green indicated live sperm bundles, red indicated dead sperm bundles. **F** Analysis the number of spermatozoa after the RNAi knockdown. **G** Schematic of TSSKs gene structure and sgRNA target sites. The gray box indicates the exon. Red arrows indicate the target sites of sgRNA. The target sequence and PAM sequence are highlighted in black and red, respectively. **H** Representative chromatograms of direct sequencing of the PCR products for genotyping the TSSKs mutation. **I** Verification of TSSKs$^{-/-}$ mutations by Sanger sequencing. PCR products spanning the target site were amplified from gDNA of TSSK$^{-/-}$ mutations and subjected to Sanger sequencing. The target sequence was in red font, while the PAM sequence was shown in purple, deleted bases are underlined in red. **J** The effect of TSSK-KO on fecundity. **K** The effect of TSSK-KO on hatching rates. The asterisks on the bars indicate the significant differences analyzed by the independent samples *t*-test. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns not significant).

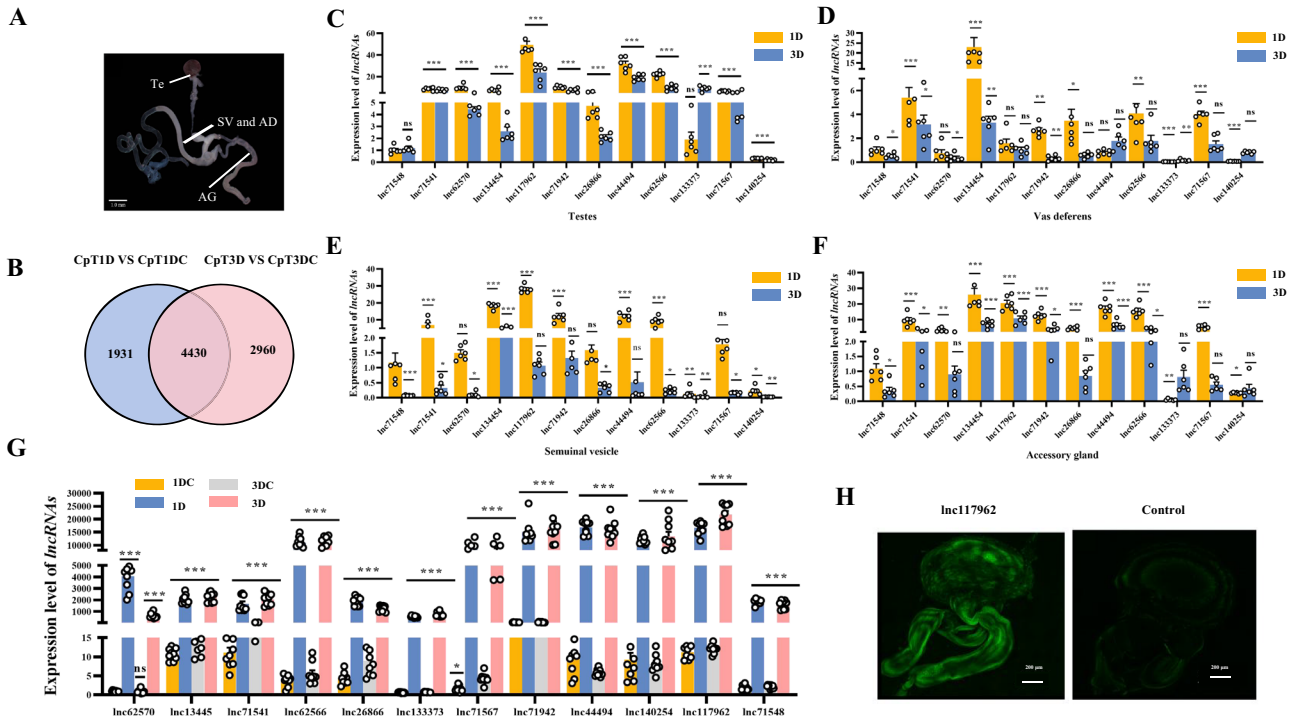

Fig. 3 | Identification of testis-specific expression of lncRNA. A Reproductive system of *C. pomonella*. 1: testis; 2: accessory gland; 3: seminal vesicles and vas deferens. **B** The number of differentially expressed genes (DEGs) in the testis compared to body from adult males of *C. pomonella* excluding the testis. RT-qPCR verification of 12 testis-specific lncRNAs expression in testes (**C**), vas deferens (**D**), seminal vesicles (**E**) and accessory gland (**F**) and development stages (**G**) of *C. pomonella*. All data were normalized using the log2 ratio. **H** Localization of lnc117962 in the testis of *C. pomonella* by fluorescence in situ hybridization; D1, D3: first and third-day adult female. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns not significant).

fluorescent in FISH revealed a localization signal in the testes of *C. pomonella* (Fig. 3H; Fig. S8). Results from Gene Ontology (GO) pathway analysis suggest that lnc117962 has the potential to modulate various gene enrichments associated with ATP activity and serine/threonine protein kinase pathways. This indicates that lnc117962 might play a crucial role in regulating the expression of TSSKs and could be significant in the post-transcriptional control of male fertility (Fig. S7).

Loss function of lnc117962 influences male fertility of *C. pomonella*

The role of lnc117962 in the regulation of male fertility was investigated using RNAi technology. Silencing lnc117962 with ds117962 resulted in a 74.83% efficacy after 48 h (Fig. 4A). Assessment of *TSSK* family gene expression levels following ds117962 treatment showed reductions of 71.94%, 42.87%, 47.96%, 91.58%, and 63.36% compared to dsGFP control (Fig. 4B). Overexpression of lnc117962 with pcDNA3.1-lnc117962 resulted in a 2.47-fold increase in lnc117962 expression compared to pcDNA3.1-GFP (Fig. 4C). Additionally, *TSSK1*, *TSSK1a*, and *TSSK4* expression decreased by 39.28%, 26.61%, and 19.93%, respectively, while *TSSK2a* expression increased by 1.26-fold with no significant change in *TSSK2* expression (Fig. 4D). Fertility response to dslnc117962 treatment was assessed based on fertilization and hatching rates, showing no significant difference in egg laying, but a 28.50% reduction in hatching rate (Fig. 4E, F). Male longevity was unaffected (Fig. S9). The insignificance of the overall fitness of dslnc117962 knockdown males was expected and assessed through a mating competitiveness assay. The dslnc117962 treatment group displayed a higher presence of deceased sperm in eupyrene (Fig. 4G) and apyrene sperm (Fig. 4H) bundles compared to the control group, indicating the involvement of lnc117962 in *C. pomonella* male spermatogenesis and its impact on in male fertility. Upon conducting the mating competitiveness assay, it was observed that dslnc117962 knockdown males exhibited courtship abilities, successful mating behaviors, and competitive interactions with wt females. Notably, the egg hatch rate was determined to be 54.47% ± 15.96% for a pairing of one wt male, one dslnc117962 knockdown male, and one wt female, in contrast to 75.26% ± 4.28% for a pairing of one wt male and one wt female, and 25.52% ± 9.55% for a pairing of one dslnc117962 treated male and one wt female, confirming the mating competitiveness (0.84) of dslnc117962 knockdown males (Fig. 4I; Table 1). Furthermore, the mating performance of lnc117962 knockdown males was assessed through field cage experiments conducted in the first two weeks of June 2023, under controlled humidity and temperature conditions (Fig. S10). Results obtained from the apple orchard (Fig. 4J) indicated no significant disparity in egg production between 10 wt females mated with 10 dslnc117962 knockdown males and those mated with 20 wt males (Fig. 4K). However, the hatch rates were 39.91% ± 7.46% and 83.13% ± 2.70%, respectively (Fig. 4L). Notably, the hatching rate of the F1 generation was determined to be 67.56 ± 5.41% ($p = 0.0029$), suggesting that the RNAi treatment predominantly affected the parents and had a lasting impact on the offspring (Fig. S11). These results indicate that lnc117962 regulates the activity of *TSSKs* through post-transcriptional mechanisms, exerting a suppressive effect on the population development of *C. pomonella*.

Lnc117962 regulates *TSSKs* through competitive bindings to miR-3960

In order to further elucidate the regulatory mechanism of lnc117962 on *TSSKs* gene expression, we predicted its potential miRNA partner. The miR-3960 emerged as the top candidate among the predicted targets (Table S6; Fig. S12), suggesting its possible interaction with lnc17962. The resemblance in their seed sequences indicates that miR-3960 could plausibly act as a target of lnc117962 (Fig. 5A). To confirm this interaction, a dual luciferase assay demonstrated a 44.33% reduction in luciferase activity in cells transfected with miR-3960 and pmirGLO-lnc117962 mimics compared to mimic negative control (Fig. 5B). Additionally, computational tools including miRanda, PITA, and RNAhybrid were utilized to predict the binding potential of miR-3960 to the 3'UTR of *TSSKs*, revealing it capability

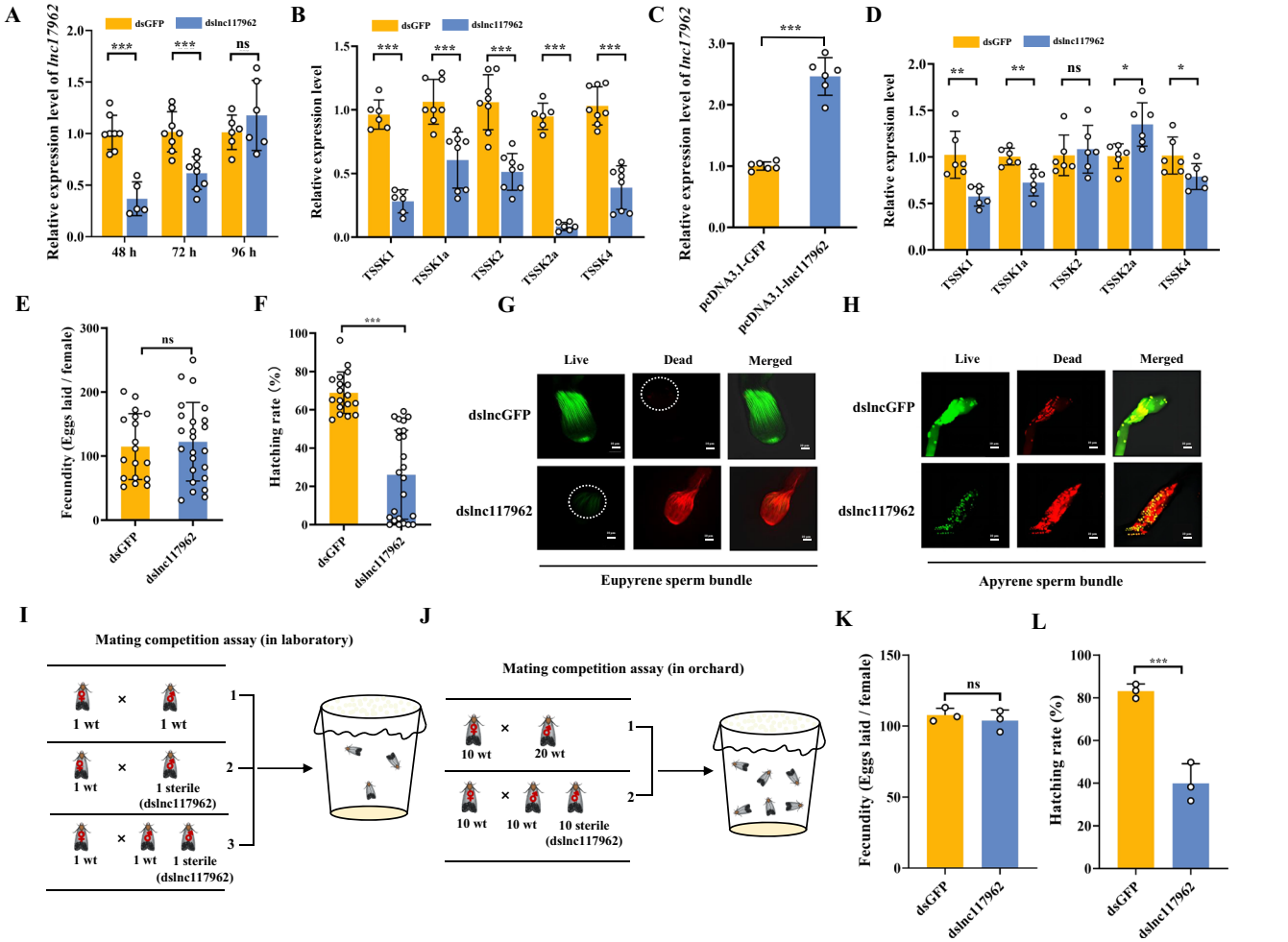

Fig. 4 | Lnc117962 in males limit fecundity and fertility. A The expression level of *lnc117962* after RNAi knockdown 48 h, 72 h, and 96 h. **B** The expression level of *TSSKs* after RNAi knockdown of *lnc117962*. **C** Effect of pcDNA3.1-lnc117962 injection on the relative expression level of *lnc117962* in *C. pomonella*. **D** Effect of pcDNA3.1-lnc117962 injection on the relative expression level of *TSSKs* in *C. pomonella*. **E** Analysis of female fecundity after RNAi knockdown of *lnc117962*. **F** Analysis of fertility after RNAi knockdown of *lnc117962*. ($n = 18$ for dsGFP; $n = 24$ for lnc117962). **G** The number of eupyrene sperm bundle per male were observed after injection ds117962. **H** The number of apyrene sperm bundle per male were observed after injection ds117962. Dead and live sperm bundle are indicated by red and green, respectively. **I** An experimental design to assess the competitiveness of ds117962 males competing with wt males. The competitiveness of ds117962 males were assessed by hatching rate. **J** An experimental design to assess the competitiveness of ds117962 males in the cage. **K** Analysis of female fecundity of ds117962 males competing with wt males. **L** Analysis of fertility of ds117962 males competing with wt males.The asterisks on the bars indicate the significant differences analyzed by the independent samples *t*-test. ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns not significant).

Table 1 | Effect of dslnc117962 knockdown males on the mating competitiveness of *C. pomonella*

Matching ratio (wtM:lnc117962KM:wtF)	Egg laid per female	Hatching rate (%)	competition mating index (C)
1:0:1	103.33 ± 18.67 a	75.26 ± 4.28 a	0.84
1:1:1	101.00 ± 17.59 a	54.47 ± 15.96 b	
0:1:1	105.40 ± 22.64 a	25.52 ± 9.55 c	

Lnc117962 knockdown males (lnc117962KM), wild-type males (wtM) and wild-type females (wtF) were introduced for mating in the ratios 0:1:1, 1:0:1 and 1:1:1, respectively. A total of 15 replicate experiments were evaluated. The table shows mean ± standard deviation (SD) data. Letters following data indicate significant differences analyzed by one-way analysis of variance (ANOVA) using Duncan's test ($p < 0.05$).

to interact with the 3'UTR of *TSSKs* (Table S7; Fig. S13). Further analysis was conducted to ascertain if lnc117962 functions as a "sponge" or decoy ceRNA of miR-3960. The synthesis of agomir/antagomir-miR-3960 and subsequent RT-qPCR assessment of miR-3960, lnc117962, and TSSKs revealed noteworthy. Following the injection of agomir-miR-3960, the expression level of miR-3960 increased by 3.4 times, along with a significant increase in the expression level of lnc117962, *TSSK1a*, and *TSSK2a*, while *TSSK1*, *TSSK2*, and *TSSK4* levels decreased ($P < 0.05$, Fig. 5C). Conversely, antagomir-miR-3960 injection led to reduced expression levels of miR-3960, lnc117962, *TSSK1*, *TSSK1a*, *TSSK2a*, and *TSSK4* ($P < 0.05$, Fig. 5D). These findings indicate that lnc117962 acts as a ceRNA by suppressing miR-3960, thereby positively regulating the expression of the *TSSK2a* (Fig. 5E). However, further research is required to explore the regulatory mechanisms of *TSSK1*, *TSSK1a*, *TSSK2*, and *TSSK4*.

Discussion

The utilization of SIT has been proven to be effective and eco-friendly for managing the invasive pest, *C. pomonella*[13,15]. In this study, we discovered

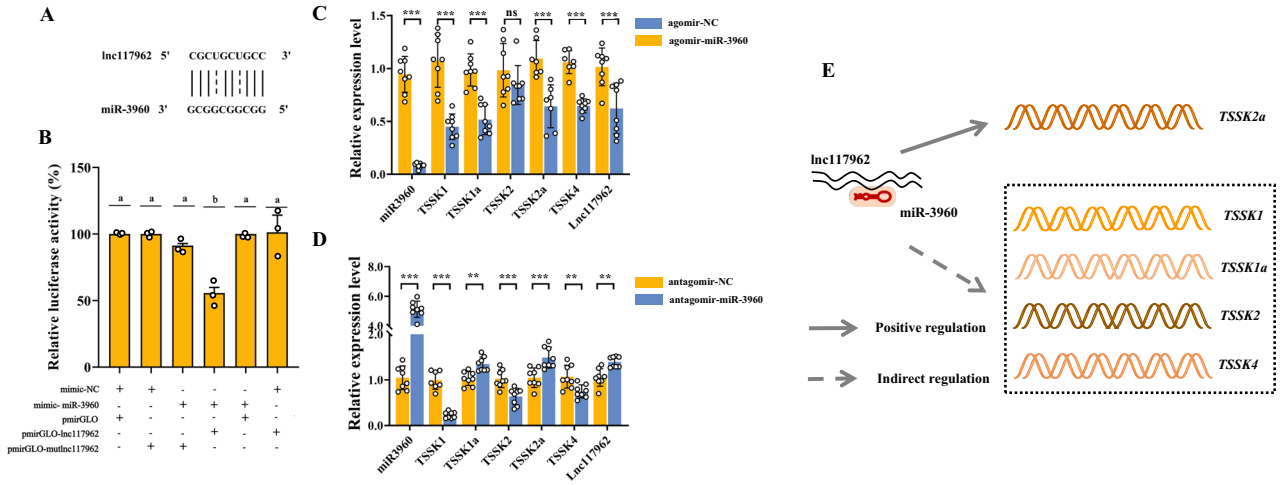

Fig. 5 | Lnc117962 regulates TSSKs through competitive bindings to miR-3960. **A** Target prediction of lnc117962. **B** Validation of the interaction of miR-3960 and lnc117962 by luciferase assay. NC: negative control of miR-3960 mimics; miR-3960 mimics: 293Tcells that were co-transfected with miR-3960 and pmiGlo-lnc117962; miR-3960 mutant: 293Tcells that were co-transfected with mutant mimics of miR-3960 and pmiGlo-lnc117962. Data were presented as the relative ratio of firefly to Renilla luciferase activity. Significance analysis was conducted with independent samples *t*-test. **C** Effect of miRNA agomir injection on the relative expression of lnc117962 and *TSSKs* in *C. pomonella*. **D** Effect of miRNA antagomir injection on the relative expression of lnc117962 and *TSSKs* in *C. pomonella*. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns not significant). **E** Schematic diagram of *TSSKs* post-transcriptional regulation. Three independent experiments were performed.

TSSKs genes which are exclusively expressed in the testis, serving as molecular targets for the development of the gSIT strategy in managing this invasive pest.

Spermatogenesis is a meticulously orchestrated process involving a series of maturation stages that induce notable structural and biochemical changes in spermatogonia within the mature testis[44,45]. This intricate process encompasses both mitotic and meiotic divisions, alongside cellular remodeling throughout the cell cycle[28]. This remodeling necessitates the synchronized activation and deactivation of specific serine/threonine protein kinase (s) that regulate the recombination of sperm chromatin[45,46]. Previous studies consistently affirms the pivotal role of serine/threonine protein kinases in spermatogenesis[44]. The *TSSKs* belong to the 5'-adenosine monophosphate-activated protein kinase (AMPK) family, characterized by the serine/threonine protein kinase catalytic (S-TKc) domain. Although typically comprising five members, *TSSK1*, *TSSK2*, *TSSK3*, *TSSK4*, and *TSSK6*, the exact number of *TSSK* family members in insects remains undisclosed. In this study, 5 *TSSK* genes were identified in the genome of *C. pomonella*, namely *TSSK1*, *TSSK1a*, *TSSK2*, *TSSK2a*, and *TSSK4*. The emergence of *TSSK1a* and *TSSK2a* as novel *TSSK* copies in distinct regulatory contexts may elucidate the substitution of *TSSK3, TSSK5*, and *TSSK6* within this group[47]. Shang et al.[48] posit that the greater frequency of sequence mutations in the C-terminal domain of *TSSK1/TSSK1B* compared to *TSSK2* could be attributed to positive evolutionary selection, potentially influenced by variations in protein partner and substrate preferences. The C-terminal domain plays a crucial role in determining the accurate spatial configuration of the serine/threonine protein kinase activity[49], facilitating the transfer of γ-phosphate residues from ATP to the hydroxyl group of serine, threonine, or tyrosine residues on the target protein[50]. Through bioinformatics analysis, it was revealed that *TSSK1*, *TSSK1a*, *TSSK2*, *TSSK2a*, and *TSSK4* genes exhibit homology, with a highly conserved S-TKc domain. All TSSK members have essential lysine residues crucial for ATP binding, as well as conserved aspartate residues vital for catalytic function. Phylogenetic analysis supports the close relationship between *C. pomonella* TSSKs and other Lepidoptera TSSK proteins, underscoring their significant involvement in spermatogenesis.

The progress in genomics, gSIT technology, and the implementation of a large-scale sterile system have become profoundly influential in current research endeavors[16]. Nevertheless, challenges such as off-target effects and the insufficient knockdown efficacy of RNAi have acted as constraining factors, despite the productive application of RNAi in numerous studies on Lepidoptera[51,52]. Enhancing RNAi efficiency in Lepidoptera is essential, the RNAi efficiency of *TSSKs* was successfully improved through double injections during the pupal and adult stages, making the first attempt in *C. pomonella*. In *Rhodnius prolixus* and *Nilaparvata lugens*, double injections of dsRNA increase RNAi efficiency from 38% to 75% and 25% to 50%, respectively, indicating that double injection is a suitable method to improve RNAi efficiency[53,54]. In recent years, the utilization of CRISPR/Cas9 technology has emerged as a prominent method for investigating gene functionality model insects have made significant advancements, particularly through the implementation of the precision-guided sterile insect technique (pgSIT), as evidenced in studies involving *D.melanogaster*[16] and *Anopheles gambiae*[55]. Nevertheless, it is important to recognize that investigations pertaining to gene editing in *C. pomonella* are still in the nascent stages. In 2017, demonstrated that the introduction of a combination of sgRNA and Cas9, targeting the *CpomOR1* gene in *C. pomonella* resulted in notable impacts on fertility, with female moths producing inviable eggs[56]. In this study, we have successfully established a functional *TSSKs* genes system using CRISPR/Cas9. *TSSKs* genes were knocked out through the introduction of a combination of sgRNA and Cas9 protein, resulting in an efficiency rate ranging from 9.38% to 12.46%. Prior studies by Chen et al.[57] and Ye et al.[58] demonstrated knockout efficiencies of 6.5% to 13.9% for opsin genes in *Plutella xylostella* and a 36% efficiency for the pheromone binding protein 1 (*PBP1*) gene in *Helicoverpa armigera*, respectively. However, the effectiveness of this system in facilitating targeted gene disruption varies significantly among species due to species-specific distinctions[52]. Research on mice has revealed that mutations in the *TSSK1* and *TSSK2* genes lead to male sterility, attributed to issues in spermatogenesis, absence of elongated spermatids, elevated apoptosis rate, and increased round spermatogenic cells in the epididymis[59]. The loss of *TSSK* expression in mice results in defective sperm cell development, abnormal sperm cell structure, and an inability of these cells to fertilize eggs[60]. In this study, we observed a marked rise in deceased spermatozoa and a decline in both spermatozoa count and offspring fertility following *TSSK* gene dysfunction. These findings align with prior investigations on *B. tryoni* and *B. dorsalis*[31]. Phenotypic abnormalities during spermatogenesis have been also observed, including spermatogonial membrane rupture due to the *TSSK4* mutation and sperm chromosome disarray caused by *TSSK6* mutation in mice[61]. Interestingly, studies on *TSSK* mutants in *D.melanogaster* indicated unsuccessful formation of the individualization complex (IC) during spermatogenesis[10]. Furthermore, the absence of the N-terminal or C-terminal domain of *TSSK*

hindered the typical IC structure formation, resulting in male infertility[16]. Nevertheless, gaps remain in understanding the post-transcriptional regulation of *TSSKs* in spermatids during spermiogenesis.

The application of advanced deep transcript sequencing methodologies has spurred an escalation in the exploration of post-transcriptional regulation in insects[35]. At the post-transcriptional level, lncRNAs and miRNAs play crucial roles as regulators of gene expression. Notably, lncRNAs demonstrate tissues-specific expression, particularly exhibiting higher abundance in the testicular tissues[41]. Studies conducted on *Z. cucurbitae* have identified a considerable number of highly expressed lncRNAs in the testis[55]. Similarly, examination of RNA-seq data in *B. mori* has unveiled a predominant expression of lncRNAs in the testicular tissues[62]. In this study, a comprehensive analysis of lncRNAs expression profiles in the testes of *C. pomonella* was carried out, leading to the identification of an upregulation of a specific lncRNA, named lnc17962. Through FISH analysis, the precise localization of lnc117962 in the apical region of the testis, characterized by the presence of mature spermatozoa, was successfully achieved. This localization pattern mirrors the findings from a prior study on *Bactrocera cucurbitae*, where mature sperm was observed[52]. The involvement of lncRNAs in the development of male germ cells in diverse species has been postulated, with some displaying distinctive expression patterns in fertile and infertile spermatozoa[63]. Notably, the knockout of murine testis-specific lncRNA *Tslrn1* using CRISPR/Cas9 technology resulted in a significant decrease in spermatozoa count[64]. In this study, RNAi was utilized to silence lnc117962, resulting in a marked reduction in sperm count and diminished embryo hatching rate compared to the control group. The findings presented in this study provide additional evidence supporting the involvement of lnc117962 in the spermatogenesis process in *C. pomonella*. Furthermore, the depletion of *CR42858*, a testis-specific lncRNA in *Drosophila*, led to a decline in sperm count and male fertility[41]. The findings reported in *B. dorsalis* also indicate impaired male fertility, possibly due to defective spermatogenesis and reduced sperm count[31]. It is widely recognized that many lncRNAs have binding sites for miRNA[65]. This observation suggests that numerous lncRNA may regulate gene expression by sequestering miRNAs, thereby reducing their cellular concentration[66]. Known as the "competing endogenous RNA (ceRNA)" hypothesis, this mechanism proposes that lncRNAs act as negative regulators of miRNA activity, ultimately promoting gene expression[67]. Additionally, our research indicates that lnc117962 can act as a ceRNA for miR-3960, controlling the expression of *TSSK2a* and impacting spermatogenesis in *C. pomonella*. Interestingly, this regulatory pattern resembles the reproductive mechanism involving lncR26319/miR-2834/EndophilinA in *B. mori*[68], suggesting a potential universality of this regulatory mode. It is noteworthy that miR-3960 has been found to directly regulate the expression of *TSSK1a*, although other factors may be involved in the regulatory process of lnc117962 on *TSSK1a*. While our study identifies lnc117962 as a ceRNA of miR-3960, the precise mechanism by which it regulates *TSSK1*, *TSSK2*, and *TSSK4* remains unclear, and there may be multiple pathways through which their expression is controlled.

The conventional irradiated-based SIT is known to generate sterile males with reduced mating competitiveness as a result of mutations across multiple genes[69]. Consequently, an alternative approach focusing on the production of highly competitive sterile males has emerged as an appealing strategy for managing *C. pomonella* populations[29]. In this study, we evaluated low-fertility males exhibiting comparable traits to wt males under both laboratory and apple orchard settings, following the suppression or elimination of *TSSK* genes. The findings indicate that these males cold potentially possess the same level of safety and resilience as wt counterparts. Furthermore, unlike the previous SIT method where maintaining sterile males as homozygous lines was challenging, mutants with *TSSKs* could generate offspring with decreased hatchability rates. This parallels the disruption of leucine aminopeptidase 1 in *Aedes aegypti*, resulting in reduced hatching rates among descendants while preserving their competitiveness edge relative to wt males[70]. Ideally, genetic modifications induced by sterility-inducing genes should naturally disseminate through pest populations without necessitating continuous mass releases of sterile insects[71]. Consequently, mating with $TSSKs^{-/-}$ males offers an effective means to propagate genetic alterations within *C. pomonella* populations through a reduced number of viable offspring. As a result, successful population management was achieved through the utilization of $TSSKs^{-/-}$ males in mating scenarios.

While the safety of CRISPR/Cas9 technology is well-established, necessitating genetic modification, the use of CRISPR-edited insects carries the potential to influence entire populations and ecosystems[72]. Prior to the release of CRISPR-edited insects, thorough risk assessments must be conducted to mitigate non-target effects and prevent unintended ecological impacts[73]. Additionally, in cases where gene flow between the target species and other organisms is possible, there exists a risk of transferred modified sequences leading to unfavorable traits in non-target species[74]. Although this may be perceived as a constraint, it is not expected to pose an insurmountable obstacle once approved[75]. The regulation of pgSIT is anticipated to follow a similar framework as Oxitec's RIDL technology, which has been effectively implemented in various regions, including the United States[17].

Overall, *TSSKs* were identified as promising candidates for gSIT owing to their notable level of conservation, rendering them applicable across various insect species. The utilization of gSIT stands to offer a secure, eco-friendly, and effective approach for managing field pest populations, thereby facilitating the adoption of sustainable agricultural practices.

Materials and Methods

Insect

The experimental insects were reared in a controlled environment with a temperature of 26 ± 1 °C, relative humidity of 60 ± 5% RH, and a photoperiod of 16 h light and 8 h dark. The adult was fed with 10% honey water. A detailed description of the feeding method has been previously described by Wang et al[76].

RNA sample preparation and sequencing

Freshly emerged adult males of *C.pomonella* were placed in an 85 mL plastic cup (40 mm in diameter × 60 mm in height), and testes were collected on the second day (CpT2D) after emergence using phosphate-buffered saline (PBS) at pH 7.5. Controls were established using the body adult male excluding testes (CpT2DC). On the fifth day after emergence, the testes were dissected (CpT5D), the body exclusive of the testes as the control (CpT5DC) as a control. Each sample comprised of tissue from 50 individuals and with a total of three replicates. Total RNA was extracted from the samples using Trizol (TaKaRa, Tokyo, Japan) following the provided instructions. The quality of the RNA samples was assessed using NanoDrop (Thermo Scientific, Wilmington, USA), and the integrity of the RNA samples was evaluated through 1% agar gel electrophoresis. The RNA-seq datasets were obtained and analyzed using the aforementioned four samples.

RNA sequencing

An Illumina TruseqTM RNA sample preparation kit (Illumina, San Diego, CA, USA) was used for library preparation. Before library construction, the quality of the RNA samples was assessed using an Agilent 2100 instrument (Agilent Technologies, Santa Clara, CA, USA). Paired-end sequencing was performed on an Illumina Novaseq 6000 sequencing platform. The library was then subjected to the removal of N-containing error bases and reads of low quality.

Annotation of testis-specific expression genes and quantitative analyses

The clean read was obtained by analyzing the library with the default parameter of the HISAT2 analysis using the reference genome (http://v2.insect-genome.com/Pcg). Clean reads were defined as those that had a unique match with a maximum of two mismatched bases and no deletions or insertions. The quality of each RNA read was evaluated by comparing its localization ratio to the reference genome. Subsequently, RNA sequencing (RNA-seq) was performed on the clean reads and aligned to the

genomes using the expectation-maximization method (RSEM, version 1.3.3). These aligned reads were then used for gene expression analysis. The expression levels of all genes were quantified as transcripts per million (TPM) in repeats. Standardized average TPM values were employed to compare the relative expression of each gene in the tissues. Differentially expressed genes (DEGs) were identified using DESeq (version 1.30.0) based on the criteria of corrected $P < 0.05$ and log2 expression ratio > 2. By comparing gene expression levels across all tissues, genes with high expression in the testes were identified, suggesting their potential role in fertility.

Full-length confirmation and sequence analysis of TSSKs

The complete open reading frame (ORF) of the *TSSKs* genes (Accession of *TSSK1*, *TSSK1a*, *TSSK2*, *TSSK2a*, and *TSSK4* are ON391477.1, XM06185049.1, XM063771875.1, XM061864474.1, XM618678.1) were verified using reverse transcriptase polymerase chain reaction (RT-PCR) with primers designed by Oligo7. Each PCR reaction was conducted in a 20 μL volume, consisting of 10 μL 2x Primer STAR Max Premix (TaKaRa), 7.4 μL RNase-free water, 1.0 μL testicular cDNA, and 0.8 μL (10 μmol L^{-1}) forward and reverse primers (Table S3). The PCR procedure as followings: 98 °C for 2 min, followed by 39 cycles of denaturation at 98°C for 5 s, annealing at 55°C for 15 s, and extension at 72°C for 1 min. Subsequently, electrophoresis analysis on a 1% agarose gel was performed on the PCR-amplified products. The resulting target products were then ligated to the pESI-Blunt simple vector (YEASEN, shanghai, China) and transformed into *Escherichia coli* DH5α. After transformation, the bacteria were cultured in an LB medium, and positive colonies were confirmed through PCR and Sanger sequencing executed by GENEWIZ (Tianjin, China). Homologous protein amino acid sequences from *TSSKs* and other insect species were obtained using NCBI BlasP, and sequence comparison analysis was conducted using DNAMAN. The results were visualized graphically using espript3 (https://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). Additionally, a phylogenetic tree was generated using MEGA11.0 software with the neighbor-joining (NJ) method and 1000 bootstrap replicates for increased reliability of the results.

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis of *TSSKs* in *C. pomonella*

The expression of the *TSSKs* genes was validated through real-time quantitative polymerase chain reaction (RT-qPCR). The primer used for this purpose can be found in Table S3. Briefly, in accordance with the procedures outlined in a previous study[77], tissues and stages were collected. Following the extraction of total RNA, the first strand of complementary DNA (cDNA) was synthesized using 1 μg of RNA following the instructions provided by the manufacturer of the PrimeScriptTM RT reagent kit witn gDNA eraser (TaKaRa, Dalian, China). As previously described[77], the qPCR reaction was conducted with a total volume of 20 μL, comprising 10 μL TB Green® Premix Ex Taq™ (TaKaRa, Dalian, China), 1 μL template cDNA, 0.8 μL of each primer (10 μM), and 7.4 μL nuclease-free water. The CFX 384 Real-Time System (Bio-Rad, Singapore) was employed for all qPCR analyses, with the following settings: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 30 s. To ensure the specificity of the amplification reaction within the temperature range of 60 ~ 95 °C, a melting curve analysis was performed on all samples. Each qPCR included three biological replicates, with three technical replicates for each of the following. The expression level of the target gene was normalized with the geometric mean of *EF-1α* and *RP12* using the $2^{-\triangle\triangle ct}$ method[77,78].

Functional analysis of the role of *TSSKs* in male fertility of *C. pomonella*

The dsRNA was synthesized and purified following the protocol described in the T7 RiboMAXTM Express RNAi system (Promega, USA). The concentration of dsRNA was determined using a Nanodrop (Thermo Scientific, Wilmington, USA), while the integrity of the dsRNA was evaluated through 1% agarose gel electrophoresis. Male pupae in the advanced stage of development, distinguished by their dark pigmentation, were selected for the experimental procedure. These pupae were injected with 1 μL of dsRNA at a concentration of 3000 ng/μL. To enhance the durability of RNAi following injection, the same dosage of dsRNA was administered once more after the emergence of the adults (double injections should be administered within a 24 h period). Insects injected with double-stranded green fluorescent protein (dsGFP) were used as negative control. The interference efficiency of the *TSSKs* genes was quantified at 24, 48, and 72 h post-injection, with nine moths selected at each time interval. The experiment included three biological replicates. In addition, sperm quantification was conducted according to established methods[31,42]. Specifically, the testicles of each male were in 100 μL of 1× PBS solution, gently shaken, and then stained with 10 μL of 4',6-diamidino-2-phenylindole (DAPI) for 15 min to stain the nuclei of the resulting sperm solution. The sperm suspension (10 μL) was placed on a slide and the sperm were counted using a Zeiss LSM780 confocal microscope (Zeiss, Jena, Germany). Sperm quantification was performed using Image J software, with the total sperm count derived from averaging the counts from five random microscope fields. The testicles from male moths aged 2 day were dissected in petri dishes containing 2 x HEPES solution (Solarbio, Beijing, China). The motility of sperm was assessed using a sperm motility kit (Thermofisher, Vilnius, Lithuania), which utilizes two fluorescent dyes to differentiate between dead (red propyl iodide) and live (SYBR-14, green glow) sperm cells. Spermatozoa were diluted 5 μL and incubated with 5 μL SYBR-14 working solution (2 μL SYBR-14 stock solution plus 98 μL Hayes solution) on a glass slide at 25 °C for 10 min, followed by 7 min of incubation with propyl iodide. All experiments were performed in triplicate. The 45 male moths injected with dsRNA were placed in an 85 mL plastic cup (40 mm in diameter × 60 mm in height) and allowed to mate with female moths of the same age to lay eggs. The hatching rate of the eggs was subsequently determined.

sgRNA synthesis and embryo microinjection

Following the screening criteria "5-GG-N18-NGG-3" (where N represents one of the four foundations), target locations were selected by the CRISPOR based on a comprehensive score calculation. Template was synthesized by PCR amplification using a set of primers (Table S3) and sgRNA was synthesized using the MEGA shortscriptTM T7 transcription kit (Invitrogen, Carlsbad, CA). The PCR was carried out using PrimeSTAR® Max DNA Polymerase (TaKaRa, Dalian, China). Eggs were promptly collected within one hour of being laid and securely placed on a microscope slide with double-sided adhesive tape. The embryos were then injected using a Leica DM2700 M stereomicroscope (Leica, Berlin, Germany) equipped with a FemtoJet4i microinjector (Eppendorf, Berlin, Germany). The injection involved 300 ng/μL sgRNA and 150 ng/μL TrueCut™Cas9 protein v2 (Thermofisher, Vilnius, Lithuania). All procedures, including embryo collection and microinjection, needed to be completed within 2 h. Following microinjection, the eggs remained attached to the slide and were incubated at 26 °C until hatching, after which they were transferred to an artificial diet.

lncRNA sequencing and analyses of lncRNA expression

Transcriptome sequencing was employed to generate four RNA-seq libraries, which consisted of testes samples from 1-day-old (CpT1D) and 3-day-old (CpT3D), the whole body of the moth from 1-day-old (CpT1DC) and 3-day-old (CpT3DC) excluding the testis. The expression levels of lncRNAs were quantified using the Fragments Per Kilobase of exon model per Million mapped fragments (FPKM) value and Cuffdiff software (version 2.1.1). To identify specific lncRNA expression, a screening process was conducted on the four testis samples, utilizing the criteria of log2FoldChange > 5 and Q-value < 0.05. To validate the expression of 12 candidates testicular-specific lncRNAs, the expression profiles in various tissues were analyzed by RT-qPCR from the tissues including accessory glands, vas deferens, seminal vesicles, and testes of both 1-day and 3-day male adults.

All samples were replicated three times for biological accuracy. Total RNA was isolated using Trizol (TaKaRa, Tokyo, Japan) following the manufacturer's instructions. The cDNA of lncRNA was synthesized using PrimeScript™ RT reagent Kit (TaKaRa, Japan). RT-qPCR primers (Table S3) were designed using Primer 5 software. *EF-1α* and *RPL12* were used as internal reference genes, and data analysis was conducted using the $2^{-\triangle\triangle ct}$ method[77,78].

Fluorescence in situ hybridization

The FAM-labeled probe was synthesized through the in vitro synthesis method and obtained from GefanBio (Shanghai, China). To conduct fluorescence in situ hybridization (FISH) analysis, the testes of 5-day-old male moths were dissected in 1xPBS and fixed overnight at 4 °C using 4% paraformaldehyde fix solution. The WISH in situ hybridization kit (GefanBio, Shanghai, China) was used for this analysis, following the procedure described below: the samples were dehydrated using an alcohol gradient (50%, 60%, 70%, 80%, 90%, 100% alcohol for 30 min), dried with DEPC-PBST (0.1% Tween-20) for 1 h, bleached with 6% H_2O_2 for 1 h, washed three times with PBS, and then washed three times with PBST (5 min/wash). Subsequently, the samples were permeated with protease K at 37 °C for 20 min. Probes (diluted 1000 times) were added and incubated for 48 h. The slides were rinsed three times with the wash solution provided in the kit and stained with DAPI (Sigma, St. Louis, Missouri, USA) for 10 min. Finally, the samples were examined using a Zeiss LSM780 confocal microscope (Zeiss, Jena, Germany).

Functional analysis of the role of lnc117962 in male fertility

The primer design, synthesis, quality detection, and RNAi of lncRNA were executed following the procedures described in the preceding section. The full-length lnc117962 cDNA was subcloned into the expression vector pcDNA3.1, and the expression vector is being constructed by GenePharma (Shanghai, China). These adults were injected with 1 μL of pcDNA3.1-lnc117962. The expression level of *lnc117962* and *TSSKs* were quantified at 48 h post-injection with 15 moths. The experiment was set up with three biological replicates. To begin, male moths were subjected to continuous injection of dsRNA, following which their testes were dissected in petri dishes containing PBS after a 48 h interval. The testes were then punctured using forceps, and 10 μL of motile spermatozoa were expeditiously collected. Subsequently, the motility and hatchability of the spermatozoa were assessed using the aforementioned methodologies.

Mating competitiveness analysis of sterile males

To evaluate the mating competitiveness of lnc117962 knockdown males in the presence of wt males, the latter were chosen as an appropriate control group due to their shared genetic background with the lnc117962 knockdown males. For this purpose, an 85 mL plastic cup (40 mm in diameter × 60 mm in height) was employed to accommodate one wt male, one lnc117962 knockdown male, and one wt virgin female. This configuration, covered with plastic wrap, facilitated the process of egg laying. Likewise, a plastic box (10.4 cm × 17.3 cm × 6.6 cm) containing 10 wt males, 10 lnc117962 knockdown males, and 20 wt virgin male moths, was employed in an apple orchard (41.83'N, 123.57'E) to ensure mating and egg laying. The competition mating index was calculated by analyzing statistical data about fecundity per female and hatching rates (Zhang et al. 2023).

Detection of the interaction of miRNAs with lnc117962 and TSSKs

To identify potential miRNA that may target lnc117962 using pre-existing miRNA, we employed three established miRNA target gene prediction software programs: miRanda, PITA, and RNAhybrid. The final prediction result was obtained by comparing the outcomes from these three software programs, taking into account their individual preferences during the prediction process. The candidate miRNAs were screened to ensure that no more than 2 GUs were matched between 2 and 8 binding positions, and that the absolute critical strength exceeded 25. The mature miRNA sequences were then sent to GenePharma (Shanghai, China) for the design and synthesis of agomir/antagomir, as well as the synthesis of the corresponding stable negative control and miRNA inhibitor negative control. After synthesizing the agomir and antagomir, RNase-free H2O was added to achieve a final concentration of 20 μM. Male adults of newly emerged were collected and injected with agomir and antagomir in their abdomens, using an injection volume of 1 μL/moth. A stable negative control and microRNA inhibitor were injected as a negative control. Total RNA was extracted from randomly selected samples 48 h after the injection. Three biological replicates were obtained for each treatment, and 10 samples were obtained for each replicate. The Mir-X miRNA first-strand Synthesis Kit (Promega, Madison, USA) was used to synthesize the first cDNA of the miRNA, following the provided instructions.

To examine the interaction between miRNA and lncRNA, a dual luciferase reporter system was employed. This experimental procedure adhered to the protocol provided by GenePharma (Shanghai, China). Specifically, a luciferase reporter vector containing the target gene was constructed. Subsequently, lnc117962 and miR3960 were linked to the pmirGLO dual luciferase reporter vector (Promega, Madison, USA). The resulting vector was designated as pmirGLO-Lnc117962-3960-mutant. Well-conditioned 293 T cells were digested, resuspended, and plated in 12-well plates at appropriate cell densities. The cells were then incubated overnight at 37°C. The 293 T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) (1.5 mg/mL glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin). The cells were maintained in a 5% CO_2-saturated humidity incubator at 37 °C. Subsequently, the interaction between miR-3960 and lnc17962 was examined. Four groups of cells were transfected, including (1) pmirGlO-Lnc117962-3960-wt and mimic negative control, (2) pmirGlO-Lnc117962-3960-wt and miR-3960 mimic, (3) pmirGlO-Lnc117962-3960-mut and mimic NC, (4) pmirGlO-Lnc117962-3960-mut and miR-3960 mimic. After 24 h of transfection, the cells were lysed and the fluorescence values of the samples were measured using the Dual-Glo® Luciferase Assay System (Promega, Wisconsin, USA). The relative activity was determined by normalizing to the Renilla luciferase. Three independent experiments were performed, with each sample being replicated three times.

Statistics and reproducibility

The statistical analysis was conducted using JMP 8.0.2 by IBM SPSS Statistics 26. Biological replicates were used to generate statistical means for the purpose of comparisons. *P* values were calculated through a two-sample Student's t-test with unequal variance, where the significance level was set at of $P < 0.05$. The results are presented as the mean value of the triplicates ± standard deviation (SD). Sample numbers and repeat numbers are indicated in experiment methods.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

RNA-seq data have been uploaded to the NCBI database in sequence read archive (SRA) with the accession number PRJNA1102100. All data in this study is available in Supplementary Data or Figshare[79].

Received: 4 April 2024; Accepted: 25 September 2024;
Published online: 04 October 2024

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Acknowledgements

This work was supported by the National Key R&D Program of China (2021YFD1400200). OSA was supported by funding from an NIH award (R01AI51004).

Author contributions

Xueqing Yang conceived and designed experiments, and writing; ZihanWei performed molecular analysis of *TSSKs* and writing; Yaqi Wang performed molecular analysis of lncRNA; Kangwu Zheng Pengcheng Wang and Ronghua Liu performed feeder population; Zhiping Wang performed gene editing experiments; Yuting Li and Ping Gao performed experimental guidance. Omar S. Akbari analyzed data, and writing and proofread manuscript. All authors discussed the results and declared that they had no conflicts of interest.

Competing interests

S.A. is a founder of Agragene, Inc. and Synvect, Inc. with equity interest. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. All other authors declare no competing interests. All other authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42003-024-06961-5.

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Peer review information *Communications Biology* thanks Qiang Xu and the other, anonymous, reviewers for their contribution to the peer review of this work. Primary Handling Editors: Aylin Bircan and Tobias Goris. A peer review file is available.