Identification of miRNAs with potential roles in regulation of anther development and male-sterility in 7B-1 male-sterile tomato mutant

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Abstract

Background: The 7B-1 tomato line (Solanum lycopersicum cv. Rutgers) is a photoperiod-sensitive male-sterile mutant, with potential application in hybrid seed production. Small RNAs (sRNAs) in tomato have been mainly characterized in fruit development and ripening, but none have been studied with respect to flower development and regulation of male-sterility. Using sRNA sequencing, we identified miRNAs that are potentially involved in anther development and regulation of male-sterility in 7B-1 mutant.

Results: Two sRNA libraries from 7B-1 and wild type (WT) anthers were sequenced and thirty two families of known miRNAs and 23 new miRNAs were identified in both libraries. MiR390, miR166, miR159 were up-regulated and miR530, miR167, miR164, miR396, miR8006 and two new miRNAs, miR#W and miR#M were down-regulated in 7B-1 anthers. Ta-siRNAs were not differentially expressed and likely not associated with 7B-1 male-sterility. miRNA targets with potential roles in anther development were validated using 5′-RACE. QPCR analysis showed differential expression of miRNA/target pairs of interest in anthers and stem of 7B-1, suggesting that they may regulate different biological processes in these tissues. Expression level of most miRNA/target pairs showed negative correlation, except for few. In situ hybridization showed predominant expression of miR159, GAMYBL1, PMEI and cystatin in tapetum, tetrads and microspores.

Conclusion: Overall, we identified miRNAs with potential roles in anther development and regulation of male-sterility in 7B-1. A number of new miRNAs were also identified from tomato for the first time. Our data could be used as a benchmark for future studies of the molecular mechanisms of male-sterility in other crops.

Keywords: 7B-1 mutation, Solanum lycopersicum, Male-sterility, Abiotic stress, Another development, Meiosis

Background

The spontaneous 7B-1 mutant in tomato (Solanum lycopersicum cv. Rutgers) is a photoperiod-dependent male-sterile line, where in long days flowers are male-sterile with stamens that are shrunken and produce non-viable microspores [1]. In short days, flowers are fertile and produce normal stamens and viable pollen. A proteomic study suggested that microsporogenesis in 7B-1 breaks down prior to the meiosis in microspore mother cells (MMCs), which was associated with altered levels of several important proteins involved in tapetum degeneration and MMCs development [2]. Compared to the WT, 7B-1 has a higher tolerance to various abiotic stresses, specifically under blue light, is less sensitive to light-induced inhibition (i.e., de-etiolation) of hypocotyl growth, to blue light-induced stomata opening [3], and has an elevated level of endogenous ABA, but less GAs, IAA, and CKs [4–6]. Fellner and Sawhney, [5] demonstrated that there was a defect in blue light perception in 7B-1, which affected hormonal sensitivity and their endogenous levels. This information adds on to the fact that the 7B-1 is a complex mutation with its primary effect still unknown. As a stress-tolerant male-sterile mutant, 7B-1 is a valuable germplasm for hybrid tomato breeding [7].

Recent studies have documented important regulatory functions of sRNAs in plant growth and development.
There are two main types of sRNAs based on their biogenesis: small interfering RNAs (siRNAs), and micro-RNAs (miRNAs). siRNAs are processed from perfectly double-stranded RNA (dsRNA) and comprise different classes; those produced from dsRNA synthesized by RNA dependent RNA polymerase 6 (RDR6) (trans-acting or ta-siRNAs) [8, 9], by RDR2 (heterochromatin siRNAs) [10], or by overlapping antisense mRNAs (natural antisense siRNAs) [11]. While ta-siRNAs target mRNAs similarly to miRNAs, heterochromatin siRNAs cause DNA methylation and/or heterochromatin formation that lead to transcriptional gene silencing [12].

Ta-siRNAs play essential roles in regulating plant development, metabolism and responses to biotic and abiotic stresses [13]. Four families of TAS genes comprising eight loci have been identified in Arabidopsis thaliana, among which miR173 targets both TAS1a/b/c family and TAS2 locus, miR390 targets TAS3a/b/c family, while miR828 triggers the production of TAS4-derived ta-siRNAs [14–16]. TAS3a-derived tasiARFs, 5‘D7(+) and 5‘D8(+), target several ARFs, including ARF2, 3 and 4 [14, 17], which were proposed to act as suppressors in auxin signaling pathway [18]. Recently, a fifth TAS family (TASS) was identified from tomato, which is triggered by miR482 [19].

Plant miRNAs are typically 21 nucleotides (nt) long, which are derived from single-stranded RNA transcripts that have the ability to fold into imperfect stem-loop secondary structures. These hairpins are processed by the ribonuclease III-like enzyme Dicer (DCL1) into miRNA/miRNA* duplexes. One of the strands of the miRNA/miRNA* duplex (usually mature miRNA) is incorporated into the RNA induced silencing complex (RISC), which guides the RISC to recognize target mRNA based on sequence complementarity. Mature miRNAs suppress the expression of their target genes by cleavage of the target mRNAs or translational repression [20]. Many plant miRNAs are conserved among species and have been implicated in processes, such as development, signal transduction, abiotic stress tolerance, and resistance to pathogens [21–23]. High-throughput sequencing was first used to identify tomato sRNAs, including miRNAs from young green fruits and more recently from several developmental stages of the fruits [24–27].

Recently, there is a growing interest towards understanding the role of miRNAs in regulation of male-sterility in plants. Even though there is no evidence that any miRNAs directly cause male-sterility in plants, it was suggested that miRNAs and their target genes play important roles in regulation of male-sterility. Wei et al., [28] identified several conserved and new miRNAs, which were differentially expressed during anther development in a male-sterile cotton mutant. These miRNAs targeted HD-Zip III-like, ARF4, AP2 and ACC oxidase 3 genes, which are key genes involved in hormone signaling, cell patterning, and anti-oxidant metabolism.

Zhang et al., [29] reported that miR156, miR159, miR164, miR166, miR172 and miR319 were differentially expressed in sporogenous cell, MMCs and microspores between a male-sterile cotton and its maintainer line. The predicted targets for these miRNAs were involved in cotton growth and development, signal transduction and metabolism pathways. Jiang et al., [30] identified 15 miRNAs, which were differentially expressed in pollens of a male-sterile line of Brassica campestris and its wild type. There are increasing evidences showing that the function of miRNAs, including miR156, miR159, miR164, miR167, miR172 and miR319 is crucial during flower development and microsporogenesis [31–33]. MiR159 directs the cleavage of GAMYB-related transcripts, which are involved in regulation of anther development and microsporogenesis [34, 35]. Overexpression of miR159 in Arabidopsis thaliana resulted in sterile anthers [36]. MiR167 targets ARF6/8, which regulate ovule and anther development [31]. In Arabidopsis thaliana, overexpression of miR167 led to male-sterility [37]. Mutation in a sRNA locus in rice which produced a 21-nt sRNA has led to environment-conditioned male-sterility [38].

sRNAs in tomato have been mainly studied in fruits development and ripening process, and no report has yet documented their involvement in regulation of male-sterility. The main goal of our study was to investigate whether sRNA biogenesis is affected by 7B-1 mutation, and if sRNAs, particularly miRNAs, are associated or involved in anther development and regulation of male-sterility in 7B-1. Known and new miRNAs, ta-siRNAs and their targets were identified and their expressions were studied in 7B-1 and WT anthers. Targets of miRNAs with potential roles in anther development and microsporogenesis were validated using 5’-RACE and localization of miRNAs was further analyzed by in situ hybridization.

Results

Deep sequencing of sRNAs

Anthers from the flower buds at three stages of pre-meiosis, meiosis, and post-meiosis (stages 1, 2, and 3) were dissected and pooled as described by Sheoran et al., [2]. Two sRNA libraries were constructed from WT and 7B-1 pooled anthers and sequenced, which produced about 108 and 52 million raw reads, respectively. After adapter trimming, reads were mapped to the tomato (cv. Heinz) genome with no mis-matches allowed (Table 1). Size class distribution of total and unique sRNAs and their complexities in each library is presented in Fig. 1. The majority of sRNAs in both 7B-1 and WT libraries were 21–24 nt, and formed a bi-modal size distribution typical for plant sRNAs. In general, the peak represented by 24-nt size class is greater than the 21-nt class as reported in several
plant species [25, 26, 29, 39–42] with a few exceptions, as in grapevine, where the 21-nt class was more abundant than the 24-nt class in total read numbers, but not at unique read level [43], and in *Brassica juncea*, where the 21-nt class had the major peak in total and unique reads [44].

In our study, the 21-nt class had a bigger peak than 24-nt class in total reads in both libraries; however, for the non-redundant distribution, the 24-nt class had the major peak in both libraries. Two key distinguishing features of sRNA libraries are the size distribution and population complexity as defined by the ratio of unique/total reads. The lower complexity of the 21-nt class in comparison to the 24-nt class in our study indicated that a relatively small number of unique reads were highly expressed in the 21-nt class, while the 24-nt class high complexity indicated the presence of more unique reads with less redundancy, which is also a typical feature of the 24-nt hcRNAs, where often are present in a more chaotic dicing pattern [45].

**Identification of known and new miRNAs**

Known miRNAs from 32 known families of miRNAs were identified, which were present in both WT and 7B-1 libraries (Additional file 1: Table S1). The composition of miRNA families was quite similar in the two libraries, and none showed a clear 7B-1 or WT-specific

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**Table 1 Statistics of sRNA reads**

| Type          | WT anthers |          | 7B-1 anthers |          |
|---------------|------------|----------|--------------|----------|
|               | Total      | Unique   | Total        | Unique   |
| Raw reads     | 108,515,964|          | 52,194,436   |          |
| Quality filtered | 108,322,391|          | 52,054,239   |          |
| Adaptor removed | 45,255,262 |          | 19,253,757   |          |
| Genome matched | 28,187,399 | 2,062,422| 12,010,196   | 1,018,385|
| rRNA          | 1,171,580 (4.16 %) | 6,480 (0.31 %) | 466,773 (3.89 %) | 4,796 (0.47 %) |
| tRNA          | 2,218,468 (7.87 %) | 8,460 (0.41 %) | 656,434 (5.47 %) | 5,911 (0.58 %) |
| snoRNA        | 117,074 (0.42 %) | 2,958 (0.14 %) | 58,061 (0.48 %) | 2,277 (0.22 %) |
| snRNA         | 107,893 (0.38 %) | 8,235 (0.40 %) | 46,202 (0.38 %) | 5,783 (0.57 %) |

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**Fig. 1** Size distribution of redundant (R) and non-redundant (NR) sRNA reads and their complexity in WT (panel a) and 7B-1 (panel b) anthers.
RT-qPCR validation of miRNAs expression

Expressions of known and new miRNAs of interest were further analyzed by RT-qPCR (Fig. 2) in 7B-I and WT anthers (stages 1, 2, and 3) and stem. MiR159 and miR390 were up-regulated at similar extent in all stages of 7B-I anthers. MiR167 and miR396 were down-regulated in all stages of 7B-I anthers; more strongly at stage 1. MiR#M was strongly down-regulated in all stages of 7B-I anthers, while miR#A and miR#B were not differentially expressed and their sequenced miRNA* strands were not detected by our RT-qPCR analysis. The RT-qPCR results were in general agreement with those from sequencing data. In 7B-I stem, miR159, miR390, miR167 and miR#M were all up-regulated, while miR396, miR#A and miR#B were not differentially expressed. MiR#B* was not detected by RT-qPCR. The results showed a very distinct anther- or stem-specific expression pattern of miR167, miR396, and miR#M, which suggests that they may regulate different biological processes in these tissues.

Target prediction for known and new miRNAs

To better understand the biological roles of miRNAs in regulation of 7B-I male-sterility, putative targets of differentially expressed known and new miRNAs were identified (Tables 3 and 4; cleavage-sites are listed in Additional file 1: Table S4). Target genes for many of known conserved miRNAs have been experimentally validated [9, 14, 17, 31, 32, 49–52]. Predicted targets for known miRNAs in our study were also in agreement with those from the literature, however a number of new putative target genes were also computationally identified, which has yet to be validated. The miRNA targets were categorized into fifteen different biological classes (Fig. 3) with the three most frequent being metabolic process, cellular process, and single-organism process. Among the targets worth mentioning (Table 3) were those, including
auxin-responsive factor 8 (ARF8), ABC transporters, G protein, F-box and no apical meristem (NAM) proteins, b-ZIP, SBP-box, MAD-box and MYB transcription factors with potential roles in anther development [53–60], cystatin, receptor-like kinase, and bHLH proteins involved in tapetum development/degeneration [61–63], kinesin-like protein and GRAS transcription factor involved in meiosis regulation [64, 65], and WD-40 protein with roles in autophagy and apoptosis [66]. Among the targets of new miRNAs (Table 4), a gene encoding pectinmethylesterase (PME) inhibitor (PMEI) was identified as the putative target of miR#M. PME is a cell wall modifying enzyme, which catalyzes de-esterification of pectin [67]. Identification of miRNA targets with potential roles in anther development and microsporogenesis regulation in our study, suggested that these miRNAs are likely associated or involved in regulation of 7B-1 male-sterility.

Identification of ta-siRNAs and prediction of their targets

TAS loci and their associated ta-siRNAs were predicted based on the phased 21 nt sRNAs characteristic of ta-siRNA loci [46]. Ta-siRNAs with abundance below 10 reads were excluded from the analysis. Twenty-five ta-siRNAs from WT, 7 from 7B-1 and 7 common in both libraries were identified and their target genes were predicted (Additional file 1: Table S5). As these ta-siRNAs had generally low abundances, those identified only in WT or 7B-1 could not be considered as WT- or 7B-1-specific and were not further analyzed. Instead, we focused on those which have been found in both libraries; however none were differentially expressed (Additional file 1: Table S5). Ta-siRNAs with phased expressions matching to the tomato TAS3 (Solyc01g058100.2.1) were also identified (Additional file 1: Table S6 and Table S7), however TAS3-derived 5’D7(+) and 5’D8(+) tasiARFs were not differentially regulated between the two libraries. The results suggested that ta-siRNAs were not likely associated or involved in regulation of male-sterility in 7B-1 as they were not differentially regulated.

5’-RACE validation of miRNA and ta-siRNA targets

The predicted targets of interest were validated in 7B-1 anthers (stage 1; where changes of the expression were strongest) and stem using 5’-RACE. Sequence analysis showed (Fig. 4) that the 5’ ends for most of the cleaved mRNA fragments corresponded to nucleotide complementary to the 10th nucleotide of the corresponding miRNA. MiR159 and miR319 could both cleave MYB transcripts [32]; however sequence analysis of the cleavage products showed that MYB cleavage in anthers and stem was directed by miR159, not miR319. Cleavage products of miR159 and miR319 can be readily distinguished as they differ in one base. Cleavage products of ARF8, target of miR167, were identified from anther, but not stem. Among the predicted targets of miR396, cleavage products of cystatin were identified in both anther and stem libraries,
### Table 3  List of the predicted targets of differentially expressed known miRNAs

| miRNA | Target gene | SGN accession no | Annotation |
|-------|-------------|------------------|------------|
| miR166 | SGN accession no | Annotation |
| miR390 | Receptor-like kinase | SGN accession no |
| miR159 | SGN accession no | Annotation |
| miR530 | SGN accession no | Annotation |
| miR30 | SGN accession no | Annotation |
| miR167 | SGN accession no | Annotation |
| miR168 | SGN accession no | Annotation |
| miR8006 | SGN accession no | Annotation |

(Continued)
but not MAD-box. Cleavage products of PMEI, predicted target of miR#M, were also identified in both anther and stem libraries. Cleavage products of ARF2, 3 and 4, targets of D7 and D8 tasiARFs, were identified from both anther and stem libraries (Additional file 2: Figure S2). These results showed that the above mentioned miRNAs, including the newly identified miR#M as well as tasiARFs were functionally active in 7B-1 anther and stem, where directed the cleavage of their predicted target transcripts. Furthermore, it provided experimental evidence to support our target predictions.

**RT-qPCR analysis of miRNA and ta-siRNA targets**

Figure 5 shows RT-qPCR analysis of miRNA targets of interest and TasiARFs in 7B-1 anthers and stem. GAMYBLI was down-regulated in anthers of all stages and more strongly in stem. ARF8 was up-regulated in anthers of all stages and stem. Cystatin and PMEI were both up-regulated in anthers of all stages, but down-regulated in stem. TasiARFs (D7 and D8) were not differentially expressed in anthers and stem. ARF2/3/4 were not differentially expressed in anthers, but up-regulated in stem (Additional file 2: Figure S3). Analysis showed a consistent negative correlation between miRNAs expression and accumulation level of their targets, except for miR390-TAS3-tasiARFs (in anthers and stem), and miR167-ARF8 (in stem). In addition to miRNA and their targets, expression of two cell wall modifying enzymes, cysteine protease and polygalacturonase, were also analyzed in 7B-1 anthers and stem (Additional file 2: Figure S4). Cysteine protease was down-regulated in anthers, more strongly at stage 3, while up-regulated in stem. Polygalacturonase was strongly down-regulated in anthers at stage 2, but not differentially regulated at stage 3, nor in stem.

**In situ hybridization**

Figure 6 shows in situ localization of miR159, GAMYBLI, PMEI and cystatin in WT and 7B-1 anthers in the late meiotic (where still some tetrads could be seen together with the newly formed microspores) and in binucleate microspores stages as for the cases of PMEI and cystatin. MiR159, GAMYBLI and cystatin were all predominantly expressed in tapetum, tetrads and free microspores in WT and more strongly in 7B-1 as the case of miR159 and cystatin. Panels L and M of the figure show WT and 7B-1 anthers, respectively at binucleate microspores stage, where tapetum was degenerated in WT, but not degenerated and vacuolated in 7B-1. Cystatin was strongly expressed in vacuolated tapetal cells in 7B-1 (Panel M). PMEI was strongly expressed in tapetum, tetrads and free microspores in 7B-1 anthers, but its expression was mainly restricted to tapetum at binucleate microspores stage (Panel H). As mentioned earlier, in some of the 7B-1 anthers, microspores were not separated and remained attached after meiosis. PMEI was strongly expressed in arrested binucleate microspores in 7B-1 (Panel I). No detectable hybridization signal was observed for the murine miR122a probe, which served as a negative control.

**Discussion**

The main goal of our study was to investigate the sRNA profiles, particularly miRNAs, between the 7B-1 mutant and WT anthers and to identify differentially expressed miRNAs and their targets with potential roles in anther development and regulation of male-sterility in 7B-1. Analysis showed that the overall size distribution and population complexity of sRNAs were quite similar between the 7B-1 and WT anther libraries. Composition of sRNAs could indicate the roles and activity level of different categories of sRNAs in a particular tissue or species or associated biogenetic machineries. In our study, the 21-nt class had a larger peak than the 24-nt class in total reads in both libraries; however, in non-redundant format, the 24-nt class had the major peak in both libraries. The 24-nt class was also found to be the major peak in anthers of a male-sterile cotton [28]. Higher abundance of the 21-nt class in total reads in anthers of 7B-1 and WT in our study, could suggest a more active roles for this class of sRNAs and an intensified contribution from miRNA biogenesis pathways that produce the more precisely defined sRNA species at those stages compared to those of other crops having the 24-nt class with higher redundancy.

Even though there is no direct evidence that any miRNAs are causative genes for male-sterility in plants, we hypothesized that differential expression of miRNAs could be associated with the regulation of male-sterility in 7B-1. Thirty-two families of known miRNAs were identified in both 7B-1 and WT anther libraries. Three and seven miRNA families were up and down-regulated, respectively in 7B-1 anthers. Out of the 23 putative new miRNAs only two, so-called miR#W and miR#M, were differentially expressed in 7B-1 anther. Expressions of miRNAs of interest were further analyzed at different
stages of 7B-1 anthers and stem. In general, changes of the expression were strongest in anthers at stage 1, where the MMCs were about to undergo the meiosis. MiR167, miR396, and miR#M had distinctively different expression patterns in anthers and stem, which strongly suggested that these miRNAs regulate different biological processes in these tissues. The predicted targets of differentially expressed miRNAs were categorized into fifteen different biological classes, with metabolic process, cellular process, and single-organism process being the three most frequent classes. Among these miRNAs, miR159, miR167, miR396, miR390 and miR#M were those of particular interest as their targets had potential roles in anther development, microsporogenesis and production of ta-siRNAs as the case of miR390. A schematic presentation of miRNA-target pairs and their role in regulation of male-sterility in 7B-1 mutant is illustrated in Fig. 7. 5′-RACE validation of these miRNA targets in 7B-1 anther and stem showed that they were active and directed the cleavage of their targets in these tissues, except for miR167 in 7B-1 stem. MiR159 targets several GAMYBs [55, 68, 69], among them AtMYB33, AtMYB65 act redundantly in regulation of anther development [55]. Overexpression of miR159 disrupted anther development and led to male-sterility in Arabidopsis thaliana [36]. 5′-RACE analysis in our study showed that miR159 directed the cleavage of GAMYBL1 transcripts out of the 4 predicted MYB targets in anther and stem. OsGAMYBL1 and 2 are expressed in rice anthers and regulated by miR159, however functions of these gene are not characterized [69]. LeGAMYBL1 plays a role in seed development in tomato [70], but it is not functionally characterized with respect to anther development. Analysis showed that the expression of GAMYBL1 was negatively correlated with the miR159 level in 7B-1 during anther development and in stem. In situ hybridization analysis in 7B-1 anthers also showed that miR159 and GAMYBL1 were both expressed mainly in tapetum, tetrad and microspores. MiR159 and GAMYBL1 were down- and up-regulated, respectively in GA-treated 7B-1 anthers (Additional file 2: Figure S5). These observations indicated that miR159-GAMYBL1 cleavage cascade and Table 4 List of the predicted targets of new miRNAs

| miRNA | Target gene | SGN accession no | Annotation |
|-------|-------------|------------------|------------|
| miR#W | Solyc01g008240.2.1 | Sugar/inositol transporter |
|       | Solyc01g015110.1.1 | Ulp1 protease^a |
|       | Solyc01g079390.2.1 | Histone-lysine N-methyltransferase MEDEA |
|       | Solyc01g095910.1.1 | Cytochrome b561/ferric reductase transmembrane |
|       | Solyc01g103670.2.1 | Alpha/beta hydrolase fold-1 domain-containing protein |
|       | Solyc01g103800.2.1 | Ribosomal protein S12e |
|       | Solyc01g104990.2.1 | Trimethylguanosine synthase |
|       | Solyc02g028270.2.1 | EMB1611/MEE22 |
|       | Solyc02g069410.2.1 | HAD-superfamily hydrolase subfamily IA variant 3 |
|       | Solyc02g069740.2.1 | Jumonji transcription facto |
|       | Solyc02g092780.1.1 | Tetratricopeptide-like helical |
|       | Solyc03g078240.1.1 | UDP-glucosyltransferase |
|       | Solyc03g115860.2.1 | Endoplasmic reticulum membrane protein |
|       | Solyc04g074600.1.1 | Folate-sensitive fragile site protein Fra10Ac1 |
|       | Solyc04g079240.2.1 | Patatin |
|       | Solyc05g007850.1.1 | Tir-nbs-irr, resistance protein |
|       | Solyc06g016750.2.1 | CBF transcription factor |
|       | Solyc06g065180.2.1 | SLL1 protein |
|       | Solyc06g083070.2.1 | Actin filament bundling protein |
|       | Solyc08g014530.1.1 | Subtilisin-like protein |
|       | Solyc08g069000.2.1 | Zinc transport protein zntB |
|       | Solyc08g081290.2.1 | ARID/BRIGHT DNA-binding protein |
|       | Solyc09g007370.2.1 | Ribonuclease P protein subunit p29 |
|       | Solyc09g083200.2.1 | Nod factor receptor protein |
|       | Solyc09g089530.2.1 | Zinc finger-homeodomain protein 2 |
|       | Solyc10g084410.1.1 | Phosphatase 2C family protein |
|       | Solyc12g027580.1.1 | Exportin 4 |
|       | Solyc12g077660.1.1 | Nucleosome assembly protein (NAP) |
| miR#M | Solyc03g112990.1.1 | Pectinesterase inhibitor |
| miR#A | Solyc03g119580.1.1 | Ethylene-responsive transcription factor 4 |
|       | Solyc10g078230.1.1 | Cytochrome P450 |
| miR#B | Solyc01g044350.2.1 | Zinc finger (Ran-binding) family protein |
|       | Solyc02g089170.2.1 | Alpha-1,4-glucan-protein synthase |
|       | Solyc03g113250.2.1 | Nitrate transporter |
|       | Solyc04g007260.2.1 | Thiosterase superfamily |
|       | Solyc04g009620.2.1 | Chorismate synthase |
|       | Solyc04g071160.2.1 | b-ZIP transcription factor |
|       | Solyc05g054890.2.1 | ABC transporter G family member 1 |
|       | Solyc06g084100.2.1 | Protein phosphatase 2C |

^a indicates targets which have identified from multiple loci

Table 4 List of the predicted targets of new miRNAs (Continued)
GA level in flower buds are tightly linked to the regulation of anther development and male-sterility in 7B-1. Most of the elongated mutants have been reported as GA-overproducers [71–73]; however elongated stem of 7B-1 had lower levels of GAs compared to WT in long days [4]. Several MYBs have been identified, which positively regulate stem elongation in light via interaction with phytochromes and/or regulation of light-responsive genes [74, 75]. Down-regulation of GAMYBL1 in 7B-1 stem is likely associated with the elongated stem phenotype, however functional analysis are needed to understand the actual function of this gene.

MiR167 cleaves ARF6/8 transcripts [76]; however we only identified ARF8 in our target prediction, which was also validated by 5′-RACE in 7B-1 anther. Tomato overexpressing miR167 and arf6-arf8 double-null mutant of Arabidopsis thaliana both had flowers with severe defects associated with female and male-sterilities, respectively [76, 77]. Therefore, proper regulation of miR167-ARF6/8 is normal female and male organ development. RT-qPCR analysis showed strong down-regulation of miR167 in 7B-1 anthers, which was negatively correlated with regulation of ARF8 in anthers. Although differential regulation of miR167-ARF8 cleavage cascade in 7B-1 anthers could be linked and due to 7B-1 mutation, its actual function with respect to anther development and male-sterility in 7B-1 remains to be further characterized.

In 7B-1 stem, neither the ARF8 expression was correlated with miR167 level. Tomato overexpressing miR167 had lower levels of ARF6/8 transcripts and a shorter hypocotyl [76], while other researchers reported that light-grown Arabidopsis thaliana arf8-1 mutant had elongated hypocotyl [78]. Although up-regulation of miR167 and ARF8 in 7B-1 stem could be independently associated and/or affected by 7B-1 mutation, understanding their functions with respect to anther development and male-sterility in 7B-1 requires further functional analysis.

To the best of our knowledge no reports have yet documented ta-siRNAs in regulation of male-sterility in plants. Ta-siRNAs including TAS3-derived tasiARFs were not differentially expressed between 7B-1 and WT; however miR390 was up-regulated in 7B-1 anther and stem. This suggested that although miR390 expression was affected by 7B-1 mutation, ta-siRNA biogenesis and tasiARFs-guided regulation of ARF2/3/4 were not related to anther development and male-sterility in 7B-1. Nevertheless, 5′-RACE analysis showed that tasiARFs are active in 7B-1 anther and stem, where directed the cleavage of ARF2/3/4 transcripts. Target genes of ARF2/3/4 are largely unknown,
thus the mechanisms linking these ARFs to a context-specific role in regulation of 7B-1 stem growth if any are poorly understood.

Our cytological studies showed (Additional file 2: Figure S6; unpublished data) that anther development in 7B-1 (between anther lobes and/or among anthers) was not synchronized in contrast to the WT. We observed that MMCs did undergo meiosis and formed tetrads despite the previous report suggested a breakdown of meiosis in MMCs [2]. More importantly, we found that in some anther lobes, the newly formed microspores were not separated, while in others they formed free microspores. In qrt1 and qrt2 mutants of Arabidopsis thaliana, microspores failed to separate from tetrads as pectin was not degraded in the primary cell wall [79]. PMEI (miR#M target) was up-regulated in 7B-1 anthers, where the expression was mainly localized in tapetum, tetrads and microspores. PMEI was strongly expressed in the arrested binucleate microspores, compared to the free binucleate microspores, where a basal expression was detected. PMEs and PMEIs are key regulators of pollen cell wall and pollen tube development [79–81]. Up-regulation of PMEI in 7B-1 anthers may have suppressed the PME activity and impaired the subsequent enzymatic degradation of pectin in the primary cell walls around tetrads. In qrt3, mutation in a gene encoding a polygalacturonase (pectin modifying enzyme) impaired the degradation of pectin in the primary cell wall, similar to qrt1 and qrt2 mutants [82]. Polygalacturonase was also down-regulated in 7B-1 anthers in our study. Based on our cytological and transcriptional data, we suggest a potential association between the regulation of miR#M-PMEI and polygalacturonase and anther development in 7B-1 as inhibition of PME and polygalacturonase activities could have disrupted pectin degradation and proper separation of tetrads and/or tapetum degeneration. However, it could not be the cause of male-sterility in 7B-1 as some of the anthers were still able to produce mature pollens.

Arabidopsis thaliana overexpressing PME showed reduced cell elongation in hypocotyl [83]. Al-Qsous et al., [84] suggested that higher level of PME transcript in elongated hypocotyl of flax was associated with cell wall stiffening. Overexpression of a polygalacturonase gene, PGX1, enhanced hypocotyl elongation in etiolated Arabidopsis thaliana [85]. Understating the function of PME and polygalacturonase in regulation of stem elongation in 7B-1 if they have indeed altered the pectin level and how they are connected to the cell expansion requires further analysis.

While conserved miRNAs regulate expression of the genes involved in basic developmental processes, the non-conserved or new miRNAs may be involved in the development of traits that are specific for certain species. New miRNAs are being continually identified from different species, including tomato [24, 25]. In addition to miR#M, we identified a number of new miRNAs, two with sequenced miRNAs*, which could form a near perfect hairpin structures.

Sheoran et al., [2] identified a number of differentially expressed proteins between 7B-1 and WT anthers, where cystatin showed the strongest up-regulation of all in 7B-1 anthers. The cystatin inhibitory activity was also higher in 7B-1 anthers relative to WT [2]. In plants, cys...
proteases act as key regulators of programmed cell death in tapetal cells and pollen development, and suppression of their expression has often resulted in delay or failure in tapetum degeneration, pollen abortion and male-sterility [62, 86–88]. MiR396 directed the cleavage of cystatin in 7B-1 anthers and stem. Cystatin was up-regulated and cysteine protease was strongly down-regulated as a result in 7B-1 anthers, with a pattern closely correlated to the tapetum degeneration during anther development. These observations provided a strong evidence to suggest that suppression of cysteine protease could have caused a delay or failure in tapetum degeneration, thereby affecting microsporogenesis and anther development in 7B-1. The length of stem in 7B-1 is tightly

**Fig. 6** In situ localization of miR159, GAMYBL1, PMEI and cystatin. a and b localization of miR159 in WT and 7B-1 anthers, respectively. c and d GAMYBL1 in WT and 7B-1 anthers, respectively. e and f PMEI in WT anthers at late meiotic and binucleate microspores stages, respectively. g, h, and i PMEI in 7B-1 anthers at late meiotic stage, free binucleate microspores, and arrested binucleate microspores, respectively. j and k cystatin in WT and 7B-1 anthers, respectively. l and m cystatin in WT and 7B-1 anthers at binucleate microspores stage, respectively. n negative control, where a murine miR122a-specific probe was used. Arrows indicate the localization sites.
correlated with the length of epidermal cells [6]. Minic et al., [89] identified several cysteine proteases in developing stems of *Arabidopsis thaliana*, suggested to be involved in cell expansion and secondary wall formation. Higher expression of cysteine protease in our study could be associated with the higher cell expansion rate and longer epidermis cells in 7B-1 stem. Overall in our study, we found that miRNA-mediated regulation of gene expression was perturbed in the 7B-1 mutant line. The findings strongly supported that miR159-GAMYBL1, miR396-cystatin and miR#M-PMEI cleavage cascades were tightly connected to the regulation of microsporogenesis and anther development in 7B-1. Stem elongation in 7B-1 may be regulated via a complex web of molecular components and interaction between miRNAs and hormones, which requires further functional studies to be understood. A number of new miRNAs were also identified for the first time from tomato, which provides new opportunities to study the unexplored functions of these miRNAs in tomato. Our data could be used as a benchmark for future studies of the molecular mechanisms of male-sterility in other crops.

**Conclusion**

Using sRNA sequencing, we studied miRNA profiles during anther development between 7B-1 mutant and WT. Comparison of expression of miRNAs and their targets between 7B-1 and WT and in situ localization analysis suggested potential involvement of several miRNA-target pairs in regulation of anther development and male-sterility in 7B-1. In addition a number of new miRNAs were identified and validated for the first time from tomato.

**Availability of supporting data**

The sequences could be found in NCBI database under accession number GSE65788.

**Methods**

**Plant materials**

The 7B-1 mutant and WT seedlings (*Solanum lycopersicum* L., cv. Rutgers) were grown in temperature controlled growth chamber set for long days condition (16/8 h light/dark). Flower buds of different sizes smaller, equal and bigger than 4-5 mm (referred as stages 1, 2, and 3 hereafter) were collected and stamens were dissected under a microscope. It should be noted that stamens at these stages were mostly consisted of anthers, with little or on filament growth. Stages of flower buds were based on those described by Sheoran et al., [2] and also further confirmed by analysis of anther squashes. Flower buds at stage 1 represents pre-meiotic anthers, stage 2 is where tetrads are formed in WT anthers (meiotic anthers), but meiosis breaks down in MMCs in 7B-1 [2]. Stage 3 represents post-meiotic anthers. Stems from three-month old seedlings were used for qPCR and 5’-RACE analysis.

**RNA analysis**

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) from 7B-1 and WT anthers of different stages and pooled separately in equimolar ratio. Two sRNA libraries were constructed using the TruSeq Small RNA Sample Preparation Kit (Illumina). In brief, sRNA fractions of 18-40 nt were isolated from 15 % denaturing polyacrylamide gels, ligated to the 5’ and 3’ TruSeq adaptors and then converted to DNA by RT-PCR following the kit protocol. The final PCR products were purified from
the gel and sequenced using Illumina Hiseq2000 platform (Illumina)

**Sequence analysis**
Adaptor sequences trimmed and reads were mapped (no mismatch allowed) to the tomato genome ITAG v2.5 Release using PatMaN [90] and a customized Perl script. Reads mapping to tomato repeats, transposons, intron, CDs, and promoter regions were identified. Sequences were searched against rRNA, tRNA, snRNAs and snoRNAs from Rfam v.12 and NCBI nt/nr databases. Known miRNAs were identified using miRprof [47], (available from the UEA sRNA workbench: http://srna-tools.cmp.uea.ac.uk/) allowing two mismatches with the mature miRNAs in miRBase database release 21 [91]. New miRNAs were predicted using miRcat (UEA sRNA toolkit), and their secondary structures were analyzed using a RNA hairpin folding and annotation tool (UEA sRNA toolkit). The parameters for miRcat included a minimum of 17 nucleotides paired, a maximum of two gaps between the miRNA and miRNA*, a maximum of 10 genomic hits, a minimum hairpin length of 70 nt, a minimum GC content of 20 %, a maximum of 60 % unpaired nucleotides and a strand bias of 80 % of sequences on one strand (regardless of the strand). These parameters were determined empirically on plant data- sets [46, 47]. The significance testing for the secondary structure was conducted with RandFold [92].

Targets of miRNAs were predicted (allowing 4 mismatches) using the tomato ITAG cDNA v2.5. Expression values of miRNAs were normalized against the total number of genome-mapping reads [48] and changes in the expression were calculated as offset-fold change as described in Mohorianu et al., [25]. The p-values were calculated based on the standardized distribution of differential expression for all genome-matching sRNAs. TAS loci were predicted based on phased 21-nt sRNAs characteristic of ta-siRNA loci using a TASI prediction tool (UEA sRNA toolkit). Gene ontologies were assigned using the Blast2go tool (http://www.blast2go.com/b2ghome).

**Quantitative PCR**
Expressions of miRNAs and ta-siRNAs were validated using the Mir-X™ miRNA First-Strand Synthesis and SYBR® RT-qPCR kit (Clontech). In a single reaction, sRNA molecules were polyadenylated and reverse transcribed using poly(A) polymerase and SMART™ MMLV Reverse Transcriptase provided by the kit. List of miRNA and ta-siRNA forward primers is provided in Additional file 1: Table S8. U6 small nuclear RNA was used as a reference for data normalization. QPCR conditions were set at 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, and annealing/extension at 60 °C for 20 s. Changes of expressions were calculated as normalized fold ratios using the ΔΔCT method [93]. QPCR validations of miRNA target genes were carried out using the SensiFAST SYBR Lo-ROX kit (Bioline). First-strand cDNAs were synthesized using the PrimeScript First Strand cDNA Synthesis kit (Takara). Gene-specific primers spanning the miRNA cleavage sites were designed and listed in Additional file 1: Table S8. Housekeeping a-tubulin and CAC genes were used as reference genes for data normalization (data were shown only for a-tubulin). PCR conditions were set at 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, and annealing/extension at 60 °C for 20 s.

**5'-RACE analysis**
miRNA targets of interest were functionally validated using the GeneRacer kit (Invitrogen) through RNA ligase-mediated rapid amplification of 5’ cDNA ends (RLM-5’ RACE). In brief, 5 µg of total RNA was used to purify the miRNA. The 5’ RNA adaptor was ligated to the degraded mRNA with a 5’ free phosphoric acid by T4 RNA ligase, followed by a reverse transcription reaction. Subsequently, 1 µl of 10X diluted reverse transcription product was used to amplify the 5’ end of the corresponding targets using the 5’ GeneRacer and 3’ gene-specific primers. Final PCR products were analyzed by gel electrophoresis and cloned into the pCR®4-TOPO vector (Invitrogen). Ten different colonies were subjected to sequence analysis. The reverse gene-specific primers are listed in Additional file 1: Table S9.

**In situ hybridization**
Flower buds were fixed overnight in 4 % paraformaldehyde, then dehydrated using a graded ethanol series (50, 70, 95 and 100 %) and embedded in Paraplast® Plus™ chips. Transverse sections of 8 µm thick were prepared from the embedded blocks using a Leica Ultracut R ultramicrotome (Leica Bensheim, Germany). In situ hybridization was carried out following the protocol described by Javelle and Timmermans, [94]. 5’-end DIG-labeled oligo-probes (Additional file 1: Table S10) with sequences complementary to miR159, GAMYBL1, PMEI, cystatin, and murine miR122a (as a negative control) were synthesized by Eastport (Eastport, Czech Republic). Probe concentration, 10 nM and hybridization temperature of 50 °C were experimentally identified as optimums. In situ localization signals were detected in colorimetric reactions using DIG-specific antibody coupled to alkaline phosphatase.

**Experimental design and statistical analysis**
The experiments were arranged in a completely randomized design with three biological replications. Data were subjected to analysis of the variance (ANOVA) and duncan new multiple range test (DNMRT p = 0.05) for comparison of the means using the SAS software version 9.2.
Additional files

**Additional file 1: Table S1.** List of the identified known miRNAs in WT and 7B-1 anthers. **Table S2.** List of the new miRNAs identified from WT anthers. **Table S3.** List of the new miRNAs identified from 7B-1 anthers. **Table S4.** List of miRNA-target cleavage sites. **Table S5.** List of the identified TAS3-derived tasiRNAs from WT library. **Table S6.** List of the identified TAS3-derived tasiRNAs from 7B-1 library. **Table S7.** List of the identified TASI-derived tasiRNAs from WT library. **Table S8.** List of the identified TASI-derived tasiRNAs from 7B-1 library. **Table S9.** List of the primers used for 5' RACE analysis. **Table S10.** List of the DIG-labeled oligo-probes used for in situ hybridization. (DOCX 676 kb)

**Additional file 2: Figure S1.** Hairpin structures of new miRNA precursors. **Figure S2.** 5' RACE validation of tasiARFs target genes in 7B-1 anther and stem. **Figure S3.** RT-qPCR validation of tasiARFs target genes in 7B-1 anther and stem. **Figure S4.** RT-qPCR validation of cysteine protease and polygalacturonase in 7B-1 anthers and stem. **Figure S5.** RT-qPCR validation of miR159 and GA3ox1B in GA-treated 7B-1 anthers. **Figure S6.** Cytological study of anther development in 7B-1 and WT. (DOCX 4243 kb)

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
VO designed, carried out the experiments and data analysis and drafted the manuscript. IM and TD contributed to the bioinformatics analysis of the sequencing data. MF contributed to the experimental design and management and supervised the research. All authors have read and approved the final manuscript.

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