Linkage mapping and expression analysis of miRNAs and their target genes during fiber development in cotton

Xuemei Chen1, Wenhui Gao1, Jinfa Zhang2, Xianlong Zhang1 and Zhongxu Lin1*

Abstract

Background: MicroRNAs (miRNAs) are small, endogenously expressed, non-coding RNA molecules involved in gene transcription and expression that combine with specific mRNA site of target genes to inhibit protein synthesis or degrade miRNA. Since the first plant miRNA was reported in 2002, numerous new miRNAs and their targets have been discovered via high-throughput sequencing and computational approaches. However, the genetic variation of miRNA genes is poorly understood due to the lack of miRNA-specific DNA markers.

Results: To study the genetic variation and map miRNAs and their putative target genes in cotton, we designed specific primers based on pre-miRNAs and published putative target genes. A total of 83 pre-miRNA primers and 1,255 putative target gene primers were surveyed, and 9 pre-miRNA polymorphic loci were mapped on 7 of the 26 tetraploid cotton chromosomes. Furthermore, 156 polymorphic loci of the target genes were mapped on the cotton genome. To map more miRNA loci, miRNA-based SRAP (sequence-related amplified polymorphism) markers were used to map an additional 54 polymorphic loci on the cotton genome with the exception of Chr01, Chr22, and Chr24. Finally, a network between miRNAs and their targets was constructed. All pre-miRNAs and 98 putative target genes were selected for RT-PCR analysis, revealing unique expression patterns across different fiber development stages between the mapping parents.

Conclusions: Our data provide an overview of miRNAs, their putative targets, and their network in cotton as well as comparative expression analyses between Gossypium hirsutum and G. barbadense. These data provide a foundation for understanding miRNA regulation during cotton fiber development.

Keywords: Cotton, miRNA, Target genes, Chromosome mapping, RT-PCR

Background

MicroRNAs (miRNAs) are a class of non-coding small RNA molecules, generally 21 nucleotides (nt) in length that regulate critical functions in plant and animal development. miRNAs arise from an ~70-nt stem-loop RNA precursor that is cleaved and modified by Ribonuclease III to create an ~21-nt single-stranded miRNA [1,2]. Generally, every mature miRNA derives from one of the precursor’s arms and all are located in intergenic regions, indicating that miRNA transcription is independent of other genes and has its own transcriptional regulatory mechanisms. miRNAs regulate gene expression at the post-transcriptional level through mRNA cleavage or translational inhibition in both animals and plants [3-7]. In plant cells, the miRNA sequence is almost completely complementary with target gene mRNA, through a mechanism similar to RNA interference (RNAi), leading to the degradation of target gene mRNA [8]. Recently, numerous miRNAs have been reported through cloning, high-throughput small RNA sequencing, and computational approaches based on sequence similarities and secondary structure predictions [4]. Because plant miRNAs are almost exactly complementary to their corresponding target sequences [5], target gene prediction is relatively straightforward and mainly through bioinformatics method.

* Correspondence: linzhongxu@mail.hzau.edu.cn
1 National Key Laboratory of Crop Genetic Improvement & National Centre of Plant Gene Research (Wuhan), Huazhong Agricultural University, 430070 Wuhan, Hubei, China
Full list of author information is available at the end of the article

© 2013 Chen et al.; licensee BioMed Central Ltd. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
In 1993, the first miRNA (lin-4 gene) was reported in *Caenorhabditis elegans* [9]. Then, Reinhart et al. [10] found the second metachronous switch gene, let-7, in *C. elegans*. Recently, with bioinformatics, molecular cloning technology improvements, and the establishment of model species cDNA libraries, hundreds of small RNA molecules have been successively reported in *C. elegans* [11,12], Arabidopsis [13,14], rice [15], maize [16], wheat [17], moss [18] and in cotton [19-22]. Almost 3,600 miRNAs have been annotated in Magnoliophyta including 291 from *Arabidopsis thaliana* and 40 from *Gossypium* species, as of April 21, 2012 (http://www.mirbase.org/cgi-bin/browse.pl). Recently, with the sequencing of the *G. raimondii* (*D. titanum*) genome, many miRNA precursors and miRNAs were identified [23-25]. However, their distribution on the tetraploid cotton genome is unknown. To date, few miRNAs have been functionally characterized in cotton, and almost no predicted target genes have been verified by experiments in cotton.

Cotton is not only a fundamental world commodity that provides an important natural material, it is also an important oil crop. Since the first miRNA was reported, studies suggest that plant miRNAs negatively regulate target genes involved in plant development, organ morphogenesis, auxin signaling, and environmental stress responses [14,26-30]. MiRNAs may also play an important role in cotton fiber development and the study of miRNAs has far-reaching significance to improve fiber quality and yield [31].

At this time, most miRNAs and target gene research is focused on functional verification through gene overexpression, gene interference, and related methods, but there is paucity in development of miRNA markers and their relationship with phenotypes. Only one report in the literature described miRNA markers- miRNA-AFLP (amplified fragment length polymorphism) [32]. It is a highly effective strategy to examine miRNA and their complementary sequences combining with SRAP primers, but no miRNAs or their target genes have been genetically mapped in cotton.

To address this gap and to map miRNAs to understand their genomic distribution in cotton, we downloaded pre-miRNA sequences reported in the literature and within the miRBase database (Additional file 1: Table S1). Putative target genes were predicted by psRNATarget (http://plantgrn.noble.org/psRNATarget). Specific primers were then designed based on the pre-miRNA sequences and the target sites of the target genes, and miRNA-based SRAP marker analysis was performed. Primers were also designed surrounding the target sites in the putative target genes to indicate their chromosomal distribution. Then, a network diagram between miRNAs and their putative targets was constructed to reveal regulatory relationships. Finally, RT-PCR analysis was conducted to detect expression differences between *G. hirsutum* and *G. barbadense* in selected pre-miRNAs and putative target genes.

**Results and discussion**

**Strategies of development markers for miRNAs and their target genes**

Primers were designed for 123 pre-miRNA sequences and identical primers were eliminated with a nucleotide BLAST search. Finally, 83 pairs of pre-miRNA primers were obtained (Additional file 2: Table S2). Then, these primers were screened using single-stranded conformation polymorphism (SSCP), of which 12 primers yielding 13 polymorphic loci. The rate of primer polymorphism was 14.5%.

A total of 1,255 primer pairs were obtained for the 1,399 target genes of the miRNAs after eliminating identical primers as described above (Additional file 3: Table S3). Of these, 147 primer pairs produced 161 polymorphic loci after SSCP analysis. The rate of primer polymorphism was 11.6%.

Based on miRNA-AFLP technology, the miRNA-SRAP marker technique was performed to enrich miRNA markers. Among the 2,944 miRNA-SRAP primer combinations, ~8 scorable bands per primer combination were typically produced. Exceptions were 59 miR319-SRAP primer combinations which generated only 0–2 scorable bands. These results are consistent with those obtained from miRNA-based AFLP marker techniques [32]. Fifty-five primers produced 59 polymorphic loci (Additional file 4: Table S4), and the rate of primer polymorphism was 1.87%. No polymorphic amplification products were obtained for miR156, miR319, miR390, miR398, and miR399 when combined with SRAP primers.

Pang et al. [32] explored the possibility of using miRNA genes as markers and identified DNA sequences of potential pre-miRNA. miRNA-AFLP analysis provides a reliable targeted genotyping strategy to assess genetic diversity among cotton species. The SRAP primers can target exons and introns respectively and generate polymorphism based on them [33]; they also can combine with primers designed from candidate gene regions, which is also called TRAP (Target Region Amplification Polymorphism) [34]. Therefore, when primers designed from conservative miRNAs and their complementary sequences combining with SRAP primers, miRNAs or their flanking sequences can be amplified. This is a highly effective strategy to examine miRNA gene distribution as well as more feasible, simple, and efficient than the miRNA-AFLP technique with respect to typing miRNA markers. Also, miRNA-SRAP markers may be functional genes; therefore, the miRNA-SRAP technique has important significance to the study of miRNAs.

**Verification of miRNA-SRAP marker techniques**

To verify whether the miRNA-SRAP marker technique reliably amplified miRNA sequences, PCR products were cloned and sequenced from a sample of 7 random polymorphic miRNA-SRAP primer combinations. Then, miRNA primers and SRAP primers were identified as
depicted in Figure 1. The figure depicts reliably amplified products using the primer combinations instead of amplified products from individual miRNA primers or SRAP primers.

Even distribution of miRNA markers and preferential distribution of target gene markers in the cotton genome

After linkage analysis, 9 of the 13 polymorphic loci of pre-miRNAs were mapped on 7 cotton chromosomes (Chr01, Chr04, Chr13, Chr15, Chr16, Chr23 and Chr26) based on SSCP analysis which can discover minor sequence mutations and reveal more polymorphisms to map genes [35]. For miRNA-based SRAP markers, 54 of the 59 polymorphic loci were mapped on 23 cotton chromosomes, except for Chr01, Chr22, and Chr24. These data suggest that miRNA markers are widely distributed on 24 chromosomes, except for Chr22 and Chr24, for which no miRNA markers were identified. Apparently, relatively more loci occurred on Chr10 (5 loci) and Chr19 (6 loci). Thirty-two loci were mapped on 13 chromosomes of the At sub-genome, and 31 loci were mapped on 11 chromosomes of the Dt sub-genome (Additional file 5: Figure S1, Additional file 6: Table S5). In conclusion, these 63 miRNA loci were evenly distributed on 24 chromosomes, except Chr22 and Chr24.

Of the 161 polymorphic loci for the putative miRNA target genes, 156 were mapped on the cotton genome: 59 loci were mapped on 13 chromosomes of the At sub-genome, and 97 loci were mapped on 13 chromosomes of the Dt sub-genome (Additional file 5: Figure S1, Additional file 6: Table S5). Overall, unlike miRNA markers, target gene markers were preferentially distributed on the Dt sub-genome. Specifically, chromosomes Chr13, Chr18, Chr19 and Chr21 contained a total of 47 target gene marker loci, i.e., 30% of the total target gene marker loci. Comparatively fewer less loci were on Chr01, Chr03, Chr06, Chr07, Chr08 and Chr09. Surprisingly, 95 of the 156 target gene polymorphic loci belonged to target genes from the miR414 family, whereas 61 loci belonged to 26 other miRNAs families.

To date, no miRNA genes and their target genes have been genetically mapped in cotton. Pang et al. [32] developed conserved miRNA gene markers and explored the possibility of an miRNA-AFLP marker technique. Then, the amplified products were sequenced and compared for homology using cotton expressed sequence tags. However, genetic mapping of miRNA markers has not been reported. Thus, miRNAs and their target gene markers reported here were genetically mapped in our interspecific G. hirsutum × G. barbadense population. Such genetic mapping of a segregating population provided an overview of the chromosomal distribution of miRNAs and their target genes in cotton.

Network diagram revealed by relationships between miRNAs and their target genes

In order to excavate the valuable miRNAs and putative targets for further research, miRNAs and putative target genes, mapped on the interspecific BC1 genetic linkage map, were used to create a network diagram based on their regulatory relationships (Figure 2, Table 1) by Circos (http://circos.ca/). Table 1 depicts 39 miRNAs and 24 target genes that are contained in this network diagram.
Some miRNAs were associated with more than one target gene, and some target genes were regulated by more than one miRNA family. Individual miRNAs and their target genes are clearly and intuitively depicted in the network diagram (Figure 2, Additional file 7: Figure S2). As shown in Additional file 7: Figure S2, miR393 and its target TC38219 (Additional file 7: Figure S2I), and miR172 and its target TC30742 were located on the same chromosome (Additional file 7: Figure S2H). Also, miR393 and its target TC38219 were proximal on the same chromosome, separated only by 1.78 cM; whereas, miR172-Me10 and its target TC30742 were separated by 30.72 cM. miR172-Me49b and its target TC30742 were separated by 11.41 cM (Table 1). Additional file 7H shows that miR172 and its target TC30742 (both located on Chr10), and another target TC41731 (located on Chr20), are found on homologous chromosomes (Chr10 and Chr20). The miR396 (Additional file 7: Figure S2K) and miR396a...
| miRNAs name       | Chromosome | Subgenome | Genetic map distance (cM) | Target name | Chromosome | Subgenome | Genetic map distance (cM) |
|-------------------|------------|-----------|---------------------------|-------------|------------|-----------|---------------------------|
| miR156c-DX535560★ | Chr23      | D         | 154.258                   | T-TC29212   | Chr11      | A         | 112.712                   |
|                   |            |           |                           | T-CO079328  | Chr21      | D         | 56.729                    |
|                   |            |           |                           | T-TC37339   | Chr21      | D         | 220.676                   |
| miR156c-DX535560★ | Chr23      | D         | 154.258                   | T-TC35884▲  | Chr11      | A         | 214.024                   |
|                   |            |           |                           | T-TC36356▲  | Chr15      | D         | 118.218                   |
| miR157b-CO076888★ | Chr23      | D         | 150.354                   | T-TC35884▲  | Chr11      | A         | 214.024                   |
|                   |            |           |                           | T-TC36356▲  | Chr15      | D         | 118.218                   |
| miR159-Me29       | Chr05      | A         | 165.2                     | T-TC32762   | Chr11      | A         | 94.707                    |
| miR159-Me24       | Chr12      | A         | 156.471                   | T-TC32762   | Chr11      | A         | 94.707                    |
| miR164-Me4a★      | Chr11      | A         | 146.281                   | T-TC41649   | Chr05      | A         | 70.705                    |
| miR164-Me4b★      | Chr20      | D         | 26.305                    | T-TC41649   | Chr05      | A         | 70.705                    |
| miR164-Me12★      | Chr14      | D         | 157.964                   | T-TC41649   | Chr05      | A         | 70.705                    |
| miR164-Me29★      | Chr17      | D         | 79.157                    | T-TC41649   | Chr05      | A         | 70.705                    |
| miR164-Me40★      | Chr13      | A         | 91.878                    | T-TC41649   | Chr05      | A         | 70.705                    |
| miR164-Em33★      | Chr17      | D         | 82.728                    | T-TC41649   | Chr05      | A         | 70.705                    |
| miR164-Em20★      | Chr26      | D         | 143.941                   | T-TC41649   | Chr05      | A         | 70.705                    |
| miR167-Me7★       | Chr25      | D         | 126.206                   | T-TC31104   | Chr12      | A         | 169.342                   |
| miR167-Me49a★     | Chr03      | A         | 67.893                    | T-TC31104   | Chr12      | A         | 169.342                   |
| miR167-Me49b★     | Chr05      | A         | 198.84                    | T-TC31104   | Chr12      | A         | 169.342                   |
| miR169a-Me27      | Chr19      | D         | 18.195                    | T-TC29763▲  | Chr13      | A         | 153.525                   |
| miR169a-Em5       | Chr18      | D         | 84.73                     | T-TC29763▲  | Chr13      | A         | 153.525                   |
| miR169a-Em10      | Chr03      | A         | 75.625                    | T-TC29763▲  | Chr13      | A         | 153.525                   |
| miR169a-Em12      | Chr15      | D         | 134.58                    | T-TC29763▲  | Chr13      | A         | 153.525                   |
| miR169b-Me45      | Chr05      | A         | 131.477                   | T-TC29763▲  | Chr13      | A         | 153.525                   |
| miR169b-Em10      | Chr19      | D         | 64.043                    | T-TC29763▲  | Chr13      | A         | 153.525                   |
| miR169b-Em29b     | Chr10      | A         | 87.599                    | T-TC29763▲  | Chr13      | A         | 153.525                   |
| miR172-Me6★       | Chr21      | D         | 149.272                   | T-TC30742   | Chr10      | A         | 123.541                   |
| miR172-Me10★      | Chr10      | A         | 92.823                    | T-TC30742   | Chr10      | A         | 123.541                   |

Chen et al. BMC Genomics 2013, 14:706
http://www.biomedcentral.com/1471-2164/14/706
families (Additional file 7: Figure S2L) have the same targets as depicted in the network diagram: TC35055 and TC39199. Chromosomes on which miR396 and miR396a are located are also on homologous chromosomes (Chr07 and Chr16), and their target, TC35055, has two sites. Furthermore, TC350055a, located on Chr19, and TC350055b, located on Chr05, are found on homologous chromosomes. Additional file 7: Figure S2G depicts the same information: miR196b-Me45 was located on Chr05 and miR196b-Em10 was located on Chr19. For miR156c-DX53560 (Additional file 7: Figure S2A), its target, TC37339 was located on Chr21 and another target TC29212 was located on Chr11, which are homologous chromosomes. Additional file 7: Figure S2F depicts the location of miR169a (Chr18) and TC29763 (Chr13), which are also located on homologous chromosomes. Other miRNAs and their target genes were located on different chromosomes.

In our network diagram, the relationship and distribution of miRNAs and their putative target genes on the interspecific BC$_1$ genetic linkage map are clearly and intuitively revealed. This diagram will facilitate our understanding of the regulatory mechanisms and networks for target genes and cell development in cotton. Allen et al. [36] stated that plant miRNAs are thought to be derived from their target sequences after gene duplication, inverted duplication, and divergence in miRNA evolution. Additional file 7: Figure S2 shows that many miRNAs and

| miRNAs     | Chromosome | Gene   | Location | Distance | Chromosome | Gene   | Location |
|------------|------------|--------|----------|----------|------------|--------|----------|
| miR172-Me49a | Chr13      | A      | 76.047   | T-TC30742 | Chr10      | A      | 123.541  |
|            |            |        |          |          |            |        |          |
| miR172-Me49b | Chr10      | A      | 134.949  | T-TC30742 | Chr10      | A      | 123.541  |
|            |            |        |          |          |            |        |          |
| miR172-Em20 | Chr21      | D      | 229.243  | T-TC30742 | Chr10      | A      | 123.541  |
|            |            |        |          |          |            |        |          |
| miR393-Me4 | Chr08      | A      | 67.257   | T-TC38219 | Chr08      | A      | 65.478   |
| miR393-Me42 | Chr12      | A      | 154.801  | T-TC38219 | Chr08      | A      | 65.478   |
| miR393-Em1 | Chr16      | D      | 44.796   | T-TC38219 | Chr08      | A      | 65.478   |
| miR395-Me4 | Chr19      | D      | 18.742   | T-CO128820| Chr18      | D      | 65.452   |
| miR396-Me7 | Chr07      | A      | 85.357   | T-TC35055b | Chr05      | A      | 155.907  |
|            |            |        |          |          |            |        |          |
| miR396-Me43 |Chr10      | A      | 53.687   | T-TC35055b | Chr05      | A      | 155.907  |
|            |            |        |          |          |            |        |          |
| miR396a-DX562190 | Chr16 | D | 73.216 | T-TC35055b | Chr05 | A | 155.907 |
|            |            |        |          |          |            |        |          |
| miR397-Me49 | Chr09      | A      | 145.396  | T-AW730829| Chr11      | A      | 226.873  |
|            |            |        |          |          |            |        |          |
| miR397a-DW490000 | Chr04 | A | 59.354 | T-TC31021 |Chr19 | D | 0 |
| miR403-Me35 | Chr02      | A      | 71.74    | T-CO078480| Chr13      | A      | 194.48   |
| miR403-Em27 | Chr11      | A      | 146.949  | T-CO078480| Chr13      | A      | 194.48   |
| miR403-Me20 | Chr17      | D      | 84.782   | T-CO078480| Chr13      | A      | 194.48   |
| miR479-ES5809290a | Chr26 | D | 44.775 | T-TC31154 |Chr18 | D | 121.25 |

★: miRNA was associated with more than one target on the interspecific BC$_1$ linkage map.
▲: Target was regulated by more than one miRNAs family on the interspecific BC$_1$ linkage map.
their targets, miRNAs with the same target, and targets of the same miRNA family were located on homologous chromosomes or the same chromosome. These data are consistent with Allen’s published findings [36]. Other miRNAs and their targets did not reside on the same or homologous chromosomes, which may be due to evolutionary mutations or deletions or insufficient marker numbers. It was noteworthy that homologous chromosomes, Chr5 and Chr19, contained more targets and miRNAs as depicted in the network diagram, and this may have implications for future studies of miRNAs and their targets.

Expression differences between parents by RT-PCR and qRT-PCR

We used RT-PCR analysis to investigate pre-miRNAs and their putative target genes during fiber development between *G. hirsutum* and *G. barbadense*. A total of 181 primer pairs were used for RT-PCR analysis, including 83 pre-miRNA primers and 98 randomly selected target gene primers. Twenty-five pre-miRNA genes (30.1%) were expressed in cotton fibers. Among them, 88% of these expressed pre-miRNAs were significantly different among various stages of fiber development (0, 5, 10, 15, 20, and 25 DPA) between Emian22 and 3–79. Ten pre-miRNAs had weak or no expression in both Emian22 and 3–79 (defined as no expression differences in this study). Furthermore, 13 pre-miRNAs had similar expression patterns, and 9 pre-miRNAs had different expression patterns (Figure 3). Figure 3 also shows that 40% of the pre-miRNA genes had higher expression in early ovary development.

Regarding target genes, 66 (67.3%) were expressed in cotton fiber (Additional file 8: Table S6). Among them, 50 were obviously differentially expressed; 14 had minor differences; and 2 were not different (Figure 4). Overall, 75.8% of the expressed target genes were significantly different at various fiber development stages (0, 5, 10, 15, 20, and 25 DPA), when comparing Emian22 and 3–79. Three target genes were weakly expressed or not expressed at all stages in both Emian22 and 3–79 (defined as no expression differences in this study). Furthermore, 39 target genes had similar expression patterns. Of the 25 target genes with different expression patterns, 13 were up-regulated and 12 were down regulated in 3–79, compared with Emian22. To further confirm RT-PCR data, target genes belonging to different categories were randomly chosen for qRT-PCR analysis (Additional file 9: Figure S3). Consistent results were observed between both RT-PCR and qRT-PCR analyses.

In this study, a diagram was constructed to depict pre-miRNAs and their corresponding target genes (Additional file 10: Figure S4). Additional file 10: Figure S4 shows that miR156c-DX535560, miR472-DT527030, and miR482a-DR457519 were similar with respect to their targets in various fiber development stages of Emian22. However, no correlation was observed between
pre-miRNA and its target expression in various fiber development stages of 3–79. MiR157-DT558905, miR395a-DT567568 and miR395a-DT567568 had contrasting expression tendencies with their targets in various stages of Emian22, but no correlation was observed between the expression of pre-miRNA and its targets in various fiber development stages of 3–79. MiR172-TC124126, miR413-AW187128, and miR414a-DR452834 and their targets had similar expression tendencies in various parent stages. MiR414d-DW514431 had contrasting expression tendencies with its targets in various stages of Emian22 and had similar expression tendencies in various stages of 3–79.

In summary, we report dynamic expression of pre-miRNAs and putative target genes at various fiber development stages in G. hirsutum and G. barbadense. Most expressed pre-miRNAs (88%) and their putative targets (75.8%) were obviously different with respect to expressions...
at different stages between the two parents. Unique expression patterns of pre-miRNAs and their putative targets may be connected with a particular function. We also found that pre-miRNA families and their putative targets had correlated expression tendencies in various growth stages, suggesting that miRNA genes may regulate target gene expression. However, the biological relevance of this must be investigated further to better understand regulatory mechanisms and the overall network of plant growth control.

Conclusions
In this study, miRNAs and target markers were developed. We found that although the primers were specific, pre-miRNA primer polymorphisms were low (14.5%), and target primer polymorphisms were even lower (11.7%). The 63 miRNA loci were evenly distributed on 24 chromosomes, except Chr22 and Chr24. Also, 156 target gene loci were preferentially distributed on the Dt sub-genome and some chromosomes. Genetic mapping in a segregating population provides an opportunity to examine distribution of miRNAs and their target genes. A network diagram depicting miRNAs and their targets confirmed that plant miRNAs may be derived from their target sequences after gene duplication or inverted duplication [36]. Comparative expression analyses of Gossypium hirsutum and G. barbadense revealed that miRNAs and their target genes play a role in cotton fiber development.

Methods
Marker development
Pre-miRNA sequences reported in the literature and a database (http://www.mirbase.org) were downloaded [37-43] (Additional file 1: Table S1). Putative target genes were predicted and downloaded with psRNATarget (http://plantgrn.noble.org/psRNATarget/). Because one target gene may relate to more than one miRNA, repeat target sequences were deleted. Finally, a total of 123 pre-miRNA sequences and 1,399 putative target sequences were obtained. Primers were then designed based on the pre-miRNAs and the target sites of the putative target genes by Primer 3.0 (examples in Figure 5). Criteria for primer design were a primer length of 18–25 bp (20 bp optimum), GC content of 35–70% (50% optimum), annealing temperature of 50–65°C (55°C optimum), and PCR product size of 100–1,000 bp.

Because the polymorphism is low in cotton, to genetically map more miRNAs, SRAP primers, including 64 forward primers and 64 reverse primers [44] were combined with 23 miRNA degenerate primers [32] in the form of miRNA-SRAP analysis. Finally, a total of 2,944 primer combinations were applied in this study.

PCR amplification and electrophoresis
PCR of pre-miRNAs and their putative target genes was performed in a solution (10 μL) containing 25 ng DNA template, 1 x Buffer, 2.0 mmol L\(^{-1}\) MgCl\(_2\), 0.25 mmol L\(^{-1}\) dNTPs, 0.16 μmol L\(^{-1}\) of forward primer, 0.16 μmol L\(^{-1}\) of reverse primer, 0.8 units of Taq DNA polymerase, and ddH\(_2\)O was added to 10 μL. The PCR program was performed using the following profile: 95°C for 5 min, followed by 34 cycles consisting of 94°C for 50 sec, 56°C for 45 sec, and 72°C for 60 sec; and a final extension step of 5 min at 72°C. Then, PCR products were separated on an 8% non-denaturing polyacrylamide gel at a constant voltage (15 W) for about 4 h at room temperature. After electrophoresis, DNA fragments were detected by silver staining, coloring in a sodium hydroxide and formaldehyde solution.

PCR of an miRNA-based SRAP marker was performed in a solution (10 μL) containing 30 ng DNA template, 1 x Buffer, 2.0 mmol L\(^{-1}\) MgCl\(_2\), 0.2 mmol L\(^{-1}\) dNTPs, 0.1 μmol L\(^{-1}\) of forward primer, 0.1 μmol L\(^{-1}\) of reverse primer, 0.5 units of Taq DNA polymerase, and ddH\(_2\)O was added to 10 μL. The PCR program was performed

![Figure 5 Primer design strategies for miRNAs and target genes. (a) miR414d from DW514431 (Genbank acc No. DW514431); (b) target gene of miR414d from TC31963 (Cotton Gene Index No. TC31963, http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=cotton). *: Target sequence; >>>>>>: Primer region.](http://www.biomedcentral.com/1471-2164/14/706)
using the following profile: 94°C for 5 min, followed by 4 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; then by 34 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min; and a final extension step of 10 min at 72°C. Then, PCR products were separated on a 6% denaturing polyacrylamide gel (29:1 acrylamide and N, N-methylene bisacrylamide) at a constant voltage (80 W) for about 2 h at room temperature. After electrophoresis, DNA fragments were detected by silver staining, coloring in a sodium hydroxide and formaldehyde solution.

Genetic mapping
Our BC1 mapping population (\[Emian22 × 3–79\] × Emian22) \[45,46\] was used to map miRNAs and their putative target genes. All primers with polymorphisms between parents were used to enrich the interspecific BC1 genetic linkage map \[46\]. Polymorphic loci were integrated into the interspecific BC1 linkage map using JoinMap V3.0 \[47\]. The logarithm of odds (LOD) threshold was 5.0. Map distances in centi Morgans (cM) were calculated using the Kosambi mapping function \[48\]. The linkage map was drawn by MapChart V2.2 software \[49\].

Drawing method of network diagram
In this study, miRNAs and their putative target genes were mapped in cotton chromosomes. The distribution of miRNAs and their putative target markers was obtained from the genetic linkage map. MiRNA loci and their putative target loci that were mapped on the chromosomes were then “connected” using individually colored lines to indicate each miRNA family’s connection to their putative target genes. Then, a network diagram was obtained based on the distribution of miRNAs and their target markers on genetic linkage map by Circos (http://circos.ca/).

RT-PCR and qRT-PCR analysis
To determine the expression difference of pre-miRNAs and their putative target genes between \(G. \text{hirsutum}\) and \(G. \text{barbadense}\), RNAs were extracted from developing fibers at 0 days post anthesis (DPA), 5 DPA, 10 DPA, 15 DPA, 20 DPA and 25 DPA, and RNAs (4 μg) were reverse-transcribed to cDNA by the M-MLV-RT Reverse Transcriptase (Invitrogen). RT-PCR and qRT-PCR analyses were performed according to the methods described by Munis et al. \[50\] with minor modifications. Ubiquitin (GenBank acc No.: DQ116441) primer pair (forward 5′-GAAGGCATTCCACCTGACA AC3′, reverse 5′-CTTGACCTTCTTCTTGTGCTTG 3′) \[51\] was used as an internal standard showing the equal amounts of first-stranded cDNA in each sample. In addition, qRT-PCR experiments were biologically repeated three times.

Additional files

Additional file 1: Table S1. Source of miRNA sequences and distribution in the scientific literature.

Additional file 2: Table S2. Characteristics of 83 pre-miRNA primers in this study. √: Polymorphic primers; ×: No polymorphic primers. The primer was not mapped on the chromosome.

Additional file 3: Table S3. Characteristics of 1,255 target primers in this study. √: Polymorphic primers; ×: No polymorphic primers. The primer was not mapped on the chromosome.

Additional file 4: Table S4. Characteristics of 55 miRNA-5'-SRAP polymorphic primer combinations in this study. The primer was not mapped on the chromosome.

Additional file 5: Figure S1. Locations of polymorphic markers on the BC1 genetic linkage map. miRNA markers are underlined and in bold. Target markers are underlined. miRNA-based SRAP markers are italic and in bold.

Additional file 6: Table S5. Marker distribution on the interspecific BC1 genetic linkage map.

Additional file 7: Figure S2. Network diagram revealed relationships between one miRNA family and their target genes. Families are as follows: A: miR156c family; B: miR157b family; C: miR159 family; D: miR164 family; E: miR167 family; F: miR169a family; G: miR169b family; H: miR172 family; I: miR393 family; J: miR395 family; K: miR396 family; L: miR396a family; M: miR397 family; N: miR397a family; O: miR403 family; P: miR479 family; Red circle: MiRNAs; black circle: Target genes. The scale marked on each chromosome represented a genetic map distance (cM).

Additional file 8: Table S6. Target gene primers and their corresponding miRNA families.

Additional file 9: Figure S3. qRT-PCR analysis of eight primers randomly chosen from pre-miRNAs and targets. Expression levels of Emian22 and 3–79 are shown. ** represents P<0.05, and *** represents P<0.001. Primers are as follows: A: miR164-BF270853; B: miR393-TC18241; C: miR434-AW187128; D: T-2F275322; E: T-8M13579942; F: T-TC27422; G: T-TC157575; H: T-TC83767.

Additional file 10: Figure S4. Wiring diagram of target gene primers and their corresponding miRNA families. (a) RT-PCR analysis of miRNA genes; (b) RT-PCR analysis of target genes.

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

Authors’ contributions
XC designed the primers, performed the experiments, and drafted the manuscript. WG made the network diagram. JZ provided the miRNA degenerate primers. XD participated in study design and manuscript proofreading. ZL directed the experiments, proofread the manuscript, and advised the scientific writing. All authors contributed to data interpretation, read, and approved the final manuscript.

Acknowledgements
This work was financially supported by the National Basic Research Program (No. 2010CB126001), and Genetically Modified Organisms Breeding Major Project of China (No. 2008ZX08009-001).

Author details
1National Key Laboratory of Crop Genetic Improvement & National Centre of Plant Gene Research (Wuhan), Huazhong Agricultural University, 430070 Wuhan, Hubei, China. 2Department of Plant and Environmental Sciences, New Mexico State University, Box 30003, Las Cruces 88083, NM, USA.

Received: 24 June 2013 Accepted: 8 October 2013 Published: 16 October 2013

References
1. Reinharz BJ, Weinstein EG, Rhodes MW, Bartel B, Bartel DP. MicroRNAs in plants. Genetics Dev 2002, 16(7):2313–2313.
1. Lee EC: MicroRNAs: runts of the genome assert themselves. Cur Riol Biol 2003, 13(23):R925–R936.

2. Carrington JC, Ambros V: Role of microRNAs in plant and animal development. Science 2003, 301(5631):336–338.

3. Ambros V, Bartel B, Bartel DP, Burge CB, Chen XM, Dreyfuss G,玉SD, Fire AL, Fox CT, Garrett-Engele P, Gardner EL, Gravina G, Hannon GJ, Hughes TR, Kang AS, Kennerdell JS, Lambeth JD, Lee RC, Lewis BP, Matsunami H, Meister G, Morin PJ, Bartel DP, Bartel BD: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004, 116(2):281–297.

4. Yu ZR, Raabe T, Hect NB: MicroRNA Mir122a reduces expression of the posttranscriptionally regulated germ cell transition protein 2 (Tip2) messenger RNA (miRNA) by miRNA cleavage. Biol Reprod 2005, 73(4):427–433.

5. Bushati N, Cohen SW: MicroRNA functions. Annu Rev Cell Dev Biol 2007, 23:175–205.

6. Jones-Rhoades MW, Bartel DP, Bartel B: MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 2006, 57:19–53.

7. Lee RC, Feinbaum RL, Ambros V: An extensive class of small RNAs in Caenorhabditis elegans. Science 2001, 294(5543):862–864.

8. Llave C, Xie XZ, Kasschau KD, Carrington JC: Cleavage of scarecrow-like miRNA target directed by a class of Arabidopsis miRNAs. Science 2002, 297(5589):2053–2056.

9. Sunkar R, Zhu JK: Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. Plant Cell 2004, 16(8):2001–2019.

10. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G: The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 2004, 430(7001):373–378.

11. Chen XL, Schneeberger C, Friesen C, Liu F, Wang Q, Zhai J, Wang K, Yi L, Wang D: Novel microRNAs and their targets including a stress-induced miRNA. Proc Natl Acad Sci USA 2004, 101(24):8771–8776.

12. Emery JF, Floyd SK, Alvarez J, Eshed Y, Hawker NP, Izhaki A, Baum SF, Bowman JL: Radial patterning of Arabidopsis shoots by class IIIId-ZIP and KANADI genes. Cur Riol Biol 2003, 13(20):1768–1774.

13. Mica E, Gianfranceschi L, Pe ME: MicroRNAs and their roles in plants. Annu Rev Cell Dev Biol 2006, 22:1–22.

14. Wei M, Wei H, Wu M, Song M, Zhang J, Yu J, Fan S, Yu S: Comparative expression profiling of miRNA during another development in genetic male sterile and wild type cotton. BMC Plant Biol 2013, 13(1):66.

15. Paterson AH, Wendel JF, Gundlach H, Guo H, Jenkins J, Jin D, Llewellyn D, Showmaker KC, Shu S, Uddall J, Yoo MI, Byers R, Chen W, Doran-Faigenboim A, Duke MV, Gong L, Grimwood J, Grover C, Gruppo K, Hu G, Lee TH, Li J, Lin L, Liu T, Marler BS, Page JT, Roberts AW, Romanel E, Sanders WS, Szadkowski E, et al: Repeated polyploidization of Gossypium genomes and the evolution of spinnable cotton fibres. Nature 2012, 492(7429):423–427.

16. Wang KB, Wang ZW, Li FG, Ye WW, Wang JT, Song GL, Yue Z, Gong L, Chang L, Shang H, Shi Z, Liu LS, Yang YL, Lu CR, Wei HL, Gou CY, Zheng QZ, Yin Y, Zhang XY, Liu K, Wang B, Song C, Shi N, Kohel Rl, Percy RG, Yu JZ, Zhu YX, Yu SX: The draft genome of a diploid cotton Gossypium raimondii. Nat Genet 2012, 44(10):1098–1103.

17. Li Q, Lin X, Zhu YX: Identification and analyses of miRNA genes in allotetraploid Gossypium hirsutum fiber cells based on the sequenced diploid G. raimondii genome. J Genet Genomics 2012, 39(7):351–360.

18. Emery JF, Floyd SK, Alvarez J, Eshed Y, Hawker NP, Izhaki A, Baum SF, Bowman JL: Radial patterning of Arabidopsis shoots by class IIIId-ZIP and KANADI genes. Cur Riol Biol 2003, 13(20):1768–1774.

19. Mica E, Gianfranceschi L, Pe ME: MicroRNAs and their roles in plants. Annu Rev Cell Dev Biol 2006, 22:1–22.

20. Wei M, Wei H, Wu M, Song M, Zhang J, Yu J, Fan S, Yu S: Comparative expression profiling of miRNA during another development in genetic male sterile and wild type cotton. BMC Plant Biol 2013, 13(1):66.

21. Paterson AH, Wendel JF, Gundlach H, Guo H, Jenkins J, Jin D, Llewellyn D, Showmaker KC, Shu S, Uddall J, Yoo MI, Byers R, Chen W, Doran-Faigenboim A, Duke MV, Gong L, Grimwood J, Grover C, Gruppo K, Hu G, Lee TH, Li J, Lin L, Liu T, Marler BS, Page JT, Roberts AW, Romanel E, Sanders WS, Szadkowski E, et al: Repeated polyploidization of Gossypium genomes and the evolution of spinnable cotton fibres. Nature 2012, 492(7429):423–427.

22. Wang KB, Wang ZW, Li FG, Ye WW, Wang JT, Song GL, Yue Z, Gong L, Chang L, Shang H, Shi Z, Liu LS, Yang YL, Lu CR, Wei HL, Gou CY, Zheng QZ, Yin Y, Zhang XY, Liu K, Wang B, Song C, Shi N, Kohel Rl, Percy RG, Yu JZ, Zhu YX, Yu SX: The draft genome of a diploid cotton Gossypium raimondii. Nat Genet 2012, 44(10):1098–1103.

23. Li Q, Lin X, Zhu YX: Identification and analyses of miRNA genes in allotetraploid Gossypium hirsutum fiber cells based on the sequenced diploid G. raimondii genome. J Genet Genomics 2012, 39(7):351–360.

24. Emery JF, Floyd SK, Alvarez J, Eshed Y, Hawker NP, Izhaki A, Baum SF, Bowman JL: Radial patterning of Arabidopsis shoots by class IIIId-ZIP and KANADI genes. Cur Riol Biol 2003, 13(20):1768–1774.

25. Mica E, Gianfranceschi L, Pe ME: MicroRNAs and their roles in plants. Annu Rev Cell Dev Biol 2006, 22:1–22.

26. Wei M, Wei H, Wu M, Song M, Zhang J, Yu J, Fan S, Yu S: Comparative expression profiling of miRNA during another development in genetic male sterile and wild type cotton. BMC Plant Biol 2013, 13(1):66.

27. Paterson AH, Wendel JF, Gundlach H, Guo H, Jenkins J, Jin D, Llewellyn D, Showmaker KC, Shu S, Uddall J, Yoo MI, Byers R, Chen W, Doran-Faigenboim A, Duke MV, Gong L, Grimwood J, Grover C, Gruppo K, Hu G, Lee TH, Li J, Lin L, Liu T, Marler BS, Page JT, Roberts AW, Romanel E, Sanders WS, Szadkowski E, et al: Repeated polyploidization of Gossypium genomes and the evolution of spinnable cotton fibres. Nature 2012, 492(7429):423–427.
49. Voorrips RE: MapChart: software for the graphical presentation of linkage maps and QTLs. *J Hered* 2002, 93(1):77–78.

50. Munis MFH, Tu LL, Deng FL, Tan JF, Xu L, Xu SC, Long L, Zhang XL: A thaumatin-like protein gene involved in cotton fiber secondary cell wall development enhances resistance against *Verticillium dahliae* and other stresses in transgenic tobacco. *Biochem Bioph Res Co* 2010, 393(1):38–44.

51. Tu LL, Zhang XL, Liu DQ, Jin SX, Cao JL, Zhu LF, Deng FL, Tan JF, Zhang CB: Suitable internal control genes for qRT-PCR normalization in cotton fiber development and somatic embryogenesis. *Chinese Sci Bull* 2007, 52:3110–3117.