Hindawi Publishing Corporation
Journal of Chemistry
Volume 2014, Article ID 536134, 8 pages
http://dx.doi.org/10.1155/2014/536134

Research Article

Construction of Differential-Methylation Subtractive Library

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Received 16 May 2014; Accepted 23 June 2014; Published 9 July 2014

Academic Editor: Wang Zhenhua

Stress-induced ROS changes DNA methylation patterns. A protocol combining methylation-sensitive restriction endonuclease (MS-RE) digestion with suppression subtractive hybridization (SSH) to construct the differential-methylation subtractive library was developed for finding genes regulated by methylation mechanism under cold stress. The total efficiency of target fragment detection was 74.64%. DNA methylation analysis demonstrated the methylation status of target fragments changed after low temperature or DNA methyltransferase inhibitor treatment. Transcription level analysis indicated that demethylation of DNA promotes gene expression level. The results proved that our protocol was reliable and efficient to obtain gene fragments in differential-methylation status.

1. Introduction

DNA methylation exists extensively in the genomes of bacteria, animals, and plants. In plants, about 20~30% cytosines are methylated in the nuclear genome [1]. In prokaryotes, modification of DNA prevents cleavage by the cognate restriction endonucleases [2]. In higher eukaryotes, DNA methylation is one of the epigenetic modifications which plays an important role in regulating development and developmental processes [3]. *Arabidopsis* decreased DNA methylation (ddm) mutants show pleiotropic phenotypes in development, such as early flowering, dwarfism, and irregular organ number [4]. In pumpkin, DNA methylation status changes were observed during somatic embryogenesis. DNA methylation reached the highest level in early embryo stages and decreased during embryo maturation [5]. Hypermethylation of promoter sequences is associated with transcriptional gene silencing while hypermethylation of transcribed or coding sequences is linked with posttranscriptional gene silencing [6]. In all, DNA methylation has been associated with many gene regulatory mechanisms including genomic imprinting, transcriptional regulation of genes and transposable elements, and gene silencing [6–10].

Animals and plants are constantly exposed to environmental stresses (biotic or abiotic). These stresses often lead to the increase in ROS level in cell. Growing evidence supports that stress-induced oxidative damage changes DNA methylation patterns, which in turn modulates gene expression [11]. Recent studies indicate that epigenetic mechanisms, such as DNA methylation, play a key role in regulating gene expression in response to environmental stresses [12]. When exposed to osmotic stress, a reversible hypermethylation of cytosine was observed in tobacco [13]. Long exposure to cold stress resulted in a stable transcription silencing of *FLC*, leading to flowering inhibition [14]. Dyachenko et al. [15] showed a twofold increase in CpNpG methylation level in nuclear genome of *M. crystallinum* plants exposed to high salinity. Choi and Sano [16] reported that environmental stimuli such as salinity, cold, and aluminum stress could cause demethylation in the coding region of *NtGPDL* and subsequently activated its expression. In rice, DNA methylation pattern changed in response to drought stress: the methylation level decreased and there was a large difference in DNA methylation/demethylation site in drought-tolerant and drought-sensitive lines under drought stress [12]. In wheat, salinity stress triggered the decrease in methylation

level [17]. Furthermore, changes in DNA methylation pattern can affect tolerance to stress. It was found that treatment with demethylating reagent, 5-azacytidine (5-Aza), resulted in wheat seedlings more tolerant to salt stress [18]. Exposure to zinc stress led to change in methylation patterns. This change could be stably inherited to progeny and caused progeny more tolerant to zinc stress [19].

Since DNA methylation plays an important role in numerous biological processes, more and more techniques such as MSAP, MS-RDA, bisulfite sequencing, and microarray analysis [20–23] are developed to analyze methylation patterns or to screen for genes regulated by methylation mechanism. However, MSAP may lead to loss of target genes because it is difficult to distinguish different sequences with the same length using these techniques. MS-RDA is technically complicated. Although bisulfite sequencing and microarray analysis can get more detailed information about the methylation site, these methods are relatively expensive. Therefore, a more efficient and economical method is necessary. Here, we described an approach combining methylation-sensitive restriction endonuclease (MS-RE) digestion with suppression subtractive hybridization (SSH) to generate a library for screening methylation-regulated genes.

2. Protocol Design

DNA methylation status is associated with gene expression. Methylation of DNA cytosine bases can repress the gene transcription, while demethylation can upregulate the gene transcription. Here, we design a protocol that combined the methylation-sensitive restriction endonuclease (MS-RE) digestion with suppression subtractive hybridization (SSH) to construct a differential-methylation subtractive library for finding genes upregulated by DNA demethylation.

2.1. Preparation of Drivers and Testers. Genomic DNA was extracted from *Arabidopsis* seedlings grown in different conditions and then was digested with Mse I, which recognizes and cuts the -TTAA- sequence in genome, to cut the genomic DNA into smaller fragments. The digested fragments from the plants grown in normal condition were ligated to different adapters to make two types of testers. The digested fragments from the plant grown in low temperature were ligated to the linker to produce the predrivers. Both testers and predrivers were digested with Hha I/Hpa II and then retrieved. The predrivers were amplified for 20 cycles to get drivers. All linkers, adapters, and primers used for SSH are listed in Table 1.

2.2. Subtractive Hybridization. Drivers were mixed with two groups of testers, respectively, at a ratio of 40 : 1 or higher. The mixtures were heat-denatured at 98°C for 15 min and allowed to anneal at 68°C for 9 h. After the first hybridization, the two mixtures were mixed and an equal volume of fresh hybridization buffer containing heat-denatured driver was added. The final mixture was allowed to hybridize at 68°C for another 9 h. At last, the mixture was diluted to 100 μL with dilution buffer (containing 20 mM Hepes (pH 8.3), 50 mM NaCl, and 0.2 mM EDTA) and heated at 68°C for 10 min and then stored at −20°C.

2.3. PCR Amplification and Cloning of Target. A nest-PCR was employed for the target amplification. The primary PCR contained 2 μL of diluted subtracted DNA, 1 μL of MP1 and MP2 (10 μM each), 2.5 μL of 10x PCR buffer, 1 μL of dNTP, and 0.5 μL of Taq in 25 μL volume. PCR was performed at 72°C for 15 min and then 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 66°C for 30 s, and 72°C for 1.5 min, with extension at 72°C for 10 min. The first PCR product was diluted 10 times with deionized water and used as the template in the secondary PCR under a similar condition to the primary PCR except for the primers replaced with MPN1 and MPN2, and the anneal temperature was set to 68°C. The secondary PCR products were ligated into the PMD-18 simple vector and then transformed into *E. coli* strain DH5α. The ampicillin resistant clones were selected for colony PCR test under a similar condition to the secondary PCR. The PCR positive clones were collected to generate the library.

3. Materials and Methods

3.1. Plant Material and Growth Condition. Seeds of wild type *Arabidopsis thaliana* (Columbia background, Col-0) were surface sterilized with 15% NaClO for 15 min, rinsed with sterilized water for 3 times, vernalized at 4°C for 2 days, and then transferred to Murashige and Skoog (MS) solid medium containing 3% sugar for germination. Plants were grown under long-day conditions (16 hours light/8 hours dark) at 23°C. For cold treatment, 7-day-old seedlings were transferred to a 4°C chamber with the same light condition for 3 days. For demethylation treatment, 7-day-old seedlings were transferred to MS medium containing 50 μM 5-azacytidine for 3 days.

3.2. DNA Isolation and Digestion with Restriction Enzymes. A modified CTAB method [24] was employed to isolate genomic DNA. A 5 μg aliquot of DNA was digested with MseI (Fermentas ER0981). Fragments retrieved using TIAN quick Midi Purification Kit (Tiangen DP204-02) were separated into two groups: group I (from plants grown at 4°C) was ligated to the linker and group II was ligated (from plants grown at 23°C) to adapters. After double digestion with two methylation-sensitive restriction enzymes, Hha I/Hpa II, group I fragments were retrieved for preamplification. Group II DNA was used as the tester.

3.3. Preamplification. 1 μg of digestion products was amplified by 20 cycles of PCR with oligonucleotide H24 as a primer. After retrieval, the PCR product was used as the driver.

3.4. Subtractive Library Construction and Sequencing. A subtractive library was constructed according to Diatchenko's protocol [25]. The ratio of driver to tester was about 60 : 1. The final PCR products were linked to PMD-18T simple vector (Takara, D-103A) and then transformed into *E. coli* strain DH5α. Ampicillin resistant single clones were selected. The

Table 1: Linkers, adapters, and primers used for SSH.

Group	Oligonucleotides	DNA sequence
Linker	Linker H24	AGGCAACTGTGCTATCCGAGGGAT
	Linker H12	TAATCCCTCGGA
Adapter	Ad1	CTACTACGTCGCACTGTTGGTTGGCGGGACGGCGCTCGTTAAGTC
	Ad1c	TAGACTTAACGAGC
	Ad2	TGTAGCGTGAAGACGACAGAAACCGCGTGGTGCTGAGGGCGTGAC
	Ad2c	TAGTCACGCCCT
Primer	MP1	CTACTACGTCGCACTGTTGGT
	MPN1	GCGGGACGGCGCTCGTTAAGTC
	MP2	TGTAGCGTGAAGACGACAGAA
	MPN2	CGCGTGGTGCTGAGGGCGTGAC

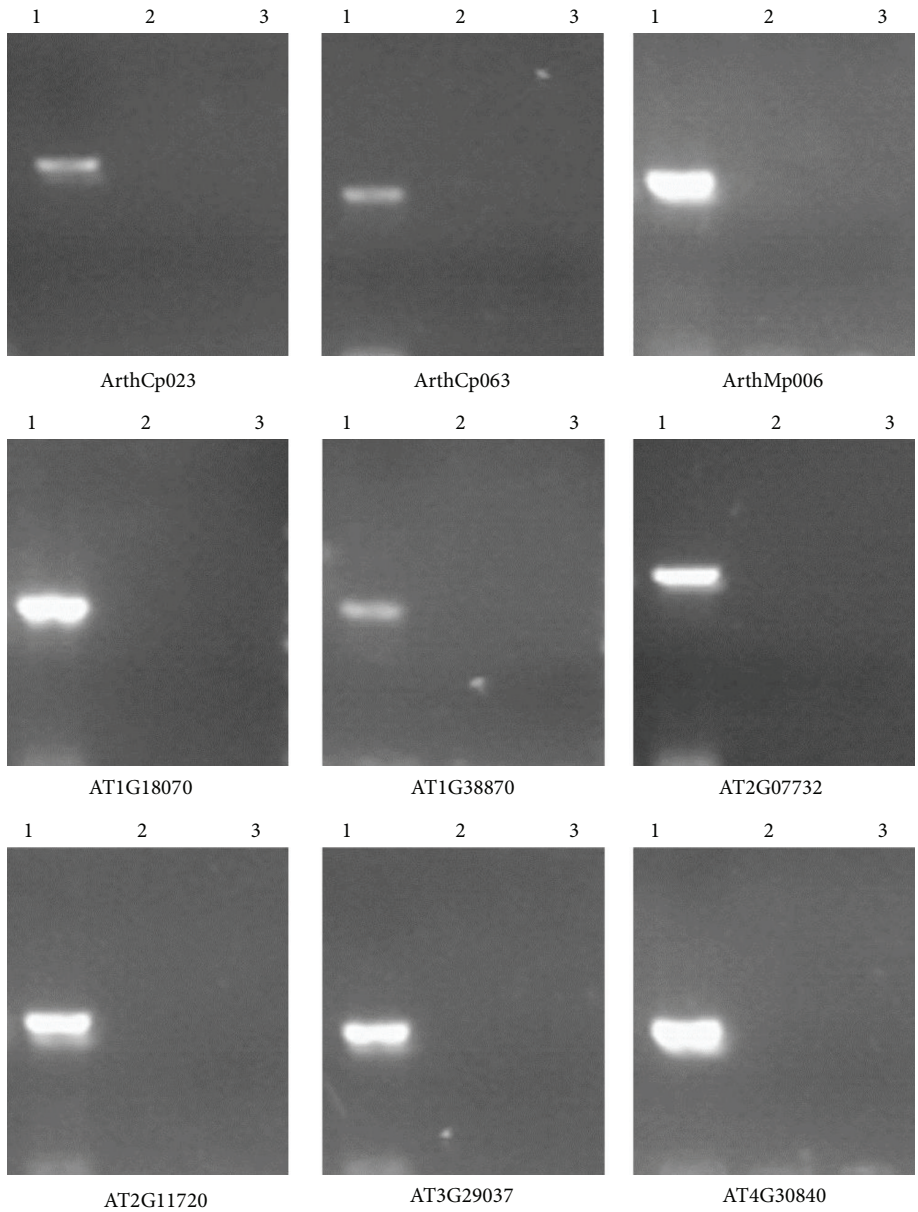

Figure 1: MS-RE PCR results of some genes under different conditions. Genomic DNA extracted from plants treated with cold (4°C) (Lane 2), 5-Aza (50 μM) (Lane 3) or normal conditions (Lane 1) were digested with Hha I/Hpa II, then taken PCR amplification.

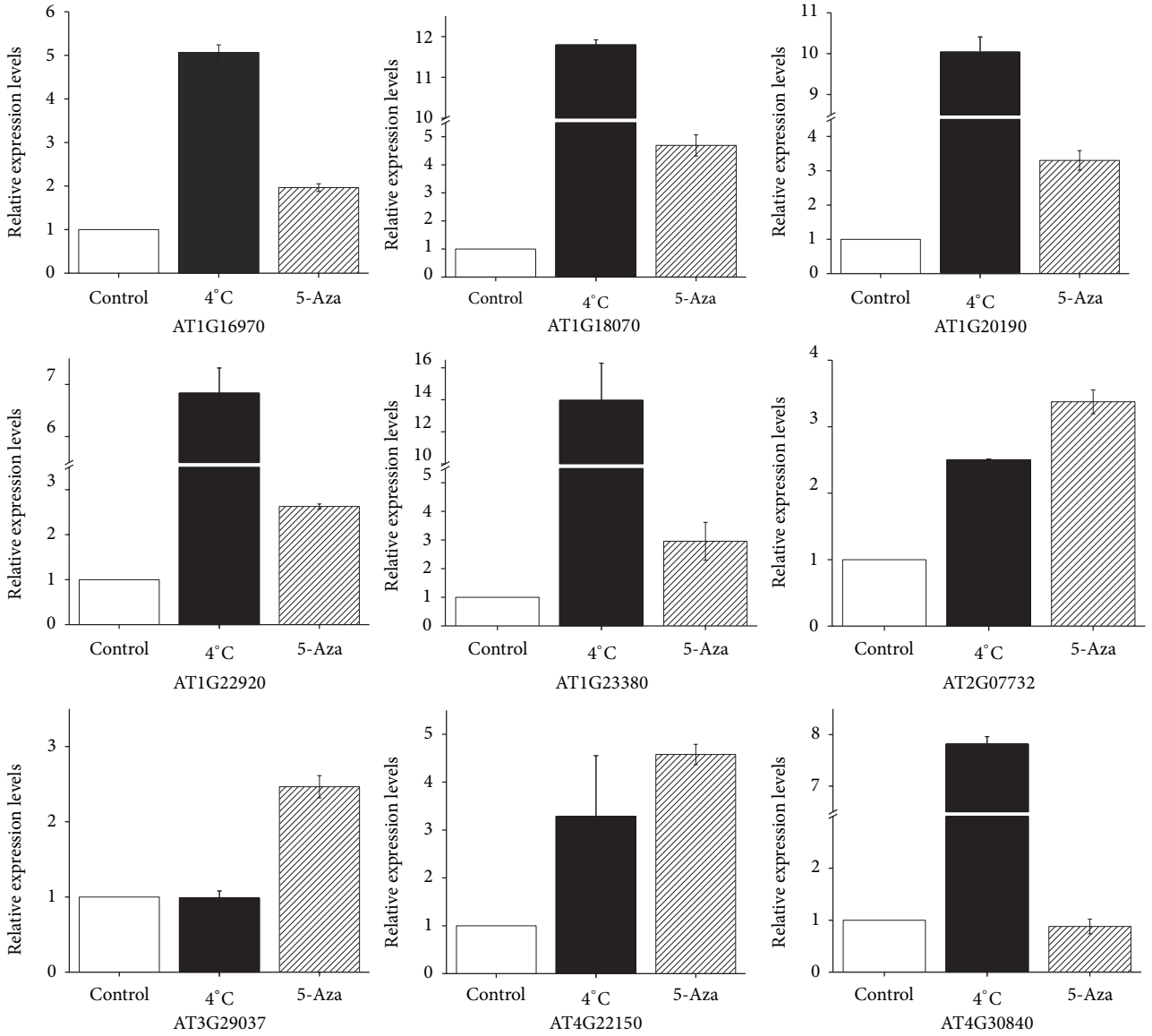

Figure 2: Transcript levels of some genes under different conditions.

second PCR primers were employed for bacteria liquid PCR test. The positive clones were stored to get the subtractive library and sent for sequencing.

3.5. Sequencing Results Analysis. Sequencing results were identified by BLAST searches against the *Arabidopsis thaliana* genome database from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=OGP__3702). Primers were designed according to the BLAST search results.

3.6. DNA Methylation Analysis. A MS-RE PCR was performed for DNA methylation analysis. Genomic DNA was digested with Hha I/Hpa II and then retrieved for PCR amplification. The primers for MS-RE PCR are listed in Table S1 (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/536134).

3.7. Quantitative Real-Time RT-PCR Analysis. A two-step quantitative real-time RT-PCR was carried out for the expression analysis. Total RNA was extracted from 100 mg of fresh tissues with Trizol reagent (Invitrogen, 15596-026) and pretreated with RNA-free DNase (Promega, M6101) to eliminate gnomic DNA contamination. First strand cDNA was synthesized from 2 μg of total RNA using Takara primer script^{RT} master mix (Drr036A) according to the user's manual. The Takara SYBR Premix Ex Taq II (RR820A) was used for the PCR reaction. All reactions were carried out in 20 μL reaction mixtures with 2 μL of template DNAs, 1 μL of forward primer, and 1 μL of reverse primer (10 μM each). The running conditions were as follows: 95°C for 3 min,

Table 2: DNA sequences of some fragments from the differential-methylation subtractive library.

Clone	DNA sequence
1–35	ATGATTGAGTATAACAACTTAAACTGCAACCGG*ATCTTAAAGGCGTAAG AACTGTATCCTTGTTAGAA
2–26	GTTGTAAGGTCAGCATTTTCACTTTTCCCGGAATGAGAGATTATCAGACT TTACATGTTGATAAGGGTAATGTTTTT
2–32	ATGATCATGGACTTATCCTACATGAGAACGCCAATGAGGATCAA*GCGCGC* CTCAAT*GCGC***AATTAGCTGCAAGAGATGCAGCAGGGCTCGGTGTC
3–32	TAAATTAGGCATGGAACGGAGCCACTACGAAGAAGTTCCGGGGGGTTACG AAGGAAACTTCGAGTTCATATTGGTCAT
4–1	ATACAACACGAGGACTTCCCGGGAGGTGACCCATCCTAGTACTACTCCC TCCCAAGCATGTTAACTGTGGAGTTCTGATGGGATCCGGTGCATTAGTGC TGGTATGATTGCATCCGTTA
5–86	TTCCGAATCAATCATTGTTAACGTCACCGGAGATGATAATAAGTCGGCTT TGTTCGGTAAGTACGATCTCGGTAAGCTCCTCGGTAGCG*GCGC*GTTTGCC AAAGTCTACCAAGCGGAGGA
6–14	TTGGTGGATCCCTTAAC*GCGC*TTCTTCTTCTTCTTTCGCGAGATAACCTA GAAACCCTTTTCCTTTCGTTCGCTTCTTCAATCCGCCATGGGTACGGGTT CTCGAGCTCTCTCTTTCTAGTCATTGGTCTATGTGTCTTCTTTCTCTCTCG ATTTCTCGATTAT*GCGC*TTACTACTTTTGTTGGTCGATCGGATCTTTTCTTC AATTTTTGTTGAATT
7–19	TCTCTCGGGTTACAGGAAAGCTTCCACTTATCAGTCCTCCGGTGAGTTTC AATTGCATTCATTAGTTGCCCACATAT
9–57	TAATGCCTATTGCTTTCTGATCAACTGGAATTTGAGCCAAGACATTTCC*G* *CGC*CCAAAAGGTGTTCGATGAAATGTCTGAACCACTAATTCCAGA
10–66	TTATTAAGGCAATAGCAATG*GCGC*TACCGGTCTACTCGATGAACTGCTTC TTACTTCCTACTTTAA
11–21	AGTATAAGACATAGAACCGCAACCGGATCATGAAAGCCTAAGTAGTGTTT CCTTGTTAGAAGATAGAAAGCCAAAGACTCATACGGACTTCGGCTACA CAATCAAAGCTATGAGAAGCAAGAAGAAGCTTTGTTAGATTTTTGTAGT CAAATATGACTAGATGTCATGTGTATGATTGAGTATAAGAACTAGAATCGC AACCGGATCTTAAAA

-CCGG-*: recognizing and cutting site of Hpa II.
-*GCGC*-**: recognizing and cutting site of Hha I.

followed by 40 cycles of 95°C for 5 s and 60°C for 30 s (CFX96 Real-Time PCR Detection System, Bio-Rad). The results were analyzed by the Bio-Rad CFX Manager software 6.541.1028. The primer sequences are listed in Table S2.

4. Results

4.1. A Differential-Methylation Subtractive Library Was Constructed. By the methylation-sensitive restriction enzymes digestion of genomic DNA, tester and driver preparation, suppression subtractive hybridization, and target cloning, we constructed a differential-methylation subtractive library consisting of about 8,000 clones (Figures S1–S5). To test our protocol, 426 clones were sent to sequence. In these clones, 363 (about 85.21% of total) were PCR2 primers positive. Among these primer-positive clones, 318 (about 87.60%) were -CCGG- or -GCGC- containing fragments (Table 2). The total efficiency was 74.64%.

4.2. Sequencing Results Analysis. 54 gene fragments were obtained from the 318 clones by BLAST searching the *Arabidopsis* genome database (Table 3). The distribution of the methylation site (-CCGG-/-GCGC-) was listed as follows: 11 in 5′ regions, 4 in 3′ regions, and 26 in the gene coding regions. We next investigated the relationship of methylation site with the gene expression. The result showed that DNA methylation, wherever in 5′ region, 3′ region of gene or in coding regions (including intron), suppresses gene expression and demethylation of these sites activates transcription (data not shown), which supported the recent reports [26].

4.3. DNA Methylation Analysis. To check the methylation status of the genome, a methylation-sensitive restriction endonuclease PCR (MS-RE PCR) was performed for 9 out of the 54 genes. The results showed that the PCR products can be found in plants grown in normal condition but not in plants treated with low temperature (4°C) or 5-Aza (50 μM) (Figure 1), suggesting that the -CCGG-/-GCGC- sites were methylated in plants grown under normal condition and were demethylated in plants treated with low temperature or 5-Aza.

Table 3: Analysis of the sequencing results of some clones from the differential-methylation subtractive library.

Clone	Size	Locus tag	Position	Gene identification
1–35	340	AT4G06700	5′	Pseudogene
1–69	227	ArthCp029	In gene	ATPB
2–10	128	ArthMp006	In gene	NAD5
2–26	188	AT4G30840	5′	Transducing/WD40 domain-containing protein
2–32	227	AT5G29041	5′	Pseudogene
2–37	307	AT2G04730	In gene	Pseudogene, F-box protein related
2–51	342	ArthCp064	In gene	RPL2
2–53	274	AT2G11720	In gene	Pseudogene
2–63	291	ArthCp079	In gene	NDHA
2–68	346	AT4G06477	3′	Pseudogene
2–71	278	AT5G34623	In gene	Pseudogene
3-4	129	AT2G06810	In gene	Pseudogene
3–23	394	AT1G42300	In gene	Transposable element gene
3–32	160	Arthct120	5′	trnN; tRNA-Asn
3–35	158	AT2G23720	In gene	Transposable element gene
3–43	315	AT3G30330	In gene	Pseudogene
3–48	275	AT3G30587	In gene	Pseudogene
3–64	315	AT2G07732	In gene	Ribulose-bisphosphate carboxylase
3–81	157	AT4G09380	In gene	Transposable element gene
3–83	247	ArthCp022	In gene	PSAA
3–85	158	AT4G28970	In gene	Transposable element gene
4–1	195	AT1G43624	5′	Unknown protein
4-5	235	AT3G33004	In gene	Pseudogene
4–16	261	AT3G43990	In gene	BAH domain-containing protein
4–32	438	ArthCp023	5′	YCF3
4–40	280	ArthCp069	In gene	RPS7
4–49	338	AT1G63360	In gene	Disease resistance protein (CC-NBS-LRR class), putative
4–62	218	AT1G16970	In gene	KU70
4–81	418	AT4G22150	5′	PUX3
5–16	186	AT5G38280	In gene	PR5K
5–38	378	AT1G22920	In gene	CSN5A
5–44	192	AT4G06678	3′	Pseudogene
5–53	229	ArthCp077	In gene	NDHG
5–58	364	ArthCp014	In gene	RPOB
5–76	311	ArthCt089	3′	trnK; tRNA-Lys
5–86	169	AT2G38490	In gene	CIPK22
5–88	288	AT3G30763	In gene	Transposable element gene
6–14	224	AT1G18070	In gene	G1 to S phase transition protein
6–15	676	ArthCr088	5′	16S ribosomal RNA
6–27	243	ArthCp062	5′	RPL22
6–28	258	AT1G23380	In gene	KNAT6
6–31	165	AT1G18370	In gene	HIK
6–87	105	ArthCp065	In gene	RPL23
7–19	386	AT4G33360	In gene	FLDH
7–44	411	ArthCp026	In gene	NDHK
9–57	213	AT4G06574	5′	Transposable element gene
9–91	273	AT1G38870	3′	Pseudogene
10–66	187	AT2G01840	In gene	Transposable element gene
11–21	212	AT1G38790	5′	Unknown protein
11–29	237	AT5G01080	In gene	Beta-galactosidase related protein
11–44	174	AT1G38630	3′	Unknown protein

Table 3: Continued.

Clone	Size	Locus tag	Position	Gene identification
11–92	407	AT3G29037	5′	Pseudogene of AT5G35760; beta-galactosidase
13–35	125	AT1G20190	In gene	EXPA11
13–56	213	ArthCp063	In gene	RPS19

4.4. Transcription Levels Analysis. The transcription levels of some genes mentioned above were examined. Comparing to the control, the plants treated with low temperature or 5-Aza showed higher expression level (Figure 2). It was notable that the genes expression levels of plants treated with low temperature were not the same as those from plants treated with 5-Aza, suggesting that demethylation and other mechanisms could play a combined role in regulating the gene expression in response to low temperature.

5. Discussion

In the present study, we described a protocol combining methylation-sensitive restriction endonuclease (MS-RE) digestion with suppression subtractive hybridization (SSH) to construct a differential-methylation subtractive library for finding genes upregulated by DNA demethylation.

We obtained a library consisting of about 8,000 clones in this study. To test the protocol, we sent some clones for sequencing. The sequencing results showed that about 85.21% of total samples were PCR2 primers positive and about 87.60% in the PCR2 primer-positive clones were -CCGG- or -GCGC- fragment containing (Table 2). The total efficiency was about 74.64%. DNA methylation status analysis indicated that DNA demethylation appeared in plants treated with low temperature but kept methylation in plants grown in normal conditions (Figure 1). These results suggest that this protocol enables efficient isolation of differentially methylated fragments from a genome and constructing the differential-methylation DNA library.

It has been reported that DNA methylation plays an important role in regulating gene expression [4, 27–29]. DNA hypomethylation leads to enhancing the gene expression [30–32]. We examined the transcription level of some genes. The results indicated that higher expression levels were observed in plants treated with low temperature or 5-Aza compared to those grown under normal conditions (Figure 2). Considering the methylation status of these genes, we could make a conclusion that the demethylation of some genes appeared in the low temperature treatment which subsequently activates gene expression. The results showed credibility of using this protocol to find genes regulated by methylation/demethylation mechanism.

A potential disadvantage of this protocol is that incomplete digestion of DNA by Hha I/Hpa II may lead to an unreliable library. To avoid this problem, we conducted Hha I/Hpa II double digestion twice with excess enzyme every time. Furthermore, high ratio of driver to tester (40 : 1 or higher) in SSH procedure is desirable [25, 33]. In this study, the ratio of driver to tester was about 60 : 1. This protocol can be modified to isolate genes downregulated by DNA hypermethylation as well.

Abbreviations

MS-RE: Methylation-sensitive restriction endonuclease
SSH: Suppression subtractive hybridization
MSAP: Methylation-sensitive amplified polymorphism.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Wei Hu and Xiaolei Liang contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31170225; 31201145), the National High Technology Research and Development Program (2007AA021401), Major State Basic Research Development Program of China (973 Program) (2012CB026105), Foundation of Science and Technology Program of Gansu Province (1107RJYA005), Foundation of Study and Application Program of Biological Technology in Gansu Province (GNSW-2013-15), and Fundamental Research Funds for the Central Universities (lzujbky-2012-104).

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