INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
15–655/2016/18–1–160–167
DOI: 10.17957/IJAB/15.0081
http://www.fspublishers.org

Full Length Article

DNA Methylation in *Rehmannia glutinosa* Roots Suffering from Replanting Disease

Yanhui Yang[1*] and Mingjie Li[2]
[1]*College of Bioengineering, Henan University of Technology, Zhengzhou, 450001, China*
[2]*College of Crop Sciences, Fujian Agriculture and Forestry University, Fuzhou, 350002, China*
[*]For correspondence: yyhui2004@126.com

Abstract

"Replanting disease" is a serious constraint to root growth in the medicinal species *Rehmannia glutinosa* (Gaertn.) Libosch. ex Fisch. and C.A. Mey. The syndrome involves an array of morphological, physiological and biochemical changes to the plant, which culminates in a major loss in tuberous root growth. Here, the tendency of replanting disease to induce differential cytosine methylation in the root DNA was explored via the methylation-sensitive amplified polymorphism (MSAP) method. Exposure to the disease measurably altered the global methylation level. Of the 231 differentially methylated MSAP fragments identified, 136 involved replanting disease-induced methylation and 95 demethylation. A set of 31 differentially methylated fragments was isolated and sequenced. The sequences were used to analyze the function of the genes involved and to investigate whether any were differentially transcribed as a result of exposure to replanting disease. Of the eight genes subjected to transcription profiling, the three which were demethylated in the diseased roots were transcribed more abundantly in these roots, and the five which were methylated were down-regulated by a real-time quantitative PCR (qPCR) method. Our study gives an insight into the DNA methylation of *R. glutinosa* subjected to replanting disease and provides valuable information for further exploring epigenetic regulation of responses to the disease in the species and other plants.

Keywords: *Rehmannia glutinosa* roots; Replanting disease; Epigenetic regulation; Differentially methylated fragment analysis

Introduction

The continuous monoculture of many crops leads to reduced levels of yield and/or end-use quality (Zhang *et al.*, 2011; Yang *et al.*, 2014; 2015). A common basis for this phenomenon lies in the build-up of soil pathogens and pests, but in some cases the effect appears to be physiological rather than pathological; such examples are commonly referred to as "replanting disease". An estimated 70% of medicinal plants grown for their roots are thought to suffer from this syndrome (Zhang *et al.*, 2011). Among these is the perennial herbaceous species *Rehmannia glutinosa* (a member of *Scrophulariaceae*), the tuberous roots of which are a widely-used raw ingredient of a number of traditional Chinese medicines (Wen *et al.*, 2002). As a result of replanting disease, land cultivated for this crop has to be rested for some 8-10 years after just a single season, since if replanted in the following season, many of the plants' fibrous roots fail to develop into the desired tuberous ones (Gu *et al.*, 2013; Yang *et al.*, 2015). This effect becomes more pronounced in the third and subsequent seasons (Wu *et al.*, 2011). Replanting disease thus represents a strong constraint on the sustainable and economically viable production of this valuable plant.

The epigenetic regulation of many eukaryotic genes is accomplished by differential DNA methylation (Dowen *et al.*, 2012; Nicotra *et al.*, 2015). There are many documented effects of DNA methylation on plant phenotype, which has an impact on the response to various biotic and abiotic stresses (Li *et al.*, 2012a; Liang *et al.*, 2014; Naydenov *et al.*, 2015). Certain stresses which disturb plant growth and development have been shown to induce alterations in the pattern of methylation of genomic DNA (Ding *et al.*, 2014; Zhong *et al.*, 2015). Replanting disease in *R. glutinosa* is thought to be largely caused by the presence of its own root exudate in the soil (Wu *et al.*, 2011; Li *et al.*, 2012b; Ru *et al.*, 2014), but it has not yet been established whether this acts as an agent of epigenetic alteration.

The so-called "methylation-sensitive amplified polymorphism (MSAP)" method relies on the contrasting sensitivity to the presence of a methylated cytosine in the recognition sequence of a pair of isoschizomeric restriction enzymes (*Hpa* II and *Msp* I) (Reyna-López *et al.*, 1997). *Hpa* II cleaves the hemi-methylated sequence (only one strand 5′-^{5m}CCGG-3′ is methylated) at the external cytosine site (5′-^{5m}CCGG-3′). *Msp* I is active if the internal cytosines

To cite this paper: Yang, Y. and M. Li, 2016. DNA methylation in *Rehmannia glutinosa* roots suffering from replanting disease. *Int. J. Agric. Biol.*, 18: 160–167

are fully methylated, digesting 5′-C^{5m}CGG-3′ (Chakrabarty *et al.*, 2003; Chen *et al.*, 2009). In addition, both of the two enzymes can digest the non-methylated 5′-CCGG-3′ site (Cervera *et al.*, 2002). The convenience and informativeness of this assay have encouraged its wide utilization (Meng *et al.*, 2012; Shan *et al.*, 2013; Cao *et al.*, 2014). Here, the effect on DNA methylation of replanting disease has been studied by applying MASP method. The successful isolation of a number of informative MSAP fragments is reported, and their sequences are used to identify potential candidate epigenes involved in replanting disease. For these sequences, the relationship between their DNA methylation status and their transcription is then explored through a real-time quantitative PCR (qPCR) assay.

Materials and Methods

Plant Material and the Measurement of Biomass

One group of *R. glutinosa* plants (cultivar "Wen 85-5") was grown in the field at the Wen Agricultural Institute, Jiaozuo City, Henan Province, China, over the period from April 22 to November 30, 2014 at a site where no *R. glutinosa* had been grown for at least ten years (hereafter R1 as control). A second group was grown in a nearby field where the same cultivar had been grown in the previous year (R2 as treatment). Root biomass was assessed at six time points: the seedling stage (May 22), the root elongation stage (June 22), the early (July 22), mid (Aug 22) and late (Sep 22) root expansion stages and maturity stage (Oct 22). Both R1 and R2 samples were represented by roots harvested from five plants at each time point. Fresh root volume was measured by the water displacement method (Niu *et al.*, 2011). Both the fresh and dry (0% moisture) weights of the roots were determined by electronic balance. All samples were performed with at least three replicates.

To provide samples for both the MSAP and qPCR analyses, the roots of five plants per treatment were harvested at the early root expansion stage.

Genomic DNA Extraction and MSAP Analysis

Total genomic DNA was extracted using cetrimonium bromide (CTAB) protocol (Murray and Thompson, 1980) with slight modifications. The quality and concentration of DNA were measured by both agarose gel electrophoresis (1.2%) and spectrophotometric assays (Agilent 2100 bioanalyzer, USA). The DNA samples were stored at −20°C.

The genomic DNA was double-digested with *Hpa* II/*EcoR* I or *Msp*I/*EcoR*I (TaKaRa Co., Tokyo, Japan). For each sample, 400 ng of genomic DNA was incubated for 8 h at 37°C in a solution containing 2 μL of 10× NE Buffer 4, 10 U of *EcoR* I, and 10 U of *Hpa* II in a final volume of 20 μL, whereas the other 400 ng of genomic DNA was incubated for 10 h at 37°C in a solution containing 2 μL of 10 × NE Buffer 1, 10 U of *Eco*R I, and 10 U of *Msp* I in a final volume of 20 μL. The reactions were terminated by incubating the samples at 65°C for 10 min.

The digested DNA fragments (10 μL) were ligated with the double-stranded *EcoR* I adapter and the *Hpa* II/*Msp* I adapter simultaneously using T4 DNA ligase (TaKaRa Co., Tokyo, Japan) according to the manufacturer's instructions. Subsequently, the ligation products were used as templates in the preamplification reaction. The adapters, preamplification primers, and selective amplification primers are listed in Table 1.

A preamplification reaction was carried out in a total volume of 20 μL, containing 0.4 μL of 10 mM dNTPs, 2 μL of 10× buffer, 0.5 μL of 5 U/μL Taq polymerase (TaKaRa Co., Tokyo, Japan), 0.5 μL of 10 μM E00-primer, 0.5 μL of 10 μM HM00-primer, and 2 μL of the ligation products. The preamplification PCR reaction protocol consisted of 24 cycles at 94°C for 0.5 min, 55°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min. The preamplification products were checked by agarose gel electrophoresis, and the fragments were 100-600 bp in length. The pre-amplification products were diluted 1: 40 with sterilized double-distilled water for further selective amplification.

Selective amplification was conducted with a touchdown PCR in a volume of 25 μL, containing 0.4 μL of 10 mM dNTPs, 2 μL of 10× buffer, 0.5 μL of 5 U/μL Taq polymerase, 0.5 μL of 10 μM *EcoR* I selective amplification primers, 0.5 μL of 10 μM *Hpa* II/*Msp* I selective amplification primers, and 2 μL of diluted preamplification product; then, sterilized double-distilled water was added to obtain a final volume of 25 μL. In total, 36 selective primer combinations were employed. The selective amplification PCR protocol consisted of 13 cycles for the touchdown program at 94°C for 0.5 min, dropping 0.7°C per cycle from 65 to 55°C for 0.5 min, 72°C for 1 min. This procedure was followed by another 24 cycles of PCR amplification, denaturing at 94°C for 0.5 min, annealing at 55°C for 0.5 min, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Selective amplification products were mixed with loading buffer and denatured at 94°C for 10 min. The samples were then resolved by electrophoresis on a denaturing polyarylamide gel (PAGE, 6% polyacrylamide, 8 M urea). The gel was silver-stained and photographed.

Sequencing of Amplified Fragments

After the MSAP assay, 31 specific bands named N1-N31 were selected for sequencing to identify the genes related to the changes in DNA methylation. First, the specific bands were excised from the gel, hydrated in 100 μL of water, and incubated at 95°C for 30 min. The eluted DNA was amplified with the same selective primers under the same conditions as the selective amplification. The PCR products were ligated to the PMD18-T vector (TaKaRa Co., Tokyo, Japan) and transformed into the competent *E. coli* DH*5α*.

Table 1: Sequences of adapters and primers used for MASP analysis

Primers/adapters	Oligonucotide sequence (5'-3')
EcoR I adapter	CTCGTAGACTGCGTACC
	AATTGGTACGCAGTCTAC
Hpa II/*Msp* I adapter	GATCATGAGTCCTGCT
	CGAGCAGGACTCATGA
Preamplification primer	
E00	GACTGCGTACCAATTCA
HM00	ATCATGAGTCCTGCTCGGT
EcoR I selective amplification primers	
E1(E00 +AGG)	GACTGCGTACCAATTCAAGG
E2(E00 +TAC)	GACTGCGTACCAATTCATAG
E3(E00 +TTT)	GACTGCGTACCAATTCATTT
E4(E00 +TGA)	GACTGCGTACCAATTCATGA
E5(E00 +TGT)	GACTGCGTACCAATTCATGT
E6(E00 +GAC)	GACTGCGTACCAATTCAGAC
Hpa II/*Msp* I selective amplification primers	
HM1(HM00 +ACA)	ATCATGAGTCCTGCTCGGTACA
HM2(HM00 +TGG)	ATCATGAGTCCTGCTCGGTTGG
HM3(HM00 +GTC)	ATCATGAGTCCTGCTCGGTGTC
HM4(HM00 +GGA)	ATCATGAGTCCTGCTCGGTGGA
HM5(HM00 +GCC)	ATCATGAGTCCTGCTCGGTGCC
HM6(HM00 +CAC)	ATCATGAGTCCTGCTCGGTCAC

Table 2: Premier sequences of 8 genes by qPCR analysis

Genes		Premier sequences (5' to 3')	Tm (°C)	Product size (bp)
N2	Forward	CGAGATGCTTTGAGTGATGAAG	60.0	147
	Reverse	CGCCTTTCTCCAATCCGTA	60.1	
N9	Forward	TATTTACCAACGGGAGATGC	57.8	159
N10	Forward	TCCCTGCTCCAATCTGAACTA	59.8	92
	Reverse	CGACTGCCGATATTGAAAGAG	59.9	
N14	Reverse	TGGTCTATGGTGTGACTCGTG	59.6	93
N18	Forward	TGTGGAGGAACATCATTGGT	58.8	181
	Reverse	TCCATGTCACGGCTGTTTAT	59.0	
N19	Forward	ACTTGTGGTGGTGCTTGCT	55.8	144
	Reverse	GACATGGCCTCTGTTCCTT	54.3	
N20	Forward	CCCAGTTCTCATTCTTCCACA	60.1	137
	Reverse	CTGCTATCCAGGGGTAAATCC	59.8	
18S	Forward	GAGCTAATACGTGCAACAAACC	58.8	166
	Reverse	CGAAAGTTGATAGGGCAGAAAT	59.6	

The recombinants were screened to sequencing according to the Sanger method (Sangon, Shanghai, China). The sequences were analyzed by NCBI BLAST (www.ncbi.nlm.nih.gov).

Total RNA Extraction and qPCR Analysis

Total RNA of each sample was extracted using the TriZOL reagent (TaKaRa Co., Tokyo, Japan), following the manufacturer's instructions. RNA solution of each sample was subjected to RNase-free DNase I (Qiagen Co., Shanghai, China) treatment, RNA concentration was measured spectrophotometrically (Agilent 2100 bioanalyzer, USA) and its integrity checked by agarose gel electrophoresis.

For the purpose of qPCR, 5 μg RNase-free DNase I treated RNA was processed with M-MLV reverse transcriptase (TaKaRa Co., Tokyo, Japan) in accordance with the manufacturer's instructions. Five-fold dilutions of the cDNA template were tested as the samples. Relevant PCR primers (Table 2), directed against a selection of different fragments by MSAP analysis, were designed using Beacon designer 8.0 software (Premier Biosoft International, Palo Alto, CA, USA). A fragment of the gene encoding 18S rRNA was used as a reference. The PCRs were performed using a Bio-Rad IQ5 instrument (Bio-Rad, Hercules, CA, USA), based on SYBR-Green to detect transcript abundance. Each 25 μL reaction contained 0.5 μM of each primer, 20 ng cDNA and 2×SYBR Green Mix (Beinjing BLKW Biotechnology Co., Ltd., China). Negative control reactions contained no cDNA. The PCR regime comprised an initial denaturing step (95°C/10 s), followed by 38 cycles of 95°C/5 s, 60°C/10 s, 72°C/15 s and a final stage of 55°C to 95°C to determine dissociation curves of the amplified products. 3 technical replicates were used for each tested sample. The data were analyzed using Bio-Rad iQ5 Optical System Software v2.1 and normalized on the basis of 18S rRNA CT value. The relative transcription level of each gene was calculated using the method of $2^{-\Delta\Delta CT}$, which meant

$\Delta\Delta CT.gene_{R2}$ = ($CTgene_{R2}$ -$CT.18S_{R2}$) - ($CT.gene_{R1}$-$CT.18S_{R1}$), or $\Delta\Delta CT.geneR1$ = (CT.geneR1-CT.18SR1) - ($CT.gene_{R1}$-$CT.18S_{R1}$) (Livak and Schmittgen, 2001).

Results

Root Biomass Accumulation in Plants Exposed to Replanting Disease

The biomass of R1 roots was clearly higher than that of R2 roots at each of the sampling points, except for the seedling stage (Fig. 1). Moreover, the difference between the R1 and R2 root biomass increased as the plants continued to grow, reaching a maximum by maturity, when the yield of R2 roots was close to zero. The outcome illustrated the severe effect that replanting disease had on *R. glutinosa* root biomass accumulation.

MSAP Profiling of *R. glutinosa* Root DNA

The 36 chosen MSAP primer combinations (Table 1) amplified a total of 592 *Eco*R I/*Hpa* II fragments and the same number of *Eco*R I/*Msp* I fragments (Table 3). Four classes of fragment were recognized: (1) Class I fragments were those present in both the *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I profiles, signifying the non-methylated state of the recognition site, (2) Class II fragments were those present in the *Eco*R I/*Hpa* II, profile but absent in the *Eco*R I/*Msp* I profile, signifying the hemi-methylated state, (3) Class III fragments were those present in the *Eco*R I/*Msp* I profile but absent in the *Eco*R I/*Hpa* II profile, signifying the fully methylated state (inner methylation of both strands) and (4) Class IV fragments were those absent from both the *EcoR* I/*Hpa* II and *EcoR* I/*Msp* I profiles, signifying the fully methylated state (outer methylation of both strands). The number (and proportion) of methylated fragments (Classes II + III + IV) identified was 197 (33.28%) in R1 root DNA and 236 (39.86%) in R2 root DNA (Table 3). The proportion of fully methylated fragments (Classes III + IV) present was 19.59% in R1 and 23.14% in R2. There were 81 (13.68%) hemi-methylated fragments (Class II) in the R1 and 99 (16.72%) in the R2 DNA. The evidence is therefore that the disease increased the global methylation of root DNA.

The MSAP fragments were divided into 15 classes based on their pattern of methylation (Table 4). Classes A-C comprised fragments in which exposure to replanting disease did not induce changes in cytosine methylation, classes D-I fragments which had become methylated and classes J-O fragments which had become demethylated. In R2 DNA, the first group consisted of 361 (60.98%) of the set of MSAP fragments, the second group 136 (22.97%) fragments and the third group 95 (16.05%) fragments.

The Sequence of Fragments Experiencing Changes in Methylation

A set of 31 informative fragments (numbered N1 through N31) was isolated from the gel, purified, cloned and sequenced (Table 5). Twelve of these fragments (N1-N12) became demethylated following exposure to replanting disease and the other 19 (N13-N31) became methylated.

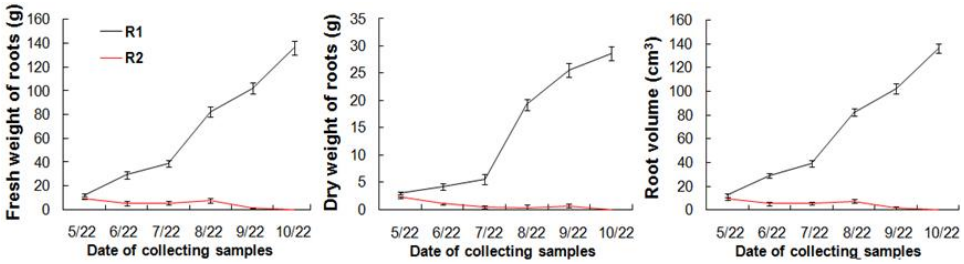

Fig. 1: The accumulation changes of root biomass from R1 and R2 smples at six time points

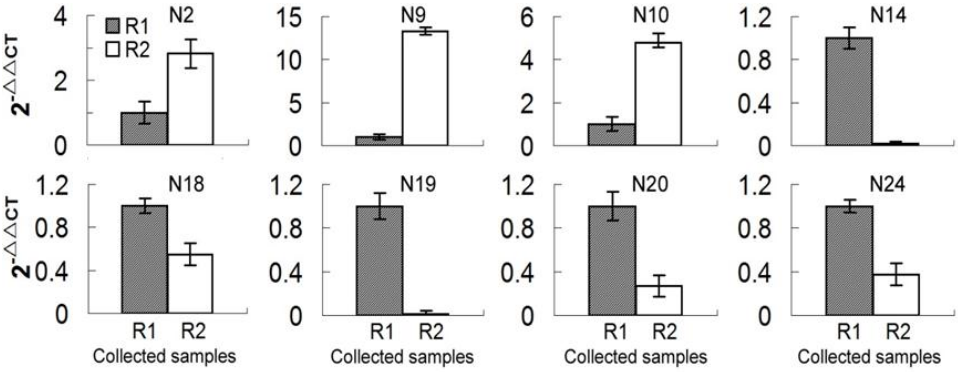

Fig. 2: Transcription analysis of genes represented by eight differentially methylated fragments

Table 3: Methylation in the genomic DNA extracted from *R. glutinosa* roots

Patterns		Methylated classes	*R. glutinosa* roots	
Hpa II	*Msp* I		R1	R2
1	1	I	395	356
1	0	II	81	99
0	1	III	64	78
0	0	IV	52	59
Total amplified bands			592	592
Total methylated bands			197	236
MASP (%)			33.28	39.86
Fully methylated bands			116	137
Fully methylated ratio (%)			19.59	23.14
Hemi-methylated ratio (%)			13.68	16.72

Note: A score of 1 represents the presence, and 0 the absence of a given fragment. Global methylation ratio is given by (II + III + IV)/(I + II + III + IV), the full methylation ratio by (III + IV)/(I + II + III + IV) and the hemi-methylation ratio by (II)/(I + II + III + IV). Class I: non-methylated fragments, Class II: hemi-methylated fragments; Classes III and IV: fully methylated fragments

Table 4: The different patterns of changes induced by replanting disease

Patterns	Classes (Methylation changes)	Banding patterns				Site number	Frequency
		R1		R2			
		Hpa II	*Msp* I	*Hpa* II	*Msp* I		
No change	A (I to I)	1	1	1	1	307	
	B (II to II)	1	0	1	0	31	
	C (III to III)	0	1	0	1	23	
	Total					361	60.98%
Methylation	D(I to II)	1	1	1	0	37	
	E(I to III)	1	1	0	1	19	
	F(II to III)	1	0	0	1	21	
	G(I to IV)	1	1	0	0	32	
	H(II to IV)	1	0	0	0	15	
	I(III to IV)	0	1	0	0	12	
	Total					136	22.97%
Demethylation	J (II to I)	1	0	1	1	14	
	K(III to I)	0	1	1	1	12	
	L(IV to I)	0	0	1	1	23	
	M(III to II)	0	1	1	0	17	
	N(IV to II)	0	0	1	0	14	
	O(IV to III)	0	0	0	1	15	
	Total					95	16.05%

Note: A score of 1 represents the presence and 0 the absence of a given fragment. I-IV: Class I-IV fragments

The 31 sequences varied in length from 103 to 546 bp. When subjected to a BLAST search against the NCBI non-redundant plant sequence database (www.ncbi.nlm.nih.gov), 19 were identified as sharing homology with various mRNA sequences, seven with genomic sequences while the remaining five recorded no significant hits.

The sequence of N2 resembled that of an *Arabidopsis thaliana* MYB transcription factor, that of N4 a multisubstrate pseudouridine synthase 7-like gene from *Malus x domestica*, N5 a *Gossypium hirsutum* putative leucine zipper protein.

N8 a *Nicotiana tomentosiformis* mucin-5B-like transcript variant X3, N9 a *Beta vulgaris subsp. Vulgaris* Ty3-gypsy retrotransposon and N10 a *Pyrus x bretschneideri* gene encoding subunit 6 of the SWR1 complex. Among the 19 fragments which experienced methylation, N14 was homologous to a *Solanum tuberosum* phytochrome B gene, N18 to a *Glycine max* gene encoding a small ubiquitin-related modifier, N19 to a *Solanum tuberosum* gene encoding a kinesin-related protein, N21 to a *Solanum lycopersicum* putative white-brown complex homolog and N24 to a *Glycine max* gene encoding a 3-ketoacyl-CoA synthase (Table 5).

Transcription Profiling of Differentially Methylated Fragments

The eight fragments homologous to plant mRNA sequences which were >250 nt in length were subjected to qPCR analysis to determine whether they were differentially transcribed in R1 and R2 roots (Fig. 2). The genes represented by fragments N2, N9 and N10 (all demethylated in R2 roots) were transcribed more abundantly in R2 than in R1 roots. The abundance of the N9 gene (homologous to a retrotransponson) was particularly high. In contrast, the genes represented by the five methylated fragments were down-regulated in R2 roots; transcript of both the N14 gene (phytochrome B homolog) and the N19 gene (kinesin homolog) was almost undetectable in R2 roots.

Discussion

It has been documented that the continuous monoculture of

Table 5: Homology of differentially methylated fragments obtained by BLAST analysis

MASP fragments Name	Primers	Size (bp)	Methylation changes	Reference accession ID	Sequence homology
N1	E3/HM3	103	Demethylation (IV to I)	KM390021.1	*Corallorhiza odontorhiza* plastid, complete genome
N2	E2/HM4	513	Demethylation (IV to I)	AY519638.1	*Arabidopsis thaliana* MYB transcription factor mRNA
N3	E4/HM3	172	Demethylation (IV to III)	KJ872515.1	*Brassica napus* strain DH366 chloroplast, complete genome
N4	E3/HM6	120	Demethylation (IV to II)	XM_008390442.1	*Malus x domestica* multisubstrate pseudouridine synthase 7-like, mRNA
N5	E3/HM2	120	Demethylation (II to I)	AY456957.2	*Gossypium hirsutum* putative leucine zipper protein (ZIP) mRNA
N6	E2/HM4	131	Demethylation (III to I)	-	Unannotation
N7	E2/HM1	245	Demethylation (IV to II)	BT137938.1	*Medicago truncatula* clone JCVI-FLMt-15G6 unknown mRNA
N8	E1/HM5	158	Demethylation (II to II)	XM_009605038.1	*Nicotiana tomentosiformis* mucin-5B-like, transcript variant X3, mRNA
N9	E1/HM5	285	Demethylation (IV to I)	XM_008365147.1	*Beta vulgaris subsp. vulgaris* Ty3-gypsy retrotransposon env-like Elbe4-5
N10	E2/HM4	546	Demethylation (III to I)	XM_009356304.1	*Pyrus x bretschneideri* SWR1 complex subunit 6, mRNA
N11	E4/HM3	252	Demethylation (IV to I)	JN710470.1	*Solanum tuberosum* isolate DM1-3-516-R44 chloroplast, complete genome
N12	E6/HM6	268	Demethylation (IV to I)	KC208619.1	*Butomus umbellatus* mitochondrion, complete genome
N13	E2/HM4	439	Methylation (II to III)	JN098455.1	*Mimulus guttatus* mitochondrion, complete genome
N14	E3/HM2	297	Methylation (I to II)	NM_001287857.1	*Solanum tuberosum* phytochrome B, mRNA
N15	E3/HM4	435	Methylation (II to IV)	JN098455.1	*Mimulus guttatus* mitochondrion, complete genome
N16	E3/HM3	187	Methylation (I to III)	BT012944.1	*Lycopersicon esculentum* clone 114112F, mRNA
N17	E1/HM3	106	Methylation (II to III)	XM_002309498.2	*Populus trichocarpa* hypothetical protein mRNA
N18	E3/HM2	334	Methylation (I to IV)	XM_003552073.2	*Glycine max* small ubiquitin-related modifier 2-like, mRNA
Continued Table 5					
MASP fragments Name	Primers	Size (bp)	Methylation changes	Reference accession ID	Sequence homology
N19	E3/HM2	303	Methylation (I to II)	XM_006339072.1	*Solanum tuberosum* 125 kDa kinesin-related protein-like, mRNA
N20	E1/HM3	264	Methylation (IIII to IV)	BT108820.1	*Picea glauca* clone GQ03201_H19 mRNA
N21	E6/HM3	219	Methylation (II to III)	XM_004246598.1	*Solanum lycopersicum* putative white-brown complex homolog protein 30-like, mRNA
N22	E1/HM2	181	Methylation (II to III)	-	Unannotation
N23	E3/HM2	203	Methylation (IV to I)	XM_004235865.1	*Solanum lycopersicum* uncharacterized, mRNA
N24	E1/HM2	408	Methylation (III to IV)	XM_006581056.1	*Glycine max* 3-ketoacyl-CoA synthase 4-like, mRNA
N25	E6/HM3	153	Methylation (II to I)	XM_007210217.1	*Prunus persica* hypothetical protein mRNA
N26	E1/HM3	259	Methylation (III to I)	-	Unannotation
N27	E5/HM5	139	Methylation (IV to III)	CP000999.1	*Borrelia recurrentis* A1 plasmid pl53, complete sequence
N28	E2/HM1	129	Methylation (III to II)	KF177345.1	*Salvia miltiorrhiza* mitochondrion, complete genome
N29	E5/HM2	159	Methylation (III to II)	-	Unannotation
N30	E2/HM3	131	Methylation (III to II)	XM_007204575.1	*Prunus persica* hypothetical protein mRNA
N31	E6/HM6	197	Methylation (II to I)	-	Unannotation

R. glutinosa to lead to a pronounced decline in the yield of tuberous roots (Zhang *et al.*, 2011), an effect which was reproduced in the present experiment. The replanting disease syndrome is thought to reflect the modulation of gene expression (Richards 1997; Ding *et al.*, 2014; Yang *et al.*, 2015; Naydenov *et al.*, 2015), most likely arising from epigenetic mechanisms (Fan *et al.*, 2012; Ding *et al.*, 2014). The present MSAP profiling showed that the global level of methylation level was somewhat (about 6.58%) higher in the R2 than in the R1 material. A number of environmental stresses have been suggested as being able to induce alterations in DNA methylation profiles (Dowen *et al.*, 2012; Liang *et al.*, 2014). In some cases, the stress lowers methylation –such as stress by drought in *Lolium perenne* and cold in *Cicer arietinum* L. (Tang *et al.*, 2014; Rakei *et al.*, 2016). While in other cases, the stress increases the levels of cytosine methylation –such as stress by salinity in *Jatropha curcas* L., water in pea (*Pisum sativum* L.), and heavy metal in maize (*Zea mays* L.) (Labra *et al.*, 2002; Mastan *et al.*, 2012; Erturk *et al.*, 2015). The study also indicates that replanting disease had an impact on genomic methylation alterations in *R. glutinosa*.

It has been proposed that methylation alterations affect a gene's transcription, with methylation tending to repress it and demethylation to activate it (Richards, 1997; Yu *et al.*, 2013; Nicotra *et al.*, 2015). Of the 31 differentially methylated MSAP fragments (representing 12 classes of methylation/demethylation changes) isolated and sequenced, twelve were demethylated in the plants exposed to replanting disease, and the other 19 were methylated. In the former group, one was a MYB transcription factor, one was a retrotransposon and one encoded a SWR1 complex subunit, and these were all up-regulated by exposure to replanting disease. MYB transcription factors are prominent in the plant response to stress (Jyothi *et al.*, 2015; Ding *et al.*, 2015). Our previous report also suggested up-regulated of MYB transcription factor was apparently instinctive reaction of *R. glutinosa* responding to replanting disease (Yang *et al.*, 2014). Here, the MYB transcriptional factor gene might be induced and demethylated by the disease, leading to its higher expression level with the adaption of continuous monoculture from *R. glutinosa* roots.

Previous reports showed that transposons were the changes in methylation status during plant the stress (Boyko and Kovalchuk, 2008; Tang *et al.*, 2014). Here the up-regulation of a retrotransposon in the R2 material coincided with its demethylated status. Activated transposons could reshape the *R. glutinosa* root transcriptome by demethylating (and hence up-regulating) other genes as previously described (Boyko and Kovalchuk, 2008; Tang *et al.*, 2014). In addition, a demethylated gene encoding a SWR1 complex subunit 6, which involved in plant flower development processes (Hurtado *et al.*, 2006), was over-expressed in the R2 roots, promoting continuous monocultured *R. glutinosa* earlier flowering. Oppositely, the functions of the genes identified by the 19 fragments, which became more methylated in plants exposed to replanting disease ranged from cell division to protein synthesis and degradation, and lipid metabolism. Phytochrome B (the product of the gene identified by N14) is a negative regulator of flowering time in *A. thaliana* (Franklin and Quail, 2010). The reduced abundance of phytochrome B in the R2 plant can therefore be expected to hinder reproductive growth, so it has a negative impact on tuberous root biomass. The gene identified by N19 encodes a KAC family kinesin-like protein, essential for the association of chloroplasts with the plasma membrane (Suetsugu *et al.*, 2012; Shen *et al.*, 2015). We inferred that a reduced abundance of this gene product in the R2 roots could repress the chloroplast membrane formations with the decrease of the cell to capture photosynthetic light efficiency, disturbing its plant normal development with the inhibition of tuberous root expansion (Yang *et al.*, 2015).

In summary, replanting disease had a measurable effect on the methylation status of the root DNA; while some sequences were demethylated by the disease, its global effect was to increase the DNA methylation level. The activation of demethylated genes and the repression of methylated ones may explain the phenotype associated with replanting disease. Although how these epigenetic changes are induced at the molecular level remains to be elucidated, and the study will provide valuable information for unfolding the regulatory mechanism for the species or other plants in response the disease.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 81403037), the Science and Technology Research Key Project of Henan Educational Committee (No. 13A180160) and High-level Personnel Scientific Research Start-up Foundation of Henan University of Technology (No. 150512).

References

Boyko, A. and I. Kovalchuk, 2008. Epigenetic control of plant stress response. *Environ. Mol. Mutagen.*, 49: 61–72

Cao, X., G. Fan, M. Deng, Z. Zhao and Y. Dong, 2014. Identification of genes related to Paulownia witches' broom by AFLP and MSAP. *Int. J. Mol. Sci*., 15: 14669–14683

Cervera, M.T., L. Ruiz-Garcia and J. Martinez-Zapater, 2002. Analysis of DNA methylation in *Arabidopsis thaliana* based on methylation sensitive AFLP markers. *Mol. Genet. Genom.*, 268: 543–552

Chakrabarty, D., K. Yu and K. Paek, 2003. Detection of DNA methylation changes during somatic embryogenesis of Siberian ginseng (*Eleuterococcus senticosus*). *Plant Sci.*, 165: 61–68

Chen, X., Y. Ma, F. Chen, W. Song and L. Zhang, 2009. Analysis of DNA methylation patterns of PLBs derived from *Cymbidium hybridium* based on MSAP. *Plant Cell Tissue Organ Cult.*, 98: 67–77

Ding, H., J. Gao, C. Qin, H. Ma, H. Huang, P. Song, X. Luo, H. Lin, Y. Shen, G. Pan and Z. Zhang, 2014. The dynamics of DNA methylation in maize roots under Pb stress. *Int. J. Mol. Sci.*, 15: 23537–23554

Dowen, R.H., M. Pelizzola, R.J. Schmitz, R. Lister, J.M. Dowen, J.R. Nery, J.E. Dixon and J.R. Ecker, 2012. Widespread dynamic DNA methylation in response to biotic stress. *Proc. Natl Acad. Sci. USA*, 109: E2183–E2191

Erturk, F.A., G. Agar, E. Arslan and G. Nardemir, 2015. Analysis of genetic and epigenetic effects of maize seeds in response to heavy metal (Zn) stress. *Environ. Sci. Pollut. Res*., 22:10291–10297

Fan, H., T. Li, L. Guan, Z. Li, N. Guo, Y. Cai and Y. Lin, 2012. Effects of exogenous nitric oxide on antioxidation and DNA methylation of *Dendrobium huoshanense* grown under drought stress. *Plant Cell Tissue Organ Cult.*, 109: 307–314

Franklin, K.A. and P.H. Quail, 2010. Phytochrome functions in *Arabidopsis* development. *J. Exp. Bot*., 61: 11–24

Gu, L., M.M. Niu, H.Y. Zheng, J.M. Wang, L.K. Wu, Z.F. Li and Z.Y. Zhang, 2013. Effect of continuous cropping of Rehmannia on its morphological and physiological characteristics. *J. Chin. Mate. Med.*, 36: 691–695

Hurtado, L., S. Farrona and J.C. Reyes, 2006. The putative SWI/SNF complex subunit BRAHMA activates flower homeotic genes in *Arabidopsis thaliana*. *Plant Mol. Biol*., 62: 291–304

Jyothi, M.N., D.V. Rai and R. Nagesh Babu, 2015. Identification and Characterization of high temperature stress responsive novel miRNAs in French bean (*Phaseolus vulgaris*). *Appl. Biochem. Biotechnol.*, DOI: 10.1007/s12010-015-1614-2

Labra, M., A. Ghiani, S. Citterio, S. Sgorbati, F. Sala, C. Vannini, M. Ruffini-Castiglione and M. Bracale, 2002. Analysis of cytosine methylation pattern in response to water deficit in pea root tips. *Plant Biol*., 4: 694–699

Li, X., J. Zhu, F. Hu, S. Ge, M. Ye, H. Xiang, G. Zhang, X. Zheng, H. Zhang, S. Zhang, Li. Qiong, R. Luo, J. Yu, J. Sun, X. Zou, X. Cao, X. Xie, J. Wang and W. Wang, 2012a. Single-base resolution maps of cultivated and wild rice methylomes and regulatory roles of DNA methylation in plant gene expression. *BMC Genom.*, DOI: 10.1186/1471-2164-13-300

Li, Z.F., Y.Q. Yang, D.F. Xie, L.F. Zhu, Z.G. Zhang and W.X. Lin, 2012b. Identification of autotoxic compounds in fibrous roots of Rehmannia (*Rehmannia glutinosa* Libosch.). *PLoS One*, 7: e28806

Liang, D., Z. Zhang, H. Wu, C. Huang, P. Shuai, C.Y. Ye, S. Tang, Y. Wang, L. Yang, J. Wang, W. Yin and X. Xia, 2014. Single-base-resolution methylomes of Populus trichocarpa reveal the association between DNA methylation and drought stress. *BMC Genet.*, 15 Suppl 1: S9

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C (T)). *Method*, 25: 402–408

Mastan, S.G., M.S. Rathore, V.D. Bhatt, P. Yadav and J. Chikara, 2012. Assessment of changes in DNA methylation by methylation-sensitive amplification polymorphism in *Jatropha curcas* L. subjected to salinity stress. *Gene*, 508: 125–129

Meng, F.R., Li Y.C., Yin J., Liu H., X.J. Chen, Z.F. Ni and Q.X. Sun, 2012. Analysis of DNA methylation during the germination of wheat seeds. *Biol. Plant.*, 56: 269–275

Murray, M. and W.F. Thompson, 1980. Rapid isolation of high molecular weight plant DNA. *Nucl. Acids Res.*, 8: 4321–4326

Naydenov, M., V. Baev, E. Apostolova, N. Gospodinova, G. Sablok, M. Gozmanova and G. Yahubyan, 2015. High-temperature effect on genes engaged in DNA methylation and affected by DNA methylation in *Arabidopsis*. *Plant Physiol. Biochem.*, 87: 102–108

Nicotra, A.B., D.L. Segal, G.L. Hoyle, A.W. Schrey, K.J. Verhoeven and C.L. Richards, 2015. Adaptive plasticity and epigenetic variation in response to warming in an Alpine plant. *Ecol. Evol.*, 5: 634–647

Niu, M., H. Fan, J. Li, J. Du, X. Chen and Z. Zhang, 2011. Effects of leaf removal on growth and physiological characteristics of *Rehmannia glutinosa*. *J. Chin. Mater. Medica*, 36: 107–111

Rakei, A., R. Maali-Amiri, H. Zeinali and M. 2016. DNA methylation and physio-biochemical analysis of chickpea in response to cold stress. *Protoplasma*, 253: 61–76

Reyna-López, G.E., J. Simpson and J. Ruiz-Herrera, 1997. Differences in DNA methylation pattern are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. *Mol. Gen. Genet.*, 253: 703–710

Richards, E.J., 1997. DNA methylation and plant development. *Trends Genet.*, 13: 319–322

Ru, R.H., X.Z. Li, X.S. Hunag, F. Gao, J.M. Wang, B.Y. Li and Z.Y. Zhang, 2014. Effect of substrate of edible mushroom on continuously cropping obstacle of *Rehmannia glutinosa*. *J. Chin. Mater. Medica*, 39: 3036–3041

Shan, X., X. Wang, G. Yang, Y. Wu, S. Su, S. Li, H. Liu and Y. Yuan, 2013. Analysis of the DNA methylation of maize (*Zea mays* L.) in response to cold stress based on methylation-sensitive amplified polymorphisms. *J. Plant Biol.*, 56: 32–38

Shen, Z., Y.C. Liu, J.P. Bibeau, K.P. Lemoi, E. Tüzel and L. Vidali, 2015. The kinesin-like proteins, KAC1/2, regulate actin dynamics underlying chloroplast light-avoidance in Physcomitrella patens. *J. Integr. Plant Biol.*, 57: 106–119

Suetsugu, N., Y. Sato, H. Tsuboi, M. Kasahara, T. Imaizumi, T. Kagawa, Y. Hiwatashi, M. Hasebe and M. Wada, 2012. The KAC family of kinesin-like proteins is essential for the association of chloroplasts with the plasma membrane in land plants. *Plant Cell Physiol.*, 53: 1854–1865

Tang, X.M., X. Tao, Y. Wang, D.W. Ma, D. Li , H. Yang and X.R. Ma, 2014. Analysis of DNA methylation of perennial ryegrass under drought using the methylation-sensitive amplification polymorphism (MSAP) technique. *Mol. Genet. Genom.*, 289: 1075–1084

Wen, X.S., S.L. Yang, J.H. Wei and J.H. Zheng, 2002. Textual research on planting history of *Rehmannia glutinosa* and its cultivated varieties. *Chin. Tradit. Herb. Drugs*, 33: 946–949

Wu, L., H. Wang, Z. Zhang, R. Lin, Z. Zhang and W. Lin, 2011. Comparative metaproteomic analysis on consecutively *Rehmannia glutinosa*-monocultured rhizosphere soil. *PLoS One*, 6: e20611

Yang, Y.H., M..J. Li, X..J. Chen, P.F. Wang, F.Q. Wang, W.X. Lin, Y.J. Yi, Z.W. Zang and Z.Y. Zhang, 2014. De novo characterization of the *Rehmannia glutinosa* leaf transcriptome and analysis of gene expression associated with replanting disease. *Mol. Breed.*, 34: 905–915

Yang, Y.H., M.J. Li, X.Y. Li, X.J. Chen, W.X. Lin and Z.Y. Zhang, 2015. Transcriptome-wide identification of the genes responding to replanting disease in *Rehmannia glutinosa* L. roots. *Mol. Biol. Rep.*, 42: 881–892

Yu, Y., X. Yang, H. Wang, F. Shi, Y. Liu, J. Liu, L. Li, D. Wang and B. Liu, 2013. Cytosine methylation alteration in natural populations of *Leymus chinensis* induced by multiple abiotic stresses. *PloS One*, 8: e55772

Zhang, Z., W. Lin, Y. Yang, H. Chen and X. Chen, 2011. Effects of continuous cropping *Rehmannia glutinosa* L. on diversity of fungal community in rhizospheric soil. *Agr. Sci. China*, 10: 1374–1384

Zhong, X., C.J. Hale, M. Nguyen, I. Ausin, M. Groth, J. Hetzel, A.A. Vashisht, I.R. Henderson, J.A. Wohlschlegel and S.E. Jacobsen, 2015. DOMAINS REARRANGED METHYLTRANSFERASE3 controls DNA methylation and regulates RNA polymerase V transcript abundance in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA.*, 112: 911–916

(Received 26 June 2015; Accepted 29 July 2015)