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RESEARCH PAPERS

Analysis of DNA Methylation during Spontaneous Rooting from the Stem Apex in *Rubus idaeus*[1]

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Abstract—*Rubus idaeus* L. is of great economic value. Some varieties of *Rubus idaeus* have a unique feature of spontaneous rooting from the stem apex. To determine whether DNA methylation is associated with the spontaneous rooting process, variations in the methylation at stem apex during four root developmental stages were investigated, using the methylation-sensitive amplification polymorphism (MSAP) technique and the bisulfite sequencing analysis (BSA). The results showed that the DNA methylation levels and patterns were significantly different between the four developmental stages. A total of 824 CCGG amplified sites were detected by MSAP. MSAP screening revealed that the level of DNA methylation at stages I to IV was 34.95, 36.04, 36.29, and 37.50%, respectively. The number of methylated sites and their methylation levels tended to decrease at stages III and IV (root differentiation and elongation) compared with those at stage I (stem elongation). After cloning and sequencing of the 16 polymorphic differentially methylated DNA fragments, BLAST search results indicated that they might be involved in differentiation of the lateral root primordium, plant defense, signal transduction, and energy metabolism. Results of the qRT-PCR and BSA analyses confirmed that methylation of some key genes was closely associated with their expression at the different developmental stages. These findings could be useful for future studies on the potential role of DNA methylation in spontaneous rooting from the stem apex, implying its importance in rooting regulation and rapid expansion of raspberry populations.

Keywords: Rubus idaeus, spontaneous rooting, stem apex, DNA methylation

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INTRODUCTION

Rubus idaeus L. is a perennial deciduous shrub species in the genus *Rubus* of the rose family. It is particularly used in juice processing industry, and has a very high nutritional value in terms of the antioxidant content [1]. *Rubus* family plants have a distinct biological feature of spontaneous rooting from the stem apex. The rooting process involves a series of changes, beginning at the time of the equinox with an acceleration of extension growth and the acquisition of positive geotropism. After entering into the ground, the stem apex forms a root primordium, from which a short negatively geotropic shoot arises, which terminates in a resting bud. In the next spring, this resting bud develops to establish an independent daughter plant [2]. This property of rooting from stem apex is beneficial for rapid expansion of raspberry populations in the wild environment. Although fast vegetative propagation under artificial culture conditions leads to high plant density, it causes rapid degradation of the raspberry orchard in a short time. Most of the *Rubus* species presently grown under controlled conditions are invasive and pose environmental concerns in many countries. However, the molecular mechanisms of spontaneous rooting in *R. idaeus* are not well known.

DNA methylation is a biochemical process that involves the addition of a methyl group to the cytosines or adenines of DNA. It has both epigenetic and mutagenic effects on many biological processes, and is believed to play an important role in regulating the gene expression during plant growth and development [3, 4], including adventitious rooting (AR) [5]. Specific changes in DNA methylation and gene expression may regulate the process of AR [6, 7]; however, no information on DNA methylation during spontaneous rooting from the stem apex in *R. idaeus* has been published currently. Therefore, the main aim of the present study was to investigate the relationship between DNA methylation and gene expression changes at different developmental stages, and the findings of this study could be useful for future study

[1] The article is published in the original.

Abbreviations: AR—adventitious rooting; BSA—bisulfite sequencing analysis; MSAP—methylation-sensitive amplification polymorphism; NR—non-redundant database; RLKs—receptor-like protein kinases.

Table 1. Primer sequences for real-time quantitative PCR analysis

Fragment	Primer sequences (5'-3')
Actin	Forward: GAGGAGCACCCAGTTCTTCTT
	Backward: GAACGGCCTGGATAGCAACA
L01	Forward: CTGCGTACCAATTCTCGCAA
	Backward: GGGGAGGAGCCTTTGAAATCTA
L14	Forward: CCTCTTGCTGCGGTCATAATT
	Backward: AAGCGTTTGGAATTTCACTCG
L16	Forward: GCACTGGTTGAGTGACCTCT
	Backward: AGTCTTGACAGTCTTCCGGC

on the potential role of DNA methylation in spontaneous rooting from the stem apex, implying its importance in rooting regulation and rapid expansion of raspberry populations.

MATERIALS AND METHODS

Morphological changes of the stem apex. The study was conducted at a raspberry (*Rubus idaeus* L.) orchard near Siping, Jilin Province, China. Morphological changes in the stem apex were observed throughout the growth season. Terminal tips that were 0.5 cm long and in good condition were sampled from the stem apex at different developmental stages. Twelve samples were acquired (three biological replicates from each of the four developmental stages) and immediately frozen in liquid nitrogen and stored at –80°C.

Total genomic DNA preparation and MSAP analysis. Total genomic DNA from the terminal tips was isolated using cetyl trimethyl ammonium bromide (CTAB) (Beijing Chemical, China) method as described earlier [8]. MSAP analysis was performed as described by Cheng et al. [9]. Briefly, genomic DNA samples (200 ng) were double-digested with *Eco*RI/*Hpa*II or *Eco*RI/*Msp*I, and then the ligation of two different adapters was performed followed by a pre-amplification reaction. Diluted pre-amplified fragments were used as the starting material for selective amplification using specific primers. Selective amplification products were mixed with loading buffer and, after denaturation at 94°C for 10 min, were fractionated on a 6% denaturing polyacrylamide gel. The silver staining was performed as described by Bassam et al. [10].

Sequencing of the MSAP fragment. After silver staining, a statistical analysis was conducted on the visualized bands. A total of 20 differentially amplified MSAP bands (L01–L16) that represented at least one demethylation or methylation change were precisely excised from the polyacrylamide gel. Of these, 16 were successfully cloned in pMD18-T vector (TaKaRa, China) and sequenced. BLASTN and BLASTX programs of NCBI [11] were used for homology searches of the cloned DNA sequences against the GenBank nucleotide and protein sequence databases.

Quantitative real-time PCR analysis (qRT-PCR). Total RNA samples were isolated using Aidlab Total RNA Extraction Kit (Aidlab, China). The RNase-free DNase I (Invitrogen, China) was used to ensure that the extracted RNA was free from genomic DNA contamination. cDNA was synthesized using PrimeScript RT Reagent Kit (TaKaRa, Japan) following the manufacturer's instructions. qRT-PCR was conducted with gene-specific primers, that were designed using Premier 6.0 software (PREMIER Biosoft International, United States) and were synthesized commercially (Invitrogen). The final volume of the reaction mixture was 25 μL; it contained 1.0 μL cDNA template, 1.0 μL of each primer (10 μM), and 12.5 μL 2× SYBR Premix Ex Taq (Takara Bio, Japan), according to the manufacturer's instructions. The qRT-PCR was performed using a Rotor-Gene 2000 thermocycler (Corbett Research, Australia). All the reactions were performed in triplicates, and three independent sets of samples were used in each experiment. PCR conditions used were as follows: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Amplification data were analyzed using real-time analysis software (Corbett Research, Australia). CT values for each gene were then normalized with the housekeeping gene β-Actin, using the formula (GENE-ACTIN), where ACTIN is the mean CT of the triplicate β-Actin gene PCR runs and GENE is the mean CT of the triplicate runs of the gene of interest. Transcript fold changes, which describe the change in the expression of the target gene, in samples from stages II to IV, relative to that from the first stage transcript, were calculated using the $2^{-\Delta\Delta Ct}$ method [12]. Primer sequences for qRT-PCR are listed in Table 1.

BS-Seq library construction and sequencing. EZ DNA Methylation Gold Kit (Zymo Research, United States) was used for bisulfite-conversion of twelve genomic DNA samples (three biological replicates from each of the developmental stages), following the manufacturer's instructions. The CpG islands of the L16 fragment were predicted using MethPrimer (http://www.urogene.org/cgi-bin/

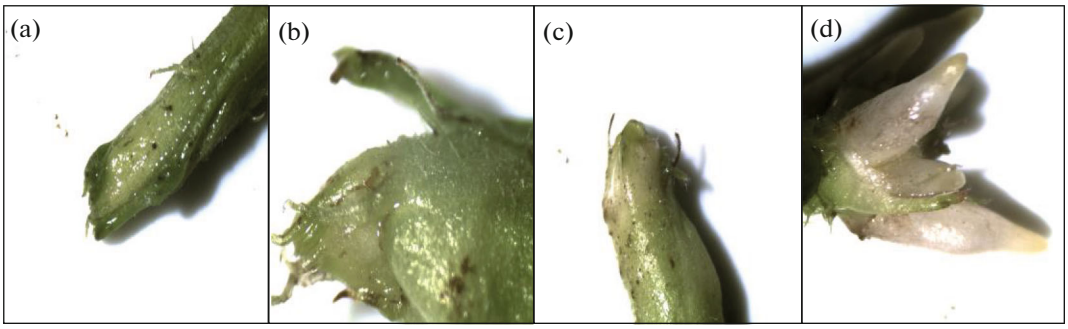

Fig 1. Morphological changes of stem apex in raspberry. (a) Stem elongation, stage I; (b) growth cessation of stem apex, stage II; (c) differentiation of root primordium, stage III; (d) root elongation of stem tip, stage IV.

methprimer/methprimer.cgi) and amplified by PCR. The PCR primers used were as follows: For-5'-ATTTAGGTAATAGATGGTTTTTGATAGT-3' and Rev-5'-ATAAACATAACCTACCTCTCTCTCC-3'. The bisulfite-converted DNA was amplified by 40 PCR cycles and purified using a PCR purification kit (Qiagen). All the qRT-PCR products were cloned into pMD18-T vector (Takara, Japan) and sequenced. Three independent amplification experiments were performed for the L16 fragment from each developmental stage. We sequenced six clones from each set of amplification and cloning. A total of 18 clones of the L16 fragment were sequenced. Methylation levels were calculated by dividing the number of non-converted (methylated) cytosines with the total number of cytosines in the sequenced regions.

RESULTS

DNA Methylation Changes in the Stem Apex at Different Root Developmental Stages as Revealed by Methylation-Sensitive Amplification Polymorphism (MSAP)

The spontaneous rooting process was divided into four stages, including stem elongation (stage I), root growth cessation (stage II), primordium differentiation (stage III), and root elongation (stage IV) (Fig. 1). For each MSAP primer combination, there were eight lanes, corresponding to four root developmental stages. A total of 18 pairs of primer combinations were used for MSAP, to detect DNA methylation variations at 5'-CCGG-3' sites in the genome of stem apex. MSAP-amplified bands were classified into four types: I, II, III, and IV. Band types III and IV were classified as fully methylated, band type II as hemi-methylated, and band type I as unmethylated [9]. The results demonstrated that the DNA methylation levels and patterns were different in the four stages (Table 2). A total of 824 loci were amplified. MSAP screening revealed that the level of DNA methylation at stages I to IV was 34.95, 36.04, 36.29, and 37.50%, respectively. The total number of methylated sites was 288 at stage I (control) and ranged from 297 to 309 at stages II to IV, respectively. The total level of methylation was 34.95% at stage I, which increased by approximately 4% at stages II–IV. A total of 101 hemi-methylated DNA fragments (band type II) were detected at stage I, accounting for 12.26% of the total bands. At stages II–IV, the number of hemi-methylated DNA fragments ranged from 62 to 97, accounting for 7.52–11.77% of the total bands, which were less frequent than those at stage I.

The total number of methylated sites and therefore, the total percentage of methylation tended to increase at stages III and IV (root differentiation and elongation) compared with those at stage I (stem elongation). Chi-square analysis showed that the MSAP bands at stages II–IV were significantly different from those at stage I (Table 3). Pairwise comparisons between different stages showed that most of them showed significantly different levels of methylation. These results indicate that the methylation status varies substantially among the different developmental stages.

Changes in Methylation and Demethylation between Stem Growth and Root Differentiation

Based on the above-mentioned results, all changes in cytosine methylation were classified into three categories: no change, demethylation, and methylation [4]. The three patterns were then further classified into 15 classes (Table 4). The no-change category was characterized with no alteration in the cytosine methylation and included classes A, B, C, and D, which indicated that the same 5'-CCGG-3' sites were detected at different developmental stages. The demethylation category was characterized by cytosine demethylation at stages II–IV and included classes E, F, G, H, I, and J, showing the cytosines that were demethylated to different extents at stages II–IV compared with stage I. The third category was the methylation category, which was characterized by increased cytosine methylation at stages II–IV compared with stage I and included classes K, L, M, N, O, and P. Of these three categories, the no-change category had 606, 465, and 443 amplified sites at stages II–IV compared to stage I,

Table 2. Different types of MSAP cytosine methylation levels during spontaneous root primordium differentiation from stem apex in raspberry

Patterns			Statistics	Stage			
*Hpa*II	*Msp*I	MSAP band types		stage I	stage II	stage III	stage IV
1	1	I		536	527	525	515
1	0	II		101	73	62	97
0	1	III		127	135	161	153
0	0	IV		60	89	76	59
			Total amplified sites	824	824	824	824
			Total methylated sites	288	297	299	309
			Fully methylated sites	187	224	237	212
			Total methylated ratio	34.95%	36.04%	36.29%	37.50%
			Fully methylated ratio	22.69%	27.18%	28.76%	25.73%
			Hemi-methylated ratio	12.26%	8.86%	7.52%	11.77%

1 and 0 represent the presence and absence of bands, respectively. The following formulae were used to calculate the total number of amplified sites, total methylated sites, fully methylated sites, total methylated ratio, fully methylated ratio, and hemi-methylated ratio. Total amplified sites = ∑ (I, II, III and IV bands), total methylated sites = ∑ (II, III and IV bands), fully methylated sites = ∑ (III and IV bands), total methylated ratio = total methylated sites ×100%/total amplified sites, fully methylated ratio = fully methylated sites ×100%/total amplified sites, hemi-methylated ratio = number of II bands ×100%/total amplified sites.

representing approximately 73.54, 56.43, and 53.76% of the total amplified sites. The demethylation category included 127, 97, and 114 amplified sites, representing 15.41, 11.77, and 13.83% of the sites, respectively. The methylation category had 91, 98, and 114 amplified sites, representing 11.04, 11.89, and 13.83% of the total amplified sites, which was less than the number of amplified sites in the no-change and demethylation category (Table 4).

Sequence Analysis of Polymorphic Fragments at Different Developmental Stages

After MSAP analysis, 16 of the 20 randomly selected differentially displayed DNA fragments were successfully cloned for sequence analysis (Table 5). The length of the fragments ranged from 115 to 799 bp. The selected MSAP bands included four single methylation and eight single demethylation changes containing fragments, and four fragments containing double changes (demethylation and methylation). As the raspberry genome is yet not sequenced, BLASTN was performed against the non-redundant database (NR) in the NCBI to find the homologous sequences in other plants. Sequence analysis indicated that 13 out of the 16 sequenced fragments displayed high sequence homology with the reported coding sequences from other plants. Most of these genes were found to be protein-coding ones. Among them, L01, L06, L13, L14, L15, and L16 were found to be homologous to the NRT1/PTR 5.10-like family of proteins, mitogen-activated protein kinase 20, transmembrane protein 87A, probable receptor-like protein kinase, DNA repair protein alkB homolog 8, and lateral root primordium protein-related in *Fragaria vesca* subsp. Vesca, respectively. L04 and L10 are homologous to *Prunus mume* DNA polymerase V and *Prunus armeniaca* 40S ribosomal protein S8 (RPS8) mRNA, respectively, whereas the other fragments, L02, L05, L07, L09, and L12 are homologous to uncharacterized proteins, microsatellite or other genomic sequences (Table 5). These results demonstrate that a large number of diverse genes show alterations in DNA methylation during the transition of shoot tip to root tip.

Methylation Changes and Quantitative Real-Time PCR Analysis

Differentially displayed DNA fragments L01, L14, and L16 involved in lateral root primordium differentiation were chosen to evaluate the relationship

Table 3. Chi-square value and significance statistics analysis of MSAP bands during spontaneous root primordium differentiation from stem apex in raspberry

	Stage I	Stage II	Stage III	Stage IV
Stage I	0			
Stage II	22.43**	0		
Stage III	28.65**	9.34*	0	
Stage IV	6.32	23.52**	17.65**	0
$\chi^2_{0.01}(3)$	11.34			
$\chi^2_{0.05}(3)$	7.81			

Single and double asterisks indicate significant differences at $P < 0.05$ and $P < 0.01$ levels.

Table 4. Different categories of DNA methylation changes during spontaneous root primordium differentiation from stem apex in raspberry

Statistics	Description of patterns	Classes	Type of methylation change*	Comparison of methylation changes during root differentiation stages		
				stage II	stage III	stage IV
No changes		A	I → I	462	465	443
		B	II → II	27	31	30
		C	III → III	91	95	99
		D	IV → IV	26	38	24
Total				606	465	443
Frequency				73.54%	56.43%	53.76%
	Demethylation	E	II → I	32	17	42
		F	III → I	28	30	29
		G	IV → I	14	15	11
		H	III → II	4	12	8
		I	IV → III	40	12	10
		J	IV → III	9	11	14
Total				127	97	114
Frequency				15.41%	11.77%	13.83%
	Methylation	K	I → II	30	18	14
		L	I → III	25	27	36
		M	II → III	2	2	12
		N	I → IV	10	15	22
		O	II → IV	12	12	13
		P	III → IV	12	24	17
Total				91	98	114
Frequency				11.04%	11.89%	13.83%

* Before and after the right arrow indicates MSAP band types during stage I and the following three stages, respectively. I (type I), unmethylated bands; II (type II), hemi-methylated bands; III and IV (type III and IV), fully methylated bands (type IV is considered as hypermethylated bands).

between the expression levels and methylation changes at stages II–IV (Fig. 2). The L01 fragment is homologous to the NRT1 (PTR) transporter family that is expressed in primary and lateral root; the putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in *Medicago truncatula* [13]. L14 fragment is homologous to the probable receptor-like protein kinases (RLKs). The *Arabidopsis* WAK-RLKs have been shown to play key roles in regulation of the root cell expansion. L16 fragment is homologous to lateral root primordium gene. L01, L14, and L16 fragments were all demethylated from the stem elongation stage to root primordium differentiation and root elongation stages (Table 5). The expression of L01, L14, and L16 fragments was up-regulated from stages II to IV. During the same stages, the demethylation changes of L01, L14, and L16 fragments occurred (Table 5). Thus, the increase in the expression levels of L01, L14, and L16 fragments may be closely related to the demethylation changes during these stages.

Validation of the L16 Fragment Methylation by Bisulfite Sequencing

To avoid discrimination against methylated or unmethylated DNA in BS sequencing, the primers are required not to contain any CpG sites. The L16 fragment coding gene associated with lateral root primordium, and it may play an important role in root development in raspberry. Moreover, there was no CpG sites in the primers of L16 fragment. Therefore, L16 fragment was selected as a representative to perform bisulfite genomic sequencing at stages II–IV using MethPrimer (http://www.urogene.org/cgibin/methprimer/methprimer.cgi). Nine CpG sites were predicted in the BSP-amplified sequences of L16 (183 bp) (Fig. 3). A total of 162 CpG sites were identified after sequencing in the L16 fragment from all the four stage with signif-

Table 5. BLAST results of 16 polymorphic differentially expressed MSAP DNA fragments

MSAP fragments	Size, bp	Reference accession no.	Primers combination	Sequence homology	Types of methylation changes at different development stages
L01	115	ref|XM_011466451.1	E85/Msp39	*Fragaria vesca* subsp. *vesca* protein NRT1/PTR FAMILY 5.10-like (LOC105349283), mRNA	IV–IV–III–I (D)
L02	140	ref|XR_907918.1	E77/Msp42	*Fragaria vesca* subsp. *vesca* uncharacterized LOC105352949 (LOC105352949), ncRNA	II–II–I–II (D → M)
L03	160	emb|FQ310506.3	E49/Msp41	*Dicentrarchus labrax* chromosome sequence corresponding to linkage group 1, top part, complete sequence	I–I–III–III (M)
L04	180	ref|XM_008238823.1	E49/Msp41	*Prunus mume* DNA polymerase V, mRNA	I–I–III–III (M)
L05	202	gb|HQ884172.1	E43/Msp39	*Linum usitatissimum* clone Contig2058 microsatellite sequence	II–II–IV–IV (M)
L06	231	ref|XM_004303818.2	E32/Msp39	*Fragaria vesca* subsp. *vesca* mitogen-activated protein kinase 20	III–III–I–III (D → M)
L07	242	ref|XM_004305476.2	E40/Msp39	*Fragaria vesca* subsp. *vesca* uncharacterized LOC101304025	III–III–III–I (D)
L08	261	gb|CP003911.1	E44/Msp40	*Variovorax paradoxus* B4 chromosome 1, complete sequence	IV–IV–II–II (D)
L09	265	gb|FJ149407.1	E32/Msp39	*Daucus carota* subsp. *sativus* clone BAC C106C12 genomic sequence	IV–III–III–III (D)
L10	296	gb|AF071889.1	E77/Msp42	*Prunus armeniaca* 40S ribosomal protein S8 (RPS8) mRNA	III–III–I–III (D → M)
L11	314	gb|CP001013.1	E76/Msp42	*Leptothrix cholodnii* SP-6	IV–IV–IV–I (D)
L12	350	gb|HQ637505.1	E38/Msp42	*Rubus glaucus* clone Mora-B10 microsatellite sequence	I–III–III–IV (M)
L13	351	ref|XM_004303662.2	E49/Msp41	*Fragaria vesca* subsp. *vesca* transmembrane protein 87A	III–I–III–IV (D → M)
L14	388	LOC101314505	E32/Msp39	*Fragaria vesca* subsp. *vesca* probable receptor-like protein kinase At1g67000	II–II–II–I (D)
L15	396	ref|XM_011467112.1	E44/Msp40	*Fragaria vesca* subsp. *vesca* alkylated DNA repair protein	III–III–III–IV (M)
L16	799	gb|U24702.1|ATU24702	E35/Msp41	*Fragaria vesca* subsp. *vesca* protein LATERAL ROOT PRIMORDIUM 1 (LOC101310963), mRNA	II–II–I–I (D)

One D and one M in brackets represents once demethylation and methylation changes at different developmental stages, respectively. Demethylation concluded the following MSAP band type changes: II → I, III → I, IV → I, III → II, IV → II, and IV → III. Methylation concluded the following MSAP band type changes: I → II, I → III, II → III, I → IV, II → IV, and III → IV.

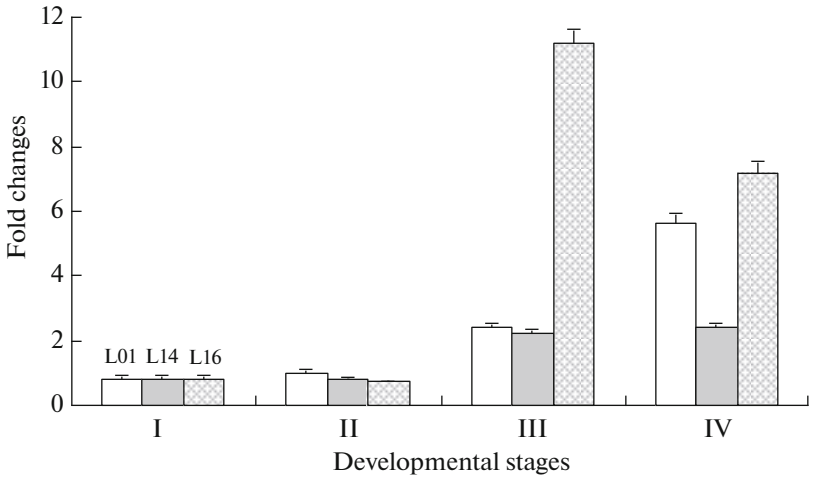

Fig. 2. Expression changes of L01, L14, and L16 MSAP fragment during the four root developmental stages.

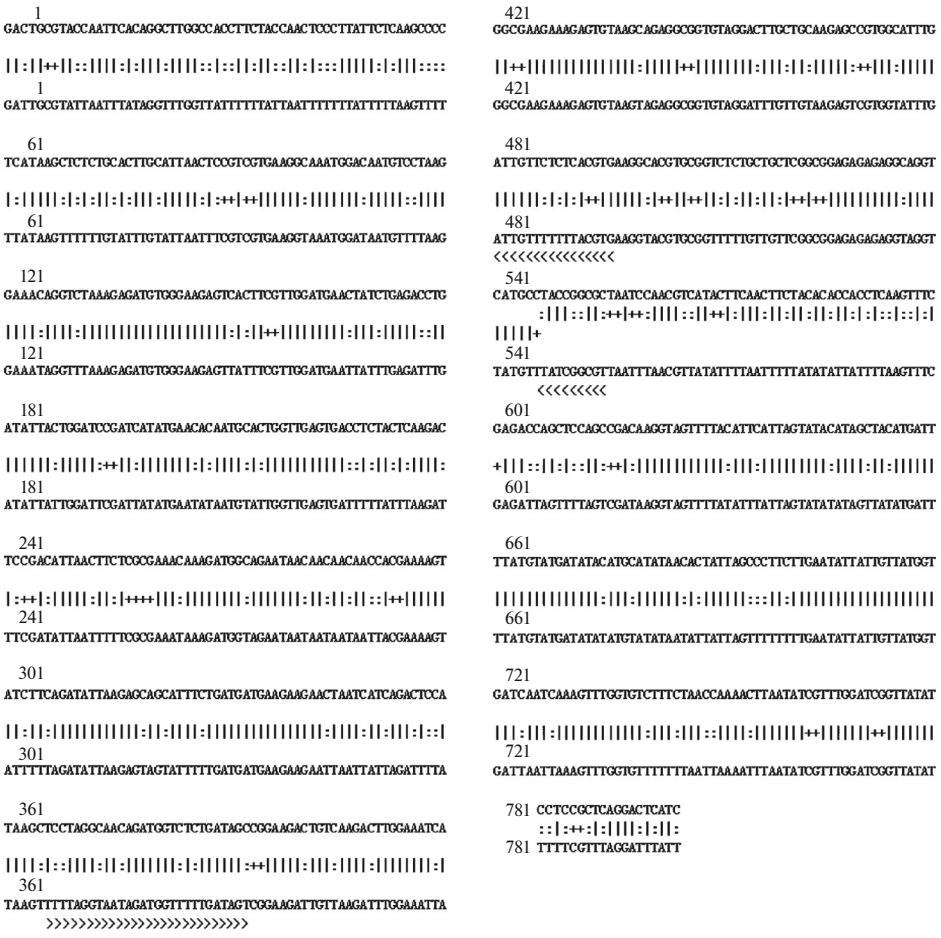

Fig. 3. Amplified nucleotide sequences of the methylation-sensitive amplification polymorphism (MSAP) DNA fragment L16 by bisulfite polymerase chain reaction. The nucleotide sequence for a 183-bp (total 9CpG sites) fragment (upper strands) and its bisulfite-converted version (lower strands) are shown. The primer sequences used are underlined. Plus signs in the figure show the position of CpG sites.

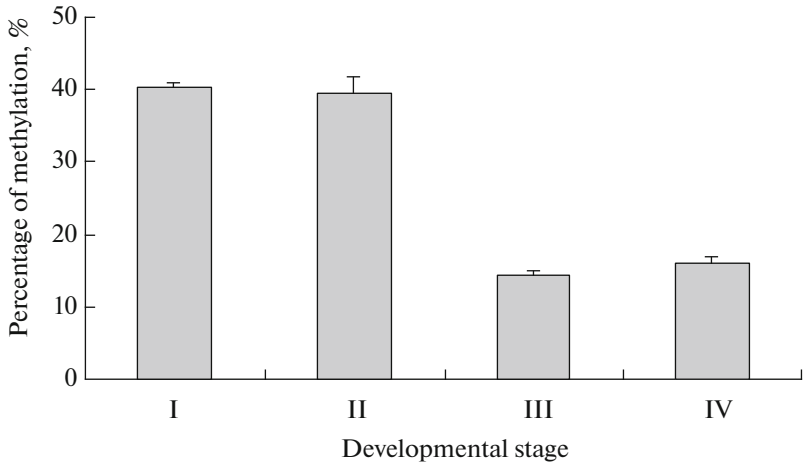

Fig. 4. DNA methylation changes in CpG sites of MSAP DNA fragment L16 during the four root developmental stages in raspberry identified by bisulfite polymerase chain reaction.

icant differences in DNA methylation. Methylation levels of 40.12 and 39.51% were found at stages I and II, respectively, and they were significantly lower than the levels at stages III and IV (Fig. 4). These results are consistent with those obtained by the MSAP analysis. Thus, *bona fide* methylation changes in L16 fragment during stage I to IV were verified by bisulfite sequencing.

DISCUSSION

Relationship between Transition of Stem Growth to Root Differentiation and DNA Methylation

DNA methylation can result in stable epigenetic changes in gene activity without alterations in the DNA sequence [14]. In the present study, the MSAP method was used to identify the methylation at different root developmental stages. The MSAP analysis, using 18 pairs of primers demonstrated that the DNA methylation levels at the root primordium differentiation and root elongation stages were higher than that at the stem elongation and cessation stages. This finding is in concordance with the results reported for the epigenetic differences between shoots and roots in *Arabidopsis*, which indicated that the DNA methylation levels are higher in *Arabidopsis* roots than in shoots [15]. Variations were noted in the root-specific genes of the extensin family, which are preferentially methylated and have at least 10-fold higher expression and lower nucleosome density in roots relative to shoots. Therefore, MSAP is an effective method for identifying genes involved in the transition from stem growth to root differentiation, although only a fraction of known DNA methylation polymorphisms are detected.

DNA Methylation and Gene Expression Changes from Stem Growth to Root Differentiation

DNA methylation is a possible mechanism for the regulation of gene expression in plant development [16]. Substantial data show a correlation between gene expression changes due to DNA methylation and adventitious root differentiation [5]. To our knowledge, the present study is the first to report changes in gene expression during the transition from stem growth to root differentiation because of the alterations in DNA methylation. We identified 16 genes related to this transition that were predicted to encode proteins involved in transcriptional regulation, plant development, signal transduction, and energy metabolism. The variations in the expression levels of these genes at different stages of root developmental might be related to the transition from stem growth to root differentiation.

The L01 fragment is homologous to NRT1 (PTR) transporter family, which was reported to participate in integrating the nutrient and hormone signaling [17]. These are the key factors directly influencing plant root differentiation, growth, and elongation [17, 18]. The two other root development-related fragments L14 and L16 are homologous to probable receptor-like protein kinase (RLKS) and lateral root primordium genes (*lrp1*) [19, 20]. L01, L14, and L16 fragments were all demethylated (Table 5) and their expression levels were higher at stages III and IV than at stages I and II (Fig. 2). In addition, lateral root primordium gene (*lrp1*) transcripts were detected only at the early stages of lateral and adventitious root primordia formation but not at the later stages of primordia development. RLKS and NRT1 have been reported to play an important role in the adventitious root development. Therefore, demethylation of these genes might occur during the transition from stem tip to root tip. It can be inferred that the transcription of methylated genes would be reduced, whereas that of the demethylated genes would be enhanced during the root primordium differentiation of the stem apex. The methylation sites are more numerous than the demethylation ones during the root primordium differentiation,

indicating that there are more inactivated genes than derepressed genes. The activation of demethylated genes and the repression of methylated genes might accelerate the transition from stem tips to root tips.

CpG Sites and Regulation of Transcription

DNA methylation represents an important epigenetic mechanism that influences chromatin structure and gene regulation, the modulation of which plays major roles in the control of plant development, especially the timing of development and tissue-specific developmental transitions [21, 22]. Global DNA demethylation was observed during root differentiation in *Arabidopsis* and *Populus trichocarpa* [13–15]. Nevertheless, the role of DNA demethylation in root differentiation from the stem apex in raspberry remains unclear. CpG island methylation in the 5'-UTR can block the binding of sequence-specific trans-acting proteins and enhance nucleosome stability by attracting histone H1 to linker regions containing methyl-CpG islands, thus leading to transcriptional repression [23]. In our BSP analysis, the methylation percentage of L16 (183 bp) sequence with nine CpG sites showed a decreasing trend from stage I and II to stage III and IV. The expression of L16 increased significantly when the methylation status changed from hypermethylation to non-methylation. These results suggest that demethylation up-regulates the expression of the related genes. Because of the difficulty in designing effective BSP amplification primers, we failed to determine the differential methylation levels of other gene fragments in this study.

In summary, this study demonstrated that MSAP is an effective technique for DNA methylation polymorphism analysis in the raspberry genome. The differences in the DNA methylation levels at different root developmental stages might be responsible for specific gene expression during root differentiation. The DNA methylation patterns mainly at the root primordium differentiation stage might be under the influence of methylation or demethylation of the key genes. The results offer preliminary but comprehensive insights into the DNA methylation associated with spontaneous rooting from the stem apex in *Rubus idaeus*, and provide a scientific basis for further studies on the mechanism of regulation of plant rooting.

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REFERENCES

1. Brennan, R. and Graham, J., Improving fruit quality in Rubus and Ribes through breeding, *Funct. Plant Sci. Biotechnol.*, 2009, vol. 3, pp. 22–29.
2. Heslop-Harrison, Y., Natural and induced rooting of the stem apex in Rubus, *Ann. Bot.*, 1959, vol. 23, pp. 307–318.
3. Cao, D., Gao, X., Liu, J., Wang, X.P., Geng, S.J., Yang, C.W., Liu, B., and Shi, D.C., Root-specific DNA methylation in *Chloris virgata*, a natural alkaline-resistant halophyte, in response to salt and alkaline stresses, *Plant Mol. Biol. Rep.*, 2012, vol. 30, pp. 1102–1109.
4. Osabe, K., Clement, J.D., Bedon, F., Pettolino, F.A., Ziolkowski, L., Llewellyn, D.J., Walford, S.A., Dennis, E.S., Llewellyn, D.J., and Wilson, I.W., Genetic and DNA methylation changes in cotton (*Gossypium*) genotypes and tissues, *PLoS One*, 2014, vol. 9, no. 1. p. e86049. doi 10.1371/journal.pone.0086049
5. Pikaard, C.S. and Scheid, O.M., Epigenetic regulation in plants, *Cold Spring Harb. Perspect. Biol.*, 2014, vol. 6. p. a019315. doi 10.1101/cshperspect.a019315
6. Kawakatsu., T., Stuart, T., Valdes, M., Breakfield, N., Schmitz, R.J., Nery, J.R., Urich, M.A., Han, X., Lister, R., Benfey, P.N., and Ecker, J.R., Unique cell-type-specific patterns of DNA methylation in the root meristem, *Nat. Plants*, 2016, vol. 2. doi 10.1038/nplants.2016.58
7. Shemer, O., Landau, U., Candela, H., Zemach, A., and Williams, L.E., Competency for shoot regeneration from Arabidopsis root explants is regulated by DNA methylation, *Plant Sci.*, 2015, vol. 238, pp. 251–261.
8. Cheng, Y.Q., Liu, J.F., Yang, X., Ma, R., Liu, C., and Liu, Q., RNA-seq analysis reveals ethylene-mediated reproductive organ development and abscission in soybean (*Glycine max* L. Merr.), *Plant Mol. Biol. Rep.*, 2013, vol. 31, pp. 607–619.
9. Cheng, Y., Wang, J., Liu, J., Zhao, Y., Geng, W., and Zhang, H., Analysis of ovary DNA methylation during delayed fertilization in hazel using the methylation-sensitive amplification polymorphism technique, *Acta Physiol. Plant.*, 2015, vol. 37, p. 231. doi 10.1007/s11738-015-1984-7
10. Bassam, B.J., Caetano-Anollés, G., and Gresshoff, P.M., Fast and sensitive silver staining of DNA in polyacrylamide gels, *Anal. Biochem.*, 1991, vol. 196, pp. 80–83.
11. Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., and Madden, T.L., NCBI BLAST: a better web interface, *Nucleic Acids Res.*, 2008, vol. 36: W5–9.
12. Schmittgen, T.D. and Livak, K.J., Analyzing real-time PCR data by the comparative C_T method, *Nat. Protoc.*, 2008, vol. 3, pp. 1101–1108.
13. Yendrek, C.R., Lee, Y.C., Morris, V., Liang, Y., Pislariu, C.I., Burkart, G., Meckfessel, M.H., Salehin, M., Kessler, H., Wessler, H., Lloyd, M., Lutton, H., Teillet, A., Sherrier, D.J., Journet, E.P., et al., Putative transporter is essential for integrating nutrient and hormone signaling with lateral root growth and nodule development in *Medicago truncatula, Plant J.*, 2010, vol. 62, pp. 100–112.
14. Jenuwein, T. and Allis, C.D., Translating the histone code, *Science*, 2001, vol. 293, pp. 1074–1080.
15. Widman, N., Feng, S., Jacobsen, S.E., and Pellegrini, M., Epigenetic differences between shoots and roots in *Arabi-*

dopsis reveals tissue-specific regulation, *Epigenetics*, 2014, vol. 9, pp. 236–242.

16. Jones, P.A., Functions of DNA methylation: islands, start sites, gene bodies and beyond, *Nat. Rev. Genet.*, 2012, vol. 13, no. 7, pp. 484–492.
17. Gojon, A., Krouk, G., Perrine-Walker, F., and Laugier, E., Nitrate transceptor(s) in plants, *J. Exp. Bot.*, 2011, vol. 62, pp. 2299–2308.
18. Léran, S., Muños, S., Brachet, C., Tillard, P., Gojon, A., and Lacombe, B., Arabidopsis NRT1.1 is a bidirectional transporter involved in root-to-shoot nitrate translocation, *Mol. Plant.*, 2013, vol. 6, pp. 1984–1987.
19. Shimizu, N., Ishida, T., Yamada, M., Shigenobu, S., Tabata, R., Kinoshita, A., Yamaguchi, K., Hasebe, M., Mitsumasu, K., and Sawa, S., BAM 1 and RECEPTOR-LIKE PROTEIN KINASE 2 constitute a signaling pathway and modulate CLE peptide-triggered growth inhibition in *Arabidopsis* root, *New Phytol.*, 2015, vol. 208, pp. 1104–1113.
20. Lavenus, J., Goh, T., Guyomarc'h, S., Hill, K., Lucas, M., Voß, U., Kenobi, K., Wilson, M.H., Farcot, E., Hagen, G., Guilfoyle, T.J., and Fukaki, H., Inference of the Arabidopsis lateral root gene regulatory network suggests a bifurcation mechanism that defines primordia flanking and central zones, *Plant Cell*, 2015, vol. 114, no. 5, pp. 1368–1388.
21. Saze, H., Tsugane, K., Kanno, T., and Nishimura, T., DNA methylation in plants: relationship to small RNAs and histone modifications, and functions in transposon inactivation, *Plant Cell Physiol.*, 2012, vol. 53, pp. 766–784.
22. Us-Camas, R., Rivera-Solís, G., Duarte-Aké, F., and De-la-Peña C., In vitro culture: an epigenetic challenge for plants, *Plant Cell Tissue Organ Cult.*, 2014, vol. 118, pp. 187–201.
23. Bird, A.P. and Wolffe, A.P., Methylation-induced repression—belts, braces, and chromatin, *Cell*, 1999, vol. 99, pp. 451–454.