DNA Methylation Analysis of Cassava (*Manihotesculenta* Crantz) SC8 and Its Autotetraploid in Response to Cold Stress

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Abstract: DNA methylation plays a vital role in the regulation of gene expression in response to environmental stress. However, little is known about the effect of DNA methylation on the cassava polyploidy. In the present study, methylation-sensitive amplified polymorphisms (MSAP) were used to investigate DNA methylation profiles of cassava polyploidy following cold treatment to identify candidate genes involved in response to cold stress. The result showed that the genome-wide DNA methylation polymorphisms accounted for 34.02%-42.56% in SC8 and its autotetraploid exposed to 5 °C for 2, 8, 24 and 48 h, respectively. The methylation levels of SC8 at 2 h-cold stress were the highest during 48h under cold treatments. With the time extension within 48 h under cold stress, the methylation levels gradually decreased to the same level as the control but DNA methylation levels of cassava autotetraploid were stable within 48 h. For future analysis of the methylation extent, the cold stress induced more DNA methylation than demethylation in SC8, where DNA methylation was consistent with demethylation in its autotetraploid. The expression analysis demonstrated increase in the transcription of one methylated gene and decrease in the transcription of two demethylated genes. The results revealed that gene methylations in specific sites would be a rapidly epigenetic response to cold stress, further elucidating the methylation functions in its autotetraploid.

Key words: Cassava SC8, tissue culture seedling, MSAP, cold stress.

1. Introduction

DNA cytosine methylation is an important epigenetic mechanism involved in transposon proliferation, genomic imprinting and the regulation of gene expression at both the transcriptional and post-transcriptional levels [1, 2]. Stresses can induce changes in gene expression through hypomethylation or hypermethylation of DNA, at least in part, increasing plant adaptation in response to abiotic stresses [3]. Low temperature is one of the major abiotic stresses that reduce crop yield and productivity rates [4]. The mechanisms of cold acclimation have been extensively investigated in *arabidopsis* [5], rice [6], *maize* [7] and barley [8].

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Cassava (*Manihotesculenta* Crantz) is a starchy root belonging to the *Euphorbiaceae* family, and is a staple food and animal feeding for tropical areas in the world [9]. Low temperatures and frozen conditions limit cassava growth and root yield. Furthermore, to ensure a prolonged growth period (i.e., early planting and late harvesting) in the high latitude regions, novel cassava cultivars with improved cold tolerance are in demand [10]. It would be important to increase understanding of the physiological, biochemical, and molecular characteristics that are associated with cold stress in cassava. However, little is known about the role of the epigenetic process involved in the molecular regulation of cold tolerance in cassava.

In the present study, we investigated the changes of DNA methylation between cassava cultivar South China 8 (SC8) and its autotetraploid, and verified their

methylation differences under cold stress using the method of methylation-sensitive amplified polymorphisms (MSAP). MSAP fragments showed homology with stress-responsive genes and genes involved in the regulation of development. The differential expression patterns of the cold-induced genes influenced by the methylation or demethylation status in SC8 and its autotetraploid revealed a possible role for epigenetic mechanisms in cold adaptation.

2. Materials and Methods

2.1 Plant Materials and Cold-Stress Treatment

A polyploidy type of cassava cultivar SC8 was induced by applying an aqueous solution of 0.001% colchicine to lateral buds for a period of 72 h. The stem identified as autotetraploids using chromo some counting and flow cytometric analysis was propagated to produce a whole plantin Cassava Tissue Culture Room, Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences.

About 50-day-oldseedlings of SC8 and its autotetraploid from Cassava Tissue Culture Room were subjected simultaneously to 5 °C in growth

chambers for 2, 8, 24 and 48 h, respectively. The leaves exposed to cold stress at four time-points were harvested and frozen inliquid nitrogen for total genomic DNA and RNA extraction. The untreated seedlings of SC8 and its autotetraploid grown at 25 °C were used as controls. Three replicates for each time-point treatment were performed in the present study.

2.2 Analysis of Methylation Sensitive Amplified Polymorphism

Total genomic DNA was extracted using the genomic DNA extraction kit (BioTeke). MSAP including enzyme digestion, ligation, pre-amplification and selective amplification was used to analyze the effect of cold stress on DNA methylation in SC8 and its autotetraploid [11]. *EcoR* I/*Msp*I and *EcoR* I/*Hpa*II (NEB) were used for double digestion. The adapter and primer sequences are shown in Table 1. The amplification products were separated by electrophoresis at 6% PAGE. The presence of amplified DNA bands was recorded as a 1, while the absence of any bands was as a 0 [12]. Four

Table 1 Adapter and primer sequences.

Adaper and primer	Sequence
<i>Eco</i> RI adapter	5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
<i>Msp</i> I/ <i>Hpa</i> II adapter	5'-GATCATGAGTCCTGCT-3' 3'-AGTACTCAGGACGAGC-5'
PreAmp primer 1 (E0)	5'-GACTGCGTACCAATTC-3'
PreAmp primer 2 (HM0)	5'-ATCATGAGTCCTGCTCGG-3'
Selective primer of <i>Eco</i> RI	
E1	E0-AAC
E2	E0-AAG
Selective primer of <i>Msp</i> I/ <i>Hpa</i> II	
HM1	HM0-TAT
HM2	HM0-TAG
HM3	HM0-TAC
HM4	HM0-TTG
HM5	HM0-TTC
HM6	HM0-TGT
HM7	HM0-TGC
HM8	HM0-TCT
HM9	HM0-TCG
HM10	HM0-TCC

types of MSAP patterns were detected in the present study, including type I (Digestion with the combinations of *EcoRI/MspI* and *EcoRI/HpaII*); Type II (Digestion with *EcoRI/HpaII*); Type III (Digestion with *EcoRI/MspI*) and type IV (Digestion without the combinations of *EcoRI/MspI* and *EcoRI/HpaII*). Here, type II represents cases of hemi-methylated bands while types III and IV represent situations of full methylation [13].

2.3 Recover, Sequencing and Analysis for Special Bands

The differentially amplified fragments (differentially methylated DNA fragments) were extracted from the gels using high salt method [12], and then, re-amplified and purified according to the instruction manual described in DNA Purification Kit (Omega). The purified DNA fragments were cloned with T-vector (Takara) and *E. coli* DH5 α , and then sequenced in Shanghai Life Tech Biological Technology Company. The sequences analysis was performed using Phytozome 11.0 (<http://phytozome.jgi.doe.gov/pz/portal.html>).

2.4 Quantitative RT-PCR

Total RNA was extracted using RNA prep Pure Plant Kit (Tiangen). The synthesis of the first-strand cDNA using the TransScript First-Strand cDNA Synthesis SuperMix (TransGen) and Amplification of First-Strand cDNA using qRT-PCR with SYBR Premix Ex TaqII (TliRNase H Plus) (TaKaRa) was conducted in the present study. Specific primers were designed using Primer Premier 5 software within the sequences CC13 (Forward 5'-ATCTGAAGCGTTGACTGCTGTAAGG-3', Reverse 5'-TCCAACCATCACCCCTTCC-TCTAT-3'), CC19 (Forward 5'-TTTAGATGAAAGCAGAGGATAGG-3', Reverse 5'-GTA-TGAACCCTAATAAAATCAATCC-3') and

CC23-1 (Forward 5'-TTGCTGCCAGGATTCA-CAGGATG-3', Reverse 5'-TCTTCAGCACCATCCTCCTCCA-3'). Cassava actin was amplified as endogenous controls. The qRT-PCR was performed using the CFX384 Touch™ Real-Time PCR Detection System according to the instructions (Bio Rad).

3. Results

3.1 Pattern of DNA Methylation under Cold Stress

The number of methylated (hemi-methylated and fully methylated) DNA polymorphic bands was from 199 to 249 bands (Type II + Type III + Type IV bands), accounting for 34.02% to 42.56% of all bands. The determination of total methylated fragments showed that full methylation (Type III + Type IV) sites accounted for 22.91% to 29.4%, hemi-methylation (Type II) sites were from 4.62% to 15.9% at all time-points. Type III fragments were the main source of the changes in the DNA methylation banding pattern induced by cold (Table 2).

Compared with SC8 autotetraploid was exposed to cold stress for 2 h and 8 h, the DNA methylation level in SC8 increased 2.56% and 2.39%, respectively. Exposed to cold stress for 24 h and 48 h, the DNA methylation level decreased 1.2% and 4.95% (Table 2).

3.2 Cold-induced Methylation and Demethylation Changes

Under cold stress, it showed that the methylation changes were detected on 191 sites in SC8 at four time-points, accounting for 32.65%, as well as 145 sites in autotetraploid, accounting for 24.79%. Fifteen different banding patterns between the control and cold stress conditions were determined in the MSAP gels (Tables 3 and 4). The patterns A-C represented the monomorphic class in which the methylation pattern was the same between control and cold stress treatments. The patterns D-I showed cytosine demethylation patterns, whereas possible cytosine

DNA Methylation Analysis of Cassava (*Manihotesculenta* Crantz) SC8 and Its Autotetraploid in Response to Cold Stress

Table 2 DNA methylation levels in SC8 and its autotetraploid at different time-points under cold stress.

Type ^a	Cold stress time-point									
	SC8					autotetraploid				
	Control	2 h	8 h	24 h	48 h	Control	2 h	8 h	24 h	48 h
I	377	336	342	358	386	354	351	364	351	357
II	40	82	75	93	27	74	66	72	88	92
III	135	130	149	119	118	140	148	131	129	115
IV	33	37	19	15	54	17	20	26	17	21
Total sites	585	585	585	585	585	585	585	585	585	585
MSAPR/% ^b	35.56	42.56	41.54	38.80	34.02	39.49	40	39.15	40	38.97
FMR/% ^c	28.72	28.55	28.72	22.91	29.4	26.84	28.72	26.8	24.96	23.25
HMR/% ^d	6.84	14.02	12.82	15.9	4.62	12.65	11.28	12.3	15.04	15.73

Type I: no methylated cytosine on double strands of DNA or inner methylated cytosine on a single strand; Type II: hemi-methylation, i.e., outer methylated cytosine on a single DNA strand; Type III: full methylation with inner methylated cytosine on double DNA strands; Type IV: fully methylated cytosine and externally methylated cytosine on double DNA strands. b. MSAPR/%: methylation-sensitive amplification polymorphism ratio = $[(II+III+IV)/(I+II+III+IV)] \times 100$; c. FMR/%: Full methylated ratio = $[(III+IV)/(I+II+III+IV)] \times 100$; d. HMR/%: Hemi-methylated ratio = $[(II)/(I+II+III+IV)] \times 100$.

Table 3 Analysis of DNA methylation patterns under cold stress with respect to control conditions in SC8 at four time-points.

Description of pattern	Class	Banding pattern				Different treatment			
		Control		Cold					
		<i>HpaII</i>	<i>MspI</i>	<i>HpaII</i>	<i>MspI</i>	2 h	8 h	24 h	48 h
No change	A	1	1	1	1	335	330	324	360
	B	1	0	1	0	37	34	34	21
	C	0	1	0	1	114	126	100	98
	Total					486	490	458	479
						83.08%	83.76%	78.29%	81.88%
Demethylation	D	1	0	1	1	0	2	2	15
	E	0	1	1	1	9	8	29	11
	F	0	0	1	1	4	5	9	11
	G	0	1	1	0	0	0	2	0
	H	0	0	1	0	2	4	5	3
	I	0	0	0	1	10	16	14	5
	Total					25	35	61	45
						4.27%	5.98%	10.43%	7.69%
Methylation	J	1	1	1	0	37	34	47	6
	K	1	1	0	1	7	7	3	10
	L	1	0	0	1	0	2	2	7
	M	1	1	0	0	4	6	2	9
	N	1	0	0	0	3	1	2	1
	O	0	1	0	0	14	3	5	28
	Total					65	53	61	61
						11.11%	9.06%	10.43%	10.43%

The percentage of bands in each pattern was determined by the number of bands in each pattern divided by the total number of bands in all three patterns.

Table 4 Analysis of DNA methylation patterns under cold stress with respect to control conditions in SC8 autotetraploid at four time-points.

Description of pattern	Class	Banding pattern				Different treatment			
		Control		Cold					
		<i>Hpa</i> II	<i>Msp</i> I	<i>Hpa</i> II	<i>Msp</i> I	2 h	8 h	24 h	48 h
No change	A	1	1	1	1	322	324	315	321
	B	1	0	1	0	52	55	61	60
	C	0	1	0	1	136	113	115	112
	Total					510	492	491	493
						87.18%	84.1%	83.93%	84.27%
Demethylation	D	1	0	1	1	7	9	3	8
	E	0	1	1	1	1	18	22	25
	F	0	0	1	1	6	4	1	1
	G	0	1	1	0	0	1	1	1
	H	0	0	1	0	5	8	11	9
	I	0	0	0	1	1	2	3	3
	Total					20	42	41	47
						3.42%	7.18%	7.01%	8.03%
Methylation	J	1	1	1	0	5	7	15	21
	K	1	1	0	1	14	7	9	4
	L	1	0	0	1	1	2	2	1
	M	1	1	0	0	0	3	2	3
	N	1	0	0	0	5	3	3	3
	O	0	1	0	0	5	12	6	10
	Total					30	34	37	42
						5.13%	5.81%	6.32%	7.18%

The percentage of bands in each pattern was determined by the number of bands in each pattern divided by the total number of bands in all three patterns.

methylation events induced by cold stress were represented by the patterns J-O. The test showed that cold stress induced DNA demethylation and methylation in SC8 more than that in its autotetraploid. However, the DNA methylation of SC8 under cold stress was more than the demethylation, where DNA methylation was consistent with demethylation in its autotetraploid. These data indicated that SC8 had more obvious changes of methylation and demethylation to regulate gene expression in order to adapt low temperature compared to its autotetraploid in a short time.

3.3 Annotation of the DNA Methylated Sites

In order to understand the functions of DNA methylation in SC8 and its autotetraploid under cold stress, 63 DNA fragments were collected from the gels and sequenced. Ten sequences have been mapped

with the genes in Phytozome 11.0 (Table 5). They are involved in DNA binding protein, ring finger protein, and heat shock protein and so on. These results show that the cold-induced methylation/demethylation bands detected involved gene of a wide range of functions.

3.4 Expression Analysis of Polymorphic Genes

CC13 and CC19 were demethylated in SC8 and its autotetraploid at 2 h time-points under cold stress; whereas CC23-1 was methylated at 24 h in SC8, and was methylated at 24 h and 48 h in autotetraploid. The expression of CC13 and CC19 demethylated genes were continually up regulated in SC8 and its autotetraploid exposed to cold stress from 8 h to 48 h. The expression of the polymorphic genes in SC8 was higher than its autotetraploid. Contrarily, the expression of CC23-1 methylated gene in SC8 and its autotetraploid was continually down-regulated (Fig. 1).

Table 5 Annotation of the DNA methylated sites.

Sequence No.	Length/bp	Sequence ID	Function	E-value
CC13	376	Cassava 4.1_004882 m	Ring finger containing protein	6.3E-175
CC15-1	472	Cassava 4.1_030766 m	KR domain (KR)	0
CC15-2	388	Cassava 4.1_002100 m	Nucleobase-ascorbate transporter 11	7.4E-168
CC15-3	618	Cassava 4.1_004359 m	15-cis-phytoene desaturase/Plant-type phytoene desaturase	5.2E-14
CC15-11	187	Manes.05G138800.1	CCAAT displacement protein-related	3.1E-67
CC19	474	Cassava 4.1_018438 m	ADP-ribosylation factor b1c	0
CC22	178	Cassava 4.1_015716 m	Helix-loop-helix DNA-binding domain (HLH)	2.2E-62
CC23-1	499	Cassava 4.1_002747 m	Heat shock protein 90	5.4E-171
CC23-2	259	Cassava 4.1_010123 m	Transcription factor smif decapping enzyme dcp1	2.2E-115
CC27	261	Cassava 4.1_010747 m	Ring finger domain-containing	6.7E-103

Fig. 1 Relative transcription levels of selected genes under cold stress.

CC13 and CC19 were demethylated in SC8 and its autotetraploid at 2 h time-points under cold stress; CC23-1 was methylated at 24 h time-point in SC8, and was methylated at 24 h and 48 h time-points in autotetraploid.

4. Discussion

DNA methylation, unlike genetic modifications, may open a rapid potential way to cope with environmental stresses [14]. In the present study, we firstly used the MSAP technique to investigate DNA methylation changes in response to cold stress between SC8 and its autotetraploid. The results indicated that cold stress could induce the significant changes in cassava DNA methylation, and further

showed that the methylation polymorphisms in SC8 (35.56%) was lower than its autotetraploid (39.49%). Further analysis indicated that the majority of cold-induced cytosine methylation changes occurred symmetrically on both DNA strands of SC8. The result is different from the level of DNA methylation reported in watermelon [15], *stevia* [16], but is similar to the reported in *Arabidopsis* [17].

The present study showed that cold stress can induce higher DNA methylation and demethylation in

SC8. These findings indicate that some gene expression of SC8 was inhibited and related to increase methylation to adapt low temperature compared to its autotetraploid in a short time. These results were consistent with the tetraploid genotype owning higher tolerance to cold stress than cassava diploid in production [18]. The previous report showed that cold stress induced DNA demethylation more than methylation in maize [7]. However, cold stress induced DNA methylation more than demethylation in SC8 and its autotetraploid. Both results were opposite, it may be caused by genetic reasons. The previous reports showed that the levels of DNA methylation were generally decreased in the biotic stresses such as chilling, planting density, rubbing, cutting, and successive rounds of subculture [19]. In the present study, the methylation levels of SC8 at 2h-cold stress were the highest. With the time extension within 48 h under cold stress, the methylation levels gradually decreased to the same level as the control but DNA methylation levels of cassava autotetraploid were stable within 48 h. Another report showed that cold stress induced DNA methylation levels increased in rice [6]. We speculated that these differences may be dependent on the genotypes and the developmental stage. The difference of DNA methylation levels to cold tolerance in cassava needs to be further testified. These results may increase the understanding of the stress-induced epigenetic impact in plants and to provide more efficient strategies to cassava breeders for the selection of resistant cultivars.

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DNA Methylation Analysis of Cassava (*Manihotesculenta* Crantz) SC8 and Its Autotetraploid in Response to Cold Stress

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