Analysis of phytoplasma-infected Chinese cherry (Prunus pseudocerasus Lindl.) based on intersimple sequence repeat (ISSR) and methylation sensitive amplification polymorphism (MSAP)

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Abstract

Background

Chinese cherry (*Prunus pseudocerasus* Lindl.) is a fruit crop that is susceptible to phytoplasma infection, which causes symptoms such as virescence, phyllody, sterility and stiff fruit. To investigate the effects of phytoplasma infection on the genome and DNA methylation of Chinese cherry, we performed inter-simple sequence repeat (ISSR) and methylation-sensitive amplified polymorphism (MSAP) analyses on the leaves and floral organs of healthy and infected plants from Qijiang District of Chongqing.

Results

ISSR analysis revealed no significant differences in the genomic DNA of leaves and floral organs between healthy and infected plants, suggesting that phytoplasma infection did not induce genomic mutations. MSAP analysis showed that phytoplasma infection caused epigenetic variations in both leaves and floral organs, with different degrees of DNA methylation and demethylation. These epigenetic changes may affect gene expression and lead to abnormal plant development.

Conclusions

This study provides insights into the molecular mechanisms of Chinese cherry phytoplasma disease and fruit development. Potential candidate genes associated with hard fruit formation were also identified, which may be useful for future research in this area.

Background

Chinese cherry (*Prunus pseudocerasus* Lindl.) belongs to the genus Cerasus of the Rosaceae family, and is a dioecious perennial woody fruit crop [1]. It is an economically and culturally important fruit crop that has been cultivated in China for more than 3000 years [2].Phytoplasmas are obligate parasitic bacteria that lack cell walls, cannot be cultured artificially, and inhabit the phloem sieve cells of plants[3]. They are mainly transmitted by hemipteran insects such as leafhoppers and planthoppers and can also be spread by dodder and grafting, causing various symptoms such as phyllody, arbuscular, cluster, and yellowing in infected plants [4, 5]. Phytoplasmas are classified into 17 groups, 24 species, and at least 40 subgroups based on the criteria developed by the International Research Program on Comparative Mycoplasmology (IRPCM)[6]. By 2014, more than 110 subgroups and 35 candidate species of phytoplasmas had been reported. Cherry phytoplasma disease was first described by Granett and Gilmer (1971)[7] and has been associated with leaf yellowing[8], plant decline [9], flower greening [10], and other fatal diseases in cherry trees. In China, cherry phytoplasma disease has rarely been reported, and only a few cases have been reported in Shanxi [11] and Shandong[12] provinces. The disease was promptly eliminated to prevent its spread, so it did not attract much attention from researchers and producers. However, from 2016 to 2018, large-scale outbreaks of phytoplasma disease were observed in the Chinese cherry (*P. pseudocerasus*) planting area in Chongqing city, which resulted in widespread plant death or typical symptoms in the cherry orchards, posing a huge threat to the local and even national cherry industry.

Research on cherry phytoplasma diseases is currently focused on the isolation and genomic analysis of pathogenic bacteria. Six different phytoplasmas have been reported to cause cherry phytoplasma disease, and these known cherry phytoplasmas belong to five different ribosome groups[13, 14]. Earlier studies suggested that several species of leafhoppers (Cicadellidae) and woodlice (Psyllidae) may be involved in the transmission of 16SrIII and 16SrX phytoplasmas in drupe fruit trees, including cherries [15, 16]. Initial progress has been made in the study of cherry phytoplasma by pathogen isolation and genomic analysis of cherry phytoplasma diseases at home and abroad, but little research has been done on DNA methylation analysis, and little research has been done on its pathogenesis and effective treatment. At present, it is unclear whether phytoplasma alters the genome and DNA methylation levels of Chinese cherries.

DNA methylation is an important epigenetic phenomenon that plays an important role in maintaining genome stability, regulating gene expression, and responding to biotic and abiotic stresses[17, 18]. The main methods for detecting DNA methylation include enzyme-linked immunosorbent assay (ELISA) [19], Methlation specific PCR (MSP)[20], Methylation Sensitive Restriction Enzymes (MSRE)[21], Methylation-sensitive high-resolution melting analysis (MS-HRMA) [22], High Performance Liquid Chromatography (HPLC)[23], Mass Spectrometry (MS)[24], etc. MSAP mainly uses genomic DNA with Msp and Hpa with endonuclease for double digestion to obtain fragments of different sizes, ligates the junction, pre-amplifies and selectively amplifies according to the junction with corresponding primers, and detects and amplifies the differential fragments using polyacrylamide gel electrophoresis or other methods[25]. This method is low cost and does not depend on the reference genome, which makes MSAP analysis known as the first choice for DNA methylation analysis, but it also has some drawbacks, MSAP analysis cannot separate the methylation of CG, CHG, CHH and other forms. Intersimple sequence repeat (ISSR) is one of the molecular markers that has the advantages of low cost, high polymorphism, simple method, good stability and repeatability[26–28]. ISSRs have been widely used in plant genetic diversity assessments, including in Jatropha[29], pineapple[30], Cymbopogon[31], etc.In this study, simple sequence internal repeat (ISSR) and methylation-sensitive amplification polymorphism (MSAP) analyses were performed on leaves and floral organs of healthy and infected plants from the Qijiang district of Chongqing, providing insights into the molecular mechanisms underlying protoplast disease and fruit development in Chinese cherry plants. Potential candidate genes associated with hard fruit formation were also identified, which may be useful for future research in this field.

Results

ISSR analysis of genomic DNA

Twelve primers with clear amplification bands, good polymorphism and stability were screened from 28 ISSR primers for PCR amplification (Table 4). The ISSR amplification results showed that there were a large number of ISSR markers and abundant polymorphisms (Fig. 2 and S1). A total of 71 bands ranging from 0.15 to 2.0 kb were amplified by 12 primers, with an average of 5.92 markers per primer, of which 64 were polymorphic markers, and the polymorphism rate was 90.14%. The polymorphism of different primers varied, and the number of polymorphic bands ranged from 3 to 9. Repeated experiments showed that ISSR markers had strong stability and abundant polymorphic bands. The Jaccard similarity coefficient of the tested materials was between 0.83 and 1[32]. ISSR analysis was performed on the leaves of susceptible and nonsusceptible plants and the DNA of floral organs with different degrees of susceptibility. The results showed that ISSR bands could be amplified from the genomic DNA of leaves and floral organs of the tested materials, and each pair of primers generated approximately seven bands. The genetic similarity coefficient analysis indicated that there was no significant difference in the genomic DNA sequence of leaves and floral organs between susceptible and nonsusceptible plants, suggesting that the disease did not cause changes in the genomic DNA sequence of plants.

Table 4
Annealing temperature and amplification results of the 12 primers

Primer	Sequence	Number of loci	Number of polymorphic loci	Polymorphic proportion
810	GAG AGA GAG AGA GAG AT	6	6	100.00
813	CTC TCT CTC TCT CTC TT	9	8	88.89
816	CAC ACA CAC ACA CAC AT	6	5	83.33
817	CAC ACA CAC ACA CAC AA	5	5	100.00
818	CAC ACA CAC ACA CAC AG	6	6	100.00
819	GTG TGT GTG TGT GTG TA	8	8	100.00
820	GTG TGT GTG TGT GTG TC	5	5	100 00
821	GTG TGT GTG TGT GTG TT	4	4	100.00
823	TCT CTC TCT CTC TCT CC	7	5	71.42
824	TCT CTC TCT CTC TCT CG	6	5	83.33
825	ACA CAC ACA CAC ACA CT	4	3	75.00
826	ACA CAC ACA CAC ACA CC	5	4	80.00
Total		71	64	
Average		5.92	5.33	90.14

MSAP analysis of DNA methylation in leaves and floral organs of susceptible and nonsusceptible plants

Both HpaII and MspI are restriction enzymes that recognize the same site, CCGG, but they differ in their sensitivity to methylation sites. HpaII cannot digest the sites containing mCCGG, CmCGG and mCmCGG, but it can recognize the sites where only one cytosine is methylated, while MspI can recognize the internal cytosine methylation on single or double strands, that is, it cannot digest the sites containing mCCGG. Because the two enzymes have different reactions to cytosine methylation at this site, the polymorphic fragment amplified by PCR reflects the methylation status and degree of the modified site. There may be four types of fragments (Table 5). The total number of amplified bands was the sum of the bands of types I, II and III, and the total number of methylated bands was the sum of the bands of types II and III. The total methylation ratio was calculated as the number of bands of type III divided by the total number of amplified bands, and the semimethylation ratio was calculated as the number of bands of type II divided by the total number of amplified bands. The MSAP ratio was calculated as the total number of methylated bands divided by the total number of amplified bands.

Table 5
Methylation status of CCGG loci based on differential sensitivity to both isoschizomers

Types	E + M band patterns	E + H band patterns	CCGG pattern	CCGG methylation pattern
	1	1	CCGG GGCC	Unmethylated
	0	1	^{5m}CCGG GGCC	lateral side of single-stranded DNA (Hemi-methylation)
	1	0	C^{5m} CGG GGC^{5m} C	medial side of double stranded (Full-methylation)
	0	0	1. ^{5m} C^{5m} CGG GGC^{5m} C^{5m} 2. ^{5m} CCGG GGCC^{5m} 3. CCGG	1. Double-chain medial and lateral cytosine methylation 2. Double-stranded extrinsic cytosine methylation 3. absence of CCGG site

Type I: the banding of DNA sample lane by Hpa II digestion, and the banding of DNA sample lane by Msp I digestion, which is unmethylated; Type II: Hpa II digested DNA sample lane with band, Msp I digested DNA sample lane without band, this type is hemimethylation; type III: the use of Hpa II enzyme digestion DNA sample lane without band, the use of Msp I digestion DNA sample lane with band, this type is full methylation; type IV: no band, there are three cases, representing the presence of double-stranded methylation, double-stranded methylation or no CCGG sequence.

Preamplification and selective amplification of genomic DNA

The genomic DNA of cherry susceptible and nonsusceptible plants was digested with EcoRI/HpaII and EcoRI/MSpI enzyme systems, and the digestion products were detected by 1% agarose gel electrophoresis. After 6 h of digestion, the DNA was completely digested without the main band, and the fragments were concentrated in 100–5000 bp. The digested products were ligated for 12 h, and the preamplification reaction was carried out according to the preamplification reaction system. The products were detected by 1% agarose gel electrophoresis. The preamplification effect was good, and the amount of preamplification products was large, mainly concentrated in the 100–2000 bp range (Fig. 3 and S2). A total of 16 pairs of primers were selected for selective amplification. Among them, 12 pairs of primers had good amplification effects, clear and abundant bands, and good amplification repeatability. Each pair of primers showed a certain degree of amplification polymorphism(Fig. 4,S3 and S5).

Analysis of genomic DNA methylation in susceptible and nonsusceptible leaves

We used MSAP to compare the genomic DNA methylation patterns of susceptible and nonsusceptible leaves. We obtained 671 fragments from 12 primer pairs, of which 97 were fully methylated, 85 were semimethylated and 182 were methylated (Table 6). The total methylation rate was 27.30% in susceptible leaves and 26.96% in nonsusceptible leaves. The full methylation rate was 18.40% in susceptible leaves and 10.72% in nonsusceptible leaves. The semimethylation rate was 8.89% in susceptible leaves and 16.23% in nonsusceptible leaves. These results indicated that susceptible leaves had slightly higher levels of total and full methylation and lower levels of semimethylation than nonsusceptible leaves.

We classified the MSAP fragments into four types according to the differences in methylation patterns between susceptible and nonsusceptible leaves: Type A, monomorphic loci, where both leaves had the same methylation status, either single-stranded (A1) or double-stranded (A2); Type B, demethylation loci, where nonsusceptible leaves were methylated but susceptible leaves were demethylated; Type C, hypermethylation loci, where susceptible leaves had higher methylation levels than nonsusceptible leaves; and Type D, hypomethylation loci, where susceptible leaves had lower methylation levels than nonsusceptible leaves but still had some methylation (Fig. 5,S4 and S5).

We identified 14 different MSAP fragment types (Table 7). Among them, type A accounted for 12.8% (19 fragments), type B accounted for 16.8% (25 fragments), type C accounted for 57.6% (86 fragments) and type D accounted for 12.8% (19 fragments). In type A, A2 (double-stranded methylation) was more frequent than A1 (single-stranded methylation), accounting for 57.9%. (Table 8)

Table 6
Genomic DNA methylation of pathological and healthy Chinese cherry plants

	LCK	L1
Total number of amplified bands	345	326
Full-methylation	37	60
Hemi-methylation	56	29
Total methylation	93	89
Full-methylation rate	10.7%	18.4%
Hemi-methylation rate	16.2%	8.9%
Total methylation rate	26.9%	27.3%

Table 7
Number of different methylation type bands of genomic DNA of pathological and nonpathogenic plants Type A: monomorphic site, type B: demethylation type, type C: hypermethylation type, type D: hypomethylation type.

Types	LCK	L1
A	2	2
B	3	3
C	6	6
D	3	3
Total	14	14

Table 8
Different methylation patterns in infected cherry and healthy cherry

Types	Patterns of band				Numbers of polymorphic bands	Number of polymorphic	Polymorphism rate	Number of total loci
	LCK		L1		2			
	M	H	M	H				
A1	1	0	1	0	8	19	12.8%	149
A2	0	1	0	1	11			
B1	0	0	1	1	2	25	16.8%	
B2	0	1	1	1	10			
B3	1	0	1	1	13			
C1	0	1	0	0	13	86	57.6%	
C2	1	0	0	0	20			
C3	1	1	0	0	10			
C4	1	1	1	0	4			
C5	1	1	0	1	21			
C6	1	0	0	1	18			
D1	0	0	1	0	10	19	12.8%	
D2	0	0	0	1	6			
D3	0	1	1	0	3			

DNA methylation analysis of susceptible and nonsusceptible floral organs

We performed MSAP analysis to compare the DNA methylation patterns of susceptible and nonsusceptible floral organs. We used 12 primer pairs and obtained 674 fragments, of which 96 were fully methylated, 79 were hemimethylated and 175 were nonmethylated (Table 9). The global methylation level was 28.32% in susceptible floral organs and 23.58% in nonsusceptible floral organs. The fully methylated fragments accounted for 17.99% in susceptible floral organs and 11.04% in nonsusceptible floral organs. The hemimethylated fragments accounted for 10.32% in susceptible floral organs and 12.53% in nonsusceptible floral organs. These results suggested that susceptible floral organs had higher levels of total and full methylation and lower levels of hemimethylation than nonsusceptible floral organs.

We classified the MSAP fragments into four categories based on the differences in methylation patterns between susceptible and nonsusceptible floral organs: Type A, monomorphic fragments, where both floral organs had the same methylation status, either single-stranded (A1) or double-stranded (A2); Type B, demethylated fragments, where nonsusceptible floral organs were methylated but susceptible floral organs were not; Type C, hypermethylated fragments, where susceptible floral organs had higher methylation levels than nonsusceptible floral organs; and Type D, hypomethylated fragments, where susceptible floral organs had lower methylation levels than nonsusceptible floral organs but still had some methylation (Fig. 6 and S3). We identified 14 different MSAP fragment types (Table 7). Among them, type A represented 19.1% (29 fragments), type B represented 29.6% (25 fragments), type C represented 34.9% (86 fragments) and type D represented 16.4% (19 fragments). In type A, A2 (double-stranded methylation) was more prevalent than A1 (single-stranded methylation), representing 55.2%. (Table 10)

Table 9
Genomic DNA methylation levels of susceptible and nonsympathetic flower organs

	FCK	F1
Total number of amplified bands	339	335
Full-methylation	61	37
Hemi-methylation	35	42
Total methylation	96	79
Full-methylation rate	17.99%	11.04%
Hemi-methylation rate	10.32%	12.53%
Total methylation rate	28.32%	23.58%

Table 10
Different methylation types of genomic DNA in susceptible and nonpathogenic plants

Types	Patterns of band				Number of polymorphic bands	Number of polymorphic	The ratios of polymorphic	Number of total polymerphicloci
	FCK		F1		4			
	M	H	M	H	F1			
A1	1	0	1	0	13	29	19.1%	152
A2	0	1	0	1	16			
B1	0	0	1	1	8	45	29.6%	
B2	0	1	1	1	20			
B3	1	0	1	1	17			
C1	0	1	0	0	9	53	34.9%	
C2	1	0	0	0	12			
C3	1	1	0	0	7			
C4	1	1	1	0	14			
C5	1	1	0	1	4			
C6	1	0	0	1	7			
D1	0	0	1	0	16	25	16.4%	
D2	0	0	0	1	9			
D3	0	1	1	0	3			

Genomic DNA methylation analysis of floral organs with different degrees of susceptibility in the same plant

We performed methylation-sensitive amplified polymorphism (MSAP) analysis to compare the genomic DNA methylation patterns of floral organs with different degrees of susceptibility in the same plant. We amplified 1744 fragments from 12 primer pairs, of which 154 were fully methylated, 199 were

semimethylated and 353 were methylated (Table 11). The total methylation rate varied from 15.61–25.21%. The full methylation rate ranged from 6.07–11.81%. The semimethylation rate varied from 9.54–13.45%. These results indicated that the total methylation rate and full methylation rate decreased, while the semimethylation rate increased with increasing susceptibility.

Different degrees of susceptibility of the same plant exhibited different methylation types. We identified 14 different methylation types in the DNA of floral organs, which could be classified into 4 methylation difference types, namely, A, B, C and D, as shown in Table 12. The DNA methylation types of floral organs with different degrees of susceptibility in the same plant are shown in Table 13. The results of the pairwise comparison between different degrees of susceptibility are shown in Table 14. We detected a total of 1504 methylation differential sites in different degrees of susceptibility in the same plant, of which 273, 345, 583 and 303 were A, B, C and D methylation types, respectively. The frequency of occurrence was 11.2%, 22.9%, 38.8% and 20.1%. Among all methylation types, the C type accounted for the highest proportion, followed by the B type, D type and A type. These results suggested that a large number of hypermethylation or hypermethylation and demethylation mutations occurred in the genome of the same plant when different symptoms appeared. In addition, the frequency of submethylation mutations was also high.

Table 11
Genomic DNA methylation levels of the same susceptible plants

	F1a	F1b	F1c	F1d	F1e
Total number of amplified bands	349	330	333	386	346
Full-methylation	41	39	25	28	21
Hemi-methylation	47	41	37	41	33
Total methylation	88	80	62	69	54
Full-methylation rate	11.75%	11.81%	7.51%	7.25%	6.07%
Hemi-methylation rate	13.47%	12.42%	11.11%	10.62%	9.54%
Total methylation rate	25.21%	24.24%	18.61%	17.88%	15.61%

a-f indicates that the degree of susceptibility gradually deepened.

Table 12
Number of different methylation types of different genomic DNAs in the same plant

Types	Varieties of band patterns				
	5	6	7	8	9
	F1a	F1b	F1c	F1d	F1e
A	2	2	2	2	2
B	3	3	3	3	3
C	6	6	6	6	6
D	3	3	3	3	3
Total	14	14	14	14	14

A: monomorphic site; B: demethylation type; C: hypermethylation type; D: hypomethylation type.

Table 13
Different methylation types of different genomic DNAs of the same plant

Types	Patterns of band			
	F1a		F1b	
A1	1	0	1	0
A2	0	1	0	1
B1	0	0	1	1
B2	0	1	1	1
B3	1	0	1	1
C1	0	1	0	0
C2	1	0	0	0
C3	1	1	0	0
C4	1	1	1	0
C5	1	1	0	1
C6	1	0	0	1
D1	0	0	1	0
D2	0	0	0	1
D3	0	1	1	0

Table 14
Different planting genomic DNA different methylation types in the same plant

Types	Numbers of polymorphic bands										Number of polymorphic		The ratios of polymorphic	Number of total polymerphicloci
	F1a F1b	F1a F1c	F1a F1d	F1a F1e	F1b F1c	F1b F1d	F1b F1e	F1c F1d	F1c F1e	F1d F1e				
A1	7	11	11	15	18	17	17	22	20	26	164	273	11.2%	1504
A2	11	8	10	14	16	10	11	10	8	11	109			
B1	5	7	9	9	7	12	15	9	10	10	93	345	22.9%	
B2	16	17	18	21	10	12	12	7	7	12	132			
B3	15	17	14	14	11		7	14	13	3	120			
C1	13	16	15	5	10	15	8	7	4	8	101	583	38.8%	
C2	16	15	17	10	13	12	7	11	18	9	128			
C3	8	8	9	14	3	4	6	8	5	3	68			
C4	11	16	10	11	11	7	10	6	9	13	104			
C5	17	7	10	10	4	13	9	14	17	12	113			
C6	11	6	7	10	2	3	13	8	4	5	69			
D1	17	20	16	19	22	16	18	15	20	13	176	303	20.1%	
D2	1	3	6	10	2	7	11	1	15	16	72			
D3	9	8	6	9	4	3	9	1	5	2	55			

Results of DNA methylation analysis of the genome and floral organs of susceptible and nonsusceptible plants

We compared the differences in DNA methylation levels and patterns of leaves, floral organs of susceptible and nonsusceptible plants and floral organs of the same plant with different degrees of susceptibility. We found that there were some differences in DNA methylation levels and patterns between susceptible and nonsusceptible leaves, floral organs and floral organs of the same plant with different degrees of susceptibility. The total methylation rate and full methylation rate of DNA in leaves and floral organs of susceptible plants were higher than those in leaves and floral organs of nonsusceptible plants, while the semimethylation rate was lower than that of the control. Among the floral organs of the same plant with different degrees of susceptibility, the total

methylation rate and semimethylation rate showed a downward trend with increasing susceptibility. Our analysis suggested that the occurrence of phytoplasma disease could cause a decrease in the semimethylation rate of plants, resulting in gene expression variation and plant morphological variation. In addition, we identified 14 different methylation types in the DNA of floral organs, which could be classified into 4 methylation difference types, namely, A, B, C and D. We detected a total of 1504 methylation differential sites in different degrees of susceptibility in the same plant, of which the C type accounted for the highest proportion, followed by the B type, D type and A type. These results indicated that a large number of hypermethylation or hypermethylation and demethylation mutations occurred in the genome of the same plant when different symptoms appeared. In addition, the frequency of submethylation mutations was also high.

Discussion

ISSR is a molecular marker technique based on the information of simple sequence repeats (SSR) in the genome [33]. MSAP is a modified AFLP technique that can detect the methylation status of cytosine in genomic DNA[34, 35] .

In ISSR, the amplified bands were analyzed using genetic similarity coefficients, and it was concluded that there were no significant differences in the genomic DNA sequences of leaves and floral organs between susceptible and non-susceptible plants, and the disease did not cause changes in the genomic DNA sequences of the plants.Previous study[36] reported that PaWB infection did not cause changes in the DNA sequences of puffballs at the AFLP level; however, the DNA methylation levels and patterns were altered. In MSAP, the analysis of statistical results could reveal that the total methylation rate and hemimethylation rate gradually became smaller with the degree and deepening of susceptibility. The categorization of different methylation types revealed that by comparing the methylation differences between the genomes of susceptible and non-susceptible leaves, flowering organs of susceptible and non-susceptible plants, and genomic DNA of the same plant with different degrees of susceptibility, it was found that among all methylation types, hypermethylation and hypermethylation types occurred with the highest frequency, followed by demethylation types and hypermethylation types, while monomorphic loci The lowest frequency was observed. These indicate that cherry phytoplasma diseases can cause epigenetic variation in plants, and therefore it can be hypothesized that DNA methylation-induced variation in gene expression leads to abnormal plant growth and development. This study showed are in agreement with previous studies that have shown that plant infection by pathogens leads to changes in DNA methylation patterns. For example, Verma et al[37] studied the cytosine methylation status of healthy and infected sesame plants and found that most differentially methylated genes were hypermethylated; Liu et al[38] showed that although the mean methylation levels of infected leaves were not significantly different from those of healthy leaves, the presence of 1,253 differentially methylated genes (DMGs) in infected leaves and 1,168 differentially expressed genes (DEGs), while 51 genes were found to be differentially methylated and expressed. However, to our knowledge, this is the first report to use ISSR and MSAP techniques to study genomic and epigenetic changes in cherry plants with different disease susceptibilities. Our study provides new insights into the possible role of DNA methylation in regulating gene expression and plant development in response to disease stress.

However, this study also has some limitations that need to be addressed.We used only two techniques (ISSR and MSAP) to analyze genomic and epigenetic variation in cherry plants. Other techniques, such as transcriptome sequencing or bisulfite sequencing, could provide more comprehensive and accurate information on gene expression and DNA methylation changes in response to disease. Therefore, future studies can use more techniques to further explore the molecular mechanisms of cherry plant diseases.

Our study has important implications for understanding and improving disease resistance in cherry plants. It suggests that DNA methylation may be a potential target for manipulating gene expression and plant development in response to phytoplasma disease pressure.Ahmad et al[39] showed that the LEAFY (LFY) gene, which controls flower organ development and maintenance and is directly involved in controlling homologous gene expression, was affected in infected Brassica juncea plants; Tian et al[40] showed that Nicotiana NbOMT1 in Nicotiana benthamiana can catalyze IBHP O-methylation in the presence of S-adenosyl-L-methionine. However, the exact mechanism of how DNA methylation regulates gene expression and plant development in cherry plants remains unclear. Therefore, future studies could focus on elucidating the molecular mechanisms of DNA methylation in cherry plants under phytoplasma stress.

Conclusions

In this study, we found that the genomes of leaves and floral organs of susceptible and non-susceptible plants did not differ significantly in DNA sequences; therefore, phytoplasma disease did not cause DNA sequence variation in cherries. However, the total and holomethylation rates of DNA in leaves and floral organs of susceptible plants were higher than those in leaves and floral organs of non-susceptible plants, respectively, whereas the hemimethylation rates were lower than those of controls, while the total and hemimethylation rates of DNA in floral organs of susceptible and non-susceptible plants tended to decrease with increasing susceptibility. These results indicate that DNA methylation patterns differ between susceptible and non-susceptible plants and between different levels of susceptibility, and also suggest that cherry plant diseases can cause epigenetic variation in plants, so we hypothesize that DNA methylation-induced gene expression variation can lead to abnormal plant growth and development. In this study, we applied ISSR markers and MSAP techniques to cherry plant protoplasm diseases for the first time in China and abroad, providing new clues and methods to reveal the pathogenesis of cherry mosaic disease phytoplasm, as well as identifying potential candidate genes involved in Chinese cherry sclerotia, which can be used as a reference for researchers in related fields.

Materials and Methods

Plant materials

Five-year-old Chinese cherry trees (Prunus pseudocerasus Lindl.) growing in Qijiang District, Chongqing city, China, were selected as experimental materials. The average annual temperature in this area is 18.8°C, with an average precipitation of 1070 mm. Nine sample groups were collected in the second week of March 2021 from three phytoplasma-infected trees and three healthy trees within a range of 0.1 km. The sample groups included LCK (healthy leaves), L1 (infected leaves), F1 (all flowers of one tree showed phyllody) and F1a-e (some flowers of one tree showed phyllody, a to e indicate the gradual deepening of symptoms) (Fig. 1). These two groups of trees were 1 km away from each other. The collected samples were immediately frozen in liquid nitrogen after being harvested from trees and were stored at -80°C until further analysis.

DNA and RNA extraction

Total DNA was extracted from cherry samples using the Plant Genomic DNA Extraction Kit (TIANGEN, CN, Catalog No. DP305-03) following the manufacturer's instructions as previously described in Wang et al. [41]. The quality of DNA was assessed by 1% agarose gel electrophoresis, and the purity of DNA was measured by a nanodrop spectrophotometer (Thermo Fisher Scientific, USA, model ND-1000). The DNA used for ISSR amplification was diluted to 20 ng/μL[42], and that used for MSAP amplification was diluted to 150 ng/μL[43].

Total RNA was extracted from cherry samples using RNA Plant Plus Reagent (TIANGEN, CN https://www.tiangen.com/asset/imsupload/up0031254001467350989.pdf). RNA purity was assessed spectrophotometrically using the model NC2000 Nanodrop (Thermo Fisher Scientific, USA). Concentrations of the RNA preparations were measured using the Qubit RNA Assay Kit (ThermoFisher Scientific, USA) with the model NC2000 Nanodrop (ThermoFisher Scientific, USA). RNA degradation and contamination were first examined on a 1% agarose gel (Biowest, FRA)[44, 45]. Further assessment of RNA integrity was performed using the model 2100 Bioanalyzer system (Agilent Technologies, CA) with the RNA 6,000 Nano Kit (model 5067 – 1511, Agilent Technologies, CA).

ISSR analysis

Preliminary assays were conducted to determine the optimum primers for analysis. Twelve primers with clear amplification bands were selected from 28 primers for PCR (Table 1). The 20 μL ISSR amplification system consisted of 10 μL 2×Ftaq PCR MasterMix, 1 μL ISSR primer, 1 μL DNA template and 8 μL ddH_2O. The PCR was carried out using a thermo cycler (ABI, PCR System 2080, Perkin-Elmer Corp, Norwalk, CT, USA). The following cycling protocol was set for amplification: 2 min initial denaturation at 94°C; 10 cycles of 2 min denaturation at 94°C, 2 min annealing at 50–60°C, and 30 s extension at 72°C; 25 cycles of 30 s denaturation at 94°C, 1 min annealing at 50°C, and 1 min extension at 72°C; and a final extension step of 10 min at 72°C[46, 47]. Amplification products were stored at -4°C. The primers used were based on the ninth set of primer sequences published by UBC and synthesized by Jin Wei Zhi Biotechnology Co., CN (http://www.biotech.ubc.ca/services/naps/primers/Primers.pdf). A total of nine samples were analyzed by ISSR (Table 2).

The 4 μl PCR amplification products were separated by electrophoresis on a 2% agarose gel prepared with 1×TBE buffer. The Direct-loadTM D2000 DNA Marker (Tiangen Biochemical Technology Beijing Co., Ltd.) was used as the standard molecular weight control. Electrophoresis was performed at 110 V for 1 h and photographed by a UV gel imaging system (DYV6-RR).

Table 1
Primer sequences for ISSR.

Primer	Sequence	Primer	Sequence
801	ATA TAT ATA TAT ATA TT	916	CAC ACA CAC ACA CAC AT
802	ATA TAT ATA TAT ATA TG	917	CAC ACA CAC ACA CAC AA
803	ATA TAT ATA TAT ATA TC	818	CAC ACA CAC ACA CAC AG
804	TAT ATA TAT ATA TAT AA	819	GTG TGT GTG TGT GTG TA
805	TAT ATA TAT ATA TAT AC	820	GTG TGT GTG TGT GTG TC
806	TAT ATA TAT ATA TAT AG	821	GTG TGT GTG TGT GTG TT
807	AGA GAG AGA GAG AGA GT	822	TCT CTC TCT CTC TCT CA
808	AGA GAG AGA GAG AGA GC	823	TCT CTC TCT CTC TCT CC
809	AGA GAG AGA GAG AGA GG	824	TCT CTC TCT CTC TCT CG
810	GAG AGA GAG AGA GAG AT	825	ACA CAC ACA CAC ACA CT
811	GAG AGA GAG AGA GAG AC	826	ACA CAC ACA CAC ACA CC
812	GAG AGA GAG AGA GAG A	828	TGT GTG TGT GTG TGT GA
813	CTC TCT CTC TCT CTC TT	829	TGT GTG TGT GTG TGT GC
814	CTC TCT CTC TCT CTC TA	830	TGT GTG TGT GTG TGT GG

Table 2
Sample number

Numbering	sample name
1	LCK, Nonsusceptible leaves
2	L1, susceptible leaves
3	FCK, Nonsusceptible floral organs
4	F1, susceptible floral organs
5	Different degrees of disease susceptibility in the same plant, F1a indicates normal floral organs of the same plant
6	Different degrees of disease susceptibility in the same plant, F1b indicates the same plant susceptible floral organ with slightly green petals
7	Different degrees of disease susceptibility in the same plant, F1c indicates susceptible floral organs of the same plant with further greening of petals
8	Different degrees of disease susceptibility in the same plant, F1d indicates that the susceptible floral organs of the same plant completely change to leaves (green)
9	Different degrees of disease susceptibility in the same plant, F1e indicates the same plant susceptible floral organs do not show petal-like, filament, ovary, etc. degeneration

MSAP analysis

Cherry genomic DNA was digested with EcoR I + Msp I/EcoR I + Hpa II (NEB, USA) enzyme systems according to the method of Reyna-Lopez et al.[48, 49]. The digestion products were verified by 1% agarose gel electrophoresis. After methylation-sensitive restriction digestion, adapters were ligated to the digested DNA fragments in a 50 µl reaction system. Preselective PCR amplification and selective PCR amplification were performed to amplify the EcoR I + Hpa II and EcoR I + Msp I DNA fragments. All reaction volumes were 25 µl. The preselective PCR amplification reaction consisted of 25 cycles of 94°C for 2 min, 56°C for 1 min, and 72°C for 1 min. Selective amplification reactions were carried out on preamplified DNA that had been diluted 40-fold using a touchdown PCR protocol of 12 cycles of 94°C for 30 s, 65°C -55.9°C for 30 s (− 0.7°C per cycle), and 72°C for 1 min and 22 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. Electrophoresis loading buffer was added to the PCR products, and then the samples were denatured at 95°C for 8 min and subsequently separated by electrophoresis on a 6% (w/v) polyacrylamide gel in 1× TBE buffer. Separated bands were visualized by silver staining. The adapters and primers used in the experiment are listed in Table 3.

Table 3
Adapters and primers for MSAP

		5'-3'Sequences
EcoR	Adapter 1	CTCGTAGACTGCGTACC
	Adapter 2	AATTGGTACGCAGTC
Hpa /Msp	Adapter 1	GACGATGAGTCTAGAA
	Adapter 2	CGTTCTAGACTCATA
Primer to preamplification	E00	GACTACGTACCAATTC
	M00	GATGAGTCCTGAGTAA
selective PCR amplification	E32	GACTGCGTACCAATTCAAC
	E35	GACTGCGTACCAATTCACA
	E39	GACTGCGTACCAATTCAGC
	E50	GACTGCGTACCAATTCCAT
	H23	GATGAGTCTAGAACGGTA
	H45	GATGAGTCTAGAACGGATG
	H60	GATGAGTCTAGAACGGCTC
	H83	GATGAGTCTAGAACGGTCA

Band scoring and data analysis

The ISSR marker was dominant, and the presence or absence of a band was recorded as 1 or 0, respectively. These scores were then used to create a binary data matrix to facilitate the statistical analysis. The Nei-Li similarity coefficients were calculated using NTSYSpc 2.1 software [50].

All of the bands generated by MSAP were visually scored as 1/0 binary matrices. ‘1 or ‘0 indicates the presence or absence of a fragment, respectively. The scores were then used to create a binary data matrix to facilitate the statistical analysis of methylation polymorphism. To ensure the reliability of the data, only clear and reproducible bands were counted in this experiment

Declarations

Ethics approval and consent to participate: Plant materials in the study complied with relevant institutional, national, and international guidelines and legislation.

Consent for publication: Not applicable.

Availability of data and material: The data sets supporting the results of this article are included within the article [and its supplementary information files].

Competing interests: The authors declare no conflict of interest.

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Figures

Figure 1

The symptoms of flower mutation

a: healthy cherry flowers; b: part of the flower organs become green; c: further greening of the flower organs; d: the flower organs completely become leaves; e: the flower organs do not show petals, filaments, ovaries, etc. Degenerate.

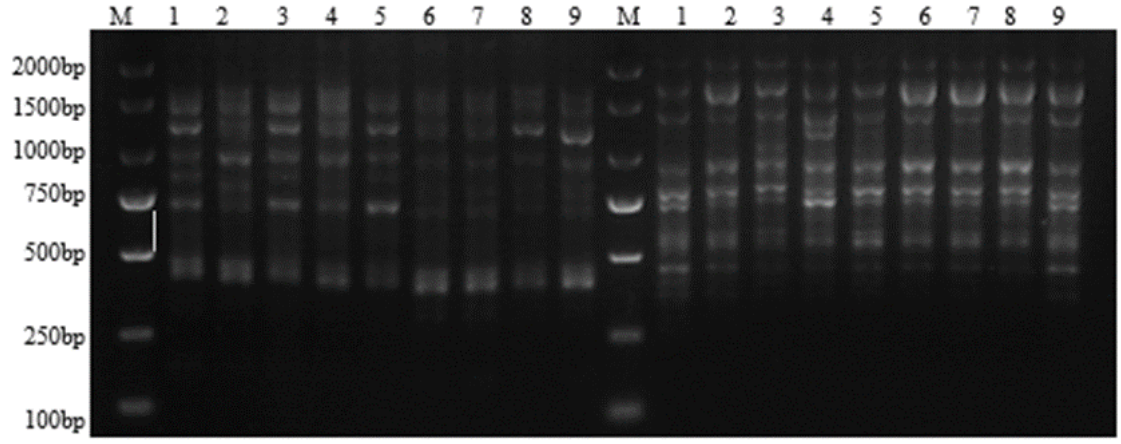

Figure 2

Amplification results of graph primers ISSR 810 and 813

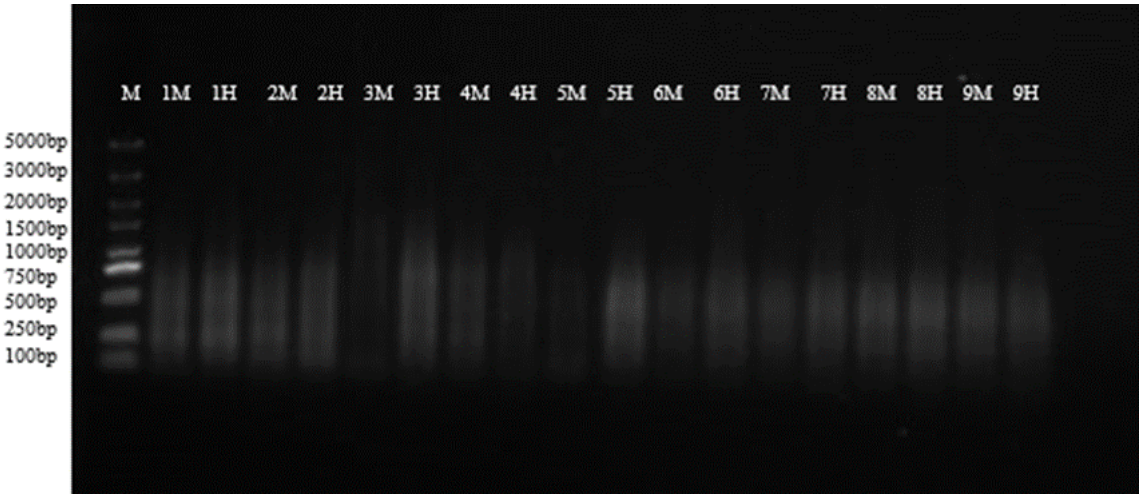

Figure 3

Preamplification fragments of genomic DNA EcoR I/Hpa II and EcoR I/Msp in susceptible and nonsusceptible cherry plants

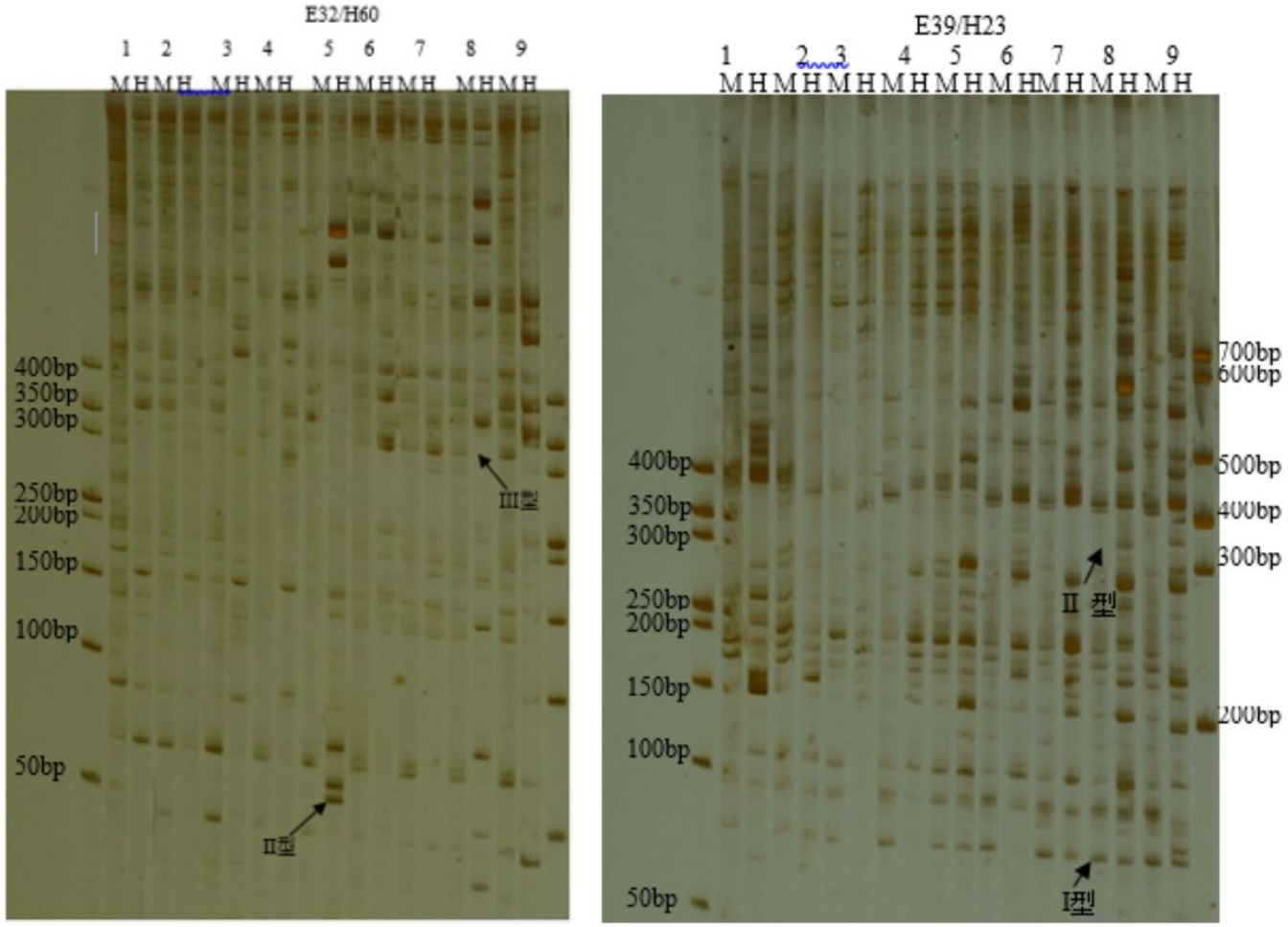

Figure 4 Amplification fragments of genomic DNA EcoR I/Hpa II and EcoR I/Msp from susceptible and nonsusceptible cherry plants by MSAP

Figure 4

Amplification fragments of genomic DNA EcoR I/Hpa II and EcoR I/Msp from susceptible and nonsusceptible cherry plants by MSAP

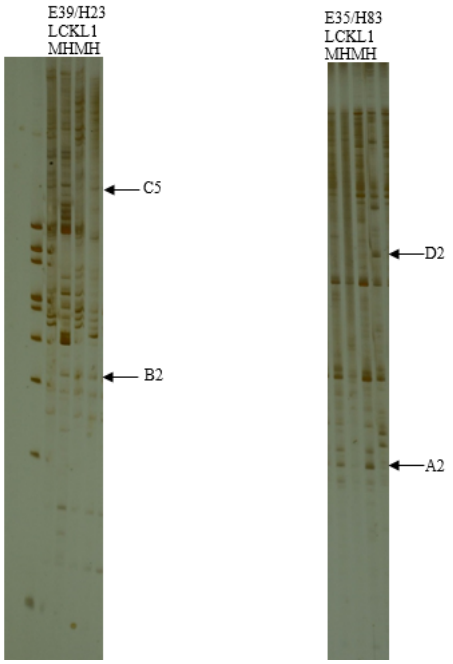

Figure 5

Different types of genomic DNA methylation in susceptible cherry and nonpathogenic cherry leaves

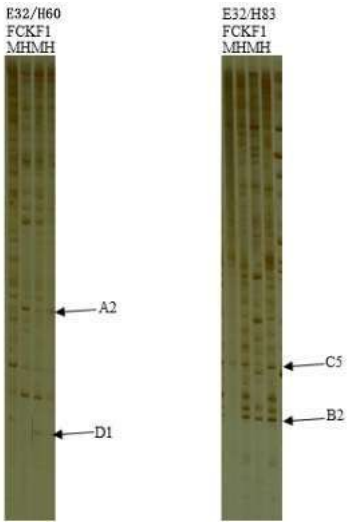

Figure 6

Different methylation types of genomic DNA of susceptible cherry and nonsympathetic cherry flower organs

Supplementary Files

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