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Role of hydrogen peroxide pretreatment in heat-induced alteration of DNA methylation in cucumber leaves

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ABSTRACT

To investigate whether exogenous hydrogen peroxide (H_2O_2) affects the heat-induced alteration of DNA methylation in H_2O_2-pretreated heat-stressed plants, cucumber seedlings were exposed to heat (42/38 °C, day/night) for 3 d after pretreatment with 1.5 mM H_2O_2 for 12 h, and then DNA methylation patterns were detected through a method of methylation sensitive amplified polymorphism (MSAP). Exogenous H_2O_2 mitigated growth suppression caused by heat. Meanwhile, using 120 pairs of primers, small percentages (16.2–16.4%) of DNA sequences were methylated in leaves when cucumbers were subjected to heat or pretreated with H_2O_2, and 20 MSAP loci were selected for differentially amplified fragments. Among which sequences of three MSAP fragments had identity with coding sequences of gene *Csa026131*, *Csa012834* and *Csa015520* in cucumbers, respectively. Compared to control, heat treatment caused methylation in 6 MSAP loci and led to demethylation in 4 loci, while the combined effect of H_2O_2 pretreatment and heat aroused methylation in 8 loci and induced demethylation in 6 loci. In comparison to heat treatment alone, the combination of H_2O_2 pretreatment and heat aroused 4 MSAP loci to be demethylated and caused 4 MSAP loci to be methylated, and it as well enhanced the transcript levels of gene *Csa026131* and *Csa012834* and decreased the expression of gene *Csa015520*. Results of bisulfite sequencing PCR showed that the cytosine methylation levels in gene *Csa026131* and *Csa015520* were different between heat and H_2O_2 + heat treatments. We propose H_2O_2 changes the heat-induced alteration of DNA methylation when it is used to mitigate heat stress of cucumbers.

1. Introduction

In recent years, growing attention has been focused on DNA methylation in higher plants (Portis et al., 2004; Verhoeven et al., 2010) that usually occurs in cytosine bases of CpG or CpNpG sequences (Saze et al., 2003). DNA methylation can cause stable alterations in gene activities without changes in DNA sequences and will play a central role in epigenetic control of genome and gene expression (Salmon et al., 2008). The changes of DNA methylation in plants can be triggered by environmental stresses (Chinnusamy and Zhu, 2009). It has been shown that cold stress results in global demethylation of root genomic DNA (Steward et al., 2002), while stresses of drought (Labra et al., 2002), salt (Verhoeven et al., 2010) and cadmium (Filek et al., 2008) induce methylation-sensitive amplified polymorphism (MSAP) fragments, suggesting that DNA methylation variations may be a part of plant adaptation mechanisms to environmental stresses.

Heat stress can result in inhibition of photosynthesis, damage of cell membranes and even death of cells (Xu et al., 2006) and has been identified as one of the major agricultural problems in many areas of the world (Huang and Xu, 2008). To protect plants from heat stress, considerable efforts have addressed on application of exogenous materials to change the physiological and biochemical parameters of heat-stressed plants and thus to induce heat tolerance of plant seedlings (Wahid et al., 2007). Hydrogen peroxide (H_2O_2) at a low concentration acts as a signaling molecular and thereby is involved in several protective pathways under heat stress (Saidi et al., 2011). Addition of H_2O_2 causes an increase in intracellular free Ca^{2+} (Rentel and Knight, 2004). Treatment with H_2O_2 induces transcription of heat-shock proteins (HSPs) and heat-shock transcription factors (HSFs) (Volkov et al., 2006; Banti et al., 2010), and application of H_2O_2 inhibitors reduces HSP expression (Volkov et al., 2006; Konigshofer et al., 2008) and blocks the DNA-binding ability of HSFs (Volkov et al., 2006) at high temperatures. Moreover, pretreatment with H_2O_2 influences antioxidant enzyme activities under heat stress (Uchida et al., 2002; Larkindale and Huang, 2004) and thereby induces heat tolerance of seedlings and leads to a higher survival percentage of plants (Gong et al., 2001). Our research team (Gao et al., 2010) found that exogenous H_2O_2 alleviates the damage of heat stress to chloroplast ultrastructure through regulating antioxidant enzymes. However, to our knowledge, the alteration of DNA methylation in plants has not been

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reported when exogenous H_2O_2 is used to mitigate heat stress. We hypothesize that heat stress induces DNA methylation variations in plants, and exogenous H_2O_2 affects the heat-induced alteration of DNA methylation in H_2O_2-pretreated heat-stressed leaves.

A technique of MSAP is highly efficient for detection of cytosine methylation in plant genomes by using two enzyme combinations *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I (Xiong et al., 1999). The principle of this technique is as follows: *Hpa*II and *Msp*I are isoschizomers and both recognize the tetranucleotide sequence 5′-CCGG-3′, but they differ in the sensitivity to methylated cytosines (Portis et al., 2004). *Hpa*II is inactive when either or both of the two cytosines in 5′-CCGG-3′ is methylated in both strands (fully methylated), and it is active while either of the cytosines is methylated in one strand (hemi-methylated). Meantime, *Msp*I acts in hemi- or fully methylated $C^{5m}CGG$ but not $^{5m}CCGG$ (McClelland et al., 1994). Therefore, four types (I, II, III and IV) of MSAP band patterns will be obtained in plant genomic DNA (Table S1). On the other hand, bisulfite sequencing PCR (BSP) is used to analyze the methylation status in specific genes of interest (Henderson et al., 2010). This technique converts unmethylated cytosine into thymine and thus determines the methylation change of individual CpG dinucleotides.

In this study, DNA methylation variations were detected using the MSAP technique when H_2O_2-pretreated cucumbers were exposed to heat. Then, we analyzed the transcript levels of three selected cucumber genes, which respectively show homology to three MSAP fragments. Meantime, the methylation status of two selected genes was studied using the BSP technique. Our aims are to investigate whether heat induces DNA methylation variations, and to detect whether exogenous H_2O_2 affects the heat-induced alteration of DNA methylation in H_2O_2-pretreated heat-stressed cucumbers. Thus, we can further study the different molecular effects between heat treatment alone and the combination of H_2O_2 pretreatment and heat. Our work in this study might contribute to understanding why H_2O_2 application induces heat tolerance of plants in view of DNA methylation and help to elucidate the mechanism of heat stress further.

2. Materials and methods

2.1. Plant materials and treatments

Five mM H_2O_2 has been applied to a cucumber cultivar "Jinyan no. 4" (Cui et al., 2011), while pretreatment with 1.5 mM H_2O_2 can protect the second leaves of cucumber cultivars "Lvfeng no. 6" and "Jinchun no. 4" from stresses (Gao et al., 2010; Liu et al., 2010; Zhang et al., 2011). In this study, a cucumber cultivar "Lvfeng no. 6" was used, and four groups of cucumber seedlings (8 plants per group) at two-leaf stage were chosen and treated for heat experiments according to Gao et al. (2010). Therefore, two groups of seedlings were pretreated with 1.5 mM H_2O_2 for 12 h and then were separately kept at 25/18 and 42/38 °C (day/night) for 3 d, and they were designated as "H_2O_2 pretreatment" and "H_2O_2 + heat treatment", respectively. Meanwhile, two groups of cucumbers were pretreated with distilled H_2O for 12 h and then were separately exposed to two temperatures indicated above for 3 d, and they were respectively named "control" and "heat treatment". Three different sets of plants grown at different times were carried out for treatments. After performed for evaluation of plant growth, the second leaves from one treatment group were mixed by grinding with liquid nitrogen and were used for extraction of genomic DNA and total RNA.

Seedlings of *Cucumis sativus* cv. Lvfeng no. 6 were also cultured and treated for PEG-induced osmotic stress experiments according to Liu et al. (2010). At two-leaf stage, four groups of cucumber seedlings (8 plantlets per group) were selected for treatments. Among which, two groups of seedlings were sprayed with 1.5 mM H_2O_2 for 8 h and then were watered for 2 d with either the Hoagland nutrient solution only or the Hoagland nutrient solution containing 10% polyethylene glycol (PEG) 6000, and they were designated as "H_2O_2 pretreatment" and "H_2O_2 + PEG treatment", respectively. Meanwhile, two groups of cucumbers were sprayed with distilled H_2O for 8 h and then were separately watered for 2 d with either of the two types of nutrient solutions indicated above, and they were respectively named "control" and "PEG treatment". Three different sets of plants grown at different times were used for treatments. And the second leaves from each treatment were collected for extraction of total RNA.

2.2. Evaluation of plant growth

Morphological observation was performed in four treatment groups (control, H_2O_2 pretreatment, heat and H_2O_2 + heat treatments) of heat experiments to investigate whether the edges of the second leaves were soft and withering, and the percentage of leaf withering in one treatment group was evaluated from the number of withered leaves divided by the total number of the second leaves. Fresh weight of the second leaves or roots was the mean value from plantlets of each treatment. Differences were analyzed using two-way ANOVA and the least significant difference (LSD) test. *P*-values of <0.05 were considered significant.

2.3. Genomic DNA extraction

Genomic DNA from the second leaves of four treatment groups (control, H_2O_2 pretreatment, heat and H_2O_2 + heat treatments) in heat experiments was separately extracted according to the CTAB method (Murray and Thompson, 1980). Three biological replicates were used for the extraction.

2.4. MSAP analysis

The level and pattern of genomic methylation were investigated by using MSAP according to Portis et al. (2004) with modifications. In a final volume of 25 μl, aliquots of genomic DNA (1 μg) were digested at 37 °C for 8 h with *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I, respectively. Then, 22 μl of digested DNA solutions were separately ligated to adapters at 16 °C by adding 8 μl of ligation mixture that contained 1× T4 DNA ligase buffer, 2 U T4 DNA ligase, 5 pmol *Eco*RI adapter and 50 pmol *Hpa*II/*Msp*I adapter (Table S2).

Two consecutive PCRs were used to amplify the *Eco*RI-*Hpa*II and *Eco*RI-*Msp*I DNA fragments. A pre-selective amplification (the first PCR) was performed using 2 μl of ligation products and 23 μl of mixture [containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 50 ng of *Eco*RI primer for pre-selective amplification, 50 ng of *Hpa*II/*Msp*I primer for pre-selective amplification (Table S2) and 1 U Taq DNA polymerase (Takara, Japan)]. The PCR parameters included 94 °C for 2 min, 20 cycles of 30 s denaturing at 94 °C, 60 s annealing at 56 °C and 60 s extension at 72 °C, ending with 5 min at 72 °C to complete extension. After checked by agarose electrophoresis, the amplification product was diluted 40 times in double distilled H_2O.

Mixtures of a selective amplification (the second PCR) contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 50 ng of *Eco*RI primer for selective amplification, 50 ng of *Hpa*II/*Msp*I primer for selective amplification (Table S2), 1 U Taq DNA polymerase (Takara, Japan) and 5 μl of pre-selective amplification product in a 25 μl reaction system. PCR reactions were performed with the following profile: 94 °C for 60 s, 13 cycles at 94 °C for 30 s, 65 °C for 30 s (reducing 0.7 °C per cycle) and 72 °C for 1 min; and another 23 cycles of PCR amplification were used following the touchdown program: the denaturing step was done at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.