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Research paper

Gene network underlying the response of harvested pepper to chilling stress

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ABSTRACT

Cold storage is an effective postharvest control strategy to maintain the freshness of vegetables by suppressing respiration. However, subtropical plants including pepper (*Capsicum annuum* L.) undergo chilling injury. To better understand the molecular mechanisms involved in preventing chilling injury, transcriptome profiling analysis of peppers stored in a cold chamber and treated with 50 μM methyl jasmonate (MeJA) and 1 μL L^{-1} 1-methylcyclopropene as an ethylene reaction inhibitor was performed. A total of 240, 470, and 290 genes were upregulated and 184, 291, and 219 genes down-regulated in cold-, MeJA- and 1-methylcyclopropene-treated peppers, respectively. MeJA-treated peppers had significant transcriptome changes compared to cold- and 1-MCP-treated peppers after 24 h of storage. MeJA treatment upregulated the genes for peroxidase and catalase related to stress responses, as well as the ethylene-responsive factor (ERF) family and MAP kinase involved in ethylene signaling factors in peppers. Functional analysis revealed that in comparison with wild type plants, *ERF1*-expressing plants showed a higher antioxidant capacity and enhanced expression levels of oxidative stress-related and jasmonic acid synthesis-related genes during chilling storage conditions. Additionally, ERFs and JA biosynthesis gene expression in peppers during long-term cold storage was upregulated by MeJA. Thus, MeJA enables peppers to respond to cold stress and ethylene signaling, and this could help to prevent chilling injury. Our results suggest that ethylene signaling and JA synthesis share the reactive oxygen species (ROS) scavenger-mediated stress adaption system during chilling stress in pepper. In addition, these findings provide a global insight into the genetic basis for preventing chilling injury in subtropical crops.

1. Introduction

Peppers (*Capsicum annuum* L.) originated in subtropical regions and are important crops from nutritional and commercial standpoints because of their high vitamin C and capsaicin contents. They are thus a very popular food for consumers. However, at temperatures below 7 °C, the temperature commonly used for long-term cold storage, peppers can experience chilling injury that results in deterioration of the calyx, seed browning, sheet pitting, and development of *Alternaria* rot on the pods (Özden and Bayindirli, 2002). Susceptibility to damage during cold storage increases postharvest loss and reduces the storage life of chilling-sensitive crops, which, like pepper, are often subtropical or tropical in origin (Paull, 1990). However, cold storage is generally the most effective technology to maintain the quality of postharvest horticultural crops by inhibiting respiration. Thus, it is important to overcome the susceptibility to chilling stress in commercially important sensitive crops.

Plant responses to biotic and abiotic stresses such as cold, ultraviolet radiation, and drought depend on interactions between hormone signaling pathways rather than on the independent contributions of individual hormones (Glazebrook, 2001). Plant hormones play a role in modulating developmental processes during growth and postharvest storage. The hormone ethylene regulates plant growth, development, and senescence. Ethylene affects postharvest quality by, for example, promoting ripening in climacteric fruit (Theologis, 1992). Exposure of horticultural products to ethylene is generally avoided, and efforts are made to minimize ethylene production during ripening and storage (Watkins, 2006). By controlling ethylene production, senescence and ripening can be delayed, and the duration of freshness can be extended. The synthetic plant growth regulator 1-methylcyclopropene (1-MCP) is thought to inhibit ethylene-dependent processes by interacting with ethylene receptors. 1-MCP delays ripening and extends the shelf life of climacteric fruits by affecting ethylene production, color changes, and respiration (Ziliotto et al., 2008). In addition, 1-MCP is a useful tool to investigate the role of ethylene in the senescence of horticulture crops. Several reports have shown that application of 1-MCP reduced chilling

Abbreviations: MeJA, methyl jasmonate; 1MCP, 1-methylcyclopropene; ROS, reactive oxygen species; *E. coli*, Escherichia coli; ERF, ethylene-responsive factor; GO, gene ontology; JA, jasmonate; PR, pathogen related; qRT-PCR, quantitative reverse transcription polymerase chain reaction

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injury and activated antioxidant enzymes (Cao et al., 2009; Yuan et al., 2010). In this study, we used 1-MCP to investigate the function of ethylene in chilling stress in pepper.

Jasmonates (JAs), a class of plant signaling hormones, are important to developmental processes such as germination, fertility, and senescence. In addition, JA contributes to defense mechanisms against stresses including wounding, extreme temperature, and pathogen infection (Anderson et al., 2004). Methyl jasmonate (MeJA) activates antioxidant metabolic pathways and defense mechanisms in various crops (Parra-Lobato et al., 2009; Sasaki-Sekimoto et al., 2005; Sasaki et al., 2001) and can confer chilling tolerance and reduced surface pitting (Fung et al., 2004). The C-repeat binding factor (CBF) pathway during cold stress and freezing tolerance in plants is regulated by JAs (Sharma and Laxmi, 2016). *MaMYC2*, a basic helix-loop-helix (bHLH) transcription factor, is involved in MeJA-induced chilling tolerance in banana through functional coordination with *MaICE1* (Zhao et al., 2013). However, few studies have examined the genetic mechanism by which MeJA confers chilling tolerance.

Interactions between ethylene and jasmonate may fine-tune plant responses to environmental stress such as ozone-induced cell death (Rao et al., 2000), wounding (Rojo et al., 1999), and pathogens (Lorenzo et al., 2003; Wang et al., 2002; Zhu et al., 2011). The transcriptome profiles of peer fruit revealed that the genes related to jasmonic acid biosynthesis and signaling could be involved in the low temperature-mediated enhancement of ripening independently or upstream of ethylene (Nham et al., 2017). However, the investigation of interactions between their pathways on chilling stress at a molecular level would improve the understanding of crop responses to chilling stress during cold storage. Global expression profiling of peppers maintained in cold storage, as well as gene expression changes in relation to chilling tolerance after hormone treatment have not previously been reported.

In this study, we identified global transcriptome changes in cold-, MeJA-, 1-MCP-treated, and non-treated peppers during cold storage. The functional analysis of candidate genes related to chilling injury, such as ethylene response factor genes (ERFs), was performed. We discuss the participation of ethylene signaling in jasmonate-mediated chilling resistance of peppers.

2. Materials and methods

2.1. Plant materials and treatments

Pepper (*Capsicum annuum* L. 'Super Bigarim') plants were grown in the greenhouse of the National Institute of Horticultural and Herbal Science for use in the experiments. Freshly harvested pepper fruits were divided into three groups. One group was treated with 50 μM MeJA (MeJA) and one with 1 μL L^{-1} 1-MCP (1-MCP), at 25 °C for 24 h. A third group (Cold) was exposed to cold without chemical treatment. All three groups were maintained in a cold chamber (0 °C). Peppers immediately harvested from the farm were used as a non-treated control (Non).

2.2. Microarray, data analysis, and gene enrichment analysis

To assess the reproducibility of the microarray analysis, total RNA was isolated from three individual samples from each treatment. For each treatment, total RNA was extracted from peppers stored at 0 °C for 24 h using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. To synthesize double-stranded cDNAs, we used a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, USA), and purification was conducted using a MinElute Reaction Cleanup Kit (Qiagen). Ten milligrams of DNA was used for microarray hybridization. Chip hybridizations were performed by GreenGene Bio Tech Inc. (Korea) using the *Capsicum annuum* 135 K GeneChip manufactured by NimbleGen (http://www.nimblegen.com). The microarray was designed from 29,580 unigenes and a total of 138,050 probes.

The microarray was scanned with a Genepix 4000 B (Axon Instrument) preset with 5-μm resolution and for Cy3 signal. Signals were analyzed by NimbleScan (NimbleGen, USA). To adjust the signal variation between chips, the data were normalized with cubic spline normalization using quantiles (Workman et al., 2002). Robust Multi-Chip Analysis using a median polish algorithm summarized the probe-level. Multiple analyses were performed with the limma package in the R computing environment (Smyth, 2004). Differentially expressed genes were identified using Student'st test, and multiple test corrections were performed using the false discovery rate (FDR) (Benjamini and Hochberg, 1995). Significantly changed genes were identified using the One-sided Fisher significance test, P value < 0.05 and a fold change > 2. Genes with FDR < 0.05 were selected for GO term analysis. Multivariate statistical tests such as clustering, principal component analysis, and multi-dimensional scaling were performed with Acuity 3.1 (Axon Instruments). Hierarchical clustering was performed with similarity metrics based on the squared Euclidean correlation, and average linkage clustering was used to calculate the genetic distance. Gene-set enrichment analysis was performed using the AgriGO analysis tools (http://bioinfo.cau.edu.cn/agriGO/).

2.3. Analysis of quantitative RT-PCR

To verify the microarray data, real-time qRT-PCR was performed on a Rotor-Gene Q real-time PCR system (Qiagen) using a Rotor-gene SYBR Green RT-PCR Kit (Qiagen) according to the manufacturer's instructions. cDNA (50 ng) synthesized as described above was used as a template for amplification with gene-specific primer sets (Supplementary Table S1). The thermal cycling conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s as a two-step condition. To standardize the data, the ratios of the expression levels of initiation factor 4E(IF4E), glyceraldehyde-3-phosphate dehydrogenase (Wan et al., 2011), elongation factor 1-α (EF1 α) (Bin et al., 2012), and ubiquitin-conjugating enzyme E2 (Alós et al., 2013) were used as internal controls (reference genes) in this study. The relative expression levels of the target genes in each sample were calibrated as fold changes relative to non-treated sample using the $^{\Delta\Delta}$Ct method (Livak and Schimittgen, 2001).

For the transcript level analysis of ERFs and JA-related genes in pepper during long-term storage and JA and antioxidant-related genes in *Arabidopsis,* real-time qRT-PCR was conducted using a CFX96 TouchTM Real-Time PCR detection system (Bio-Rad). Total RNA was isolated from the pepper fruit and the leaf tissues of *Arabidopsis* using an RNeasy Mini kit (Qiagen). Five hundred nanograms of total RNA was used to synthesize cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). The resulting cDNA was amplified using iQ™ SYBR Green Supermix and specific primers (Supplementary Table S1). The PCR was programmed using the following conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 51 °C or 55 °C for 40 s. The relative expression value of each gene was calculated by the $^{\Delta\Delta}$Ct method (Livak and Schimittgen, 2001). To ensure accurate measurement of target gene expression, three internal control genes, namely *IF4E*, *EF1α* and *Actin,* were used for pepper (Bin et al., 2012; Wan et al., 2011) and *Actin* was used for Arabidopsis (Dai et al., 2007). A qRT–PCR analysis was carried out with at least three biological and two technical replicates.

2.4. Construction of gene expression in transformed E. coli

Total RNA was extracted from peppers using an RNeasy Plant Mini Kit (Qiagen), and 1 μg of RNA was synthesized to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). Subsequently, the region including the open reading frame of the genes of interest was amplified by an *amfiFusion* High Fidelity PCR Master Mix (2X) (GenDEPOT) with gene-specific primer sets. The primers were designed with particular restriction enzyme sites as described in Supplementary