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Transcriptome profiling reveals candidate genes associated with sex differentiation induced by night temperature in cucumber

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ABSTRACT

The sex differentiation of cucumber directly affects cucumber fruit yield, and low night temperature (LT) promotes female flower differentiation. However, studies on the effects of LT on sex differentiation are scarce. To better understand this mechanism, we used transcriptomics to examine the expression patterns of genes related to female flower differentiation in the cucumber cultivar 'C09-123' under LT and high night temperature (HT). We identified a total of 1654 differentially expressed genes in response to LT. There were 1147 up-regulated genes and 507 down-regulated genes under LT compared with their expression under HT. Gene ontology (GO) analysis revealed that the differentially expressed genes were mainly involved in cellular process, metabolic process, response to stimulus process and other processes (biological process). Many of these differentially expressed genes (DEGs) were related to glucose metabolism, cysteine and methionine metabolism and the ethylene and auxin signal transduction pathway and were up-regulated under LT, which promotes female differentiation, compared with their expression level under HT. These results suggest that changes in these genes are likely to affect the corresponding metabolism, thus inducing the female flower differentiation of cucumber under LT. Ethylene and auxin play important roles in the induction of female flower differentiation under LT. This study provides valuable resources for further exploring the molecular basis of LT induction of female flower differentiation and for enabling targeted breeding strategies for developing varieties with superior strong female differentiation to achieve yield potential.

1. Introduction

The sex differentiation of cucumber (*Cucumis sativus* L.) is directly related to cucumber yield. The early emergence, abundance and uniform distribution of female flowers on a plant are the basis for the early, high and stable yield of cucumber. Therefore, studying the sex differentiation of cucumber is of great significance in the field of developmental biology and for production purposes.

The development of floral sex differentiation in plants is a very complex process. Cucumber is a model for the study of plant unisexual flower development and has been used for breakthroughs in research. At the early stages of development, cucumber floral primordia are initially bisexual, containing initials of both anthers and pistils, and sex determination occurs following the selective arrest of the development of either the staminate or pistillate primordia cells (Bai et al., 2004). Sex expression in cucumber is mainly determined by three major genes: *female* (*F*), *andromonoecious* (*M*) and *androecious* (*A*). The *F* gene has been cloned and encodes 1-aminocyclopropane-1-carboxylic acid synthase (ACS) (Trebitsh et al., 1997; Mibus and Tatlioglu, 2004; Knopf and Trebitsh, 2006), which is involved in the regulation of female flower differentiation (FFD). The *M* gene has also been cloned and encodes another member of the ACS gene family, *CsACS2* (Boualem et al., 2009; Li et al., 2009), which controls bisexual flower expression. The *A* gene increases maleness (Pierce and Wehner, 1990).

Plant hormones and environmental factors induce sex differentiation in cucumber. These factors include hormones, temperature, light, and molecular genetic pathways (Atsmon and Galun, 1962; Galun, 1962; Bachman and McMahon, 1997). Phytohormones such as

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-L-carboxylic acid; ACS, 1-aminocyclopropane-L-carboxylate synthase; ACO, ACC oxidase; Asp, aspartic acid; BR, brassinolide; cDNA, complementary DNA; CK, cytokinin; CTR1, constitutive triple response 1; DAS, days after sowing; DEGs, differentially expressed genes; EIN2, ethylene insensitive 2; FFD, female flower differentiation; GA, gibberellin; HT, high night temperature; IAA, indole-3-acetic acid; LT, low night temperature; Met, methionine; MFD, male flower differentiation; KEGG, Kyoto Encyclopedia of Genes and Genomes; SA, shoot apex; TF, transcription factor

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ethylene, auxin, gibberellins (GAs), abscisic acid (ABA) and brassinolide (BR) are involved in sex differentiation in cucumber. However, ethylene is the main hormone that determines FFD in cucumber. For example, gynoecious plants evolve greater quantities of ethylene than do monoecious plants (Rudich et al., 1972a; Trebitsh et al., 1997). The exogenous application of ethylene or ethylene-releasing compounds to monoecious cucumber plants enhances the formation of female flowers (Yamasaki et al., 2003). Ethylene synthesis and perception as well as ethylene-induced genes are also involved in sex differentiation in cucumber (Yamasaki et al., 2000; Ando and Sakai, 2002). GAs negatively affect female flower formation in cucumber. Monoecious cucumber plants contain higher GA levels than do gynoecious lines (Atsmon and Galun, 1962) and exogenous GAs can increase maleness and delay female flower formation (Peterson and Anhder, 1960; Galun, 1961; Wittwer and Bukovac, 1962; Pike and Peterson, 1969). GAs regulate stamen and anther development via the transcriptional regulation of *GAMYB*. Knockdown of *CsGAMYB1* in cucumber decreases the ratio of nodes having male and female flowers (Zhang et al., 2014). ABA and BR may also participate in the regulation of sex expression in cucumber. Rudich et al. (1972a) reported that the ABA content of gynoecious plants is higher than that of monoecious plants. Also, BR promotes FFD in cucumber, probably by promoting ethylene synthesis (Papadopoulou and Grumet, 2005).

Light and temperature affect sex differentiation in cucumber. Li et al. (2011) showed that sex differentiation in cucumber is a quantitative trait controlled by multiple genes, and the environment (season) has a considerable influence on the percentage of female flowers. Cheng et al. (2012) used 'C09-123' cultivar as a test material to further explore the influence of light and temperature on sex differentiation in cucumber. The results showed that night temperature was the dominant factor affecting the formation of 'C09-123' females, but the influence of photoperiod was not obvious. A low night temperature (LT) of 12 °C was beneficial for the formation of female flowers of 'C09-123'. At 24 °C (high night temperature, LT), 'C09-123' formed only male flowers.

In other crops, temperature has also been shown to affect female differentiation. The sterility of a Chinese cabbage male sterile line has temperature-sensitive characteristics typical of the high-temperature sterile type (Shao et al., 2008). Temperature plays a dominant role in the fertility transformation of thermosensitive genic male sterile rice, and the expression condition of the male sterile gene was mainly temperature-dependent (Xiao et al., 2005).

Although female flower formation has been positively correlated with the level of ethylene under different growth conditions (Rudich et al., 1972a, 1972b; Yamasaki et al., 2003), the mechanism by which LT affects ethylene biosynthesis is not clear, and genes associated with the promotion of sex differentiation in cucumber by LT/HT have not been identified. Here, we subjected 'C09-123' cultivar to LT (12 °C) and HT (24 °C) treatments and applied comparative transcriptomic analyses to reveal the molecular genetic events related to sex differentiation in cucumber.

2. Materials and methods

2.1. Plant growth conditions

The monoecious cucumber cultivar 'C09-123' was used in this study (Cheng et al., 2012). Seeds were sown in 8 × 8-cm plastic pots containing a soil, peat and organic fertilizer mixture (5:4:1, v/v/v). The pots were placed in a growth chamber under a photoperiod of 16 h/8 h at temperature of 26 °C/18 °C (day/night). When the cotyledons were flattened, we placed the seedlings into 2 separate growth chambers. Two day/night temperature regimes were designed: LT, consisting of 26 °C/12 °C (12 h/12 h, day/night), and HT, consisting of 26 °C/24 °C (12 h/12 h, day/night) (Cheng et al., 2012). LT and HT were both applied 10 days after sowing (DAS) (cotyledon stage) until 33 DAS (approximately the 2.5- to 3-leaf stage). Shoot apex (SA) samples containing immature leaves shorter than 2.0 cm were collected for use in RNA-seq sequencing and qRT-PCR. Samples were frozen in liquid nitrogen immediately after harvest and stored at −80 °C.

2.2. Transcriptomic analysis

The SA samples of 'C09-123' from both treatments were used for total RNA extraction with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. RNA-seq libraries were sequenced on an Illumina

HiSeq™ 2000 System by BGI-Tech (Shenzhen, China). The raw data, which contained adapter and low-quality cycle reads, were filtered. For annotation, all clean tags were mapped to the cucumber genome database (http://cmb.bnu.edu.cn/Cucumis_sativus_v20/) (Huang et al., 2009), and the maximum nucleotide mismatch allowed was 1 bp. The number of raw clean tags in both libraries was normalized to the number of transcripts per million clean tags (TPM) to obtain normalized gene expression levels. Genes were deemed significantly differentially expressed based on the following criteria: P value < 0.005, false discovery rate (FDR) < 0.01, and relative fold change > 2 in the sequence counts across libraries. For pathway enrichment analysis, we mapped all differentially expressed genes (DEGs) in terms of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Pathways with a P value < 0.05 were identified as pathways with significant enrichment.

2.3. Real-time quantitative reverse-transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA from the SA samples under LT and HT was reverse-transcribed to obtain cDNA using the ReverTra Ace qPCR RT Kit (Toyobo) in accordance with the manufacturer's instructions. qRT-PCR was performed using SYBR Green Master Mix (Toyobo, Japan), and the reaction system consisted of 10 μL of SYBR Green PCR Master Mix, 0.5 μL of upstream primer (10 μmol L^{-1}), 0.5 μL of downstream primer (10 μmol L^{-1}), 2 μL of cDNA template, and ddH_2O added to the mixture for a total volume of 20 μL. Relative quantitation of gene expression was calculated and normalized to that of *EF1a* (Wan et al., 2010) in accordance with the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). The sequences of the primers used for qRT-PCR are listed in Supplementary Table S1.

3. Results

3.1. RNA-seq library sequencing and differentially expressed genes (DEGs) analysis

Transcriptome libraries of cucumber SA under LT and HT were constructed for double-end sequencing using an Illumina RNA-Seq 2000 system. The raw reads were deposited in the NCBI Sequence Read Archive database (Accession SRP110559). A total of 4873471 and 4765885 raw tags were identified in the LT and HT samples of the "CO9-123" cultivar respectively. The poor-quality tags were removed, after which 4.72 million (LT) and 4.62 million (HT) clean tags were obtained, which accounted for 96.83% and 96.94% of the total tags, respectively (Fig. S1). The total number of DEGs was 1654 (Table S2). There were 1147 up-regulated genes and 507 down-regulated genes in LT compared with HT respectively (Fig. 1A).

3.2. qRT-PCR confirmation

To confirm our findings based on the RNA-seq data, we conducted a validation experiment by quantitative real-time PCR (qRT-PCR). We screened 29 DEGs, including those related to plant carbon and nitrogen metabolism, amino acid metabolism, hormone synthesis and signal transduction and other genes (random screening). As shown in Table S3, the qRT-PCR results show that the expression patterns of the 29