Scientia Horticulturae 240 (2018) 81–88

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Analysis of *CsPAP-fib* regulation of cucumber female differentiation in response to low night temperature conditions

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ARTICLE INFO

Keywords:
Cucumber female differentiation
CsPAP-fib
Low night temperature
Functional analysis
Transformed plants

ABSTRACT

In this study, the expression pattern of CsPAP-fib under different night temperature conditions (12 °C, 18 °C, and 24 °C) was analyzed using qRT-PCR, the subcellular localization of CsPAP-fib protein was determined, and sense and antisense CsPAP-fib were transformed into cucumber. The first node with female flowers and the female flower rate were calculated and recorded. Next, the ethylene and gibberellin contents were determined. The expression of CsPAP-fib in cucumber leaves was the highest under the low night temperature (12 °C) condition and was significantly higher than that in the roots, stems and shoot tips; however, under the high night temperature (24 °C) and normal night temperature (18 °C) conditions, no significant differences in CsPAP-fib expression in the roots, stems, leaves, shoot tips and other organs were observed. The expression of CsPAP-fib was the highest in cucumber leaves during the two-leaf stage and was significantly higher than that during the four-leaf stage. CsPAP-fib played a functional role mainly in chloroplasts. The overexpression of CsPAP-fib significantly improved the rate of cucumber female flowering and reduced the first node with a female flower. Thus, CsPAP-fib is a relevant gene for the regulation of cucumber female differentiation under low night temperature conditions. Altogether, CsPAP-fib is induced by a low night temperature (12 °C) and promotes the formation of cucumber femaleness by increasing the female flowering rate and decreasing the first node with a female flower. CsPAP-fib may be a relevant gene for cucumber femaleness differentiation at a low night temperature.

1. Introduction

Cucumber is used as a model plant in sex determination studies due to its diversity in sex differentiation (Bai and Xu, 2013; Barak and Trebitsh, 2007; Malepszy and Niemirowicz-Szczytt, 1991; Yamasaki et al., 2003a, b). Most cucumber plants are monoecious and become dioecious or remain hermaphroditic according to their genotype (Tanurdzic and Banks, 2004). Genetic studies have shown that the main regulatory loci of cucumber sex expression include F, M and A loci, each of which plays a different role. The F locus mainly promotes female expression. In contrast, the A locus focuses on male expression. The M locus controls the process of flower primordium to male or female. However, genetic control and environmental factors (e.g., photoperiod, temperature, light and nutritional conditions), particularly temperature, also influence cucumber sex expression (Atsmon, 1968; Frankel and Galun, 1977; Takahashi et al., 1983; Yamasaki et al., 2003a, b). Low temperatures promote femaleness in most cucumber cultivars (Ito and Saito, 1958; Yamasaki et al., 2003a, b), while high temperatures inhibit femaleness by accelerating senescence processes (Miu and Li, 2001).

The influence of photoperiod and temperature on femaleness in cucumber has been determined, and the results indicate that a low night temperature, rather than the photoperiod, is the leading factor that promotes femaleness in the cucumber cultivar "C09-123." Female flowers preferentially develop under a low night temperature of 12 °C and, in contrast, male flowers are promoted by a high night temperature of 24 °C (Cheng et al., 2012). Furthermore, phytohormones have always been considered a determinant of cucumber sexual differentiation and expression (Fujita and Fujieda, 1981; Rudich et al., 1972a, b; Trebitsh et al., 1987). The regulatory roles of ethylene and gibberellin (GA) are the most significant determinants. Ethylene is known as the "sex hormone" in cucumber because it induces female formation

Abbreviations: AS, antisense; GA, gibberellin; S, sense

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https://doi.org/10.1016/j.scienta.2018.05.011
Received 18 December 2017; Received in revised form 28 April 2018; Accepted 7 May 2018
Available online 07 June 2018

Table 1
Primer sequences.

Primer name	Sequence (5'-3')	Used for
qCsPAP-fib-F	GGCTACTTCCTATTACTCTTGGTC	qRT-PCR
qCsPAP-fib-R	TGTTTTCACGGTCGTTTTCT	qRT-PCR
qEF1a-F	CCAAGGCAAGGTACGATGAAA	qRT-PCR
qEF1a-R	AGAGATGGGAACGAAGGGGAT	qRT-PCR
GFP-CsPAP-fib-F	CCAAGCTTATGGCTGCTCTAGCCAGCTC	Subcellular localization
GFP-CsPAP-fib-R	GGGGATCCTTTGAGATAACAAACACCCTGAGTT	Subcellular localization
PCXSN-1250-F	CGGCAACAGGATTCAATCTTA	PCR detection
PCXSN-1250-R	CAAGCATTCTACTTCTATTGCAGC	PCR detection

(Malepszy and Niemirowicz-Szczytt, 1991). The ethylene content in the shoot apices of a gynoecious cucumber is higher than that in the counterpart monoecious cucumber. Thus, the ethylene concentration is likely involved in the regulation of sex expression (Fujita and Fujieda, 1981; Rudich et al., 1972a, b; Trebitsh et al., 1987). In contrast, the regulation of sex expression in cucumber by GA exhibits the opposite pattern (Atsmon and Tabbak, 1979). The application of exogenous GA induces maleness in gynoecious cucumber plants and increases the ratio of transformation from male to female flowers in monoecious plants (Pike and Peterson, 1969; Wittwer and Bukovac, 1962).

Further experiments on the "C09-123″ cultivar found an unknown protein that was upregulated at a low night temperature of 12 °C by differential protein analysis (Liu et al., 2016). The homologous gene, known as *CsPAP-fib,* of the unknown protein was found by DNA sequence alignment against the Cucumber Gene Database and encodes members of the fibrillin protein family. The fibrillin family is widely present in photosynthetic organism plastids as a conserved lipid-binding protein family (Laizet et al., 2004). Since 1976, fibrillins have been found in the rugose rose (*Rosa rugosa*) (Wuttke, 1976) and the garden nasturtium (*Tropaeolum majus*) (Winkenbach et al., 1976). Other members have also been continually discovered in other species. By 1994, fibrillin was isolated from bell pepper fruit and named (Deruère et al., 1994). Genes encoding fibrillin are induced by drought, low temperatures, high temperatures, salt damage and oxidative damage (Zhou et al., 2014) and are also involved in plant growth and stress tolerance, as shown by transgenic studies (Gillet et al., 1998; Pruvot et al., 1996; Vishnevetsky et al., 1996). Previous studies have determined that the expression of fibrillin is involved in plant organ development and the response to environmental stress (Teng, 2006).

Although the functions of a few members of the fibrillin protein family have been determined, no relevant studies investigating the function of *CsPAP-fib* in cucumber have been reported. In our previous study, cloning and sequence analysis revealed that the full-length cucumber *CsPAP-fib* was 870 bp and encoded by 289 amino acids. ATG was the initiation codon and TAA was the termination codon. The protein encoded by *CsPAP-fib* is an unstable hydrophobic protein present in chloroplasts that lacks a signal peptide or transmembrane structure and has the same evolution embranchment and homology as *Cucumis melo* L. (Liu et al., 2017a, b).

Cucumber sex expression is directly related to the yield and quality of fruits; thus, research in this area can bring economic and social benefits. Therefore, both in theory and in production, it is important to study the effects of temperature and photoperiod on the sex differentiation of cucumber. Based on the previous study concerning the *CsPAP-fib* cloning, this study aims to investigate whether *CsPAP-fib* is involved in cucumber female formation under a low night temperature. The expression pattern analysis and the overexpression of *CsPAP-fib* in cucumber were performed, to investigate how *CsPAP-fib* functioning in cucumber femaleness formation under low night temperature conditions.

2. Materials and methods

2.1. Plant material

The strong male phenotype cucumber cultivar "D06103″ (Li et al., 2011) and the temperature-sensitive cultivar "C09-123″ (Cheng et al., 2012) were obtained from Northeast Agricultural University (Harbin, China) and used in this study. Seeds of the "C09-123″ cultivar were sown in 9 × 9 cm plastic pots with a peat-vermiculite mixture (1:1, v/v) and maintained in growth chambers at the Biotron of Northeast Agricultural University until the cotyledon stage. The obtained seedlings were divided into three groups using the same day temperature (26 °C) with various night temperatures: 12 °C (low), 18 °C (normal), and 24 °C (high). The photoperiod was maintained at 12 h day/12 h night.

2.2. Expression analysis by qRT-PCR

Total RNA was extracted from cucumber leaves, roots, stems and shoot tips collected at the two true-leaf and four true-leaf stages using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The two true-leaf and four true-leaf stages were chosen because they constitute the initiation and termination period, respectively (Zhang et al., 2015). RNA was reverse transcribed into the first stable strand of cDNA using a QuantiNova Reverse Transcription Kit (Qiagen, Hilden, Germany). cDNA was stored at −20 °C after diluting to a concentration of 200–400 ng·μL^{-1} for subsequent experiments.

The *CsPAP-fib* primers for qRT-PCR (Table 1) were designed using an online website (https://www.genscript.com) based on the *CsPAP-fib* sequence obtained by PCR in a previous study. *CsEF1α* (XM_004138916) was used as the housekeeping gene. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

qRT-PCR was performed on a Bio-Rad iQ5 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR® Green PCR Master Mix (ToYoBo Co., Ltd., Osaka, Japan). The qRT-PCR system included 10 μL SYBR® Green PCR Master Mix, 0.5 μL upstream primer (10 μmol·L^{-1}), 0.5 μL downstream primer (10 μmol·L^{-1}), and 2 μL cDNA template, with ddH_2O up to 20 μL. The cycling conditions used for qRT-PCR were 95 °C for 3 min; 95 °C for 10 s, 56 °C for 30 s, 72 °C for 30 s for 40 cycles; and a 72 °C extension.

Three biological replicates were performed. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ relative quantitative method, and the variance and significance were analyzed using DPS 7.051 data processing system software (Li et al., 2016).

2.3. Subcellular localization in Arabidopsis thaliana protoplast cells

Primers containing restriction enzyme digestion sites for *Hind*III and *Bam*HI (Table 1) were designed to PCR amplify the *CsPAP-fib* open reading frame without its stop codon. The PCR product was fused upstream of green fluorescent protein (GFP) between the *Hind*III and *Bam*HI sites in the pGII-EGFP vector. A plasmid kit (Axygen, Inc., Union City, CA, USA) was used for plasmid extraction and purification. The CsPAP-fib-GFP plasmid taken up by *Arabidopsis* protoplasts was