

Gene expression analysis related to ethylene induced female flowers of cucumber (*Cucumis sativus* L.) at different photoperiod

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Received: 12 May 2017 / Revised: 27 July 2017 / Accepted: 22 August 2017
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Abstract Photoperiod is one of the factors affecting productivity of cucumber plant by inducing ethylene hormone production and so triggering flower sex differentiation into female flower. However, only few studies have been performed in order to reveal the effect of photoperiod in molecular level in relation to the flower differentiation. Therefore, in this study, Mercy cultivar of cucumber (*andromonoecious*) was treated with photoperiod of 8, 12, 16 hours of light, while control received no treatment of additional light. Photoperiod of 8 hours was achieved by blocking the sunlight with shade net and 16 hours by giving longer light exposure using white LEDs. Cucumber's flowers were quantified and the apical and lateral shoots were extracted to evaluate the gene profile related to the photoperiod, ethylene production, and female flower differentiation, which were *CsACS2*, *CsETR1*, *CsCaN*, and *CsPIF4* using PCR method. Photoperiod of 8 hours affected the production of female flower with average number of 6.7 flowers in main stem and 8.0 flowers in lateral stem, compared to photoperiod of 12 and 16 hours which produced 3.7 and 2.0 flowers in main stem with 7.0 and 11.3 in lateral stem, respectively. *In silico* studies in this experiment resulted in proposed model of signal transduction that showed the connection between ethylene production and flower differentiation. PCR analysis confirmed the expression of *CsACS2*, *CsETR1*, and *CsCaN*, that was positively correlated with numbers of female flowers in cucumber, but the expression of *CsPIF4* that represent photoperiod haven't been confirmed correlated with the ethylene production and flower differentiation.

Keywords *CsACS2*, *CsCaN*, *CsETR*, *Cucumis sativus*, Female flower, Photoperiod

Introduction

Production of cucumber (*Cucumis sativus* L.) in 2015 was 447.677 ton/year in plantations covering 43.573 ha, so that it was one of the biggest horticultural plants in Indonesia (Indonesia Ministry of Agriculture, 2016). Other than commonly consumed as fruit-vegetable food, cucumber has been used traditionally to cure sore throat and fever. Furthermore, cucumber is also used as raw materials to produce facial cleansing agent in cosmetic industries, and contain cucurbitacin that is potential as a cure for cancer in pharmaceutical industries (Scout and Myers 2010; Lee et al. 2010; Chen et al. 2005).

Conventional cultivation of some cucumber cultivars resulted a very low productivity due to low numbers of female flowers produced in the early stage of flowering (Johnson 2008). One of the factors that affects flowering is photoperiod. Generally, cucumber is known as a neutral day plant, in which its flowering process is independent of daylength (Savonen 2003; Reece, et al. 2012). However, based on other study, *andromonoecious*, *gynomonoecious*, and hermaphrodite cultivar that were treated with low temperature and short-day condition promoted femaleness in cucumber plants, while high temperature condition and long-day photoperiod promoted maleness (Pessaraki 2016).

Other factor that can affect flowering process in cucumber is the production of ethylene, which is affected by the presence of sucrose inside the plant. High sucrose content will correlate to higher production of ethylene (Miao et al. 2010). According to Yamasaki et al (2010), the increase of ethylene in short-day plants is a result of an increase in expression of *CsACS2*, which then increases ethylene response through ethylene recognition by ethylene receptor *CsETR1*. As a result, complex CTR1 and *CsETR1* cannot be formed, which cease negative regulation from CTR1, hence resulting in the increase of ethylene response (Wang et al. 2010). Ethylene signal triggers a response to the synthesis of enzyme(s)

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that lead to DNA degradation of primordial anther. Therefore, the previously hermaphrodite flower with two sex organs develops only into stigma, the female organ of flower (Wang et al. 2010). This study was performed to evaluate the effect of photoperiod on female flower production on molecular level, in order to optimize the factors in flowering mechanism that can affect the productivity of cucumber.

Materials and Methods

Plant Materials

Cucumber's seeds used in this experiment were from Mercy cultivar that represent *andromonoecious*. Seeds were germinated on charcoal husks for 7 days until the seedling's height was 3~5 cm. The charcoal husks were watered daily. Subsequently, seedlings were transferred to polybag containing soil, charcoal husks, compost, and maintained in screenhouse for acclimatization for 14 days.

Photoperiod treatment

Photoperiod used in this experiment represent the short-day (8 h irradiation), neutral-day (12 h irradiation), and long-day plants (16 h irradiation). Photoperiod for short-day treatment was achieved by using 2-ply 75% shade net. Neutral and long day photoperiod were achieved by using white LEDs with Photosynthetic Active Radiation (PAR) of 100~220 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and light intensity 600~10.000 lux with total power input 74 watt. LEDs light was set to turned on for neutral-day and long-day photoperiod at 5.00~6.00 PM and 5.00~10.00 PM, respectively. For control treatment, cucumbers were not given any light treatment. Plants were watered using half liter of tap water each day until 3 weeks, and

then watered with a liter/day until harvest. Plants were fertilized with NPK fertilizer (16:16:16). Experiment were conducted in ITB Jatinangor screen house.

Flower quantification

Observation and quantification of flowers was conducted manually by the end of the treatments. The flowers that were counted were male and female flowers from main and lateral stems.

Statistical analysis

Data were analyzed using one way ANOVA and further by using Duncan Test at the level of $p \leq 0,05$ using SPSS Software version 22nd.

In silico studies

In silico studies in this experiment consisted of primer synthesis and modelling of signal transduction from photoperiod to female flower differentiation in cucumber. Genes used as a basis for primer synthesis were *CsACS2* (Accession number: KM272632.1), *CsETR1* (Accession number: NM_001280633.1), *CsCaN* (Accession number: GQ149069.1), *CsPIF4* (Accession number: XM_008466464.2). Primer synthesis of *CsACS2*, *CsETR1*, *CsCaN*, and *CsPIF4* were based on NCBI's Primer-Blast (Table 1). Primers quality were checked using oligoanalyzer software based on its T_m value, GC percentage, and predicted conformation of hairpin and dimer structure. Domain prediction was conducted with NCBI's CDD search to understand the basic function of enzymes involved in cucumber flower differentiation. Suggested model of signal transduction from phytochrome-ethylene-differentiation of female flowers was presented in Figure 2.

Table 1 Primers used in PCR reaction

Primers	Nucleotide sequence (5' < 3')	Amplicon (bp)
CsGaDPH F	TCAACGACCCCTTCATCAC	236
CsGaDPH R	AGCAGCCTTGTCCTTGTC	
CsACS2 F	CTCACAAACGCAACGGTTTT	167
CsACS2 R	CTCAAATTCATCGGCATTCCCT	
CsETR1 F	GTTGTTGCTGTCCGTGTTCC	670
CsETR1 R	ACCCACAACATTAAGAATAGCTTC	
CsCaN F	TCACGGATGCTGGTTACAGG	836
CsCaN R	TGGTCTTAGGCGGCAAGATT	
CsPIF4 F	TGTCTCGAATGGCTATGGGC	533
CsPIF4 R	CTTCTACTTGGTGGAGAGCAAGG	

PCR Analysis

Sampling method was modified based on Yamasaki's research. Apical and lateral shoot of cucumber were excised and used for total RNA extraction with Promega SV Total RNA Isolation System (Catalog No: Z3100) based on manufacturer's instructions. Isolated RNAs were cleaned up from DNA contaminant using DNase I Thermo Scientific (Catalog No: #EN0521). Isolated RNA was treated with with Bio-rad iScript™ cDNA Synthesis Kit (Catalog No: 170-8890) to produce First strand cDNA by previously calculating RNA concentration and RNA quality using spectrophotometer at 260, 280, 320, and 230 nm. Synthesized cDNA was used as a template for PCR confirmation with Promega GoTaq Green Master Mix (Catalog No: M7122) and ABI Veriti® thermocycler. *GaDPH* was used as a control gene (*Accession Number*: NM_001305758.1). Procedure in PCR was modified based on manufacturer's instruction with cDNA used in the reaction was 2 μ L. Primers used in PCR are listed in Table 1.

Results and Discussion

Total Flowers Analysis

Total male and female flowers produced from photoperiod treatments was shown in Figure 1A. This figure showed that Mercy was cucumber from *andromonoecious* line that produced dominant male flowers. However, eight hours treatment resulted in the highest number of female flowers (14.7 flowers/plant). This treatment produced lower total male flowers compared to 12 and 16 hours photoperiod, in which eight hours treatment produced 54.7 male flowers/plant while other treatments produced around 80 male flowers/plant. Lower number of total flowers indicated that the flowering capability was correlated with photoperiod that might relate to sugar production, thus affected the total number of flowers (Taiz and Zeiger 2010; Ikram et al. 2015). These total flowers data was similar to Yamasaki's research that showed conversion between male and female flowers in the main stem that was affected by photoperiod, longer photoperiod produced higher male number and lower female number, while shorter photoperiod produced higher female number and lower male number (Yamasaki et al. 2003).

Another finding in this experiment was that the main and lateral stems produced different numbers of female flowers (Figure 1B). Eight hours irradiation produced more female flowers in the main stem, while 12 and 16 hours irradiation showed more female flowers in their lateral stem.

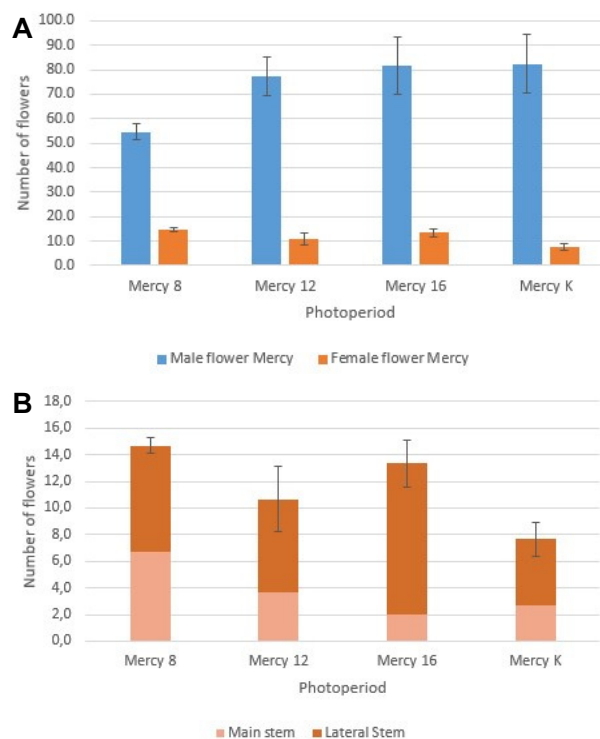


Fig. 1 (A) Total number of flowers produced by Mercy cultivar. (B) Comparison of female flowers produced by Mercy cultivar in the main stem (light orange) and lateral stem (dark orange)

The maximum number of female flowers in the main stem could be explained by the production of ethylene in apical shoot induced by shorter photoperiod. This finding was supported by Yamasaki research that showed the same effect of photoperiod (Yamasaki et al. 2003). High female flowers on cucumber main stem was also related to the increase of ethylene production that induced by shorter photoperiod (Ikram et al. 2015; Yamasaki et al. 2003). Interestingly, prolonged photoperiod to 16 hours significantly increased the female flowers in lateral stem, while the total number of female flowers in the plant was similar compared to 8 hours. Longer photoperiod increased sugar content in cucumber (Mayorazaki et al. 2015). Escalation of sugar content affected the lateral bud development (Mason et al. 2016). Apical dominance known to be affected by the role of auxin, but Mason (2014) also suggested that it may not be controlled solely by auxin, it was also controlled by the needs of sugar from shoot tip. Excised shoot tip's canceled apical dominance as a result from distributed sugar content to axillary buds (Mason et al. 2014). Therefore, higher sugar content induced by longer photoperiod influenced the development of lateral buds. Increased sugar content affected the production of lateral stem, the development of flowers, and induced ethylene production to stimulate flower differentiation to female flower (Miao et al. 2010; Ikram et

al. 2015; Mason et al. 2014).

In spite of its difference in flower differentiation, photoperiod did not play a major role in initiation of flowering, all treatment conditions induced flowering. Therefore, cucumber could be categorized as neutral-day plant. This statement was supported by other research that showed cucumber was into neutral-day plant (Savonen 2003). It is suggested that flower production might be induced by autonomous pathway or sugar content (Taiz and Zeiger 2010; Ikram et al. 2015). In addition, Figure 1 showed that photoperiod on cucumber played a major role in sex determination. This result supported by other research such as Yamasaki (2003), Wang (2010), and Pessaraki (2016). Flower differentiation in Melon (*Cucumis melo*) and Watermelon (*Citrullus lanatus*) was also affected by photoperiod that lead to the presence of ethylene which induced the female flower determination (Girek et al. 2013; Manzano et al. 2016). However, this phenomenon was only appeared in *monoecious* and *andro-monoecious* cultivar. Female-dominant cultivar (*gynomonoecious*) in cucumber did not shown to be affected by ethylene presence (Ikram et al. 2015).

Probability of cucumber production in Mercy cultivar depends on the capability of cucumber plant to produce female flowers (Pessaraki 2016). Even though the total number of female flowers in 8 hours and 16 hours photoperiod did not differ significantly, the energy consumption to produce each female flowers was different. 16 hours light treatment need an additional light supply in the cultivation, 8 hours photoperiod did not need any additional energy. Hence, the best photoperiod for producing female flower in cucumber

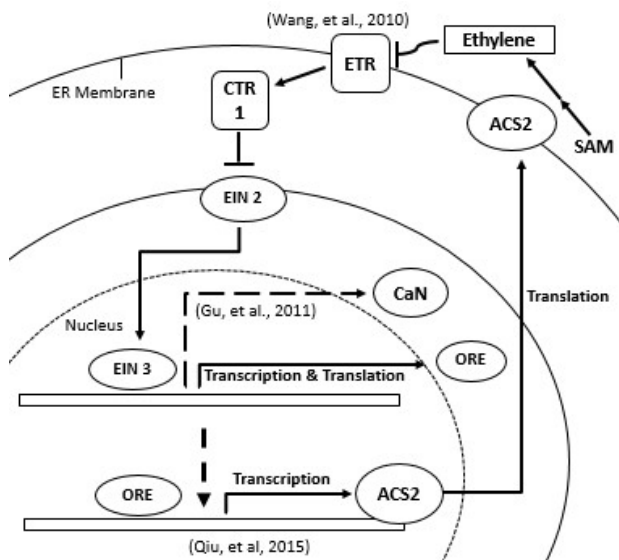


Fig. 2 Proposed model of ethylene affecting flower differentiation in cucumber

Mercy cultivar was 8 hours irradiation.

PCR Analysis and *in silico* studies

Result from RNA extraction was shown by electrophoregram in Figure 3A. cDNA was synthesized from the RNA and kept at -20°C . Gene expression analysis to confirm the model was conducted by using *CsACS2*, *CsETR1*, *CsCaN*, and *CsPIF4* genes, started with primer synthesis from *in silico* study. Each gene represented the function in model signal transduction, *CsACS2* for ethylene expression, *CsETR1* for ethylene response, *CsCaN* for flower differentiation, and *CsPIF4* for photoperiod. Electrophoregram from PCR analysis was shown in Figure 3. Brightness in electrophoregram (Fig. 3) did not correlate to the concentration of gene expression. *In silico* studies showed that *CsACS2* have ATT_like domain (Aspartate aminotransferase). ATT_like function in ethylene synthesis is to transfer aspartate chain to form ACC (Marchler-Bauer et al. 2015). *CsETR* has HTPase_C and Histidine Kinase A domain. These domain shows that *CsETR* function as receptor that accepts ethylene and act as two-component system with CTR1 (Marchler-Bauer et al. 2015). *CsCaN* is homologous with Staphylococcal nuclease that works as nuclease for eukaryotes and *CsPIF4* has Helix-Loop-Helix domain that characterized it as transcription factor (Marchler-Bauer et al. 2015).

Figure 3B showed the result of Mercy that was given 8 hours and 16 hours light treatments expressed *CsACS2*, *CsCaN*, and *CsETR*. These gene expression profiles showed positive correlation with total number of female flowers produced in photoperiod treatments. However, *CsPIF4* was not expressed in all treatments, which might be caused by

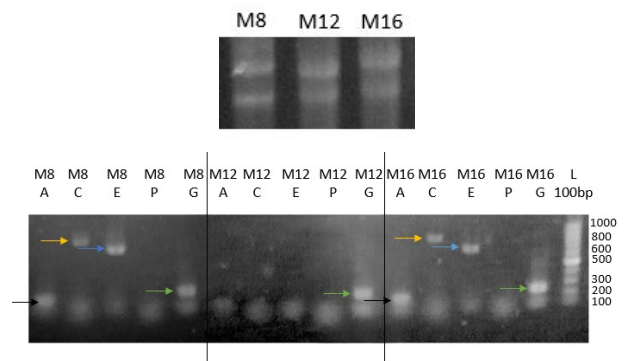


Fig. 3 (A) Electrophoregram for RNA extraction (Note : M = Mercy; 8,12,16 = Photoperiod). (B) Electrophoregram for gene expression analysis related to photoperiod, ethylene, and flower differentiation. (Note : M = Mercy; 8,12,16 = photoperiod; A = ACS; C = CaN; E = ETR; P = PIF4; G = GaDPH; L = Ladder; Black arrow = ACS gene; Orange arrow = CaN gene; Blue arrow = ETR gene; Green arrow: GaDPH gene)

inappropriate sampling time and organ, because gene is expressed at time-specific and organ-specific (Shedden and Cooper 2002). Alternatively, the primer did not match with the cDNA template, because the CsPIF4 primer was based on honeydew (*Cucumis melo*) (Abd-Elsalam 2003). Despite the *CsACS2* expression in short day plant treatment, the expression of *CsACS2* in 16 hours irradiation showed a difference to the result of Yamasaki's research (Yamasaki et al. 2003) and that explained by Miao et al. (2010) as the effect of increasing sugar produced by prolong photosynthesis that induced the ethylene, in this case *CsACS2* production.

Ethylene is commonly known as plant hormone that plays important role in fruit ripening, plant growth, maturation, and signalling for self-defense mechanism (Taiz and Zeiger 2010). However, limited study has described the signal transduction pathway of ethylene in the process of sexual differentiation of flowers. Nevertheless there were studies that explained that ethylene was received by ETR receptor in hermaphrodite flowers and the response was transduced to the activation of calcium-dependent nuclease in order to stop the development of primordial anther (Gu et al. 2011; Bai and Xu 2013). Therefore, alternative/additional signal transduction process that can complement the data basis on KEGG to relate phytochrome and ethylene and ethylene with flower sex determination is needed.

Based on results from this research, it was confirmed that *CsACS*, *CsETR*, dan *CsCaN* played a role in the female flowers differentiation. Therefore, the proposed model (Figure 2) in this research of flower differentiation could be combined with KEGG database to complement the information about the ethylene response in plants. *CsCaN* expression also confirmed Gu's research that *CsCaN* played a role in flowers organ development, where cucumber's flowers, which initially were hermaphrodite, differentiated into female flowers upon the exposure of ethylene by the function of *CsCaN* as specific nuclease to primordial anther, thus arrested stamen development. This proposed model could be applied to another *monoecious* and *andromonoecious* species that showed similar effect in the presence of ethylene, such as melon and watermelon, but may not be applied to *gynomonoecious* cultivar, even in the same cucumber species (Girek et al. 2013; Ikram et al. 2015; Manzano et al. 2016)

Best photoperiod to achieve optimum cucumber productivity depends on its cultivar, as it based on its sex differences in flowers production. In Mercy cultivar, female flower production affected the productivity more than male flowers. The condition to achieve the highest productivity was 8 hours treatment which produced 14.7 female flowers

and 54.7 male flowers. *In silico* study resulted on proposed model related to signal transduction model through the light-induced *CsACS2* to produce ethylene, which then followed by ethylene response through *CsETR1*, and finally affected *CsCaN* in the determination process of female flower differentiation.

This proposed model was confirmed by electrophoresis that showed bands of *CsACS2*, *CsETR1*, and *CsCaN* genes from plants with high number of female flowers following 8 and 16 hours treatments of photoperiod. However, expression of *CsPIF4* was not found in all treatments. This PCR result confirmed that *CsACS2*, *CsETR1*, and *CsCaN* genes played a role in ethylene production and affected female flower determination. These gene expressions may be affected by photoperiod, yet still not known which genes that related photoperiod and flowering differentiation.

Acknowledgement

The researchers would like to thank Lembaga Pengelola Dana Pendidikan (LPDP) for giving research grant and PT. East West Seed Indonesia for providing cucumber's seeds used in this research.

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