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Research article

Biosynthesis and accumulation of 20-hydroxyecdysone in individual male and female spinach plants during the reproductive stage

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ABSTRACT

The steroid 20-hydroxyecdysone (20E) is a major component of phytoecdysteroid in plants and may play a defensive role against insect pests in higher plants. In spinach, the biosynthesis and accumulation of 20E have been investigated during the vegetative stage; however, these processes have not been clearly studied during the reproductive stage, particularly in male and female individuals. In this study, we analyzed the level and distribution of 20E in individual male and female spinach plants during the reproductive stage via high performance liquid chromatography (HPLC). We found that 20E biosynthesis and accumulation were markedly different between male and female spinach during the late flowering stage. Compared with the male plant, biosynthesis of 20E in the leaves was more active and its accumulation in the floral parts was higher in female plants during the late flowering stage. These results indicate that the female reproductive organs at least in PE-positive plants could be effectively protected against harmful insects via active biosynthesis and accumulation of PE during the late flowering stage to protect floral parts from harmful insects for seed formation and store the available 20E in seeds for the next generation.

1. Introduction

Spinach (*Spinacia oleracea* L.) is a largely dioecious species with separate male and female individuals. Similar to a monoecious plant, both the male and female flowers are occasionally produced on the same plant in spinach plants, although this is not common. Morphologically, the floral organs of male and female spinach are different, although the plants are indistinguishable by the naked eye at the vegetative stage before flowering (Onyekwelu and Harper, 1979; Rosa, 1925).

Male and female individuals of a dioecious plant species have specialized biological characteristics at morphological, physiological, biochemical and ecological levels (Espírito-Santo et al., 2003). The primary differences between male and female plants are the sexual organs and seed and/or fruit production ability. Male plants contain only male sexual organs (anther and filament) in their flowers and cannot produce seeds and/or fruit, whereas female plants contain only female sexual organs (stigma, style and ovary) in their flowers and can produce seeds and/or fruit (Ainsworth et al., 1995; Barrett, 2002). Sex determination in dioecious plants is affected by genetics, plant hormones and environmental factors, which can cause biological differences between male and female plants (Mao et al., 2017). Moreover, differences in the biological characteristics between male and female plants are related to metabolomic differences associated with nutrients and secondary metabolites (Al-Obaidi et al., 2017).

20-hydroxyecdysone (20E) is the most common steroid hormone and acts as a trigger or initiator of the molting process in insects (Henrich et al., 1998). This steroid is also found as a secondary metabolite in various plants, including spinach (Dinan, 1995), and provides protection against herbivorous insects (Bergamasco and Horn, 1983; Lafont et al., 1991). The effects of 20E on insects include insect invasion inhibition, abnormal molting, immobility and death (Soriano et al., 2004). Currently, secondary metabolites have a critical economic impact and present interesting effects on plant breeding and agriculture. To improve pest resistance in plants, a better understanding of the complete 20E metabolism in both vegetative and reproductive stages of plant development is required.

The characteristics of 20E biosynthesis and accumulation in plants have been studied to determine the fluctuation and distribution of this phytoecdysteroid within individual plants. The fluctuation of 20E within plants at different growth stages has been demonstrated in *Taxus cuspidata*, *Rhaponticum carthamoides*, *Leuzea carthamoides*, *S. oleracea*, *Lamium album* and *Limnanthes alba* Hartw. ex Benth (Preston-Mafham and Dinan, 2002; Ripa et al., 1990; Robert and Adler, 1991; Savchenko

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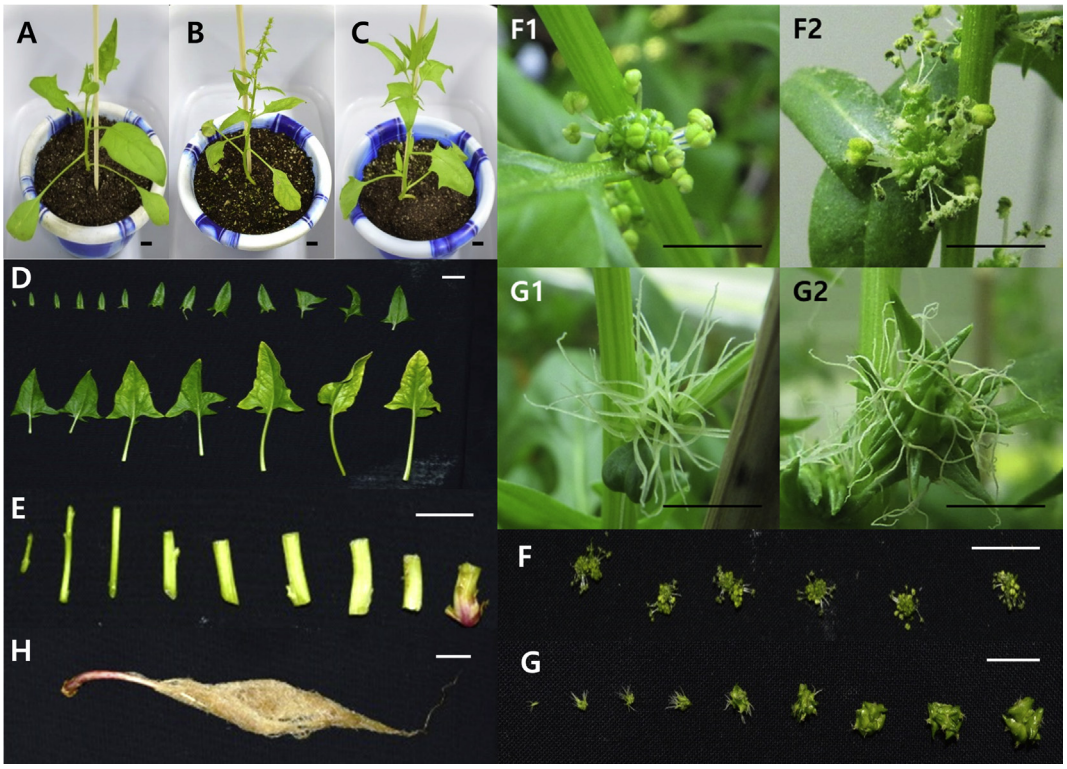

Fig. 1. Spinach plants at the late vegetative and reproductive stages. (A) Plant at the late vegetative stage, (B) female plant at the reproductive stage, (C) male plant at the reproductive stage, (D) leaves, (E) stems, (F) male flowers (male flower at 10 DAF (F1) and 20 DAF (F2)), (G) female flowers (female flower at 10 DAF (G1) and 20 DAF (G2)), and (H) roots. Bar 1 cm.

et al., 2001; Varga et al., 1986; Vereskovskii et al., 1983). The distribution of 20E within an individual plant has also been reported in certain species and varies according to the organ types and the position and development state of organs. Furthermore, the fluctuation and distribution of 20E within a plant involve biosynthesis and transport, and the highest concentration of 20E is accumulated in the most important tissues for survival and reproduction.

In spinach, several recent studies indicate that the concentration of 20E is different in the individual organs during the vegetative stage (Bakrim et al., 2008; Robert and Adler, 1991, 1993). At this stage, 20E is always highly concentrated in leaves, and the distribution varies in old leaves and young leaves that serve as a source and sink of 20E, respectively (Bakrim et al., 2008). Roots are also a 20E biosynthetic organ; however, the 20E concentration in roots is low (Robert and Adler, 1993). Moreover, 20E is transported to the root from the shoot when the root is damaged (Schmelz et al., 1999). However, the biosynthesis and accumulation of 20E in individual male and female spinach plants during the reproductive stage have not been reported yet.

In this study, we analyzed the 20E concentration (μg/g dry weight (DW)) and content (μg/plant or organ) in the plant or individual organs of both male and female spinach during the reproductive stage to provide a better understanding of 20E biosynthesis, accumulation and function in spinach.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade methanol, hexane, 2-propanol and water were purchased from Fisher Scientific (Pittsburgh, PA, USA) and used for 20E extraction and analysis. Extra pure trifluoroacetic acid (TFA) was purchased from Daejung Chemicals and Metals (Siheung, Korea) and used as mobile phase additive for the HPLC analysis. The 20E standard was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plant materials

The Korean spinach (*Spinacia oleracea* L.) cultivar Gyeowoonae was used in this study. The plants were grown from seeds in the greenhouse at Jeju National University. Organs of the plant, including the leaf, stem, root and flower, were collected in three periods: late vegetative, early flowering (10 days after flowering, DAF) and late flowering (20 DAF) (Fig. 1).

2.3. 20E extraction

The plant material was dried at 55 °C for 2 days. After grinding by mortar and pestle, samples (approximately 100 mg) were extracted in methanol (1 mL at 55 °C for 1 h, 3 times). The extracts were mixed with 0.75 mL of water and then partitioned with hexane (3 mL, 2 times). The methanolic extracts were dried at 55 °C overnight and dissolved with 200 μL of 11% 2-propanol.

2.4. Analysis of 20E

The concentration (μg/g DW) of 20E in each sample was analyzed via HPLC with an ultraviolet detector (HPLC-UVD) (Shimadzu CBM-20A, Tokyo, Japan), which consisted of a DGU-20A3R degassing unit, LC-20AD solvent delivery unit, SIL-20A autosampler, CTO-20A column oven, SPD-M20A diode array detector and CBM-20A system controller. The extracts (20 μL) were separated on a C18 Luna 5 μm column (250 × 4.6 mm ID, 5 μm) at 40 °C with a mobile phase of 11% 2-propanol containing 0.1% TFA. The flow rate was 1.0 mL/min, and the detection wavelength was 242 nm. The 20E content per single plant (μg/plant) or whole individual organs within single plant (μg/organ) was measured based on the 20E concentration (μg/g DW) and total dry weight of each sample.