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Research article

Ecdysteroids in spinach (*Spinacia oleracea* L.): Biosynthesis, transport and regulation of levels

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Abstract

Many plant species produce phytoecdysteroids (PEs: i.e. analogues of insect steroid hormones). There is increasing evidence that PEs are used as a chemical defence by plants against non-adapted insects and nematodes. PEs are good candidates for the development of an environmentally safe approach to crop protection. Most crop species do not accumulate PEs. However, many arguments support the idea that most, if not all, plant species have the genetic ability to produce PEs, but the biosynthetic pathway is not active. A better understanding of the PE biosynthetic pathway and its regulation is consequently necessary. Spinach is one of the very few crop plants which produce large amounts of PEs, of which 20-hydroxyecdysone is the major component. Labeling experiments with radiolabeled precursor (mevalonic acid), putative ecdysteroid intermediates and 20-hydroxyecdysone itself have allowed investigation of PE biosynthesis and transport during spinach development.

Biosynthesis takes place in older leaf sets (''sources''), but not in the young developing ones, which in contrast accumulate (acting as ''sinks'') the PEs produced by the older leaves. PEs are thus continuously redistributed within the developing plant, as its leaf set number increases. The biosynthetic pathway has been analyzed using excised leaves and various labeled precursors, and a preferential sequence of the last steps has been established. Although they do not produce PEs, apical leaf sets are nevertheless able to perform several putative terminal steps of PE biosynthesis.

The regulatory mechanisms of PE synthesis appear to involve a direct negative feedback of 20-hydroxyecdysone (the major PE in spinach) on its own synthesis; thus, a sustained synthesis in older leaves requires that they can export the PE they produce.

Keywords: 20-Hydroxyedysone; Development; Metabolism; Phytoecdysteroid; Regulation; Spinach; Transport

1. Introduction

Ecdysteroids are polar steroid hormones controlling arthropod development and reproduction [1]. They are also secondary metabolites produced by a wide range of vascular plant species and termed phytoecdysteroids (PEs) [2,3]. More than 300 different molecules have been described from plants [4], and their levels can reach 3% of plant dry weight [5]. A given plant species usually accumulates a complex cocktail of PEs, where 20-hydroxyecdysone (20E: the major insect ecdysteroid) is usually the major component [3,6].

Whether PEs function as plant hormones has been investigated using different (growth, flowering, etc.) bioassays

Abbreviations: E, ecdysone; 20E, 20-hydroxyecdysone; PolB, polypodine B; 2dE, 2-deoxyecdysone; 2d20E, 2-deoxy-20-hydroxyecdysone; 2,22dE, 2,22-dideoxyecdysone; 2,22d20E, 2,22-dideoxy-20-hydroxyecdysone; MVA, mevalonic acid; COT, cotyledon; L1, L1 leaf; L2, L2 leaf; EIA, enzymoimmunoassay; f.w., fresh weight; HPLC, high-performance liquid chromatography; MS, mass spectroscopy; NP-HPLC, normal-phase high-performance liquid chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; PE(s), phytoecdysteroid(s); UV, ultra-violet; V, volume.

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[7–12], but these experiments did not allow clear-cut conclusions. However, PEs share some structural resemblance with brassinosteroids, which in turn show some biological activity on insects as weak ecdysteroid antagonists [13]. Furthermore, the uneven distribution of PEs in vascular plant species, the huge concentrations found in some of them, and the metabolic stability of 20E do not support a phytohormonal function for 20E [2,3,14]. On the other hand, there is increasing evidence supporting a defensive function of PEs towards (non-adapted) phytophagous insects and soil nematodes. Indeed, feeding experiments have demonstrated the endocrine-disrupting activity of dietary ecdysteroids on phytophagous insects or nematodes [15,16]. Alternatively, they may also deter insects, which avoid feeding on ecdysteroid-containing diets [17]. Moreover, the concentrations of PE in plants are increased by mechanical damage, insect herbivory or methyl jasmonate treatment [18–20]. Additional support for the defensive function of PEs comes from their spatial distribution: according to the optimal defence theory, higher PE levels are usually found in tissues with higher value for plant fitness, i.e. actively growing or reproductive tissues in annual plants, or roots in perennial species [3,21]. For example, gradients of PE concentrations towards the apex of aerial parts and root tips have been observed in spinach [22–24] and the related species *Chenopodium album* [24,25]. The cyclical accumulation of high levels of PEs has also been described in the roots of perennial species, e.g. *Leuzea carthamoides* [26] and *Ajuga reptans* [27].

Public opinion is presently highly sensitive to excessive chemical pesticide treatments. Alternative approaches to crop protection using natural resources have to be found. As PEs are not toxic to humans [28], they are good candidates for the development of new and safer strategies for crop protection, provided that they would be produced by the plant themselves, as topical applications appear unrealistic for many reasons. Most crop species do not contain detectable levels of PEs, but the wide and uneven distribution of PE-containing species in the plant kingdom [2,3], the presence of significant PE levels in some specimens of *Arabidopsis thaliana*, a non-accumulating species [29], and the ability of another non-accumulating plant species, maize, to convert radioactive precursors into 20E [30] support the idea that most, if not all, plant species have the genetic potential to produce PEs, but that the biosynthetic pathway is only weakly active (or inactive) in non-accumulating species [3]. A crop protection approach respectful of the environment and human health could therefore be based on the activation of genes involved in PE biosynthesis and accumulation.

However, our knowledge of the PE biosynthetic pathway and of its regulatory mechanisms is still fragmentary [3]. Previous metabolic studies showed that spinach leaves synthesize PEs from mevalonic acid (MVA): PE synthesis proceeds through lathosterol and is developmentally regulated [31]. The occurrence of ecdysteroid (poly)phosphate conjugates as intermediates in the biosynthetic pathway and their involvement in its down-regulation has been suggested [30,32], and it was proposed that the stability of these polyphosphates would prevent further PE synthesis in non-accumulating species.

Spinach is one of the few cultivated PE-accumulating species, together with other Chenopodiaceae (beets and quinoa) [24], and therefore represents an interesting model system for a detailed analysis of PE biosynthesis and transport, and their level regulation. The present study has been performed in order to provide a better understanding of some of these topics.

2. Materials and methods

2.1. Plants

Spinach (*Spinacia oleracea* L., var. ''Géant d'hiver''), purchased from Truffaut (France), was grown in the laboratory at 20 ± 1 °C and under a short (8L:16D) photoperiod and at 50% relative humidity. Seeds were sown on a damp paper and germinated seedlings were transplanted individually into compost at cotyledons (COT) stage and grown until the appropriate developmental stage. COT, I, II, III, IV, V and VI indicate developmental stages of a plant, corresponding to the presence of only cotyledons, first (L1), second (L2), third (L3), fourth (L4), fifth (L5) and sixth (L6) leaves, respectively (Fig. 1). Leaves are numbered from the basal set to the youngest apical ones.

2.2. Chemicals

Reference ecdysteroids were purified in the laboratory from various plant or insect materials.

Radiochemicals: [2-^{14}C]MVA DBED (dibenzylethylenediamine) salt (1.92 GBq mmol^{-1} specific activity) and [23,23,24,24-$^{3}H_4$]ecdysone (E, 3.3 TBq mmol^{-1} specific activity) were purchased from Perkin Elmer Life Sciences, Inc; [1α,2α-$^{3}H_2$]2-deoxy-20-hydroxyecdysone (2d20E, 1.7 TBq mmol^{-1} initial specific activity) was custom-made by Moravek Biochemicals (CA, USA). [1α,2α-$^{3}H_2$]20E (1.5 TBq mmol^{-1} specific activity) was prepared by Dr. C. Dauphin-Villemant and Dr. C. Blais from [1a,2 a-$^{3}H_2$]2d20E using a recombinant 2-hydroxylase from *Drosophila melanogaster*. [23,23,24,24-$^{3}H_4$]2-deoxyecdysone (2dE, 6.6 TBq mmol^{-1} initial specific activity [33]) and [22,23-$^{3}H_2$]2,22-dideoxyecdysone (2,22dE, 2.2 TBq mmol^{-1} initial specific activity [34]) were kindly provided by Dr. Hétru (Strasbourg). All radioactive molecules were purified by normal-phase HPLC (NP-HPLC) prior to use.

2.3. Leaf and plant radiolabeling

Substrates in a methanol/water (1/9, v/v) mixture were applied topically (10 μL total volume) for labeling in vivo or exposed to the cut petiole of excised leaves (50 μL total volume). In the latter, substrates were taken up by leaves by photo-transpiration, which lasted generally 1 h or less. Excised leaves were then incubated in water at 20 °C.