J Plant Growth Regul (2008) 27:93–98
DOI 10.1007/s00344-007-9028-1

SHORT COMMUNICATION

Endogenous Hormonal Profiles in Hop Development

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Received: 1 March 2007 / Accepted: 12 July 2007 / Published online: 16 October 2007

Abstract Changes in gibberellins (GAs), indole-3-acetic acid (IAA), and cytokinins associated with the transition from vegetative growth to reproductive growth in *Humulus lupulus* L. buds and leaves harvested at fortnight intervals were studied. During vegetative growth, GA_1 increased gradually and the lowest content was observed during flower development. Both GA_3 and GA_4 showed a dramatic increase in the samples taken from the apical part of axillary branches from plants 4–5 m high, which corresponds to the maximum vegetative development prior to macroscopically visible inflorescences. Notable increases in the cytokinins *trans*-zeatin (*t*-Z), isopentenyladenine (iP), and the riboside and ribotide forms of iP were also obtained. The auxin, indole-3-acetic acid, was the most abundant plant hormone, and its content was highest during vegetative growth. These results show for the first time a relationship between endogenous hormone profiles and both vegetative and reproductive development in hop plants, which may be relevant for future research on the control of the flowering by exogenous hormone applications.

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Keywords *Humulus lupulus* · Gibberellins · Cytokinins · Indole-3-acetic acid · Flowering · Mass spectrometry

Introduction

European hop (*Humulus lupulus* L.) is a dioecious, climbing, herbaceous plant that produces new shoots in early spring and senesces to the perennial rootstock in autumn. The cultivated hop is a short-day plant that initiates flowering when the plant is at a critical size (about 6 m, 20–24 nodes) and the "critical" daylength is about 16 h, beyond which flowering cannot be induced. The plant also has a "minimum daylength" (8–10 h), below which the plant ceases vegetative growth and forms dormant terminal buds. The value of female plants lies in the lupulin glands that contain resins and essential oils required for the flavor and aroma of beer.

Plant growth regulators are chemical messengers connecting environmental changes and plant responses. The involvement of gibberellins in processes such as internode elongation, flower induction, and modulation of sexual expression is widely known (Durand and Durand 1984; Zeevart and others 1993; King and others 2003). Indole-3-acetic acid (IAA), a natural auxin, is a signaling substance involved in almost every plant developmental process. In particular, IAA is involved in the vegetative growth of hops (Galovic and others 2001), but its role in the reproductive development in this species has not been studied. Finally, cytokinins are also involved in many physiologic processes via cell proliferation, including shoot formation, delay in leaf senescence, chlorophyll production, phase change, and sexual modulation (Durand and Durand 1984; Corbesier and others 2003).

Such an extensive analysis of changes in endogenous plant growth regulators in hop has not been reported earlier. Thus, the main objective of this study was to characterize hormonal changes associated with both vegetative and reproductive development in hop.

Material and Methods

Plant Material

Samples were collected from female plants of *Humulus lupulus* cv. 'Nugget' growing in the fields of S. A. E. de Fomento del Lúpulo (Villanueva de Carrizo, León, Spain) approximately every 15 days during plant development from initial shoot sprout time on April 29 (V1), during vegetative development on May 18 (V2) and June 7 (V3), and after development of the flowers on June 21 (F1) and July 6 (F2). Samples V1 and V2 consisted of the apical bud and the first pair of fully expanded leaves of the main shoot. V3 consisted of the apical part of axillary branches. F1 and F2 were two initial stages of inflorescence development. Upon harvest, plant material was immediately submerged in liquid nitrogen, crushed, lyophilized, and stored at –20°C until analyzed. Every sample was analyzed in duplicate.

Identification of Gibberellins

Four hundred milligrams of sample from nodes belonging to the third collection were used for the identification of gibberellins; they were subjected to the same purification protocol described later. Five fragmentation ions plus the molecular ion of the Me-TMS derivative of GA_1, GA_3, GA_4, GA_7, GA_9, and GA_{20} were monitored. The gibberellins chromatography column was first calibrated with an injection of C_{22} and C_{23} alkanes, and gibberellins' chromatography-mass spectrometry (GC-MS) conditions are described in the next paragraph.

Analyses of Gibberellins and Indole-3-Acetic Acid

Samples (100 mg DW) were homogenized in 15 ml of 80% methanol and extracted overnight in darkness at 4°C; prior to extraction, 50 ng of $[^2H_2]GA_1$, $[^2H_2]GA_3$, $[^2H_2]GA_4$, $[^2H_2]GA_9$ (L. N. Mander, Canberra, Australia), and $[^2H_5]$IAA (OlChemIm®, Olomouc, Czech Republic) was added. Each extract was centrifuged (4000 rpm, 10 min) and filtered (Whatman® GF/A 3 glass microfiber). Samples were re-extracted in 5 ml of 80% methanol for 1 h, and methanol from the pooled extract was removed under reduced pressure at 40°C. After acidifying the sample (pH 2.5) with 1 N HCl, the aqueous extract was extracted thrice using ethyl acetate (1:2); the pooled organic phase was evaporated until dry and redissolved in 10 ml of 80% methanol. The extract was purified through a C18 Sep-Pack cartridge (Waters, Milford, MA) and adsorbed onto 0.5 g of celite 545 (Prolabo®, Paris, France), dried under a stream of hot air, purified throughout a column of silicic acid (ICN Silica 32–100, active 60 Å, ICN Biochemicals GmbH) (5 g of SiO_2 in 50 ml of ethyl acetate:*n*-hexane 95:5 saturated with 0.5 M aqueous formic acid), and brought to a dry state. Samples were dissolved in 30 µl of methanol and 270 µl of 1% (v/v) aqueous acetic acid, passed through a 0.45-µm PVDF filter (Millex r-HV, Millipore®), and then fractionated by reverse-phase HPLC (Kromasil 100 C18 250 × 4.6 mm, 5 µm, Scharlab Science). The HPLC system used was a Waters 600 chromatograph coupled to a Waters 717 plus autosampler and connected to a Waters 996 PDA. Prior to injection, 2 µg of kinetin was added to every sample as internal standard to corroborate chromatographic retention times. The mobile phase was a linear gradient from 10% to 73% of methanol in aqueous solution with 1% (v/v) acetic acid for 45 min at 1.5-ml min^{-1} flow rate. Retention times of gibberellins and IAA at the HPLC separation were determined by injecting templates containing 10,000 dpm of $[^3H_2]GA_1$ and $[^3H_2]GA_4$ and 1 µg of IAA. Forty-five 1.5-ml fractions were collected and the radioactivity was measured in each fraction with a Packard 2500 TR scintillation counter using an aliquot of 100 µl of each fraction mixed with 500 µl of scintillation cocktail (Ultima Gold XR; PerkinElmer, Waltham, MA). Thus, fractions containing gibberellins and IAA from samples were collected, pooled, and evaporated until dry, methylated using 200 µl of ethereal diazomethane, and trimethylsilylated using 50 µl of N,O-bis(trimethylsilyl)trifluoroacetamide with 1% (v:v) trimethylchlorosilane (Sigma Aldrich, St. Louis, MO) for 30 min at 65°C. Fractions were dried under a steam of N_2 and dissolved in 5 µl of chloroform. Samples (1 µl) were injected into a Hewlett Packard 6890 GC equipped with a HP-5MS capillary column (60 m × 250 µm i.d. × 0.25 µm nominal) and coupled to a Hewlett Packard 5973 mass detector. For gibberellin fractions, injector and interface temperatures were 270°C and 280°C, respectively. The column temperature was maintained at 60°C for 1 min, then increased by 20°C min^{-1} to 200°C, followed by 4°C min^{-1} to 250°C. An aliquot of IAA was injected using injector and interface temperatures of 250°C and 280°C, respectively. The column temperature was raised from 60°C to 165°C at 20°C min^{-1} then to 220°C at 5°C min^{-1}, and finally to 270°C at 10°C min^{-1}. Levels of endogenous phytohormones were determined by measuring the relative abundance of the following ion pairs: m/z = 506/508 and 493/491 for GA_1, m/z = 504/506 and 491/489 for GA_3, m/z = 418/420 and

284/286 for GA_4, m/z = 298/300 and 270/272 for GA_9, and m/z = 261/266 and 202/207 for IAA.

Analyses of Cytokinins

Twenty milligrams of sample (DW) were extracted overnight in 1.8 ml of Bieleski solvent (chloroform/methanol/formic acid/water, 25:60:5:10, by volume) (Bieleski 1964) at –20°C. Ten picomoles of deuterated cytokinins ([$^2H_5$]DHZ, [$^2H_5$][9R]DHZ, [$^2H_5$](9G)DHZ, [$^2H_6$]iP, [$^2H_6$][9R]iP, [$^2H_6$](9G)iP, [$^2H_5$][9R-MP]Z, and [$^2H_6$][9R-MP]iP; OlChemIm®, Olomouc, Czech Republic) was added. Samples were centrifuged (20,000*g*, 20 min, 4°C), re-extracted for 1 h in 1 ml of 80% methanol, and recentrifuged. Both supernatant fractions were pooled and passed through a C18 SPE (500 mg of solid phase) to remove pigments. Organic solvents were removed under reduced pressure at 40°C, the pH of the water phase was adjusted to 7, and distilled water was added until a final volume of 20 ml was obtained. The sample was applied onto a DEAE-Sephadex A-25 (Amersham Pharmacia, Uppsala, Sweden) anion exchange cartridge (2 ml, NH_4HCO_3 form) with a C18 SPE cartridge (BondElut® Varian, Hamburg, Germany) coupled underneath. The cartridges were rinsed with an additional 20 ml of distilled water. The DEAE cartridge was eluted with 10 ml of 2 M NH_4HCO_3, recovering the ribotide fraction, whereas the C18 cartridge was eluted with 10 ml of 80% methanol, recovering the free bases and ribosides. Cytokinin ribotides were hydrolyzed by alkaline phosphatase (pH 8, 1 h at 37°C; Roche Diagnostics, Mannheim, Germany) to obtain the corresponding ribosides. Both fractions were further purified separately using an immunoaffinity column for isoprenoid cytokinins (OlChemIm®, Olomouc, Czech Republic). All cytokinins were dissolved in 20% methanol and analyzed by micro liquid chromatography coupled to electrospray tandem mass spectrometry [LC-(ES+)-MS/MS]. The LC system used was a Kontron 325 pump; samples (30 µl) were injected using a Kontron 465 injector. The LC system was coupled to a Quattro II triple-quadrupole mass spectrometer (VG Micromass, Manchester, UK) equipped with an electrospray interface [(+)ES LC-MS/MS] under multiple reaction monitoring mode and a Z-spray source (source temperature = 80°C, capillary voltage = +3.5 kV, cone voltage = 20 V). The column was a Phenomenex Synergy Max-RP 80 Å (10 cm × 1 mm, 4 µm). Cytokinins were eluted using a gradient from 20% MeOH in 0.01 M $AcNH_4O$ to 90% MeOH for 2 min, held in those conditions for 3.5 min, and returned to initial conditions in isocratic mode for 5 min (flow rate = 60 µl/min). Quantification was done by multiple reaction monitoring of $[M+H]^+$ ion (dwell time = 0.1 s) and the appropriate product ion. All data were processed by Masslynx software (VG Micromass, Manchester, UK).

Statistical Analyses

Statistical analyses of data were performed using STATISTICA® software (StatSoft Inc., Tulsa, OK). The Kruskal–Wallis nonparametric test was used to test differences for each phytohormone in apical buds and leaves along the studied development. To detect differences among collections, group comparison was made for each plant growth regulator using the Mann-Whitney *U* test. It is worth mentioning that in this time-series analysis, statistical differences make sense only between contiguous collections in time for each analyzed hormone.

Results and Discussion

Among the tested gibberellins GA_1, GA_3, GA_4, and GA_9, only GA_1 and GA_4 were fully identified. Similar gibberellin profiles were found for leaves and buds at different stages of vegetative and reproductive development of the hop plant, but higher contents of GA_3 and GA_4 were found in buds (Figure 1). A dramatic increase of GA_3 and GA_4 was obtained in samples taken from the apical part of axillary branches of plants 4–5 m high (V3). The highest levels of GA_9 corresponded to V1 samples when the plant was less than 50 cm high.

The endogenous content of the isoprenoid cytokinins is shown in Figures 2 and 3. In general, there was a higher cytokinin content in buds than in leaves, as occurred with gibberellins. Conjugated forms (ribosides or ribotides) were found for iP and *t*-Z in both leaves and buds. However, free forms of cytokinins were found only in buds. During vegetative development, *t*-Z increased gradually, reaching the maximum content in the transition phase (V3) and very low content in the flower developmental stage (F1 and F2). Free and conjugated forms of iP also showed a maximum content in the V3 stage and low content in the other stages. Conjugated forms of *t*-Z showed the highest content in samples collected in April when the plant begins to sprout (V1), or during the second vegetative development stage (V2), showing low content in the V3 stage, and during the floral development (F1 and F2).

The endogenous IAA content is shown in Figure 4. Indole-3-acetic acid content is remarkably higher than gibberellin and cytokinin content, and is also higher in buds than in leaves. They present a profile similar to GA_1 in buds; in leaves, IAA content decreases during development.

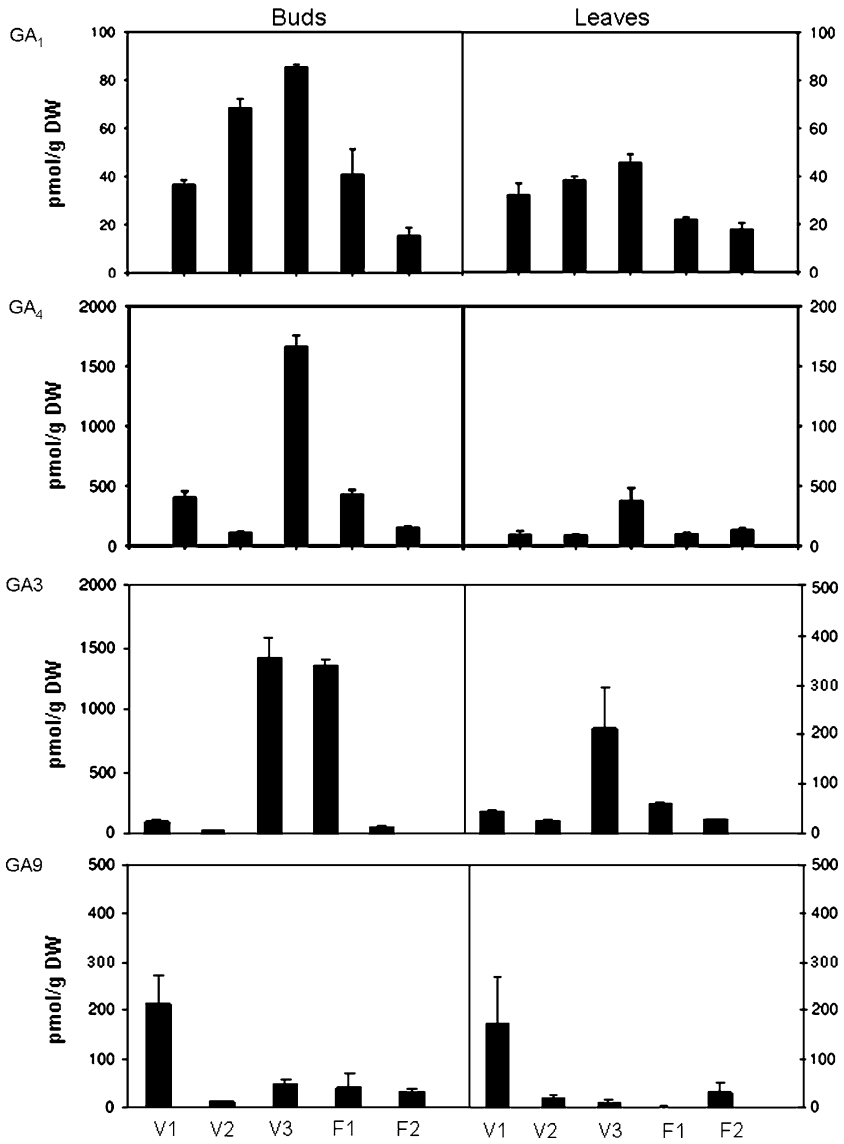

Fig. 1 Endogenous gibberellin content in buds and leaves of *Humulus lupulus* L. (cv. 'Nugget') during vegetative growth (V1, V2, and V3) and floral development (F1 and F2). Bars represent means with standard error. Characters over bars group nonsignificant changes in GA content during the studied development

In potato, Suttle (2004) found an increase in some gibberellins during initial shoot growth after the arrest from dormancy. Gibberellin A19, along with iPR, Z, and ZR, has been previously found in young shoots of hop, but iPR and Z were found only in the cone (Watanabe and others 1978a). Dramatic increases in GA_3 (data not shown) and GA_4 content together with the highest content of *t*-Z and iP-free forms and some conjugated forms of cytokinins obtained in V3 samples could be considered indicators of a phase change in hop that would correspond to a maturation stage prior to flowering. Several papers connect the levels of gibberellins and cytokinins with the floral transition. Thus, Evans and Poethig (1995) established the influence of GA_1, GA_3, and GA_5 in floral development by means of analyses done in maize mutants. Indeed, drastic changes in cytokinin levels have been observed and connected to floral development in *A. thaliana* (Corbesier and others 2003; Watanabe and others 1978b, c). Contrary to the results in buds, Galovic and others (2001) presented data indicating that free IAA was not detected in apical buds of several hop cultivars undergoing this same active vegetative growth. On the other hand, Ding and others (1999) observed reduced levels of IAA in *Polianthes tuberosa* corms during early floral development, which increased during subsequent stages. The decreasing IAA content found in leaves during hop development agrees with the results of Kowalczyk and Sandberg (2001) who reported that IAA concentrations were high during leaf development in *Arabidopsis thaliana* and declined when they reached its final size.

In conclusion, analyses of endogenous plant growth regulators in hop reveal changes in their levels during both vegetative and reproductive development. The results from this study suggest that both GA_3, an active form of the early 13-hydroxylation pathway, and GA_4, an active gibberellin from the early non-13-hydroxylation pathway, seem to be the main gibberellins involved during onset of

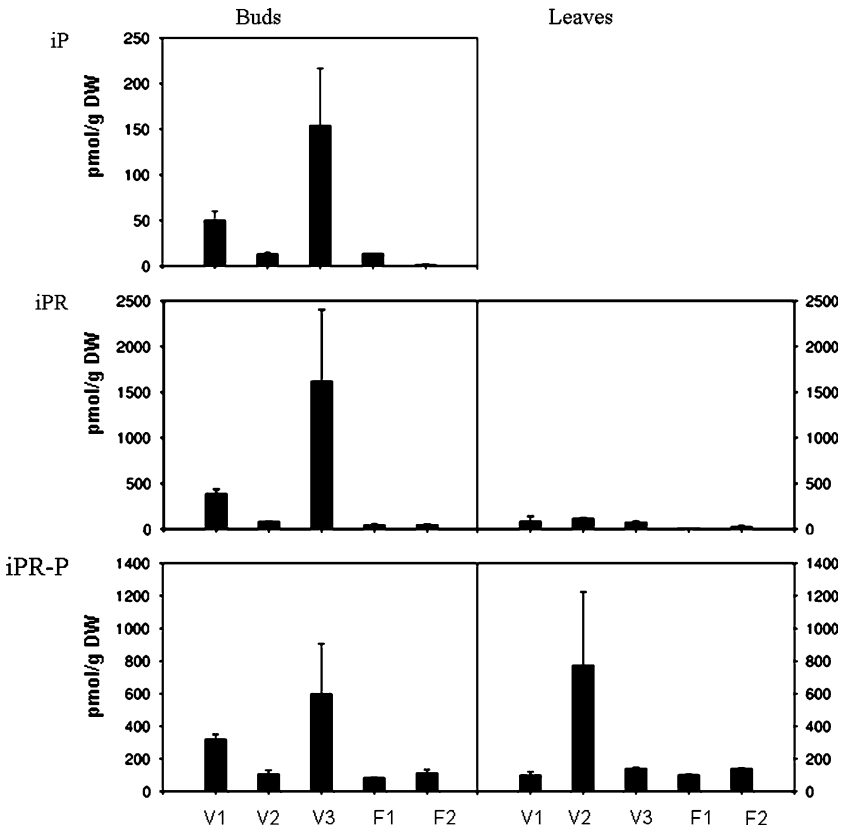

Fig. 2 Endogenous iP-type cytokinin content in buds and leaves of *Humulus lupulus* L. (cv. 'Nugget') during vegetative growth (V1, V2, and V3) and floral development (F1 and F2). Bars represent means with standard error. Characters over bars group nonsignificant changes in CK content during the studied development

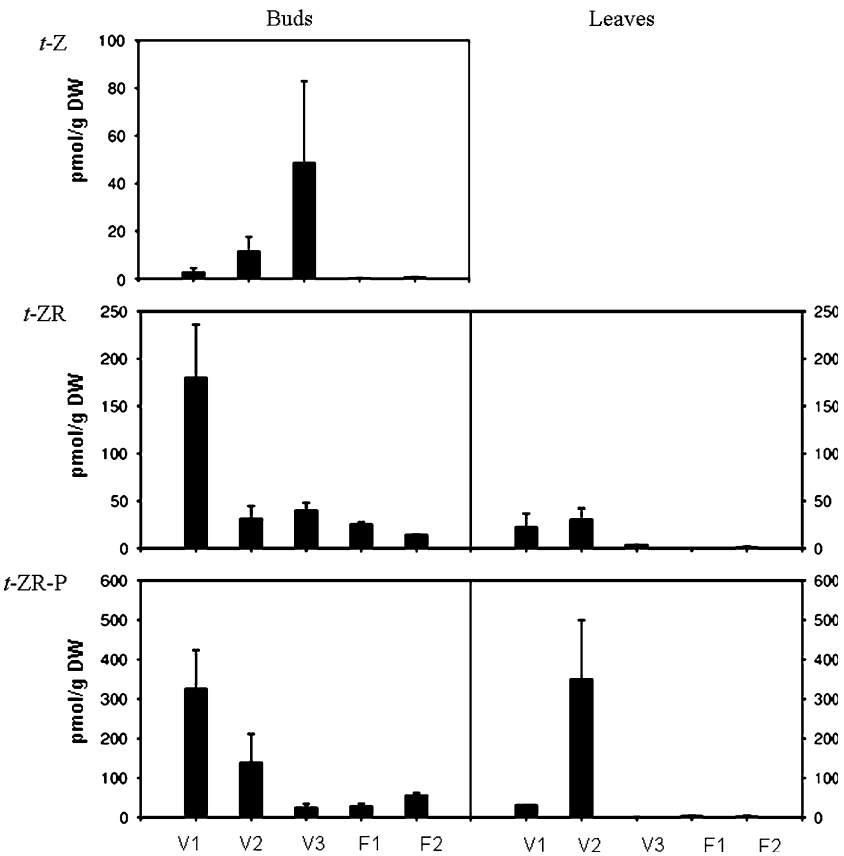

Fig. 3 Endogenous Z-type cytokinin content in buds and leaves of *Humulus lupulus* L. (cv. 'Nugget') during vegetative growth (V1, V2, and V3) and floral development (F1 and F2). Bars represent means with standard error. Characters over bars group nonsignificant changes in CK content during the studied development

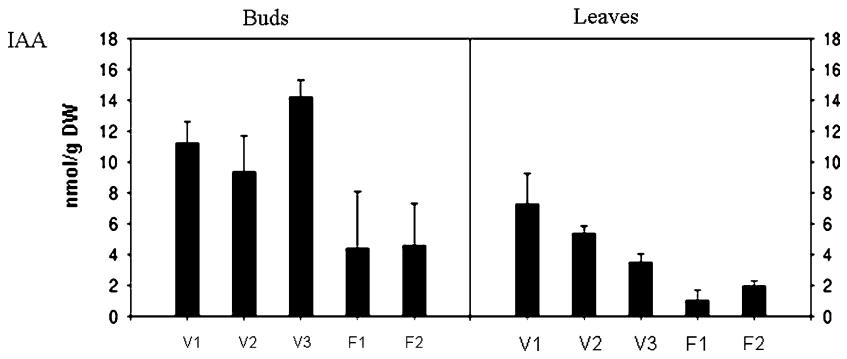

Fig. 4 IAA endogenous content in buds and leaves of *Humulus lupulus* L. (cv. 'Nugget') during vegetative growth (V1, V2, and V3) and floral development (F1 and F2). Bars represent means with standard error. Characters over bars group nonsignificant changes in GA content during the studied development

flowering but not during the visible flower development. Also, the high levels of iPR suggests a possible role of this cytokinin in promoting flowering similar to IAA, but evidence now points to *t*-Z and perhaps iP being more important because they are the compounds for which AHK receptors have highest affinity.

Acknowledgments The authors thank the Spanish Ministry of Science and Technology and FEDER (project MCT-02-AGL-02472) for the financial support and S.A.E. Fomento del Lúpulo (León, Spain) for the technical assistance.

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