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Histomorphological changes in shoot apices of *Lactuca sativa* treated with gibberellic acid

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Abstract

Lettuce plants were treated with gibberellic acid (GA_3) and uniconazole (UZ; a gibberellin synthesis inhibitor) to investigate the influence of GA_3 on cell division frequency in the shoot apical meristem (SAM) during stem elongation and flower initiation in lettuce (*Lactuca sativa*) grown in a greenhouse. GA_3 (0.1 mM) was sprayed on the surface of outer leaves and uniconazole solution (0.86 mM) was applied to the soil. GA_3 increased cell division frequency in the peripheral zone and the rib meristem of shoot apices, and this was associated with the stimulation of stem elongation. UZ treatment decreased cell division frequency in the peripheral zone, rib meristem and subapical pith, and this was associated with restricted stem elongation. Treatment with UZ and GA_3 together induced minor stem elongation. Flower induction occurred 3 d earlier in the GA_3 and UZ+GA_3 treatments than in the control, while the UZ treatment delayed flower initiation for more than 9 d relative to the control.

Additional key words: bolting, floral initiation, histone *H4* gene, *in situ* hybridization, lettuce, stem elongation.

Introduction

Gibberellins (GA) are endogenous plant hormones that promote cell division and/or cell elongation. Rapid stem elongation is their universal and direct effect on plant growth. In lettuce (*Lactuca sativa*) gibberellic acid (GA_3) induced hypocotyl elongation through increased cell elongation (Stuart *et al.* 1977). In Japanese radish GA_3 content increased under long day, which led to stem elongation, while the application of uniconazole (UZ; a GA synthesis inhibitor) retarded stem elongation (Nishijima 2000). Hypocotyl elongation was depressed and plant height reduced in GA-deficient mutants of *Arabidopsis thaliana* (Peng and Harberd 1997). Tubulins are components of microfibrils, which are involved in cell division, cell elongation and cell shape. GA enhanced the transcription of tubulin genes as well as internode elongation in oat (Mendu and Silflow 1993). Recently, Yang *et al.* (2009) reported enhanced β-tubulin transcription by GA and in contrast low transcription of β-tubulin in GA-deficient mutant.

The role of GA in flowering is variable and species-specific (Mutasa-Göttgens and Hedden 2009). In the long day grass species *Lolium temulentum*, GA acts as a direct signal for flower induction (King *et al.* 2006). Some GA-deficient mutants of *Brassica rapa* and *Thlaspi arvense* take longer to flower than wild type plants, suggesting that GA accelerated flower initiation (Metzger and Hassebrock 1990, Zanewich *et al.* 1990). However, in *A. thaliana* GAs exert minor influences on flowering time under long day conditions but play a major role in flower induction under short day conditions (Mutasa-Göttgens and Hedden 2009). An *A. thaliana* mutant deficient in GA synthesis failed to flower under short day unless supplied with exogenous GA (Wilson *et al.* 1992). In *Arabidopsis* GAs induce flowering through activation of flower integrator genes encoding *SUPPRESSOR OF OVER EXPRESSION OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*) and *FLOWERING LOCUS T* (*FT*) in meristems and/or leaves. Under short day conditions *FT* expression is low, and GAs possibly play a principal role in flower induction through stimulation of *FT* expression (Hisamatsu and King 2008). However, GAs and FT act independently on flower induction in *Lolium temulentum*, because GA

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Abbreviations: BCIP - 5-bromo-4-chloro-3-indolyl phosphate; CZ - central zone; DAS - days after sowing; *FT* - *FLOWERING LOCUS T*; GA - gibberellin; GA_3 - gibberellic acid; *LFY* - *LEAFY*; NBT - nitroblue tetrazolium; PZ - peripheral zone; RM - rib meristem; SAM - shoot apical meristem; *SOC1* - *SUPPRESSOR OF OVER EXPRESSION OF CONSTANS 1*; UZ - uniconazole.

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treatment does not increase *FT* gene expression (King *et al.* 2006).

In rosette plants the phase transition from the vegetative to the reproductive stage coincides with the start of bolting (rapid elongation of flower stalks), which is involved in GA-dependent cell division and/or cell elongation (Sachs and Lang 1957, Silverstone and Sun 2000, Mutasa-Göttgens and Hedden 2009). Histones, which participate in chromatin formation in the nucleus, include five distinct subtypes (H1, H2A, H2B, H3 and H4), amongst which expression of subtype H4 in plants is dependent on DNA replication. The *in situ* pattern of histone *H4* gene expression is an excellent marker for floral initiation. An increase in gene expression in the central zone (CZ), the peripheral zone (PZ) and the rib meristem (RM) has been reported in flowering-induced plants including lettuce, tomato, soybean and snapdragon (Bandstädter *et al.* 1994, Kouchi *et al.* 1995, Fobert *et al.* 1996, Lee *et al.* 2005).

For seed production in lettuce GAs are often used to induce early seed stalk formation, prior to head formation. However, it is not clear how GAs affect cell division and morphological changes in the shoot apical meristem (SAM) during stem elongation and flower initiation. In this study, we investigated the influence of GA_3 on cell division frequency in the SAM of lettuce during stem elongation and flower initiation, using *in situ* hybridization of the histone *H4* gene.

Materials and methods

Lactuca sativa L. cv. Queen Crown (*Sakata Seed Co*., Yokohama, Japan) was grown in 2 000 cm^3 pots in a temperature-controlled greenhouse from 3 June to 25 July 2003 (experiment 1), 22 April to 26 June 2004 (experiment 2), and 16 May to 4 July 2005 (experiment 3). Air temperature was maintained at 25 °C during the day and 20 °C at night. In experiment 1 GA_3 (0.1 mM; *Wako*, Osaka, Japan) was sprayed onto the surface of outer leaves of 31-d-old plants (20 cm^3 per plant). We had previously demonstrated that this concentration of GA_3 induces seed stalk elongation prior to heading (data not shown). In experiment 2, 1 cm^3 of the GA synthesis inhibitor, *Uniconazole-P* (UZ; *Sumitomo Chemical*, Tokyo, Japan), was applied to the soil of 38-d-old plants. We had previously demonstrated that 0.86 mM UZ significantly reduces stem elongation and delays flower initiation (data not shown). In experiment 3 we investigated the effect of exogenous GA_3 on stem elongation and flower initiation at an extremely low level of endogenous GA_3 by applying UZ in combination with GA_3 (UZ+GA_3; 0.25 and 0.1 mM, respectively) or UZ alone (0.86 mM) to 34-d-old plants. In all experiments, control plants were treated identically with water. Five plants were harvested at 3-d intervals from 31 d after sowing (DAS) in experiment 1 and 35 DAS in experiment 3, while in experiment 2 three plants were harvested at 6-d intervals from 34 DAS. Stem length was measured from the cotyledons to the uppermost nodes at which leaves larger than 1 cm were attached. The stem diameter measurement was made at the thickest part of the stem. The number of leaves larger than 1 cm in length was counted.

The DNA replication frequency in SAMs was evaluated by *in situ* hybridization using a lettuce histone *H4* antisense probe. In each experiment, five plants were harvested at 3-d intervals, and for each the apical region of the vegetative shoot was excised and fixed overnight in FAA (formalin + acetic acid + 50 % ethanol; 5:5:90). The fixed tissues were dehydrated through a tertiary butyl alcohol series and embedded in paraffin wax (Sass 1958). Sections (10 µm thick) were cut using a rotary microtome, mounted on silane-coated slides and used for *in situ* hybridization (Chono *et al.* 2001). Briefly, the sections were dewaxed, rehydrated, pretreated with proteinase K and probe-hybridized. The hybridized sections were washed and treated with RNase. The signal was detected immunohistochemically using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) as coloring substances. The slides were dehydrated, mounted in *Eukitt* (*Kindler*, Freiburg, Germany) mounting medium and photographed using an optical microscope (model *BX51*, *Olympus*, Tokyo, Japan).

Results

Effect of GA_3 application on stem elongation and flower initiation: Stems started to elongate 3 d after GA_3 treatment and continued to elongate throughout the experiment (Fig. 1*A*). After 21 d, stem length was 348 ± 32 mm in GA_3-treated plants but only 72 ± 6 mm in controls. The stem diameter was 17 ± 1 mm for control plants and 9 ± 1 mm for GA_3-treated plants. The number of leaves longer than 1 cm was 26 for the control and 28 for the GA_3-treated plants.

The cell division frequency at the PZ and the RM of the SAM began to increase 3 d after the GA_3 treatment (Fig. 2*A*,*B*). In control plants, DNA replication in the CZ became detectable 49 DAS, although the apical meristem was still flat at this time (Fig. 2*C*). The shoot apices became dome-shaped, and cells expressing the histone *H4* gene were more common and uniformly distributed at

52 DAS (Fig. 2*D*). In the GA$_3$ treatment, the CZ was clearly distinguishable as a group of cells, and no *H4* gene expression was detected until 43 DAS, but it was no longer recognizable at 46 DAS, 3 d earlier than in the control, because the *H4* gene was expressed at the CZ as well as the PZ and RM (Fig. 2*E*). The apical meristems of GA$_3$-treated plants were enlarged and dome-like at 52 DAS similar to control apices. In the GA$_3$ treatment the morphology of the SAM and leaf primordia was more sharp, narrow and elongated than in control plants (Fig. 2*A,B,C,E*).

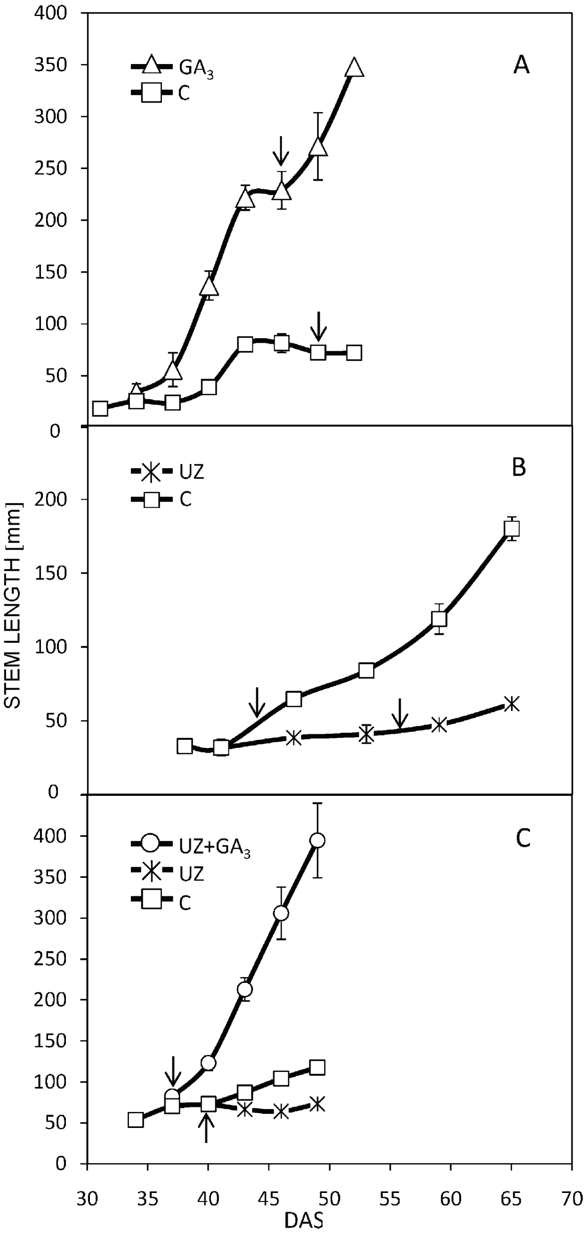

Fig. 1. Changes in lettuce stem length with GA$_3$ treatment (*A*), UZ treatment (*B*), and UZ + GA$_3$ or UZ treatments (*C*). Treatments were applied at 31, 38 and 34 DAS, respectively. Flower initiation assessed on the basis of histone *H4* gene expression in the central zone of the apical meristem were indicated by *arrows*. Means ± SE, *n* = 5; C - control.

Effect of UZ application on stem elongation and flower initiation: Stem elongation was almost completely inhibited by UZ treatment (Fig. 1*B*). In the UZ treatment the stem length was 61 ± 2.9 mm at 62 DAS (*i.e.*, 27 d after UZ treatment), but was 180 ± 8.0 mm in the control. The stem diameter was 25 ± 0.5 mm in the control and 20 ± 0.6 mm in the UZ-treated plants. At the same time, the number of leaves longer than 1 cm was significantly less in the UZ-treated plants (33 ± 1) than in the control (49 ± 7).

UZ treatment suppressed internodal elongation and leaf elongation, and retarded flower induction (Fig. 1*B*). In control plants, the CZ could be distinguished at 41 DAS, but the frequency of DNA replication increased in CZ at 44 DAS, leading to equal expression of the *H4* gene in the dome-like apex at 47 DAS (data not shown). A decrease in cell division at the base of the PZ, RM, subapical pith and vascular bundle was observed in UZ-treated plants (Fig. 2*F*). The CZ could be distinguished from the PZ and RM at 53 DAS, *i.e.*, 15 d after treatment (Fig. 2*F*), and DNA replicating cells were distributed more uniformly and commonly in the CZ of UZ-treated plants at 56 DAS (Fig. 2*G*). A dome-like apex was apparent in UZ-treated plants at 65 DAS (data not shown).

Effect of combined UZ and GA$_3$ application: Stem elongation increased 6 d after UZ+GA$_3$ treatment and continued throughout the experiment (Fig. 1*C*). However as in experiment 2, UZ treatment suppressed stem elongation. At 49 DAS (15 d after treatment) the stem length was 395 ± 102 mm in the UZ+GA$_3$ treatment, 118 ± 8 mm in the control, and 73 ± 13 mm in the UZ treatment. The stem diameter was not affected by the UZ+GA$_3$ or UZ treatments. The number of leaves longer than 1 cm was significantly greater in the UZ+GA$_3$ treatment (32 ± 2) than in the control (25 ± 1) and the UZ treatment (22 ± 1).

In the control, DNA replication was not detected in the CZ, and the apical meristem was still flat at 34 DAS. *H4* gene expression became detectable in the CZ at 40 DAS. Thereafter, shoot apices became dome-shaped, and cells expressing the *H4* gene were common and uniformly distributed at 46 DAS. In the UZ+GA$_3$ treatment, *H4* gene expression in the CZ was detected at 37 DAS, *i.e.*, 3 d after treatment (Fig. 2*H*). An increase in expression of *H4* gene was also observed at the base of leaf primordia and in the subapical pith and vascular bundle. At 40 DAS (*i.e.*, 6 d after treatment), the apices became dome-shaped, and DNA replicating cells were more common and distributed uniformly (data not shown). In the UZ treatment, histone *H4* gene expression at the SAM was low, and was not detected in the CZ until 49 DAS (data not shown).

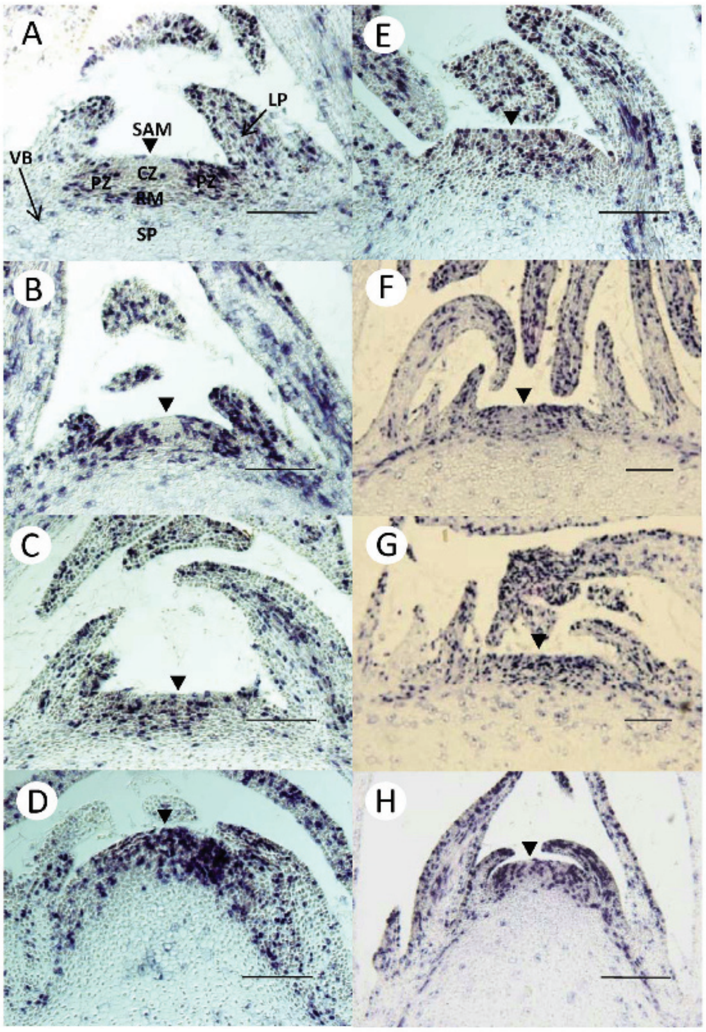

Fig. 2. *In situ* hybridization analysis of median longitudinal sections of lettuce apices using the DIG-labeled histone *H4* probe in GA_3 experiment (*A* - *E*), UZ experiment (*F*,*G*), and UZ + GA_3 experiment (*H*). Cells expressing the histone *H4* gene are stained blue. *A*,*B* - control and GA_3-treated apices at 34 DAS; *C*,*D* - control apices at 49 and 52 DAS;. *E* - GA_3-treated apex at 46 DAS; *F*,*G* - UZ-treated apices at 53 and 56 DAS. *H* - UZ + GA_3-treated apex at 37 DAS. Apices of *A*,*B*,*F* are considered to be at vegetative phase because the central zone is clearly recognized as a group of cells with a low *H4* expression frequency. Apices of *C*,*E*,*G*,*H* are considered to be flower induced because *H4* expression becomes detectable at central zone. Apex of *D* is dome-shaped, being the first morphological sign of flower induction. *Arrowheads* indicated the central zone at which *H4* expression was detected. SAM - shoot apical meristem, CZ - central zone, PZ - peripheral zone, RM - rib meristem, SP - subapical pith, LP - leaf primordium, VB - vascular bundle. *Bar* = 200 µm.

Discussion

Sachs *et al.* (1959) and Chouard (1960) demonstrated that GAs hasten bolting. GA_3 accelerates stem elongation in *Samoulus parviflorus*, *Hyoscyamus niger*, *Rudbeckia bicolor* and *Raphanus sativus*, (Sachs 1965, Jacqmard 1968, Nishijima 2000, Gocal *et al*. 2001, Jacqmard *et al.* 2003) and shoot elongation of *Asparagus officinalis* (Saharan 2010). In our experiments GA_3 induced stem elongation in lettuce 3 d after application, and the effect lasted to the end of the experiment (21 d after application; Fig. 1*A*). However, UZ reduced internodal and stem elongation (Fig. 1*B*). The application of GA_3 in combination with UZ nullified the effect of UZ, and accelerated stem elongation (Fig. 1*C*). These results provide direct evidence of a role for GAs in stem elongation.

In situ hybridization using the *H4* gene enabled us to detect cytological changes at the SAM during stem elongation and flower initiation after GA_3, UZ and UZ+GA_3 application. In a previous study, GA_3 induced hypocotyl elongation in lettuce by increasing cell elongation but did not increase cell division (Stuart *et al.* 1977). This is in contrast to our study, in which GA_3 markedly increased mitotic activity in the PZ, RM and leaf primordia 3 d after treatment (Fig. 2*A*,*B*). The UZ treatment suppressed *H4* expression at the PZ, RM and subapical pith (Fig. 2*F*). However, the UZ+GA_3 treatment increased mitotic activity at the shoot apex, particularly in the PZ, RM and subapical pith, resulting in enhanced stem elongation (Fig. 2*H*). These results suggest that GA_3 plays a role in stem elongation in part through DNA replication and cell division activity in the shoot apex and subapical pith.

In this study the first sign of flower induction (*i.e.*, an increase in DNA replication in the CZ) preceded a change in the morphology of the SAM from flat to dome-like, except in GA_3-treated plants in experiment 1 (Figs. 1,2). The GA_3 treatment resulted in the SAM becoming more sharpened, suggesting that the morphological changes in the SAM may have been apparent earlier in the GA_3-treated plants in experiment 1. The GA_3 and UZ+GA_3 treatments hastened uniform expression of the *H4* gene in the CZ by 3 d relative to the control (Fig. 1*A*,*C*). The UZ treatment suppressed cell division frequency at the CZ, PZ and RM, and delayed uniform expression of the *H4* gene at the CZ by 9 d relative to the control (Figs. 1*B*,2*F*,*G*).

In *Arabidopsis*, four flowering time genetic pathways have been proposed: the GA, light-dependent, autonomous and vernalization pathways (Roux *et al.* 2006). The GA pathway regulates flowering time through hormone inputs. The light-dependent pathway is related to perception of the photoperiod, irradiance and spectral composition. The autonomous pathway accelerates flowering in response to internal developmental signals, and long periods of cold temperature induce flower induction *via* the vernalization pathway. These pathways promote flowering through upregulation of the integrator genes *SOC1*, *FT* and *LFY*. The extent to which GA induces flowering varies among species and with environmental conditions. In the long day plant *Lolium temulentum*, GAs act as a significant flowering signal. However, GAs have little influence on flowering in other annual long day plants and inhibit flower induction in many perennials (Mutasa- Göttgens and Hedden 2009). In this study, flower induction in experiment 3 was inhibited by UZ treatment, possibly because the endogenous GA concentration was extremely low. However, flower induction occurred with UZ treatment in experiment 2. These results suggest that the GA pathway and the light-dependent or autonomous pathways control flower induction in lettuce under long day conditions, as occurs in *Arabidopsis* (Roux *et al.* 2006).

The timing of stem elongation and flower initiation is important in lettuce because these processes have major impacts on yield and head quality. In *Arabidopsis*, flower induction precedes bolting and the light-dependent pathway in flower induction appears to dominate under long day conditions (Kobayashi and Weigel 2007). However, in biennial species including sugar beet, vernalized plants initiate rapid stem elongation, which precedes the development of indeterminate inflorescences with floral shoots (Mutasa-Göttgens *et al.* 2009). In our study marked stem elongation (bolting) in lettuce was preceded by the development of an inflorescence meristem, at least when GA_3 was applied (Fig. 1*A*,*C*).

In the *in situ* hybridization experiments using lettuce apices, the histone *H4* probe detected cytological changes in the shoot apex during the transition to flowering and stem elongation after GA_3 and UZ treatment. Consequently, stem elongation and flower initiation in lettuce are related to an increase in cell division frequency in the CZ, PZ, RM and subapical pith in the SAM, and GA_3 may accelerate flower initiation. Further research is necessary to clarify the role of GAs in flower induction in lettuce, through the study of flower integrator genes.

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