Estimation of endogenous contents of phytohormones during internode development in *Merremia emarginata*

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Abstract

During the entire period of internode growth of *Merremia emarginata* contents of gibberellic acid (GA_3), phenyl-acetic acid (PAA), indole-3-acetic acid (IAA, free and conjugated) and abscisic acid (ABA, free and conjugated) were estimated by ELISA using polyclonal antibodies raised against each hormones. At the time of internode elongation free auxin content was low and increased with the decrease in the rate of elongation. In contrast, conjugated IAA showed declining trend where free IAA content was remarkably high, suggesting thereby that conjugated IAA might have mobilized during the later phase of internode development. The endogenous GA_3 contents were high as compared to other hormones; however, no significant role of GA_3 was discernible in elongation growth. Conjugated ABA contents remained very low during the elongation growth and increased thereafter.

Additional key words: abscisic acid, auxins, ELISA, elongation growth, gibberellins.

Introduction

Auxins, including indole-3-acetic acid (IAA), stimulate elongation by increasing cell wall extensibility (Lüthen *et al*. 1990, Rayle and Cleland 1992). IAA content in a cell is the sum of free, conjugated and oxidized forms. Free forms are utilized during elongation. Conjugated IAA is a storage form that can release free IAA on cellular or metabolic demand. It is protected against oxidase (Cohen and Banduraski 1982). Oxidised IAA does not participate in elongation growth. Phenyl acetic acid (PAA) is also a natural auxin and can perform many auxin like functions (Wightman and Lighty 1982).

GA_3 also promotes cell elongation in either excised or intact internode tissue. Endogenous contents of GA_3 positively correlated with root growth rates in certain dwarf and normal plants (Yaxley *et al*. 2001). Gibberellins (GAs) are also involved in regulation of stem elongation (Fujioka *et al*. 1990) and cotton fiber elongation (Gokani and Thaker 2001). GA_3 and auxin act synergistically or additively in stimulating cell elongation (Miyamoto and Kamisaka 1988). On the contrary, ABA reduces GA-promoted processes in plant development (Anker 1975). There are also several reports indicating that ABA inhibits the enhancement of elongation by IAA and there is evident that ABA can inhibit IAA biosynthesis (Pilet 1975a,b). Therefore, it is the ratio of different phytohormones rather than content of any single hormone, which influence the overall growth and development of the plant tissue (Millborrow 1970).

In the present study, *Merremia emarginata* (family *Convolvulaceae*) is used as an experimental plant because of its exceptional to stretch the internodes. Probable role(s) of phytohormones in this property has not been worked out. Therefore, endogenous content of plant growth regulators were estimated using antibodies against each plant growth regulators (*i.e*. IAA, PAA, GA_3 and ABA) during the entire period of internode growth.

Materials and methods

The plants were grown up to flowering, *i.e.* nearly 30 d, in the botanical garden of the Department of Biosciences, Saurashtra University, Rajkot. The internodes were separated from each other at the center of the connecting nodes. The age of the internodes was considered according to its position from the apex. The one at the

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Abbreviations: ABA - abscisic acid; ELISA - enzyme link immunonosorbant assay; GA_3 - gibberellic acid; IAA - indole-3-acetic acid; PAA - phenyl-acetic acid.

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apex, next to the primordia, were considered to be the youngest and the internodes near to the root as mature. The young internodes *i.e*. No. 1 through No. 5 were considered individually while internode Nos. 6 - 8, 9 - 11, 12 - 14 and ≥15 were considered as a pooled group because of their similarity in biochemical and other characteristics revealed by pilot experiments. At least 10 - 15 replicates in each of these 9 groups were taken for the experimental analysis to eliminate the probable variations due to biological differences among individual plants.

The length of individual internodes, their fresh and dry masses were measured and the means ± standard deviations of 25 - 30 replicates were calculated.

Freshly harvested samples were crushed in liquid nitrogen. Approximately 100 mg of crushed material was suspended in 80 % methanol containing 0.01 % of ascorbic acid as an antioxidant (Kara *et al*. 1997). After 48 h at 4 °C the homogenate was centrifuged at 5 000 *g* for 10 min. The supernatant was collected and the residue was washed twice with the same solution. These supernatants were pooled and allowed to concentrate by evaporation up to desired volume in dark. The final volume was made using 80 % methanol containing 0.01 % ascorbic acid.

Antibodies against plant growth hormones were prepared in this laboratory. The plant growth hormones (*i.e*. IAA, PAA, GA_3 and ABA) were tagged with a bovine serum albumin (BSA) as described earlier (Gokani and Thaker 2001, 2002, Sharma and Thaker 2001). The conjugates of each hormone were mixed thoroughly with adjuvant and injected to the rabbits by intramuscular route. Booster injections were given periodically to raise the titer of antibodies. Antisera were separated and purified using DEAE-cellulose. To amplify the reaction, indirect ELISA using polyclonal antibodies was performed.

The casein conjugated form of antigen prepared in coating buffer (100 mM carbonate buffer, pH 9.7) was added to each well (0.35 cm^3) of the microtiter trays and were incubated for 3 h at 37 °C followed by incubation at 4 °C for over night to allow complete binding of the antigen to the wells. Then the wells were washed with phosphate buffered saline containing 0.05 % *Tween-20* (PBS-T, pH 7.2) for three washes.

With intention to prevent direct binding of antibodies on the well, the plates were coated with blocking buffer (PBS-T+0.5 % egg albumin). Blocking buffer (0.35 cm^3) was added to each well and the plates were incubated at 37 °C for 1 h. At the end of the incubation the blocking buffer was removed and the wells are washed thrice using PBS-T.

The wells were then added with 0.35 cm^3 of the hormone extract mixed with the diluted antibodies against the respective antigen. The positive control was prepared by coating the antibodies directly into the wells without any prior mixing with samples. The accuracy of the assay was confirmed by reacting the antibodies with different known concentrations of IAA, PAA, GA_3 or ABA *in vitro* before coating on the wells. The wells after addition of the antibodies, were incubated for 3 h at 37 °C and then over night at 4 °C. The antibodies were then removed and the wells were washed thrice with PBS-T.

Anti-rabbit goat I_gG tagged with peroxidase (*Bangalore Genei*, Bangalore, India) was used as second antibodies. The original stock was diluted using PBS-T in a ratio of 1:5000. Each well was added with 0.35 cm^3 of antibodies. The plates were then incubated for 2 h at 37 °C and then washed thrice with PBS-T.

The peroxidase present with the second antibodies was reacted with *o*-phenelene-diamine (OPD) as a hydrogen donor. The substrate was prepared by adding 8 mg OPD and 1 cm^3 hydrogen peroxide solution in 20 cm^3 30 mM Na-phosphate buffer (pH 5.0) in dark. Freshly prepared substrate (0.28 cm^3) was added to each well and incubated in dark at room temperature for 15 min. H_2SO_4, (3 M, 0.07 cm^3) was added to the reaction mixture in the wells to terminate the reaction. The intensity of brown colour developed was measured at 470 nm using microtiter plate reader (*Bio-Tek*, Vermont, USA).

Equal amount of the extract and the respective antibody (diluted up to a pre-determined concentration using PBS-T solution), was mixed and incubated at 37 °C for 3 h and then at 4 °C for overnight in a microcentrifuge tube after thorough mixing. The mixture was then used for coating the wells.

In order to remove contaminants that may interfere with quantification of endogenous IAA, *e.g*. putative tryptophan, indole-acetaldehyde, indole-carboxylic acid, indole species, *etc.*, the hormone extract was purified by column chromatography using insoluble polyvinyl-polypyrrolidone (PVPP) column. PVPP column (1.5 × 2.0 cm) was prepared and equilibrated with 5 cm^3 of 0.05 M acetic acid and pH was adjusted to 3.0 with concentrated acetic acid (Kara *et al*. 1997). Hormone extract (1 cm^3) was loaded on to these columns and allowed to react over night at room temperature. The supernatant was thrown and the columns were then washed with 4 cm^3 60 mM potassium phosphate buffer (pH 5.0). Then 3.0 cm^3 of phosphate buffer saline was added to the column to elute the immobilized IAA. These supernatant was then collected and evaporated to concentrate. The final volume of the concentrate was made using 80 % methanol containing 0.01% ascorbic acid and used for the sample preparation for loading the wells of the microtiter plates.

The estimation of the conjugated hormones was done by calculating the same on the basis of free and total hormones. For estimation of the total hormones (free and conjugated forms), the sample was prepared by taking equal amount of the hormone extract and 14 M NaOH, which was then incubated after thorough mixing at 100 °C for 3 h. At the end of the incubation the pH of the mixture was set to 5.0 using HCl (Bandurski and Schulze 1977). The resultant mixture was then reacted with equal

amount of antibodies (of appropriate dilution) and then incubated at 37 °C for 1 h followed by at 4 °C for 3 h. Finally it was used for loading the antigen coated microtitre plates.

Different known concentrations of commercially available hormones were used to prepare a standard curves and the quantity of the endogenous hormone was expressed as ng hormone internode^{-1}.

Results and discussion

The length of the internode showed a gradual increase from number 1 to 5 and then stabilized in subsequent internodes (Fig. 1). The rate of elongation was higher up to 2nd internode and decline thereafter. Minimum rate was observed in 5th internode thereafter. With a lag for about 2 to 3 initial internodes a gradual increase in dry matter accumulation was observed up to 9th-11th internode and then it stabilized in subsequent internodes (Fig. 1). The rate of dry mass accumulation (DMA) was maximum at 3rd internode and declined thereafter. Water content increased gradually from 2nd internode to 9th - 11th internode and declined later on (Fig. 1). Observed positive correlation between cell elongation and water content ($P \leq 0.001$) is well documented (Cosgrove 1993, Rabadia *et al*. 1999).

Free IAA content remained low initially and increased from 3rd internode. A decline was observed on 6th internode and then the levels was maintained higher during the subsequent stages of the growth (Fig. 2). Conjugated IAA increased gradually up to 4th internode and then declined gradually up to 9th - 11th internode and a rise was observed during later phases (Fig. 2). It indicates that higher free IAA content during later stages (Fig. 2) might have been released from the conjugated ones.

The characteristic feature of auxins is their ability to promote cell elongation in coleoptiles and stem tissues

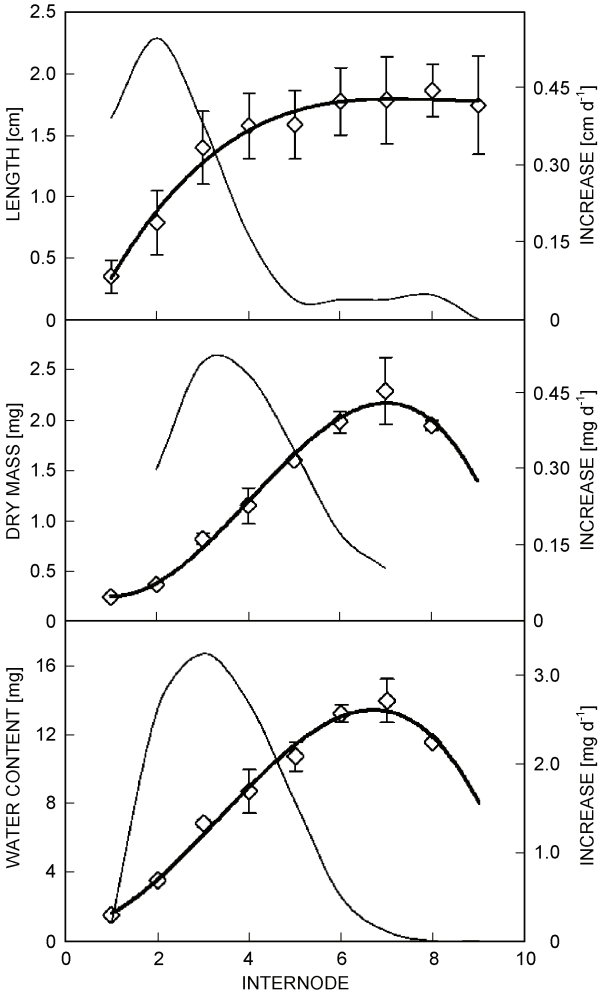

Fig. 1. Changes in length (*rhombs*) and its increase, dry mass (*rhombs*) and its increase, and water content (*rhombs*) and its increase in internodes in relation to their age. *Vertical bars* represent ± SD, or SD are within the symbols.

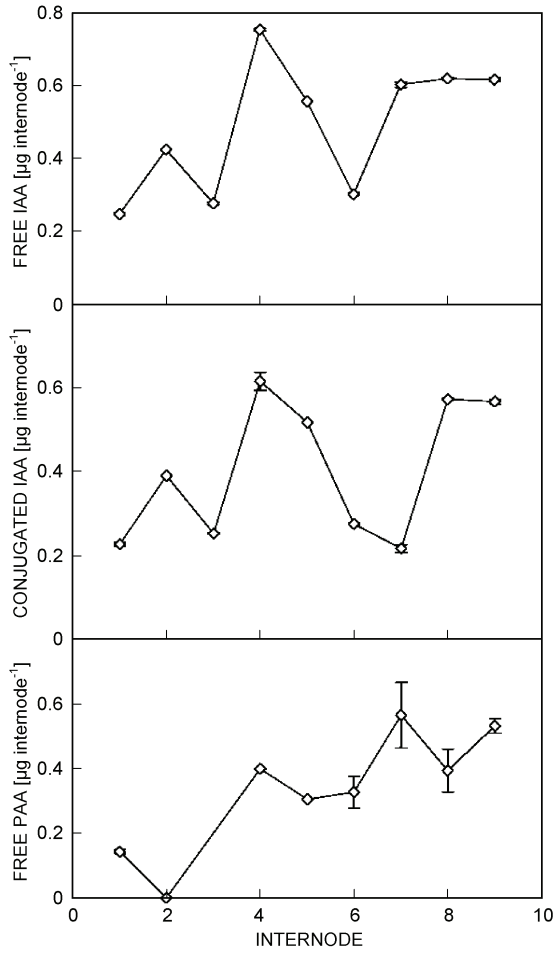

Fig. 2. Changes in free IAA, conjugated IAA, and free PAA contents in internodes in relation to their age. *Vertical bars* represent ± SD, or SD are within the symbols.

(Rayle and Cleland 1992, Turusaki *et al*. 1997). Auxins are reported to enhance growth of either excised or intact stem segments (Yang *et al*. 1993, 1996). A positive and significant correlation was observed between fiber length and endogenous as well as exogenously supplied auxins (Gokani and Thaker 2002). In the present study also at the time of internode elongation free auxin content was low and increased with the decrease in the rate of elongation (Figs. 1,2). In contrast, conjugated IAA showed a declining trend where free IAA content was remarkably high, suggesting thereby that conjugated IAA might have mobilized during the later phase of internode development (Fig. 2).

PAA contents showed an increasing trend with the internode growth except at 2nd internode where a sudden fall was observed (Fig. 2). PAA mimics IAA in bioassays for auxins but is found to be active only at much higher concentrations than IAA (Wightman and Lighty 1982). Considerably higher endogenous contents of PAA and IAA in rapidly growing regions than in mature portions of shoot have been reported (Wightman and Lighty 1982). Further, it has been found that PAA is appreciably more active than IAA in lateral root formation of pea (Wightman *et al.* 1980). Gokani and Thaker (2002) also reported a positive correlation between PAA content and cotton fiber length.

In addition to auxin, GA_3 is also known to promote cell elongation in various plant tissues. In the present

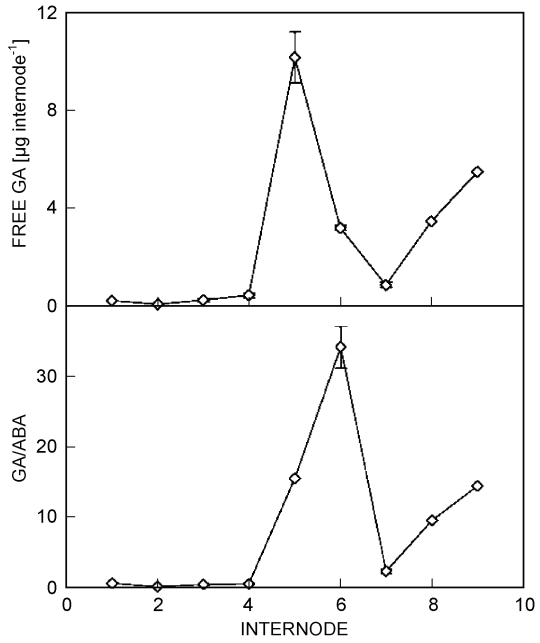

Fig. 3. Changes in free GA content and GA to ABA ratio in internodes in relation to their age. *Vertical bars* represent ± SD or SD are within the symbols.

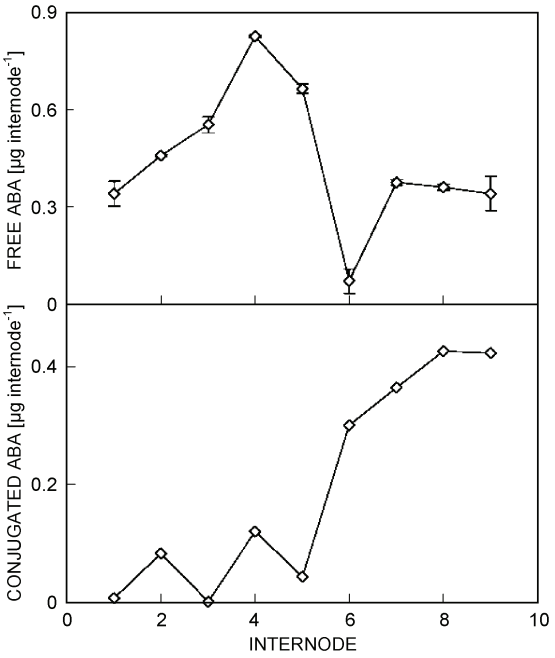

Fig. 4. Changes in free ABA and conjugated ABA contents in internodes in relation to their age. *Vertical bars* represents ± SD or SD are within the symbols.

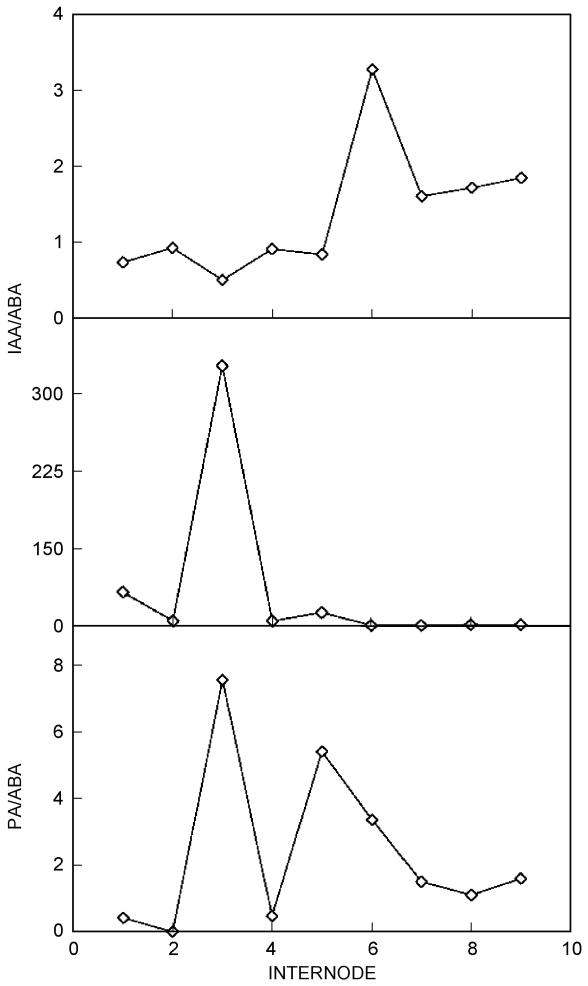

Fig. 5. Changes in free IAA to ABA (*upper part*), conjugated IAA to ABA (*middle part*), and free PAA to ABA ratios in internodes in relation to their age.

study, GA_3 content remained low up to 4th internode and increased in the later stages (Fig. 3). The levels declined in latter stages after a sharp increase on 5th internode. Although the endogenous GA_3 contents were high as compared to those of other hormones, remarkably low contents during elongation phase of developing internodes in the present study, suggesting that GA_3 might have been utilized for elongation growth, in which no specific role of GA_3 was distinctly discernible.

Free ABA content gradually increased up to 4th internode and declined thereafter in subsequent periods. Peak value was recorded at 4th internode (Fig. 4). In contrast to free, conjugated ABA content remained very low during the elongation growth and increased thereafter (Fig. 4). Thus, an negative correlation was observed between free and conjugated ABA. In contrast to auxins and GA_3, ABA inhibits stem and root elongation and counteracts the promotive effect of other growth regulators (Kaufman and Jones 1974, Pilet 1975a,b). Inhibitory effect of ABA in cell elongation of cotton fiber and seed development was observed. ABA content remained low during the elongation period of fiber development and maximum accumulation of ABA was observed at maturation (Gokani *et al*. 1998). ABA is also regarded as a regulator of GA_3 promoted processes in plant development (Anker 1975). In the present study the ratio of GA_3 to ABA remained near to one throughout the development. The values were exceptionally higher at the time when internode length stabilized and declined in subsequent stages. Remarkably higher ratio of conjugated IAA to ABA was observed at 3rd internode, which declined thereafter (Fig. 5) whereas changes in free IAA to ABA ratio showed higher values at later stages (Fig. 5). The ratio of PAA to ABA remained high (Fig. 5). Thus, ratio of IAA or GA_3 or PAA to ABA remained more than one throughout the period of internode development. It suggests that regulation of development may be controlled by the balance between promotory and inhibitory group of hormones, by opposing the effects of other group or by altering the effective concentration of one group of hormones by other group.

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