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SHORT COMMUNICATION

Involvement of Growth Promoting Hormones in *Rho1ps* Gene Expression in Garden Pea Shoot Apexes

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In order to examine the possible involvements of plant hormones in actin filament organization in a garden pea shoot apex, the effect of growth promoting hormones such as IAA, GA_3, brassinolide (BL), and zeatin (Zea) on the gene expression of *Rho1ps* was investigated by northern blot analyses. Compared with the level of *Rho1ps* transcripts in hormone-untreated segments of the apex, the application of 10^{-6} M IAA, GA_3, and BL activated the accumulation of *Rho1ps* transcripts as much as 2, 2, and 4 times, respectively. Also the application of 10^{-6} M Zea showed accumulation of the transcripts 7 times higher than the control, which is about 2 to 3 times higher than that induced by the other three hormones. Thus, it is thought that actin filament organization mediated by *Rho1ps* is closely associated with growth of the segments induced by the hormones tested, and that the organization is more necessary for a process related to cell division rather than that to cell expansion (elongation and/or enlargement) in the segment. The increase of *Rho1ps* transcripts induced by each hormone was additive when another hormone was simultaneously applied, indicating that the four hormones, IAA, GA_3, BL, and Zea, participate independently in regulating the gene expression.

Keywords: gardenpea, plant hormones, *Rho1ps*, small GTP-binding protein

INTRODUCTION

Cellular morphogenesis in plants is a function of asymmetric cell division and expansion. A number of studies have suggested that the processes are spatially controlled by a dynamic cytoskeleton that consists of dozens of interacting proteins (Lloyd, 1991). Actin is a major component of the plant cytoskeleton and is thought to be required for various cellular morphogeneses such as cell-plate synthesis, cell shape determination, cell-polarity establishment, cytoplasmic streaming, organelle movement, and tip growth (Staiger and Schliwa, 1987; Staiger and Lloyd, 1991; Meagher and Williamson, 1994). However, very little about how plant actin microfilaments are organized and how they are regulated during cellular morphogenesis has been elucidated.

In fungi and animals, actin microfilament organization is controlled by small GTP-binding proteins, rho proteins. Also the cellular functions mediated by the actin cytoskeleton are regulated by actions of rho proteins as a key component of signal transduction pathways (Ridley, 1995). Because the functions of rho proteins in regulating the organization of actin microfilament are highly conserved in such a diverse range of organisms, from yeast to the human, it is likely that plant actin microfilament organization is also controlled by the rho proteins in a similar manner (Yang and Watson, 1993). Recently, Yang and Watson (1993) isolated a gene for a member of the rho protein family, *Rho1ps*, from the garden pea using PCR-based cloning that involves degenerated primers corresponding to conserved domains within the yeast and mammalian rho proteins. Sequence analysis of *Rho1ps* indicated that *Rho1ps* possesses all of the key structural features unique to members of the rho family. In addition, the *Rho1ps* fusion proteins expressed in *Escherichia coli* exhibited specific GTP-binding activity, indicating that *Rho1ps* encodes a member of rho proteins in the garden pea. furthermore, tissue specific analysis of *Rho1ps* showed that transcription levels of *Rho1ps* are higher in the apical buds and root tips than in the stems and roots, indicating that the *Rho1ps* gene is preferentially expressed in the regions of the pea seedlings that are active in cell division. It is thought, thus, that *Rho 1ps* may play an important role for regulating an

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actin-mediated function in meristematic tissues of the pea.

Plant meristematic tissues consist of actively dividing cells which are sensitive to plant growth regulators, mainly plant hormones (McDowell *et al.*, 1996). Thus, the preferential expression of *Rholps* in meristematic tissues suggested that the gene expression of *Rholps* could be influenced by plant hormones (McDowell *et al.*, 1996). In order to examine the possible involvement of plant hormones in *Rholps* gene expression, we previously investigated the effect of a synthetic cytokinin, BAP, on the gene expression of *Rholps* in a shoot apical meristem of the pea by northern blot analysis. In result, a strong activation of the transcription of *Rholps* by BAP was observed, which proposed that other plant hormones are also involved in the gene expression of *Rholps* (Nam *et al.*, 1998). Our continuous interest in relationship between plant hormones and *Rholps* gene expression in the tissue prompted us to examine the effect of other plant hormones. Herein, the effects and the interactions of plant hormones on the expression of *Rholps* in the tissues of the pea plant are reported.

MATERIALS AND METHODS

Plant Materials

Garden pea (*Pisum sativum* L.) seeds were germinated and grown in the dark as described previously (Nam *et al.*, 1998). Then, 1.5 cm segments containing shoot apexes obtained from 7-day-old dark-grown seedlings were collected and shaking-cultured in a liquid MS medium with or without hormones at 25°C under 3,000 lux light. After incubation, the segments were collected into liquid nitrogen and stored at −70°C until analysed.

Chemicals

Brassinolide (BL) [(2α, 3α, 22*R*, 23*R*)-tetrahydroxy-24S-methyl-7-oxa-5α-cholestan-6-one] was kindly provided by Prof. Takao Yokota in Teikyo University, Ustumomiya, Japan. Indole-3-acetic acid (IAA), gibberellic acid (GA_3) and zeatin (Zea) were purchased from Sigma Chemical Co., St. Louis, USA.

Synthesis of Probe

Two specific oligonucleotide sequences in the conserved regions of *Rholps* gene were selected for synthesis of primers. The sense primer was 27 nucleotides (nt) from 294 to 321, and the anti-sense primer was 27 nt from 774 to 801 in the sequence numbers of the *Rholps* gene (Yang and Watson, 1993). The PCR for amplifying the 510 bp cDNA of the *Rholps* gene was performed as follows: pre-denaturation for 5 min at 94°C, 35 cycles with denaturing for 1.5 min at 94°C, annealing for 1 min at 60°C, extension for 2 min at 72°C, and post-elongation for 30 min at 72°C. The reaction solution contained 5 μL Mg^{++}-free buffer, 1.25 mM of $MgCl_2$, 200 μM of each dNTP, 0.5 μM of the designed primers, and 1 ng of template DNA in 50 μL. The reaction was initiated by the addition of 1 unit of *Taq* DNA polymerase. After PCR, the product was ligated on a pGEM-T vector and then introduced into *E. coli*, DH5α. The plasmids were isolated, and the insert sites were determined by digestion with *Sac I* and *Sac II*. The sequences of the resulting 560 bp cDNA fragment was determined by the dideoxynucleotide chain termination method using double strand DNA as a template (Sanger *et al.*, 1977). To obtain a probe for detecting *Rholps* transcripts, finally, cDNA was radiolabelled using a random priming kit (Boeringer Mannheim) containing 0.5 mM dNTP, 10 × hexanucleotide mixture, 1 unit of Klenow enzyme, and 50 μM of [α-^{32}P]dCTP (3,000 Ci/mmol) in total 20 μL at 37°C for 45 min.

Northern Blot Analysis

Northern blot analysis was performed using the method developed by Sambrook *et al.* (1989). Total RNA (20 μg) obtained from the segment was separated on a 1.0% agarose/formaldehyde gel and transfered to Nylon membrane (Hybond N^+: Amersham) for 4 h (semi-dry transfer unit: Hoefer). Prehybridization was performed for 4 h at 42°C in a solution containing 5 × SSPE, 50% formamide, 2 × Denhardt's, 10% SDS and 100 μg/mL denatured salmon sperm DNA. Hybridization was performed in a solution containing 50% formamide (v/v), 5 × SSC, 0.1% SDS, 5 mM EDTA, 10 × Denhardt's solution and 25 mM sodium phosphate at 42°C for 18-24 h. After washing with 2 × SSC and 0.1% SDS at 42°C for 30 min (three times), the membrane was exposed to an x-ray film for 18-20 h.

RESULTS

Effect of Hormones on the Growth of Shoot Apex Segments of the Garden Pea

In plants, the growth of a shoot apex is basically induced by two cellular processes, cell division and cell expansion (elongation and/or enlargement), in the shoot meristem (Taiz and Zeiger, 1991). In order to examine the growth pattern induced by three growth promoting hormones, 10^{-6} M of IAA, GA_3, and brassinolide (BL) were exogenously applied to segments of garden pea shoots. As shown in Fig. 1, application of IAA, BL, and GA_3 to the segments strongly promoted longitudinal and lateral growth of the segments compared with that of hormone-untreated segments. Also, the fresh weight of the segments were significantly increased by the application of IAA, BL, and GA_3 as much as 64%, 82%, and 91%, respectively. The growth pattern and significant increase of fresh weight of the segments induced by the three hormones are characteristic responses shown in a shoot apex growth by cell expansion in many plants, indicating that a main function of the three hormones for growth of the segments is expansion of cells in the apex.

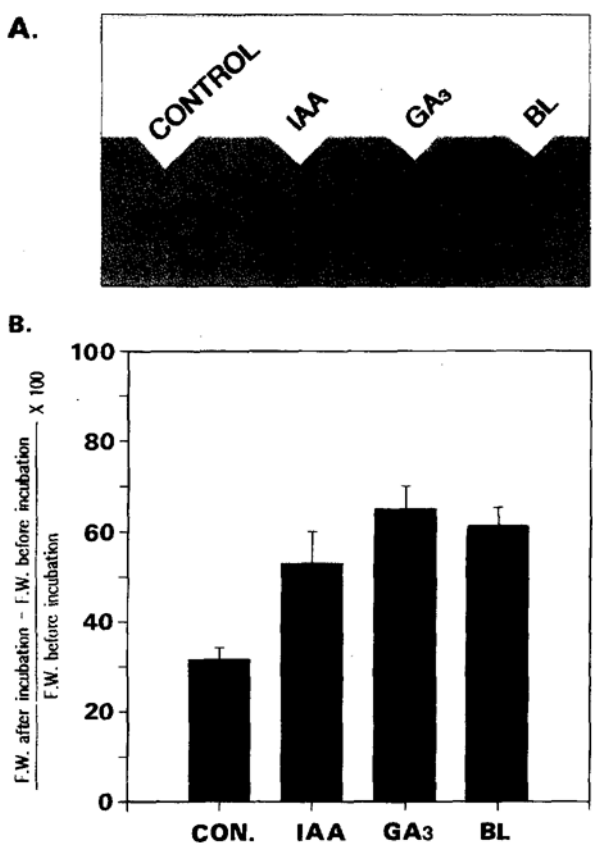

Fig. 1. Growth of a shoot apex segment of garden pea induced by application of 10^{-6} M IAA, GA_3, and BL. A, Morphological changes of hormone-treated (IAA, GA_3 and BL) and hormone-untreated (control) segments after 24 h incubation; B, The change of fresh weight in hormone-treated and hormone-untreated segments after 24 h incubation. Each value in B is the average of 3 independent experiments using 10 segments.

Effect of Plant Hormones on Gene Expression of *Rholps* in Shoot Apex of Garden Pea

Rholps gene expression in shoot apex of garden pea was analyzed by northern blot hybridization using ^{32}P-labelled cDNA (560 bp) as a probe. As shown in Fig. 2, the amount of *Rholps* transcripts in the hormone-untreated segments increased until 12 h, and then decreased. Similarly, total amount of *Rho 1ps* transcripts in hormone (10^{-6} M IAA, BL, and GA_3)-treated segments increased for 12 h, and then decreased. However, comparing the increment of the *Rholps* transcripts in the hormone-treated segments with that in the hormone-untreated segments, the effectiveness of hormones on the level of *Rholps* transcripts was continued for 24 h after the treatment.

It was previously reported that BAP, a synthetic cytokinin, strongly increased *Rholps* transcripts in the segments. In this study, as shown in the time

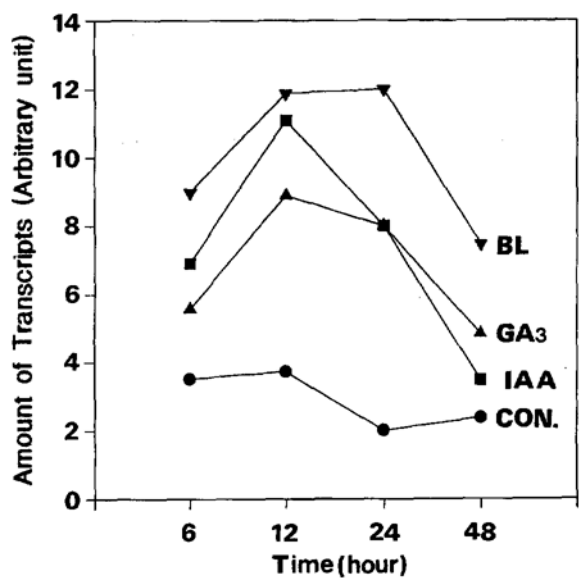

Fig. 2. A time course analysis of *RholPs* transcripts in 10^{-6} M hormone-treated and hormone-untreated segments by Northern blot hybridization. RNA from the segments was separated on a formaldehyde agarose gel and hybridized to the ^{32}P-labelled *RholPs* cDNA. The blot was rehybridized with a probe for 25S ribosomal RNA to normalize the level of *RholPs* geue expression. The 1 unit in Y-axis represents the amount of *RholPs* in the hormone-untreated segments after 24 h incubation.

course analysis of *Rholps* transcripts in the hormone-treated segments (Fig. 2), application of IAA, GA_3, and BL also increased the level of *Rholps* transcripts. To compare the relative activity of the four classes of hormones, the amount of *Rholps* transcripts induced by IAA, GA_3, BL, and zeatin (Zea, a endogenous cytokinin in the segment) was investigated. As shown in Fig. 3, application of 10^{-6} M IAA (lane 2) and GA_3 (lane 4) to the segments showed a level of *Rholps* transcripts twice as high as that of hormone-untreated segments. Also, application of 10^{-6} M BL to the segments induced a level of *Rholps* transcripts four times higher than that of the hormone-untreated segments. Application of 10^{-6} M Zeas induced a level of *Rholps* transcripts 7 times higher than that in the hormone-untreated segments.

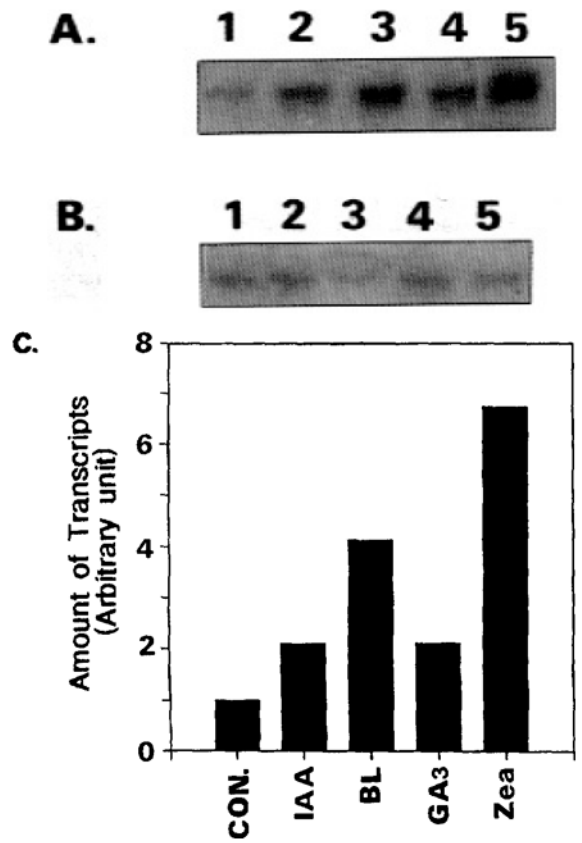

Fig. 3. Northern blot analysis of *RholPs* transcripts in 10^{-6} M hormone-treated and hormone-untreated segments after 24 h incubation. A, RNA from the segments was separated on a formaldehyde agarose gel and hybridized to ^{32}P-labelled *RholPs* cDNA; lane 1, control; 2, IAA; 3, BL; 4, GA_3; 5, Zea B, Rehybidization with a probe for 25S ribosomal RNA; C, Normalized intensity of *RholPs* transcripts signals measured by densitometric scanning of X-ray films. The 1 unit in Y-axis represents the amount of *RholPs* transcripts in the hormone-untreated segments after 24 h incubation.

Thus, it is thought that the level of *Rholps* transcripts in the segments is enhanced in order of Zea > BL > IAA and GA_3.

The interactions of the four kinds of hormones for accumulating *Rholps* transcripts were investigated by combination-treatments of the hormones to the segments. As shown in Fig. 4, the level of *Rholps* transcripts in the hormone-untreated segments was increased by application of IAA, BL, or GA_3 in the presence of Zea as much as 10, 13, and 9 times, respectively. Application of BL or IAA in the presence of GA_3 increased the level of *Rholps* as much

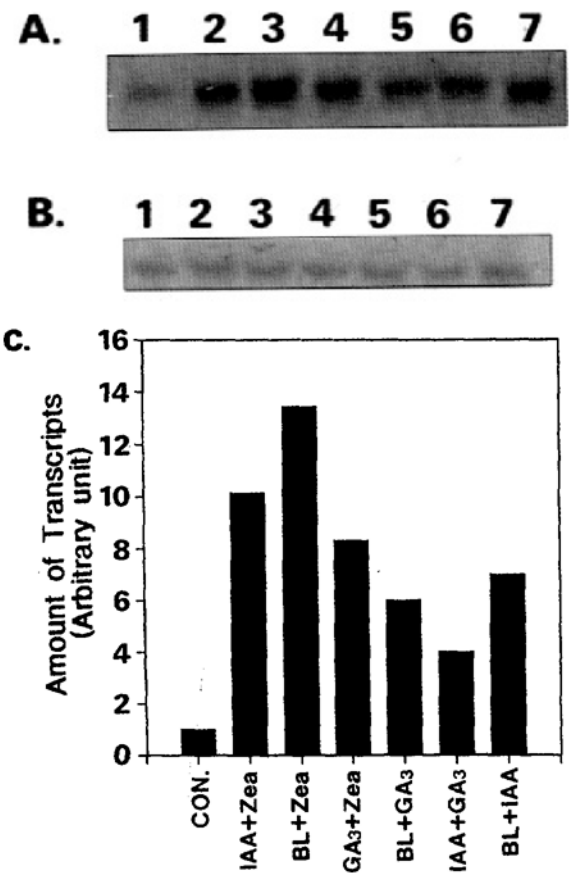

Fig. 4. Northern blot anaysis for detecting *Rholps* transcripts in the segments induced by combinations of four hormones, IAA, BL, GA_3, and Zea. A, RNA from the segments was separated on a formaldehyde agarose gel and hybridized to the ^{32}P-labelled *RholPs* cDNA; B, Rehybidization with a probe for 25S ribosomal RNA to normalize; C, Normalized intensity of *RholPs* transcripts signals measured by densitometric scanning of X-ray films. The unit 1 in Y-axis represents the amount of *RholPs* in hormone-untreated segments for 24 h incubation. Lane 1, no hormone (control); 2, IAA and Zea; 3, BL and Zea; 4, GA_3 and Zea; 5, BL and GA_3; 6, IAA and GA_3; 7, BL and IAA. The concentrations of IAA, GA_3, BL, and Zea applied were 10^{-6} M.

as 6 and 4 times, respectively. Application of BL in the presence of IAA increased the level of *Rho1ps* transcripts as much as 7 times. The level of *Rho1ps* transcripts induced by combinations of two different hormones is almost the same to the sum of the levels of the transcripts induced by two different hormones applied separately in the segments. Thus, it can be concluded that the interactions of the four hormones for controlling the gene expression are additive.

DISCUSSION

In this study, the increase of *Rho1ps* transcripts by application of IAA, GA_3, BL, and Zea was established. These results positively indicate that the four kinds of hormones play an important role in regulating the expression of *Rho1ps* in the segments. Yang and Watson (1993) have suggested that *Rho-1ps* may play a central role in controlling actin filament organization in the segments. In this respect, thus, the increase of *Rho1ps* transcripts by the four plant hormones could be closely associated with regulating processes linked to the actin filament organization in the garden pea.

It has already been demonstrated that a synthetic cytokinin, BAP, strongly increased the level of *Rho 1ps* transcripts in the segments of the garden pea plant (Nam *et al.*, 1998). In the present study, it was also demonstrated that an endogenous cytokinin, Zea, strongly increased the level of *Rho1sp* transcripts. It has been reported that the *Rho1ps* gene is highly expressed in meristematic tissues and is closely related to cell division in the tissues of the garden pea (Yang and Watson, 1993). Thus, it can be understood that the level of *Rho1ps* transcripts is remarkably enhanced by a cell division activating hormone, a cytokinin.

Application of IAA, GA_3, and BL clearly activated the accumulation of *Rho1ps* transcripts in the segments. However, the level of *Rho1ps* transcripts accumulation induced by the three hormones was obviously lower than that induced by a cytokinin. This would suggest that the organization of actin filament mediated by *Rho1ps* is necessary for cell expansion, but the actin filament organization is more necessary for the cell division process rather than the cell expansion process during the shoot apex growth.

If an activation of a gene expression induced by a hormone is synergistically enhanced by a different hormone, it is thought that perhaps the two hormones participate dependently in a route for expression of the gene. And if the activation is just additively enhanced by a different hormone, it is thought that perhaps the two hormones are participate independently in different routes for the gene expression (Katsumi, 1985). In this respect, the additive increase of *Rho1ps* transcripts by all combinations of IAA, GA_3, BL and Zea could indicate that the four hormones participate independently in different routes for *Rho1ps* gene expression.

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