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SHORT COMMUNICATION

The Effect of BAP on the Gene Expression of a Small GTP-Binding Protein, *Rho1Ps* in a Shoot Apex of Garden Pea

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The effect of a synthetic cytokinin, 6-benzylaminopurine (BAP), on gene expression of *Rho1Ps* in shoot apex segments of garden pea was examined by Northern blot hybridization using ^{32}P-labelled 560 bp partial *Rho1Ps* cDNA fragment as the probe. The application of 10 ^{12} M BAP, the lowest concentration tested, for 24h to the segments strongly activated the synthesis of *Rho1Ps* transcripts as much as 7 times compared with those in BAP-untreated segments. In addition, the *Rho1Ps* transcripts synthesis was enhanced by BAP in a concentration dependent manner, suggesting that BAP is concretly involved in the *Rho1Ps* gene expression in the segments. A time course analysis of BAP on *Rho1Ps* gene expression exhibited that BAP was closely related not only to the *Rho1Ps* transcripts synthesis but also to the degradation of *Rho1Ps* transcripts.

Keywords: small GTP-binding protein, *Rho1Ps*, benzyaminopurine, garden pea

Rho1Ps is a member of Rho proteins which has been characterized from garden pea seedlings (Yang and Watson, 1993). Tissue specific analysis of *Rho1Ps* showed that *Rho1Ps* gene is preferentially expressed in meristematic tissues of pea seedlings, suggesting that *Rho1Ps* may play a role in the regulation of cell division. These results prompt us to investigate a possible involvement of BAP, a synthetic hormone regulating cell division, in gene expression of *Rho1Ps* in the meristematic tissues of garden pea shoot apexes. Herein evidences for activation of *Rho1Ps* gene expression by BAP are reported.

MATERIALS AND METHODS

Plant Materials

Seeds of garden pea (*Pisum sativum* L) were surface-sterilized by soaking in 0.5% NaOCl solution for 30 min, and pre-germinated in an air-applied water bath for 24 h at room temperature. The pre-germinated seeds were grown in vermiculite at 25°C. Then, 1.5 cm segments containing shoot apex obtained from 7-day-old dark-grown seedlings were collected and shaking-cultured in a hormone free liquid MS medium with or without BAP at 25°C under 3000 lux light condition. After incubation, the segments were collected into liquid nitrogen, and stored at -70°C until analysed.

Synthesis of Probe

Total mRNA in the shoot apex segments was essentially extracted by the method of Chirgwin *et al.* (1979), and the 1st strand cDNA was prepared from total mRNA as a template by 1st strand cDNA synthesis kit (Pharmacia). The reaction mixture contains 1~5 μg of total mRNA in 20 μL, 5 mM Tris-HCl (pH 8.3), 68 mM KCl, 15 mM DTT, 9 mM $MgCl_2$, 0.08 mg/L BSA, 1.8 mM each dNTP and 1 unit of *Not* I-d$(T)_{18}$.

Two specific oligonucleotide sequences in the conserved regions of *Rho1Ps* gene were selected for synthesis of primers. The sense primer was 27 nucleotide (NT) from 294 to 321, and the anti-sense primer was 27 NT pairs from 774 to 801 in sequence numbers of *Rho1Ps* gene (Yang and Watson, 1993). The PCR for amplifying 510 bp cDNA of *Rho1Ps* gene performed as follow: 35 cycles with pre-denaturation for 5 min at 94°C, denaturing for 1.5 min at 94°C, annealing for 1 min at 60°C, extension for 2

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min at 72°C and post-elongation for 30 min at 72°C. The reaction solution (50 μL) contained 5 μL Mg^{2+}-free buffer, 4 units of $MgCl_2$, 200 μM of each dNTP, 0.5 μM of the designed primers and 1 ng of template DNA. The reaction was initiated by addition of 1 unit of *Taq* DNA polymerase. After PCR, the product was ligated in a pGEM-T vector and then introduced into *E. coli*, DH5α. The transformed bacterial strain was incubated, and the inserted region of PCR product was digested by *Sac*I and *Sac*II. The resulting 560 bp cDNA fragment was sequenced by the dideoxynucleotide chain termination method using double strand DNA as a template (Sanger *et al.*, 1977). Finally, ^{32}P-labelled cDNA as a probe for detecting *RholPs* transcripts was synthesized by a random priming kit (Boeringer Mannheim) containing 0.5 mM dNTP, 10X hexanucleotide mixture, 1 unit of Klenow enzyme, 50 μM of [α-^{32}P]dCTP (3000 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 electrophored in 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 5X SSPE, 50% formamide, 2X Denhardt's solution, 10% SDS and 100 μg/mL denatured salmon sperm DNA. Then hybridization was performed in the solution containing 50% formamide (v/v), 5X SSC, 0.1% SDS, 5 mM EDTA, 10X Denhardt's solution and 25 mM sodium phosphate at 42°C for 18~24 h. After washing with 2X SSC and 0.1% SDS at 42°C for 30 min (three times), autoradiography was undertaken for 18~20 h.

RESULTS AND DISCUSSION

Effect of BAP on the Growth of Shoot Apex Segments

Growth of the shoot apex segments induced by BAP was investigated. As shown in Fig. 1b, application of 10^{-6} M BAP to the segments for 24 h showed only 5% increase in growth rate than that of BAP-untreated segments, suggesting that BAP has no significant role in increasing fresh weight caused by cell elongation and/or cell enlargement in the segments. In contrast, a characteristic lateral growth of the segments was observed by application of BAP (Fig. 1A). Manos and Goldthwaite (1975) reported that the lateral growth of the shoot apex caused by cytokinin is closely related to an increased activity for cell division in the apex. Thus it was considered that the lateral growth of the segments by BAP observed in our experiment also involves increasing cell division in the segments.

Effect of BAP on Gene Expression of *RholPs* in Shoot Apex Segments

A full length cDNA of a member of Rho proteins in garden pea denoted as *RholPs* has been recently characterized (Yang and Watson, 1993). Base on the nucleotide sequences in *RholPs*, a partial cDNA was synthesized by a PCR method. Then, the pGEM-T/partial *RholPs* was introduced into *E. coli*. After digesting the plasmid by *Sac*I and *Sac*II, 560 bp (Fig. 2, lane 4; upper arrow) cDNA containing 510 bp (Fig. 2, lane 5; lower arrow) PCR products were obtained.

The effect of BAP on *RholPs* gene expression in shoot apex segments of garden pea was investigated by northern blot hybridization using the ^{32}P-labelled cDNA as a probe. As shown in Fig. 3, application of 10^{-6} M BAP to the apex segments strongly activated *RholPs* mRNA expression about 10 times

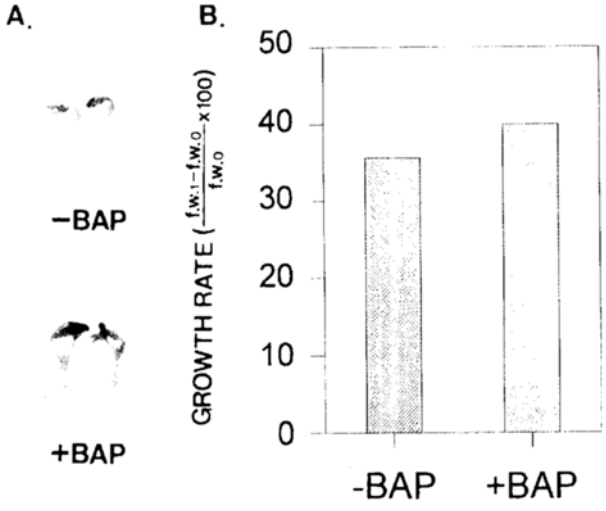

Fig. 1. The effect of 10^{-6} M BAP on the growth of shoot apex segments of garden pea. A: Morphological difference between BAP-treated (+BAP) and BAP-untreated (-BAP) segments after 24 h incubation. B: The changes of fresh weight in BAP-treated (+BAP) and BAP-untreated (-BAP) segments after 24 h incubation.

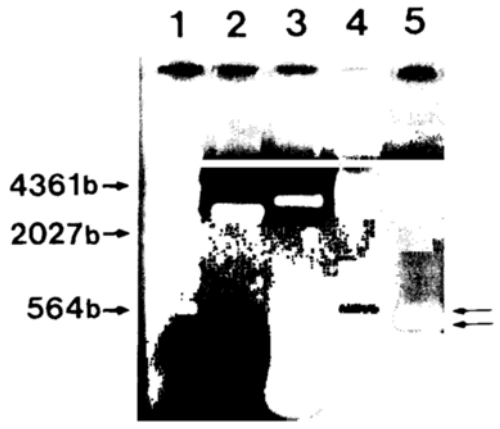

Fig. 2. Agarose gel (1.0%) electrophoresis stained with ethidium bromide. Lane 1, λ-*Hind*III digestion as size markers; lane 2, pGEM-T vector (3 kbp); lane 3, pGEM-T/partial *Rho1Ps*; lane 4, inserted fragments (upper arrow: 560 bp) by digesting with *Sac*I and *Sac*II; lane 5, PCR product (lower arrow: 510 bp).

compared with that of BAP-untreated segments. To confirm the activation of *Rho1Ps* transcription by BAP, dosage-dependent analysis was conducted at various concentrations ranged from 10^{-12} to 10^{-4} M BAP. As shown in Fig. 4, application of 10^{-12} M

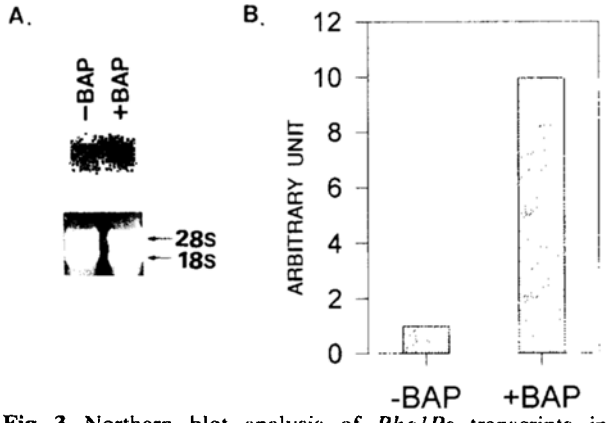

Fig. 3. Northern blot analysis of *Rho1Ps* transcripts in 10^{-6} M BAP treated (+BAP) and BAP-untreated (-BAP) segments after 24 h incubation. A; RNA from the segments was separated on a formaldehyde agarose gel and hybridized to ^{32}P-labelled *Rho1Ps* cDNA (upper gel). RNA was stained with ethidium bromide to compare the amount of ribosomal RNA (lower gel). 28S and 18S represent the positions of large and small ribosomal RNAs. B; Normalized intensity of *Rho1Ps* transcripts signals measured by densitometric scanning of X-ray film. The unit 1 in Y-axis represents the amount of *Rho1Ps* transcripts in BAP-untreated segments after 24 h incubation.

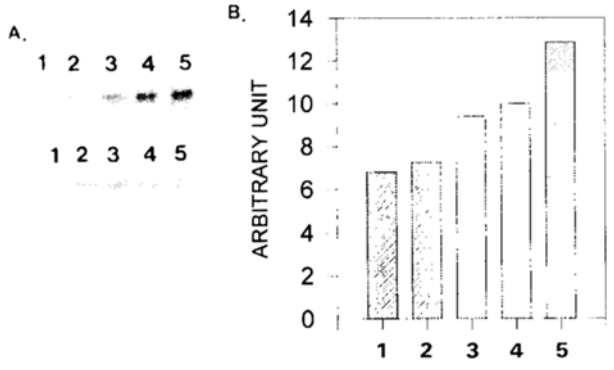

Fig. 4. A dosage-dependent analysis of *Rho1Ps* transcripts in the 24h incubated segments by Northern blot hybridization. A; RNA from the segments was separated on a formaldehyde agarose gel and hybridized to the ^{32}P-labelled *Rho1Ps* cDNA (upper gel). Rehybidization with a probe for 25S ribosomal RNA to normalize (lower gel) B; Normalized intensity of *Rho1Ps* transcripts signals measured by densitometric scanning of X-ray film. The unit 1 in Y-axis represents the amount of *Rho1Ps* in BAP-untreated segments for 24 h incubation. The data shown in B are calculated amounts based on the relative amounts of *Rho1ps* transcripts between BAP-untreated and 10^{-6} M BAP-treated segments in Fig. 3b. Lane 1, 2, 3, 4 and 5 represent the concentration of 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}, 10^{-4} M BAP, respectively.

BAP, the lowest concentration tested, enhanced the synthesis of *Rho1Ps* transcripts in the segments by approximately 7 times compared with those in BAP-untreated segments, and the enhancement of *Rho1Ps* transcripts synthesis was dependent on the concentration of BAP. Based on the strong activation activity and the well-matched dosage-dependent response, therefore, it is obvious that BAP activates the expression of *Rho1Ps* gene.

Time course analysis for *Rho1Ps* gene expression in the segments was undertaken. In BAP-treated segments, as shown in Fig. 5, total amounts of *Rho1Ps* transcripts was increased for 12 h incubation and then decreased. The pattern of increase in the total amounts of transcripts in the BAP-treated segments was similar to that in the BAP-untreated segments, implying that application of BAP to the segments may simply activate the synthesis of *Rho1Ps* transcripts. In respect to effectiveness of BAP for activating *Rho1Ps* gene expression, however, a different time course response was obtained. The activation rate of *Rho1Ps* gene by BAP was maximized in the segments for 24 h incubation (Fig. 5b). The difference in two time course responses suggests that the enhanced level of *Rho1Ps* gene expression by BAP is also due to the inhibition of *Rho1Ps* tran-

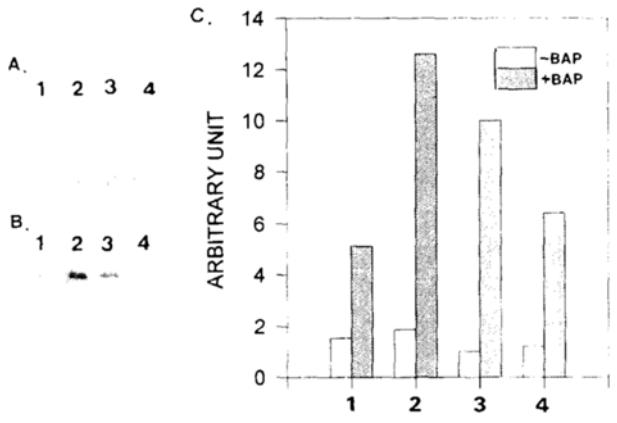

Fig. 5. A time course analysis of *Rho1Ps* transcripts in 10^{-6} M BAP-treated (+BAP) and BAP-untreated (-BAP) segments by Northern blot hybridization. A and B; RNA from the segments was separated on a formaldehyde agarose gel and hybridized to ^{32}P-labelled *Rho1Ps* cDNA (upper gels in A and B). Rehybridization with a probe for 25S ribosomal RNA to normalize (lower gels in A and B). C Normalized intensity of *Rho1Ps* transcripts signals measured by densitometric scanning of X-ray film. The unit 1 in Y-axis represents the amount of *Rho1Ps* in BAP-untreated segments for 24 h incubation. The data shown in B are calculated amounts based on the relative amount of *Rho1ps* transcripts between BAP-untreated and 10^{-6} M BAP-treated segments in Fig. 3b. Lane 1, 2, 3 and 4 represent the time incubated for 6, 12, 24, and 48 h, respectively.

scripts degradation.

In this study, a strong activation of *Rho1Ps* gene expression by application of a synthetic cytokinin, BAP in the shoot apex segments of garden pea was established. This finding positively indicate that the endogenous cytokinins in the segments play an important role in regulating expression of *Rho1Ps* gene. It has been reported that *Rho1Ps* gene is highly expressed in the meristematic tissues and is closely related to cell division in the tissues of garden pea (Yang and Watson, 1993). Thus it is quite interesting that *Rho1Ps* gene expression is remarkably enhanced by a cell division activating hormone.

Members of the Rho family of small GTP-binding proteins in yeast and animals share unique structural and functional features (Hall, 1990; Allen *et al.*, 1997; Koch *et al.*, 1997; Ridley and Hall, 1992; Tapon and Hall, 1997). *Rho1Ps* in garden pea also possesses all of the key structural features unique to members of the Rho family (Yang and Watson, 1993). Thus it is thought that *Rho1Ps* may play a central role in controling microfilament organization and is key components of signal transduction pathways regulating functions mediated by the actin cytoskeleton. In this respect, the activation of *Rho1Ps* gene expression by a cytokinin could be associated with regulating processes linked to the actin cytoskeleton in garden pea.

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