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GIBBERELLIN STIMULATES SYNTHESIS OF A PROTEIN KINASE IN DWARF PEA EPICOTYLS

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Key Word Index—*Pisum sativum*; Fabaceae; dwarf pea epicotyls; protein kinase; enzyme regulation by GA_3.

Abstract—Application of gibberellin A_3 (GA_3, 1 μM) stimulated protein kinase activity two-fold in light-grown dwarf pea epicotyls. This response was almost completely blocked by the simultaneous application of abscisic acid (ABA, 10 μM). Likewise, this GA_3-mediated increase in protein kinase activity was strongly inhibited by the application of cycloheximide (CHI, 20 $\mu g\,ml^{-1}$), indicating that *de novo* protein synthesis was necessary for the response. The enhancement of protein kinase activity was observed in crude and partially purified enzyme fractions prepared from untreated and GA_3-treated dwarf pea epicotyls. However, purified protein kinase (casein–Sepharose fraction) from untreated and GA_3-treated epicotyls showed no significant difference in their specific activities. These results clearly suggested that GA_3 stimulates protein kinase activity through *de novo* enzyme synthesis and not by enzyme activation. The M_r of the untreated and GA_3-stimulated protein kinase was 70 000. Analysis of the purified protein kinase on SDS–PAGE from untreated and GA_3-treated epicotyls showed a single protein-stained band with M_r 67 000, indicating that it is monomeric. Chemical characterization of the reaction product of the protein kinase showed ^{32}P-label in phosphoserine and phosphothreonine residues of the substrate casein. Significantly, the tall pea epicotyls contained 2.9-fold higher activity of the M_r 70 000 protein kinase than did epicotyls from dwarfs. Thus, it seems likely that the protein kinases activity is stimulated by endogenous GA_S. We have also observed a significant increase in the phosphorylation of endogenous proteins in GA_3-treated dwarf pea epicotyls over the untreated epicotyls.

INTRODUCTION

Post-translational modification of proteins by phosphorylation is crucial for the regulation of cellular metabolism in eukaryotes [1]. Proteins are phosphorylated at their serine, threonine or tyrosine residues by a variety of protein kinases. As a rule, protein kinases utilize ATP as a donor of the γ-phosphate group for the phosphorylation of proteins. Protein kinases are also known to dephosphorylate phosphoproteins through their reversed action in the presence of high concentrations of ADP [2]. Both casein kinases and histone kinases have been reported from plant cells [3, 4].

Protein kinases have been purified and characterized from our laboratory from wheat embryo [5], *Cicer* embryonic axis [6] and dwarf pea epicotyls [7]. In *Cicer*, a significant enhancement of protein kinase activity (10-fold) was observed during early germination of embryos (10 hr). This was essentially achieved through *de novo* enzyme synthesis. The M_r of the *Cicer* protein kinase was 94 000 and it was shown to be a heterodimer [6]. Likewise, a three- to four-fold increase in protein kinase activity was observed by us in germinating wheat embryos 12 hr after imbibition. This too was achieved by *de novo* enzyme synthesis as shown by labelling the enzyme with ^{35}S-labelled sulphate *in vivo*. The M_r of wheat protein kinase was 100 000 [5]. Protein kinases have also been purified from wheat germ [8–11], soya bean [12, 13] and spinach [14–16].

Phytohormones have been implicated in the regulation of protein phosphorylation in plant cells. Thus, increased phosphorylation of chromatin isolated from GA_3-treated dwarf pea plants compared with untreated plants was observed [17]. In another report, stimulation of phosphorylation of nuclear proteins by kinetin is reported in Chinese cabbage [18]. In cultured soya bean cells, zeatin brought about a three-fold increase in the phosphorylation of ribosomal proteins [19]. Increased phosphorylation of several nuclear proteins was reported in 2,4-D-treated soya bean hypocotyls with parallel increases in the nuclear protein kinase activity [20]. In *Lemna*, phosphorylation of ribosomal protein was inhibited by abscisic acid [21]. More recently, a protein kinase has been purified from dwarf pea epicotyls in our laboratory, using extensively purified RNA polymerase II as

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a substrate protein [7]. The M_r of this protein kinase was estimated to be 63 000 by SDS–PAGE and it was shown to be a monomer [7]. At present, the precise mechanism by which phosphorylation of proteins is regulated is not well understood. However, from the existing reports, it appears that several phytohormones could be involved in regulation of phosphorylation of proteins in plant cells.

In the present investigation, we report the stimulation of the activity of M_r 70 000 casein protein kinase by GA_3 in light-grown dwarf pea epicotyls. This stimulation was nullified by ABA and CHI. Additional experimental evidence indicated that GA_3 controls the *de novo* synthesis of this protein kinase. Increased phosphorylation of endogenous proteins was also observed in GA_3-treated dwarf pea epicotyls. Interestingly, light-grown tall peas showed a higher activity of a M_r 70 000 protein kinase than that observed in light-grown dwarf peas. These results suggest a role for GA_3 in the regulation of protein kinase activity in light-grown peas.

RESULTS AND DISCUSSION

Stimulation of protein kinase by GA_3 in dwarf pea

The fraction precipitated by 65% saturated ammonium sulphate, from an extract prepared from dwarf pea epicotyls 48 hr after treatment with GA_3, contained twice as much protein kinase activity as the equivalent fraction from untreated epicotyls (Table 1). Simultaneous application of abscisic acid (ABA, 10 μM) strongly inhibited the GA_3-mediated increase in protein kinase activity. Cycloheximide (CHI, 20 $\mu g\,ml^{-1}$) also completely nullified the GA_3-stimulated protein kinase activity, suggesting a requirement for *de novo* protein synthesis. Stimulation of protein kinase activity by GA_3 (1 μM) was also observed in a partially purified enzyme fraction, prepared from the ammonium sulphate ppt (0–65% satn) by molecular sieve chromatography on Sephacryl S-200. We observed three molecular forms of protein kinase in untreated and GA_3-treated tissue (data not presented). The activity of a M_r 70 000 form was stimulated three-fold by GA_3 treatment compared with untreated dwarfs (Fig. 1). This observation strongly suggested the selective role of GA_3 in the regulation of this M_r 70 000 protein kinase in pea epicotyls.

The light-grown dwarf pea showed dramatic elongation of the epicotyls in response to exogenous application of GA_3 (1 μM). In contrast, light-grown tall pea failed to respond to the external application of GA_3 in terms of further elongation of epicotyls. Since light-grown tall peas are known to possess higher levels of extractable GA_3 than do light-grown dwarf peas [22], we thought of comparing the catalytic activity of a M_r 70 000 casein protein kinase in Sephacryl S-200 fractions prepared from epicotyls of light-grown tall and dwarf peas, with the prime objective of determining the possible role for GA_3 in the regulation of protein kinase activity in nature. The specific activity of protein kinase in tall pea was significantly greater (10 nmol of Pi incorporated per mg protein) than that observed in the dwarf pea (3.4 nmol of Pi incorporated per mg protein). Clearly, there was a 2.9-fold stimulation of the M_r 70 000 protein kinase in the tall pea in comparison with the dwarfs. It may be stressed that the external application of GA_3 to light-grown tall pea failed to stimulate the activity of the M_r 70 000 protein kinase, as was witnessed in light-grown dwarf pea (data not presented). Thus, it appears that GA_3 has a physiological role in the regulation of a protein kinase in light-grown dwarf pea.

With a view to determine whether GA regulates protein kinase activity through *de novo* enzyme synthesis or by enzyme activation, we purified this kinase from both untreated and GA_3-treated dwarf pea epicotyls. This approach would enable us to compare the specific activity of purified protein kinase in untreated and GA_3-treated epicotyls. Thus, the Sephacryl S-200 fraction from untreated and GA_3-treated dwarf pea epicotyls was purified to electrophoretic homogeneity by casein–Sepharose affinity chromatography. The casein–Sepharose fraction showed a single protein stained band (M_r 67 000) on SDS–PAGE both in untreated and GA_3-treated dwarf pea epicotyls (Fig. 2). Thus, it appears that protein kinase in untreated and GA_3-treated epicotyls is a monomer. The purified protein kinase showed no significant difference in its specific activity in untreated (41 nmol of Pi incorporated per mg protein) and GA_3-treated pea epicotyls (39 nmol of Pi incorporated per mg protein). We, therefore, conclude that the GA_3-mediated increased protein kinase activity in dwarf pea epicotyls is achieved through *de novo* enzyme synthesis and not by enzyme activation. Chemical characterization of the ^{32}P-labelled reaction product of protein kinase activity from untreated and GA_3-treated tissue showed phosphorylation at the serine and threonine residues of the casein sub-

Table 1. Stimulation of protein kinase activity by GA_3 and its negation reversal by abscisic acid and cycloheximide in light-grown dwarf pea epicotyls

Treatment	Protein kinase activity (pmol Pi incorporated mg^{-1} protein)	Relative activity
Untreated	266	1.00
GA_3 (1 μM)	539	2.03
GA_3 (1 μM) + ABA (10 μM)	260	0.98
GA_3 (1 μM) + CHI (20 $\mu g\,ml^{-1}$)	285	1.07

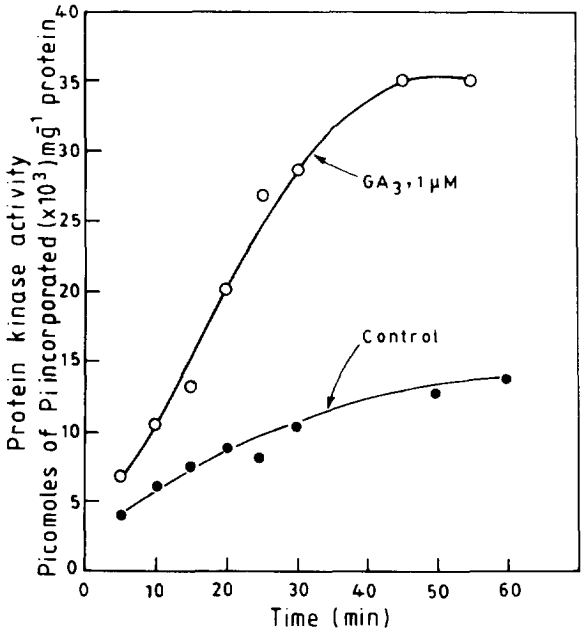

Fig. 1. Stimulation of protein kinase activity by GA_3 in light grown dwarf pea epicotyls. Ammonium sulphate ppt (0–65% satn) was fractionated by molecular sieve chromatography on Sephacryl S-200. Protein kinase was assayed in Sephacryl S-200 fr. prepared from untreated and GA_3-treated epicotyls.

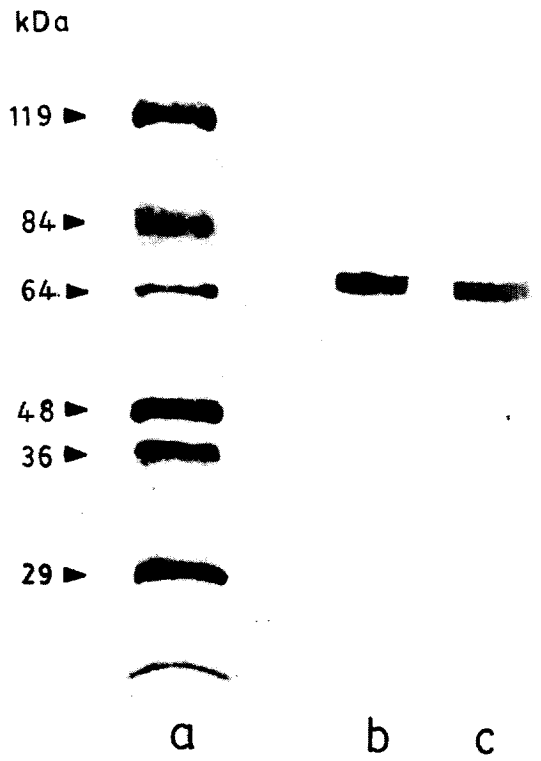

Fig. 2. Analysis of purified protein kinase on SDS–PAGE from untreated and GA_3-treated light grown dwarf pea epicotyls. Protein kinase was purified from untreated and GA_3-treated dwarf pea epicotyls by casein–Sepharose affinity chromatography. The casein–Sepharose frs were reported on SDS–PAGE (7.5%). Lane a, marker proteins; lane b, protein stained band of protein kinase from untreated epicotyls; lane c, protein stained band of protein kinase from GA_3-treated epicotyls.

strate. This was demonstrated by the chromatographic separation of hydrolysate of reaction product by paper chromatography. Two radioactive spots (^{32}P-label) were observed, coinciding with the authentic phosphoserine and phosphothreonine amino acids.

GA_3-regulated phosphorylation of endogenous proteins in vivo

Since GA_3 stimulated a single molecular form of casein protein kinase in light-grown dwarf pea epicotyls, it was of interest to us to determine its physiological significance in terms of phosphorylation of endogenous proteins. Therefore, we determined the extent of phosphorylation of endogenous proteins in untreated and GA_3-treated dwarf pea epicotyls. For this purpose, untreated and GA_3-treated pea epicotyls were incubated in a solution of [^{32}P]orthophosphate for 12 hr. The incorporation of ^{32}P-label in the total protein fraction was determined in untreated and GA_3-treated epicotyls. The specific radioactivity of ^{32}P-label in the protein fraction was significantly greater in GA_3-treated epicotyls (2.4×10^4 dpm per mg protein) as compared with the untreated (0.54×10^4 dpm per mg protein). Thus a unique feature of the present finding is the quantitative enhancement of protein phosphorylation (*ca* 4.4-fold) in GA_3-treated light-grown dwarf pea. Tentatively, we consider that the increased protein kinase activity in GA_3-treated dwarf pea epicotyls could enhance phosphorylation of endogenous proteins. There are several reports which have claimed a regulatory role for phytohormones in protein phosphorylation. In dwarf pea plants, GA_3 increased the phosphorylation of chromatin [17], although this was not shown to be associated with the regulation of a protein kinase by GA_3. In Chinese cabbage leaf disc, kinetin stimulated *in vivo* phosphorylation of proteins [18]. In the cytokinin-requiring strains of cultured soya bean cells, addition of zeatin brought about a three-fold stimulation of phosphorylation of ribosomal proteins [19]. Likewise, increased phosphorylation of several nuclear proteins was reported in 2,4-D-treated soya bean hypocotyls. A membrane fraction, isolated from soya bean hypocotyls, showed a small, but very rapid increase in ^{32}P incorporation into the acid insoluble protein fraction [23]. In *Lemna*, phosphorylation of ribosomal proteins was inhibited by abscisic acid [21]. These studies indicate a role for phytohormones in the regulation of protein phosphorylation in a variety of plants. Although induction of protein kinase was reported in germinated seeds of *Cicer* and wheat, nothing was stated about the factors that regulate kinase activity [5, 6]. The present study clearly revealed a role for GA in the regulation of a specific protein kinase in light-grown dwarf pea. However, our findings do not rule out the possibility of the

regulation of other classes of protein kinase by GA_3 that accept different substrate proteins.

EXPERIMENTAL

Materials. Dwarf pea (*Pisum sativum* L. var. arkel) and tall pea (*P. sativum* L. var. azad) seeds were purchased from the Indian Agricultural Research Institute (Experimental Station, Karnal, India).

Reagents. [γ^{32}P]ATP (3000 Ci mmol^{-1}) was purchased from Amersham and also synthesized in our laboratory by the procedure given in ref. [24]. Casein was a product of Sigma. [^{32}P]H_3PO_4 (carrier-free) was purchased from Bhabha Atomic Research Centre (Bombay, India).

Seed germination. Dwarf and tall pea seeds were surface sterilized with $HgCl_2$ (0.02%) for 10 min and then washed thoroughly with H_2O. Thereafter, the seeds were imbibed in H_2O for 6 hr. These seeds were grown in light at 25° for 6 days.

Spray application of phytohormones. Dwarf pea plants (6-days-old) were sprayed with GA_3 (1 μM), GA_3 (1 μM) + ABA (10 μM) and GA_3 (1 μM) + CHI (20 μg ml^{-1}). The spray application was repeated after 24 hr. Likewise, the light-grown tall pea plants were also sprayed with GA_3 (1 μM). The untreated plants were sprayed with sterile H_2O. Dwarf and tall pea epicotyls were harvested 48 hr after treatment.

Purification of protein kinase. Epicotyls, excised from 8-day-old untreated and GA_3-treated dwarf pea plants, were homogenized in Tris–HCl buffer (250 mM, pH 8) containing EDTA (0.2 mM), $(NH_4)_2SO_4$ (75 mM), $MgCl_2$ (5 mM), 2-mercaptoethanol (50 mM) and PVP (2%, w/v). Acid washed sand was used as an abrasive. The homogenate was passed through 4 layers of muslin and centrifuged at 17000 *g* for 15 min at 4°. The clear supernatant was subjected to polymin-P (0.7%) ppt. The pptd nucleic acid bound protein was removed by centrifugation at 17000 *g* for 10 min. The clear supernatant was pptd with $(NH_4)_2SO_4$ (0–65% satn). The pellet, obtained after centrifugation, was suspended in Tris-acetate buffer (25 mM, pH 7.6) containing Mg-acetate (3 mM) and 2-mercaptoethanol (5 mM). The same buffer was used for dialysis of this enzyme. The desalted $(NH_4)_2SO_4$ ppt. (30 mg protein) was subjected to molecular sieve chromatography on Sephacryl S-200 column. Frs (2 ml each) were collected soon after the void vol. (42 ml). The Sephacryl S-200 fr. containing protein kinase activity, was subjected to casein–Sepharose affinity chromatography (5 mg protein per ml of matrix). The matrix was equilibrated with Tris-acetate buffer (25 mM, pH 7.6) containing Mg-acetate (3 mM) and 2-mercaptoethanol (5 mM). After binding the sample (at a flow rate of 1 ml per 4 min), the column was washed with 10 bed vols of the same buffer. The protein eluted with 0.25 M KCl was discarded. The casein–Sepharose fr. eluted between 0.25 and 0.5 M KCl was dialysed and concd by lyophilization. Protein kinase activity was assayed at each step of enzyme purification.

Assay of protein kinase. The assay mixt. comprised of Tris-acetate buffer (20 mM, pH 7.6); Mg-acetate (15 mM), [γ^{32}P]ATP (40 μM, 880 dpm mol^{-1}), dephosphorylated casein (500 μg) and enzyme fr. (5–100 μg) in a final vol. of 250 μl. The reaction was carried out for 30 min at 25°. An aliquot of the reaction mixt. (40 μl) was spotted on a Whatman 3 MM disc (20 mm × 20 mm) and immediately immersed in chilled TCA (5%, w/v, at 4°, 10 ml per filter) containing Na-pyrophosphate (20 mM). The filter discs were washed with hot TCA (5%, w/v, at 80° for 20 min) followed by successive washings (× 3) with chilled TCA (5%), Et_2O–EtOH (1 : 1, 10 ml per disc) and finally with Et_2O (10 ml per disc). The filters were dried in an oven (60°) for 2 hr and radioactivity was measured by the Beckman Liquid Scintillation Spectrometer LS 1801.

PAGE. Purified protein kinase (casein–Sepharose fr.) was fractionated on SDS–PAGE (7.5%) by the method given in ref. [25] for determining the subunit structure of the enzyme. The gels were stained with Coomassie Brilliant Blue R-250 for the visualization of the protein stained band of purified protein kinase.

In vivo labelling of phosphoproteins with [^{32}P]orthophosphate. Dwarf pea epicotyls (5 g tissue), excised from untreated and GA_3-treated tissues, were incubated in a soln of [^{32}P]orthophosphate (5 mCi each) for 12 hr. The epicotyls were harvested, rinsed in H_2O and homogenized in Tris–HCl buffer (100 mM, pH 8). The homogenate was centrifuged at 15000 *g* for 20 min at 4°. An aliquot, containing 100 μg protein of the supernatant fr. from untreated and GA_3-treated tissue was spotted on a Whatman 3 MM disc and immediately immersed in chilled TCA (5%, w/v, for 10 min). The filter discs were then transferred to hot TCA (80° for 30 min) for the hydrolysis of [^{32}P]-labelled nucleic acids. This was followed by washing the filter discs (× 4) in chilled TCA (5%), Et_2O–EtOH (1 : 1) and finally with Et_2O. The filter discs were dried in an oven for 2 hr at 60° and radioactivity incorporated into the protein fr. was determined.

Characterization of [^{32}P]-labelled reaction product of protein kinase. The [^{32}P]-labelled reaction product of protein kinase was hydrolysed by 6 M HCl at 110° for 2 hr under N_2 in a sealed ampoule. Authentic samples of phosphoamino acids (phosphoserine, phosphothreonine and phosphotyrosine: 20 μg each) were added to the hydrolysed sample as carriers. The hydrolysate was neutralized and chromatographed for the separation of phosphoamino acids by the procedure given in ref. [5].

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