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INORGANIC PHOSPHATE MIMICS THE SPECIFIC ACTION OF GIBBERELLIC ACID IN REGULATING THE ACTIVITY OF MONOPHENOLASE IN EMBRYO-LESS HALF-SEEDS OF WHEAT

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Key Word Index *Triticum aestivum*, Gramineae, embryo-less wheat half-seeds, monophenolase, enzyme regulation, inorganic phosphate mimics GA_3 action

Abstract The GA_3-mediated activation of monophenolase (about 12-fold stimulation) in embryo-less half-seeds of wheat was mimicked by the addition of inorganic phosphate (Pi, 50 mM). Similarly, the hormone-induced altered molecular properties of monophenolase, such as the shift in the pH optimum towards alkalinity (pH 9.0), the relatively increased thermostability of the enzyme at 55° and the changed pattern of multiple forms of the enzyme, were also seen in Pi-treated half-seeds. Furthermore, the simultaneous addition of GA_3 and Pi to wheat half-seeds showed no cumulative effect on the enhancement of monophenolase activity. This indicated that both GA_3 and Pi regulated monophenolase activity through a common mechanism. Abscisic acid effectively blocked the GA_3- and Pi-directed stimulation of monophenolase. Since GA_3 treatment of half-seeds increased the free pool of Pi (2.5-fold), we envisage that the Pi-mediated activation of monophenolase is of physiological relevance in our system. However, the *in vitro* addition of Pi (50 mM) to water-imbibed control half-seeds (48 hr) during enzyme extraction failed to activate monophenolase. We thus consider that the high pool of Pi, generated by GA_3 in half-seeds, or even the direct addition of Pi to half-seeds *in vivo*, seems necessary for some metabolic events which eventually trigger the activation of monophenolase.

INTRODUCTION

The activation of phenolases has been reported by the *in vitro* treatment of enzyme preparations with various chemicals [1]. A short exposure of chloroplast preparations to acid (pH 3.0–3.5) and alkali (pH 11.5) resulted in the activation of latent phenolases in *Vicia faba* [2, 3]. Activation of phenolases has also been observed by treating the enzyme fractions with detergents [4], trypsin [5] and urea [6]. It has been suggested that conformational changes in the preformed enzyme molecule could be responsible for the activation of phenolases [7]. In mushroom and avocado tyrosinase was activated by the addition of hydrogen peroxide (6 mM) and hydroxylamine (1.5 mM) during the assay of the enzyme. Both chemicals are responsible for reducing the lag period of tyrosine hydroxylation [8, 9].

Phytohormones (GA_3 and auxin) have been implicated in the regulation of phenolases in plant cells [10–14]. Earlier, we showed that the stimulation of monophenolase activity by GA_3 was brought about by the activation of preformed enzyme molecules *in vivo* [10–12]. The activated monophenolase exhibited altered molecular properties, such as a shift in the pH optimum, relatively high thermostability of enzyme and altered electrophoretic mobility of its multiple forms [11]. Subsequently, we observed two activity peaks of monophenolase in reponse to GA_3-treatment and only one in control half-seeds [12]. Hormone treatment of half-seeds possibly brings about some covalent modification of the performed enzyme that could be responsible for the activation of monophenolase [11, 12].

In the present investigation, we have observed that the stimulation of monophenolase activity by GA_3 can be mimicked by the exogenous addition of Pi (50 mM) under *in vivo* conditions. Abscisic acid effectively blocked the activation of monophenolase elicited both by GA_3 and Pi. Since GA_3 enhances the free pool of Pi, we consider that the activation of monophenolase in hormone-treated half-seeds is also dependent on the high levels of Pi.

RESULTS

Stimulation of monophenolase activity by inorganic phosphate ions

In our earlier communications [11, 12], we reported the activation of monophenolase (7–9-fold at pH 9.0) by the *in vivo* application of GA_3 to embryo-less half-seeds of wheat. We have now observed that the enhancement of monophenolase activity, elicited by GA_3, can also be achieved by imbibing half-seeds of wheat in a medium containing inorganic phosphate (Pi, 50 mM; Table 1). Thus, Pi selectively mimics the action of phytohormones in regulating the activity of monophenolase in wheat system. Figure 1 depicts the activity profiles of monophenolase as a function of the Pi concentration. A significant enhancement of monophenolase (12–13-fold) was observed in half-seeds imbibed in 40 mM Pi (Fig. 1). However, addition of Pi to control half-seeds (50 mM) during enzyme extraction, or its presence during the assay

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Table 1 Stimulation of monophenolase activity by GA_3 and inorganic phosphate and its inhibition by ABA in embryo-less half-seeds of wheat

Additions	Monophenolase activity A_{420} units mg protein	Relative activity
Control	0 04	1 0
GA_3 (10^{-5} M)	0 50	12 5
Pi (50 mM)	0 52	13 0
GA_3(10^{-5} M) + Pi (50 mM)	0 48	12 0
GA_3(10^{-5} M) + ABA (5×10^{-5} M)	0 09	2 25
Pi (50 mM) + ABA (5×10^{-5} M)	0 09	2 25

The half-seeds were imbibed in the presence of GA_3, Pi, GA_3 + Pi, GA_3 + ABA and Pi + ABA for 48 hr at 25° in dark The enzyme activity was assayed in a desalted $(NH_4)_2SO_4$ fraction precipitate (30-50% satn)

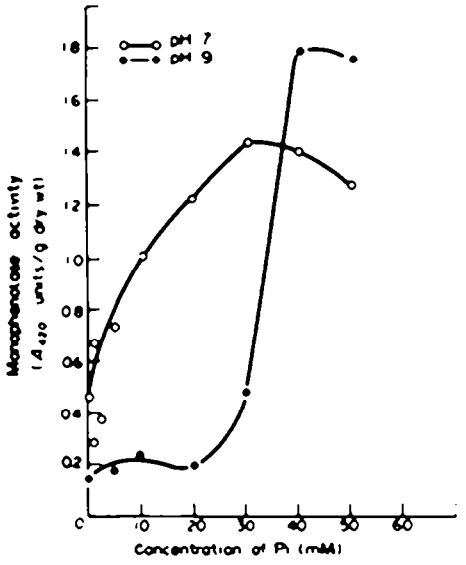

Fig 1 Stimulation of monophenolase activity as a function of Pi concentration The half-seeds were imbibed in the presence of different concentrations of Pi (0-50 mM) for 48 hr The enzyme activity was assayed in dialysed crude extracts at pH 7 0 and 9 0

of enzyme, failed to stimulate the activity of monophenolase over that of the controls On the other hand, enzyme extract (30-50% ammonium sulphate fraction precipitate) prepared from Pi-imbibed half-seeds retained high monophenolase activity even after removal of Pi by exhaustive dialysis against Tris-HCl buffer (20 mM, pH 7.0). These experiments clearly demonstrated that Pi is not directly responsible for the enzyme activation We speculate that the *in vivo* addition of Pi to half-seeds is necessary for some metabolic events that eventually result in the activation of monophenolase

Altered molecular properties of monophenolase in Pi-treated half-seeds

Since Pi mimicked the action of GA_3 in stimulating monophenolase activity in wheat half-seeds, our next experiment was to compare the molecular properties of the enzyme in GA_3- and Pi-treated half-seeds The activity profiles of monophenolase as a function of pH were remarkably similar in GA_3- and Pi-treated half-seeds (Fig 2). In both, there was a shift in the pH optimum towards alkalinity (pH 9.0) in comparison with the controls (pH 7 0, Fig. 2). We also compared the pattern of multiple forms of monophenolase in GA_3- and Pi-treated half-seeds In both, two multiple forms of monophenolase could be physically separated by Sephacryl S-200 (Fig 3) The elution profile of two activity peaks (peak I, peak II) by molecular sieving was identical in GA_3- and Pi-treated half-seeds. This is in contrast to control half-seeds where

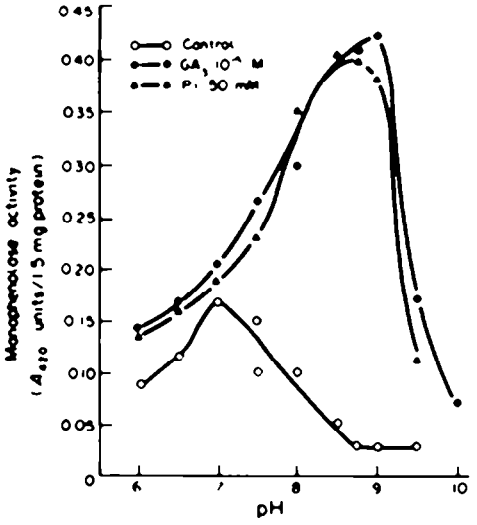

Fig 2 The pH activity profiles of monophenolase in the control, GA_3- and Pi-treated half-seeds The $(NH_4)_2SO_4$ fraction precipitate (30-50% satn) was used for the assay of monophenolase activity at different pH values (pH 6 0-10)

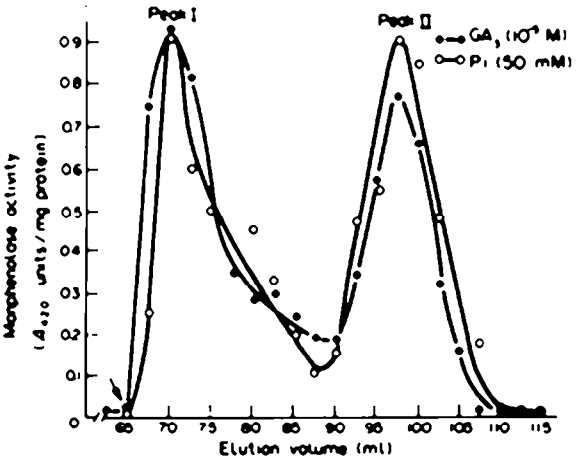

Fig 3 Multiple forms of monophenolase in GA_3- and Pi-treated half-seeds of wheat An aliquot (2 ml, 30 mg) of $(NH_4)_2SO_4$ fraction precipitate (30-50% satn) was loaded on the Sephacryl S-200 for the separation of multiple forms of monophenolase The arrow indicates the void volume (V_o = 68 ml) of the column

only a single molecular form (peak II) was reported by us [12]. The M_r of peak I is 180000, while that of peak II is 45000. Thus, in control half-seeds, only the low M_r form was observed [12] while in GA_3- and Pi-treated half-seeds both the low and high M_r forms were present. Peak I appears to be a tetrameric form and is possibly formed by the oligomerization of the low M_r form. Since both the activity peaks in GA_3- and Pi-treated half-seeds showed a shift in pH optimum (data not presented, [12]), we consider that the enzyme in both GA_3- and Pi-treated half-seeds has possibly undergone a similar covalent modification.

Since GA_3-treatment of half-seeds is known to confer heat stability to monophenolase enzyme activity [11], we compared the thermostability of this enzyme in Pi- and GA_3-treated half-seeds from their heat inactivation curves. In both treatments, the enzyme was relatively thermostable at 55°. There was no substantial decrease in the enzyme activity in Pi- and GA_3-treated (8–10%) half-seeds of wheat, while the enzyme isolated from control half-seeds showed a significant loss (70%) of activity (data not presented, [11]). Thus, it is evident that the altered molecular properties of monophenolase reported earlier in GA_3-treated half-seeds [11, 12] are also seen in Pi-treated half-seeds.

Levels of Pi in GA_3-treated half-seeds

We then went on to determine whether GA_3-mediated activation of monophenolase activity could be achieved through the modulation of Pi levels *in vivo*. This aspect was tested by measuring the free pool of Pi in the imbibition medium of control and GA_3-treated half-seeds. We observed a consistent increase in the free pool of Pi (2.5-fold) over that of the controls. Thus, it appears that stimulation of monophenolase by GA_3 is mediated by the rise in the levels of Pi. This is quite tenable since Pi alone can mimic the action of GA_3 in activating monophenolase.

Stimulation of phosphatases by GA_3 in wheat half-seeds

Since GA_3 is known to enhance the activity of many hydrolytic enzymes in wheat and barley half-seeds [15], we thought that the high pool of Pi in hormone-treated tissue could be due to increased activity of phosphatases. GA_3 treatment of half-seeds did enhance the activities of acid phosphatase (3-fold) and also that of alkaline phosphatase (7-fold). The GA_3-elicited phosphatase activity could thus account for the increased levels of Pi in wheat half-seeds. However, exogenous addition of Pi (50 mM) to the imbibition medium showed no increase in the levels of phosphatases over those of the controls.

Inhibition of monophenolase activity by ABA

Abscisic acid (5×10^{-5} M) not only inhibited the GA_3-stimulated monophenolase activity (82%) but was equally potent in blocking the Pi-activated monophenolase activity in wheat half-seeds (Table 1). We also checked the effect of ABA on the activity of phosphatases. A significant inhibition of both acid and alkaline phosphatase was observed in the presence of ABA (data not presented). Concomitantly ABA also brought about a considerable decline (72% inhibition) in the free pool of Pi in GA_3-treated half-seeds. Thus, it appears that ABA can block the activation of monophenolase by operating at multiple sites.

DISCUSSION

The present investigation provides a further clue to the understanding of the mode of action of GA_3 in the regulation of monophenolase activity in embryo-less half-seeds of wheat. Earlier, we showed that the stimulation of monophenolase activity by GA_3 in wheat half-seeds was associated with the altered molecular properties of the enzyme. The activation of monophenolase by GA_3 possibly occurs through some post-translational modification of the preformed enzyme. The precise nature of the structural modification of monophenolase is so far not known [11, 12].

The present finding has revealed that imbibition of half-seeds in a medium containing Pi (50 mM) remarkably mimics the action of GA_3 in stimulating the activity of monophenolase. The enhancement of monophenolase activity in Pi-treated half-seeds also represents a case of enzyme activation. This contention is supported by the fact that the enzyme exhibited altered molecular properties, such as a shift in the pH optimum towards alkalinity (pH 9.0), relative thermostability of the enzyme and an altered pattern of multiple forms in a manner similar to that observed in GA_3-treated half-seeds. Thus, the molecular properties of the monophenolase in Pi-treated half-seeds were in no way different from those witnessed in GA_3-treated half-seeds.

The *in vitro* addition of Pi to water-imbibed half-seeds (control) during enzyme extraction, however, failed to activate or alter the molecular properties of the enzyme. Clearly, Pi does not seem to directly activate the preformed enzyme. In contrast, enzyme extracts, isolated from Pi-imbibed half-seeds, remained in an activated form even though Pi was removed by exhaustive dialysis against Tris–HCl buffer. Apparently, the presence of Pi under *in vivo* conditions is responsible for some modification of the preformed enzyme molecules that could be responsible for enzyme activation and altered molecular properties of monophenolase. It was further observed that the extent of activation of monophenolase by GA_3 and Pi in wheat half-seeds was almost identical. However, in no instance did the simultaneous addition of GA_3 and Pi to half-seeds showed any additive effect on monophenolase activity. We therefore infer that both GA_3 and Pi regulate monophenolase activity through a common mechanism. The stimulation of monophenolase activity by GA_3 in embryo-less half-seeds of wheat represents a true biological response, since a similar enhancement of enzyme activity was also witnessed in half-seeds dissected from water-imbibed intact seedlings (48 hr). In this case, the source of GA_3 is the embryonic tissue of the seedling. Since the exogenous addition of Pi mimics the action of GA_3 in modulating monophenolase activity, we now propose that the stimulation of monophenolase activity by GA_3 is mediated through the rise in the levels of Pi. In fact a significant increase in the free pool of Pi (2.5-fold) has been observed in GA_3-treated half-seeds over that of the controls. Thus the enhancement of monophenolase activity by Pi in wheat half-seeds also depicts a course of events which normally occurs during the imbibition of seeds.

The GA_3-stimulated monophenolase activity was blocked by ABA. Curiously, ABA was equally effective in

curtailing the Pi-stimulated monophenolase activity. Thus then leads us to speculate that Pi itself is not directly responsible for enzyme activation, but must participate in some metabolic pathway before it can activate monophenolase enzyme.

Finally, we now put forth a hypothesis to explain the regulation of monophenolase by GA_3 in wheat half-seeds. The course of events appears to be as follows: to begin with, GA_3 activates phosphatases in the embryo-less half-seeds of wheat. The hormone-elicited activity of phosphatases is responsible for the increased build up of the free pool of Pi through the degradation of polyphosphates. The GA_3-induced rise in the levels of Pi seems crucial for the activation of monophenolase. The main support for this postulate is derived from the fact that exogenous addition of Pi mimics the action of GA_3 in stimulating and altering the molecular properties of monophenolase.

Experiments are in progress to purify monophenolase so that we can eventually determine the nature of the modifications to monophenolase elicited by GA_3 and Pi in embryo-less half-seeds of wheat.

EXPERIMENTAL

Material. Wheat seeds (*Triticum aestivum* L. var. HD 2009) were procured from the Division of Seed Technology, Indian Agricultural Research Institute, New Delhi-110012, India.

Imbibition of half-seeds. The embryo-less half-seeds of wheat were imbibed for 48 hr in a medium containing 10^{-5} M GA_3, 5×10^{-5} M ABA and 5–50 mM Pi (as K Pi, pH 6.6). The half-seeds were imbibed under aseptic conditions in dark at 25° for 48 hr [11, 12]. The effect of these substances was tested on the activities of monophenolase, acid and alkaline phosphatases.

Preparation of the enzyme fraction. The imbibed half-seeds were homogenized (1:5 w/v) in Pi buffer (50 mM, pH 6.6) and subjected to $(NH_4)_2SO_4$ fraction precipitation (30–50%, satn.) as described in ref. [12]. The desalted enzyme preparation was used for the assay of monophenolase.

Sephacryl S-200 CC. The $(NH_4)_2SO_4$ ppt (30–50%, satn., 30 mg protein), prepared from Pi- and GA_3-treated wheat half-seeds, was fractionated on a Sephacryl S-200 column (70 × 1.8 cm) at 6°. The fractions (2.5 ml each) were collected immediately after the void volume (V_o = 68 ml). Tris–HCl buffer (25 mM, pH 7.0) was used for the equilibration of the column and for the elution of the protein fractions. Protein was determined in each fraction by the procedure given in ref. [16].

Determination of Pi levels. The procedure of ref. [17] was adopted for the determination of Pi released into the imbibition medium of control and GA_3-treated half-seeds.

Assay of phosphatases. Acid and alkaline phosphatase activities were determined as described in refs [18, 19].

Assay of monophenolase activity. The incubation mixture contained tyramine base (2 ml; 8 mM), Pi buffer (50 mM, pH 7.0) and enzyme fraction (1 mg protein) in a final vol. of 4 ml. The assay mixture was incubated for 1 hr at 37° and A_{420} was measured. The Pi buffer was substituted by Tris–HCl buffer (50 mM, pH 9.0) when the enzyme was assayed at pH 9.0. The tyramine base was dissolved in distilled water. The enzyme activity was also assayed at different pH values (6.0–10.0). The buffers were prepared as described in ref. [20].

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