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EARLY RESPONSE TO GIBBERELLIC ACID OF MONOPHENOLASE (TYROSINASE) ACTIVITY IN DE-EMBRYONATED HALF-SEEDS OF WHEAT

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Key Word Index—*Triticum aestivum*; Gramineae; de-embryonated wheat half-seeds; monophenolase; enzyme regulation; early response to GA_3; altered molecular properties of the enzyme.

Abstract—A significant enhancement (7–8 fold) of monophenolase activity by GA_3 was observed in de-embryonated half-seeds of wheat after 2 hr of imbibition. This early response to GA_3 (2–12 hr) was apparent only after lowering the endogenous levels of phytohormone in wheat half-seeds by leaching. Cycloheximide (CHI, 20 μg/ml) failed to inhibit GA_3-stimulated (GA_3, 10 μM) monophenolase activity. This ruled out the requirement of *de novo* protein synthesis for the hormone-regulated activity of monophenolase. Abscisic acid (100 μM) effectively stopped the GA_3-mediated activation of monophenolase. Curiously, the early stimulatory response to GA_3 of monophenolase activity was completely mimicked by the administration of phosphate ions (Pi, 75 mM). Simultaneous addition of GA_3 and Pi to wheat half-seeds, however, showed no cumulative effect on the activation of monophenolase. Both GA_3 and Pi-activated monophenolase exhibited altered molecular properties, in terms of shift in the pH optimum of enzyme activity towards alkalinity (pH 9.0) and relatively increased thermostability of the enzyme at 50°. Molecular sieving of the enzyme fraction on Sephacryl S-200 revealed a single molecular form of monophenolase (M_r 45 000) both in control and GA_3-treated wheat half-seeds during the first 12 hr of imbibition. This indicated that the activation of monophenolase by GA_3 is due to some structural modification of the enzyme without its oligomerization. We envisage that the modulation of monophenolase activity by GA_3 during early imbibition of wheat half-seeds is a true primary response to the phytohormone.

INTRODUCTION

Phytohormones play an important regulatory role in the control of gene expression in plant tissues [1]. *In vivo* application of GA_3 to barley and wheat aleurones triggers the activity of many hydrolytic enzymes [2]. The enhancement of enzyme activity is achieved either by the *de novo* synthesis of enzyme proteins or by the activation of latent enzymes. Activation of β-amylase [3], monophenolase [4–7], phosphorylcholine glyceride transferase [8, 9] and acid phosphatase [10] by GA_3 has been reported in cereal aleurones and wheat half-seeds. However, the precise molecular mechanism involved in the GA_3-mediated activation of enzymes is not well understood. In our earlier communications, we reported the activation of monophenolase by GA_3 in 48 hr imbibed half-seeds of wheat. Obviously, this would represent a delayed secondary response of the phytohormone in the regulation of monophenolase. Activation of phenolases by various chemical agents has been extensively reviewed by Mayer and Harel [11]. We now provide fresh evidence to show that the activation of monophenolase by GA_3 can be detected during early imbibition of half-seeds and can be considered a true primary response to the phytohormone. Abscisic acid exerted a negative control on the GA_3-stimulated monophenolase activity.

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RESULTS

Early response of GA_3 in the stimulation of monophenolase activity

The de-embryonated half-seeds of wheat, prior to imbibition, showed negligible monophenolase activity (0.015 A_{420} units/mg protein/hr). A rapid rise in monophenolase activity (0.13 A_{420} units/mg protein/hr) was observed during the first 6 hr of imbibition of wheat half-seeds in water. Application of GA_3 during early imbibition of wheat half-seeds showed no additional enhancement of enzyme activity over the water imbibed controls. The possibility that the endogenous pool of GA_3 in wheat half-seeds was quite adequate to account for the early stimulation of monophenolase activity was tested by lowering the endogenous levels of phytohormone in half-seeds before studying the effect of exogenously added GA_3 on monophenolase activity. We designed our experiment in such a manner that the imbibition and effective washing of half-seeds occurred simultaneously. Thus the half-seeds were imbibed in relatively large volumes of water (10 g/l H_2O) with constant stirring on a shaker. This ensured the leaching out of

endogenous pool of GA_3.* The addition of large amounts of exogenous GA_3 to the incubation medium enabled us to maintain high levels of phytohormone in the test half-seeds. The results of this experiment, clearly showed that the application of GA_3 to wheat half-seeds is indeed responsible for a rapid rise in the activity of monophenolase after 2 hr of inhibition (Fig. 1). Treatment of wheat half-seeds with GA_3 also resulted in the altered molecular properties of monophenolase in comparison to the control enzyme. The pH optimum of control enzyme was 7.0, while the pH optimum in hormone-treated half-seeds was 9.0 (data not presented). Thus, there was a distinct shift in the pH optimum of monophenolase towards alkalinity even during early imbibition of half-seeds (2–12 hr). In addition, the enzyme preparation from hormone-treated tissue revealed relatively increased thermostability at 50°. However, only a single molecular form (M_r 45 000) of monophenolase was present both in control and GA_3-treated half-seeds (12 hr; Fig. 2).

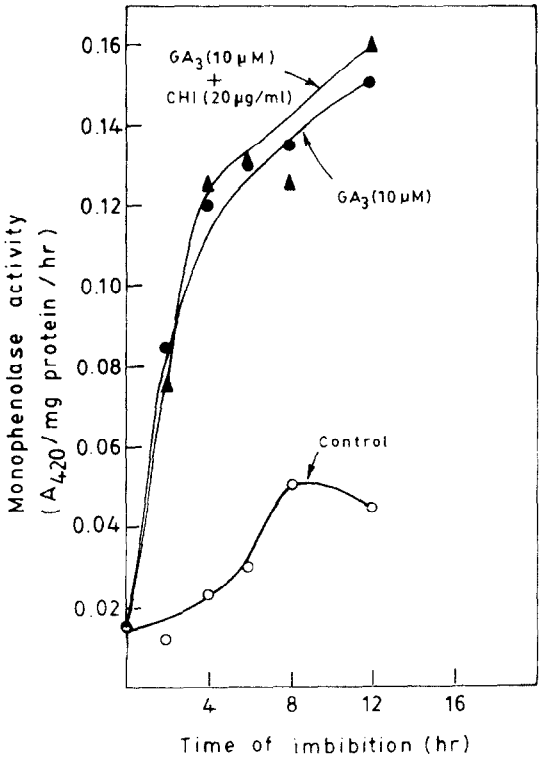

Fig. 1. Stimulation of monophenolase activity by GA_3 and its insensitivity towards the translation inhibitor CHI. The half-seeds were imbibed in H_2O, GA_3 (10 μM) and GA_3 (10 μM) + CHI (20 μg/ml) for different time intervals. The enzyme activity was assayed in the desalted $(NH_4)_2SO_4$ fraction precipitate (30–50% satn) at pH. δ9.0.

*To confirm that GA_3 was leached out of the half seeds by this treatment, we tested the effect of the leachate on the induction of α-amylase in wheat aleurones. Induction of α-amylase in wheat and barley aleurones is a well-established method for the bioassay of gibberellic acid [12]. The leachate was concentrated by lyophilization and bioassayed for GA_3. After 40 hr, a significant induction of α-amylase activity (5-fold) was observed in leachate-treated wheat aleurones over the controls. This strongly suggested that endogenous GA_3 was released on washing of wheat half-seeds.

Pi mimics the early stimulatory response to GA_3 of monophenolase activity

The early stimulatory effect of GA_3 on monophenolase activity was completely mimicked by the *in vivo* addition of Pi (75 mM) to half-seeds of wheat (Fig. 3). However, simultaneous addition of GA_3 (10 μM) and Pi (75 mM) showed neither an additive nor a synergistic effect on monophenolase activity during early stages of imbibition (Table 1). The stimulatory response of GA_3 and Pi on monophenolase activity was observed only *in vivo*. Pre-incubation of enzyme fraction [$(NH_4)_2SO_4$ fraction precipitate (30–50% satn)] from control half-seeds with GA_3 and Pi for 2–4 hr failed to enhance the activity of monophenolase.

Effect of CHI and ABA on GA_3-stimulated monophenolase activity

Cycloheximide (CHI, 20 μg/ml) failed to inhibit the GA_3-mediated increase in monophenolase activity during early imbibition of wheat half-seeds (2–12 hr). This indicated that the hormonal regulation of monophenolase is achieved by the activation of enzyme at the post-translational level (Fig. 1). Nevertheless, the antibiotic

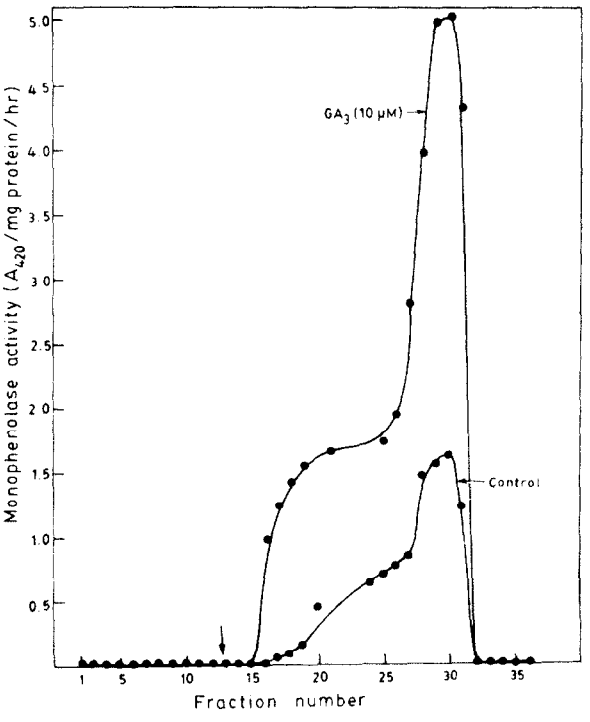

Fig. 2. Molecular form of monophenolase in control and GA_3-treated half-seeds of wheat. De-embryonated half-seeds were imbibed for 12 hr in the presence and absence of GA_3. An aliquot (1.5 ml, 30 mg protein) of the $(NH_4)_2SO_4$ fraction precipitate (30–50% satn) was fractionated on Sephacryl S-200. The arrow indicates the void volume of the column.

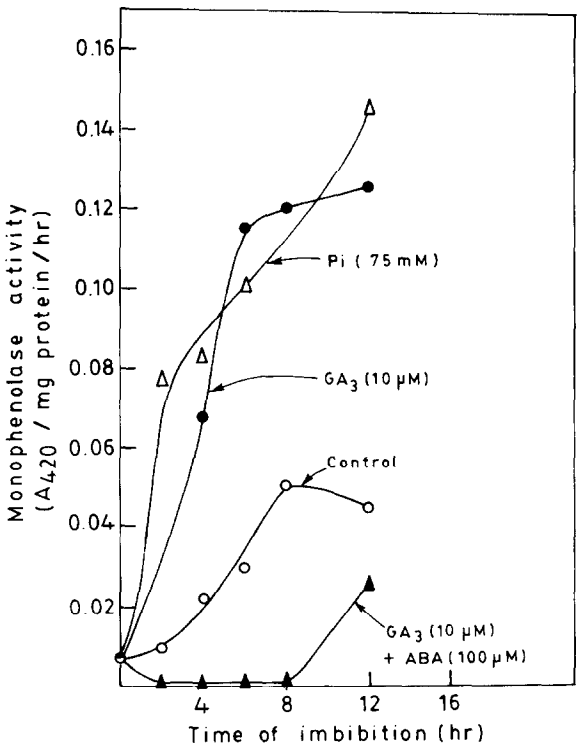

Fig. 3. Time course study depicting the stimulation of monophenolase activity by GA_3 and Pi and its inhibition by ABA in embryo-less half-seeds of wheat. The de-embryonated half-seeds were imbibed for different time intervals in the presence and absence of GA_3 (10 μM), Pi (75 mM) and GA_3 (10 μM) + ABA (100 μM). Monophenolase activity was determined in the desalted $(NH_4)_2SO_4$ fraction precipitate at pH 9.0.

Table 1. Stimulation of monophenolase activity by GA_3 and inorganic phosphate

Additions	Relative activity
Control	1.0*
GA_3 (10 μM)	9.7
Pi (75 mM)	10.0
GA_3 (10 μM) + Pi (75 mM)	8.3

*A_{420}/4 mg protein = 0.052.

The embryo-less half-seeds of wheat were imbibed in the presence and absence of GA_3, Pi, GA_3 + Pi for 12 hr in dark. Monophenolase activity was assayed in the desalted $(NH_4)_2SO_4$ fraction precipitate (30–50% satn) at pH 9.0.

proved quite effective (75%) in strongly inhibiting the GA_3-induced acid phosphatase in wheat half-seeds. We also tested the effect of ABA on GA_3-mediated activation of monophenolase in wheat half-seeds (2–12 hr). Abscisic acid (ABA, 100 μM) completely counteracted the GA_3-elicited stimulation of monophenolase activity (Fig. 3). These results indicated that ABA exerts a negative control on the GA_3-stimulated monophenolase activity.

DISCUSSION

The present investigation has revealed that the stimulation of monophenolase activity by GA_3 is indeed an early response to this hormone. The endogenous pool of GA_3 is normally sufficient to activate monophenolase during the first 12 hr of imbibition. Depletion of the internal pool of GA_3 by imbibing wheat half-seeds in a relatively large volume of water (1 g/100 ml) with constant stirring on a shaker was necessary to lower the basal level of monophenolase activity. A significant rise in the activity of monophenolase could then be elicited by the exogenous application of GA_3 (10 μM) to half-seeds imbibed for 2–12 hr. We consider that exhaustive washing of half-seeds during early imbibition probably lowers the endogenous levels of GA_3 which is normally crucial for enzyme activation. The GA_3-mediated stimulation of monophenolase activity was completely inhibited by the simultaneous addition of ABA (100 μM) to wheat half-seeds. Intriguingly, the imbibition of half-seeds in phosphate buffer (Pi, 75 mM) fully mimicked the early stimulatory response of GA_3 on monophenolase activity.

Two lines of evidence support the view that GA_3 regulates monophenolase through the process of enzyme activation. First, the GA_3-stimulated monophenolase was not blocked by a translational inhibitor (CHI 20 μg/ml), although the drug was very potent in inhibiting the activity of acid phosphatase in wheat half-seeds. Second, the enzyme preparation from GA_3-treated half-seeds exhibited altered molecular properties in comparison to the controls.

A single activity peak of monophenolase (M_r 45000) was observed by molecular sieving both in control and GA_3-treated half-seeds during initial imbibition of 12 hr. This is in contrast to our earlier observations, in which we reported an additional high M_r multiple form (M_r 180000) in GA_3-treated half-seeds imbibed for 48 hr [6]. Thus, the appearance of an additional multiple form of monophenolase could be considered a delayed response to GA_3 in wheat half-seeds. This further suggested that oligomerization of monophenolase was not obligatory for enzyme activation. Finally, we believe that the activation of monophenolase during early imbibition of wheat half-seeds is primary physiological response to GA_3. The altered molecular properties of the monophenolase witnessed as an early response to GA_3 strongly support our contention that the hormone-activated enzyme could be structurally different from that of the control enzyme. Further studies are in progress to purify monophenolase to electrophoretic homogeneity and determine the precise nature of the structural modification in the GA_3-activated enzyme.

An early stimulatory response of GA_3 (after 2 hr) has also been reported for phosphorylcholine cytidyl transferase and phosphorylcholine glyceride transferase, involved in the biosynthesis of lecithin, in barley aleurone layers [8, 9]. A maximum stimulation of 150% was reported after 8–10 hr of imbibition. Since, the administration of amino acid analogues failed to inhibit the GA_3-stimulated enzyme activities, it was considered a case of enzyme activation [8, 9].

EXPERIMENTAL

Material. Wheat seeds (*Triticum aestivum* L. var. HD-2009) were procured from the Indian Agricultural Research Institute,

Karnal, India. Tris base, GA, ABA, PVP and tyramine free base were products of Sigma, U.S.A.

Imbibition of half-seeds. Transversely cut de-embryonated half-seeds of wheat were imbibed in H_2O (10 g half-seeds/l H_2O) at 25° in a Brunswick shaker (100 rpm) for different intervals of time (2–12 hr). Wheat half-seeds were also imbibed in the medium (10 g/l of desired soln) containing GA_3 (10 μM), GA_3 (10 μM) + ABA (100 μM), phosphate ions (as K-Pi, 75 mM, pH 6.6) and GA_3 (10 μM) + cycloheximide (20 μg/ml). The half-seeds of these sets of experiments were also placed in a Brunswick shaker (100 rpm) at 25° for the same duration of time (2–12 hr). Thereafter, the control and treated wheat half-seeds were frozen in liquid N_2 and stored at −50°.

Preparation of enzyme fraction. The frozen samples of half-seeds (10 g) were homogenized in Pi buffer (50 ml, 50 mM, pH 6.6) and PVP (2% w/v). Acid-washed sand was used as an abrasive. The crude homogenate was centrifuged at 10 000 *g* for 20 min. The pellet was discarded, while the supernatant was subjected to $(NH_4)_2SO_4$ fractionation (30–50% satn) as described in our earlier refs [6, 7]. The desalted $(NH_4)_2SO_4$ fraction precipitate was used for the assay of monophenolase activity. All operations were carried out at 4°.

Sephacryl S-200 CC. The $(NH_4)_2SO_4$ fraction ppt. (30–50% satn, 30 mg protein), prepared from control and GA_3-treated wheat half-seeds, was subjected to gel permeation on Sephacryl S-200 column (50 × 1.8 cm). The fractions (2.5 ml each) were collected after discarding the first 15 ml of buffer. The void volume (V_o = 48 ml) of the column was determined experimentally from the elution profile of Blue Dextran (5 mg/ml). Tris–HCl buffer (25 mM, pH 7.0) was used for the equilibration of the column and also for the elution of the protein fractions.

Protein was determined by the procedure given in ref. [13].

Assay of monophenolase. The incubation mixture contained tyramine free base (4 mM), Tris–HCl buffer (50 mM, pH 9.0) and enzyme fraction (4 mg protein) in a final vol. of 4 ml. The assay mixture was incubated for 1 hr at 37°. The enzyme activity was expressed as A_{420}/mg protein/hr.

Bioassay of GA_3. The leachate of half-seeds of wheat was concd by lyophilization. The lyophilized sample was eluted with aq. 50% EtOH. The EtOH extract was evapd to dryness and the residue was suspended in sterile H_2O (5 ml) and bioassayed for GA_3 using α-amylase induction in excised wheat aleurones as a marker enzyme. Excised wheat aleurones (50 aleurones) were incubated in the presence and absence of leachate (5 ml) for 40 hr at 25° [14].

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REFERENCES

1. Sachar, R. C. and Berry, M. (1984) *J. Sci. Ind. Res.* **43**, 595.
2. Jacobsen, J. V. (1977) *Annu. Rev. Pl. Physiol.* **28**, 537.
3. Shinke, R. and Mugibayashi, N. (1972) *Agric. Biol. Chem.* **36**, 378.
4. Taneja, S. R. and Sachar, R. C. (1974) *Planta (Berlin)* **116**, 133.
5. Berry, M. and Sachar, R. C. (1982) *Phytochemistry* **21**, 585.
6. Saluja, D. and Sachar, R. C. (1982) *Phytochemistry* **21**, 2625.
7. Saluja, D., Berry, M. and Sachar, R. C. (1987) *Phytochemistry* **26**, 611.
8. Evins, W. H. and Varner, J. E. (1971) *Proc. Natl. Acad. Sci.* (U.S.A.) **68**, 2674.
9. Ben-Tal, Y. and Varner, J. E. (1974) *Plant Physiol.* **54**, 813.
10. Bailey, K. Y., Philips, I. D. J. and Pitt, D. (1976) *J. Exp. Botany* **27**, 324.
11. Mayer, A. M. and Harel, E. (1979) *Phytochemistry* **18**, 193.
12. Jones, R. L. and Varner, J. E. (1967) *Planta* **72**, 155.
13. Bradford, M. (1976) *Anal. Biochem.* **72**, 248.
14. Lall, S., Berry, M., Saluja, D. and Sachar, R. C. (1988) *Plant. Sci.* **55**, 185.