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Growth Regulators Have Rapid Effects on Photosynthate Unloading from Seed Coats of *Phaseolus vulgaris* L.[1]

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PAUL E. CLIFFORD[2], CHRISTINA E. OFFLER*, AND JOHN W. PATRICK
Department of Biological Sciences, The University of Newcastle, New South Wales, 2308, Australia

ABSTRACT

Of nine plant growth regulators (indoleacetic acid, 1-naphthalene acetic acid, gibberellic acid, giberellin 4/7, 6-benzylaminopurine, 6-furfurylaminopurine, abscisic acid, and 1-aminocyclopropane carboxylic acid) tested, only 6-benzylaminopurine and abscisic acid affected ^{14}C-photosynthate unloading from excised seed coats of *Phaseolus vulgaris* L. Unloading, in the presence of KCl, was stimulated by 25 to 40%. Stimulation occurred immediately for 6-benzylaminopurine and for abscisic acid within 10 to 12 minutes of application.

In recent years, a significant addition to methods suitable for studies of phloem unloading has emerged in the form of experimental systems using legume seed coats (12, 13, 19, 25, 26). Such experimental systems, involving embryo removal and its substitution with an appropriate bathing medium in the seed-coat cavity, have two important attributes: they (a) provide a valid estimation of phloem-derived photosynthate unloaded into the sink apoplast, and (b) utilize an unloading site in the plant where knowledge of the processes involved and their regulation is particularly meaningful for crop physiology. Studies indicate that unloading from seed coat to apoplast is energy-dependent (12, 19, 25) and is sensitive to extracellular osmolality (13, 26). In *Phaseolus* seed coats, it is clear that unloading necessitates plasmalemma transfer of sucrose to the apoplast. However, the role of proton pumping, the direction of proton movement, and the identification of carrier proteins (20) together with the principal site of plasmalemma transfer (9) are areas needing clarification. There are numerous investigations showing that growth regulators occur and their levels change in developing legume seeds (6 and references therein). Furthermore, it has been shown that application of growth regulators to legume fruits increases total seed weight (3), photosynthate movement into fruits (1), and total protein content of seeds (16). Thus, it seemed timely to examine the effects, if any, of growth regulators on photosynthate unloading from legume seed coats with the view to understanding the control of plasmalemma transfer to the apoplast as well as adding information to that already known about the role of growth regulators in phloem unloading at sinks (5, 8, 11). Here we report experiments testing representatives of all classes of plant growth regulators for their effects on ^{14}C-photosynthate unloading using excised seed coats of *Phaseolus* (12, 13).

MATERIALS AND METHODS

Plant Material and Selection of Seeds. Dwarf bean plants (*Phaseolus vulgaris* L. cv Redland Pioneer) raised under greenhouse conditions (14) in the summer months in Australia (September–March) were pruned to a single pod at the third trifoliate node supported by the second trifoliate and primary leaves. Plants were selected for experiments when their pods contained seeds at one of the two stages of development defined as: stage 1—pale-green seeds, fresh weight embryo = 0.43 ± 0.03 g; fresh weight seed coat = 0.18 ± 0.006 g; and stage 2—light-gray to brown seeds, fresh weight embryo = 0.66 ± 0.03 g; fresh weight seed coat = 0.22 ± 0.008 g. These stages correspond closely to stages 4 and 6, respectively, of the seed developmental stages described for *Phaseolus* (24). The fresh weight values reported may be related to other seed growth characteristics (10). Before use in experiments, plants were transferred to a growth room (14-h photoperiod, constant temperature 24°C, quantum flux density at plant level 200 μmol m^{-2} s^{-1} (400–700 μm)) for a 16-h equilibration period.

Preparation of Seed-Coat Halves and Measurement of ^{14}C-Photosynthate Unloading. The terminal leaflet of trifoliate leaf 2 was supplied with 0.375 MBq $^{14}CO_2$ generated from $Na_2{}^{14}CO_3$ (37 MBq/mmol from Radiochemical Centre Amersham) and pods harvested 2 h later. Seeds were removed from pods and the seed coat was cut laterally and gently pulled from the embryo. Matched seed-coat halves from each seed were used as a control and treatment. Aerated bathing solutions with or without growth regulator and containing 150 mM sorbital, 20 mM KCl, 10 mM sucrose, and 0.5 mM $CaCl_2$ in 5 mM Mes-Tris buffer at pH 6.0 were dispensed into the cavities of seed-coat halves. In some experiments, KCl concentration was varied and the osmolality maintained at a constant value by altering the concentration of sorbitol in the bathing medium. Bathing solutions were discarded/replaced 3 times over a 0 to 10 min period to remove free space label and thereafter collected/replaced every 2 or 5 min depending on the experiment. Collected solutions were bulked when required, reduced to dryness at 80°C, and ^{14}C activity assessed using liquid scintillation counting. Full details of methods used to prepare seed-coat halves and measure ^{14}C-photosynthate unloading have been described previously (12).

Plant Growth Regulators. These were obtained from the following sources: ACC[3], BAP, IAA, and NAA from Sigma Chemical Co.; ABA (racemate), GA_3, and KIN from Calbiochem-Behring Corp.; $GA_{4/7}$ a gift from Schering Aust. Pty. Ltd. All growth regulators (except $GA_{4/7}$ supplied as a liquid concentrate containing 20 g active ingredient/L) were dissolved in a minimum volume of 0.5 M KOH before being prepared as appropriate stock solutions.

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[2] Present address: Botany Department, The Queen's University of Belfast, Belfast, Northern Ireland BT7 1NN.

[3] Abbreviations: ACC, 1-aminocyclopropane carboxylic acid; BAP, 6-benzylaminopurine; $GA_{4/7}$, gibberellin 4/7; NAA, 1-naphthalene acetic acid; KIN, 6-furfurylaminopurine.

Statistical Treatment of Results. Treatment effects on unloading rate were expressed as a percentage of the control for the pairs of seed-coat halves. Differences between treatments in any experiment were analyzed by 1-way ANOVA using the percentage data without transformation and the difference between means assessed by partitioning of treatment sum-of-squares or LSD P = 0.05. In addition, the significance of any single treatment mean was determined when necessary by a paired-sample *t*-test using the cpm data of control and treated seed-coat halves.

RESULTS AND DISCUSSION

Any data showing that growth regulators alter photosynthate unloading in the legume seed-coat experimental system arguably provide an unequivocal demonstration that they influence unloading in sink regions in a direct manner. Such a demonstration was shown by the results for BAP and ABA, two of the growth regulators used in this study (Table I). For these growth regulators, we did not observe promotory effects of more than 40% above the control. At stage 2 of seed development, BAP effects on unloading were readily reproducible with best promotion occurring invariably at 10^{-5} M, no effect or slight promotion at 10^{-6} M, and no effect or inhibition at 10^{-4} M (*cf.* Table I). In contrast, ABA effects on unloading varied from experiment to experiment. In many experiments best promotion occurred at 10^{-5} M, but in some experiments promotions were highest at 10^{-6} M and, in a single experiment, at 10^{-4} M. For 35% of experiments, an ABA effect was completely absent. Such variability in response to ABA is possibly due to variations in its endogenous levels in the seed coats both between plants and between batches of plants (*cf.* 15). For stage 1 seeds, 10^{-5} M BAP gave a significant (P = 0.05) 10 to 15% promotion of unloading, but ABA was nonaffective at this stage of development (data not shown). Photosynthate unloading was not significantly affected by IAA, NAA, GA_3, $GA_{4/7}$, KIN, and ACC applied at concentrations of 10^{-4}, 10^{-5}, and 10^{-6} M to seed coats excised from seeds at the two stages of development (data not shown). We recognize that the latter results do not preclude the possibility that these chemicals could influence unloading. For instance, extension of the treatment times could elicit responses (*e.g.* 8). Alternatively, endogenous levels could have been optimal as indicated by the finding that 2-chlorophenoxyisobutyric acid, 4-chlorophenoxyisobutyric acid, and 2,4,6,-trichlorophenoxyacetic acid (compounds related to 2,4-dichlorophenoxyacetic acid and thought to be auxin agonists [GF Katekar, personal communication]) were all capable of promoting unloading in the order of 10 to 15% above the control (data not shown). We tentatively suggest that the reason for no significant response to KIN while the synthetic cytokinin BAP produced both inhibition and promotion is due to the higher physiological activity of BAP compared with KIN (18).

Further investigation was confined to examination of the effects of BAP and ABA on photosynthate unloading from seed coats of stage 2 seeds. Clearly, the promotory effects of both BAP and ABA on photosynthate unloading are rapid as shown by time-course data for these growth regulators (Fig. 1). For BAP (10^{-5} M), promotion of unloading was immediate and reached

Table I. *Effect of BAP and ABA on ^{14}C-Photosynthate Unloading from Excised Seed Coats of Developmental Stage 2 Seeds of Phaseolus vulgaris L*

Following the removal of free space label, bathing solutions were collected/replaced every 5 min. ^{14}C-activity was assessed for the 10 to 30 and 30 to 50 min collection periods. Unloading is expressed as a percentage of the control value. Values are the mean of 8 replicates and underlined if significantly different (P = 0.05) from 100%. For each experiment, means marked with different letters are significantly different (P = 0.05) with partitioning of treatment sum-of-squares.

Concentration of Growth Regulator	BAP % Unloading (no BAP = 100%) at		ABA % Unloading (no ABA = 100%) at	
	10–30 min	30–50 min	10–30 min	30–50 min
10^{-4} M	72[a]	75[a]	107[a]	99[a]
10^{-5} M	122[b]	126[b]	131[b]	125[b]
10^{-6} M	108[c]	106[a]	131[b]	137[b]

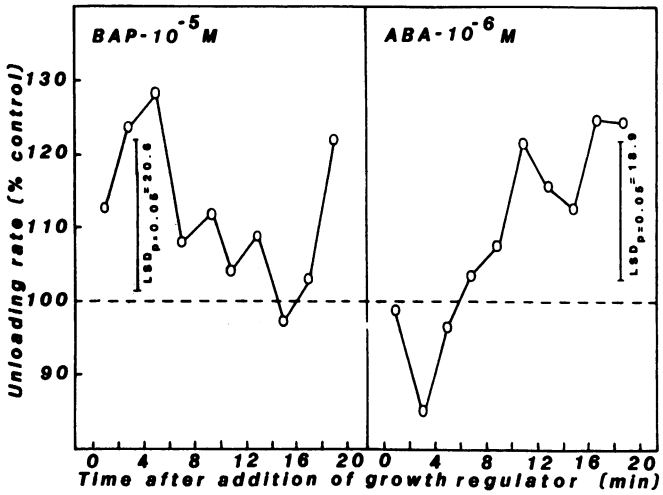

FIG. 1. Time course of BAP- or ABA-stimulated ^{14}C-photosynthate unloading from excised seed coats of developmental stage 2 seeds of *Phaseolus vulgaris* L. BAP or ABA was added at 10 min, after removal of free space label. Bathing solutions were collected/replaced every 2 min with ^{14}C activity assessed for each collection. Unloading is expressed as a percentage of the control value. Values are the mean of 10 replicates. BAP and ABA were supplied at 10^{-5} M and 10^{-6} M concentrations, respectively. For each experiment the LSD P = 0.05 is indicated.

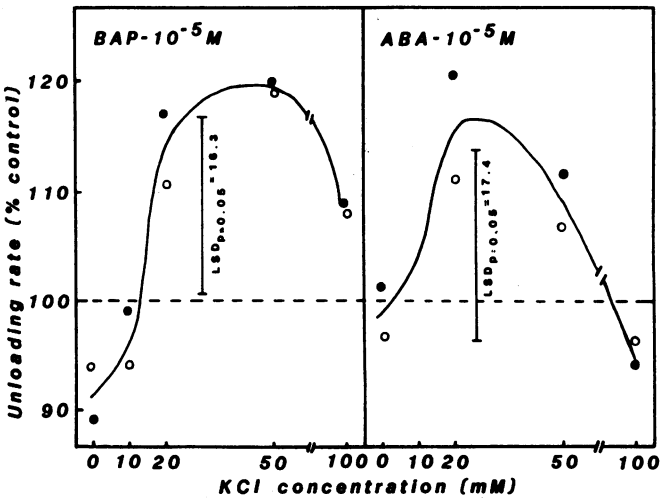

FIG. 2. Effect of KCl concentration on BAP- or ABA-stimulated ^{14}C-photosynthate unloading from excised seed-coats of developmental stage 2 seeds of *Phaseolus vulgaris* L. Osmolality was maintained at a constant value (200 mOs/kg) with changing KCl concentration by altering the concentration of sorbitol. BAP or ABA was added at the start of the experiment and KCl at 10 min, after removal of free space label. Bathing solutions were collected/replaced every 5 min and ^{14}C activity assessed for 10 to 30 (●) and 30 to 50 (○) min collecting periods. Unloading is expressed as a percentage of the control value. Values are the mean of 8 replicates. Both BAP and ABA were supplied at a 10^{-5} M concentration. For each experiment the LSD P = 0.05 is indicated.

maximal levels within 4 to 6 min. Thereafter, unloading declined to control levels before rising rapidly again by 18 to 20 min. For ABA (10^{-6} M) treatment, however, unloading was apparently inhibited between 0 to 6 min and increased only with further time so that promotion neared maximum by 10 to 12 min. Interpretation of these contrasting time-course patterns for BAP and ABA need further study. However, the effects of both growth regulators are suggestive of direct action on membrane permeability. Certainly, for ABA it is well documented that it closes stomata within minutes (27) and it is now generally accepted that many growth regulators including cytokinins and ABA can change the properties of cell membranes without the need for changes in enzyme synthesis (28).

We also examined the possible interaction of BAP and ABA with external KCl concentration since, in *Phaseolus* seed coats, K^+ is a major osmotic component of the apoplast (13) and K^+ stimulates proton extrusion (21) and photosynthate unloading (20). For BAP and ABA (both at 10^{-5} M), promotion of ^{14}C-photosynthate unloading was maximal between 20 to 50 mM KCl with unloading in the case of ABA falling to control levels when 100 mM KCl was present in the bathing solution (Fig. 2). The KCl-dependence of the growth regulator effects on photosynthate unloading are suggestive of their interaction with a membrane porter (12) whose activity may depend on charge transfer (20). Thus, BAP could stimulate K^+ influx across the plasmalemma in the same way that cytokinins stimulate K^+ accumulation by guard cells (18) which, in turn, would increase photosynthate unloading (20). Dependence of ABA promotion of unloading upon K^+ is more difficult to interpret, especially in view of reports that ABA causes K^+ efflux from guard cells (7) and K^+ release into the xylem (4). A number of possible modes of ABA action on photosynthate unloading are currently being examined. These include, an indirect effect of ABA mediated by its inhibition of proton extrusion through a plasmalemma-ATPase (21) and possible interaction with Cl^- rather than K^+ ions (23).

It is especially interesting that testing a range of growth regulators for their short-term effects on photosynthate unloading revealed promotions with a cytokinin and ABA. Current evidence suggests that a large proportion of both these growth regulators are not synthesized in the seeds themselves but are transported to seeds after synthesis elsewhere in the plant. For example, cytokinins synthesized in roots are transported to developing seeds via the xylem (22), while ABA synthesized in photosynthesizing leaves is transported to seeds via the phloem (2). It is possible, therefore, that assimilate supply from source leaves could influence indirectly the amounts of cytokinins, and influence directly the amounts of ABA, reaching the tissues of the seed coat. Some control of sink unloading residing in the source itself would provide the means for source leaves to influence sink unloading according to total photosynthate supply. Circumstantial evidence that such a mechanism operates in the whole plant for ABA is provided by a report that soybean varieties with larger seed size contain higher ABA levels in seed coats (17). Interestingly, preliminary results of our own examining the effects of BAP and ABA injection into developing *Phaseolus* seeds *in situ* indicate that higher seed weights are produced with both growth regulators only when plants are severely defoliated in order to create a limited photosynthate supply for seed growth.

In conclusion, it has been demonstrated that the synthetic cytokinin BAP and ABA are capable of rapid and significant promotions of ^{14}C-photosynthate unloading from *Phaseolus* seed coats provided that 20 to 50 mM KCl is included in the bathing medium. Our study gives direct evidence that BAP and ABA influence unloading of phloem-derived photosynthate into the apoplast of the *Phaseolus* seed coat. The possibility that cytokinins and ABA can regulate the energized component of plasmalemma transfer to the apoplast in the legume seed coat and the significance of such regulation for seed growth are worthy of further investigation.

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