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Biochemistry of a Plant Dormancy Process: Prospects for Chemical Regulation

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Abstract. The mobilization of protein nitrogen reserves in apple shoot bark is subject to abscisic acid (ABA)-mediated regulation. At the biochemical level proteolytic activities in the shoot bark are suppressed in the autumn. As endogenous ABA levels decline during the autumn and winter tissue protease levels increase in parallel with the rate of inducible breakdown of reserve protein; metabolic demand *per se* plays no significant role. This process is used as a model system to explore the possibilities for chemical regulation ("bioregulation") of ABA-determined dormancy phenomena by: manipulation of tissue ABA concentrations, substitution of synthetic growth regulators and direct interference at the site of action.

The study of dormancy phenomena in higher plants is, in addition to its contribution to the knowledge of plant growth and development, of considerable agro-economic interest. In fruit tree-growing areas in Northern temperate zones the manipulation of flowering date would be of undoubted benefit as in many years crop yield is greatly reduced by spring frosts. Conversely, the prevention of dormancy development (by defoliation) or the chemical breaking of innate dormancy is essential to apple horticulture in the tropics and semi-tropics (SAMISCH 1945, JANICK 1974, NOTODIMEDJO *et al.* 1981).

The endogenous growth inhibitor 2-*cis*-4-*trans*-abscisic acid (ABA) has been implicated as a regulatory factor in dormancy processes in both vegetative and reproductive plant tissues (WAREING 1978). These can potentially be chemically regulated at three levels: the hormonal (the movement and accumulation or disappearance of the plant growth regulator, changes in the sensitivity of target cells and changes in the levels of antagonistic regulators), the biochemical (the enzymic, membrane transport or other subcellular effects of the regulator) and the molecular (the primary interactions of the growth regulator, for example by binding to a specific receptor).

Physiological and biochemical studies have revealed a novel role of ABA in the regulation of the utilization of nitrogen reserves in the apple (*Malus domestica* BORKH.). We propose this as a model system for dormancy process where ABA suppresses development in otherwise favorable environments; theoretical aspects of chemical control of this system are explored at the hormonal and biochemical levels.

MATERIALS AND METHODS

Plant materials, growth conditions, assays for protein, amino acids and ABA and the iso-electric focusing methods have been described previously (MOUSDALE 1982, 1983a,b, MOUSDALE *et al.* 1982). Intact rooted shoots were separated into: shoot bark, shoot wood, buds (with any emergent shoot growth), underground stem and roots. These samples were freeze-dried and ground to pass a 1 mm mesh in a mill cooled with solid carbon dioxide. When buds were excised prior to transfer of rooted shoots to growth chambers the bud sites were covered with lanolin to prevent bark tissue dehydration.

Protein synthesis was monitored by the incorporation of ^{35}S-labelled methionine. 0.5 g fresh mass samples of shoot bark fragments were incubated at 25 °C for 1 h in 5 ml incubation medium: 50 mM phosphate buffer (pH 7.0) containing 45 . 9 $\pm$ 3 .6 pmol methionine (specific activity 37 . 4 — 43 . 9 TBq mmol^{-1}; The Radiochemical Centre, Amersham, U.K.) together with the other protein amino acids as in the method of HUNT and JACKSON (1974). After incubation the tissue was filtered under suction, washed with 20 ml 25 mM unlabelled methionine and ground under liquid nitrogen. Proteins were extracted with 2 $\times$ 5 ml 0.1 M Tris/HCl (pH 8.0) containing 0.4 M NaCl and 6 mM L-cysteine. The combined extracts were centrifuged at 10 000 $\times$ *g* for 20 min at 4 °C. 30 µl aliquots were pipetted on to Whatman GFC filter discs presoaked in 10 % (m/v) trichloroacetic acid in diethyl ether. The discs were washed and counted for radioactivity by the method of HALLERMAYER *et al.* (1977).

RESULTS AND DISCUSSION

ABA-Regulated Protein Metabolism in Apple Shoot Bark: Outline of the Model

Much of the nitrogen (and phosphate) in apple leaves is metabolically labile in that during autumnal senescence it is translocated from the leaves to

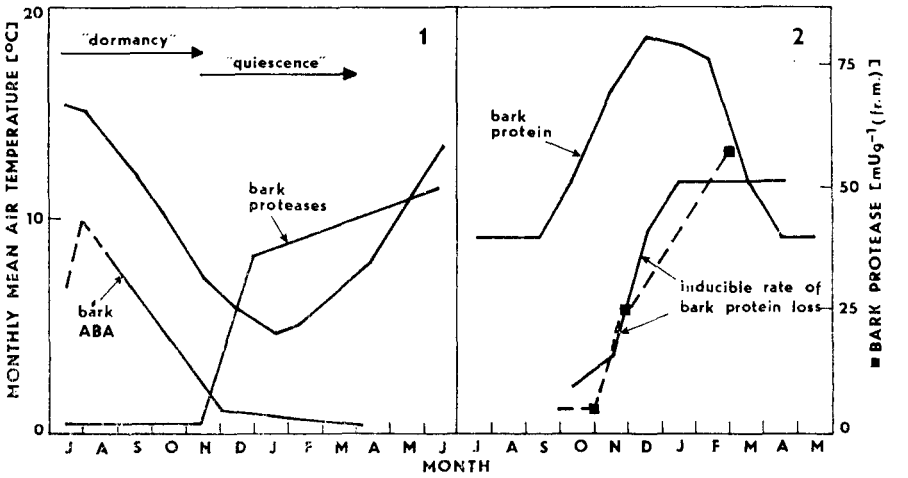

Fig. 1. Schematic outline of model of ABA-regulation of shoot bark protease levels. Monthly mean temperatures refer to Dublin (Ireland) and represent 1941—1970 averages.
Fig. 2. Schematic outline of model of control of rate of bark protein loss induced by transfer to 25 °C. Data redrawn from MOUSDALE 1983a.

perennial woody tissues (MASON and WHITFIELD 1960). Foliar-applied nitrogen (as urea) in the autumn is efficiently transferred to storage sites (O'KENNEDY *et al.* 1975, SWIETLIK and SLOWIK 1981). These reserves of nitrogen are utilized during the initial stages of spring regrowth (TAYLOR 1967).

Shoot bark contains reserve nitrogen at the highest concentration and this shows the most pronounced drop during spring mobilization (MASON and WHITFIELD 1960). Protein is the major form of stored nitrogen in apple shoot bark and wood (TROMP 1970). The physiology of reserve protein breakdown was investigated by TROMP and OVAA (1971, 1973) and the biochemistry of apple leaf-shoot bark nitrogen recycling was first outlined by Titus and coworkers (O'KENNEDY and TITUS 1979, KANG and TITUS 1980a,b, KANG *et al.* 1982).

The model of ABA-regulated accumulation-utilization of protein reserves in apple shoot bark is outlined in Figs. 1 and 2. The evidence can be summarized as:

(a) the rate of bark protein loss induced by transfer to 25 °C increases greatly in the period November-January and there is a good parallel of mobilization rate and endogenous protease levels (MOUSDALE 1982, 1983a, MOUSDALE *et al.* 1982);
(b) there is an inverse relationship between ABA levels in shoot bark and the rate of bark protein loss at 25 °C; ABA retards or suppresses increases in protease activities in excised shoot bark sections (MOUSDALE 1983a,b, MOUSDALE *et al.* 1982);
(c) most or all of the soluble proteins in shoot bark contribute to the cycle of protein accumulation-mobilization (O'KENNEDY and TITUS 1979, KANG *et al.* 1982, MOUSDALE *et al.* 1982); no polypeptides appear to function solely in nitrogen storage (as, for example, in seeds and cereal grains).

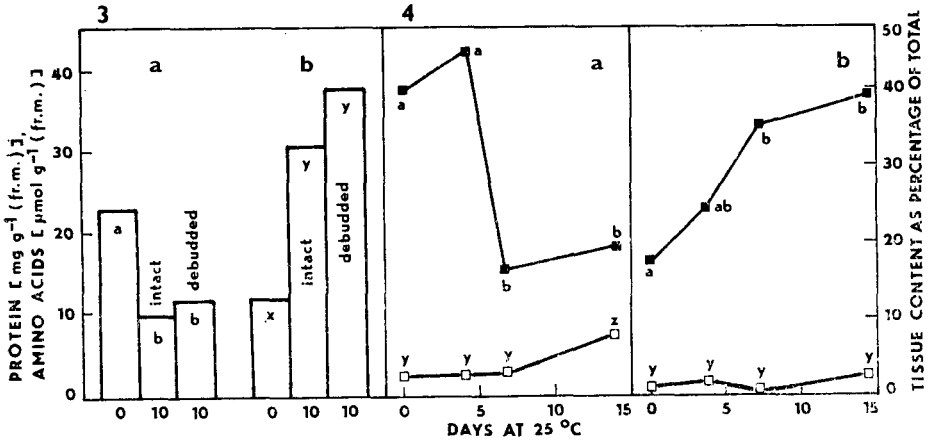

Fig. 3. Changes in total protein (a) and free amino acid (b) contents of shoot bark of apple rootstock MM106 after transfer to 25 °C growth chamber (February 1982). Means with same letter do not differ at $P \leq 0.05$ (Duncan's multiple range test).

Fig. 4. Changes in total protein (a) and free amino acid content (b) contents of shoot bark (■) and buds (□) of apple rootstock MM106 after transfer to 25 °C growth chamber (March 1982). Means with same letter (a,b bark; y,z buds) do not differ at $P \leq 0.05$ (Duncan's multiple range test).

The absence of any endogenous control of bark protein utilization in overwintered trees was shown by TROMP and OVAA (1971, 1973). In such trees no metabolic control of the rate of bark protein loss can be demonstrated: mobilization of the reserves is not retarded by the absence of any sites of shoot regrowth (Fig. 3). The nitrogen demands of new shoot growth are small in

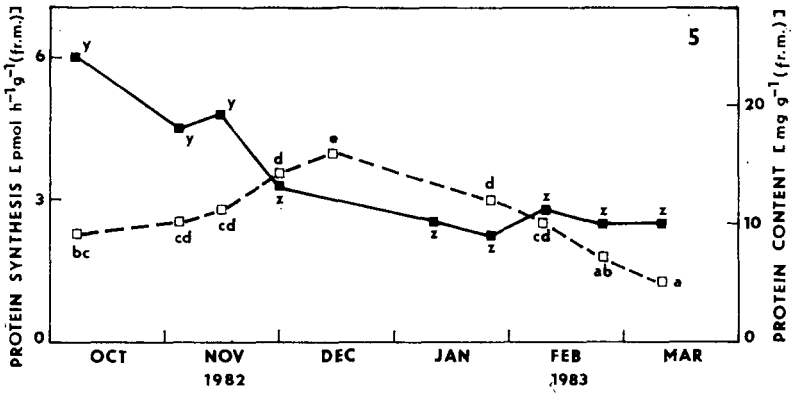

Fig. 5. Seasonal changes in shoot bark protein content (□) and rate of incorporation of ^{35}S-methionine into protein (■). Means with same letter (a-e protein content; y-z incorporation rate) do not differ at $P \leq 0.05$ (Duncan's multiple range test).

comparison with the protein nitrogen available (Fig. 4). Over a more prolonged period of shoot regrowth there is a more complete transfer of nitrogen from woody storage sites (SUZAKI 1984).

The prevention of premature (autumnal) loss of shoot bark reserve protein is analogous to processes occurring in many plant dormancy phenomena, the biological function of which is to integrate development with the sequence of growing seasons and/or plant generations. In the proposed model suppression by ABA of shoot bark proteolytic enzyme levels is considered the principal endogenous factor in regulating protein metabolism. After the rise in tissue proteases in the winter, temperature becomes the major controlling element ("quiescence").

Additional regulatory elements may be included in the model. In particular, protein synthesis rates decline by 50 % between autumn and spring (Fig. 5). This decrease may however only be apparent and result from the increasing effect of protease activity on *in vitro* estimates of protein synthesis (BECKER *et al.* 1981). In *Phaseolus vulgaris* ABA increases storage protein formation in developing seeds (SUSSEX *et al.* 1980); this effect has not been investigated directly in apple shoot bark where auxins have a promotive effect on protein synthesis in excised internode sections (MOUSDALE 1982, 1983a).

Theoretical Considerations of the Chemical Regulation of Protein Metabolism in Shoot Bark

Protein Accumulation. Increasing the effective "sink strength" of shoot bark (and other woody tissues) would result if protease levels were suppressed throughout the period of protein accumulation by autumnal

application of ABA (or a synthetic growth inhibitor with similar effects). A secondary effect would be the promotion of leaf senescence and leaf protein breakdown (WAREING 1978).

Reserve Protein Mobilization. The major conceptual difficulty in the chemical control of the rate and onset of reserve protein mobilization is that the transition from low to high potential rates occurs between two and four months before the major utilization of the reserves in the spring (Figs. 1 and 2). A delayed spring mobilization could be achieved if bark tissue proteases were maintained at the low levels found in the early autumn throughout the winter. Applications of ABA before natural leaf fall are unlikely to result in such a long-term effect because of the *in vivo* instability of free ABA (POWELL and SEELEY 1974, SEMBDNER *et al.* 1980). No stable synthetic analogue of ABA is presently known. Spring applications of ABA may have a significant retarding effect on protein breakdown as bark protease levels continue to rise throughout the spring (KANG *et al.* 1982). Penetration to the site of action would however be very limited until new foliage is produced.

A different approach to the chemical regulation is by exploiting the known biochemical site of action. Thiol-endopeptidases can be effectively inhibited by the low molecular weight peptidic compound leupeptin (acetyl-L-*leu*-L-*leu*-arginal). This inhibitor prevents enzyme inactivation by proteolysis in crude extracts of plant tissues (ALPI and BEEVERS 1981a, CAMPBELL and WRAY 1983) and strongly inhibits germination and the development of gluconeogenetic enzymes in *Ricinus communis* seed endosperm (ALPI and BEEVERS 1981b), it lacks the high toxicity of thiol reagents which were found to inhibit the acid-pH endopeptidase activity in apple shoot bark (KANG and TITUS 1980a). If leupeptin were sufficiently stable after autumnal application a concentration-dependent delay in the rise in bark tissue proteases would be expected.

The closest correlation between the change in proteolytic activity and the rate of inducible protein breakdown is with the acid-pH endopeptidase (MOUSDALE 1983a). The kinetic properties of this enzyme activity offer a potential means of bio-engineering a controllable spring reserve mobilization. This could be made to occur later or slower by a decrease, and earlier or faster by an increase, in the activation energy of the enzyme-catalyzed hydrolytic reaction (as compared with the wild-type enzyme) in the critical temperature range 4—10 °C (Fig. 1). This enzymic property, deduced from Arrhenius plots of the temperature-dependence of the proteolysis *in vitro*, may vary significantly among cultivars; tissue culture also offers a means for the rapid screening of natural variation and variation resulting from chemical or stress treatments. Techniques are presently being developed for the insertion into higher plants of novel genetic information coding for enzymes with altered properties, for example with the aim of introducing resistance to herbicides (AMRHEIN *et al.* 1983, COMAI *et al.* 1983).

Relevance to Major Plant Dormancy Phenomena

TROMP (1970) reviewing shoot bark protein metabolism suggested that "the commencement of protein hydrolysis marks the end of winter dormancy [quiescence]." There is no clear evidence that the onset of reserve protein mobilization influences the timing or rate of bud-break in fruit tree species. The kinetics of decline of endogenous ABA concentrations in vegetative

buds and shoot bark are consistent with the processes being parallel but independent. An isolated finding is that chemical dormancy-breaking treatments in Southern Africa had little effect in the absence of nitrogen application in the previous year (TERBLANCHE and STRYDOM 1973).

The role of ABA in bud dormancy in tree species — whether the principal or one of several factors — has been the subject of many investigations. The correlation found experimentally between the natural ABA decline in vegetative and floral buds and their dormancy state has generally been poor (WAREING 1978). Comparison of growth capacity with changes in bud ABA levels after transfer to conditions stimulating bud-break shows a clearer relationship (SEELEY and POWELL 1981, MOUSDALE 1983b). The attempted control of natural bud-break with ABA applications suffers from similar problems to those considered for shoot bark protein metabolism. Endogenous ABA levels can probably be influenced by autumnal applications but this may not be sufficient to affect the later events directly determining the cessation of innate dormancy. A successful approach to delaying spring bud-break in peach (*Prunus persica* L.) involved nitrogen application in the previous year; bud ABA concentrations were elevated throughout the winter and early spring (REEDER and BOWEN 1978).

The actions of ABA at the biochemical level in influencing dormancy state have not been accurately defined. In developing seeds of cotton (*Gossypium hirsutum* L.) the suppression of premature germination by ABA has been related to inhibition of the translation of mRNA present in immature seeds but not normally translated until germination (IHLE and DURE 1970); one of the derived proteins is a carboxypeptidase functioning during hydrolysis of storage proteins (IHLE and DURE 1972). In apple embryos the removal of primary dormancy by cold treatments is associated with changes in substrate-specific proteolytic activities, although the exact significance of these changes is unknown (ŽARSKA-MACIEJEWSKA and LEWAK 1983). ABA-induced turion (dormant bud) formation in *Spirodela polyrrhiza* L. has been studied in detail (SMART and TREWAVAS 1984): ABA has differential effects on mRNA translation, several novel proteins appearing in the early stages of turion development; later there is a general suppression of protein synthesis. Determination of the key steps regulated by ABA is required before accurate assessment can be made of how and when the chemical manipulation of these and other plant dormancy phenomena can be selectively achieved.

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BOOK REVIEW

JANICK, J. (ed.): PLANT BREEDING REVIEWS, Vol. 2. — Avi Publishing Company, Inc., Westport Connecticut 1984. 326 pp. US $ 45.00.

Plant breeding has been practised by man since he settled and started cultivating plants. The techniques for breeding crop plants, however, has undergone changes in the course of the progress of science, especially in the last years. The objective of Plant Breeding Reviews is to synthesize the most important information on the theory and practical achievements of plant breeding, which until now has been dispersed throughout the specialist literature. The first volume in this series was published in 1983. Each volume is opened by a life history of a remarkable plant breeder. Volume 2 is dedicated to G. F. Sprague, whose research accomplishment have been primarily in the development and evaluation of new breeding methodology and the production of corn inbred lines that have been widely used in hybrid seed production. For the benefit of plant breeders, leading experts have compiled overviews on techniques and breeding achievements in several important crops. Included are articles on breeding cassava, bananas and coffee, reviews on breeding strawberries for red stele resistance and hybrid wheat breeding. A review on induced mutations in seed-propagated plants brings data on new mutants of crop plants that have been induced by both ionizing radiation and chemical mutagens. Three papers focus on modern techniques of tissue culture and plant engineering: tissue culture of legumes for crop improvement, tissue culture techniques for virus elimination and germplasm preservation and plant genetic engineering *via* organelle transfer.

This volume should be placed, together with its predecessor in the series, among valuable reference books for readers interested in all aspects of contemporary plant breeding research.

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