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Activity of L-phenylalanine ammonia-lyase in winter rape leaves treated with cold*

Danuta Solecka and Alina Kacperska

Laboratory of Plant Resistance Physiology, Institute of Plant Experimental Biology, University of Warsaw, Krakowskie Przedmieście 26/28, Warszawa, Poland

Cold acclimation of a herbaceous plant is a complex process involving the adjustment of cell metabolism and growth to the biophysical constraints imposed by low temperature and leading to the induction of frost resistance [1]. The process involves alterations in membrane composition, changes in gene expression, increased amounts of soluble proteins and sugars, changes in isoenzyme patterns [2 - 4]. However, it is difficult to determine whether these changes are associated with plant growth at low temperature or with the development of frost tolerance.

More detailed studies are needed to understand the exact relationship between the increase in frost resistance and such growth responses as an increase in the weight of cell walls and changes in wall composition, e.g. cellulose, arabinogalactan and lignin content [5 - 7].

L-Phenylalanine ammonia-lyase (PAL[1], EC 4.3.1.5.) is the entry-point enzyme into phenylpropanoid metabolism and its activity can serve as an indicator of the activity of the pathway. We are interested in the cold induced changes in PAL activity because of the role of this enzyme in lignification, an important event in plant acclimation to different stresses.

Winter rape plants *(Brassica napus* var. *oleifera* cv Jantar) were grown in sand supplied with Hoagland solution in a temperature controlled room: 20°C (day) and 15°C (night), under 16 h photoperiod. Light, approx. 20 W $\times$ m^{-2}, was provided by "day light" fluorescent tubes (Polam, Poland). The relative humidity was 80 ± 5%. After 3 weeks of growth plants were subjected to a continuous low (+2 ± 1°C) temperature (the cold-acclimated plants); the other conditions remained unchanged. After 3 weeks of hardening at low temperature the plants were subjected to freezing temperature (–5°C, 18 h in darkness). After the treatment the plants were transferred back to cold temperature (+2°C, 30 h) for regeneration of reversible injury. Then, one part of the plants was transferred to +20°C, the other was remained at +2°C for 7 days.

Samples for analysis were taken after: 1) 0, 2, 4, 8, and 21 day of plant growth at +2°C, 2) 6 and 30 h of plant regeneration at +2°C following freezing, 3) 7 days of growth of the frost-pretreated plants at +2°C or +20°C. All analyses were performed on young blades of the 5th and 6th leaf. Determination of frost resistance (expressed as LT_{50}) were performed as described previously [8].

Tissue from 5 leaves (1 g fresh weight) was homogenized in 10 ml of extraction buffer (0.1 M borate buffer, pH 8.8, 0.11 M NaCl). Extracts were centrifuged at 14000 $\times$ *g* for 20 min and the supernatants were taken for the assay. All procedures were carried out at +4°C.

The reaction mixture of 3 ml contained 0.4 - 0.75 mg protein of crude extract, in 0.2 mM borate (Na^+) buffer, pH 8.8, containing 1.1 mM NaCl and 0.05 mM L-phenylalanine. Incuba-

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[1]Abbreviations: PAL, L-phenylalanine ammonia-lyase; LT_{50}, temperature at which 50% injury occurred

tion was performed at 37°C for 3 h. Production of cinnamic acid was measured as increase in absorbancy at 290 nm [9].

Protein concentration in the crude extract was determined by the method of Bradford [10] using bovine serum albumin (Sigma) as a standard.

It has been shown that frost tolerance of cold-grown leaves increased from – 4.7°C to 10.2°C (Fig. 1B). Dry matter content increased simultaneously about 75% (Fig. 1A). Protein content in the cold-acclimated plant leaves increased about 65% in comparison to the non-cold-acclimated plants (Fig. 2).

After the slight frost followed by 30 h of leaf regeneration at +2°C the freezing tolerance of leaves increased to –11.0°C. Prolonged acclimation of prefrozen plants at +20°C resulted in

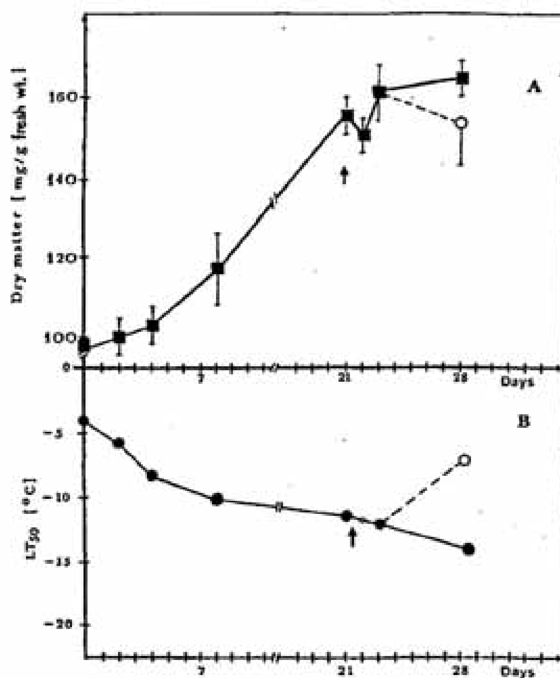

Fig. 1. Changes in (A) dry matter content and (B) LT_{50} of winter rape leaves in the course of plant acclimation.

Arrow indicates the start of plant exposure to –5°C for 18 h. Open symbols, dry matter and LT_{50} in plants transferred to 20°C. Mean of 3 experiments ± S.D. are given

increased leaf frost tolerance below the testing temperature, i.e. –12°C. In the plants transferred to +20°C the frost tolerance decreased to –6°C.

Freezing caused a significant, but transient, reduction both in dry matter and protein content in cold-grown leaves (Fig. 2). During the regeneration of leaves at +2°C, protein content in the frost-pretreated blades was maintained at a level constant but lower than that in the non-frozen ones. On the other hand, after 7 days of leaf growth at +20°C (deacclimation), total protein content fell down below the level observed at "0" day of the experiment.

A significant increase in PAL activity was evident on the 2nd day of the cold treatment (Fig. 3), irrespective of the calculation (dry weight or protein) basis. After 21 days of cold acclimation specific PAL activity was 3.5 or 2.5 times higher than that observed on the "0" day when calculated on dry weight or protein basis, respectively. The freezing treatment caused a dramatic increase in specific activity of PAL, which reached a level aproximately threefold higher than that before the treatment. However, the frost-induced increase in PAL activity was much lower when expressed on dry weight basis and did not exceed 30%. The rise in PAL activity was transient: after 30 h of regeneration the activity decreased either to the level observed before frost treatment (calculated on protein basis) or to the control level (expressed per dry weight basis). During the further 7 days of plant growth at +2°C the activity of enzyme remained unchanged. In plants growing at +20°C activity of PAL decreased by half.

The data presented here show that cold hardening of winter rape plants resulted in the increased activity of PAL in the leaves. We cannot yet explain the mechanism of induction of PAL activity during cold acclimation at low temperature, about 0°C. It is possible that the increase of the enzyme activity was caused by *de novo* enzyme protein synthesis as it was observed in plant tissue subjected to changes in illumination and wounding [11, 12]. The rapid but transient increase of PAL activity after freezing treatment point to the shock character of this reaction, similarly as it was observed in tissue infected with pathogens [13, 14]. It might be proposed that the apparent frost-induced increase in the specific activity of PAL could be

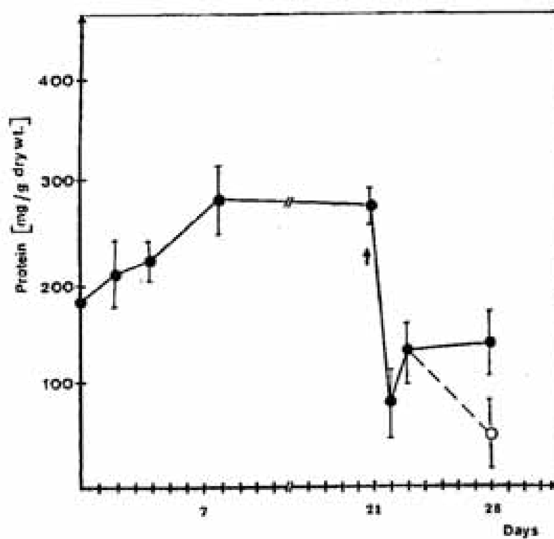

Fig. 2. Changes in protein content of winter rape leaves during the cold acclimation.
Arrow indicates the start of plant exposure to −5°C for 18 h. Open symbol, protein content in plants transferred to 20°C. Mean ± S.D.

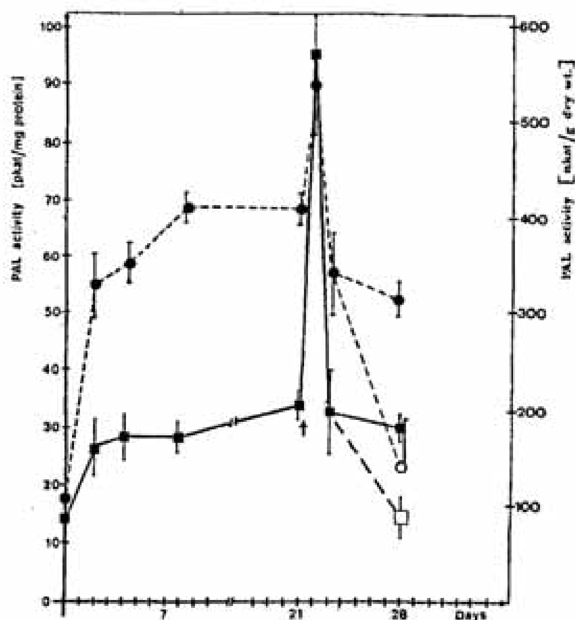

Fig. 3. The effect of cold acclimation on activity of PAL in winter rape leaves.
Specific activity (■); activity expressed on dry weight basis (●). The results are means from three independent experiments with 6 - 12 replicates in each. Arrow indicates the starts of plant exposure to −5°C for 18 h. Open symbols, activity in plants transferred to 20°C (mean ± S.D.)

due to degradation of protein other than PAL (Fig. 3). However, the fact that the increase was also observed when PAL activity was expressed on dry weight basis points to possibility that the frost treatment actually increased the enzyme activity.

We cannot exclude that in PAL responses to low temperature, selective expression of PAL genes encoding functional variants of the enzyme (as shown in bean [15]), specific types of allosteric interactions or associations with other proteins (formation of multienzyme complexes) or protein inhibitors are involved. There is evidence that a PAL inhibitor participated in the control of PAL activity by light [16].

All those possibilities should be checked in future.

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