BULG. J. PLANT PHYSIOL., 1995, 21(4), 30–38

HISTOCHEMICAL INVESTIGATION OF ALCOHOL DEHYDROGEHASE, GLUTAMATE DEHYDROGENASE AND α-ESTERASE ACTIVITY IN LOW TEMPERATURE STRESSED TOBACCO CALLUS AND THE EFFECT OF CYTOKININS

Irina Puneva*, Lubomir Iliev

Acad. M. Popov Institute of Plant Physiology, Acad. G. Bonchev Str., Bl. 21, Sofia 1113, Bulgaria

Received February 2, 1996

Summary. The influence of the synthetic phenylurea cytokinin 4-PU-30 and of the adenin cytokinin kinetin on the chilling and freezing stress of tobacco callus was studied histochemically. The investigations were carried out immediately after the low-temperature stress and after 20 days cultivation. It was established that alcohol dehydrogenase and glutamate dehydrogenase activities decreased but esterase activity rose drastically after chilling and freezing in the presence of both cytokinins in the nutrient medium. After 20 days the alcohol dehydrogenase activity was restored more rapidly in the presence of 4-PU-30 than in the presence of kinetin in the nutrient medium. A tendency of restoring normal glutamate dehydrogenase, and α-esterase activities after freezing was observed only in the presence of 4-PU-30. It was concluded that 4-PU-30 added to the nutrient medium manifests higher chilling and freezing antistress activity as compared to kinetin.

Key words: freezing and chilling stress, cytokinins, alcohol dehydrogenase, glutamate dehydrogenase, α-esterase

Abbreviations: ADH – alcohol dehydrogenase; GDH – glutamate dehydrogenase, MS – Murashige and Skoog medium

Introduction

In general green plants respond to low-temperature action by alteration in the membrane lipid phase and by change of the membrane fluidity (Lyons and Raison, 1970). These disturbances influence the associated metabolic processes and the enzymes

*Corresponding author

involved in them (Levitt, 1980). In cold sensitive plants respiration is among the first inhibited processes and ADH is one of the most rapidly responding enzymes (Guy, 1990; Perata and Alpi, 1993). There exist contradictory data concerning the behaviour of this enzyme as well as of the coding gene activity under low temperature conditions and the resulting from that anaerobiosis (Xie and Wu, 1989; Jarillo et al., 1993). Different conceptions are also published about the involvement of the enzymes in the process of adaptation and development of tolerance toward freezing. Some authors attribute the inconsistency of the data about the gene expression under low temperature stress to the character of the changes wich depends on the species, tissue and age specifity (Wilson, 1987; Xie and Wu, 1989; Christie et al., 1991).

Though the signalificance of the increased ADH activity under anaerobiosis is not quite clear (Roberts et al., 1989) the enzyme is considered as a basic one, related to the tolerance toward anaerobiosis, resulting from low temperature stress.

It is established that in the cold-sensitive plants GDH is characterized by low cold resistance (Guy, 1990). As a mitochondrial anaerobic enzyme it is affected by the low temperature damages of the mitochondrial membranes and their functions (Levitt, 1980). Furthermore the alterations in the membrane lipid phase and the cytoplasmic acidification, accompanying low-temperature stress may result in severe tissue damage (Wilson, 1987) which is thought to be connected with changes in the activities of some hydrolytic enzymes.

As known the plant hormones play an important role in the regulation of the green plant response to stress affecting the gene expression (Vojnikov and Ivanova, 1988; Zhyrmunskaya et al., 1989; Ershova et al., 1991). It is also established that low-temperature stress leads to alteration in the cytokinin hormonal status (Tantan and Dörffling, 1991). While applied exogenously they exert a protective effect, thus inducing tolerance toward low temperature, freezing and anaerobiosis (Reaney and Gusta, 1987; Hwang and Van Toai, 1991).

The purpose of this work was to evaluate the effect of two cytokinin protectors (4-PU-30 and kinetin) on the response of tobacco callus low-temperature stress by histochemical investigation of the alterations in the activities of ADH, GDH and α-esterase.

Materials and Methods

Plant material

Nicotiana tabacum L., Wisconsin 38 callus culture was cultivated on modified Murashige and Skoog (1962) medium to which 2 mg/l indolylacetic acid, 0.2 mg/l kinetin (6-furfurylaminopurin) or 0.05 mg/l 4-PU-30 (N-phenyl-N′-2-(chloro-4-pyridyl)-urea) was added. The callus was cultivated in the dark under 26°C.

Chilling and low-temperature stress

Four-week-old cultures were inoculated on fresh MS nutrient medium and cultivated 3 days under 26°C. The chilling stress was performed in a dark refrigerator under 4°C for three days. The freezing stress was realized after cold acclimation to 4°C for 3 days followed by treatment with –2°C for 2 hours and –10°C for 3 hours in the dark. Finally the flasks were left for 24 hours under 4°C to thaw gradually.

Histochemical methods

ADH and GDH were determined by the tetrazolium reductase method (Lojda et al., 1979). The positive reaction was expressed as a blue staining of cell constituents. The α-esterase was investigated by the method of simultaneous azocopulation (Beneš, 1962) using as substrate α-naphthylacetate and diazonium salt fast blue RR. The dark brown staining is characteristic for the cells with α-esterase activity. The enzymes were studied in hand made sections.

Samples for the analyses were taken immediately after the chilling and freezing treatment and also after 3 weeks recovery in order to estimate the adaptation potential of the tobacco callus to low temperature, as well as to test the protective effect of 4-PU-30 and kinetin. Callus cultivated under 26°C was used as control.

Results

Data from the histochemical investigation showed that the three enzymes under study were affected by low-temperature stress (Table 1). The activity of ADH and GDH in the callus cultured at 4°C was largely decreased and after freezing at –10°C no activity was established, while all the cells in the control manifested high activity of both enzymes (Plate I, Figs. 1, 2, 3, 6, 7, 8). These data remain valid independently of the callus cultivation on the medium containing 4-PU-30 or kinetin. After a 20-day period of recovery a tendency of restoration of ADH activity was observed (Plate I, Figs. 4, 5). While immediately after the low-temperature stress only groups of cells or single cells characterized by positive ADH reaction were observed in the callus chilled at 4°C and in the callus frozen at –10°C the enzyme reaction was negative (Plate I, Figs. 2, 3). After 20 days in all calluses that passed the temperature stress the number of the cells with positive ADH activity increased. In the –10°C treated callus single cells or groups of cells with well expressed ADH reaction were found at the common background of cells with negative ADH reaction (Plate I, Figs. 4, 5).

The inclusion of 4-PU-30 into the nutrient medium increased ADH activity to a higher extent than kinetin. This tendency was evident in the control as well as in the variants immediately after low-temperature treatments as well as 20 days after the stress (Table 1). The restoration of ADH activity after the chilling stress was observed

both in the 4-PU-30 and kinetin variants. It must be emphasized however, that a clear restoration of ADH activity after the freezing stress was established only in callus cells cultivated in the presence of 4-PU-30 (Table 1). After the freezing stress the callus cultivated in the presence of kinetin restored the activity of ADH more moderately.

While ADH activity resulted both from 4-PU-30 and kinetin treatment a tendency of normalization of the GDH activity was found only in the 4-PU-30 treated variants (Table l). Tobacco callus grown on kinetin containing nutrient medium at 4°C and –10°C remained with low or negative GDH reaction after a 20-day-recovery period (Plate I, Figs. 14, 15).

Table 1. Effect of cytokinins 4-PU-30 and kinetin on the activity of the enzimes ADH, GDH and α-esterase in tobacco callus after low-temperature stress

Enzymes	4-PU-30			Kinetin		
	control 26°C	chilling stress 4°C	freezing stress –10°C	control 26°C	chilling stress 4°C	freezing stress –10°C
ADH I	++++	++	–	+++	+	–
ADH II	++++	+++	++	+++	++	+
GDH I	++++	++	–	+++	+	–
GDH II	++++	+++	+	+++	+	–
Esterase I	++	+++	++++	++	+++	++++
Esterase II	++	++	+++	++	+++	++++

I – enzyme activity immediately after the low-temperature stress; II – enzyme activity after 20 days incubation; – negative reaction; + very pale staining; ++ light staining; +++ intensive staining; ++++ very intensive staining

Increased α-esterase activity in the tobacco callus was evident immediately after chilling and freezing stress both in the presence of 4-PU-30 and kinetin. Simultaneously much more intensive staining was established in the –10°C samples as compared to the 4°C samples.

After 20 days a clear tendency of decreased α-esterase activity and restoration of the normal levels was revealed in the 4-PU-30 variants stressed at 4°C and –10°C (Plate II, Figs. 1, 2, 3, 4, 5). However no alteration in the α-esterase activity was detected in the callus cultivated in the presence of kinetin after 20 days – the enzyme activity remained very high (Table 1).

Discussion

Responses of some plants to low temperature treatments followed a somewhat different pattern and are associated with the shift from aerobic to anaerobic metabolism (Kimmerer and Kozlovski, 1982). Our results differ from those reported previously

Plate I. (p. 34) Effect of 4-PU-30 and kinetin on the activity of ADH and GDH in tobacco callus after low-temperature stress

Figure 1 to figure 10 – treatment with 4-PU-30

Fig. 1. ADH in tobacco callus cultivated at 26°C (control); **Fig. 2**. ADH in callus immediately after chilling at 4°C; **Fig. 3**. ADH in callus immediately after freezing at –10°C; **Fig. 4**. ADH in callus which passed low-temperature stress; **Fig. 5**. ADH in callus which passed freezing stress at –10°C after 20 days incubation. Single cells or groups of cells manifesting ADH reaction are established;
Fig. 6. GDH in tobacco callus cultivated at 26°C (control); **Fig. 7**. GDH in callus immediately after chilling at 4°C; **Fig. 8**. GDH in callus immediately after freezing stress at –10°C; **Fig. 9**. GDH in callus which passed low-temperature stress at 4°C after 20 days incubation; **Fig. 10**. GDH in callus which passed freezing stress at –10°C after 20 days incubation. Single cells with positive GDH activity

Figure 11 to figure 15 – treatment with kinetin

Fig. 11. GDH in tobacco callus cultivated at 26°C (control); **Fig. 12**. GDH in callus immediately after low-temperature stress at 4°C; **Fig. 13**. GDH in callus immediately after freezing stress at –10°C; **Fig. 14**. GDH in callus which passed low-temperature stress at 4°C after 20 days incubation; **Fig. 15**. GDH in callus which passed freezing stress at –10°C after 20 days incubation

Plate II. Effect of cytokinin 4-PU-30 on α-esterase activity in tobacco callus after low-temperature stress

Fig. 1. α-esterase in tobacco callus at 26°C (control); **Fig. 2**. α-esterase in callus immediately after chilling at 4°C; **Fig. 3**. α-esterase in callus immediately after freezing at –10°C; **Fig. 4**. α-esterase in callus 20 days after chilling at 4°C; **Fig. 5**. α-esterase in callus 20 days after freezing at –10°C

that the low temperature causes rapid expression of the ADH mRNA and increases the level of the enzyme activity (Ricard et al., 1986; Vojnikov and Ivanova, 1988; Christie et al., 1991). It is known that some plants which are sensitive to freezing pass rapidly to ethanol fermentation increasing ADH activity. This, in turn, could lead to a sustaining of the energy generation and neutralization of the cytoplasmic acidosis by the regeneration of the oxidised NAD^+ (Walker et al., 1987; Christie et al., 1991). Arguments for the role of ADH in the creation of tolerance toward low temperature

and anaerobic conditions were developed (Roberts et al., 1984). In contrast, Jarillo et al. (1993) supposed that ADH is not obligatory for the developing cold resistance. Grout (1987) and Gui (1990) proved that during the freezing frost/thaw cycle many of the plant enzymes and proteins of non-enzymatic nature are inactivated. The above contradictory conceptions may be due to genotypical differences and to different experimental approach. Our data are in concert with the conception of the second group of authors as far as the decreased ADH activity at 4°C and –10°C established in this study may be attributed to the inactivation of the enzyme because of the tropical origin of tobacco. The latter can survive under 0°C. In addition it was reported that the production of ethanol under anaerobic conditions is different in various plant species, depending on their tolerance toward anoxia (Alpi and Beevers, 1983) and its decrease correlated with the tissue necrosis during freezing (Kimmerer and Kozlowski, 1982).

The established GDH lability is easily understood as far as being a mitochondrial enzyme which participates in the citric acid cycle, GDH is affected as a result of the cold treatment damage in the mitochondrial structure. Guy (1990) also characterized GDH as a cold-labile enzyme.

As a lysosomal enzyme esterase is one of the hydrolases that are related to cell death (Matile, 1975; Gupta, 1985). There are data on the increased enzyme activity in some higher plants in relation to the processes of cell autolysis and lysis of the cell wall (Flinn and Smith, 1967). Taking into account that tobacco is a tropical plant and that in tropical and subtropical plant tissues damages are observed at lower than 15°C (Wilson, 1987) we assume that the established increased esterase activity during chilling and freezing stresses is probably a result of enhanced hydrolytic processes in the severely damaged tobacco callus. The low temperature may cause cell death related to cytoplasmic acidosis, reduced glycolitic pathway and alteration in the membrane lipid phase (Wilson, 1987; Perata and Alpi, 1993). It is highly possible that the increased esterase activity could be related to the degenerative processes resulting from the low-temperature stress.

We observed a tendency of restoration of the initial activity of the enzymes under study three weeks after the stress action. In the presence of the synthetic cytokinin 4-PU-30 a stronger effect toward normalisation of the enzyme activities was established. These results support our previous data concerning higher antistress activity of 4-PU-30 as compared to that of kinetin (Iliev et al., 1994).

Similar induction of tolerance toward anaerobiosis through increased ADH activity following treatment with abcisic acid was described by other investigators (Hwang and Van Toai, 1991; Perata and Alpi, l993).

It may be concluded that phenylurea cytokinin 4-PU-30 added to the nutrient medium manifests higher chilling and freezing antistress activity as compared to adenine cytokinin – kinetin. The cytokinins of this group may be involved in plant

adaptation to low temperature, as well as in the reversibility of the changes resulting from the cold, treatment and increase of low-temperature tolerance.

Acknowledgements: This work was supported by Grant B-413/1994 from the National Science Fund, Bulgaria.

References

Alpi, A., H. Beevers, 1983. Effect of O_2 concentration on rice seedlings. Plant Physiol., 71, 30–34.

Beneš, K., l962. Histochemical demonstration of esterase in the root tip of *Vicia faba* L. with azo-coupling method. Biol. Plant., 4, 211–219.

Christie, P. J., M. Hahn, V. Walbot, 1991. Low-temperature accumulation of alcohol dehydrogenase-l mRNA and protein activity in maize and rice seedlings. Plant Physiol., 95, 699–706.

Ershova, A. N., V. V. Churikova, I. A. Sterligova, 199l. Effect of kinetin on phospholipid content in maize seedlings in modified gaseous media. Physiol. Biochem. Cult. Plants, 3, 250–256 (In Russ.).

Flinn, A., D. Smith, 1967. The localization of enzymes in the cotyledons of *Pisum arvense* L. during germination. Planta, 75, 10–22.

Grout, B. W. W., 1987. Higher plants at freezing temperatures. In: The Effects of Low Temperatures on Biological Systems. Eds. B. W. W. Grout and G. J. Morris. Edwards Arnold, 293–314.

Gupta, H., 1985. Plant lysosomes: aspects and prospects. Current Sci., 54, 554–559.

Guy, C., 1990. Cold acclimation and freezing stress tolerance: Role of protein metabolism. Annu. Rev. of Plant Physiol. and Mol. Biol., 41, 187–223.

Hwang, S. Y., T. T. Van Toai, 1991. Abcisic acid induces anaerobiosis tolerance in corn. Plant Physiol., 97, 593–597.

Iliev, L., M. Tsolova, E. Karanov, 1995. The effect of phenylurea and adenine cytokinins on the chilling and freezing tolerance of tobacco callus cultures. Compt. rend. Acad. bulg. Sci., 47, 67–70.

Jarillo, J. A., A. Leyva, J. Salinas, J. N. Martinez-Zapater, 1993. Low temperature induces the accumulation of alcohol dehydrogenase mRNA in *Arabidiopsis thaliana*, a chilling tolerant plant. Plant Physiol., 101, 833–837.

Kimmerer, T., T. Kozlowski, 1982. Ethylene, ethane, acetaldehyde and ethanol production by plants under stress. Plant Physiol., 69, 840–847.

Levitt, J., 1980. Responces of plants to environmental stresses: chilling, freezing and high temperatures. In: Physiological Ecology: A series of Monographs, Texts Treatises, Ed. 2, vol. l Ed. T. Kozlowski, Acad. Press, New York, 23–64.

Lojda, Z., R. Gossrau, T. H. Schibler, 1979. Enzyme Histochemistry. A laboratory Manual. Springer-Verlag, Berlin-Heidelberg-New York.

Lyons, J. M., J. K. Raison, 1970. Oxidative activity of mitochondria isolated from plant tissues sensitive and resistant to chilling injury. Plant Physiol., 45, 386–389.

Matile, Ph., 1975. The lytic compartment of plant cells. In: Cell Biology, Monographs, Vol. 1, Springer-Verlag, Wien-New York.

Murashige, T., F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant., 15, 473–479.

Perata, P., A. Alpi, 1993. Plant responses to anaerobiosis. Plant Sci., 93, 1–17.

Reaney, M. J. . L. V. Gusta, 1987. Factors influencing the induction of freezing tolerance by abscisic acid in cell suspension cultures of *Bromus inermis* Leyss and *Medicago sativa* L. Plant. Physiol., 83, 423–427.

Ricard, B., B. Mocquot, A. Fournier, M. Delseny, A. Pradet, 1986. Expression of alcohol dehydrogenase in rice embryos under anoxia. Plant Mol. Biol., 7, 321–329.

Roberts, J., J. Callis, O. Jardetzky, V. Walbot, M. Freeling, 1984. Cytoplasmic acidosis as a determination of flooding tolerance in plants. Proc. Nat. Acad. Sci. USA, 81, 6029–6033.

Roberts, J., K. Chang, J. Webster, J. Callis, V. Walbot, 1989. Dependence of ethanolic fermentation, cytoplasmic pH regulation and viability on the activity of dehydrogenase in hypoxic maize root tips. Plant Physiol., 89, 1275–1278.

Tantan, H., J. Dörffling, 1991. Effect of chilling on physiological responses and changes in hormone level in two *Euphorbia pulcherrima* varieties with different chilling tolerance. J. Plant. Physiol., 138, 734–740.

Vojnikov, V. K., G. G. Ivanova, 1988. Physiologicial stress and the regulation of genome activity of eocaryotic cells. Success Contemp. Biol., 105, 3–16 (In Russ.).

Walker, J. C., E. A. Howard, E. S. Dennis, W. J. Peacock, 1987. DNA sequences required for anaerobic expression of the alcohol dehydrogenase 1 gene. Proc. Nat . Acad. Sci. USA, 84, 6624–6628.

Wilson, J. M., 1987. Chilling injury in plants. In:The Effects of Low Temperatures in Biological Systems. Eds: B. W. W. Grout and G. J. Morris, Edward Arnold, 271–292.

Xie, Y., R. Wu, 1989. Rice alcohol dehydrogenase genes: anaerobic induction, organ specific expression and characterization of cDNA clones. Plant Mol. Biol., 13, 53–68.

Zhyrmunskaja, N., T. Ovsyannikova, Y. U. Shapovalov, A. Baskakov, 1989. Interrelation between the antistress activity and cytokinin like properties of synthetic biologically active compounds. Physiol. and Biochem. of Plants, 21, 446–451 (In Russ.).