BULG. J. PLANT PHYSIOL., 2003, 29(1–2), 13–20

INHIBITION OF CYTOKININ-GLUCOSE CONJUGATION IN DEROOTED RADISH, TOBACCO AND BEAN SEEDLINGS

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Received July 21, 2002

Summary. Four inhibitors of cyclin-dependent kinases, olomoucine, bohemine, roscovitine and isopropyl-olomoucine were tested for their ability to modify the conversion of exogenous tritiated dihydrozeatin ([^{3}H]DHZ) in species differing in their pattern of cytokinin sequestering. In radish (*Raphanus sativus* L.), species with predominant cytokinin N-glucosylation pathway, application of inhibitors (100 µM) decreased conversion of [^{3}H]DHZ into 7N- and 9N-glucosides. In common bean (*Phaseolus vulgaris* L.) cytokinin O-glucosylation is the prevailing deactivation mechanism and the tested inhibitors did not influence significantly the conversion of [^{3}H]DHZ. The down-regulation of active cytokinin levels in tobacco (*Nicotiana tabacum* L.) is achieved by both 7N-glucosylation and degradation by cytokinin oxidase/dehydrogenase (CKX). The inhibition of 7N-glucosylation in this species stimulated the conversion of [^{3}H]DHZ to nucleotide and riboside. The tested purine analogues have been proved to be selective inhibitors of cytokinin N-glucosylation with potential practical application.

Key words: dihydrozeatin, cyclin-dependent kinase inhibitors, cytokinin 7N-glucosylation, radish, bean, tobacco

Abbreviations: BA – N^6-benzyladenine, cdk inhibitors – cyclin-dependent kinase inhibitors, CKX – cytokinin oxidase/dehydrogenase, DHZ – dihydrozeatin, DHZR – dihydrozeatin riboside, DHZ7G – dihydrozeatin 7-glucoside, DHZ9G – dihydrozeatin 9-glucoside, DHZOG – dihydrozeatin O-glucoside,

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DHZMP – dihydrozeatin 9-riboside-5′-monophosphate, iP – N^6-(Δ^2-isopentenyl)adenine, Z – *trans*-zeatin, ZR – *trans*-zeatin riboside, ZOG – *trans*-zeatin O-glucoside, Z7G – *trans*-zeatin 7-glucoside, Z9G – *trans*-zeatin 9-glucoside

Introduction

Cytokinins play a decisive role in regulation of numerous physiological and morphological processes occurring in plant cells. The level of their active forms is subjected to tight control throughout the whole plant development. Important metabolic conversion, involved in the regulation and maintenance of cytokinin homeostasis, is the conjugation of cytokinin base or riboside to glucose. Glucosylation results in a substantial decrease of biological activity, thus reducing the active cytokinin pool. Depending on the position of glucose attachment glucosylation may be either reversible or irreversible. When the glucose moiety is attached to the side chain or to the 3N-position of the purine ring storage forms are produced, which can be converted back by β-glucosidase. Attachment of glucose to 7N- or 9N-position at the purine ring results in formation of stable compounds with low physiological activity thus representing deactivation process (Letham et al., 1983).

Cytokinin conjugation patterns differ among the plant species (Auer, 1997). In radish (*Raphanus sativus* L.) the predominant deactivation pathway of exogenous cytokinins was found to be 7N-glucosylation. After feeding of radish cotyledons with radiolabeled zeatin (Z) the major metabolite was zeatin 7-glucoside (Z7G), while only low amounts of the corresponding 9N-glucoside were detected (Parker et al., 1973). A similar metabolic conversion was reported for exogenously applied DHZ (McGaw et al., 1984). In common bean (*Phaseolus vulgaris* L.) cytokinin O-glucosylation is the prevailing type of cytokinin sequestering. The major metabolite of exogenous DHZ was identified to be its corresponding O-glucoside (DHZOG) (Palmer et al., 1981). Recently, the genes for the enzymes zeatin O-glucosyl transferase and O-xylosyl transferase were isolated also from *Phaseolus* (Martin et al., 1999a; Martin et al., 1999b). In tobacco exogenous cytokinins are accumulated mainly as ribosyl-monophosphates and as 7N-glucosides (Laloue et al., 1982). Exogenous N^6-benzyladenine (BA) and N^6-(Δ^2-isopentenyl)adenine (iP) are metabolized to nucleotides in tobacco as well as in *Acer* cell suspensions (Laloue et al., 1974).

The suppression of cytokinin deactivation mechanisms may cause an increase in the pool of active cytokinins and strengthen the action of the exogenous ones. Searching for compounds with potential N-glucosylation inhibitory activity, Parker et al. (1986) synthesized 2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (later named olomoucine). As olomoucine was reported to be O-glucosylated (Tao et al., 1991), which contributed to the weakening of its inhibitory strength, we have tested several olomoucine structural analogues – bohemine, roscovitine and isopropyl-

Table 1. Structure of the purine analogues. C2, C6 and N9 are the positions of substitution at the purine ring.

Compound	Substituent		
	C2	**C6**	**N9**
Adenine	H	Amino	H
Olomoucine	2-Hydroxyethylamino	Benzylamino	Methyl
Bohemine	2-Hydroxypropylamino	Benzylamino	Isopropyl
Roscovitine	[1-(Hydroxymethyl)propyl]amino	Benzylamino	Isopropyl
Isopropyl-Olomoucine	2-Hydroxyethylamino	Benzylamino	Isopropyl

olomoucine (Table 1), for potential inhibitory activity in exogenous cytokinin N-glucosylation. The selected compounds were proven to exhibit another important physiological effect of olomoucine – they were effective inhibitors of cyclin-dependent kinases, enzymes involved in the cell cycle progression (Vesely et al., 1991; De Azevedo et al., 1997; Havlicek et al., 1997).

In this work we report the effect of olomoucine and its structural analogues bohemine, roscovitine and isopropyl-olomoucine on the conversion of exogenous [^{3}H]DHZ in three species differing in their pattern of irreversible cytokinin glucosylation.

Material and Methods

Plant material

Seeds of radish (*Raphanus sativus* L. cv. Rampouch), common bean (*Phaseolus vulgaris* L. cv. Great Northern) and tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) were sown in trays on Perlite supplied with Knopp's solution as nutritional medium. The plants were grown in cultivation chamber at 22 ºC and 8 h light/16 h dark cycle (fluence rate 110 μmol.m^{-2}.s^{-1}). Before each experiment the 7-day old seedlings were derooted to achieve fast and efficient uptake *via* transpiration stream.

Incubation with tritiated DHZ

Derooted seedlings were incubated for 24 h in 100 μM solutions of purine analogues [2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine (olomoucine); 2-(1-(hydroxymethyl) propylamino)-6-benzylamino-9-isopropylpurine (roscovitine); 2-(3-hydroxypropylamino)-6-benzylamino-9-isopropylpurine (bohemine), 2-(2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine (isopropyl-olomoucine)]. The inhibitors were kindly provided by Prof. Miroslav Strnad (Institute of Experimental Botany,

Olomouc, Czech Republic). The substrate [^{3}H]DHZ (1.8 TBq/mmol, 500 000 dpm, 4 pmol per sample prepared by Dr. Jan Hanus, Institute of Experimental Botany AS CR, Prague, Czech Republic) was supplied 2 h after incubation initiation.

Cytokinin extraction and purification

Samples of approximately 1 g FW were frozen in liquid nitrogen and extracted overnight with methanol/water/formic acid = 15/4/1, v/v/v, pH ~ 2.5, –20 °C. The extract was passed through C_{18} to eliminate the lipophilic interferences. DHZ bases, ribosides, glucosides and nucleotides were separated stepwise on Oasis MCX mixed mode (cation exchange and reverse-phase) column (150 mg, Waters, USA) applying the method described by Dobrev and Kamínek (2002).

HPLC of radiolabeled cytokinins

Radiolabeled metabolites of [^{3}H]DHZ were analyzed using instrumental set-up of: Series 200 Quaternary HPLC Pump (Perkin Elmer, USA) coupled to 235C Diode Array Detector (Perkin Elmer, USA) and RAMONA 2000 flow-through radioactivity detector (Raytest, Germany), column: Luna C_{18}(2), 150 mm/4.6 mm/3 mm (Phenomenex, USA). The sample (100 µl) was eluted at flow rate 0.6 ml/min at 30 °C and UV detection was performed at 270 nm. Mobile phase: A = 40 mM CH_3COOH adjusted to pH 4.1 with NH_4OH; B = CH_3OH/CH_3CN =1/1 (v/v). Linear gradient: 10 to 20% B at 2 min, 20 to 45% B at 17 min, 45 to 100% B at 2 min, 100% B held for 2 min, 100 to 10% B at 2 min. The radioactive metabolites were identified on the basis of coincidence of retention times with authentic standards.

Results

The metabolism of radiolabeled DHZ was followed in derooted seedlings of radish, bean and tobacco, supplied *via* the transpiration stream with 100 µM aqueous solutions of olomoucine, bohemine, roscovitine and isopropyl-olomoucine. The [^{3}H]DHZ conversions varied significantly among the species investigated. In radish up to 50% of the supplied [^{3}H]DHZ was 7N-glucosylated (Fig. 1, control). The amounts of 9N- and O-glucosides were significantly lower, together amounting to about 15% of the radioactivity applied for HLPC radiocounting. Only 2% of the activity corresponded to DHZR.

Similar metabolic pattern was observed after supplying tobacco seedlings with [^{3}H]DHZ (Fig. 2, control). Above 50% of the radioactivity was due to DHZ7G. In contrast to radish, low amounts of DHZMP were also detected (about 4%). The activity corresponding to O-glucoside and riboside was low.

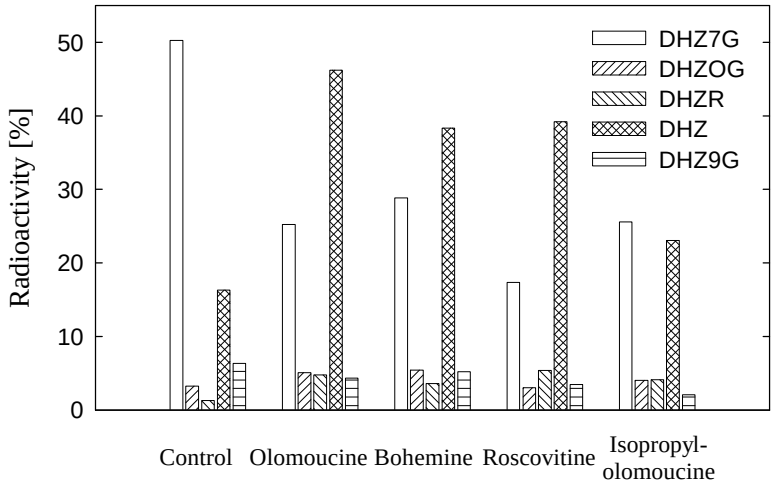

Fig.1 Effect of purine analogues (100 µM) on [^{3}H]DHZ conversions in radish seedlings. Values are expressed as percentage of radioactivity, subjected to HPLC radiocounting. Means of three independent experiments are shown.

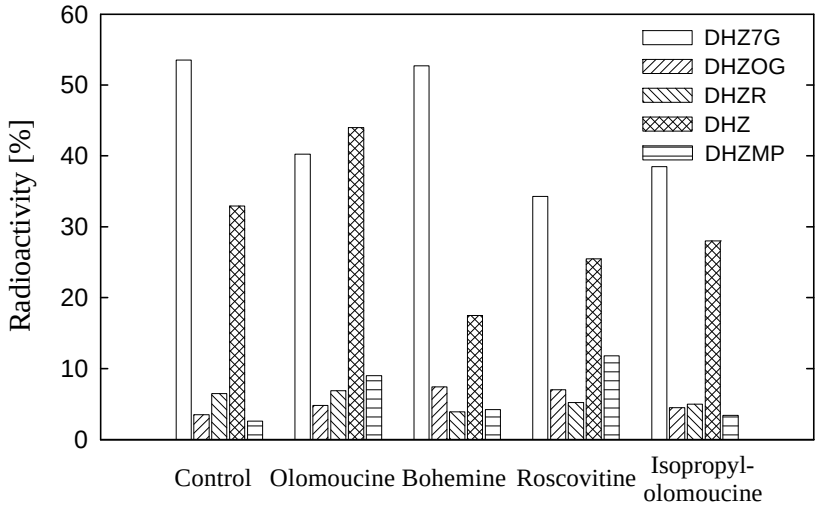

Fig. 2 Effect of purine analogues (100 µM) on [^{3}H]DHZ conversions in tobacco seedlings. Values are expressed as percentage of radioactivity, subjected to HPLC radiocounting. Means of three independent experiments are shown.

In *Phaseolus* seedlings the predominant metabolite of [^{3}H]DHZ conversion was its O-glucoside, amounting to about 8% of the injected activity (Fig. 3, control). Most of the radioactivity (about 15%) was due to [^{3}H]DHZ – both non-metabolized or resulting from the back-conversion. The levels of DHZ 7N-glucoside and riboside were assigned to 5% and 3%, respectively, of the radioactivity subjected to HPLC.

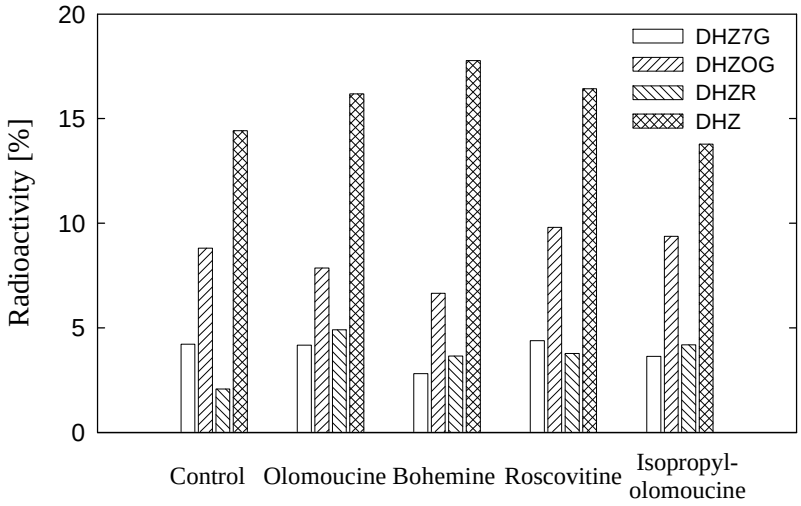

Fig. 3 Effect of purine analogues (100 µM) on [^{3}H]DHZ conversions in bean seedlings. Values are expressed as percentage of radioactivity, subjected to HPLC radiocounting. Means of three independent experiments are shown.

Treatment with purine analogues had differential effects on DHZ turnover in each individual species. In radish seedlings the substantial reduction of 7N-glucosylation caused simultaneous up to 3-fold rise in the radioactivity due to DHZ (Fig. 1). The strongest effect was exhibited by roscovitine, followed by olomoucine, isopropyl-olomoucine and bohemine. Mild reduction was observed also in the activity corresponding to DHZ9G. On the contrary, a slight increase in the accumulation of DHZOG was detected.

Application of purine analogues induced substantially the levels of DHZMP in tobacco seedlings (Fig. 2). The radioactivity corresponding to DHZMP rose from 4% in the controls to 10% in roscovitine- and olomoucine-treated seedlings. In contrast, the radioactivity accumulated as DHZ7G was reduced. Again, roscovitine had the strongest effect reducing DHZ7G level by ca 20%. Inhibitor treatment did not affect the radioactivity accumulation into O-glucosides and ribosides of DHZ.

In contrast to radish and tobacco, in bean seedlings the purine analogues had only negligible effect on [^{3}H]DHZ conversion (Fig. 3). Bohemine slightly reduced the accumulation of DHZ7G, raising simultaneously the level of the corresponding free base. A mild decrease in the radioactivity due to the principal metabolite of DHZ in *Phaseolus,* the O-glucoside, was also registered. In general, it seems that the purine analogues do not affect the metabolism of exogenous cytokinin in common bean.

Discussion

The effect of exogenous cytokinins in plants may be considerably diminished by their fast metabolic conversion into less biologically active forms. Thus, the simultaneous

application of inhibitors of their deactivation may prolong their functioning and strengthen their effect. In this communication the influence of four purine analogues on the conversion of exogenous radiolabeled DHZ was evaluated in three species differing in their pattern of cytokinin deactivation. Radish is a species in which 7N-glucosylation is the principal mechanism for down-regulation of the active cytokinin pool (McGaw et al., 1984). In common bean the predominant process of cytokinin sequestering is O-glucosylation (Palmer et al., 1981). Tobacco represents a mixed type as both N- and O-glucosylation pathways take equal part in the cytokinin deactivation in this species. The exogenous cytokinins are mostly converted to nucleotides (Laloue et al., 1974).

The applied inhibitors exerted their effect only on the species with 7N- and 9N-glucosylation type of cytokinin sequestering, but not on bean, where O-glucosylation is the principal deactivation mechanism. Hence, it seems that the purine analogues do affect the enzymes involved in N-glucosylation but do not influence O-glucosylation machinery. Among the inhibitors tested roscovitine appears to be the most effective. Most probably, its structure enables its effective binding to the catalytic site of the enzyme(s) blocking further attachment of substrates. The differential regulation of N- and O-glucosyl transferases seems to correspond to the different function of both enzyme groups. N-glucosyl transferase(s) have rather broad substrate specificity including adenine (Baumann et al., 1994), isoprenoid as well as aromatic cytokinins (Mok et al., 2001) and seems to be involved in rapid “detoxification” reactions. The O-glucosyl transferases exhibit strict substrate- and developmental stage-specificity (Martin et al., 2000).

Olomoucine, bohemine, roscovitine and isopropyl-olomoucine strongly affected DHZ metabolism in radish and tobacco plants but had only negligible effect on beans. Thus, they can be utilized as a tool for studying the mechanisms involved in the regulation of cytokinin levels in these species with potential practical application.

Acknowledgements: The authors thank Prof. Miroslav Strnad, Institute of Experimental Botany AS CR, Olomouc, Czech Republic for kindly providing the inhibitor substances and Dr. Jan Hanuš, Institute of Experimental Botany AS CR, Prague, Czech Republic for preparing the radiolabeled DHZ. This work was supported by Grant Agency of the Czech Republic (grants No. 522/99/1130 and 522/02/D058) and by the Ministry of Education, Youth and Sports (grant No. LN00A081).

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