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CYTOKININ METABOLISM AND CYTOKININ OXIDASE AND ADENINE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN MALE STERILE *BRASSICA NAPUS* LEAVES

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Key Word Index—*Brassica napus*; adenine phosphoribosyltransferase; cytokinin oxidase; cytokinin metabolism; male sterility.

Abstract—The metabolism of [^{14}C]zeatin (Z) and [^{3}H]dihydrozeatin (DZ), and the activity of two key enzymes in cytokinin (CK) metabolism, i.e. CK oxidase and adenine phosphoribosyltransferase (APRT), was studied in the leaves of wild type (WT) and genic male sterile (GMS) plants of *Brassica napus*. The leaves of the GMS plants metabolized Z and DZ less efficiently compared to WT leaves. Adenine (Ade) was the major metabolite of Z in both the leaves, and GMS leaves produced 50% less Ade in comparison with WT leaves. This correlated well with the CK oxidase activity in the two types of leaves; WT leaves show twice the activity of this enzyme in comparison with GMS leaves. GMS leaves produced higher amounts of DZ nucleotide than the WT leaves, and this correlated with 17% more APRT activity in GMS leaves. There was no difference in the amount of Z nucleotide produced by the two types of leaves. The results presented show that mutation in a gene controlling male fertility affects CK metabolism in vegetative tissues, in addition to reproductive tissues (reported earlier), and is related to the activity of at least two enzymes involved in CK metabolism. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

The involvement of plant growth substances (PGSs) in the expression of male sterility in angiosperms has been argued for in a number of genic (GMS) and cytoplasmic (CMS) male sterile systems [1]. In particular, cytokinins (CKs) have been implicated in both GMS and CMS systems [2–6].

The relationship of CKs with male sterility has primarily been based on the analysis of endogenous levels of CKs in vegetative and reproductive tissues of male sterile, male fertile and restored plants. In a few studies, the metabolism of CKs has also been shown to be affected in male sterile plants [3, 4]. However, it is not clear whether CK metabolism is affected throughout the plant body or whether it is restricted to certain tissues of male sterile plants. In order to understand the whole plant physiology of male sterile plants, a comparative analysis of the endogenous levels of CKs, their metabolism and translocation in different parts is essential.

In a single gene recessive male sterile line (3–8) of *Brassica napus*, we showed earlier that roots, stem, flowers and in particular stamens, contain low levels of a number of CKs in comparison with corresponding tissues of fertile wild type (WT) plants [5, 7]. Also, the developing flowers of this GMS line metabolize [^{3}H]dihydrozeatin (DZ) less efficiently than the WT buds; in particular, dihydrozeatin nucleotide (DZNT) is produced in lower quantities in GMS than in WT buds [7]. One interesting aspect of this study was that leaves of GMS plants contain higher concentrations of endogenous CKs than those of WT plants, the opposite of other tissues in the plant body [5]. These observations were interpreted in terms of differential metabolism, biosynthesis, or translocation of CKs in GMS and WT leaves.

This study was conducted to determine: (1) whether the leaves of male sterile plants metabolize CKs differently than the male fertile plants, and (2) whether this difference correlates with the activity of two enzymes involved in CK metabolism, i.e. CK oxidase and adeninephosphoribosyl transferase (APRT).

RESULTS

[^{14}C]*Zeatin metabolism*

The total uptake of [^{14}C]zeatin (Z) increased over a period of 12 hr in both genotypes, but there was no appreciable difference between the two types of leaves (Table 1). About 30% of the extracted radioactivity was

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Table 1. Distribution of [^{14}C]Z in bound and soluble fractions in the leaves of WT and GMS line of *B. napus* after 0, 1 and 12 hr of uptake. All values represent DPM (mg^{-1} fr. wt)

Time uptake (hr)	Bound [^{14}C]				Soluble [^{14}C]		Total	
	Total		RNA					
	WT	GMS	WT	GMS	WT	GMS	WT	GMS
0	44	38	40	34	80	70	128	114
1	62	50	58	46	128	94	198	150
12	56	54	54	52	142	116	202	172

found in the bound fraction (DNA, RNA and proteins) and the remaining was in the soluble fraction as Z and its metabolites.

The percentage of unmetabolized [^{14}C]Z was higher in GMS than in WT leaves after 12 hr (Table 2). The main metabolites of [^{14}C]Z in both types of leaves were adenine (Ade), adenosine (Ado), zeatin riboside (ZR), *O*-glucosides of Z and ZR (OGZ and OGZR) and zeatin nucleotide (ZNT). Adenine showed the maximum difference between the two lines. After 12 hr, 41% of the total extracted radioactivity in WT leaves was in Ade in comparison with 19% in GMS leaves. Similarly, 20% radioactivity was recovered as Ado in WT leaves as compared to 10% in GMS leaves. Differences were also observed in the amount of ZR produced in GMS and WT leaves. However, there was no difference in the amount of *O*-glucosides and ZNT produced in the two leaves.

[^{3}H]*Dihydrozeatin metabolism*

The total [^{3}H]DZ uptake increased with time in the leaves of both genotypes, with WT leaves showing higher uptake than GMS leaves (Table 3). Most (>95%) of the extracted radioactivity was present in the soluble fraction as DZ and its metabolites, and negligible amounts in the bound fraction (DNA, RNA and proteins).

The percentage of [^{3}H]DZ unmetabolized in GMS leaves was higher, at 0, 1 and 12 hr of incubation, than

Table 2. Metabolism of [^{14}C]Z in the leaves of WT and GMS line of *B. napus* at 0, 1 and 12 hr of incubation. Values presented are percentages of total radioactivity extracted and are the means of two samples

CK metabolites	WT			GMS line		
	0 hr	1 hr	12 hr	0 hr	1 hr	12 hr
Z	42	25	3	45	34	10
ZR	3	9	0	5	16	18
Ade	10	16	41	3	6	19
Ado	0	5	20	0	3	10
OGZ + OGZR	0	3	23	0	2	27
Z-9-G	0	0	3	0	1	5
ZNT	15	12	3	12	10	2
Total % of [^{14}C] in known metabolites	70	70	93	65	72	91

Table 3. Distribution of [^{3}H]DZ in bound and soluble fractions in the leaves of WT and GMS line of *B. napus* after 0, 1 and 12 hr of uptake. All values represent DPM (mg^{-1} fr. wt)

Time uptake (hr)	Bound [^{14}C]				Soluble [^{14}C]		Total	
	Total		RNA					
	WT	GMS	WT	GMS	WT	GMS	WT	GMS
0	37	22	22	13	2645	1210	2698	1235
1	37	32	33	27	2658	1963	2703	2013
12	70	35	43	30	2857	2138	2967	2195

Table 4. Metabolism of [^{3}H]DZ in the leaves of WT and GMS line of *B. napus* at 0, 1 and 12 hr of incubation. Values presented are percentages of total radioactivity extracted and are the means of two samples

CK metabolites	WT			GMS line		
	0 hr	1 hr	12 hr	0 hr	1 hr	12 hr
DZ	32	29	17	44	40	27
DZR	1	2	4	2	3	9
OGDZ + OGDZR	11	11	12	9	13	18
9-Glucoside	1	1	2	1	1	3
DZNT	11	10	8	16	13	11
Dihydrolupinic acid	2	3	6	2	2	9
Unknown acidic compound	10	12	14	10	12	18
Total % of [^{3}H] in known metabolites	68	68	63	84	84	95

in WT leaves (Table 4). However, the percentage of various metabolites produced was greater in GMS leaves at 12 hr of incubation (long-term metabolism) as compared to WT leaves. *O*-Glucosides (OGDZ + OGDZR) were one of the major metabolites in both types of leaves, and at 12 hr incubation their percentage was higher in GMS than in WT leaves (Table 4). The GMS leaves also produced more nucleotides than the WT leaves and their level was high initially, but decreased after 12 hr in both WT and GMS leaves (Table 4). An unknown acidic compound (λ_{max} 254–260 nm) was present in both the genotypes and its level increased with time. At 12 hr of incubation, its level was higher in GMS than in WT leaves. The percentages of dihydrozeatin riboside (DZR) and lupinic acid were also relatively more in GMS in comparison with WT leaves at 12 hr after incubation.

CK oxidase activity

The leaves of the two genotypes showed differences in CK oxidase activity. WT leaves had more than twice the CK oxidase activity as compared to the GMS leaves (Table 5). This correlated well with the greater amounts of Ade produced from [^{14}C]Z in WT leaves, at different time intervals, than in GMS leaves (Table 4).

Adenine phosphoribosyltransferase activity

GMS leaves showed *ca* 17% more APRT activity in comparison with WT leaves (Table 5). The high APRT activity in GMS leaves correlates with a greater amount of DZNT, but not ZNT, produced in GMS in comparison with WT leaves.

DISCUSSION

This study has shown that leaves of the GMS line of *B. napus* do not metabolize [^{14}C]Z and [^{3}H]DZ as efficiently as the WT leaves. This could explain the high endogenous levels of Z and DZ in GMS as compared to WT leaves [5]. In both GMS and WT leaves, the metabolism of [^{14}C]Z was greater, over the 12 hr period, than that of [^{3}H]DZ. This supports the earlier results that DZ, and not Z, is the major endogenous CK in *B. napus* tissues [5, 6]. There were a number of metabolites produced from [^{14}C]Z and [^{3}H]DZ in both the GMS and WT leaves; the major ones different in the two tissues were Ade, nucleotides and glucosides.

Ade is an important purine involved in various metabolic and developmental pathways in plants. Ade is also a major metabolite of CK oxidase activity, which catalyses the side-chain cleavage in Z-type CKs; DZ-type CKs are resistant to CK oxidase because of saturation of bonds in the side-chain [8]. Because of this property, CK oxidase plays an important role in regulating the level of endogenous CKs in plants, and thus in plant growth and development [9]. In WT leaves, Ade was the major metabolite of [^{14}C]Z and in GMS leaves its level was 50% less than in WT leaves (Table 2). This correlates well with 50% more CK

Table 5. Specific activity of CK oxidase and APRT extracted from leaves of WT (cv. Westar) and GMS line of *B. napus*. Values represent mean±S.E. ($n = 3$)

Genotype	CK oxidase (nmol mg^{-1} protein hr^{-1})	APRT (pmol mg^{-1} protein hr^{-1})
WT	1.18±0.01	51.8±1.7
GMS line	0.55±0.03	60.7±0.5

oxidase activity in WT in comparison with GMS leaves (Table 5).

Ade is one of the precursors in the synthesis of CK nucleotides [10]. CK nucleotides play an important role in the uptake of CK bases and in cell to cell movement of CKs [10, 11]. Furthermore, CK nucleotides have been implicated to have a role in male sterility [12]. In young seedlings of a male sterile, *BM3*, mutant of *Arabidopsis thaliana*, low amounts of CK nucleotide were produced and the tissues had reduced levels of APRT activity—an enzyme involved in the Ade salvage pathway [4]. In *B. napus* GMS leaves, the amount of DZNT produced was higher than in WT leaves (Table 4) and it correlated with 17% more APRT activity in GMS than in WT leaves (Table 5). High APRT activity in GMS leaves may also explain the low amount of Ade produced in these tissues. The increase in APRT activity in turn could be related to greater amount of Z available (due to low CK oxidase activity) as a substrate for APRT.

O-Glucosides (OGDZ + OGDZR and OGZ + OGZR) were the major metabolites in both the WT and GMS leaves and their levels were higher in GMS than in WT leaves after 12 hr. Palmer and co-workers [13, 14] found OGDZ as the main CK in bean leaves and suggested that leaves are one of the sites of CK glucosylation. The level of DZR and lupinic acid was relatively low in the two types of leaves, with GMS leaves showing higher levels than WT leaves. However, Palmer *et al.* [13] and Van Staden and Bossè [15] found DZR as the major metabolite of DZ in bean stem and carnation flowers, respectively. The level of ZR produced from [^{14}C]Z after 12 hr was higher in GMS leaves as compared to WT leaves. A similar profile was observed in the endogenous ZR levels in leaves of the two lines [5].

This study, along with our previous report [7], has shown that in male sterile plants of *B. napus*, the metabolism of CKs is affected in both the vegetative and reproductive tissues. Thus, although mutation in a gene controlling male fertility affects only the flower phenotype, i.e. stamen development, the hormonal physiology is affected in both the reproductive and vegetative tissues of male sterile plants. Also, mutation in the male fertility gene affects at least two keys enzymes involved in Ade cycling and in CK metabolism.

EXPERIMENTAL

Plant material. Seed source of *B. napus* (cv. Westar) and the GMS line (3–8) and the growth conditions of plants were as described earlier [5].

CK metabolism

Uptake of [^{3}H]DZ *or* [^{14}C]Z. Fourth oldest fully expanded leaf from the base of the inflorescence was excised and immediately dipped in H_2O. A 5-mm portion of petiole was removed under water and the leaf was transferred to 500 μl H_2O containing 0.37 MBq [^{3}H]DZ (Amersham, sp. act. 1.1–2.2 TBq mmol^{-1}) or 3.69 KBq [^{14}C]Z (Sigma, sp. act. 44.8 mCi mmol^{-1}). Two leaves per sampling time were allowed to take up [^{3}H]DZ–[^{14}C]Z for 1 hr. The sampling times were 0, 1 and 12 hr after 1 hr uptake.

Analysis of metabolites. The metabolites were extracted, purified and analysed in a manner similar to that described previously [7]. The samples were sepd into bound and soluble frs. Bound frs were analysed for [^{3}H]/[^{14}C] bound to DNA, RNA and proteins. Soluble frs were subjected to ion-exchange chromatography and 2D TLC. The nucleotides were chemically hydrolysed as reported in ref. [7] and *O*-glucosides were hydrolysed with β-glucosidase [16]. Frs with major radioactivity were purified by HPLC [5].

CK oxidase assay

Extraction. The extraction and assay procedure for CK oxidase was performed in a cold room and was similar to that described in ref. [17]. The leaf tissue was homogenized in an equal vol. chilled 100 mM Tris–HCl (pH 7.5). To the homogenate, 1.5 g dry wt per 5 g fr. wt tissue PVPP (hydrated with 50 mM Tris–HCl) was added and the suspension was filtered through cheese cloth, *in vacuo*. The residue was washed with 20 ml 50 mM Tris–HCl and the combined filterates were centrifuged at 10 000 *g* for 10 min. To the supernatant, 50 μl ml^{-1} 1% (v/v) Polymin P was added dropwise with constant stirring. The sample was centrifuged after 20 min at 10 000 *g* for 10 min. To the supernatant, solid $(NH_4)_2SO_4$ was added to give 80% satn and it was allowed to stand for 30 min. The sample was centrifuged at 20 000 *g* for 20 min and the ppt. was collected and stored at −20°.

Enzyme assay. The pptd enzyme was dissolved in 1 ml Tris–HCl buffer (pH 7.5) per 5 g fr. wt tissue. The assay mixt., containing 22.29 μM Z-8-^{14}C (Sigma, sp. act. 44.8 mCi mmol^{-1}) and extracted enzyme [in a total of 50 μl 100 mM Tris–HCl (pH 7.5)], was incubated at 37° for 30 min. The reaction was terminated by adding 100 μl cold EtOH (95%, v/v) containing Z and Ade (0.75 mM each), and the ppt. was removed by centrifugation in a minifuge. The sample was purified by 1D TLC, using *n*-BuOH–NH_3–H_2O (7:1:2, upper phase) and by HPLC (10% MeOH in 1% HOAc for 27 min at 2.5 ml min^{-1}). TLC and HPLC systems were as described elsewhere [5]. Radioactivity associated with Z and Ade was determined for calculation of CK oxidase activity.

Adenine phosphoribosyltransferase assay

Extraction. The extraction procedure was similar to that of ref. [12]. A crude prepn of enzyme was prepd by grinding the leaf tissue in cold 50 mM Tris–HCl (1 ml per 250 mg tissue) (pH 7.4) containing 5 mM $MgCl_2$.

The sample was centrifuged in a minifuge for 3 min to remove insoluble matter.

Enzyme assay. This procedure was as in ref. [4]. The assay mixt. contained 50 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 10 mM NaN_3, 2.4 mg ml^{-1} BSA, 1 mM PRPP, 22.29 μM Z-8-^{14}C (Sigma, sp. act. 44.8 mCi $mmol^{-1}$) and enzyme in a total vol. of 100 μl. The mixt. was incubated at 37° for 1 hr and the reaction was terminated by adding 200 μl cold EtOH containing Z and ZR (100 μg ml^{-1} each). Radioactivity associated with Z, ZNT and ZR was determined using 1D TLC and HPLC [5]. Enzyme extracts were also tested for the presence of nucleoside phosphorylase [4], but no activity was detected.

Protein assay

Protein content of the samples was estimated by the protein dye-binding method [18].

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