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D-TRYPTOPHAN AS IAA SOURCE DURING WHEAT GERMINATION

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Summary. To study the hormonal control of germination, the contents of Trp, MTrp and IAA were determined in coleoptiles, scutellum and roots of wheat seedlings (*Triticum aestivum* L.) on the 3 and 5 days of growth in the darkness. The concentration of Trp high at the 3rd day increased 1.15 and 2.5 times to the 5th day of germination for root and scutellum and coleoptile respectively. The MTrp and IAA contents decreased with seedling growth from the 3rd to the 5th day. The highest concentration of MTrp was in the upper 3 mm of coleoptile apex. The IAA concentration was high in the apex and in the elongation zone of coleoptile on the 3rd day of germination. The ratio MTrp/IAA was several times lower than the Trp/IAA ratio in almost all parts of seedlings, indicating that Trp is not a limiting factor for IAA biosynthesis. The activity of tryptophan racemase (EC 5.1.1.10) in etioplasts fraction was higher in the direction LTrp → DTrp on the 3rd day of germination. Both cytosol and etioplasts tryptophan racemase diminished with time and in the coleoptile at the 7th day the activity was less than a tenth. The activities of both L-TAT (EC 2.6.1.27) and D-TAT (EC 2.6.1.21) were also present in etioplasts fractions from upper part and roots of etiolated seedlings. Both activities were sensitive to the addition of cofactors Na pyruvate and NAD^+. Thus it was suggested that DTrp and MTrp participated in IAA biosynthesis during wheat germination and growth of seedlings and the direction LTrp → DTrp of tryptophan racemase played a key role during first days of heterotrophic growth.

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Abbreviations: DAO – D-amino acid oxidase, ME – β-mercaptoethanol, MTrp – N-malonyl-D-tryptophan, PMSF – phenylmethylsulphanylflouride, PLP – pyridoxal phosphate, L- (D-) TAT – L- (D-) tryptophan aminotransferase, Trp – L- (D-) tryptophan

Introduction

D-amino acids are present in a small amount practically in all living organisms despite of L-amino acids are normally used for protein biosynthesis. In order to facilitate the thermodynamic stability, racemization always occurs in nature and its rate is increased under acid and base conditions. Interconversion of D- and L-amino acids is widespread in microorganisms and this reaction was studied in detail according to antibiotic synthesis and the formation of peptidoglucan layer.

Racemization of amino acids is less understood in plants. D-alanine and D-tryptophan were appreciably found in free or bound to malonate forms. The possible use of D-tryptophan for IAA biosynthesis was shown in bioassay of elongation of pea stem segments (Law, 1987), in plastids of dark-grown pea seedlings (McQueen-Mason and Hamilton, 1989) and in barley coleoptiles (Tsurusaki et al., 1990).

Klambt (1961) found DTrp bound in N-malonyl-D-tryptophan (MTrp) in wheat seedlings. Then Elliott (1971) isolated MTrp from roots of wheat but did not observe any auxin activity in bioassay of stem elongation of oat. Therefore the physiological role of MTrp remained obscure. Despite these results the opportunity of DTrp participation derivatives, as well as MTrp, in IAA biosynthesis is still discussed (Markova and Gamburg, 1997). Previously, only traces of MTrp in dry seeds or in isolated embryos of wheat have been found (Gamburg et al., 1993), but the amount of MTrp increased up to 100 $nmol.g^{-1}$ of fresh weight in 5 days old wheat seedlings grown in the dark. In wilting the wheat seedlings, or excised mature leaves, accumulation of MTrp up to 300 $nmol.g^{-1}$ was reported (Rekoslavskaya et al., 1988). The participation of endogenous MTrp in IAA biosynthesis was not considered at all. Because the structure of DTrp is maintained intact in the molecule of MTrp, the latter could be used as a precursor in IAA biosynthesis. Therefore, to the present time, in case tryptophan is furthermore confirmed as IAA precursor (Müller et al., 1998), the experimental data about the physiological role of DTrp as an auxin source are not sufficient. From this point of view we continued the physiological study of a role of DTrp (MTrp). The purpose of this paper was to evaluate the presence of tryptophan racemase, and D-TAT, and the role of DTrp as precursor of IAA during the germination and growth of wheat seedlings in the dark.

Materials and Methods

Plant materials

Seeds of spring wheat (*Triticum aestivum* L. cv. Skala) were soaked for 1 h in tap water, sterilised in mixture of 1% chloramine and 0.1% saponine for 30 min, washed with sterilised water and then germinated in the dark on moistened filter paper at 26 °C.

For the estimation of tryptophan racemase and tryptophan aminotransferase activities 3, 5 or 7 days old dark-grown seedlings were used. Endogenous indole compounds were analysed in 3 and 5 days old seedlings selected for uniform coleoptile lengths of 1.5–2 cm and 5–6 cm respectively.

Extraction and assay of tryptophan racemase

For each tryptophan racemase preparation a batch of 200–250 seedlings were harvested. Seedlings were used intact or divided into upper parts (coleoptiles and primary leaves) and lower parts (roots and scutellum with surrounded zones). Both parts or whole seedlings were frozen with liquid nitrogen and homogenised immediately with 10–15 ml of buffer 0.66 M KH_2PO_4/Na_2HPO_4, pH 8.3, containing 20 µM PLP, 1 mM Na EDTA, 4 mM $MgCl_2$, 1 mM PMSF, 20% glycerol and 0.1% ME. The homogenate was passed through 4 layers of cheese cloth and the filtrate centrifuged at 12 000 *g* for 20 min at 4 °C. The pellet (etioplast fraction) and the supernatant (cytosol fraction) were used to estimate tryptophan racemase activity. The reaction mixture contained 5 µmol DTrp or LTrp and enzyme preparation in final volume of 1 ml of the same K/Na-phosphate buffer. The amount of protein in the reaction mixture was about 15–20 mg. The reaction mixture incubated at 37 °C was stopped by boiling in microwave oven during 10 s. The samples were centrifuged at 20 000×*g* and the supernatant was used for estimation of stereoisomers of tryptophan. The quantities of DTrp and LTrp were determined according to the method of Nagata et al. (1988) with the use of D-amino acid oxidase (EC 1.4.3.3) and L-amino acid oxidase (EC 1.4.3.2). The colour developed, after condensation of indolepyruvic acid with 2,4-dinitrophenylhydrazine, was measured at 492 nm.

Determination of the activities of L- and D-tryptophan aminotransferase

Activities of L-TAT and D-TAT were determined in wheat seedlings 5 days after germination according to the method of McQueen-Mason and Hamilton (1989). 25–50 seedlings were cut into upper and lower parts and were homogenised in 20 ml of buffer containing 0.5 M sucrose, 50 mM K_2HPO_4, 2 mM EDTA, 4 mM $MgCl_2$, 2 mM PMSF, pH 8.9. The homogenate was filtered through 4 layers of cheese cloth and centrifuged at 10 000×*g* for 20 min at 4 °C. The supernatant was discarded. The pellet, which was rich in etioplasts fraction, was resuspended in the same buffer containing 100 µM PLP

but without sucrose. The reaction mixture contained the fraction of etioplasts (3.8–4.0 mg equivalent of protein), 10 mM DTrp or LTrp, 0.1% ME and, where indicated, 10 mM Na pyruvate, 100 μM NAD^+, in a final volume of 2 ml. The incubation was conducted during 16–18 hours at 37 °C. The reaction was stopped by acidification to pH 2 with 3 mM H_3PO_4. The forming IAA was extracted 3 times with peroxide free diethyl ether. The ether extract was evaporated to dryness. The amount of IAA synthesised was determined with HPLC.

Determination of endogenous IAA, MTrp and Trp

After 3 or 5 days of germination 100 seedlings were cut into: coleoptiles (divided in tip, intemediate and bottom zones), scutellum with intermediate zones, roots and then fixed in boiled 96% ethanol with 0.1% DTT. At the moment of fixation 0.4 ng 3[5(n)-^{3}H]-IAA (0.8 TBK, Isotop, Russia) were added as the internal standard. The fixed plant material was extracted 3 times with 96% ethanol and bulked extracts evaporated under vacuum. The aqueous residue was adjusted with $NaHCO_3$ to pH 7.5 and extracted 3–4 times with peroxide free diethyl ether. The ether extract was discarded. The purified aqueous residue was adjusted with 20% H_3PO_4 to pH 3 and again extracted 3–4 times with peroxide free diethyl ether. The ether fraction was evaporated to dryness in water bath. The residue was redisolved and the amount of IAA was determined with HPLC. HPLC system equipped with the fluorescent detector (Ex 280 nm, Em 365 nm) and C18 microcolumn (250×0.7 mm) was used in the isocratic regime with the solvent mixture of 40% acetonitrile in 1% CH_3COOH. The amount of IAA in samples was calculated comparing to IAA standard peaks. Radioactivity recovery was checked in aliquot of redisolved samples in a scintillation counter. The losses during the extraction procedure of IAA in wheat seedlings were always in the range of 25–30%. The data in the tables are presented without correction of recovery.

The amount of MTrp was estimated with HPLC with the same C18 microcolumn (250×0.7 mm), fluorescent detector and gradient elution were used. The solvents were 40% acetonitrile in 1% CH_3COOH. The initial fraction of 40% acetonitrile being 87% and the final 20% during one cycle of 25 min. As standard in MTrp determination MTrp obtained via biotransformation of DTrp in the suspension of soybean tissue cultures (Rekoslavskaya, 1982) was used. The amount of MTrp was calculated as DTrp equivalent with the calibration curve of standard amino acid. The isomer configuration of Trp in MTrp was determined after the hydrolysis in 1 N HCl followed by neutralisation of hydrolyse with 1 N NaOH and estimation of stereoisomery with DAO. According to the reaction with DAO, more than 90% of Trp in MTrp was D-isomer.

Trp was estimated in the amino acid analyser. The amount of protein was determined by the method of Lowry et al. (1951). Experiments were conducted in 3–7 biological replicates with 3–5 analytical replicates and data in the tables are presented an mean ± standard error.

Results

IAA, MTrp and Trp contents during germination of wheat seedlings in the darkness

The IAA, MTrp and Trp contents were analysed in wheat seedlings during the rapid growth in the darkness between 3 and 5 days of germination. After 4–5 days of germination the growth of coleoptile stopped and the primary leaves began to outgrow.

The amount of IAA was higher in the coleoptile than in the root systems in three days old seedling (Table 1). The difference in concentration decreased at 5 days and the IAA total quantity present in the root can be considered the same as the IAA present in a whole coleoptile. But the mostly concentrated IAA zones were always the upper and middle coleoptile parts; zones which generally were used as a more sensitive tissue of coleoptile in the auxin biotest. As Table 2 shows, the amount of MTrp

Table 1. The distribution of IAA in wheat seedling on 3rd and 5th days of germination

Parts of seedlings	3rd day		5th day	
	nmol/g FW	nmol/seedling	nmol/g FW	nmol/seedling
Whole coleoptile	30±12	0.72±0.28	24± 3	1.34±0.19
Upper part[a]	67±16	0.13±0.03	33± 4	0.07±0.01
Middle part[a]	71±20	0.30±0.09	38±12	0.28±0.09
Lower part[a]	17±15	0.29±0.20	23± 3	1.00±0.10
Scutellum	7± 4	0.10±0.06	11± 2	0.18±0.03
Roots	15± 5	0.54±0.18	17± 7	1.36±0.56

[a] The lengths of upper, middle and lower parts were 2–3 mm, 5 mm and the rest of coleoptile length till to the base (8–13 mm and 43–53 mm respectively by 3rd and 5th day).

Table 2. Distribution of MTrp in wheat seedlings on 3 and 5 days of germination

Parts of seedlings	3rd day		5th day	
	nmol/g FW	nmol/seedling	nmol/g FW	nmol/seedling
Whole coleoptile	26± 6	0.62±0.01	34± 3	1.90±0.10
Upper part[a]	217±66	0.43±0.02	31±12	0.06±0.01
Middle part[a]	25± 2	0.10±0.01	21±10	0.16±0.01
Lower part[a]	5± 0	0.09±0.01	38± 9	1.70±0.08
Scutellum	9± 1	0.14±0.01	5± 0	0.09±0.01
Roots	9± 3	0.32±0.01	20± 7	1.06±0.05

[a] Conventional signs as in Table 1.

was predominant in coleoptiles. At the 3rd day of germination the highest amount of MTrp was determined in the apex of 2–3 mm length, both by calculation per g of fresh weight and per seedling basis. At the 5th day of germination the concentration of MTrp was sharply lowered in the apex. It was with little change in the middle 5 mm part and the highest MTrp concentration and total quantity was in the basal part of coleoptiles. The quantity of MTrp in the scutellum with surrounded zones was higher at the 3rd day of germination and was reduced untill the 5th day of germination. The amount of MTrp increased in roots from 3rd day to 5th day. It means that the concentration of MTrp was not diluted by new-forming roots because the root mass increased about 1/3rd 1/4th in the period from the 3rd day to the 5th day. The total MTrp quantity increased by the same factor either in whole coleoptile and roots form the 3rd to the 5th day of germination. The root contribution to the total MTrp present in the seedling did not change with time of germination ranging around the 30%. On the other hand, the different coleoptile parts, upper, middle and lower, drastically changed their contribution. Upper parts contributed for 35% for 3 days and decreased by 2% for 5 days, the lower part had 8% and 55% at the 3rd and 5th day respectively.

Table 3 shows that the amount of Trp was high in almost all parts of seedlings. This corresponds to the expected figure considering Trp as substrate for recemase, transaminase and at the end of IAA pathways here considered. The ratio Trp/IAA was several times higher than the ratio MTrp/IAA both at the 3rd and 5th day of germination in all seedling parts confirming the significant of IAA precursors abundance.

Table 3. Contents of Trp in wheat seedlings on the 3rd and 5th days of germination.

Parts of seedlings	3rd day		5th day	
	nmol/g FW	nmol/seedling	nmol/g FW	nmol/seedling
Whole coleoptile	165±33	3.9±0.6	417±24	32±1.8
Scutellum	90± 2	1.3±0.1	104±18	1.8±0.3
Roots	283±69	10.1±2.4	336±60	16.5±2.9

Tryptophan racemase

Tryptophan racemase activity was determined in wheat seedlings after 3, 5 and 7 days of germination (Table 4). The most active racemization was determined in etioplasts fractions in the direction L→D on the 3rd day of germination and this activity was 7–8 times higher than the activity of cytosol in L→D direction. The D→L activity of tryptophan racemase in etioplasts fraction was more then 5 times lower than the activity of L→D direction. Both D→L activities, in etioplasts and in cytosol, were approximately equal. At 5 and 7 days of germination the activity of tryptophan racemase decreased in both fractions and directions. In the etioplast of 5 days old seedlings the L→D direction activity was still 4 time higher then others. Later, at 7 days, the

Table 4. The dynamics of tryptophan racemase activity during germination of wheat seedlings in the darkness.

	Etioplast		Cytosol	
	L→D	D→L	L→D	D→L
	nmol.mg protein^{-1}.h^{-1}			
3rd day	223±9	34±4	31± 3	31±1
5th day	46±0	12±0	10± 0	16±0
7th day	18±6	11±2	27±13	12±1

differences between fractions and racemization direction became lower and quite indistinguishable.

When upper (coleoptile) and lower (scutellum and roots) parts of 5 days old wheat seedlings were extracted separately, the activity of tryptophan racemase was higher up to 25% in root and scutellum etioplasts in comparison with the upper part of seedling.

Tryptophan aminotransferase activity

The existence of Trp in the two stereoisomers allows to suggest the presence of two tryptophan amino acid transferases: L-TAT and D-TAT. The activity of aminotransferases (Table 5) was greately dependent on the addition of cofactors: both Na pyruvate and NAD^+ increased the IAA formation. Without cofactors the activities of L-TAT and D-TAT were higher in the lower part of seedling (scutellum and root system) in comparison with the upper part.

In the presence of Na pyruvate, but without NAD^+, L-TAT and D-TAT were approximately equal in the lower part of seedling. In coleoptile, the upper part, the presence of Na pyruvate showed to have strong effect on L-TAT activity only. In the pres-

Table 5. L- and D-tryptophan transaminase in the etioplast fraction of 5 days old wheat seedling.

Reaction mixture	Upper part		Lower part	
	pmol per seedling	pmol per mg protein h	pmol per seedling	pmol per mg protein h
-Na pyruvate -NAD^+ +LTrp	12± 1	78±12	91± 7	654± 49
-Na pyruvate -NAD^+ +DTrp	3± 0.2	18± 0.9	71± 20	475±134
+Na pyruvate -NAD^+ +LTrp	384± 3	2558±22	131± 22	873±147
+Na pyruvate -NAD^+ +DTrp	51± 1	343±15	125± 10	832± 40
+Na pyruvate +NAD^+ +LTrp	741±11	3429±30	982±174	4546±155
+Na pyruvate +NAD^+ +DTrp	333±24	1542±44	132± 13	603± 59

ence of both cofactors the amount of IAA formed after administration of LTrp to reaction mixture was 2 times higher in the upper part of seedling and about 7 times higher in reaction mixture with enzyme preparation from roots and scutellum in comparison to DTrp used as substrate for IAA biosynthesis. However, NAD^+ significantly affected the DTrp deamination to IAA in the wheat coleoptiles. The specific activities of both enzymes were increased about 1.5–7 times in seedling lower part, for D-TAT and L-TAT respectively, adding cofactors to the reaction mixture. In the upper part, in the presence of both cofactors, D-TAT specific activity increased up to hundred times.

These data suggest that NAD^+ increased the IAA biosynthetic pathway by formation of indoleacetaldehyde either in root and coleoptile when LTrp was used as precursors, and it, the NAD^+, was essential to rise up the DTrp $\rightarrow$ IAA pathway in coleoptile.

Discussion

MTrp is the predominant indole compound in wheat seedling during germination (Gamburg et al., 1993) excluding Trp which has an important part in protein newly synthesised during germination. Because storage proteins in wheat contain only trace amounts of Trp, therefore the conclusion about the competition for Trp during germination between protein and IAA biosyntheses comes true. It was shown that Trp appeared during germination via transamination and glutamine and other amines could be donors of the amino group for IPYrA (Kretovich and Poljanski, 1959).

Triptophan (Table 3) in 3-day-old seedlings was not the limiting factor for IAA and MTrp biosyntheses (Tables 1 and 2) and at this stage of growth the tryptophan racemase was at higher activity level (Table 4).

Highest tryptophan racemase activity was found in etioplasts and in the direction L $\rightarrow$ D during the rapid growth of etiolated coleoptiles. Later, at the 7th day both directions of tryptophan conversion were slow and comparable (Table 4). Localisation of tryptophan racemase mainly in etioplast of wheat seedlings is in agreement to McQueen-Mason's study (1989) that characterised IAA biosynthesis enzyme as plastid enzyme. During germination the transamination of amino acids greatly increased. As consequence of storage proteins hydrolysis the nitrogen of amino acids overspread between new metabolic pools, and both L-TAT and D-TAT are working during the early stage germination (Table 5). During the germination DTrp appeared in amount of 30–60 nmol per gram-equivalent of seedlings to become endogenous substrate for D-TAT. In *in vitro* transaminase activity assays (without NAD^+) D-TAT in lower seedling parts revealed the same activity as L-TAT did. In the presence of NAD^+ the production of IAA increased several times when LTrp was used as substrate for tryptophan transaminase. So in wheat roots the capability of the pathway LTrp $\rightarrow$ IAA is probably more dependent on the energy sources and donor-acceptor relations of

electrons than the DTrp→IAA pathway (Table 5). In upper seedling parts the D-TAT was lower then L-TAT but present data show an enzyme in coleoptile to convert DTrp to IAA, and it is strongly dependent of NAD^+. The different responses of L-TAT and D-TAT to cofactors, pyruvate and/or NAD^+, reported by the autors here for wheat seedling could be due to the presence of different enzymes. Indeed the presence of two different L-TAT and one D-TAT has been described in etioplast from maize (Koshiba et al., 1993).

Müller et al. (1998) in their recent work on *A. thaliana*, on the reevaluation of Trp as IAA precursor, identified LTrp as IAA precursor in roots. This is in agreement with the data here presented for wheat root. Furthermore, wheat coleoptile showed that DTrp can be IAA source in rapid elongating and highly sensitive tissue. Müller et al. (1998) did not give information if the same Trp isomer is the IAA source in green seedling part of *A. thaliana*. Examination of the role of grain or coleoptile tip as the source of auxin production in cereal seedlings revealed two extreme opinions (Jackson and McWha, 1984). The first school supports the classic hypothesis that the coleoptile tip is the source of free IAA (Wildman and Bonner, 1948). The other "seed" school argued that the free IAA in the shoot is derived from the endosperm (Trewavas, 1981). According to here presented data, the tip of wheat coleoptile has tissues with higher concentration of IAA and MTrp on the 3rd day of germination (Tables 1 and 2). The elongation zone, coleoptile middle part, which is generally used for auxin bioassay was also rich (as tip) in IAA, but not in MTrp, on the 3rd day of germination. The concentration of IAA in tip and in the zone of elongation high on the 3rd day, lowered at the 5th day of germination when coleoptile elongation stopped. Data of IAA presence in coleoptile agree with the presence of tryptophan racemase and aminotransferase in it.

That the exogenous DTrp was more active than LTrp as an auxin during early growth stage has been shown in seedlings of pea (Law, 1987), barley (Tsurusaki, 1990) and maize (Koshiba, 1993) and in preliminary experiments we obtained similar data with wheat embryos cultivated *in vitro* (data not reported). Therefore, the present results let to infer that the hormonal control of wheat germination is controlled by formation of DTrp and its conversion to IAA and allow to designate a key role of D-tryptophan racemase during germination. First data on tryptophan racemase in wheat are reported. The presence of enzyme activity either in etioplast and cytosol, the modulation of acitivty in L→D and D→L direction during the time of wheat seedling growth are of wide interest for the further characterization of the enzyme in relation to its hypothised role in IAA biosynthesis pathway.

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