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Polyamines and Anaerobic Elongation of Rice Coleoptile

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The role of polyamines in the anaerobic elongation of rice (*Oryza sativa* L.) coleoptiles was studied. The reduced growth of rice coleoptiles under anoxic conditions was accompanied by a massive accumulation of free putrescine. Putrescine was synthesized from arginine in a reaction catalyzed by arginine decarboxylase (ADC). The anoxic titer of putrescine was closely correlated with elongation of coleoptiles. In experiments in which putrescine and inhibitors [a-difluoromethylarginine (DFMA) and a-difluoromethylornithine (DFMO)] of the synthesis of polyamines were exogenously supplied, we demonstrated an absolute requirement for putrescine, synthesized by ADC, for anaerobic elongation of coleoptiles. The presence of exogenous putrescine (alone or in combination with DFMA) increased the rate of anaerobic elongation of coleoptile by 30-40%.

Key words: Anoxia — Coleoptile — *a*-Difluoromethylarginine — *a*-Difluoromethylornithine — Polyamine — Rice.

Rice, besides being the staple food of more than half of the world's population and widely cultivated in both tropical and temperate regions, represents an important tool for plant physiologists in the study of plant adaptation to an anoxic environment. Rice is able to germinate in a totally oxygen-depleted environemnt (Opik 1973). Under these conditions, rice seeds germinate producing only a shoot whose growth is limited to a white coleoptile (Kordan 1976). This type of growth is an adaptive response to the anaerobic conditions frequently encountered in flooded soils. During anaerobic germination, the rice coleoptile elongates until it reaches an aerobic layer from which oxygen can be transferred to the seed, with subsequent normal growth of radicle and leaf (Kordan 1976, Takahashi 1978).

The fastest elongation of the coleoptile occurs at an oxygen concentration of 3-3.5% (Ku et al. 1970) and, under these conditions, the growth is promoted by ethylene alone or by a combination of GA₃ and CO₂ (Suge 1984). The total dependence on oxygen of the biosynthesis of ethylene (Adams and Yang 1979) suggests that ethylene does not play a role in the anaerobic growth of the rice coleoptile.

Since the importance of auxin in anaerobic elongation of coleoptiles is uncertain (Pegoraro et al. 1988), the substance that stimulates the growth of the coleoptile in strict anoxia has yet to be ascertained.

Polyamines are a class of compounds that have been convincingly demonstrated to be growth factors in higher plants (Slocum et al. 1984). Changes in the rates of cell division and growth of plant tissues are often correlated with changes in the titer of polyamines (Dumortier et al. 1983, Shen and Galston 1985). Moreover, experiments involving inhibition of the synthesis of polyamines by DFMO or DFMA and the release of inhibition by the supply of polyamines have shed considerable light on the role of these substances in plant growth (Smith 1985). Studies on the regulation of growth by polyamines have shown that polyamine synthesis proceeds through the ODC pathway in tissues in which active cell division is occurring, and through the ADC pathway during growth of nondividing, but rapidly growing tissues (Smith 1981, Bagni et al. 1983). During the anaerobic germination of rice seeds, cell division is restricted and the coleoptile grows essentially by cell enlargement (Opik 1973). In maize coleoptiles growing in air, changes in the rates of elongation of cells were associated with gradients in titers of specific polyamines and in the activity of associated biosynthetic enzymes (Dumortier et al. 1983). Moreover, the titer of Put and ratio of Put to Spd were shown to rise with increasing rates of elongation of plant tissues (Shen and Galston 1985).

Abbreviations: ADC, arginine decarboxylase; DFMA, *a*difluoromethylarginine; DFMO, *a*-difluoromethylornithine; HPTLC, high-performance thin-layer chromatography; ODC, ornithine decarboxylase; Put, putrescine; Spd, spermidine; Spm, spermine.

We here describe a marked difference between the polyamine metabolism in anaerobically growing rice coleoptiles and in rice shoots growing in air. We examined the hypothesis that polyamines play a role in growth of rice coleoptiles under strictly anoxic conditions. The use of irreversible inhibitors of polyamine synthesis allowed us to demonstrate the necessity of this class of substances for the anaerobic elongation of the rice coleoptile.

Materials and Methods

Plant materials—Dehulled seeds of rice (*Oryza sativa* cv. Arborio) were sterilized with 70% (v/v) ethanol for 2 min and with 5% (w/v) Ca(OCl)₂ for 30 min, each treatment being followed by several rinses with distilled water. The seeds were then spread on a net, hung 0.5 cm above the surface of water in a 2.5 liter jar, and allowed to germinate in the dark at 25°C. Aerobic or anaerobic conditions were obtained by flushing humidified air or nitrogen gas (99.999% nitorgen) through a pumice stone at the bottom of the jar. The time required to obtain complete anaerobiosis was 20 min (Reggiani et al. 1985).

In one set of experiments, the seeds were anaerobically germinated under sterile water for 4 days at 25°C, and then DFMO, DFMA (Merrell-Dow Research, U.S.A.) or Put (Sigma, U.S.A.) was added at 0.5 mM, 0.5 mM and 0.2 mM, respectively. In experiments involving the reversal of the effects of DFMA, we utilized 0.5 mM DFMA together with 0.2 mM Put. After 2 days of treatment, the viability of the seedlings was checked by staining the tissues with tetrazolium salts (Bertani et al. 1981). After every treatment, no change in the viability of seedlings was found.

Determination of polyamines-The aerobic and anaerobic seedlings were immediately frozen in liquid nitrogen; the coleoptiles were excised and stored at -80° C until extracted. Samples of coleoptiles were ground in a mortar with 0.6 M HClO₄ (100 mg fresh weight per ml). The homogenate was cleared by centrifugation at $12,000 \times g$ for The bound polyamines were hydrolyzed as 15 min. described by Tiburcio et al. (1986). The non-hydrolyzed HClO₄ supernatant which contained the free polyamines (S), the hydrolyzed HClO₄ supernatant (SH) and the hydrolyzed pellet (PH) which contained polyamines liberated from conjugates were dansylated by addition of 0.2 ml of saturated Na₂CO₃ and 0.4 ml of dansyl chloride (5 mg/ml acetone). The reactions, in stoppered tubes, were stored overnight in the dark at room temperature. The dansylated amines were extracted into toluene (0.4 ml) by vortex mixing. When the two phases were separated, $20 \,\mu l$ of the toluene layer was loaded with a Linomat IV (Camag, Switzerland) on an HPTLC plate of silica gel 60 with a concentration zone (Merck, F. R. G.). The plate was run with n-hexane/ethyl aceate (11:8, v/v) as solvent. Fluorescence (excitation at 350 nm) of each lane was read

into a densitometer TLC Scanner II (Camag, Switzerland). The data were quantitated by a D-2000 integrator (Hitachi-Merck, F. R. G.).

Assay of ADC—The samples of coleoptiles were ground in a chilled mortar at a ratio of 100 mg fresh weight per ml of 0.2 M Tris-HCl (pH 8.0) that contained $20 \mu \text{M}$ pyridoxal phosphate and 2 mM DTT. The supernatant obtained after centrifugation (12,000 × g for 15 min) was used immediately for the assay. The activity of ADC in the extract was estimated by measuring the release of ¹⁴CO₂ from ¹⁴C-arginine, as described by Tiburcio et al. (1986). One unit of the enzyme was defined the amount of enzyme that catalyzed the release of 1 nmol of ¹⁴CO₂ in 1 h under the conditions of the assay.

Protein analysis—Levels of protein were determined with a Bio-Rad Protein Kit (Bio-Rad, U.S.A.) by the method of Bradford (1976). Bovine serum albumin was used as a standard.

Results

Elongation of coleoptiles—The increase in length of rice coleoptiles under aerobic and anaerobic conditions is shown in Figure 1. As can be seen from the Figure, the elongation of the anaerobic coleoptiles was much slower (only 56%) than that of the aerobic coleoptiles. The elongation of the aerobic coleoptiles was 4.5 mm/day compared with 2.5 mm/day for the anaerobic coleoptiles.

Levels of polyamines—The levels of total (free+ bound) Put, Spd and Spm in coleoptiles of aerobically and anaerobically germinated seedlings of rice are shown in



Fig. 1 Increase in length of aerobic (\bigcirc) and anaerobic (\bigcirc) rice coleoptiles. Vertical bars=SE; n=3.

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Figure 2. The titer of Put was 3- to 4-fold higher in the anaerobic coleoptiles than in the coleoptiles grown in air. However, no significant difference between the two types of coleoptile was observed in the concentration of total Spd and Spm. Table 1 shows the distribution of Put, Spd and Spm in the acid-soluble (S), the hydrolyzed acid-soluble (SH) and the hydrolyzed pellet (PH) fractions. During aerobic germination, the titer and the relative proportion of soluble conjugated polyamines (SH) increased, while the level of free polyamines (S) decreased as the germination proceeded. After 5 days of aerobic germination, 72.1%, 64.2% and 85.3% of the total Put, Spd and Spm, respectively, were present in the SH fraction. In the anaerobic coleoptiles, Put was totally absent as an acid-soluble conjugate (SH) and the high concentration of total Put in the tissue (about 2 mm at 5 days) was mainly attributable to the free form (97.0%). During anaerobic germination, the concentrations of the free and the soluble conjugates (S and SH) of Spd and Spm increased, but their relative distribution in the two fractions remained more or less unchanged. Furthermore, the pellet fraction of the anaerobically grown coleoptiles contained higher levels of Put and Spd than that of the aerobically grown coleoptiles.

Effects of exogenous DFMA, DFMO and Put on anaerobic growth of coleoptiles—Anaerobic coleoptiles, after 4 days of germination, were treated with DFMA and DFMO, irreversible inhibitors of polyamine synthesis, and with Put. After 2 days the growth (in terms of both length and fresh weight) and the levels of Put were measured (Table 2). At 0.5 mm, DFMA significantly depressed the rate of elongation of coleoptiles and the titer of Put (by 74% and 84%, respectively), but the inhibition of the in-



Fig. 2 Levels of total putrescine (Put), spermidine (Spd) and spermine (Spm) in aerobic (\odot) and anaerobic (\bullet) rice coleoptiles. Vertical bars=SE; n=3.

		Aerobic coleoptile Days of germination			Anaerobic coleoptile		
					Days of Germination		
		3	4	5	3	4	5
Put	S	357.5 (86.1)	223.9 (49.4)	145.9 (27.3)	1,150.5 (95.1)	1,905.1 (96.8)	2,231.0 (97.0)
	SH	37.7 (9.1)	223.0 (49.2)	386.1 (72.1)	nd ^{<i>a</i>}	nd	nd
	PH	19.8 (4.8)	6.3 (1.4)	3.4 (0.6)	99.6 (4.9)	63.6 (3.2)	68.8 (3.0)
Spd	S	275.9 (86.4)	162.1 (46.3)	143.1 (35.2)	165.0 (58.2)	195.0 (57.1)	220.2 (56.4)
	SH	25.7 (8.0)	183.2 (52.3)	260.8 (64.2)	84.9 (30.0)	105.7 (31.0)	120.9 (31.0)
	PH	17.8 (5.6)	4.7 (1.4)	2.4 (0.6)	53.5 (11.8)	40.6 (11.9)	49.5 (12.6)
Spm	S	94.1 (56.7)	53.2 (25.3)	37.5 (14.4)	61.0 (30.3)	66.1 (27.6)	68.8 (24.9)
	SH	58.0 (35.0)	154.8 (73.5)	222.4 (85.3)	140.4 (69.7)	173.7 (72.4)	210.7 (75.1)
	PH	13.8 (8.3)	2.6 (1.2)	0.9 (0.3)	nd	nd	nd

Table 1 Levels of polyamines (soluble, S; hydrolyzed $HClO_4$ supernatant, SH; hydrolyzed pellet, PH) in coleoptiles of aerobically and anaerobically germinated seeds of rice

^a Not detected.

Data are the mean of results of 3 independent experiments. The values are presented as nmoles/g fresh wt. The relative percentages of the total (S+SH+PH) are given in parentheses.

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Treatment (тм)	Length (mm/coleoptile)	Fr wt (mg/coleoptile)	Put titer (nmol/coleoptile)	
Control (6 days)	6.73 (100)	2.97 (100)	17.80 (100)	
DFMO, 0.5	7.16 (106)	3.18 (107)	18.69 (105)	
DFMA, 0.5	1.76 (26)	1.68 (57)	2.83 (16)	
Put, 0.2	9.41 (140)	3.24 (109)	30.41 (171)	
DFMA, 0.5+Put, 0.2	8.83 (131)	3.21 (116)	28.22 (159)	

 Table 2
 Influence of exogenous DFMO, DFMA and putrescine (Put) on the anaerobic increase in length, fresh wt and titer of free Put of rice coleoptile between 4 and 6 days of germination

Each value represents the difference from the value of the 4-day germinated control. Data are the mean of results from 3 independent replicates. Standard errors were below 10%. Percentages of control values are given in parentheses.

crease in fresh weight (43%) was less pronounced. The inhibitory effect was completely reversed by addition of 0.2 mM Put. No significant changes were observed upon addition of 0.5 mM DFMO. The presence of exogenous Put (0.2 mM), alone or in combination with DFMA, increased the rate of elongation of the coleoptile by 30–40%, but did not change the increase in fresh weight. Moreover, when Put was supplied, its level in the tissue increased by 60–70% as compared with the control.

The titer of Put and the elongation of anaerobic coleoptiles—Rice seedlings were germinated anaerobically for up to 13 days and the titer of free Put in the coleoptiles was determined. A close correlation was observed between the elongation of the coleoptile and the endogenous concentration of Put (Fig. 3). A regression line ($R^2=0.9899$, P < 0.001) indicated that an increase in the concentration of Put of 2 nmol per coleoptile was associated with an increase in length of 1 mm.

Activity of ADC—The observation that DFMA inhibited, the anaerobic accumulation of Put suggests that the ADC pathway is involved in the biosythesis of Put. The activity of ADC in the aerobic coleoptile decreased slightly as germination proceeded, whereas that in the anaerobically grown coleoptile increased by about



Fig. 3 Regression line fitted to data for lengths of anaerobic coleoptiles and titers of Put ($R^2=0.9899$, $P \le 0.001$). The data points correspond to 3, 4, 5, 7, 9 and 13 days of germination.

Fig. 4 Changes in activity of arginine decarboxylase (ADC) in aerobic ($^{\circ}$) and anaerobic ($^{\circ}$) rice coleoptiles. Vertical bars=SE; n = 3.

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1.73 units/day (Fig. 4).

Discussion

Even in the complete absence of oxygen, the rice coleoptile is still able to elongate (2.5 mm/day). Since a role of the known phytohormones in promoting this growth is unlikely (Jackson 1985, Pegoraro et al. 1988), we examined whether polyamines promote the anaerobic growth of the coleoptile.

The metabolism of putrescine was very active under anoxic conditions and appeared particularly important. Synthesis of Put through the ADC pathway is greater in anaerobic coleoptiles than in aerobic coleoptiles (Table 1, Fig. 4). This type of response resembles responses exhibited by plant tissues under other stressful conditions (DiTomaso et al. 1988, Flores et al. 1985, Weinstein et al. 1986). The accumulation of Put during anoxia, besides being the result of accelerated synthesis, could be favoured by the absence of degradation of Put. All the enzymes now known to degrade Put are, in fact, oxidases (Smith 1985). In the anaerobic coleoptile, Put was present almost entirely in the free form (Table 1); the reason for the absence of soluble, conjugated Put in the tissue when the seeds are germinated in an atmosphere of pure nitrogen is still obscure.

In this study, we found evidence that the concentration of free Put is directly related to the elongation of rice coleoptiles. The anoxic titer of Put correlates very closely with the increase in length of the coleoptile (Fig. 3). When the increase in the concentration of Put was prevented by DFMA, the increase in length and fresh weight was strongly inhibited (Table 2). These effects were not due to the death of the seedlings because they were still alive at the end of 2 days of treatment (as checked with tetrazxolium salts). The addition of exogenous Put at the time of treatment with DFMA reversed the inhibition by DFMA, a result that suggests that Put is effectively involved in this growth. The inhibitory effect of DFMA, but not of DFMO, on anaerobic growth suggests that, after 4 days of germination, the coleoptile cells did not divide but elongated. Moreover, the exogenous supply of Put (alone or in combination with DFMA) resulted both in an increase in the level of Put in the tissue and an increase of 30-40%in the elongation of the anaerobic coleoptiles. This is the first demonstration that an exogenous compound can stimulate elongation of anoxic coleoptiles.

Two roles of Put can be suggested to explain the effect of Put on anaerobic growth. The elongation of the coleoptile under anoxic conditions may be directly promoted by Put via processes yet to be elucidated (specific effect). Alternatively, the accumulation of Put could be involved in the control of the intracellular pH. In the latter case, biosynthesis of Put would serve as a homeostatic buffering mechanism to stabilize the intracellular pH (Slocum et al. 1984). A stabilized pH during the anoxic stress might permit the rice coleoptile to elongate (non-specific effect). The second hypothesis can be verified by appication of exogenous substances that induce alkalinization (i.e. benzylamine). Such experiments are in progress.

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