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Polyamine Metabolism and Osmotic Stress[1]

II. IMPROVEMENT OF OAT PROTOPLASTS BY AN INHIBITOR OF ARGININE DECARBOXYLASE

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ABSTRACT

We have attempted to improve the viability of cereal mesophyll protoplasts by pretreatment of leaves with DL-α-difluoromethylarginine (DFMA), a specific 'suicide' inhibitor of the enzyme (arginine decarboxylase) responsible for their osmotically induced putrescine accumulation. Leaf pretreatment with DFMA before a 6 hour osmotic shock caused a 45% decrease of putrescine and a 2-fold increase of spermine titer. After 136 hours of osmotic stress, putrescine titer in DFMA-pretreated leaves increased by only 50%, but spermidine and spermine titers increased dramatically by 3.2- and 6-fold, respectively. These increases in higher polyamines could account for the reduced chlorophyll loss and enhanced ability of pretreated leaves to incorporate tritiated thymidine, uridine, and leucine into macromolecules. Pretreatment with DFMA significantly improved the overall viability of the protoplasts isolated from these leaves. The results support the view that the osmotically induced rise in putrescine and blockage of its conversion to higher polyamines may contribute to the lack of sustained cell division in cereal mesophyll protoplasts, although other undefined factors must also play a major role.

In the preceding study (27), we have shown that the osmotically induced Put[2] accumulation in oats, which yield poorly dividing protoplasts, contrasts with the low Put content in species which yield readily dividing protoplasts. Moreover, osmotically stressed leaves of species which yield protoplasts capable of sustained growth show increased Spd and/or Spm levels, in contrast with the decrease of these higher PAs in osmotically stressed oat leaves (27). Since Spd and Spm are essential for cell division (11, 23), while Put accumulation is toxic to certain cells (9, 22, 24), we have suggested that this difference in response to osmotic stress may be one of the reasons for failure of cereal mesophyll protoplasts to divide *in vitro* (27).

To test this hypothesis, we have attempted to prevent the accumulation of Put in oat protoplasts by pretreating oat leaves with DL-α-difluoromethylarginine (DFMA), an irreversible inhibitor of the enzyme (ADC) responsible for the osmotically induced Put rise in cereals (5). We have estimated the short- and long-term effects of the pretreatment with DFMA on PA levels and macromolecular synthesis in osmotically shocked oat leaves and on the viability and development of protoplasts isolated from these leaves.

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[2] Abbreviations: Put, putrescine; DFMA, DL-α-difluoromethylarginine; ADC, arginine decarboxylase; ODC, ornithine decarboxylase; Spd, spermidine; Spm, spermine; PAs, polyamines.

MATERIALS AND METHODS

Plant Material and Pretreatment with DFMA. Seeds of *Avena sativa* cv Victory were sown in vermiculite in plastic pots and allowed to germinate and develop in a controlled growth room as detailed in the preceding report (27). Four-d-old seedlings (about 50 plants/pot) were sprayed, unless otherwise indicated, for 4 consecutive d with 5 ml of distilled H_2O containing 0.002% Tween-20 and adjusted to pH 8 (control), or the same solution containing 10 mM DFMA. The first leaf of 8-d-old oat seedlings was used as the experimental material.

Osmotic Treatment of Excised Leaves. The excised leaves were rinsed several times with distilled H_2O, after which the lower epidermis was removed by peeling, and peeled leaf segments (5 cm length) were floated in the dark over buffer or 0.6 M sorbitol (27) for 6 h or 136 h. In the long-term experiments (136 h), the leaves were first sterilized by immersion for 7 min in 10% Clorox containing Tween-20 (1 drop/10 ml), followed by 10 rinses with sterile-distilled H_2O. All manipulations were performed aseptically in a laminar flow hood.

Polyamine and Enzyme Analysis. Polyamine extraction, separation by TLC, and quantification by spectrophotofluorometry were performed as detailed in the preceding report (27). For ADC and ODC determinations, leaves were extracted in 100 mM K-phosphate (pH 7.6) at a ratio of 100 mg fresh weight/ml. The extracts were sonicated twice for 1 min (26) and centrifuged at 27,000*g* for 20 min. The supernatant fraction was dialyzed overnight against the same buffer and assayed for ADC and ODC activities as previously described (6, 27). Protein was determined according to the method of Bradford (2).

Incorporation Studies. DNA, RNA, and protein synthesis were estimated according to the method of Gulati *et al.* (10) by determining the incorporation of tritiated thymidine, uridine, and leucine, respectively, into the TCA-insoluble fraction of the experimental material. Six peeled leaf-segments were incubated in Petri dishes (100 × 15 mm) in the dark for 6 and 136 h with 1 μCi/leaf segment of [methyl-^{3}H]thymidine (70 Ci/mmol), or [5-^{3}H]uridine (27 Ci/mmol); or 0.1 μCi/leaf segment of [4,5-^{3}H] leucine (59.2 Ci/mmol) in the absence or presence of osmotica (0.6 M sorbitol) (27). The long-term experiments were performed under aseptic conditions as described above. After incubation, the leaf-segments were gently blotted on filter paper and transferred to stainless steel perforated microcapsules, and immediately placed in chilled 10% (w/v) TCA for 2 h. The segments in the capsules were washed: three times with 5% TCA, once with 0.25% HCOOH in methanol, and finally twice with a mixture of absolute ethanol and anhydrous ether (1:1, v/v); each for 15 min, consecutively. All steps were carried out at 5°C with constant shaking. The washed leaf-segments were removed from the capsules, gently blotted on filter paper, and placed in minivials containing 2 ml Betafluor with 5% (v/v) of the tissue solubilizer Bio-Solv 3 (Beckman). After overnight digestion of the experimental material at room temperature, radioactivity was meas-

ured in a Beckman LS 7000 scintillation counter.

Isolation and Culture of Oat Protoplasts. Leaves from control seedlings and seedlings pretreated with DFMA were rinsed several times with distilled H_2O and sterilized as described above. Protoplasts were isolated aseptically in a laminar flow hood by peeling off the lower epidermis and floating the peeled leaf surface on a filter-sterilized mixture of 1 mM phosphate buffer (pH 5.8) containing 0.5% Cellulysin (B grade, Calbiochem) and 0.5 M mannitol for 2 h at 30°C in the dark (1). The released protoplasts were collected by centrifugation at 100*g* for 6 min, then washed twice with 0.5 M mannitol in buffer (pH 5.8) by resuspension and centrifugation. The final protoplast pellet was resuspended at a concentration of about 10^5 protoplasts/ml in a modified GS-1 medium (21) containing 25 g/L glucose, 20 mg/L arginine, and 10 mg/L ascorbic acid. This medium adjusted to pH 5.8 was previously filter-sterilized. The protoplast suspensions were cultured at about 50-μl drops (about 5000 protoplasts) on the bottom of 60 × 15 mm Petri dishes (6 drops/dish) placed in moist chambers in the dark at 26°C. After 15 d, fresh medium with 0.2 g/L glucose and 0.2 g/L mannitol was added, and after 4 to 5 weeks the cultures were transferred to the same medium without mannitol but with 0.05 g/L glucose as microdrops or with 1% purified agar added (25). Two separate protoplast isolations and cultures were made from control and pretreated leaves. Some of the cultures were maintained up to 2 months in liquid-drop medium to follow developmental changes. Viability of protoplasts or developing cells was evaluated by microscopic observation of the cytoplasmic streaming and cell wall regeneration.

Staining Procedures. The fluorescent brightener, calcofluor white ST (American Cyanamid Co., Wayne, NJ) was used to stain cellulosic walls. Aliquots (0.1 ml) from the cultures were treated with 5 μl of a 0.1% solution of calcofluor white (17). The stained cells were observed using a fluorescence microscope (model 2070, American Optical Corp., Buffalo, NY). The filter used included a UG-1 filter for excitation (360 nm), a chromatic beam splitter at 450 nm, and a barrier filter at 440 nm. Nuclei were stained with modified carbolfuchsin, according to the method of Kao (12). Briefly, aliquots from the cultures were first fixed with acetic acid-ethanol (1:9, v/v) and kept at 5°C for 25 h. The fixed cells were then stained for at least 1 h and observed microscopically.

RESULTS AND DISCUSSION

The previous paper demonstrated that a high Put:Spd + Spm ratio is negatively correlated with protoplast survival and development. The diamine Put increases rapidly during isolation of cereal mesophyll protoplasts, due to osmotic stress activation of the biosynthetic pathway mediated by ADC (5), while conversion of Put to Spd and Spm is inhibited (5, 6). Under such stress conditions, the ratio of Put to Spd and Spm rises (27). Simultaneous osmotic treatment of cereal leaves with DFMA, an irreversible inhibitor of ADC, was effective in preventing the rise of Put, but did not alter the Spd and Spm levels (5, 6). To prevent Put accumulation while permitting normal Spd and Spm synthesis, we opted for *in situ* leaf pretreatment with DFMA before subjecting the excised leaf segments to osmotic stress in connection with protoplast isolation.

Table I shows the effect of spraying oat seedlings with different concentrations of DFMA on ADC and ODC activities. ADC activity was progressively inhibited by increasing concentrations of DFMA, while ODC activity was only slightly affected. The highest inhibition of ADC activity (65%), which was obtained by spraying oat seedlings for 4 consecutive d with 10 mM DFMA, was used in subsequent pretreatments.

When pretreated leaves were subjected to osmotic stress by floating them on 0.6 M sorbitol for 6 h, Put titers decreased by 45%, Spm titers increased about 2-fold, and Put:Spd + Spm ratio decreased sharply as compared with nonpretreated, osmotically stressed leaves (Table II). After 136 h of osmotic stress, Put titers in pretreated leaves increased by only about 50%, but Spd and Spm titers dramatically increased by 3.2- and 6-fold, respectively, in relation to nonpretreated, osmotically stressed leaves. As a consequence of these changes, the Put:Spd + Spm ratio in DFMA-pretreated leaves is only about one-third as high as in untreated leaves (Table II).

Table I. *Effect of DFMA on Decarboxylase Activity (ADC and ODC) in Oat Seedlings*

Numbers represent the mean ± SE of three replicates. Numbers without ± SE are the mean value of 2 replicates.

DFMA Concentration	Decarboxylase Activity	
	ADC	ODC
mM	*nmol CO_2/h·mg protein*	
0	4.99 ± 0.15 (100)	1.30 ± 0.05 (100)
1[a]	3.30 (66)[b]	1.15 (88)
5[a]	2.61 (52)	1.10 (85)
10[c]	1.76 ± 0.32[d] (35)	1.14 ± 0.08 (88)

[a] Four-d-old oat seedlings were sprayed once with DFMA (1 and 5 mM). [b] Figures in parentheses express activity in percent relative to control (−DFMA). [c] Four-d-old oat seedlings were sprayed for 4 consecutive d with 10 mM DFMA as described in "Materials and Methods." The first leaf of 8-d-old seedlings was used as the experimental material. [d] Significantly different from control at $P < 0.05$.

Table II. *Effect of Pretreatment with DFMA on PA Titer in Oat Leaves Subjected to Osmotic Stress*

Numbers represent the mean of 2 replicates from a single experiment.

Pretreatment	Osmotic Treatment[a]	Put	Spd	Spm	Put/Spd + Spm
	h	*nmol[b]/mg protein*			
−DFMA	6[c]	4.00 (100)	2.00 (100)	1.30 (100)	1.21
+DFMA	6	2.20 (55)[d]	2.10 (105)	2.70 (208)	0.45
−DFMA	136	4.70 (100)	0.90 (100)	0.60 (100)	3.13
+DFMA	136	7.10 (151)	2.90 (322)	3.60 (600)	1.09

[a] Oat leaves were floated over 0.6 M sorbitol in the dark for 6 or 136 h as indicated in "Materials and Methods." [b] PA titer are the sum of the S- and SH-fractions (27). [c] The experiment at 6 h was repeated twice. [d] Percent in relation to controls (−DFMA).

The low Put:Spd + Spm ratio in leaves pretreated with DFMA corresponds with greater vigor of the leaves after osmotic stress. For example, pretreatment with DFMA causes a marked retardation of Chl loss ordinarily occurring during the incubation period. In all the long-term experiments, the control leaves floated in the dark over buffer or osmotica appeared pale yellow, while the leaves pretreated with DFMA, especially those which were osmotically stressed, appeared green even after 136 h of incubation. Previously, we have reported that oat leaves floated in the dark over buffer showed a loss of Chl of about 71% after 48 h, and that this effect was prevented by the addition of Spd or Spm (15). Since in the leaves pretreated with DFMA, Spm titer dramatically increases after 136 h of osmotic stress, this antisenescence effect of DFMA may be related to the increase in the higher polyamines Spd and Spm.

To further examine the integrity of the leaves, we investigated the effect of the pretreatment of the seedlings with DFMA on subsequent macromolecular synthesis as affected by osmotic stress (Table III). Incorporation of [^{3}H]thymidine into macromolecules continues to increase for at least 136 h, whereas incorporation of [^{3}H]uridine or -leucine declines from 6 to 136 h of incubation under osmotic stress in both DFMA-pretreated

Table III. *Effect of the Pretreatment with DFMA on Macromolecular Synthesis in Oat Leaves Subjected to Osmotic Stress*

Numbers represent the mean ± SE of 6 replicates.

Pretreatment	Osmotic Treatment	[H³]Thymidine	[H³]Uridine	[H³]Leucine
	h		*dpm/mg protein*	
−DFMA	6	694 ± 36 (100)	2885 ± 785 (100)	803 ± 100 (100)
+DFMA	6	1090 ± 190 (157)	4037 ± 278 (140)	1212 ± 167 (151)
−DFMA	136	7469 ± 746 (100)	700 ± 110 (100)	86 ± 16 (100)
+DFMA	136	11,566 ± 1,072[a] (155)	1743 ± 227[b] (249)	110 ± 26 (128)

[a] Significantly different from control (−DFMA, 136 h) at $P < 0.05$. [b] Significantly different from control at $P < 0.01$.

and control leaves. Pretreatment with DFMA increased the incorporation of all precursors into macromolecules. At 136 h of osmotic stress, there is a significant increase of thymidine and uridine incorporation in osmotically stressed, DFMA-sprayed leaves when compared with unsprayed leaves. The incorporation of leucine was, however, severely reduced in both pretreated and nonpretreated leaves after 136 h of osmotic stress. This may be due to a breakdown of the labeled leucine induced by osmotic treatment rather than to a decrease in synthetic capacity (20). This is further supported by our observation that the incorporation of leucine in oat leaves incubated over buffer (no osmoticum) for 136 h was about 11-fold higher than in osmotically stressed leaves. Furthermore, the incorporation of thymidine and uridine into macromolecules in leaves incubated over buffer for 6 or 136 h was significantly higher than in osmotically stressed leaves (data not shown). These results suggest that (a) macromolecular synthesis in oat leaves is inhibited by osmotic stress, and that (b) this effect can be partly prevented by pretreatment of the leaves with DFMA.

The higher net macromolecular synthesis and lowered rates of senescence observed in osmotically stressed leaves pretreated with DFMA encouraged us to examine whether protoplasts isolated from such leaves will grow better in culture. Thus, we isolated and cultured protoplasts from DFMA-treated oat leaves and compared them with those isolated from control leaves. Protoplasts isolated from DFMA-pretreated leaves appeared greener, with greater cytoplasmic streaming, more uniform regeneration of the cell wall, and showing less lysis than protoplasts extracted from control leaves. Although in the early stages of culture (13 d; Table IV) no significant differences were observed in the total number of viable protoplasts, some improvement of development was observed in protoplasts isolated from leaves pretreated with DFMA. Most of the control protoplasts remained at the oval stage of development, whereas cultures obtained from leaves presprayed with DFMA showed a significant increase of 'doubles' (Table IV). With time, the pretreated cultures showed a significant increase of the total number of viable protoplasts and developing cells as compared with controls (Table IV). In 23-d-old pretreated cultures the number of doubles and 'triples' increased 2.3- and 2.2-fold, respectively, in relation to control cultures. One-month-old pretreated cultures showed an increase in all stages of protoplast development when compared with the control cultures (Table IV). Some of the cultures maintained in liquid droplets for up to 2 months were transferred to solid medium. These cells still showed active cytoplasmic streaming and continuing development, as indicated by the occurrence of doubles, triples, and occasionally 'multiples,' while cells from control cultures had senesced. Many protoplasts from pretreated cultures regenerated complete cell walls, as opposed to the usual patchy deposition of cellulose (17). Although DFMA pretreatment significantly improved the overall viability of the protoplasts, this effect is not striking, indicating that other undefined factors must also play a major role in oat protoplast viability.

Oat mesophyll protoplasts are labile and usually lyse within 24 h of incubation in various osmotica and nutrient media (3, 4). They senesce rapidly upon isolation, do not regenerate a uniform cell wall, and do not divide consistently (14, 17). Leaf pretreatment or addition to the culture medium of senescence retardants such as cycloheximide, kinetin, PAs, and their precursors arginine and lysine has been reported (a) to stabilize oat protoplasts against spontaneous or induced lysis, (b) to increase the incorporation of amino acids and nucleosides into proteins and nucleic acids in leaves and protoplasts, (c) to retard Chl breakdown in leaves and protoplasts, and (d) to decrease or prevent the postexcision and senescence induced rise of RNase and other hydrolytic enzymes (1, 8, 13–15). Of those compounds, the most active are the tri- and tetraamines Spd and Spm (15, 16), which can also induce DNA-synthesis and limited mitosis in oat mesophyll protoplasts (16). We now show that the pretreatment of oat leaves with DFMA reduces the osmotically induced rise of the diamine Put and prevents the arrest of the conversion of Put to Spd and Spm. The increase of the endogenous levels of Spd and Spm may thus be related with the increased macromolecular synthesis and the decrease of Chl loss observed in DFMA-sprayed leaves during osmotic stress, as well as with the improved viability and development of the oat protoplasts extracted from these leaves.

Genetic factors and injuries (excision and wounding) sustained during isolation of mesophyll protoplasts have been emphasized in the past as the main reasons for lack of sustained cell division and failure of plant regeneration in cereals (4). For example, it has been suggested (7, 13) that morphological instability and deterioration of oat protoplasts may be related to a complex of senescence-induced changes, especially increased RNase activity (13, 28), following leaf excision and protoplast isolation. However, tobacco protoplasts which readily divide also show a dramatic increase (12- to 15-fold) of RNase activity after 24 h of isolation (18), and this increase has been shown to be due mostly to the osmotic stress rather than the injury caused during isolation of protoplasts (19, 20). We have presented evidence suggesting that the accumulation of Put and the arrest of Spd and Spm biosynthesis may be one of the reasons for failure of sustained cell division in mesophyll cereal protoplasts cultured *in vitro*. Although the effect of the pretreatment with DFMA on oat protoplasts viability is not dramatic, the results are in the right direction to support the proposed hypothesis.

Put accumulation under osmotic stress could result not only from the ADC activation but also from the inhibition of spermidine synthase, the enzyme which catalyzes the transformation of Put to Spd. Such an inhibition would also account for the reduced levels of Spd and Spm in osmotically treated leaves. Preliminary data indicate that the activity of this enzyme in control leaves is reduced by osmotic stress, and that pretreatment of the leaf with DFMA prevents this effect.

Table IV. *Effect of Pretreatment with DFMA on Oat Protoplast Viability and Development*

Pretreatment	Age of Cultures[a]	No. of Drops Examined	Oval[b]	'Doubles'	'Triples'	Total
	d		*No. of viable[c] protoplasts or developing cells/drop*			
−DFMA	13	11	31.5 ± 2.1	1.5 ± 0.4	0	33.0 ± 1.9
+DFMA	13	11	32.7 ± 2.7	3.4 ± 0.3[d]	0	36.1 ± 3.0
−DFMA	23	11	20.1 ± 1.0	2.5 ± 0.3	0.4 ± 0.2	23.0 ± 1.2
+DFMA	23	12	20.3 ± 1.0	5.8 ± 0.5[e]	0.9 ± 0.2	27.0 ± 1.2[f]
−DFMA	30	11	14.9 ± 0.9	5.9 ± 0.3	1.0 ± 0.2	21.8 ± 0.9
+DFMA	30	11	20.5 ± 1.7[f]	7.4 ± 0.5[f]	1.5 ± 0.3	29.4 ± 1.8[d]

[a] Oat protoplasts from leaves pretreated (+DFMA) or not (−DFMA) were isolated and cultured as described in "Materials and Methods." [b] Oval: initial stages of protoplast development. Many of these cells from the cultures pretreated with DFMA showed complete cell wall regeneration as indicated by staining with Calcofluor White and plasmolysis test; 'Doubles' and 'Triples' (3) are cells undergoing karyokinesis and showing 2 or 3 nuclei, respectively, as indicated by staining with Carbolfuchsin. Some of these cells showed completion of cytokinesis as indicated by the presence of calcofluor-stained cross walls. [c] Viability of protoplasts or developing cells was evaluated as described in "Materials and Methods." [d] Significantly different from control at $P < 0.01$. [e] Significantly different from control at $P < 0.001$. [f] Significantly different from corresponding control (−DFMA) at $P < 0.05$.

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