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A Mutation Causing Proline Requirement in *Zea mays*

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Summary. A conditional seedling lethal, monogenic recessive, endosperm mutant is described. Phenotypic can be accomplished when embryos are cultured in vitro on media supplemented with proline. The efficiency of the repair is proportional to the concentration of proline in the medium. Normal growth is resumed at a dose of 160 mg/l. All the data collected are most easily interpreted by assuming that the mutant, symbolized *pro* has a genetic block in the biosynthetic route leading to proline.

This is probably the first case of a strict genetic requirement for an amino acid to be reported in *Zea mays*. The possible reasons for the difficulties encountered in isolating obligate auxotrophs in higher plants are briefly reviewed.

Introduction

Nutritional mutants in higher plants are very rare. New impetus has been given to this research by the recent advances in plant cell cultures, such as the production of haploid plants (Guha and Maheshawari 1964) and the availability of selective techniques (Carlson 1970) for the isolation of biochemical mutants.

Nevertheless, the number of established mutants with simple metabolic blocks in higher plants in still relatively low if compared to the wealth of mutants of this kind known in microorganisms (see Li et al., 1967, for a general discussion). The only obligate auxotrophs verified, in fact, are those requiring thiamine as a supplement. They have been reported in *Arabidopsis* (Langridge 1955; Rédei 1960) and *Lycopersicum* (Langridge and Brock 1961). Isolating this class of mutant is an important preliminary step for the study of the biochemical basis of the expression of mutant phenotypes, for investigating biosynthetic pathways and the genetic basis of their regulation.

Material

The mutant arose spontaneously in a genetic stock with the W22 inbred line background. According to its pedigree, the stock should be heterozygous for a translocation involving chromosome 1 and 10 (TI-10g). However the translocation was no longer present at the time of the isolation of the mutant, at least to judge from the lack of linkage between gene markers of chromosome 1 and 10, lack of pollen or ear semisterility, and the presence of a normal karyotype in root tip preparations. The mutation is recognizable as early as 20 days after pollination and the plants exhibit, at maturity, a reduced and irregular growth of the endosperm (Fig. 1). Following germination, the coleoptile forms normally but the seedling becomes necrotic and dies before emergence of the first leaf. Excised embryos occasionally show some growth, up to 10-15 mm. before necrosis ensues. In these cases growth proceeds in an irregular manner, yielding one or two small leaves with abnormal morphology. Usually embryos do not grow at all or, in the case of immature embryo cultures (25-30 days after pollination), a callus is formed from scutellar tissues.

Methods

The growth requirements of the mutant were determined from in vitro cultures of detached primary roots or excised embryos.

Asepsis was secured by leaving the seeds in a 0.1% $HgCl_2$ alcoholic solution (70%) for five minutes, rinsing them twice with sterile H_2O and leaving them for 12 minutes in a 5% calcium hypochlorite solution which contained a few drops of Tween 80. Seeds were left in sterile Petri dishes for 48 hours at room temperature. For embryo cultures, the embryos with the scutella were then excised in a sterile chamber; placed in a 5% calcium hypochlorite solution for four minutes and then transferred to test tubes containing different media and cultured in a growth chamber for 15 days at 25 ± 2 °C, under 15 hours daily light cycles.

For root culture, the tips of the primary root, 5-10 mm long, were excised one day after germination of the seeds treated as indicated above and transferred aseptically to 250 ml Erlenmeyer flasks filled with 50 ml of liquid medium. The flasks, each with three root tips, were left on a rotary shaker (80 rpm) for 12 days at 23 ± 2 °C, under 14 hours of daily light regimes. Growth parameters were determined as indicated in the tables.

Initially the weight of the entire seedling was taken as a measure of growth but later this was abandoned because the results were inconsistent. It was realized that since excised embryos together with their scutella contribute significantly to the total weight of the seedlings, variations in their initial size affect the seedling weight determination thus masking the effect of nutrients upon growth.

Each weight determination refers to three roots or two seedlings, respectively.

Pigment content determination

Pigments were extracted by grinding leaf material in 80% (v/v) acetone with a Potter homogenizer. The homogenate was centrifuged twice at 3000 g and pigment concentration was determined spectrometrically using the formulae given by Arnon (1949) for chlorophylls and by v. Wettstein (1957) for carotenoids.

Composition of growth media (in mg/l deionized water)

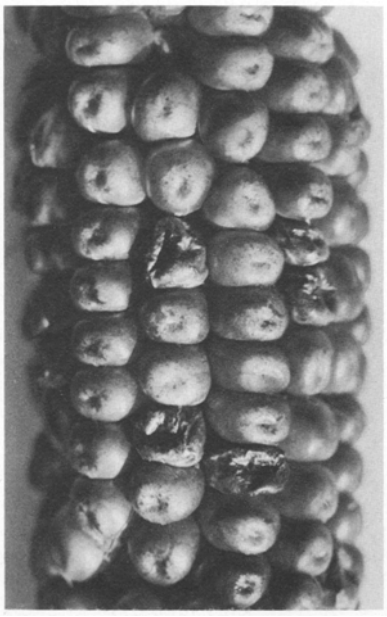

Fig. 1. Ear segregating for the lethal endosperm mutant

1. Mineral medium (D) for embryo culture: NH_4NO_3, 600; $MgSO_4$, $7H_2O$, 400; $CaH_4(PO_4).H_2O$, 400; KH_2PO_4, 400; K_2HPO_4, 160; $FeC_6H_5O_7 . 3H_2O$., 6
2. Enriched D medium (C.M.): consisted of casein hydrolysate, yeast extract, yeast hydrolysate (100, 20, 20 mg/l, respectively) and 4% coconut milk in the D medium. Both media were supplemented with 2% saccharose
3. Heller medium (H) with minor modifications (Heller 1948) for root culture.

Free amino acid determination

Young shoots were homogenized at room temperature. Cold 95% ethanol was added to the homogenate, using 10 ml for each g of fresh tissue, and then centrifuged. The alcohol-soluble supernatant was run on an amino acid analyzer.

Results

Identification of the nutritional requirement of the mutant

The initial observation that led to this analysis was the differing growth behaviour of excised mutant embryos cultured on basic (D) or enriched (C.M.) media. While shoot development on both media was arrested at an early stage when seedlings had reached a height of about 5-10 mm, the roots of the embryos were ostensibly more developed when cultured on C.M. than on D medium. The average fresh weight of the roots with the attached embryos of 48 day-old mutant grown on basic and enriched media was 161 and 304 mg respectively.

This difference could be explained by assuming the simultaneous occurrence of a nutritional requirement in the mutant and a defect in the transport of the nutrient from roots to shoots as a result of a single mutational event. The data however favour a simpler explanation, i.e. a mutation leads to a requirement for a nutrient present only in the C.M.

To identify the component of the enriched medium promoting root growth in the mutant, excised roots were cultured on either basic medium (H) or on basic media with single additions of the organic components of the enriched D medium. The results (Table 1), show that the only addition promoting growth of the roots of the mutant was vitamin free casein hydrolysate (50 mg/l). Roots of normal siblings, on the other hand, were not stimulated in their growth by casein or the other additions but were inhibited by coconut milk. The effect of increasing doses of casein hydrolysate was then measured. It can be seen from the results (Table 2) that doses

Table 1. Effect of different supplements to the basic Heller medium on the growth of excised roots of mutant and normal sibs. In this and following tables figures in brackets indicate standard error. Abbreviations: LA = mean length of primary root in mm; NL = mean No. of lateral roots; FW = mean fresh weight in mg; DW = mean dry weight in mg

Supplement (mg/l)	No. roots	LA	NL	FW	DW
Normal					
None	12	89.5(4.2)	10.6(2.6)	52.1(2.7)	3.1(0.3)
Casein hydr. 50	12	81.5(5.9)	12.7(2.8)	54.0(2.3)	3.2(0.3)
Yeast hydr. 10	6	71.3(3.0)	22.3(2.7)	53.0(6.6)	4.0(0.5)
Yeast extr. 10	6	62.3(8.7)	12.0(3.0)	36.1(15.8)	2.5(1.5)
Coconut milk 2,5%	9	19.8(1.1)	3.6(1.1)	23.0(1.5)	2.4(0.4)
Mutant					
None	9	15.0(1.6)	2.9(1.6)	14.0(1.2)	0.4(0.1)
Casein hydr. 50	6	47.0(12.0)	11.6(4.1)	35.0(5.0)	3.5(0.5)
Yeast hydr. 10	9	11.7(1.1)	2.0(0.8)	10.8(2.1)	1.0(0.4)
Yeast extr. 10	3	13.3(1.3)	1.7(1.2)	13.0 -	1.0 -
Coconut milk 2.5%	9	10.1(0.7)	0	12.0(1.9)	1.4(0.3)

Table 2. Response of excised roots of the mutant to basic Heller media supplemented with increasing doses of casein hydrolysate. Abbreviations as in Table 1

Casein hydrolysate (mg/l)	No. of roots	LA	NL	FW	DW
None	6	11.6(3.3)	4.3(3.3)	10.2(1.2)	0.7(0.1)
50	6	36.6(1.2)	11.0(1.2)	18.6(2.2)	1.0(0.0)
100	5	35.0(3.5)	9.2(2.1)	14.3(1.3)	0.9(0.3)
200	6	62.6(2.7)	16.1(2.1)	26.6(0.4)	1.9(0.1)
400	6	76.6(6.9)	15.3(2.6)	29.2(0.8)	2.1(0.1)

Table 3. Concentration of free aminoacids (μmoles/ml extract) in shoots of 2-day-old mutant and normal sibs

Aminoacid	Normal	Mutant
	*	
Asp	...	.03
Thr	.24	.18
Ser	.25	.12
Glu	.17	.07
Pro	.25	...
Gly	.11	.07
Ala	.30	.19
Val	.21	.05
Cys	...	...
Ile	.07	.02
Leu	.29	.02
Tyr	.06	.02
Phe	.04	...
Lys	no	...
His	.04	...
Arg	no	...
Ammonia	.57	.23

* traces

of 50 and 100 mg/l were equally effective in promoting growth and that the same was true for doses of 200 and 400 mg/l which accounted for a further increase in growth. The positive growth response of the mutant to the addition of casein hydrolysate suggests that a basic step in amino acid synthesis is blocked in the mutant. This assumption is supported by the observation that proline was undetectable in the free amino acids pool of the mutant shoot tissues (Table 3). Several other amino acids, notably valine and leucine, were much reduced relative to the wild type. Excised roots of the mutants were then grown on H media, with and without supplements of groups of L-amino acids (except tryptophan) combined according to their biosynthetic relationship. Their concentration in the medium corresponded to 400 mg/l of casein hydrolysate. The results (Table 4) indicated that the proline, arginine and glu-

Table 4. Effect of groups of amino acids on growth of excised roots of the endosperm mutant (Concentration equivalent to that of 400 mg casein hydrolysate/l, abbreviations as in Table 1)

Growth medium	N° roots	LA	NL	FW	DW
1st experiment					
Basic (H)	12	14.1(0.5)	4.2(0.7)	15.9(1.3)	1.2(0.2)
H+ (1)	6	68.1(0.1)	16.8(3.5)	41.7(10.5)	3.4(1.1)
H+ (2)	9	13.7(0.5)	4.1(0.6)	14.6(1.6)	0.8(0.1)
H+ (3)	11	26.8(11.3)	3.6(0.9)	14.3(1.5)	0.8(0.1)
2nd experiment					
Basic (H)	7	19.8(2.0)	2.7(0.5)	22.0(2.0)	2.4(0.2)
H+ pro, arg, glu	14	97.6(6.5)	36.4(3.4)	56.7(5.8)	6.0(0.7)
H+ val, leu, ile	8	18.4(1.4)	2.2(0.9)	22.3(1.1)	2.5(0.1)
H+ lys, ala	8	15.3(1.2)	3.1(0.9)	16.1(1.2)	1.9(0.2)

1) ala, val, leu, ile, pro, arg, lys, glu.
2) phe, tyr, his.
3) met, thr, cys, gly, ser.

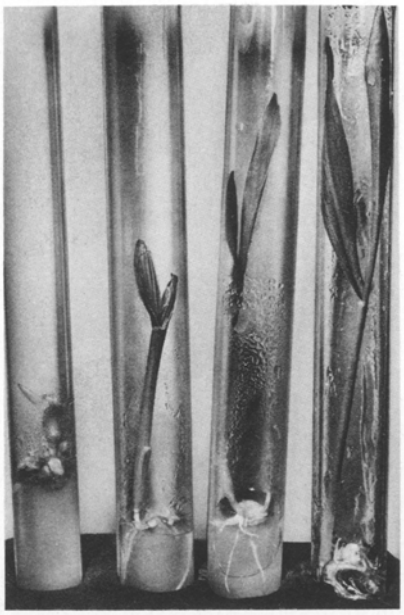

Fig.2. Effect of the medium composition on mutant growth.
Starting from left: mutant embryos cultured on M.M., on M.M. with pro, arg, glu (40, 15 80 mg/l), on M.M. with double dose of the aminoacids, and non mutant embryos on M.M. Note the pigmentation of the leaves on the second tube from the left

Table 5. Growth of excised embryos of mutants and normal sibs on basic and enriched media (LA = length of seedlings; n = No. of embryos)

Growth	Mutant		Normal	
medium	n	LA	n	LA
Basic (D)	16	13.4(2.5)	4	149.0(17.2)
D + (pro, arg, glu)*	16	62.6(2.5)	4	92.2(3.0)
D + 2 (pro, arg, glu)	14	84.0(2.7)	4	110.2(6.1)

* Concentration (mg/l): pro 40; arg 15; glu 80.

Table 6. Effect of proline, arginine and glutamic acid, at concentrations of 80, 30 and 160 mg/l respectively, on embryo growth of mutants and normal sibs (LA = length of seedlings; n = No. of embryos)

Growth	Mutant		Normal	
medium	n	LA	n	LA
Basic (D)	10	21.5(2.1)	4	134.5(10.6)
D + arg, pro, glu	10	84.5(5.8)	4	143.7(9.0)
D + arg, pro	10	65.4(6,9)	4	152.7(8.9)
D + arg, glu	10	12.3(1.5)	4	157.7(17.1)
D + pro, glu	10	83.9(8.3)	4	121.2(26.4)
D + arg	10	13.5(0.6)	4	126.5(11.9)
D + pro	10	79.0(10.9)	4	142.7(12.0)
D + glu	10	14.5(2.2)	4	152.0(9.9)

tamic acid group was responsible for the growth promoting effect. The response of the entire plant to the three amino acids was then tested. It is evident (Fig.2, Table 5) that the addition of proline, arginine and glutamic acid to the basic medium allowed the mutant embryos to bypass the lethal phase and to grow into almost normal seedlings.

Growth on the higher of the two doses of amino acids resulted in better development as well as more uniform chlorophyll distribution in the leaves. Growth of embryos on media containing either the three amino acids together or two or only one at a time (Table 6) revealed that only proline is responsible for remedy of the mutant. A statistical analysis of the data shows that the growth promoting effect of proline is highly significant while the differences observed by keeping proline in the medium and changing the composition of the other amino acids are not. The normal siblings did not disclose any significant response to either proline or to the other two amino acids (Table 5). Mutant embryos were also cultured on mineral medium (D), supplemented with a mixture of the 20 amino acids except proline at the concentration reported by Oaks and Beever (1964), without observing any growth stimulation (unpublished data). It is thus evident that proline is a critical nutrient for producing normal development of this conditional lethal mutant.

Dosage of proline

To establish whether proline can elicit normal growth of the mutant, mutant and normal embryos were cultured on media with increasing doses of proline. Since previous data had shown that proline might affect the pigments as well as growth, the chlorophyll and carotenoid content were also considered in this experiment.

The length of normal seedlings was not affected by the addition of proline, while that of mutants showed an initial lag followed by a linear increase, reaching a maximum at a dose of 160 mg/l of proline (Fig.3). At this concentration the length of the mutants was about the same as that of normal siblings grown on the same medium. No further increase was observed at higher proline doses up to 2560 mg/l (data not shown).

The weight of normal seedlings remained constant with increasing doses of proline, while that of the mutants showed a linear increase in growth up to the dose of 160 mg/l of proline, followed by a stationary phase. Both chlorophyll and carotenoid content per unit weight

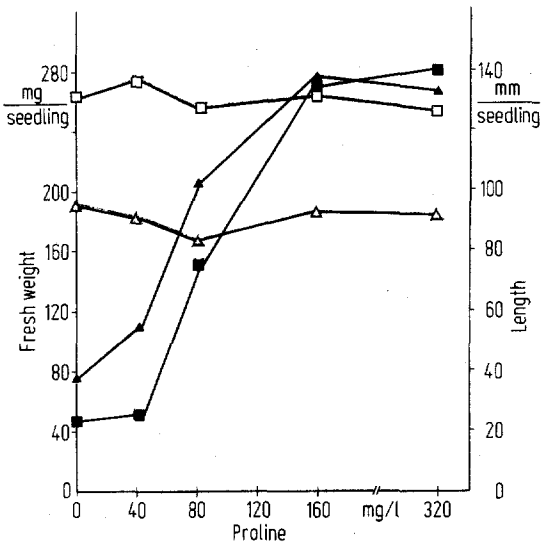

Fig.3. Changes in fresh weight (▲ mutant, Δ normal) and length (■ mutant, □ normal) of mutant and normal seedlings with increasing concentrations of proline in the medium

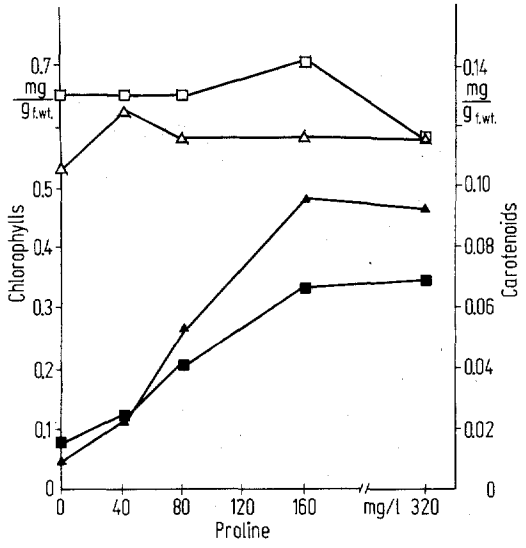

Fig.4. Changes of chlorophylls (▲ mutant, Δ normal) and carotenoids (■ mutant, □ normal) of mutant and normal seedlings with increasing concentrations of proline in the medium

in the mutant increased with addition of proline, reaching a maximum at 160 mg/l (Fig.4). Higher doses had no additional effect. The increase of chlorophyll was more striking than that of carotenoids. In neither case, however, was the pigment content of the normal seedlings achieved.

Proline supplements to the wild type plants were essentially without consequence within these ranges. Chlorophyll in the mutant remained constant while carotenoids were slightly inhibited at doses higher than 160 mg/l.

Discussion

The data indicate that the lethal mutant can be rescued by the addition of proline to the growth medium. We propose to use the gene symbol *pro* to designate this new mutant. The results obtained with root tip cultures showed that the only component of the enriched medium to stimulate growth of the mutant was casein hydrolysate, while coconut milk appeared to be inhibitory.

Further experiments showed that the casein effect was related to proline. The other organic components, even though containing amino acids, failed to induce growth, presumably because their concentration in the medium did not provide sufficient proline to allow mutant growth. The inhibitory effect of coconut milk, on the other hand, might be due to the auxins it contains. Addition of indole acetic acid to the M.M. at the dose of 5 mg/l was in fact sufficient to prevent root tip growth (unpublished results). Growth experiments using media with increasing doses of proline showed that, given the proper dose, growth of the mutant was almost completely normal at the seedling stage. When growth is expressed as increase in fresh weight the mutant seems to grow even more than the normal segregants. The increase of weight of the mutant at 160 mg/l proline was 148% that of normal siblings. At present this fact remains unexplained. An observation not disclosed by the data is the morphogenetic effect of proline. While leaves occasionally formed on MM or on media with 40 mg/l of proline were quite abnormal in their morphology, those formed on 80 mg or higher doses resumed normal development. This normalizing effect could well be related to the increased synthesis of hydroxyproline that may be required in the cell wall extension process (Lamport 1969). On the whole, the results suggest that the mutant has a metabolic block in the proline biosynthetic path. The fact that the extent of growth on MM plus proline, arginine and glutamic acid (40, 15 and 80 mg/l) is far greater than that on MM plus proline only at the same concentration (62.6 vs 20.5 mm) suggests that the mutant is using exogenous arginine to synthesize proline, thus bypassing the block in the major biosynthetic route (for a review on the biosynthesis of these three amino acids see Oaks and Bidwell 1970).

Another feature of the mutant worth considering in this context is the high variability between seeds from different ears in the degree of growth preceding necrosis. It could well be that this variability is related to the amount of proline accumulated in the seed through the maternal tissues.

Similarly, the incomplete endosperm development of the mutant could be explained by assuming that the maternally supplied proline is insufficient for normal development. Perhaps other mutants with abnormal endosperm morphology, known as defective endosperm or aborted seeds, will reveal metabolic deficiencies.

Whatever the cause of this variability and of the proline requirement, this mutant represents, to the best of our knowledge, the first case of an absolute genetic requirement of an amino acid in either *Zea mays* or other flowering plants. The presumed auxotrophs induced in haploid callus cultures of tobacco (Carlson 1970) were in fact leaky, while a chlorophyll mutant in barley first reported as a leucine auxotroph (Walles 1963) was later shown to have the ability to synthesize leucine (Land and Norton 1970). The difficulties encountered in inducing and detecting nutritional mutants in higher plants (for an extensive analysis, see Li et al., 1967; Nelson 1973, Melchers 1974) could be explained by different hypotheses:

1. Redundancy of the genetic information necessary for the metabolic functions essential to the cell (Carlson 1970; Rédei 1974).

2. Activation of secondary biosynthetic routes leading to the same end product (Kao and Puck, 1967).

3. Selection against mutant cells. A mutant cell arising in a multicellular organism could be selected against as a result of failure of intercell feeding, if the gene product is not diffusible from cell to cell (Langridge, 1958; Neuffer 1974) or, alternatively as a result of a decrease in replication time of the mutant sector.

4. Incapacity of the mutant cell or plant to take up exogenous compounds or to transport them from the roots to the above ground portion of the plants. Experimental evidence in favour of this mechanism has been recently obtained in *Chlamydomonas* (Loppes 1969). In this organism the active transporting system of arginine is impaired by ammonium ions. Accordingly the omission of ammonium chloride in the medium allows the survival of arginine-requiring mutants that would be undetectable in the usual medium. This is not intended to be an exhaustive list of all the factors that might account for the difficulty encountered in inducing and detecting auxotrophs in higher plants, but simply as a guideline for further experiments. These possibilities are amenable to experimental tests. The initial success in identifying the *pro* mutant is encouraging us to continue these investigations.

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