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Osmolyte-related recovery of the *opaque-6* lethal phenotype in *Zea mays* L

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Abstract

Endosperm growth and development is a complex phenomenon, driven by the coordinate expression of several genes. A series of endosperm mutants with altered timing and zein synthesis rate have been studied, allowing the partial unravelling of a multifarious system, integrating carbohydrate, amino acid, and storage protein metabolisms, and operating during endosperm growth and development. The exact biological function of one of these loci, the *Opaque-6* (*O6*) gene, remains to be acknowledged. The *o6* locus determines a general reduction of 19- and 22 kDa zeins as well as a number of non-zein polypeptides present in the wild type endosperm. The *o6* mutants present a collapsed, dull endosperm, leaf striations and early seedling death; however, *o6* seedlings can survive when grown in the presence of exogenous proline. It has been suggested that, in mutant seeds and in contrast with the development of the normal seeds, proline does not reach the sites of protein synthesis in adequate amounts. Yet, it has been demonstrated that amino acids other than proline are also able to restore *o6* seedling lethality, contradicting this hypothesis. In this paper, we explored the possibility that the observed proline-mediated rescue of *o6* mutant seedling lethality regarded an osmolyte-mediated mitigation of aberrant protein folding rather than the restoration of a reduced proline flux needed for protein synthesis. This hypothesis was tested by means of *in vitro* cultivation of *o6* seedlings in the presence of putative osmolytes including a series of amino acids, methylamines, and polyols. Several osmolytes were identified, which were able to restore normal growth in *o6* mutant seedlings. Root reestablishment required higher osmolyte concentrations than those necessary for the recovery of the aboveground plant parts. The results presented in this paper provide sufficient preliminary evidence to assume that proline-induced recovery of the *o6* mutant phenotype depends on the osmolytic properties of this amino acid.

Keywords: *Zea mays*, *opaque-6*, seedling lethality, osmolytes

Introduction

Over the past 30 years, the structure and biochemical properties of seed storage proteins have had the main focus of many investigations owing to their abundance, complexity, and impact on the over-all nutritional value of the seed. A great deal is now known about the compounds that are made and stored in cereal and, in particular, maize seeds, as well as how they are hydrolyzed and absorbed by the embryo (see Hannah, 2007; Holding and Larkins, 2009 for a review). The most abundant protein storage components (>60%) in developing endosperm tissues of the maize grain are prolamines called «zeins». Alcohol-soluble compounds rich in glutamine, proline, alanine, and leucine, and almost completely devoid of lysine and tryptophan (Gibbon and Larkins, 2005) constitute these proteins. Based on their evolutionary relationships, zeins are divided into four protein subfamily of α- (19- and 22-kDa), β- (15 kDa), γ- (16-, 27-, and 50-kDa), and δ-zeins (10- and 18-kDa), that are encoded by distinct classes of structural genes (Holding and Larkins, 2009).

Endosperm growth and development is a complex phenomenon, driven by the coordinate expression of several genes. Strategies using spontaneous and induced maize endosperm gene mutations has allowed the partial unravelling of the underlying multifarious system, integrating carbohydrate, amino acid, and storage protein metabolisms, and operating during endosperm growth and development. In this respect, several endosperm mutants with altered timing and zein synthesis rate have been described (reviewed by Hartings et al, 2013). These mutants exhibit a more or less defective endosperm and have a lower than normal zein content at maturity. Many of these genes have been mapped to chromosomes and their effect on zein synthesis has been described (Hartings et al, 2013).

The exact biological function of one of these loci, the *Opaque-6* (*O6*) gene, remains to be acknowledged. The *o6* locus determines a general reduction of 19- and 22 kDa zeins as well as a number of non-zein polypeptides present in the wild type endosperm, the most abundant of which is a 32 kDa cytosolic albumin, termed b-32 (Soave et al, 1981). Ajmone Marsan et al (1992), using RFLP analysis on a segregating F_3 maize population, demonstrated that the b-32 and *O6* locus are physically separated at an approximate distance of 4.0 ± 1.6 cM and ending speculations indicating the b-32 protein as the product of the *o6*

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locus. The *o6* mutation, which is functionally allelic to the *pro-1* mutation (Gavazzi et al, 1975; Manzocchi et al, 1986), was recovered as a spontaneously acquired gene alteration (Ma and Nelson, 1975). *o6* (*pro-1*) mutants present a collapsed, dull endosperm, leaf striations and early seedling death. *O6* causes seedling lethality in its double recessive state. However, *o6* seedlings can survive when grown in the presence of exogenous proline. In fact, Manzocchi et al (1986) were able to show how increasing levels of proline in the culture medium could reverse *o6* seedling lethality and promote complete recovery at the higher proline concentrations tested. The endosperm of homozygous mutant seed contains significant levels of free proline, in most cases in excess of the wild type level (Racchi et al, 1980). Racchi et al (1978) showed that *o6* mutants only responded to proline for seedling recue and not to proline precursors. Furthermore, Tonelli and Bertani (1979) studied the presence of Δ1-pyrroline-5-carboxylate reductase, one of the two enzymes effecting the final step in proline synthesis from glutamic acid, in mutant seedlings and concluded that this enzyme was present both in wild type and in mutant embryos. Taken together, these findings made Racchi et al (1980) suggest that in mutant seeds and in contrast with the development of the normal seeds, proline does not reach the sites of protein synthesis in adequate amounts. However, Balconi et al (1992) reported that amino acids other than proline were also able to restore o6 seedling lethality, contradicting this hypothesis.

Osmolytes are small organic compounds that effect protein stability and are ubiquitous in living systems. These compounds represent different chemical classes that occur naturally including amino acids (proline and glycine), methylamines (betaine and trimethylamine-N-oxide), and polyols (sorbitol and sucrose). Many osmolytes regulate plant responses to stress, including reduced growth (for a review see Khan et al, 2010). Osmolytes exert a dramatic influence on the protein folding reaction, interacting with the peptide backbone of proteins; the free energy of this interaction may be positive or negative. Protecting osmolytes push the folding equilibrium toward the native protein state, whereas denaturing osmolytes push the equilibrium toward an unfolded protein state (Kumar, 2009). Though the magnitude of the energy of interactions with each peptide bond is very small, peptide bonds are by far the most numerous structural component of a protein and the sum of such interactions can be quite large (Gekko and Timasheff, 1981).

The solution thermodynamics of protein-osmolyte mixtures has been well characterized (Schellman, 2002). However, currently, there is no universal molecular theory that can explain the mechanism by which osmolytes interact with the protein to affect protein stability. In this context, Street et al (2006) have developed a putative model based on the energy transfer of the protein backbone from water to a water/osmolyte solution, suggesting the existence of a universal mechanism involving osmolyte, backbone, and water. Considering the transfer free energy of a protein backbone from water to a water/osmolyte solution (Δg_{tr}), they measured the degree to which an osmolyte stabilizes a protein.

In this paper, we explored the possibility that the observed proline-mediated rescue of *o6* mutant seedling lethality is not the result of the restoration of a reduced proline flux needed for protein synthesis, but is rather due to an osmolyte-mediated mitigation of aberrant protein folding. This hypothesis was tested by means of *in vitro* cultivation of *o6* seedlings in the presence of different amino acids, methylamines, and polyols.

Materials and Methods

Plant Material

All maize lines used were part of the germplasm collection of the Maize Research Unit in Bergamo of the «Consiglio per la Ricerca e Sperimentazione in Agricoltura» Two *o6* alleles (*o6-1* and *o6-15*) in A69Y and B37 backgrounds, as well as the corresponding wild type strains were used. Recessive *o6* seeds were visually identified based on their opaque phenotype and subsequently verified through *in vitro* cultivation to avoid the inclusion of «escapes» in the experiments. Such «escapes» occur at an approximately 2% rate and represent opaque kernels, with an apparent normal growth cycle.

In vitro seedling growth

Seeds were surface sterilized by i) immersion in a 2% hypochlorite solution for 10 min and in a 10% ethanol solution for 10 min; ii) abundant rinsing in several changes of distilled water; iii) air drying on

Figure 1 - Proline induced rescue of *o6* mutant plants. Phenotypic differences due to different genetic backgrounds (A69Y and B3) and the effect of proline on plantlet growth are shown.

sterile filter paper. Upon surface sterilization, seeds were inserted into magenta boxes containing basal Murashige and Skoog medium (MS-zero), according to Murashige and Skoog (1962). Growth conditions applied were 16 h light – 8 h darkness at a constant temperature of 27°C. Forty-eight hours after germination, seedling growth was evaluated and *o6* mutant seedlings exhibiting a normal developmental pattern were excluded from the experiments. Verified plantlets were transferred on MS-zero medium or on MS medium containing one of the additives tested. The following additives were considered: i) all 20 principal amino acids; ii) three methylamines [betaine, trimethylamine-N-oxide (TMAO), and urea], iii) three polyols (mannitol, sarcosine, and trehalose). All additives listed were used at a final concentration of 50 mM unless otherwise indicated. After a further growth period of eight days, plantlets were removed from their containers and visually examined. All comparisons eight days after transplantation (wt vs *o6* vs MS-zero vs MS + additive) were performed on batches of at least five seedlings.

Results

Several authors have studied the *opaque-6* maize mutant in detail to gain insight into the mechanisms involved in seedling lethality associated with its recessive state. The finding that its seedling lethality could be overturned through the addition of exogenous proline opened the way to the hypothesis of an impediment in the proline flow towards the sites of active protein synthesis (Racchi et al, 1980). However, Balconi et al (1992) demonstrated that several other amino acids were able to induce a complete recovery of the o6 mutant seedlings, suggesting that the *o6* mutation is related to a more general N-metabolism bottleneck in the seed. Since the above-mentioned studies, several authors have published on the capability of proline to act as a potent protein stabilizer (Madhab et al, 2004; Fisher, 2006; see also Burg and Ferraris, 2008 for a review). In this paper, we describe our preliminary data regarding the osmolyte-induced rescue of the lethal *o6* mutation A total of 26 putative osmolytes, including 20 amino acids, 3 methylamines [betaine, trimethylamine-N-oxide (TMAO), and urea], and 3 polyols (mannitol, sarcosine, and trehalose) were analyzed regarding their ability to overcome *o6* mutant growth inhibition.

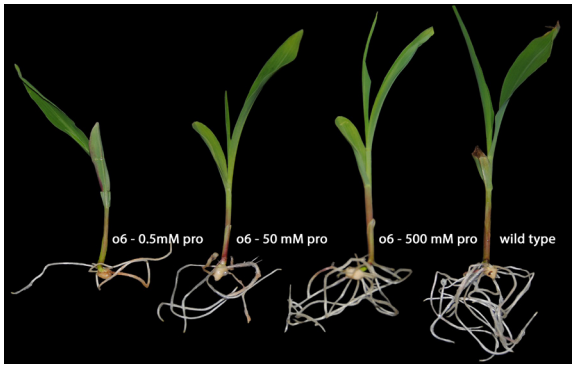

Figure 2 - Evaluation of a proline concentration effect during *o6* mutant plant rescue. Mutant plants were grown on MS-zero medium containing three increasing concentrations of proline. The wild type phenotype is shown for comparison.

As specified in Materials and Methods, two *o6* alleles (*o6-1* and *o6-15*) in two genetic backgrounds (A69Y and B37) were available for this research. Firstly, homozygous *o6-15* seedlings in both backgrounds, as well as their proline-rescued siblings were visually compared, in order to evaluate their growing patterns. In Figure 1, A69Yo6, B37o6, A69Yo6 + proline and B37o6 + proline plantlets are shown. Considering that proline-rescue of A69Yo6 and B37o6 results in visually nearly identical plantlets, the B37 background, showing a more severe mutant behavior, was selected for further analyses. In this background, both *o6* mutant alleles display identical growing patterns (data not shown), allowing for the usage of a single mutant allele (*o6-15*) in all further experiments.

Amino acid induced seedling rescue

After selection of maize background and o6 allele, a first series of experiments were performed to validate the results obtained by Balconi et al (1992) regarding the rescue of the lethal *o6* genotype using amino acid additives in the growth medium. These authors performed an experiment at a fixed final amino acid concentration of 320 mg l^{-1}, which for proline corresponds to a 2.8 mM final concentration. Due to the different molecular weights of the various amino acids, however, this constant weight determines a fluctuation between 4.2 mM (glycine) and 1.5 mM (tryptophane) in final amino acid concentration. In an attempt to verify an alleged negative effect on plant growth at higher amino acid concentrations in the growth medium, mutant plantlets were grown in MS-zero medium with increasing quantities of proline. In Figure 2, we show the behavior of such plants in the presence of 0.5 mM, 50 mM, and 500 mM of proline. Wild type seeds, grown on MS-zero medium, are included in this figure to allow for a direct comparison of plant development. As expected, the lowest proline concentration did not completely restore the wild type phenotype of the *o6* mutant plantlets, whereas both higher concentrations induced a normal development of the aboveground part of the mutant plants. As far as the root system was concerned, root mass was clearly correlating with the amount of proline present in the culture medium. In fact, recovery of the aboveground parts of the plant seemed to occur at lower proline levels than the recovery of belowground portions. Although, for an exhaustive analysis, this kind of experiment should be performed for each of the additives used in this study, in our preliminary analysis we opted for a final osmolyte concentration of 50 mM, without considering further optimization of the working concentration of the single additives.

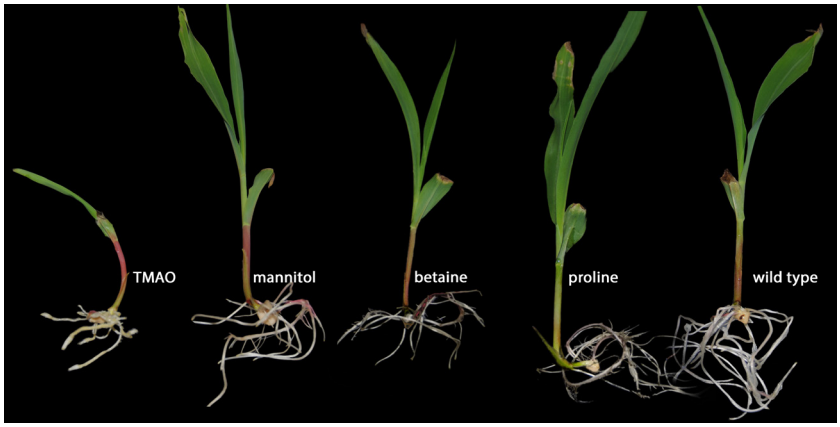

Figure 3 - Effect of osmolytes on wild type plant growth. Several osmolytes (from left to right: TMAO, mannitol, betaine, and proline) were tested for their effect on plant growth of wild type plantlets. An untreated wild type plantlet (right) is included for comparison.

At this point, and in order to verify earlier results of Balconi et al (1992), *o6* mutant seeds were germinated on MS-zero agar and transferred onto MS-zero agar containing distinct amino acids at a 50 mM final concentration. In order to rule out toxicity of the amino acids used at this concentration, we tested a parallel series of wild type plantlets under identical conditions. None of the amino acids had toxic effects on plant growth. In Figure 3, the effect of proline on wild type plantlets is shown as an example of the amino acid effect on growth (other amino acids not shown). Subsequently, the recovery of the o6 lethal phenotype was tested. In Figure 4, an example of o6 plantlet growth in the presence of glycine is shown, demonstrating the recovery of the wild type phenotype. At this amino acid concentration, growth responses were straightforward and plantlets showed either complete recovery of the aboveground parts or no recovery what so ever. Plantlets grown on recovering amino acids did show sub-optimal development of the root system in all cases tested (Figure 4 and data not shown). The results regarding amino acid induced recovery are summarized in Table 1.

Non-amino acid induced seedling rescue

Three methylamines (betaine, TMAO, and urea), and three polyols (mannitol, sarcosine, and trehalose) were used in this study. Similarly to previously described, these additives were first tested on wild type plantlets to analyze their effects on plant growth. In Figure 3, the effect of TMAO, mannitol, and betaine on the growth of wild type plantlets is shown. Clearly, mannitol and betaine did not cause any negative effect on plantlet growth. Conversely, TMAO caused growth disturbance in wild type plantlets and urea had a similar effect on plant growth (data not shown). Sarcosine did not disturb the growth pattern of wild type plantlets (data not shown), while the effect of trehalose on wild type plantlets was not tested. In all these cases, effects were equally visible on aboveground and belowground vegetative parts.

Considering the discontinuous effect of the osmolytes tested on wild type growth, the action of all non-amino acid osmolytes on *o6* mutant plantlets was tested. Figure 4 summarizes the effects registered with the non-amino acid osmolytes. In particular, betaine and trehalose induce a similar phenotypic

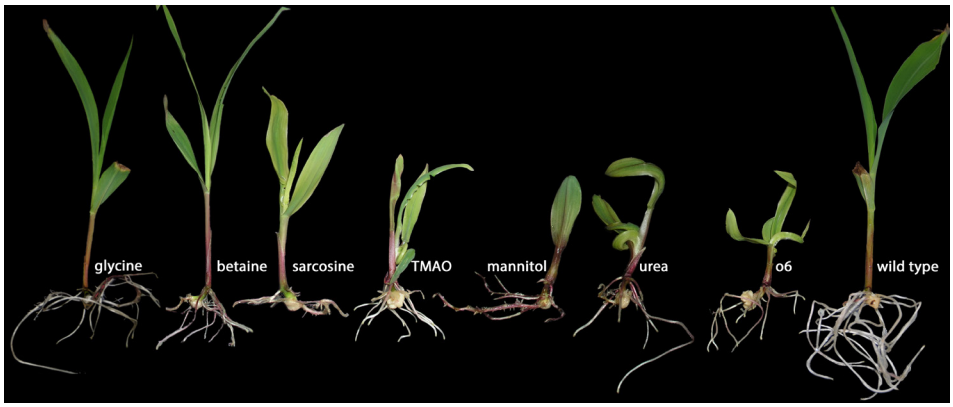

Figure 4 - Effect of osmolytes on *o6* mutant plant growth. Several osmolytes (from left to right: glycine, betaine, sarcosine, TMAO, mannitol, and urea) were tested for their effect on plant growth of o6 mutant plantlets. An untreated mutant plantlet and wild type plantlet are included for comparison.

Table 1 - Amino acid induced recovery of the lethal o6 mutant phenotype.

medium	growth mutant	wildtype
MS-zero	-	+
Arginine	+	+
Asparagine	+	+
Glycine	+	+
Leucine	+	+
Methionine	+	+
Proline	+	+
Tryptophan	+	+
Alanine	-	+
Aspartic Acid	-	+
Cystein	-	+
Glutamine	-	+
Glutamic Acid	-	+
Histidine	-	+
Isoleucine	-	+
Lysine	-	+
Phenylalanine	-	+
Serine	-	+
Threonine	-	+
Tyrosine	-	+
Valine	-	+

recovery of the *o6* mutant plantlets as the recovery observed for proline and the further recovering amino acids listed in Table 1. Sarcosine induced a partially recovered phenotype; plantlets exhibited moderately etiolated leaves and stunted growth. Urea exerted a negative effect on wild type plantlets. Its effect on *o6* mutant growth was not noticeable different; plantlets grown in the presence of urea had an identical phenotype to the mutant seedlings grown on MS-zero. Also, TMAO had a negative effect on wild type plant growth (Figure 3). Though this osmolyte was not able to compensate for the effect of the *o6* mutation on plantlet growth, it induced typical phenotypic changes in the aboveground parts of mutant seedlings (Figure 4). Finally, while the addition of mannitol to the growth medium did not induce any phenotypic effect in the wild type plantlets, its effect on mutant growth was evident and determined a deteriorating of plant growth with respect to the already severe effect of the o6 mutation on plant development (Figure 4). Again, in all tests performed on *o6* plantlets, effects revealed on aboveground plant parts correlated with visible effects on the belowground vegetative parts. The effect of the osmolytes tested on plant growth of both wild type and mutant plantlets, expressed as ranges of measured plant heights, are summarized in Table 2.

Discussion

In an attempt to augment our knowledge of the o6 maize mutation, its proline-dependent growth was investigated. In particular, in this paper, we explored the possibility that the observed proline-mediated rescue of *o6* mutant seedling lethality regarded an osmolyte-mediated mitigation of aberrant protein folding rather than the restoration of a reduced proline flux needed for protein synthesis. This hypothesis was tested by means of *in vitro* cultivation of *o6* seedlings in the presence of putative osmolytes including a series of amino acids, methylamines, and polyols.

Several amino acids were identified, which were able to restore normal growth of mutant plantlets. In particular, our data on recovering amino acids corroborated previously published preliminary data (Balconi et al, 1992). Curiously, recovery of the aboveground (mesocotyl, coleoptile, and leaf) structure was always more pronounced than the recovery observed for the belowground (root) plant parts. For proline, we showed that enhanced root recovery occurred at higher osmolyte concentrations (Figure 2). None of the other recovering amino acids was tested at higher concentrations, but it seems likely to assume that root recovery, in general, requires a higher osmolyte input. Finally, it appears worth mentioning that none of the amino acids caused visible phenotypic effects on wild type plants.

Among the remaining putative osmolytes tested, two were able to induce normal growth in mutant plantlets (betaine and trehalose), while a third osmolyte induced partial recovery of the mutant condition (sarcosine). As mentioned previously, also in this case growth recovery regarding predominantly aboveground plant tissue. Root growth appeared strongly reduced with respect to wild type plants in all cases analyzed. The remaining osmolytes tested exerted different phenotypic changes in wild type and mutant plantlets. Among these, urea and TMAO had a negative effect both on wild type and mutant seedlings. TMAO, moreover, induced additional morphological changes in the mutant phenotype. Finally, mannitol enhanced the negative effect of the *o6* mutation.

Several of the osmolytes analyzed in this paper were used by Street et al (2006). Data obtained by these authors allowed to classify the osmolytes (either stabilizing or destabilizing) according to their Δg_{tr} and polar surface areas. Interestingly, both TMAO (a strong stabilizer) and urea (a strong denaturant), considered as opposites by Street et al (2006), behave similarly in our experiments and failed to restore the o6 phenotype. Conversely, proline, betaine, and trehalose, all moderate stabilizers, were able to recover

Table 2 - Plant height (in cm) 8 days after transplantation.

medium	wild type	opaque-6
MS-zero	15.5 - 16.5	5.5 - 7.0
MS + AA1[§]	14.5 - 18.5	17.5 - 22.0
MS + AA2	14.0 - 18.5	5.0 - 6.5
MS + betaine	16.5 - 22.0	16.0 - 19.5
MS + mannitol	13.0 - 16.0	4.5 - 6.0
MS + trehalose	ND	16.0 - 17.5
MS + sarcosine	16.5 - 17.5	11.0 - 12.0
MS + TMAO	7.5 - 9.0	7.0 - 8.0
MS + urea	6.5 - 8.0	6.5 - 7.0

[§]AA1 = arg, asp, gly, leu, met, pro, trp; AA2 = asp, cys, glu, gln,his, ile, lys, phe, ser, thr, tyr, val.

the wild type phenotype. These findings seem to indicate that a strong osmolytic action is not sufficient to induce the changes necessary to overcome an o6-related growth inhibition. Nonetheless, the results presented in this paper provide sufficient preliminary evidence to assume that proline-induced recovery of the o6 mutant phenotype depends on the osmolytic properties of this amino acid. In fact, our data imply that several osmolytes are able to induce, in the *o6* mutant plantlets, state changes, probably at the protein level, sufficient to overcome the early growth inhibition associated with this mutation.

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