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Communication

Proline Is Not the Primary Determinant of Chilling Tolerance Induced by Mannitol or Abscisic Acid in Regenerable Maize Callus Cultures[1]

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ABSTRACT

Chilling sensitive regenerable maize (*Zea mays* L.) callus cultures can be induced to survive prolonged exposure to 4°C by treatments with mannitol, abscisic acid (ABA), and/or high levels of proline. Maize callus with a free proline content of about 122 micromoles/grain fresh weight survived longer exposures to 4°C than did callus with a free proline content of about 68 micromoles/grain fresh weight. The addition of 0.53 molar mannitol or 0.1 millimolar ABA to culture medium produced a free proline content in maize callus of about 136 and 145 micromoles/grain fresh weight, respectively, if the medium contained 12 millimolar proline or about 36 and 1 micromoles/grain fresh weight, respectively, if no proline was in the medium. Although these mannitol and ABA treatments produced drastically different free proline levels in maize callus, callus grown on these media survived longer exposures to 4°C than did maize callus grown on any proline treatment alone. Thus, the internal free proline level of treated callus is not the primary factor conferring chilling tolerance on these tissues.

Maize is cold sensitive and its growth and survival are limited by temperatures of 15°C or lower (4). This cold sensitivity can create many agronomic difficulties such as costly replanting or reduced yield in northern latitudes or regions subjected to occasional late spring or early fall frosts or low temperatures.

Attempts have been made, using *in vitro* tissue culture techniques, to study the chilling sensitivity of maize and to develop chilling tolerant maize material (2). This previous work demonstrated that 0.53 M mannitol, 0.1 mM ABA, or a combination of the two could induce chilling tolerance in typically chilling sensitive regenerable maize callus (2). Furthermore, this induced chilling tolerance was correlated with the accumulation of proline by the treated tissues (2). The accumulation of proline induced by the mannitol treatment was shown to occur whether or not proline was in the culture medium. However, the proline accumulation induced by the ABA treatment was shown to occur only when proline was present in the culture medium. These observations suggested that the mannitol and ABA treatments were affecting the callus by different mechanisms.

If mannitol and ABA affect maize callus by different mechanisms, it is possible that the induction of proline accumulation by these compounds is not the primary callus response that confers chilling tolerance to the treated tissue. This concept is addressed in the present work.

MATERIALS AND METHODS

Plant Material

Type I maize (*Zea mays* L.) callus cultures capable of plant regeneration were developed from pooled immature embryos of field-grown and self-pollinated Pa91, as described by Duncan *et al.* (1). Cultures were maintained in the dark at 28°C on D medium by selectively subculturing only regenerable tissue at 21-d intervals (1).

Cold Treatment

Callus was grown on D medium with or without 12 mM proline and maintained on each medium for at least one 14-d subculture period before being used in further experiments. Callus (20 pieces of callus/treatment, 0.01 gfwt[2]/piece) used in cold experiments was transferred to fresh medium and incubated at 28°C for 7 d, then transferred to 4°C. For each experiment, callus was removed from 4°C at 7- or 10-d intervals and incubated at 28°C for 3 weeks before the callus was examined for growth. The doubling time for Pa91 callus is approximately 12 d at 28°C and 144 d at 4°C (Fig. 1). Cold treatments used in this study were so brief that no appreciable growth occurred during the 4°C treatment. Consequently, the 3 week growth period after the 4°C treatment was necessary to observe medium treatment effects.

All experiments were conducted in the dark. Variations on this experimental procedure consisted of supplementing the medium with ABA, mannitol, proline, or combinations of these compounds (concentrations listed in Tables and Figures).

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[2] Abbreviation: gfwt, grain fresh weight.

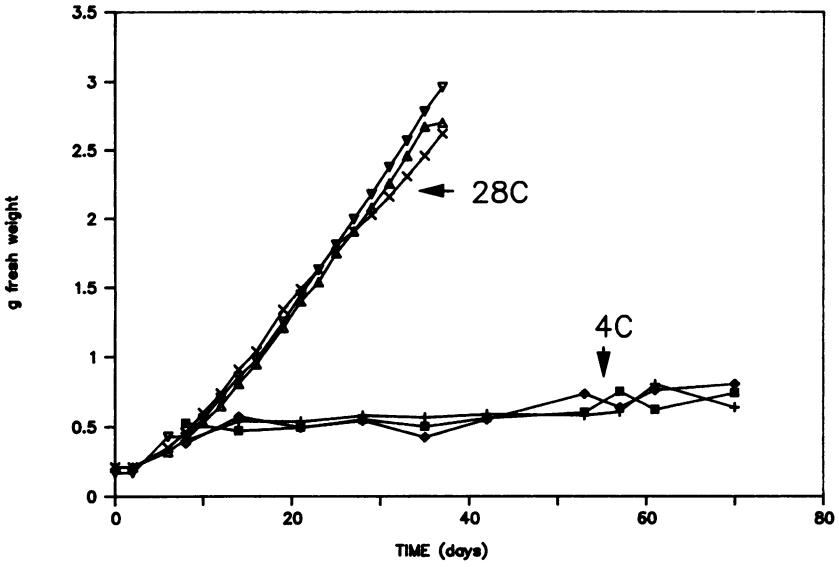

Figure 1. Fresh weight gain of Pa91 type I callus grown on D medium (1) at 4 and 28°C. Initial inoculum consisted of 0.2 gfwt (20 callus pieces). Each data point represents a different set of 20 callus pieces. Experiment repeated three times and all three experiments are represented in the Figure.

Proline Measurements

To produce callus of comparable age and temperature exposure, callus was grown for 17 d at 28°C on medium containing various concentrations of proline with or without the addition of 0.53 M mannitol or 0.1 mM ABA. After this culture period, 1 gfwt of each treatment was pulverized in 5 mL of 70% (v/v) ethanol in a glass hand homogenizer. The homogenate was left standing for 1 h at room temperature then centrifuged at 150*g* for 10 min and the supernatant collected. The pellet was reextracted in 70% ethanol. The combined supernatants were dried with a rotary evaporator, washed twice with 5 mL of petroleum ether (b.p. 37–54°C), dissolved in lithium citrate buffer (pH 2.2), and subjected to physiological fluid amino acid analysis using a Beckman model 119 CL amino acid analyzer.

RESULTS AND DISCUSSION

Pa91 callus, when grown on culture medium containing 12 mM proline, had a free proline level about 24-fold greater than similar callus grown on culture medium lacking proline, 62.9 and 3.1 μmol/gfwt, respectively (Table I and ref. 2). In another experiment where the proline content of the culture medium was increased from 0 to 50 mM, the free proline level of 17-d-old Pa91 callus was increased approximately 45-fold, from a 2.7 to 121.8 μmol/gfwt (Table II). These data show that by adding different concentrations of proline to the culture medium, a gradient of internal free proline levels can be created.

Other compounds, besides proline, can also change the internal free proline content of maize callus. When 0.53 M mannitol was added to the culture medium with and without proline, callus grown on culture medium containing 12 mM proline accumulated fourfold more free proline than callus grown on culture medium lacking proline, 135.8 and 36.2

Table I. *Free Proline Content of Pa91 Callus Exposed to Mannitol or ABA and Grown on Culture Medium with or without Proline*

Data presented in this table were compiled from 15 experiments over a 2-year period. Callus (0.01 gfwt/piece, 20 pieces/treatment) which was subcultured onto treatment medium was first maintained for two 21-d growth cycles on medium lacking proline.

Proline Content of Medium[a]	D Medium	D Medium + 530 mM Mannitol[a]	D Medium + 0.1 mM ABA[b]
mM		μmol/gfwt of free proline[c]	
0.0	3.1 ± 1.3 (n = 12)	36.2 ± 4.1 (n = 5)	1.3 ± 0.5 (n = 6)
12.0	62.9 ± 19.4 (n = 16)	135.8 ± 14.7 (n = 7)	145.1 ± 11.0 (n = 5)

[a] Proline and mannitol were added to D medium (1) before autoclaving. [b] ABA was filter sterilized into medium after autoclaving. [c] Data were collected from a Beckman model 119 CL amino acid analyzer and a 70% (v/v) ethanol extraction of callus grown 17-d at 28°C.

Table II. *Proline Accumulation by Pa91 Callus when Exposed for 17 d to Several Concentrations of Proline in the Culture Medium*

Callus (0.01 gfwt/piece, 20 pieces/treatment) which was subcultured onto treatment medium, was first maintained for two 21-d growth cycles on medium lacking proline. Experiment was repeated twice.

Proline Content of Medium[a]	Proline Content of Callus[b]
mM	μmol/gfwt
0.0	2.7
12.5	68.4
25.0	97.9
50.0	121.9

[a] Proline was added to D medium (1) before autoclaving. [b] Data were collected from a Beckman model 119 CL amino acid analyzer and a 70% (v/v) ethanol extraction of callus grown 17-d at 28°C.

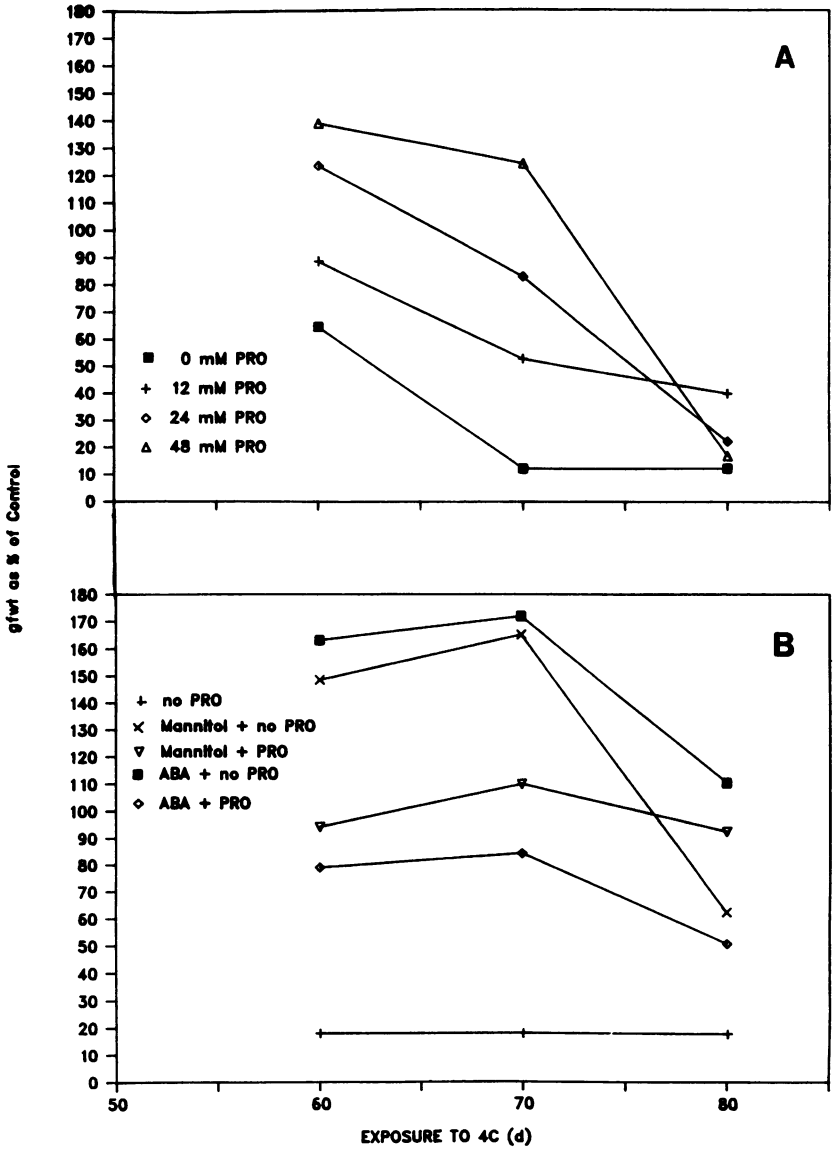

Figure 2. Effect of several media treatments on the survival of Pa91 type one callus at three exposure times at 4°C. The control used for calculating percentage of control was callus exposed 50-d to 4°C, an exposer all treated callus survived. Internal free proline (PRO) content induced by each treatment were: ABA + no PRO = 1.3 μmol/gfwt, 0 mM Pro = 3.1 μmol/gfwt, Mannitol + no PRO = 36.2 μmol/gfwt, 12.0 mM PRO = 62.9 μmol/gfwt, 24 mM PRO = 97.9 μmol/gfwt, 48 mM PRO = 121.9 μmol/gfwt, Mannitol + PRO = 135.8 μmol/gfwt, and ABA + PRO = 145.1 μmol/gfwt. The accumulated gfwt were from tissues grown 28-d at 28°C after a exposure to 4°C and a 7-d exposure to 28°C prior to the 4°C exposure. The treatments listed refer to proline and mannitol (0.53 M), added before autoclaving the D medium (1), and ABA (0.1 mM) filter sterilized and added after autoclaving the medium. All proline levels were determined using a Beckman model 119 Cl amino acid analyzer with 70% ethanol extracts of additional callus grown 17-d at 28°C. Experiments were repeated twice.

μmol/gfwt, respectively (Table I and ref. 2). The free proline level of callus grown on culture medium containing mannitol and no proline was still only about one-half the free proline level in callus grown on culture medium lacking mannitol but containing 12 mM proline, 36.2 and 64.0 μmol/gfwt, respectively (Table I and ref. 2). Callus exposed to 0.1 mM ABA alone contained only 1.3 μmol/gfwt free proline while the level increased to 145.1 when both 0.1 mM ABA and 12 mM proline were in the medium. The free proline accumulation patterns of callus just described have been seen in all the experiments conducted thus far and the free proline level of callus resulting from exposure to culture medium containing 50 mM proline was comparable to that induced by 0.53 M mannitol or 0.1 mM ABA in culture medium containing 12 mM proline (Tables I, II and ref. 2).

Thus, by using proline, mannitol, and/or ABA containing media a wide range of internal proline levels can be induced in maize callus with which to test the effect of internal proline levels on the chilling tolerance of the callus.

In this paper, growth is used as the sole measure of cell survival. Other methods of determining cell survival, such as the reduction of 2,3,5-triphenyltetrazolium chloride, have proved to be less reliable than growth due to treatment, assay-component interactions (3). Furthermore, growth *versus* no growth is the obvious delineation between successful and nonsuccessful treatments. However, in these experiments variation between these extremes was noted. Because a callus piece is a large collection of totipotent cells, we intrepret this variation in growth after prolonged exposure to 4°C as an indication that some but not all of the cells in the callus are alive and capable of growth.

When callus was exposed to 4°C, the proline treatments increased callus longevity from 50 d for the callus treated with 0 mM proline to 70 d for callus treated with either 24 or 48 mM proline (Fig. 2A). These results indicate that a free proline level of at least about 98 μmol/gfwt is needed for all callus pieces to survive a 70-d exposure to 4°C. There were no appreciable differences in growth when the callus was grown

at 28°C on a proline concentration range from 0 to 48 mM (data not included) indicating that there was little or no water stress effects induced by these proline treatments.

When callus was exposed to mannitol or ABA in the culture medium with or without proline, callus longevity at 4°C was substantially increased with all treatments but there was no correlation with tissue free proline levels. This is most evident with the ABA treatment medium lacking proline where only 1.3 μmol/gfwt of proline accumulated (Fig. 2B). Furthermore, callus treated with mannitol or ABA survived an 80-d (Fig. 2B) to 100-d (2) exposure to 4°C, whereas callus exposed to proline treatments alone survived only up to a 70-d exposure to 4°C (Fig. 2A). These results indicate that, although proline can itself increase callus longevity at 4°C, mannitol and ABA treatments can increase callus longevity to a greater degree than would be expected by the amount of free proline accumulated in the treated tissues.

ACKNOWLEDGMENT

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