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TRIGONELLINE ACCUMULATION IN SALT-STRESSED LEGUMES AND THE ROLE OF OTHER OSMOREGULATORS AS CELL CYCLE CONTROL AGENTS

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Key Word Index—*Glycine max*; *Pisum sativum*; Leguminosae; salt stress; trigonelline; proline; glycine betaine; osmoregulator; cell cycle

Abstract—Plants, when exposed to excess salt, accumulate osmoregulators such as glycine betaine, proline and trigonelline to prevent water loss. Alfalfa (*Medicago sativa*) shows a five-fold proline increase and a two-fold trigonelline increase after salt-stress. Trigonelline also has a second function as a cell cycle regulator during the early growth of many legume root meristems. In this study, greenhouse grown soybeans (*Glycine max*) were exposed to salt-stress and levels ($\mu g\ g^{-1}$, dry wt) of trigonelline were determined in leaf samples until senescence. Significant trigonelline increases occurred, beginning several weeks after salt exposure. Further experiments determined if other known osmoregulators (proline, glycine betaine) could affect the cell cycle parameters in cultured root meristems of peas (*Pisum sativum*), in a manner similar to trigonelline. Mitotic values remained near 4% of controls. At concentrations of 10^{-4}–10^{-7} M trigonelline, an accumulation of G2 nuclei occurred, whereas proline was ineffective and glycine betaine only slightly effective in promoting G2 nuclei accumulation. These results demonstrate that the cell cycle effects of the trigonelline molecule are indeed specific and that its role as an osmoregulator in salt-stressed legumes is strengthened. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Environmental pressures, such as extreme temperatures, water availability and excess salinity in the soil, limit the growth and production yield of crops. Stresses such as these have triggered the development of mechanisms which regulate or counteract the stress-induced effects in plant tissues. The amino acids, proline and glycine betaine, are the two compounds that widely accumulate in stressed plants. In alfalfa (*Medicago sativa*), the accumulation of proline and glycine betaine was specifically associated with water and salt-stress [1]. Trigonelline, a pyridine betaine, which also functions as a cell cycle regulator, may also accumulate in response to various stresses.

Water stress, in the primary leaves of barley seedlings, induced an increase in proline up to two to three times the control [2]. In alfalfa, among the various stresses such as salt, water and cold, water stress was the strongest effector in proline accumulation. Under a 48 hr water stress, proline increased 20-fold [1]. Proline accumulation has also been recorded in bacteria and algae when water levels or the osmotic potential decreased [3].

Cold stress also seems to have an effect in amino acid build up in plant tissues. In wheat seedlings, proline and glycine betaine levels increased in response to a cold stress of 4°. The total amino acid content increased nearly four-fold on a dry weight basis with plants grown at 20° during the same period. Proline concentration accounted for a 52-fold increase, while glycine betaine increased more than two-fold [4].

Salinity poses a threat to plant production and is a limitation in many parts of the world [5]. Salt-stress induced decreases in leaf water potential and in osmotic potential in barley [6]. When alfalfa plants were stressed with 0.2 M NaCl for 48 hr, the trigonelline concentration in the plant tissues increased 5-fold [1]. *In vitro* studies with proline, glycine betaine and other low M_r compatible solutes have shown that they can protect enzyme structure and function, and also protect membranes against other deleterious conditions [4].

In legumes, salt-stress results in a significant limitation of productivity when related to the adverse effects on the growth of the host plant, the root nodule bacteria and, finally, the nitrogen-fixation capacity [7]. When alfalfa plants were subjected to 0.15 M NaCl

Table 1. Trigonelline concentrations ($\mu g\ g^{-1}$) in leaves of *G. max* grown under greenhouse conditions and exposed to salt-stress from week 8 to 14. Samples were taken from week 11 to 17 (senescence)

	Trigonelline concentrations ($\mu g\ g^{-1}$ dry wt)	
Plant age (weeks)	Control	Salt-stress
11	32.1 ± 4.1	53.1 ± 7.3
12	75.7 ± 2.3	98.7 ± 5.6
13	90.8 ± 7.8	114.0 ± 6.1
14	83.3 ± 7.1	124.4 ± 5.8
15	44.2 ± 6.1	159.6 ± 6.4
16	45.6 ± 9.1	73.8 ± 8.8
17	25.4 ± 1.9	24.1 ± 4.5

Values represent mean and standard error of at least two determinations per treatment.

for two weeks proline showed the highest increase among the amino acids in the roots and nodules [7]. Amino acid accumulation due to water deficit and salinity, may play a physiological function, such as osmoregulation whereby the compounds can provide a sink for energy and nitrogen, a signal for senescence and an indicator of drought resistance [3]. Thus, interest in the metabolic concentrations in nodules are essential to understanding the consequence of salt-stress on the function of legume nodules at the molecular level [7].

The pyridine trigonelline, among the betaines, is believed to act as an osmoregulator. In *in vitro* plants of *Popolus trichocarpa X deltoides*, trigonelline was found to have increased twice over the control in response to 0.1 M NaCl. Trigonelline, which is preferentially located in the cytoplasm, may play some other role such as modification of membrane transport properties. However, it may also be a sensitive indicator of salt-stress in poplars [5].

Since trigonelline is another betaine involved in the osmoregulatory mechanism in stressed plants, experiments were performed to determine trigonelline concentrations in the foliage of salt-stressed soybean plants over a continuous growing period. In addition, since trigonelline has been shown to regulate the cell cycle of meristematic legume roots [8], other experiments were performed utilizing pea roots to determine if the osmoregulators glycine betaine and proline could affect the cell cycle of the pea root meristem in a manner similar to trigonelline.

RESULTS AND DISCUSSION

When 9-week-old soybean plants were subjected to 100 ml of 0.1 M NaCl three times a week for six weeks, foliage trigonelline levels increased throughout the exposure period until the plants began to senesce (Table 1). At 11 weeks (2 weeks exposure) the trigonelline concentration in the control was 32.1 $\mu g\ g^{-1}$, while the trigonelline concentration of the salt-stressed plant was 53.1 $\mu g\ g^{-1}$. Trigonelline levels in both the control and the salt-stressed plants increased throughout the next 3 weeks, but at a faster rate in the salt-stressed plants. There was a marked increase in trigonelline levels of 14-week-old salt-stressed plants, as compared to the control plants, from 83.3 to 124.4 $\mu g\ g^{-1}$. As the plants aged, and leaf abscission began, trigonelline levels in the salt-stressed plants decreased until they approached the controls. The 16 and 17 week salt-stress soybeans, had 73.8 and 24.1 $\mu g\ g^{-1}$ of trigonelline, whereas the control had 45.6 and 25.4 $\mu g\ g^{-1}$, respectively.

None of the osmoregulators at any concentration affected the mitotic index in cultured root meristems of *Pisum sativum* (Table 2). Exposure to trigonelline, glycine betaine or proline kept the mitotic index values near 4% obtained in the controls.

When analysing the effects of varying concentrations of trigonelline, glycine betaine and proline on the relative DNA content of the root meristems cells of garden peas, trigonelline showed the highest increase in promoting G2 accumulation (Table 3). At 10^{-4}–10^{-6} M trigonelline, over 45% of the cells were in G2, compared to the control value of 16%. Glycine betaine was only slightly effective at 10^{-4} M, inducing 26% of the cells in G2. Proline was ineffective at all concentrations, with G2 values remaining on or near the control values.

Trigonelline's potential role as a stress metabolite is of particular interest to this laboratory since we have investigated trigonelline's role as a cell cycle regulator for several years. Trigonelline naturally accumulates in the leaf and is translocated to the developing pod for deposition in the seed [8]. When seeds germinate the trigonelline is again translocated to the root meristem where it induces G2 nuclei accumulation. Since trigonelline may also play a role in salt adaptation and salt tolerance as does proline and glycine betaine, perhaps these two other solutes could also function as cell cycle regulators? When tested on pea root meristems in culture at concentrations from 10^{-4} to 10^{-8} M, proline and glycine betaine showed no significant increase of nuclei in G2. Control soybean

Table 2. Effects of the salt-stress solutes trigonelline, glycine betaine and proline on the mitotic index of meristems of excised roots of the garden pea (*P. sativum*) grown in White's medium with sucrose for 24 hr in the presence of the test compound

	Compound (μ)		
Concentration in medium (M)	Trigonelline	Glycine betaine	Proline
10^{-4}	2.5 ± 0.4	3.7 ± 0.5	3.9 ± 0.4
10^{-5}	3.2 ± 0.3	3.4 ± 0.3	3.5 ± 0.1
10^{-6}	4.1 ± 0.5	5.5 ± 1.0	4.0 ± 0.2
10^{-7}	2.9 ± 0.4	3.9 ± 0.4	3.9 ± 0.4
10^{-8}	4.1 ± 0.3	4.0 ± 0.1	4.0 ± 0.1

Mean and standard error of at least three slides per treatment. Mitotic indices for roots grown with no additives are 3.9 ± 0.3.

Table 3. Effects of the salt-stress solutes trigonelline, glycine betaine and proline on the proportion of G2 nuclei in stationary phase meristems of garden pea (*P. sativum*) exposed to the test compound for 3 days in White's medium

	Proportion of cells in G2 in presence of		
Concentration in medium (M)	Trigonelline	Glycine betaine	Proline
10^{-4}	0.50 ± 0.02	0.26 ± 0.06	0.12 ± 0.03
10^{-5}	0.46 ± 0.09	0.15 ± 0.05	0.14 ± 0.03
10^{-6}	0.48 ± 0.02	0.17 ± 0.03	0.19 ± 0.04
10^{-7}	0.32 ± 0.02	0.13 ± 0.01	0.16 ± 0.04
10^{-8}	0.15 ± 0.03	0.19 ± 0.05	0.19 ± 0.02

Mean and standard error of three slides per treatment, 50 nuclei were scored per slide. Controls roots subjected to stationary phase conditions without additives to the medium had a value of 0.16 ± 0.02.

leaves accumulate trigonelline between weeks 12 and 14. Salt-stress accentuates the already rising levels of trigonelline in the leaves.

Results presented herein demonstrate that trigonelline accumulates in salt-stressed leaves of soybeans. Since many legumes seeds have high endogenous trigonelline concentrations [9], hence the ability to synthesize trigonelline, this is not an unexpected result. Tissue levels of trigonelline can be used as an indicator of stress. However, with respect to its role as a cell cycle regulator, trigonelline appears to be specific, in that other known osmoregulators that accumulate in legumes, such as proline and glycine betaine, do not mimic trigonelline's cell cycle regulating abilities.

EXPERIMENTAL

Growth and maintenance of greenhouse salt-stressed soybeans. Soybeans (2 per pot) were planted in a 2:2:1 (topsoil, perlite, peat) mix and grown in 6 inch pots under standard greenhouse conditions for 9 weeks, after which test pots were treated with 100 ml NaCl soln three times per week for an additional 7 weeks. Leaf samples were harvested at appropriate dates and dried at 45°.

Determination of trigonelline concentration by HPLC. Dried leaf tissue (2 g) was boiled in 50 ml MeOH for 2–3 min, and the extract was treated with 80 and 40% aq. MeOH. The MeOH soln was extracted (×2) with $CHCl_3$ to remove lipids. The aq. MeOH was condensed to 2 ml and subjected to rep. TLC on 5 silica gel UV plates (250 μm, (Analtech, Newark, DE); 150 μl of sample/plate, Me_2CO–H_2O, 1:1). Trigonelline bands (located under UV) were eluted with 95% aq. MeOH vortexed and centrifuged. The eluate was Millpore filtered and evapd to dryness. The extracts were taken up in 200 μl MeOH–H_2O (1:1) and trigonelline was estimated by HPLC [Perkin Elmer/Analytical C-18 column, using MeOH–H_2O (1:1) as mobile phase (1 ml min^{-1})].

Determination of mitotic index in pea root meristems exposed to various osmoregulators. Pisum sativum seeds were surface-sterilized with undiluted Clorox, stirred for 10 min, washed with sterile water and germinated in sterile vermiculite for 3 days. Under aseptic conditions, roots were excised and cultured in 50 ml White's medium for 24 hr. Roots were fixed in 95% EtOH–HOAc (3:1) for 30 min and transferred to 70% EtOH. The roots were then hydrolysed with 5 N HCl for 20 min and stained with Feulgen. The roots were squashed on to slides, and run through an alcohol series and cover slipped with Canada Balsam. At least three slides per treatment, 1000 cells per slide, were scored for mitotic index.

Determination of relative DNA content in pea root meristem exposed to various osmoregulators. Similar culture procedures as for the determination of mitotic

index were used. However, roots were first grown in 50 ml White's medium with sucrose for 3 days, then transferred to 50 ml White's medium without sucrose for an additional 3 days. Roots were harvested, Feulgen stained and prepd for scoring. DNA measurements were obtained via microfluorimetry.

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