Biochemistry (Moscow), Vol. 67, No. 4, 2002, pp. 491-497. Translated from Biokhimiya, Vol. 67, No. 4, 2002, pp. 587-594.
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Role of Ca^{2+} in Drought Stress Signaling in Wheat Seedlings

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Received April 23, 2001
Revision received September 27, 2001

Abstract—Plants use complex signal transduction pathways to perceive and react to various biotic and/or abiotic stresses. As a consequence of this signaling, plants can modify their metabolism to adapt themselves to new conditions. One such change is the accumulation of proline in response to drought and salinity stresses. We have studied drought and salinity induced proline accumulation and the roles of Ca^{2+} (10 mM) and indoleacetic acid (IAA, 0.3 mM) in this response. Subjecting seedlings to both drought (6% polyethylene glycol, PEG) and salinity (150 mM NaCl) stress resulted in a dramatic increase in proline accumulation (7-fold higher than control level). However, the application of Ca^{2+} along with these stress factors had different effects. Unlike the salinity stress, Ca^{2+} prevented the drought induced proline accumulation indicating that these stress factors use distinct signaling pathways to induce similar responses. Experiments with IAA and EGTA (10 mM) supported this interpretation and suggested that Ca^{2+} and auxin participate in signaling mechanisms of drought-induced proline accumulation. Drought and salt stress-induced proline accumulation was compared on salt resistant (cv. Gerek 79) and salt sensitive (cv. Bezostaya) wheat varieties. Although proline level of the first was twofold lower than that of the second in control, relative proline accumulation was dramatically higher in the case of the salt resistant wheat variety under stress conditions.

Key words: stress, salinity, drought, signal transduction, proline accumulation, Ca^{2+}

Approximately half of the irrigated soils in the world are under salinity and drought stresses, and these are the major constraints limiting crop production. Since osmotic stresses are a major abiotic factors affecting crop production, attention has been focused on elucidation of gene expression related to osmotic stress, particularly genes involved in proline biosynthesis, with the ultimate goal of producing osmotic stress tolerant transgenic plants [1, 2].

Osmotic stress severely limits plant growth and agricultural productivity [2]. In response to drought and salinity stress, plants accumulate high levels of proline [3-5]. It is thought that accumulation of proline is achieved through induction of a gene encoding Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) [6] and repression of the proline oxidase gene (*POX*) [1, 7]. Accumulated proline is rapidly oxidized to glutamate when stress conditions are reverted. It is thought that proline accumulation has a key role during adaptation to stress conditions [6, 7]. However, the lack of correlation between proline level and osmotic tolerance in some plant species suggested that proline accumulation is simply a consequence of stress and does not provide salt tolerance [8]. Proline accumulation was also observed in plants exposed to UV irradiation, and a protective role of proline against UV irradiation was speculated [9].

When investigating stress, perceiving and transmitting stress signals, primary metabolic changes, changes in gene expression, and secondary metabolic responses due to the changes in gene expressions have to be taken into consideration [10]. If we consider proline accumulation as a clear response to stress conditions, it can be speculated that this might occur through transmitting signals to genome and induction of the *P5CS* gene. Indeed a mutant *Arabidopsis thaliana* (*SOS1*) which is hypersensitive to salt stress accumulating ten times higher amount of proline compared to wild type has been identified [11].

Since plants cannot escape from environmental stresses (osmotic stress, heat, anoxia, oxidative stress, mechanical injuries), they need a mechanism to identify those abiotic effects and respond to them. Most of those mechanisms are related to intracellular Ca^{2+} concentration. Changes in cytosolic Ca^{2+} concentrations are accomplished through mobilization of Ca^{2+} from outside of the cell and from intracellular stocks (ER, vacuoles) to cytosol. Changes in cytosolic Ca^{2+} concentrations produce signal related to defense and adaptations to environmental conditions through involvement of calmodulin,

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Ca^{2+} dependent kinases, and other Ca^{2+} dependent proteins [12, 13], and this signal causes high expression of stress induced and defense response genes [14].

Drought and salt sensing receptors have not been identified in plants yet, but they might be similar to osmotic sensors in yeast [15]. To understand which signal transduction pathways are operating, it might be useful to elucidate the role of Ca^{2+} as a second messenger in molecular regulatory mechanism and important effector enzymes such as protein kinases and protein phosphatases.

In this study, the role Ca^{2+}, EGTA, and IAA on signal transduction during salt and drought stresses in wheat have been investigated.

MATERIALS AND METHODS

Plant material. *Triticum aestivum* L. cv. Gerek 79 and cv. Bezostaya seeds were obtained from the Kayseri Agricultural Extension Center.

Experimental setup. Salt (NaCl) and drought (PEG) resistance of wheat cultivars was determined under laboratory conditions [16]. Seeds were grouped according to experimental setup, keeping one group as a control, and germinated on Petri plates. On the seventh day of germination, seedlings were treated with stress factors (150 mM NaCl solution or 6% PEG for drought) and other agents (10 mM EGTA, 10 mM Ca^{2+}, 300 μM IAA). The amount of proline was determined in 0.25 g samples taken before stress and 24, 48, and 72 h after stress. In some experiments, after the treatment with stress factors seedlings were reversed to normal conditions and the amount of proline was determined one day later. In addition, samples were taken from all experimental groups and dried in an oven to constant weight. Samples were extracted with 3% sulfosalicylic acid, and proline level was determined according to Bates et al. [17].

Statistical tests were done using the Minitab program. Student's *t*-test was used to compare mean differences among the groups. Mean difference was considered statistically significant at $p < 0.1$.

RESULTS

The level of proline in experiments with 150 mM NaCl and 6% PEG depending on the time of treatment is shown in Fig. 1. The proline level varied between 67 and 84 μmol/g dry weight in the control group. The proline level started to increase 24 h after the beginning of stress treatment and continued to increase during the next 48 h of treatment approaching 290 and 395 μmol/g dry weight for salt and drought treatment, respectively. The proline level started to decrease 24 h after reversion to normal conditions (Table 1) showing a tendency to return to control values 48 h after reversion.

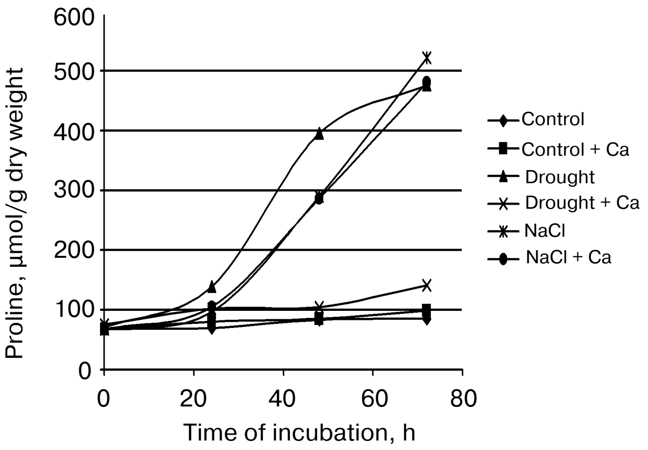

Fig. 1. Effect of Ca^{2+} (10 mM) on proline accumulation in wheat seedlings (Gerek 79 cv.) under salt (150 mM NaCl) and drought (6% PEG) stresses. All treatments were applied at time zero.

The results of this experiment indicated that the level of proline could be a good monitor of salt and drought stresses in wheat. In this way, wheat can be used as an appropriate experimental system for investigating several factors operating during stress conditions.

After establishing a reliable experimental setup for studying the effect of stress, 10 mM Ca^{2+} was added to each experimental group to investigate the role of Ca^{2+} in signal transduction during stress conditions. The results are presented in Fig. 1. In these serial experiments sampling was not done before stress treatment and the stress condition was removed after 48 h. As shown by the data, there was no significant change in proline level of control group treated with Ca^{2+}, but there was an increase in the proline level of salt treated group both in the presence and absence of Ca^{2+}. However, application of drought treatment (6% PEG) in the presence of Ca^{2+} decreased the proline level close to the control group value.

As seen from Table 1, when EGTA (chelating agent for Ca^{2+}) was applied to control group, there was no significant change in proline level. However, when EGTA was applied together with drought stress, proline level was slightly higher than in the case of drought treatment alone (5.6- and 4.7-fold increase compared to control). The presence of Ca^{2+} during drought stress significantly decreased the level of proline accumulation.

Salt stress significantly (3.8-fold) increased proline content compared to control, neither Ca^{2+} nor EGTA having significant effect on proline accumulation under these conditions. These data support the previous results and show the lesser effect of Ca^{2+} on signal transduction

Table 1. Effects of Ca^{2+} and EGTA on the proline accumulation (μmol/g dry weight) in wheat seedlings (Gerek 79 cv.) under salt and drought stresses

Treatment	Treatment interval, h			
	0	24	48	Reversion to normal conditions[1]
Control	80.74 ± 4.39	80.82 ± 1.25	83.00 ± 0.41	83.25 ± 0.83
Control + EGTA	76.08 ± 2.19	87.45 ± 1.77	91.82 ± 2.65	95.84 ± 2.21
Drought	79.18 ± 2.2	157.82 ± 8.07*	371.54 ± 7.44*	293.2 ± 18.5*
Drought + Ca^{2+}	84.47 ± 0.87	135.4 ± 7.98*	151.0 ± 9.62*	128.0 ± 1.8*
Drought + EGTA	81.35 ± 5.27	163.96 ± 5.27**	454.1 ± 22.7*	363.1 ± 7.0*
Salt	84.46 ± 0.87	104.64 ± 0.63*	254.0 ± 3.73*	230.12 ± 6.64*
Salt + Ca^{2+}	80.74 ± 0.0	103.44 ± 1.63*	227.9 ± 3.51*	218.73 ± 8.73*
Salt + EGTA	77.64 ± 4.38	116.63 ± 1.18*	303.5 ± 22.6*	288.6 ± 18.1*

Note: Here and in Tables 2-4, * $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

[1] Content of proline was measured 24 h after the removal of the stress factor.

during salt stress. There was some decrease in proline accumulation in all treatments 24 h after removing the stress conditions.

Based on the literature on Ca^{2+} and auxin interactions and their effect on gene expression, another series of experiments was designed to investigate the effect of drought stress, IAA, and EGTA on proline accumulation. In this particular series of experiments reversion to normal conditions was not performed but stress treatment was continued up to 72 h. As seen from the data, IAA decreases the effect of drought stress on proline accumulation only at 48 h (Table 2). The results presented in Table 2 are obtained using dry tissue samples. But, when fresh tissue was used for proline measurements, the effect of IAA on drought stress-induced proline accumulation was more clearly demonstrated (data not presented). In combined application of IAA and EGTA under drought stress, proline accumulation is closer to the level of drought treatment alone. No positive effect on proline accumulation was observed in combined application of IAA and EGTA under the salt stress. Although the effect of combined application of IAA and EGTA under salt stress was not manifested during 48 h of incubation, there was a considerable increase in proline level 72 h after the beginning of treatment. In addition, there was a significant increase in proline content compared to control in drought and also in the combined application of IAA and EGTA under drought stress 72 h after the beginning of treatment. All the values presented so far were obtained using dry tissue samples. When calculations were done

Table 2. Effects of IAA and EGTA on the proline accumulation (μmol/g dry weight) in wheat seedlings (Gerek 79 cv.) under salt and drought stresses

Treatment	Treatment interval, h			
	0	24	48	72
Control	79.19 ± 6.59	87.88 ± 1.32	88.2 ± 0.0	92.85 ± 4.82
Drought	83.22 ± 3.51	185.67 ± 9.38*	384.61 ± 2.52**	459.85 ± 2.99**
Drought + IAA	85.32 ± 2.23	202.1 ± 7.82*	270.8 ± 29.0*	467.42 ± 7.46**
Drought + IAA + EGTA	74.52 ± 4.39	180.82 ± 2.41*	364.39 ± 6.01*	617.1 ± 43.7*
NaCl	76.08 ± 2.19	116.5 ± 0.88*	280.22 ± 4.08*	497.85 ± 33.7*
NaCl + IAA	79.18 ± 2.2	156.26 ± 9.21*	256.0 ± 5.4*	538.3 ± 23.6*
NaCl + IAA + EGTA	69.87 ± 2.19	141.6 ± 27.4	295.46 ± 9.45*	738.3 ± 38.4*

Table 3. Effects of salt and drought stress degree on the proline accumulation (μmol/g dry weight) in Gerek 79 cv. seedlings

Treatment	Treatment interval, h			
	0	24	48	72
Control	85.08 ± 8.12	100.07 ± 2.49*	105.68 ± 7.89*	110.25 ± 4.58*
Drought (3% PEG)	86.81 ± 9.34	134.8 ± 20.2*	169.2 ± 11.8**	205.8 ± 12.7**
Drought (6% PEG)	81.65 ± 4.83	170.44 ± 6.05***	351.38 ± 9.78***	433.77 ± 2.52****
Drought (9% PEG)	83.62 ± 9.63	224.8 ± 7.65***	727.5 ± 24.1***	795.0 ± 29.6***
75 mM NaCl	80.08 ± 7.98	110.7 ± 10.7*	195.79 ± 9.07***	380.6 ± 28.6**
150 mM NaCl	90.59 ± 4.96	133.9 ± 15.6*	255.7 ± 14.3**	439.83 ± 3.36****
300 mM NaCl	92.88 ± 3.17	161.0 ± 13.9*	276.8 ± 11.0**	450.46 ± 8.22***

per 1 g of fresh tissue, changes in proline content were more expressed.

To determine the effect of the degree of drought and salt stress on proline accumulation, three different levels of drought (3, 6, and 9% PEG) and three different levels of salt stress (75, 150, and 300 mM NaCl) were applied. The results are shown in Table 3. As can be seen from the table, when the level of stress increased, proline accumulation was also increased correspondingly. Lower proline accumulation in salt stress treated plants compared to drought stressed ones was interesting, this obviously being due to weakness of salt stress for wheat. The proline level obtained at 300 mM NaCl treatment was close to that at mid drought treatment (6% PEG). The level of proline was much higher at 9% PEG treatment (10-fold increase compared to 5-fold increase at 300 mM NaCl treatment).

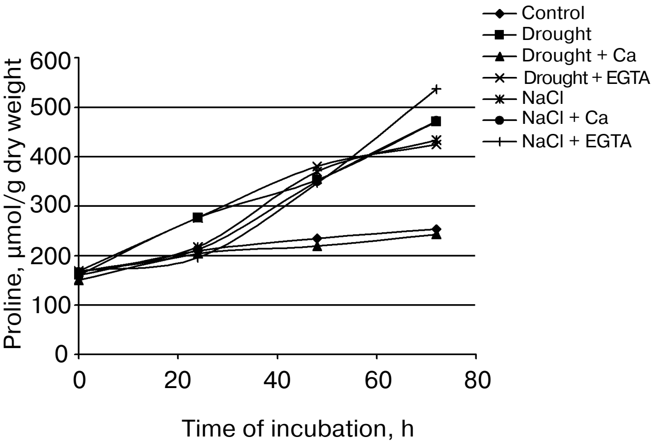

Fig. 2. Effect of Ca^{2+} (10 mM) and EGTA (10 mM) on proline accumulation in wheat seedlings (Bezostaya cv.) under salt (150 mM NaCl) and drought (6% PEG) stresses. All treatments were applied at time zero.

Beside relatively salt resistant Gerek 79 variety, the same experiments were also carried out using relatively salt sensitive Bezostaya variety. The results are shown in Fig. 2 and Table 4. As seen from Fig. 2 and Table 4, the proline level in the control group of Bezostaya variety was slightly higher than that in the control group of Gerek 79 variety and showed a slight increase in the course of experiment. An increase in proline level due to drought effect was also observed at 72 h in Bezostaya variety (3-fold), but the increase was not as big as in Gerek 79. The presence of Ca^{2+} during drought stress decreased the proline content close to the level of the control group. The presence of EGTA during drought stress did not increase the proline level significantly compared to that in drought stress alone.

The highest levels of proline accumulation in Bezostaya variety were observed in salt treatment and combined application of EGTA and Ca^{2+} during the salt stress. However, in contrast to drought stress, the presence of Ca^{2+} during the salt stress did not decrease the proline accumulation. The presence of EGTA during the salt stress increased the proline level compared to the salt treatment alone.

Another series of experiments was designed to investigate the interaction of drought and salt stresses with IAA and EGTA in Bezostaya variety (Table 4). Interestingly, proline level was decreased in the presence of IAA during drought compared to drought treatment alone, but that was not the case for salt stress.

Salt resistant (Gerek 79 cv.) and salt sensitive (Bezostaya cv.) varieties clearly differed by proline levels in control groups and in groups subjected to stress. Although basal proline level of the salt resistant variety was lower than that of the salt sensitive one, relative stress induced proline accumulation was higher in salt resistant variety (Tables 2 and 4).

Table 4. Effects of IAA and EGTA on proline accumulation (μmol/g dry weight) in wheat seedlings (Bezostaya cv.) under salt and drought stresses

Treatment	Treatment interval, h			
	0	24	48	72
Control	140.4 ± 21.2	205.19 ± 4.4*	224.8 ± 12.4**	240.4 ± 12.4**
Drought	126.12 ± 1.68	274.96 ± 7.62***	316.29 ± 2.03****	518.1 ± 17.7***
Drought + IAA	139.6 ± 11.5	229.64 ± 7.9**	263.9 ± 23.51*	408.06 ± 1.36***
Drought + IAA + EGTA	146.99 ± 7.17	256.77 ± 9.44***	267.51 ± 2.49**	517.7 ± 23.9**
NaCl	143.9 ± 22.5	228.52 ± 4.89*	395.6 ± 26.8**	442.84 ± 5.48**
NaCl + IAA	154.86 ± 5.6	224.5 ± 14.8*	381.8 ± 10.1***	430.46 ± 0.49***
NaCl + IAA + EGTA	153.41 ± 3.78	188.1 ± 13.8*	404.9 ± 10.6***	539.21 ± 3.84****

DISCUSSION

Although a vast amount of research is done on the adaptive physiological and molecular responses of plants to osmotic stress, knowledge about molecular mechanism that operates in the signal transduction pathway of cellular response to osmotic stress is still quite limited [18-21].

Many genes that are regulated by salt stress are also responsive to drought or cold stress [22]. Because salt stress can be applied accurately and reproducibly, many drought stress studies in the laboratory use salt stress instead of actual drought [23].

In this study, accumulation of proline under drought stress mediated by 6% PEG solution and salt stress mediated by 150 mM NaCl on Gerek 79 wheat seedlings (starting from the 7-th day) has been investigated. High level of proline accumulation was observed in stress treated seedlings (Fig. 1).

Based on the literature, a higher proline accumulation in a salt resistant wheat variety compared to a salt sensitive one might be expected [7], and the data fulfilled this expectation. Although basal proline level of the salt resistant variety (Gerek 79 cv., Table 2) was lower then that of the salt sensitive one (Bezostaya cv., Table 4), salt stress induced proline accumulation was dramatically higher in the case of the salt resistant variety.

Effects of Ca^{2+} and EGTA during drought or salt stress depended significantly on combination of these factors (Fig. 1). Proline accumulation was not significantly different from control when EGTA was used alone. Although drought stress increased the proline accumulation considerably (3-fold at 72 h), in the presence of Ca^{2+} this effect was not manifested. That means that 10 mM Ca^{2+} prevents the drought stress induced proline accumulation. It can be speculated that Ca^{2+} level in cytoplasm is decreased in drought stress and exogenous Ca^{2+} might be repressing the mechanism triggering the proline biosynthesis. The results obtained from EGTA treatments support this hypothesis. However, these results are controversial with respect to some literature findings. For instance, induction by mannitol of the *P5CS* gene encoding Δ^1-pyrroline-5-carboxylate synthetase, which is the key enzyme of proline biosynthesis, is inhibited by Ca^{2+} channel blockers gadolinium and verapamil and also by EGTA. Though, these experiments were carried out with *A. thaliana* [14].

The situation was somewhat different in salt stress treatment. A large increase in proline accumulation, although lower compared to drought treatment, was observed in salt treatments alone. This observation is in agreement with the literature data showing that the induction of *P5CS* during the drought is higher than during the salt stress [14].

We showed that the presence of Ca^{2+} during the salt stress did not influence proline level. These findings support the existence of two different signal transduction pathways for salt and drought stresses and suggest that salt induced signal transduction pathway for proline accumulation might not be Ca^{2+} dependent. Obtaining similar results in combined application of salt stress and EGTA supports the previous results, thus indicating the existence of a Ca^{2+} independent signal transduction pathway for proline accumulation during salt stress. However, it is known that salt stress induced repression of root growth and DNA fragmentation in barley seedlings were prevented by Ca^{2+} [24]. There are other data indicating the protective role of Ca^{2+} in salt stress [25].

Na^+ stress is known to cause Ca^{2+} depletion in the extracellular space and on the outer surface of the cytoplasmic membrane [23]. External Ca^{2+} enhances plant salt tolerance [23]. The *SOS* genes have been proposed to be involved in mediating the beneficial effect of Ca^{2+} [26, 27]. The *SOS3* gene encodes a Ca^{2+} binding protein with three predicted EF-hands [26], which is similar to the β-subunit of calcineurin [28] and animal neuronal sensors

[29]. The *SOS2* gene encodes a serine-threonine protein kinase [30].

Induction of the *P5CS* gene [6, 7, 11] and corresponding repression of the *POX* gene [1] by transmitting stress signals to the genome is a crucial step for proline accumulation.

It is thought that abscisic acid (ABA) has a role in the signal transduction mechanism during stress [31]. Tissue specific expression of the *P5CS* gene in *A. thaliana* is induced by drought, cold, ABA, and to a lesser extent by auxins [32]. However, it is known that the polypeptide products of the *AXR1* and *AXR2* genes could play a role both in the auxin and drought response pathways. Auxin dependent drought stress response in *A. thaliana* is disrupted when a mutation occurs in the *AXR1* gene [33]. Protein phosphorylation is involved in the signal transduction pathways for the cellular response to dehydration. In addition, it is postulated that Ca^{2+} dependent protein kinase is involved in the ABA independent signal transduction pathway in *A. thaliana* [34].

ABA independent induction of the *RPK1* gene by drought stress indicates that the receptor-like protein kinases encoded by the *RPK1* gene may also be involved in an ABA independent dehydration pathway [21].

It is known that binding of auxins to their cytoplasmic receptors and their transfer to nucleus and transcription of related genes are mediated by Ca^{2+} [35]. In our experiments, the presence of IAA during drought stress decreased proline content in Bezostaya variety to the level observed during drought stress in the presence of EGTA. These results suggest the involvement of IAA and Ca^{2+} during stress induced proline accumulation.

The simultaneous presence of IAA and EGTA during drought stress did not affect significantly proline accumulation. These data support the results obtained in the presence of Ca^{2+}, since binding of IAA to its specific receptors depends on Ca^{2+} mediated phosphorylation of those receptors by protein kinase [35].

Although our results are clearly repeatable, there is some controversy with the literature. The ineffectiveness of Ca^{2+} on salt stress induced proline accumulation shown in our experiments is one of the controversial results. Some literature especially shows the role of Ca^{2+} in salt stress in *A. thaliana* [12-14]. Positive correlation of decreased Ca^{2+} with proline accumulation in the marine green microalgae *Ulva fasciata* via inhibition of proline dehydrogenase (PDH) activity was also demonstrated [36]. This finding shows that Ca^{2+} might induce the activity of *POX* (PDH), which supports our data.

Although we have preliminary results about the effect of Ca^{2+} on stress related signal transduction pathways, it needs to be elucidated what triggers the changes of Ca^{2+} concentration in cells regulating *POX* activity—drought stress or proline concentration increase.

Since stress induced gene expression in plants is very rapid, clear, and reproducible [37], which was also observed in our experiments, this experimental system could be used as a model to study stress related signal transduction pathways and the factors affecting them.

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