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Short Communication

Influence of Water Stress on the Vacuole/Extravacuole Distribution of Proline in Protoplasts of *Nicotiana rustica*

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ABSTRACT

Tobacco (*Nicotiana rustica*) plants were stressed by addition of polyethylene glycol solution (−20 bar) to the growth medium. The proline contents and concentrations in total protoplasts, vacuoles, and extravacuolar fractions of these plants have been determined and compared with protoplasts and cell fractions of well-watered plants. As compared to the control, the stress treatment of intact plants results in a 7-fold increase of the proline content in the extravacuolar fraction while the vacuolar content was enriched only 2.6-fold. In protoplasts of control plants, a proline concentration ratio (extravacuole to vacuole) of 1 was measured. In protoplasts of stressed plants, this ratio was nearly 3. Thus, water stress seems to have an effect on a tonoplast-localized transport system for proline.

It has been shown that proline and other amino acids are compartmentalized in higher plant cells. Major portions of these compounds are located in the vacuole (4, 7, 8, 11, 15, 16). These vacuolated fractions of the amino acids are separated from the cytoplasmic metabolism. Therefore, the existence of a proline pool in the vacuole cannot be ignored when working on the mechanism of stress-induced proline accumulation.

Stress-induced proline accumulation results from altered rates of biosynthesis and degradation of this amino acid (2, 14). But rates of synthesis and degradation are also functions of metabolically available pool sizes (9, 10). To get insight into the mechanisms of stress effects, it is necessary to look not only at the respective enzyme activities but also at the metabolically effective concentrations of precursors and products.

In this paper, we present results which show that water stress applied to *Nicotiana* plants changes considerably the vacuolar/extravacuolar concentrations of proline.

MATERIALS AND METHODS

Nicotiana rustica was grown in a greenhouse (photoperiod 12 h, 23–25°C). The plants were harvested at an age of 90 d. Only healthy leaves were used for protoplast isolations. Water-stress was applied by keeping the plants for varying time spans in PEG solutions (PEG 20,000, −20 bar, according to Steuter *et al.* [13]). Protoplasts and vacuoles were isolated according to the procedure of Saunders (12). Counting and measurements of the diameters of protoplasts and vacuoles (stained with neutral red) occurred in a counting chamber. For volume calculations, the relation $v = d^3\pi/6$ was used.

Proline was determined with the procedure of Bates *et al.* (3). The water potential was measured with the isopiestic method (5).

RESULTS AND DISCUSSION

Starting with 3 to 4 g of leaf material, we isolated 9×10^6 to 2×10^7 protoplasts. From these protoplasts, 4×10^5 to 3×10^6 vacuoles were isolated. Assuming one vacuole per protoplast, this amount resembles up to approximately 15% of total vacuoles. For the calculation, only vacuoles with a diameter of ≥ 15 μm were regarded. Based on ten determinations, we obtained the volumes of protoplasts ($v = 1.23 \times 10^{-4}$ μl, 100%), vacuoles ($v = 0.87 \times 10^{-4}$ μl, 71%), and the cytoplasm ($v = 0.36 \times 10^{-4}$ μl, 29%). These volumes were used to calculate the proline concentrations in the protoplasts and the cell fractions (Table I). The stress treatment increases the proline content of the protoplasts (Table I). This finding was expected and is in agreement with reports on proline accumulation in intact water-stressed plants. Furthermore, the table shows that proline accumulates to higher amounts in the extravacuolar fraction (cytosol) than in the vacuole itself. The proline content in the control samples is significantly higher in the vacuoles as compared to the cytoplasm (71% to 29%). In stressed protoplasts, however, the extravacuolar fraction of proline outweighs that of the vacuolar content (54% to 46%).

Using the concentrations as a measure (right side of Table I) the picture is somewhat different. The extravacuolar/vacuolar proline concentrations are close to a ratio of 1 in the control samples. This ratio is shifted towards higher values in the course of stress treatment. The concentration of proline increases in the cytoplasm more rapidly than in the vacuole.

From these data, it might be concluded that a tonoplast-localized transport system for proline exists which is altered with increasing water stress. It is worth noting that proline accumulation in *Staphylococcus aureus* at low water potential is primarily by transport (1). The salt-stimulated proline accumulation is thought to require metabolic energy (6) while the efflux is none-nergy dependent (6). As yet, it is not known whether comparable transport systems are functioning at the cytoplasmic/extra-cytoplasmic membrane interfaces of higher plants. Irrespective of transport mechanisms, these stress-induced shifts of the intracellular pools of proline indicate that the rates of proline metabolizing sequences should also be changed in accordance with the respective concentrations. Translocation of proline has to be considered an important event in proline metabolism.

The isolation of protoplast from leaves imposes a strong stress on the cells. We have measured a water potential of −22 bar in the isolation medium. Therefore, our 'control' should represent stressed protoplasts.

Table I. *Proline Contents and Concentrations of Protoplasts Vacuoles, and Extravacuolar Fractions (Cytoplasm) from Water-Stressed and Well-Watered Plants*

The values refer to 10^6 protoplasts and vacuoles ($n = 4$).

	Proline Content			Proline Conc.			
	Protoplasts	Vacuoles	Extravacuolar fraction	Protoplasts	Vacuoles	Extravacuolar fraction	Extravacuole/Vacuole
		$\mu M \times 10^2$			$\mu M\ l^{-1} \times 10^4$		
Control Exp.	4.26	3.13	1.13	3.46	3.6	3.14	0.87
	5.56	4.00	1.56	4.52	4.59	4.34	0.95
	6.17	4.34	1.83	5.02	4.99	5.07	1.02
	7.04	4.87	2.17	5.72	5.59	6.03	1.08
Mean value	5.76 (100%)	4.09 (71%)[b]	1.67 (29%)[b]	4.68	4.92	4.65	0.98
12 h stress	7.65	5.21	2.44	6.22	5.99	6.76	1.13
	8.25	5.65	2.60	6.71	6.49	7.24	1.22
	8.43	5.91	2.52	6.85	6.79	7.00	1.03
	8.95	5.99	2.96	7.28	6.89	8.21	1.19
Mean value	8.32 (144%)[a]	5.69 (139%)[a] (68%)[b]	2.63 (157%)[a] (32%)[b]	6.77	6.54	7.30	1.12
20 h stress	23.11	9.21	13.90	18.79	10.59	38.61	3.65
	23.11	9.30	13.81	18.79	10.69	38.37	3.59
	23.11	10.60	12.57	18.79	12.18	34.75	2.85
	23.11	13.55	9.56	18.79	15.58	26.55	1.70
Mean value	23.11 (401%)[a]	10.67 (261%)[a] (46%)[b]	12.46 (747%)[a] (54%)[b]	18.79	12.26	34.57	2.95

[a] As compared to the control.

[b] % of total proline content of protoplasts.

Thus, 'experimental stress' might explain the small differences between the control and 12-h stressed plants. In fact, leaf discs which were incubated in 0.7 M mannitol for 4 h (the time used to digest cell walls in the experiments) increased their proline content by a factor of 2.3. Nevertheless, the results shown in Table I indicate that the intracellular distribution of proline depends on the stress duration. Transport phenomena have to be imposed on models which try to explain the mechanism of proline accumulation. At least three compartments have to be considered important: the cytoplasm (synthesis), the mitochondria (degradation), and the vacuole (deposition). As yet, knowledge is missing concerning stress-dependent changes of mitochondrial, tonoplast, (and plasmalemma) proline transport systems.

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