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SIMULATED HYPOGRAVITY AND PROLINE INCORPORATION INTO SALT-EXTRACTABLE MACROMOLECULES FROM CELL WALLS

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Abstract—Proline [U-^{14}C] was fed to shoots of intact *Tagetes patula* grown normally, on horizontal clinostats, or on vertical clinostats rotating at 15 rev/hr. After various periods the incorporation of ^{14}C into salt-extractable material from the cell walls of stems, petioles, leaves and flowers was determined. The cell walls of the gravity-compensated plants (grown on horizontal clinostats) has the highest amount of salt-extractable radioactivity. A 2- to 9-fold increase was observed in comparison to either the normal or vertical clinostat plant controls. Some physico-chemical properties of the salt-extractable fraction suggest that it consists of highly charged, low MW entities, possibly short chain peptides. On acid hydrolysis this material yields radioactive aspartic acid, glutamic acid and proline. The presence of labelled hydroxyproline is suggested. After acid hydrolysis of the cell walls of leaves, it was found that *ca* 4 times the amount of ^{14}C was incorporated in the hypogravity-grown plant compared to the controls. It appears likely that extensibility changes in tissues under simulated hypogravity required additional cell wall protein.

INTRODUCTION

The biosynthesis of the protein component of the primary cell wall in higher plants has been under intensive investigation for a number of years [1]. The presence of a cell wall protein precursor which can be extracted from the cell wall by the use of salt solutions has been well-documented [2–4]. The kinetics of formation of this precursor has the typical characteristics of a component in transit [4].

When plants are rotated horizontally on clinostats a typical response is the appearance of epinastic curvatures of the leaves. A similar response was observed under hypogravity conditions on orbital flights [5]. It appears reasonable that during epinasty, changes in extensibility would occur in the leaf or petiole cell wall, and therefore a biochemical change correlated with this phenomenon would be an increase in the amount of cell wall protein. The present report gives the results of a study comparing the synthesis of salt-extractable cell wall macromolecules in normal plants of *Tagetes patula* with those on a horizontal clinostat under simulated hypogravity. These macromolecules could be precursors of new cell wall protein.

RESULTS

The stems and leaves of *T. patula* exhibited typical epinastic curvatures when plants were subjected to simulated hypogravity by rotation on horizontal clinostats. After infiltration with proline-[^{14}C] the radioactivity in the salt-extractable macromolecules (SEM) in the cell walls from shoots of 68-day-old plants subjected to hypogravity amounted to 192 000 cpm per g fr. wt as compared to 57 500 in shoots of normal control plants, about a 3-fold increase. The amount of radioactivity in the SEM of control plants and plants rotated on vertical clinostats (47 000 cpm/g fr.wt) was essentially the same.

The incorporation of radioactivity into the SEM of horizontal plants was higher in every tissue than the corresponding tissue of control plants (Table 1). Differences of 2- to 9-fold between the two treatments were found depending on the particular tissue. Differences between treatments tend to be more marked in tissues from older plants compared to younger plants. This is evidenced by a comparison of the data in Tables 1 and 2.

The minimum time necessary for hypogravity to exert its effect on the SEM appears to be 36 hr (Table 2). In shorter times, e.g. 24 hr, the SEM in horizontal plants is only slightly higher if at all than in the control. After a presentation time of 36 hr, however, the effect of hypogravity is evident and all tissues of the plants on horizontal clinostats show considerably higher amounts of SEM than those of the controls.

After acid hydrolysis of the cell walls of the leaves, the amount of label recovered was *ca* 4 times greater

Table 1. ^{14}C incorporation into SEM in different plant tissues. 50-day-old *T. patula* plants were infiltrated with L-proline [U-^{14}C] for 68 hr as described in 'experimental' and SEM was isolated from each tissue.

Treatment	^{14}C Incorporation into SEM (cpm × 10^{-4}/g fr. wt)			
	Stem	Petiole	Leaf	Flower
Normal control	7.4	1.4	Trace	0.3
Horizontal clinostat	31.9	3.5	0.8	1.9

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Table 2. Incorporation of ^{14}C from L-proline [^{14}C] into SEM as a function of incubation time

Incubation time (hr)	^{14}C Incorporated (cpm × 10^3/g fr. wt)					
	Stem		Petiole		Leaf	
	Control	Horizontal clinostat	Control	Horizontal clinostat	Control	Horizontal clinostat
24	10.3	9.5	14.2	18.7	10.3	18.7
36	15.0	53.3	14.5	347	16.6	51.1

28-day-old *T. patula* plants were infiltrated with L-proline [U-^{14}C] as described in 'Experimental'. SEM was isolated from the specific tissue as described in the text

from plants under hypogravity than the corresponding tissue of control plants. In an experiment with 28-day-old plants the recovery was 69 × 10^4 cpm/g fr. wt from normal plants compared to 284 × 10^4 cpm/g for plants on the horizontal clinostat.

Physicochemical properties of SEM

The salt extractable cell wall material binds firmly to a Dowex 50 W (H^+) column and is easily eluted by ethanolic ammonia. It migrates with the buffer front in disc gel electrophoresis. It is non-dialysable for the most part since only a trace of label appears in the medium. The labelled material, however, passes through an ultrafilter with a MW cut off at 1000. It is ninhydrin positive. Scanning of PC of the acid hydrolysate revealed the presence of labelled proline, aspartic acid, glutamic acid, and some indication of hydroxyproline.

DISCUSSION

The salt-extractable fraction has been considered to consist of protein precursors that are ionically bound to the cell walls [1, 2]. It presumably represents components in transit from the cytoplasm to the cell walls [4] where they are eventually incorporated into the structural protein matrix. The physico-chemical properties of the SEM were incompletely characterized in this study. However, based on its rapid electrophoretic mobility, its behaviour on ultrafilters, ion exchange chromatography and PC one may conclude that it consists of highly charged low MW entities, possibly short chain peptides. The low incorporation of ^{14}C into the hydroxyproline residues of the 'peptides' suggests that only a few proline molecules in these peptides were hydroxylated. In intact systems, hydroxyproline synthesis is not as rapid as in loose proliferating cells in suspension cultures [6].

Old tissues were more responsive than young tissues, possibly because polar transport of auxin in old tissues is more sluggish [7, 8] and hence the chances for lateral displacement of auxin by hypogravity are increased. Our data suggest that hypogravity induces the synthesis of SEM which may be cell wall protein precursors. This induction may be ethylene-mediated [9] since hypogravity enhances ethylene production [10]. Ethylene action may also involve increased permeability of the plasmalemma [11]. Thus the high amount of SEM in hypogravity tissues may be due not only to ethylene-mediated induction of protein synthesis but also to higher rate of diffusion of the SEM across the plasmalemma. Exactly how any newly synthesized proteins participate in the characteristic epinastic curvatures of gravity-compensated plants is a matter for conjecture. It is conceivable that new protein becomes bonded to polysaccharide chains, and such interaction might alter the microfibrillar arrangements of these polysaccharides so as to allow extensibility changes.

Apart from effects on ethylene production, hypogravity may effect epinastic curvatures through other auxin dependent processes such as: (a) activation of cell wall loosening enzymes: For example, Bara and Gordon [12], have reported an increase in cellulase activity when seedlings of *Helianthus annuus* were subjected to hypogravity. (b) stimulation of a H^+ pump on the plasmalemma [13, 14]: Secretion of H^+ from the cytoplasm to the cell wall leads to a lowered pH in the latter. The more acid pH may exert an effect directly on certain chemical bonds or indirectly by providing a more optimum pH [15] for the activity of certain enzymes. (c) osmotic effects: Hypogravity enhances the synthesis of starch and sugars [16] which results in an increased osmotic potential. The subsequent absorption of water would result in cell elongation.

Although the exact sequence of events resulting in epinastic curvature is yet to be clarified, it appears likely that the primary motive force is provided by either auxin transport, synthesis or a combination of both.

EXPERIMENTAL

Growth conditions. Seeds of *Tagetes patula* L. var. Helen Chapman A were sown in plastic pots filled with peat moss, sand and sawdust (1:1:1) in a greenhouse at 80°/70° day/night temp. and 16 hr day length. The pots were irrigated daily to field capacity with a modified half-strength Hoagland's soln I. The culture soln modification consisted of supplying no additional micronutrients and Fe was furnished in the chelated form 'Fe-138' at a concentration of 2 ppm. The ages of plants used for experiments ranged from 28–68 days.

Radioactive material. L-Proline [U-^{14}C] with a sp. act. of 200 mCi/mmol was supplied by International Chemical and Nuclear Corporation.

Incubation procedure. A modification of the cotton wick method described in ref. [17] was followed. In our method, the wick was threaded through capillary tubing which is directly inserted into the stem of the plants. This device reduces evaporative loss. Aliquots of the proline-[^{14}C] soln containing 5, 10 or 15 μCi were used. After the radioactive soln had been absorbed, the vial was rinsed twice with H_2O. Plants were then mounted on the horizontal or vertical clinostats. Control plants were left on the bench. The incubation period was 24, 36, 68 or 72 hr. The plants were under fluorescent light with an intensity of 20 to 40 μeinsteins/m^2/sec in the spectrum between 400 and 700 nm.

Isolation of cell walls and extraction of ionically bound wall material. Tissue were weighed and frozen in solid CO_2 and ground in H_2O by mortar and pestle. The homogenate was centrifuged at 120 *g* for 30 sec and the pellet was washed 10 × with H_2O. The cell walls were packed into columns and eluted with 0.125 M NaCl. Preliminary expts showed that this soln was a more effective extractant than a concn of 0.25 M or 0.50 M. The eluted material contained the SEM. Aliquots of the eluates were assayed for radioactivity in a liquid scintillation counter using Bray's soln [18].

Hydrolysis of cell wall material and SEM. Cell walls were hydrolysed with 6 M HCl in sealed tubes at 100° for 18 hr. Hydrolysate was centrifuged and the supernatant soln assayed for radioactivity. The SEM was hydrolysed with 6 M HCl and assayed by the same procedure described for the cell walls.

Chromatographic procedures. (a) *Ion-exchange methods.* The SEM was dialysed 18 hr against a large vol. of deionized H_2O in the cold. The internal dialysate and the external dialysis medium were each assayed for radioactivity. A portion of the former was placed on a column of Dowex 50 (H^+) 20–50 mesh (1 × 10 cm) and eluted with 80% EtOH followed by EtOH–NH_4OH (3:1). Aliquots of the eluate from each solvent were assayed for radioactivity. (b) PC. The remaining portion of the dialysed hydrolysate of the SEM was evaporated at 100° under a stream of air. Dried residue was dissolved in H_2O and dried again as before to remove HCl. The dried sample was then resuspended in H_2O, spotted on filter paper and irrigated with PhOH–H_2O (5:1). Chromatograms were treated with either 0.2% isatin in Me_2CO or 0.2% ninhydrin in 95% EtOH. After being heated at 100° for 10 min the spots obtained were compared with proline and hydroxyproline standards. The radioactive components of the chromatographed hydrolysate of SEM were located by use of a strip counter.

Ultrafiltration. The SEM was passed through an ultrafilter under a N_2 pressure of *ca* > 1.5 kg/cm². All compounds having a MW less than 1000 passed through the filter membrane.

Disc gel electrophoresis. Performed according to the procedure of ref. [19] and the protein bands visualized by use of Coomassie blue.

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