Plant Physiol. (1984) 75, 551–555
0032-0889/84/75/0551/05/$01.00/0

Induction of Aluminum Tolerance in Wheat Seedlings by Low Doses of Aluminum in the Nutrient Solution[1]

Received for publication October 11, 1983 and in revised form March 7, 1984

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ABSTRACT

Preincubation of wheat (*Triticum aestivum* L. Thell.) seedlings in a nutrient solution containing low doses of aluminum (0.5 microgram per milliliter for tolerant cultivar Atlas 66 and 0.1 microgram per milliliter for the sensitive cultivar Grana) enabled substantial root regrowth of varieties grown in a lethal aluminum concentration, despite an increased accumulation of aluminum in root tissue of the pretreated seedlings. The distribution of aluminum in the subcellular fractions remained unchanged. The increase in tolerance was completely abolished by the addition of cycloheximide. Aluminum ions at sublethal concentrations significantly increased the incorporation of [^{14}C]valine and [^{3}H]thymidine in roots. The possible role of the synthesis of the inducible aluminum binding protein in the mechanism of aluminum tolerance is discussed.

Aluminum ions are regarded as the main toxic factor in mineral acid soils of pH below 5.0 (9). Plants differ in their reaction to Al toxicity, and variability was found between plant species as well as between cultivars of cultivated plants (1, 7, 10). The plant reaction to Al was found to be genetically controlled (5, 23).

Studies have concentrated mainly on the mechanisms of Al toxicity, especially on the inhibitory effect of Al on root elongation. According to Matsumoto *et al.* (17) binding to nuclear DNA is a primary effect of Al in the plant cell. However, it is not certain whether disturbances of the uptake, transport, and utilization of mineral nutrients (6, 9) or the effect on oxidation processes (13) are the direct consequences of the decreased template activity of DNA. Still less is known about the mechanism of Al tolerance. Recent suggestions are summarized by Foy *et al.* (9).

The results presented in this paper suggest that there is induction of an Al-binding protein in the process of Al detoxication in plants.

MATERIALS AND METHODS

The inhibition of elongation of primary roots by Al has been used in various screening techniques for Al tolerance (12, 14, 18). The modified 'pulse' method was used for screening varieties and hybrid populations of wheat (2). The Al concentration in nutrient solution that caused irreversible damage of root apical meristem in 4-d-old seedlings during 24 h incubation at 25°C was used for differentiation of genotypes tested, and the same method was applied to further studies.

Plant Material. Two winter wheat (*Triticum aestivum* L. Thell.) cultivars, Al-tolerant Atlas 66 and sensitive Grana, were used. Seeds of Atlas 66 were obtained from the Department of Radiation Biology of our Institute while seeds of Grana cultivar were kindly supplied by Plant Breeding Station Choryń. Seeds were sterilized with 0.1% Hg_2Cl_2 aqueous solution for 10 min, rinsed excessively with water, and germinated overnight on filter paper in Petri dishes. Sprouted seeds were sown next day on polyethylene net fixed in lucite frames. Styrofoam blocks were attached to the frames with rubber bands and floated on the surface of vigorously aerated nutrient solution. Containers with nutrient solution were placed in water bath at 25°C under continuous incandescent light (12 w/m^2). Nutrient solution of the following composition was used: 0.4 mM $CaCl_2$; 0.65 mM KNO_3; 0.25 mM $MgCl_2 \cdot 6H_2O$; 0.01 mM $(NH_4)_2SO_4$ and 0.04 mM NH_4NO_3. Four-d-old seedlings were transfered to the same nutrient medium supplied with Al in the form of $AlKSO_4 \cdot 12H_2O$ at the concentration indicated in the experiments. After 24 h of incubation in the medium containing aluminum, seedlings were throughly washed for 2 to 3 min in running tap water and stained with 0.1% aqueous solution of Eriochrome cyanine R for 10 min. The excess dye was washed after staining with tap water. The seedlings after staining were transferred to the nutrient medium without Al for 48 h. Then the root regrowth after aluminum shock (or additional root growth) was easily assessed. The dye is nontoxic to roots at concentration and time of staining applied. During all stages of growth, and particularly during Al treatment, the nutrient solution was maintained at pH 4.0 ± 0.02 adjusted with 0.1 N HCl. At the ratio of approximately 20 ml of nutrient solution per seedling changes of pH of the medium did not exceed 0.02 during the 24 h of aluminum treatment. Root apical meristems of the sensitive cultivar Grana were irreversibly damaged at 4 μg/ml Al, while the same effect in tolerant Atlas 66 was observed only after incubation in 27 μg/ml Al.

Induction. The seedlings of both varieties were pretreated or pretested for 48 h at low levels of Al in the medium: 0.5 μg/ml and 0.1 μg/ml Al for Atlas 66 and Grana, respectively. After such pretreatment the seedlings were subjected to lethal doses of Al as assessed before. In addition, cycloheximide, an antibiotic which blocks protein synthesis, was added to the nutrient medium at concentration of 10 μg/ml for 6 to 24 h before or during pretreatment.

Protein and DNA Synthesis. DNA and protein syntheses in roots under Al stress were studied by measuring incorporation of [^{14}C]valine and [^{3}H]thymidine. Labeled valine and thymidine were added at concentrations of 2.5 μCi/ml (4 μg/ml) and 10 μCi/ml (10 μg/ml), respectively, to the nutrient solution and seedlings were incubated in this medium for 24 h at 25°C, the

[1] Supported by United States Department of Agriculture Agricultural Research Service, University of Missouri, Columbia, is gratefully acknowledged.

same as in the 'pulse' test. Approximately 5-mm long root tips were excised and homogenized in glass homogenizer. Samples of 50 mg of fresh root tips were homogenized in 2 ml of ice cold buffer pH 7.2, containing: 0.15 M NaCl, 0.1 M EDTA, 2.5% SDS, and 0.005 M mercaptoethanol. Homogenates were centrifuged for 10 min at 15,000*g*. Incorporation of the labels into protein and DNA was measured in precipitates obtained by treating root homogenates with 10% TCA with nonlabeled valine and thymidine added. Samples were analyzed in the Beckman Liquid Scintillation System, Model CS-350.

Subcellular Distribution of Al in Roots. Root tips (5 mm long) were cut off from treated roots and homogenized. The homogenate was fractionated by the method of Bonner (4) with some modifications; about 1 g of root tissue was ground in an ice cold mortar with 6 ml of the medium containing 0.3 M mannitol, 0.01 M EDTA, 0.1% BSA, 0.05% cysteine, and 0.025 M phosphate buffer, pH 7.2. The homogenate was squeezed through 8 layers of cheesecloth and centrifuged at 480*g* for 6 min. The supernatant was centrifuged at 15,000*g* for 15 min. The pellets of crude nuclei and mitochondria were washed twice with the homogenization medium devoid of cysteine. The pellets were resuspended in approximately 1 ml of the washing medium. All operations were carried out at 2 to 4°C. Purity of the nuclear and mitochondrial fractions was checked with the mitochondrial and cytoplasmic enzyme markers Cyt *c* oxidase and glucose-6-P dehydrogenase, respectively. The enzyme activities were assayed according to Smith (22) and Kronberg and Horecker (15), respectively. Contamination of mitochondrial and nuclear fractions with cytosol did not exceed 1 and 6%, respectively. The nuclei did not show any Cyt *c* oxidase activity.

The homogenates and isolated fractions were mineralized in the mixture of H_2SO_4, HNO_3, $HClO_4$ (1:7:2 v/v) as described by Jones and Thurman (11). After mineralization, the samples were transferred into 50-ml graduated flasks, pH was adjusted to 3.0 ± 0.02 with ammonia solution, and diluted to the mark with distilled water. Aluminum was determined with Eriochrome Cyanine R (11).

RESULTS

The seedlings of Atlas 66 incubated for 24 h at 25°C in the nutrient solution containing 27 μg/ml Al showed irreversible inhibition of root growth. For sensitive Grana the lethal dose was 4 μg/ml (Tables I and II). However, the seedlings of both varieties, when pretreated with Al at low concentrations, showed significant root regrowth at the lethal level of Al. Higher tolerance at the sublethal and lethal concentrations of Al was manifested as increase in the percentage of the seedlings with root regrowth. Approximately 80% of the pretreated seedlings of Atlas 66 survived a lethal concentration of Al and, at a concentration twice as high, roots of 21% of the seedlings remained alive (Table I). About 70% of the Grana pretreated seedlings showed root regrowth after treatment at lethal Al concentration and at double the higher concentration roots of about 45% of the seedlings showed regrowth (Table II).

It is of considerable importance that at sublethal and lethal concentrations of Al, accumulation of Al in root tissue was distinctly higher in the pretreated seedlings of Grana, especially in root tips. The same tendency was observed in Atlas 66, but only at the lethal concentration of Al. Thus, despite higher accumulation of Al in root tissue, the apical meristems of pretreated seedlings were still viable and able to grow, while at the lower level of Al, in control seedlings, meristems were irreversibly damaged. The lack of correlation between Al concentration in roots and Al tolerance was observed previously in different wheat genotypes (1, 2).

Induction of Al tolerance was completely prevented when wheat seedlings were pretreated with Al in the presence of cycloheximide, an antibiotic that blocks protein synthesis. At the same time, addition of the antibiotic to the inducing medium significantly increased Al accumulation, particularly in root tips (Table III).

I concluded that the level of Al found in root tissue was not crucial for Al toxicity, but its subcellular distribution and perhaps the form in which Al was present inside the cell were important. The increased tolerance to Al induced by low doses of Al might be the result of an induced increase in the efficiency of the mechanism responsible for sequestering or transport of Al inside the root cell.

The distribution of Al in root subcellular fractions from induced and control Atlas 66 seedlings was studied using the methods described earlier (20). Significantly more Al was found in all fractions from pretreated roots than in controls (Table IV), but the relative distribution of Al between subcellular fractions was unchanged. Similar results were obtained when the whole root tissue was homogenized and extracted with Tris-HCl buffer (Table V).

Increased Al content in preincubated roots was proportionally distributed between particulate and soluble fractions. However, the antibiotic significantly affected this distribution pattern, where a much higher increase in Al content was observed in the particulate fraction than in the soluble one. It seems that cycloheximide severely affected the mechanism of Al compartmentation in root cells. The Al found in subcellular fractions of roots treated with cycloheximide seemed to be bound differently to

Table I. *Induction of Al Tolerance in Roots of the Tolerant Winter Wheat Cultivar Atlas 66*

The seedlings were preincubated with Al at a concentration of 0.5 μg/ml for 48 h at 25°C. Data are averages from three independent experiments (± SD). The calculation of percentages of seedlings with regrowth above and below 10 mm is based only on seedlings with some regrowth.

Al Concentration in the Testing Medium	Treatment	No. of Seedlings Tested	Seedlings with Root Regrowth			Mean Regrowth	Al Content in Roots	
			Total	Above 10 mm	Below 10 mm		Tips	Upper part
μg/ml				*%*		*mm*	*mg/g dry wt*	
16	Preincubation	230	85 ± 10.4	73	27	19	0.3	1.0
	Control	146	45 ± 5.5	28	72	10	1.0	0.9
24	Preincubation	305	80 ± 7.1	83	17	16	1.7	1.6
	Control	278	44 ± 4.2	28	72	10	1.8	1.7
27	Preincubation	175	78 ± 9.2	46	54	15	3.3	2.0
	Control	185	0	0	0	0	2.8	1.2
54	Preincubation	101	21 ± 4.2	8	92	6	6.0	4.0
	Control	159	0	0	0	0	5.2	3.3

Table II. *Introduction of Al Tolerance in Roots of the Sensitive Winter Wheat Cultivar Grana*

The seedlings were preincubated with Al at a concentration of 0.1 μg/ml for 48 h at 25°C. Data are averages from three independent experiments (±SD). The calculation of percentages of seedlings with regrowth above and below 10 mm is based only on seedlings with some regrowth.

Al Concentration in the Testing Medium	Treatment	No. of Seedlings Tested	Seedlings with Root Regrowth			Mean Regrowth	Al Content in Roots	
			Total	Above 10 mm	Below 10 mm		Tips	Upper part
μg/ml				%		*mm*	*mg/g dry wt*	
2	Preincubation	139	91 ± 6.8	84	16	21	2.4	0.7
	Control	132	42 ± 4.5	48	52	12	0.2	0.6
4	Preincubation	125	68 ± 8.7	10	90	15	2.5	0.7
	Control	135	0	0	0	0	0.9	0.6
8	Preincubation	87	43 ± 5.6	15	85	14	2.5	1.3
	Control	91	0	0	0	0	2.4	1.6
12	Preincubation	89	0	0	0	0	3.1	1.0
	Control	119	0	0	0	0	2.2	1.3

Table III. *Effect of Cycloheximide on the Induction of Al Tolerance by Al*

Antibiotic was added for the last 12 h of preincubation at the concentration of 10 μg/ml to the medium containing 0.5 μg/ml and 0.1 μg/ml Al for Atlas 66 and Grana, respectively. After preincubation, both cultivars were tested at toxic levels of Al, i.e., 27 μg/ml and 4 μg/ml for Atlas 66 and Grana, respectively. Pretreatment with cycloheximide *per se* did not affect root growth of both varieties. Data are averages from three independent experiments.

Variety	Pretreatment	No. of Seedlings Tested	Seedlings with Root Regrowth	Length of Root Regrowth	Al Content in Roots		
					Tips	Upper part	Whole
			%	*mm*	*mg/g dry wt*		
Atlas 66	None	324	0	0	2.5	0.9	1.2
	Al	286	84	15	3.2	0.9	1.1
	Al + cycloheximide	326	0	0	11.3	2.4	3.2
Grana	None	263	0	0			
	Al	292	60	12			
	Al + cycloheximide	253	0	0			

Table IV. *Aluminum Distribution in the Cellular Fractions of Root Tips of Atlas 66 Seedlings with and without Induced Al Tolerance*

Al tolerance was induced by preincubation of the seedlings with Al (0.5 μg/ml) for 48 h at 25°C. Preincubated seedlings were then tested at 27 μg/ml Al for 24 h at 25°C. Control seedlings were tested at 27 μg/ml Al without pretreatment.

Al Tolerance	Root Regrowth	Root Fractionation Al Content				Cytosol Fractionation		
		Total	Nuclei	Mitochondria	Cytosol	Total	TCA Precip.	Sediment on 05 Amicon membrane
	%	*μg/g fresh wt*				*μg/g fresh wt*		
Induced	93	190.4	40.4	29.0	121.0	121.0	90.0	116.0
		(100)	(21.2)	(15.2)	(63.6)	(100)	(74.4)	(95.9)
Control	0	132.0	28.0	21.0	83.0	83.0	59.0	76.0
		(100)	(21.2)	(15.9)	(62.9)	(100)	(71.1)	(91.6)

cellular components than Al found in root tissue not treated with cycloheximide. Approximately 50% of the Al from particulate and soluble fractions from the cycloheximide-treated roots 'leaked out' during the 48-h period of regrowth while in control roots Al content remained the same, only a shift from the soluble to the particulate fraction was observed.

Because the majority of Al found in root tissue was accumulated in the cytosol fraction (Table IV), this fraction was further studied. Approximately 70% of Al found in cytosol was precipitated with TCA and practically no Al was found in the low-molecular fraction filtered through an Amicon 05 membrane. This distribution was similar in the controls and plants with induced Al tolerance. The aliquots of cytosol were also dialyzed against H_2O and acetate buffer and no Al was detected in the dialysates. However, almost all Al added to the cytosol and incubated at 25°C for 6 h passed through the membrane and was found in dialysate. This clearly indicates that Al in the cytosol, in both induced and control plants, was bound with high mol wt

compounds, probably proteins and/or nucleic acids. The assumption that Al inside root cells might be bound with proteins was further corroborated by the results of studies on incorporation of labeled valine and thymidine into proteins and DNA in wheat roots exposed to Al (Table VI). Sublethal and lethal doses of Al in the nutrient solution increased the incorporation of both labeled substances and incorporation was significantly higher in tolerant Atlas 66 than in sensitive Grana. Incorporation of valine increased over four times in Atlas 66 roots treated with Al at a sublethal dose (16 μg/ml), whereas the highest increase of valine incorporation observed in roots of sensitive Grana was approximately 80%. Differences in incorporation of thymidine were smaller, but in both varieties DNA synthesis continued even when roots were in the medium containing Al at concentrations above the lethal dose.

DISCUSSION

The data presented suggest an inducible mechanism of Al detoxication in wheat. Its operation is greatly enhanced by low Al concentration in the medium. This inducible mechanism was present in both tolerant and sensitive cultivars, but its efficiency in the tolerant genotype was much higher. The mechanism allows increased accumulation of Al in root cellular components without damage to their function and is severely disturbed by blocking the protein synthesis in root cells. Therefore, it seems that the synthesis of Al-binding proteins protects cellular components from Al damage.

These results are in agreement with observations of other authors. Sampson *et al.* (21) found increased DNA synthesis in barley roots treated with Al, even when cell division was halted. Matsumoto *et al.* (17) found that Al content in the nuclei of pea root cells increased up to 24 h following Al treatment, and did not decrease after transfer of Al-treated plants to water. Al in nuclei was associated preferentially with DNA. But, when the phosphorus in DNA was masked by histone, the association of Al with DNA was considerably reduced. Binding of Al to nuclear proteins could explain the higher accumulation of Al in the tolerant wheat cultivar Atlas 66 than in the sensitive cultivar Grana. In fact, despite a higher Al content, root cells in the tolerant cultivar were able to divide and roots were able to elongate, whereas at a much lower Al content in the nuclei of root cells from the sensitive cultivar Grana an irreversible damage of root apical meristems was observed (20). A similar phenomenon was observed in nuclei from pretreated *versus* control roots of Atlas 66 (Table IV). A 100% higher accumulation of Al was also found in nuclei from tolerant snapbean (*Phaseolus vulgaris* L.) cultivar Dato than in sensitive cultivar Romano (19).

Blocking of the protein synthesis by cycloheximide destroyed the proposed protective mechanism and allowed the direct action of Al on its targets inside the root cell. One of such targets is phosphorus in nucleic acids (17). This assumption is corroborated by the observed shift of Al from cytosol to nuclei when the Al concentration in the medium was increased from sublethal to a lethal dose for Atlas 66 wheat seedlings (20). The above data and a very high degree of specificity in the response of plants to particulate metals (8) suggest that proteins play a role in tolerance mechanisms. These mechanisms can function in two ways: either the metal is metabolized into stable metaloproteins, like cadmium in cabbage (*Brassica oleracea* L.) (24) or it is complexed. In the latter case, proteins can serve as specific carriers. The binding of Al with proteins was previously found in Al-corrosive bacteria (3), so it is possible that such mechanisms also exist in plants.

Inducible synthesis of Al binding proteins can be localized in different root cell compartments and, therefore, could be controlled by different genes. In fact, Al tolerance in wheat was found to be determined by several genes (16) and can explain the existence of different degrees of Al tolerance in wheat (1, 10).

It can be concluded that proteins play an important role in the mechanism of Al tolerance in wheat. Aluminum-tolerant and -sensitive genotypes may differ in the number of genes determining synthesis of protective proteins and probably also in their efficiency. Further studies on genetically elaborated tolerant and sensitive plants are needed for full elucidation of the mechanism of Al tolerance in wheat.

Table V. *Effect of Cycloheximide on Al Binding in Root Tissue*

Roots of Atlas 66 were crushed in liquid nitrogen and extracted with Tris-HCl buffer, pH 6.6 with 10 mM EDTA, and 0.1% mercaptoethanol. Homogenates were centrifuged at 27,000*g* for 15 min. Al content expressed in μg/g fresh wt of root tissue; per cent of total content given in parentheses.

Al Tolerance	Root Regrowth	Pellet	Supernatant
	%		
Control	0	15 (42)	21 (58)
Induced	90	52 (40)	77 (60)
Induction blocked (Cycloheximide)	0	131 (62)	80 (38)

Acknowledgments—The author would like to thank Drs. J. P. Gustafson and D. G. Blevins for review of the manuscript. The technical assistance of E. Kulińska is gratefully acknowledged. I am grateful to S. Kowalewski for producing the manuscript.

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Table VI. *Incorporation of [^{14}C]Valine and [^{3}H]Thymidine into TCA Precipitates of Root Homogenates of Wheat*

Seedlings incubated in the nutrient medium containing Al for 24 h/25°C. Data are averages from three experiments. Homogenates from 50 mg of fresh root tips were analyzed.

Al Concentration	Root Regrowth		[^{14}C]Valine		[^{3}H]Thymidine	
	Altas 66	Grana	Atlas 66	Grana	Atlas 66	Grana
μg/ml	%		*μmol/g fresh wt*			
0.0	100	100	1.7 (100%)	1.5 (100%)	4.6 (100%)	2.7 (100%)
0.5	100	86	1.6 (94%)	2.7 (180%)	4.4 (97%)	3.9 (144%)
2.0	100	75	2.2 (129%)	2.0 (133%)	14.0 (304%)	8.4 (311%)
4.0	100	0	5.6 (329%)	1.3 (87%)	12.4 (270%)	2.7 (100%)
16.0	76	0	7.3 (429%)	1.3 (87%)	17.8 (387%)	2.3 (85%)
27.0	0	0	1.2 (71%)		7.1 (154%)	

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