Plant Physiol. (1997) 114: 363–372

# Al-Induced, 51-Kilodalton, Membrane-Bound Proteins Are Associated with Resistance to Al in a Segregating Population of Wheat[1]

**Gregory J. Taylor*, Atanu Basu, Urmila Basu, Jan J. Ślaski, Guichang Zhang, and Allen Good**

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

**Incorporation of $^{35}S$ into protein is reduced by exposure to Al in wheat (*Triticum aestivum*), but the effects are genotype-specific. Exposure to 10 to 75 μM Al had little effect on $^{35}S$ incorporation into total protein, nuclear and mitochondrial protein, microsomal protein, and cytosolic protein in the Al-resistant cultivar PT741. In contrast, 10 μM Al reduced incorporation by 21 to 38% in the Al-sensitive cultivar Katepwa, with effects becoming more pronounced (31–62%) as concentrations of Al increased. We previously reported that a pair of 51-kD membrane-bound proteins accumulated in root tips of PT741 under conditions of Al stress. We now report that the 51-kD band is labeled with $^{35}S$ after 24 h of exposure to 75 μM Al. The specific induction of the 51-kD band in PT741 suggested a potential role of one or both of these proteins in mediating resistance to Al. Therefore, we analyzed their expression in single plants from an $F_2$ population arising from a cross between the PT741 and Katepwa cultivars. Accumulation of 1,3-β-glucans (callose) in root tips after 24 h of exposure to 100 μM Al indicated that this population segregated for Al resistance in about a 3:1 ratio. A close correlation between resistance to Al (low callose content of root tips) and accumulation of the 51-kD band was observed, indicating that at least one of these proteins cosegregates with the Al-resistance phenotype. As a first step in identifying a possible function, we have demonstrated that the 51-kD band is most clearly associated with the tonoplast. Whereas Al has been reported to stimulate the activity of the tonoplast $H^+$-ATPase and $H^+$-PPase, antibodies raised against these proteins did not cross-react with the 51-kD band. Efforts are now under way to purify this protein from tonoplast-enriched fractions.**

Cultivars of wheat (*Triticum aestivum* L.) vary dramatically in their resistance to Al (Taylor and Foy, 1985a, 1985b). This differential resistance has been the focus of numerous studies in recent years. From a physiological perspective, a simple, well-defined genetic system can provide a powerful tool for investigating mechanisms of Al resistance. Unfortunately, early investigations of the genetic basis of Al resistance yielded contradictory results. Several studies reported that resistance to Al might be mediated by a single dominant gene (Kerridge and Kronstad, 1968; Camargo, 1981; Delhaize et al., 1993a, 1993b), whereas other studies indicated polygenic control of this character (Campbell and Lafever, 1981; Aniol, 1984, 1990; Aniol and Gustafson, 1984; Berzonsky, 1992; Bona et al., 1994; Ślaski, 1995). To some extent, differences in the patterns of inheritance reported in the literature could reflect the unique characteristics of germ plasm used in individual experiments. Even if resistance is mediated by a suite of adaptive traits, careful selection of genetic material may provide a system in which a single component of Al resistance behaves as a single dominant gene.

The possibility that Al resistance is mediated by an integrated suite of adaptive traits provides a powerful incentive for the use of near-isogenic or isogenic plant material (Taylor, 1995). With a truly isogenic system, in which differences between genotypes are directly related to the presence or absence of a single gene, the task of identifying resistance mechanisms is greatly simplified. Although separating cause and effect remains a concern, the experimental effort required to achieve this objective is considerably less demanding (Taylor, 1995). Whereas a truly isogenic Al-resistant system will be difficult to develop, genetic material that segregates as a single gene can provide an equally important experimental tool. If a specific physiological trait fails to segregate with the resistance phenotype, that trait cannot play a role in mediating resistance. For example, Delhaize et al. (1991) identified several Al-induced polypeptides that were specific to an Al-resistant genotype of wheat (Carazinho) under conditions of Al stress. However, in $F_2$ progeny derived from a cross between the Al-resistant cv Carazinho and the Al-sensitive cv Egret, none of the Al-induced polypeptides cosegregated solely with the Al-resistant phenotype (Delhaize et al., 1991). In contrast, Delhaize et al. (1993b) demonstrated that enhanced exudation of malate from roots cosegregated with resistance to Al in a similar segregating population. This observation, plus the fact that malate present in root exudates is specifically labeled upon exposure to Al and $^{14}C$-acetate (Basu et al., 1994c), provides strong support for the hypothesis that enhanced synthesis and export of malate may play an important role in mediating resistance to Al.

[1] This work was supported by the University of Alberta, Central Research Fund, and the Research Grants Program of the Natural Sciences and Engineering Research Council of Canada (NSERC). Financial support for A.B. and J.J.Ś. was provided by NSERC's International Fellowship Program.

* Corresponding author; e-mail gjtaylor@gpu.srv.ualberta.ca; fax 1-403-492-9234.

Abbreviations: $H^+$-ATPase, $H^+$-translocating ATPase; $H^+$-PPase, $H^+$-translocating pyrophosphatase; PE, pachyman equivalents.

We recently identified a pair of Al-induced, 51-kD, membrane-bound proteins in roots of an Al-resistant cultivar of *T. aestivum*, PT741, which was only weakly expressed in the Al-sensitive cv Neepawa (Basu et al., 1994a). In cv PT741 these proteins were localized at the root tip (2 cm) and accumulated in a dose- and time-dependent fashion. Accumulation of these proteins in the presence of Al, and their subsequent disappearance after removal of Al from the growth medium, suggested a possible involvement in Al resistance (Basu et al., 1994a). In the present study we show that the 51-kD band in cv PT741 is specifically labeled with $^{35}S$, despite a general reduction in protein synthesis. Furthermore, accumulation of the band is a trait that cosegregates with the resistance phenotype in $F_2$ progeny arising from a cross between cvs PT741 and Katepwa. The 51-kD band is most clearly associated with membrane material enriched in the tonoplast, and is not observed in plasma membrane-enriched fractions. Although assignment of a putative functional role awaits identification of genes encoding these proteins, our results suggest a possible role in limiting Al-induced injury within the cytoplasm or in regulating the intracellular distribution of Al.

## MATERIALS AND METHODS

An Al-resistant (PT741) and an Al-sensitive cultivar (Katepwa) of wheat (*Triticum aestivum* L.) provided genetic material for all of the experiments reported here. Based upon its pedigree (cv PT741 = Toropi//Ciano "S"/Noroeste 66/3/Bluebird/Ciano "S"/4/Grajo "S"; cv Katepwa = Neepawa*6/RL2938/3/Neepawa*6//CI81542/2*Frocor; Moroni et al., 1991), Al tolerance in cv PT741 could arise from the Brazilian cv Frontana. In addition to these cultivars, a population segregating for Al resistance was prepared by selfing single $F_1$ plants arising from a cross between cvs PT741 and Katepwa. The resulting $F_2$ seed was used directly in physiological experiments.

In all experiments seeds were surface-sterilized in 1% (w/v) sodium hypochlorite for 20 min and soaked for 24 h in double-distilled water containing 0.005 g $L^{-1}$ of Vitavax (Uniroyal, Calgary, Alberta, Canada) to limit fungal growth. Seedlings were then grown for 3 to 7 d on nylon mesh suspended over 15 L of an aerated nutrient solution containing (in $\mu$M) 2900 $NO_3^-$, 300 $NH_4^+$, 100 $PO_4^{3-}$, 800 K, 1000 Ca, 300 Mg, 101 $SO_4^{2-}$, 34 Cl, 60 Na, 10 Fe, 6 B, 2 Mn, 0.15 Cu, 0.5 Zn, 0.1 Mo, and 10 EDTA (pH 4.3). Plants were grown in a growth chamber with 16 h of light (23°C, 68% RH) and 8 h of darkness (16°C, 85% RH). For most experiments, these seedlings were used directly for experimentation (using the same exposure solutions described below). In experiments with 54 single $F_2$ plants and their parents, 8-d-old plants of uniform size were selected and mounted on Plexiglas covers of 10-L polyethylene buckets (1 plant per bucket), which contained an aerated solution (pH 4.3) as described above. Plants were grown for 3 weeks in a controlled-environment room with a 16-h photoperiod. Air temperatures at leaf height ranged from 21 to 23°C during the light period and from 15 to 16°C during the dark period. Solution temperatures were maintained between 19 and 21°C in light and darkness by standing all of the buckets in a common water bath. RH varied from 58 to 66% in the light period and from 92 to 96% in the dark period. Light was provided by 25 cool-white fluorescent lamps yielding a PPFD at the plant bases of 342.7 ± 7.3 $\mu$mol $m^{-2}$ $s^{-1}$ (mean ± SE). The pH of the growth solutions was monitored throughout the treatment period. After 3 weeks of growth plants were transferred to new buckets containing (in $\mu$M) 2900 $NO_3^-$, 300 $NH_4^+$, 100 Ca, 300 Mg, and 100 $\mu$M $AlCl_3$, and grown for another 24 h.

### [$^{35}$S]Met Labeling

After 3 d of growth as described above, seedlings were transferred to fresh nutrient solutions containing 0, 10, 25, 50, or 75 $\mu$M $AlCl_3$ (pH 4.5). After 24 h of treatment, 20 to 30 seedlings were transferred to each of a series of beakers containing 5 to 10 mL of treatment solution in the presence of 33 $\mu$L $L^{-1}$ [$^{35}$S]Met (370 MBq $mL^{-1}$, specific activity 37.0 TBq $mmol^{-1}$, Amersham). After 3 to 6 h of labeling at 25°C, seedlings were washed in 1 mM Met (pH 4.5) for 10 min, and then collected and kept on ice in sealed plastic bags during excision of root tips.

Root tips (5 mm) were excised from seedlings, blotted dry, weighed, and homogenized in 5 mL of a homogenization buffer with a mortar and a pestle. The homogenization buffer contained 50 mM Mops (pH 6.5), 1 mM EDTA, 2 $\mu$M PMSF, and 1 mM DTT (Basu et al., 1994a). The homogenate was filtered through four layers of cheesecloth, and the mortar and pestle were rinsed with 5 mL of the homogenization buffer that was filtered through the same cheesecloth. An aliquot (0.5 mL) of the crude filtrate was removed for determination of total incorporation of [$^{35}$S]Met, and the remainder was centrifuged at 20,000*g* for 10 min. The resulting pellet was collected as the mitochondrial and nuclear pellet, and the supernatant was centrifuged at 100,000*g* for 45 min. The 100,000*g* pellet was collected as the microsomal membrane fraction and the supernatant was collected as the cytoplasmic fraction. The crude filtrate, 20,000*g* pellets, microsomal membrane pellets, and accompanying supernatants were processed for determination of $^{35}S$ incorporation using TCA precipitation, according to the method of Zhang et al. (1995). In experiments in which further use of the microsomal membrane fraction was required (SDS-PAGE analysis), the microsomal membrane fraction was resuspended in a dilution buffer containing 50 mM Mops (pH 6.5), 1 mM EDTA, 1 mM DTT, and 1 $\mu$M PMSF. Protein concentrations were determined by the method of Lowry et al. (1951).

### Membrane Isolation and Purification

The plasma membrane, tonoplast, and intracellular membranes were isolated from approximately 20 g of 1.0-cm root tips after exposure to 100 $\mu$M Al for 72 h. Root tips were homogenized immediately after harvest using a polytron homogenizer in 30 mL of a homogenization buffer containing 0.25 M Suc, 50 mM Mops-Tris (pH 7.5), 5 mM EDTA, 3 mM DTT, 1 mM PMSF, 0.2% BSA, and 5 mM ascorbic acid. The homogenate was filtered through four

layers of cheesecloth and centrifuged at 20,000*g* for 15 min, and the supernatant was collected and centrifuged at 100,000*g* for 45 min. The resulting microsomal membrane pellet was resuspended in 10 mL of a resuspension buffer containing 0.25 M Suc, 5 mM phosphate buffer (pH 7.8), 2 mM KCl, 1 mM DTT, and 0.1 mM EDTA. Nine milliliters of the microsomal membrane fraction was set aside for further purification of membranes (see below). The remainder (approximately 1 mL) was diluted 13-fold with a dilution buffer containing 0.25 M Suc, 5 mM Mes-Tris (pH 7.0), 2 mM DTT, 1 mM PMSF, and 5 mM EDTA, centrifuged at 120,000*g* for 45 min, and resuspended in the dilution buffer, and 100-$\mu$L aliquots were frozen at −80°C.

Membrane fractions enriched in the plasma membrane and intracellular membranes were prepared by aqueous two-phase partitioning (Larsson et al., 1994). Nine milliliters of the microsomal membrane fraction was added to a 27-g phase mixture to give a phase system with a final composition of 6.5% (w/w) Dextran T 500, 6.5% (w/w) PEG, 0.25 M Suc, 5 mM phosphate buffer (pH 7.8), 2 mM KCl, 1 mM DTT, and 0.1 mM EDTA. The plasma membrane-enriched fraction ($U_3$ + $U_{3'}$) and an intracellular membrane-enriched fraction ($L_2$) were diluted 6 to 10 times with the dilution medium and pelleted at 120,000*g* for 45 min. The plasma membrane pellet was resuspended in dilution medium and frozen at −80°C. The 120,000*g* pellet obtained from the $L_2$ was resuspended in 10 mL of a gradient buffer containing 0.25 M sorbitol, 5 mM Hepes-bis-Tris (pH 7.0), and 1 mM DTT, and loaded on a two-step gradient composed of 5 mL each of 2 and 10% (w/w) Dextran T70 prepared in the gradient buffer (Kasai et al., 1992). The Dextran T70 gradient was centrifuged at 70,000*g* for 120 min, and the interface containing the tonoplast was collected, diluted with gradient buffer, and spun at 120,000*g* for 60 min. The resulting tonoplast pellet was resuspended in the gradient buffer and frozen at −80°C.

Activities of the $NO_3^-$-sensitive ATPase, the vanadate-sensitive ATPase, Cyt *c* oxidase, and NADH-Cyt *c* reductase were used as markers for the tonoplast, plasma membrane, mitochondria, and ER, respectively (Briskin et al., 1987).

### SDS-Gel Analysis and Immunoblotting

SDS-PAGE was performed according to the method of Laemmli (1970) using an electrophoresis cell (Mini-Protean II, Bio-Rad). Proteins were electrophoresed at 10 mA in the stacking gel (4% total monomer concentration, 2.7% cross-linking monomer solution), followed by 20 mA in the separating gel (10% total monomer concentration, 2.7% cross-linking monomer solution). Gels were stained with Coomassie blue or silver stain and band intensity was estimated using a densitometer (model FB910, Fisher Scientific) and a data reduction system (GS365W version 3.02, Hoefer Scientific Instruments, San Francisco, CA). Microsomal proteins separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane (0.45 $\mu$m) for 2 h at 80 V (Mini Trans-Blot cell, Bio-Rad) with a transfer buffer containing 25 mM Tris, 192 mM Gly (pH 8.3), 0.02% SDS (w/v), and 20% (v/v) methanol. Nitrocellulose membranes were blocked overnight, incubated for 1 h with primary antibody, and washed five times (5 min each), with each step using a blocking solution containing TBS (20 mM Tris, pH 7.5, 140 mM NaCl), 0.05% Triton X-100, and 5% skim milk (Difco, Detroit, MI). Membranes labeled with primary antibodies were incubated for 1 h in horseradish peroxidase-linked goat anti-rabbit IgG antibody (Amersham) in a 1:4000 dilution of a blocking solution. Blots were washed three times in a blocking solution (15 min, 2 × 5 min), twice in TBS (5 min), and developed using chemiluminescent detection (Amersham ECL reagent) according to the manufacturer's instructions. Antibodies against the tonoplast $H^+$-PPase (Maeshima and Yoshida, 1989) and the two largest subunits (68 and 57 kD) of the tonoplast $H^+$-ATPase from *Vigna radiata* (Matsuura-Endo et al., 1992) were provided by M. Maeshima at the Laboratory of Biochemistry, School of Agricultural Sciences, Nagoya University, Nagoya, Japan.

### Spectrofluorometric Estimation of Callose

Synthesis of 1,3-$\beta$-glucans (callose) was used as a marker for Al-induced injury in both short- and long-term experiments. At the end of Al treatments, five root tips were harvested and weighed, and their callose content was measured according to the procedure of Zhang et al. (1994). Pachyman (Calbiochem) was used as an external standard and callose contents were expressed as milligrams PE per gram root fresh weight.

## RESULTS

We previously demonstrated that a pair of 51-kD, membrane-bound proteins accumulated in microsomal membranes isolated from root tips of the Al-resistant cv PT741 under conditions of Al stress (Basu et al., 1994a). These proteins, which can be separated using two-dimensional SDS-PAGE, appear as a single band at 51 kD in one-dimensional gels. In this study we have investigated the effect of Al on synthesis of these proteins by labeling roots with $^{35}S$. After exposing intact roots to Al (0 or 75 $\mu$M) for 24 h, the 51-kD band was strongly labeled after 3 and 6 h exposure to [$^{35}S$]Met (Fig. 1). Enhanced synthesis of proteins, however, was not a general response to Al stress. Whereas a 67-kD protein was also more prominently labeled after exposure to 75 $\mu$M Al, all other proteins resolved on our gels were either not affected by Al or were more clearly labeled under control conditions (Fig. 1). Reduced labeling of bands at 29 and 35 kD were the most dramatic, although a general reduction in labeling was apparent in all bands below 40 kD.

Analysis of $^{35}S$ incorporation into a crude root filtrate (total protein), a low-speed pellet containing nuclear and mitochondrial proteins, a high-speed pellet containing microsomal proteins, and a high-speed supernatant containing cytosolic proteins confirmed that enhanced protein synthesis was not a general response to Al stress. The effect of Al on protein synthesis, however, differed between genotypes (Fig. 2). In the Al-resistant cv PT741 exposure to 10 $\mu$M Al had little effect on total $^{35}S$ incorporation. Increasing Al concentrations to 25 and 50 $\mu$M increased $^{35}S$ incorporation

slightly (13 and 22%, respectively), whereas $^{35}S$ incorporation returned to control levels at 75 $\mu$M Al (Fig. 2A). In contrast, marked reductions in $^{35}S$ incorporation were observed in the Al-sensitive cv Katepwa at all concentrations tested. Incorporation was reduced by 38 and 62% after exposure to 10 and 25 $\mu$M, respectively. No further reduction was observed with increasing Al concentrations (Fig. 2A). Similar changes in $^{35}S$ incorporation (both qualitatively and quantitatively) were observed in high-speed supernatants containing cytosolic proteins (Fig. 2D).

Differential effects of Al on protein synthesis were also observed in microsomal membrane fractions and in low-speed pellets containing nuclear and mitochondrial proteins, although the extent of change was not as dramatic as that observed in the cytosolic and total fractions. In the Al-resistant cv PT741 exposure to 10 to 50 $\mu$M Al had no significant effect on $^{35}S$ incorporation into microsomal proteins, whereas a 21% reduction was observed at 75 $\mu$M (Fig. 2C). In the Al-sensitive cv Katepwa $^{35}S$ incorporation was reduced by 21% at 10 $\mu$M Al. At 25, 50, and 75 $\mu$M, $^{35}S$ incorporation declined to 53, 47, and 38%, respectively, of the control (Fig. 2C). Similar changes were observed in the low-speed pellets containing nuclear and mitochondrial proteins (Fig. 2B).

The results of our $^{35}S$ labeling studies indicated that Al had a dramatic effect on protein synthesis in roots of the Al-sensitive cv Katepwa, whereas protein synthesis in the Al-resistant cv PT741 was only slightly affected. Although this pattern of response could be explained by a variety of internal or external resistance mechanisms (Taylor, 1988, 1991, 1995), the specific induction of a 51-kD, membrane-bound protein in the Al-resistant cv PT741 suggested a possible role of this protein in mediating resistance to Al (Basu et al., 1994a). This possibility was further tested by analyzing Al-induced synthesis of 1,3-$\beta$-glucans (callose) and Al-induced expression of the 51-kD band in an $F_2$ population segregating for Al resistance. Synthesis of 1,3-$\beta$-glucans provides an accurate and sensitive short-term marker for Al injury that correlates well with the long-term effects of Al on root growth (Zhang et al., 1994). Synthesis of callose in both cv PT741 and cv Katepwa was stimulated in 5-d-old seedlings after 24 h of exposure to Al, with the Al-sensitive cv Katepwa producing approximately three to four times more callose than the Al-resistant cv PT741 over a broad range of Al concentrations (0–100 $\mu$M, Fig. 3A). At 100 $\mu$M Al the pattern of response was consistent throughout the duration of a 24-h exposure (Fig. 3B).

Analysis of callose content in root tips from 54 single $F_2$ plants (24-d-old) arising from a cross between cv PT741 and cv Katepwa yielded values ranging from 0.9 to 3.3 mg PE $g^{-1}$ fresh weight (Fig. 4A), values somewhat higher than those reported for the 5-d-old plants in Figure 3. The frequency distribution of callose contents showed two distinct populations. A group of 40 plants had callose contents below 1.7 mg PE $g^{-1}$ fresh weight (2–3-fold higher than independent controls), whereas the remaining 14 plants had callose contents in excess of 2.0 mg PE $g^{-1}$ fresh weight (5–8-fold higher than independent controls; Fig. 4A). On the basis of this distribution and the callose content of parental plants (Al-resistant parents produced an average

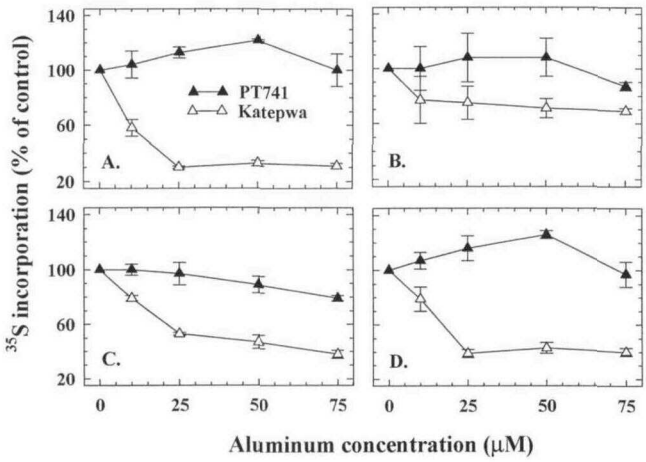


**Figure 2.** Incorporation of $^{35}S$-Met into a crude root filtrate (total protein, A), a low-speed pellet containing nuclear and mitochondrial proteins (B), a high-speed pellet containing microsomal proteins (C), and a high-speed supernatant containing cytosolic proteins (D) in 5-mm root tips of the Al-resistant cv PT741 and the Al-sensitive cv Katepwa of wheat in the presence of different concentrations of $AlCl_3$. $^{35}S$ incorporation was calculated per unit of fresh weight and expressed as a percentage of control (+Al/Al). Values represent means ± SE of three replicates.

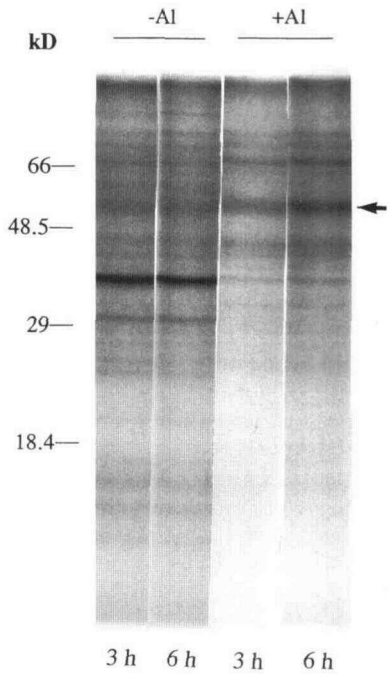


**Figure 1.** SDS-PAGE analysis of $^{35}S$-Met-labeled microsomal membrane proteins from cv PT741. Three-day-old seedlings were grown in the presence (+Al) and absence (−Al) of Al (75 $\mu$M) for 24 h and labeled with L-[$^{35}S$]Met for 3 and 6 h. Microsomal membrane fractions were isolated, proteins (equal CPM per lane) were separated on 10% polyacrylamide gels, and gels were exposed to film (X-Omat, Kodak). The molecular masses of marker proteins are indicated on the left in kilodaltons. The 51-kD band, which is more prominently labeled under conditions of Al stress, is indicated by the arrow.

of 1.4 ± 0.3 mg PE $g^{-1}$ fresh weight, Al-sensitive parents produced 2.7 ± 0.2 mg PE $g^{-1}$ fresh weight of callose), 40 plants were classified as Al-resistant and 14 as Al-sensitive. Thus, this population segregated for Al resistance in about a 3:1 ratio (Fig. 4A). When the relative abundance of the 51-kD protein in these same plants was estimated by densitometric analysis of SDS-PAGE gels (Fig. 4B), the 40 plants classified as Al-resistant also showed show the highest levels of the 51-kD protein (ranging from 1.1–2.5 arbitrary units). The 14 plants classified as Al-sensitive showed relative abundance values ranging from 0.3 to 0.8 arbitrary units. Thus, enhanced production of the 51-kD, membrane-bound protein(s) is a trait that cosegregates with the Al-resistance phenotype.

Although the close association between resistance to Al and accumulation of the 51-kD, membrane-bound protein(s) suggests that one or both of these proteins cosegregates with the Al-resistance phenotype, it remains possible that enhanced expression might reflect a general Al-stress response that is only observed under conditions of mild (perceived) Al stress. If this were true, enhanced expression may not be observed in Al-sensitive plants under the conditions used in our assay. To examine this possibility, expression of the 51-kD band was investigated over a broad range of Al concentrations and at times 0 to 24 h after exposure (Figs. 5 and 6). In the Al-resistant cv PT741 en-

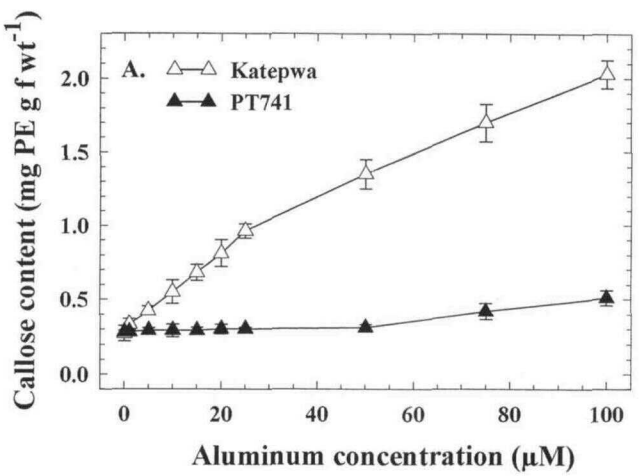


**Figure 3.** The effect of increasing concentrations of Al (A) and increasing time of exposure (B) on synthesis of 1,3-β-glucans (callose) in root tips of the Al-resistant cv PT741 and the Al-sensitive cv Katepwa. Five-day-old seedlings were treated with different concentrations of Al (0, 1, 5, 10, 15, 25, 50, 75, and 100 μM) for 24 h, or with 100 μM Al for 0, 3, 6, 10, 18, and 24 h. Values represent means ± SE of three replicates. f wt, Fresh weight.

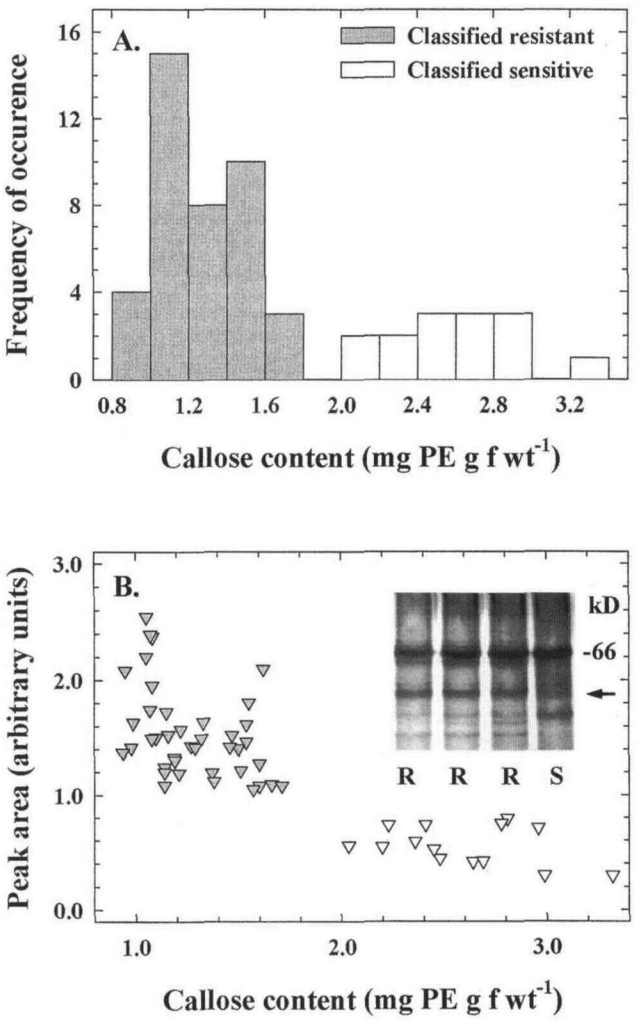


**Figure 4.** The frequency distribution of root tip callose content, a sensitive marker for Al injury (A), and the effect of Al on callose content and accumulation of the 51-kD band in 5-mm root tips (B) from 54 single $F_2$ plants arising from a cross between the Al-resistant cv PT741 and the Al-sensitive cv Katepwa. Individual seedlings were grown for 24 d in a complete nutrient medium without Al and were then transferred to solutions containing 100 μM Al. After 24 h of exposure to Al, five root tips from each plant were harvested for callose estimation and the remaining ones were used for isolation of microsomal membranes, SDS-PAGE, and estimation of band intensity by densitometric analysis. Under these conditions parental genotypes produced an average of 1.4 ± 0.3 mg PE $g^{-1}$ fresh weight (Al-resistant) and 2.7 ± 0.2 mg PE $g^{-1}$ fresh weight (Al-sensitive) of callose. The inset in B provides representative gels from three resistant (R) plants and one sensitive (S) plant. The molecular mass of a 66-kD marker protein is indicated. The 51-kD band is indicated with an arrow. f wt, Fresh weight.

hanced expression of the 51-kD band was observed at concentrations as low as 25 $\mu$M for 24 h (Fig. 5), or as early as 6 h after exposure to 100 $\mu$M Al (Fig. 6), and continued through to exposure to 100 $\mu$M Al for 24 h. In the Al-sensitive cv Katepwa the intensity of the 51-kD band remained unchanged, irrespective of the level of Al stress applied (Figs. 5 and 6). Our data on Al-induced synthesis of 1,3-$\beta$-glucans (Fig. 3, A and B) suggest that exposure of the Al-sensitive cv Katepwa to 1 to 5 $\mu$M Al for 24 h or 100 $\mu$M Al for 3 h should have provided a similar level of perceived stress as exposure of cv PT741 to 50 to 100 $\mu$M Al for 24 h. At equal levels of perceived stress, enhanced expression of the 51-kD band was not observed in cv Katepwa. Since mild or moderate Al stress failed to induce the expression of the 51-kD band, we conclude that one or both of the 51-kD proteins cosegregates with the Al-resistance phenotype.

In addition to the changes in levels of the 51-kD band, several other Al-induced changes were observed in the Al-resistant cv PT741. In Figure 5 bands migrating at 35, 43, and 45 kD are observed to increase after exposure (24 h) to Al, an effect that was clearly dose-dependent (Fig. 5). These bands, however, were not consistently expressed in all of our experiments. Enhanced expression of the 35- and 45-kD bands were not as pronounced in the time-course experiment, and the intensity of the 43-kD band declined between 6 and 24 h of exposure (Fig. 6). None of these bands was observed in previous experiments reported by Basu et al. (1994a), so more work is required before we can confidently conclude that these are also Al-induced proteins.

As a first step in identifying a possible function for the 51-kD protein, we have attempted to determine if it is associated with a specific membrane system. Microsomal membrane material from cv PT741 was used to isolate membrane fractions enriched in the plasma membrane, tonoplast, and intracellular membranes. Marker analysis of our membrane fractions (Table I) showed that activity of the plasma membrane-bound, $K^+$-stimulated, $Mg^{2+}$-dependent ATPase was enriched more than 6-fold in plasma membrane preparations, whereas the $NO_3^-$-sensitive ATPase was enriched 1.9-fold in tonoplast fractions. Intracellular membranes were enriched 2-fold in Cyt *c*

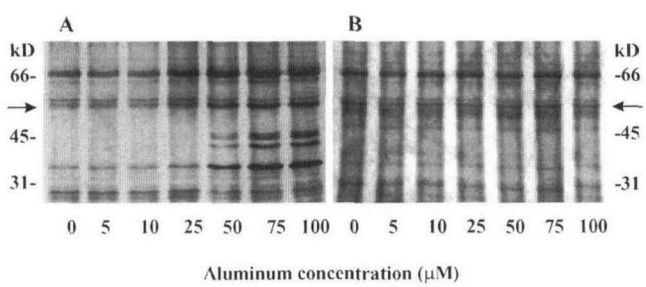


**Figure 5.** The effect of increasing concentrations of Al on the polypeptide profile of microsomal membrane fractions from root tips of the Al-resistant cv PT741 (A) and the Al-sensitive cv Katepwa (B). Five-day-old seedlings were treated with different concentrations of Al (0, 5, 10, 25, 50, 75, and 100 $\mu$M) for 24 h. Polypeptide bands were visualized by silver-staining. The 51-kD band is shown with an arrow.

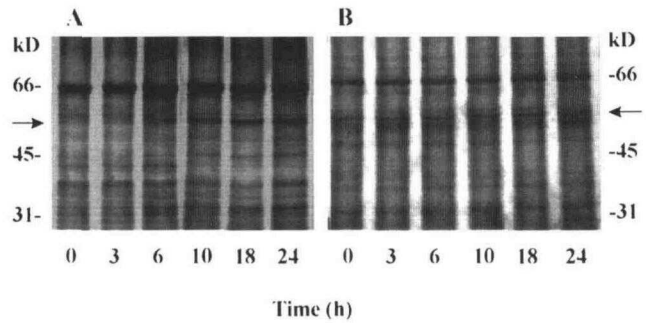


**Figure 6.** The effect of time of exposure on the polypeptide profile of microsomal membrane fractions from root tips of the Al-resistant cv PT741 (A) and the Al-sensitive cv Katepwa (B). Five-day-old seedlings were treated with 100 $\mu$M Al for 0, 3, 6, 10, 18, and 24 h. Polypeptide bands were visualized by silver-staining. The 51-kD band is indicated with an arrow.

oxidase activity (a marker for the mitochondria), but showed no enrichment in NADH-Cyt *c* reductase activity (a marker for the ER). Electrophoretic analysis of protein profiles from membranes isolated from root tips of 3-d-old seedlings showed that the 51-kD band was most clearly associated with the tonoplast fraction (Fig. 7). The 51-kD band was also observed in the intracellular membranes, but not in the plasma membrane fraction. Analysis of purified mitochondrial fractions showed no enrichment of the 51-kD band under Al stress (data not presented). Although the 51-kD band was most clearly associated with the tonoplast, it did not cross-react with antibodies raised against the 57- and 68-kD subunits of the tonoplast $H^+$-ATPase or the tonoplast $H^+$-PPase from *V. radiata* (Fig. 8).

## DISCUSSION

Surprisingly little quantitative information about the effects of Al on protein synthesis in plants is available, and that which is available is somewhat contradictory. Aniol (1984) reported that treatment with moderate to high concentrations of Al increased incorporation of $^{14}$C-Val into a TCA-insoluble fraction isolated from root tips of an Al-resistant (Atlas 66) and an Al-sensitive (Grana) cultivar of *T. aestivum*. Higher concentrations (>600 $\mu$M in cv Atlas 66, >74 $\mu$M in cv Grana) reduced incorporation of $^{14}$C in both cultivars. In contrast, Rincon and Gonzales (1991) reported that concentrations as low as 4 $\mu$M reduced incorporation of $^{35}$S into a TCA-insoluble fraction isolated from root tips of the Al-sensitive cv TAM 105. Although experimental data were not provided to support their claim, observations by Rincon and Gonzales (1991) are in accord with several qualitative reports of reduced protein synthesis in *T. aestivum* (Ownby and Hruschka, 1991) and *Medicago sativa* (Campbell et al., 1994), as well as a quantitative report of reduced protein export to the apoplasm in *T. aestivum* (Basu et al., 1994b). The results presented here provide the first quantitative report, to our knowledge, of decreased protein synthesis over a broad range of stress conditions. Analysis of $^{35}$S incorporation into TCA-insoluble fractions isolated from a crude root filtrate (total

**Table I.** *Distribution of specific activities and enrichments of marker enzymes in membrane fractions isolated from root tips of T. aestivum*

Plasma membrane and intracellular membranes were isolated from microsomal membrane preparations using aqueous two-phase partitioning (6.5% Dextran T500 and 6.5% PEG). The tonoplast was isolated from intracellular membranes using a two-step Dextran T70 gradient (2 and 10%). Data are representative for six independent membrane preparations from control and Al-treated plants. Although absolute values varied among preparations, triplicate analysis within one experiment deviated less than 10% from the mean value.

| Marker Enzyme (Cellular Location) | Membrane Fractions | | | | | | |
|---|---|---|---|---|---|---|---|
| | Microsomal Activity | Plasma Membrane | | Intracellular Membranes | | Tonoplast | |
| | | Activity | Enrichment | Activity | Enrichment | Activity | Enrichment |
| | | *μmol mg⁻¹ protein min⁻¹* | | | | | |
| $K^+$, $Mg^{2+}$, ATPase (plasma membrane) | 0.021 | 0.14 | 6.6 | 0.02 | 0.9 | 0.024 | 1.1 |
| Cyt *c* oxidase (mitochondria) | 0.031 | 0.0032 | 0.1 | 0.063 | 2.0 | 0.0045 | 0.1 |
| NADH-Cyt *c* reductase (ER) | 0.025 | 0.018 | 0.7 | 0.026 | 1.0 | 0.022 | 0.9 |
| $NO_3^-$-sensitive ATPase (tonoplast) | 0.011 | 0.001 | 0.1 | 0.015 | 1.4 | 0.021 | 1.9 |

protein), a low-speed pellet (containing nuclear and mitochondrial proteins), a high-speed pellet (containing microsomal proteins), and a high-speed supernatant (containing cytosolic proteins) demonstrated that 10 μM Al was sufficient to reduce protein synthesis in the Al-sensitive cv Katepwa. In the Al-resistant cv PT741, $^{35}S$ incorporation was unaffected by treatment with 10 μM Al, and little or no effect was observed at 75 μM Al (Fig. 2).

Despite the general lack of information about the effect of Al on protein synthesis, Aniol (1984) speculated that resistance to Al in *T. aestivum* might be mediated by Al-induced proteins that are encoded by nuclear genes. Unfortunately, experimental support for the existence of such resistance proteins is lacking. The appearance of Al-induced or Al-enhanced proteins is a common feature of several recent studies (Ownby and Hruschka, 1991; Picton et al., 1991; Rincon and Gonzales, 1991; Cruz-Ortega and Ownby, 1993; Basu et al., 1994a, 1994b; Campbell et al., 1994); however, many of these changes in protein expression are probably a consequence of Al toxicity. Although it is possible that enhanced expression of the 51-kD proteins also reflects the toxic effects of Al, several characteristics of these proteins suggest a potential role in mediating resistance. Basu et al. (1994a) demonstrated that these proteins are strongly induced in the Al-resistant cv PT741, but only weakly expressed in the Al-sensitive cv Neepawa. They are localized at the root tip (2 cm), the region of the root most sensitive to Al stress (Ryan et al., 1993), and accumulate in a dose- and time-dependent fashion. After removal of Al from the growth medium, the proteins return to control levels over a period of 72 h (Basu et al., 1994a). We now report that these proteins are enhanced only in resistant progeny of an $F_2$ population segregating for resistance to Al (Fig. 4), the first report to our knowledge of a protein that cosegregates with the resistance phenotype. Induction is not observed in the Al-sensitive cv Katepwa over a broad

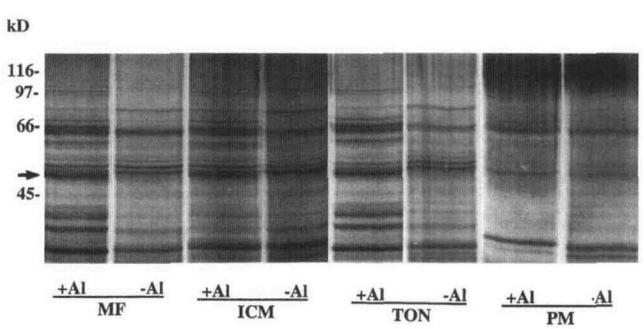


**Figure 7.** Polypeptide profiles of the microsomal (MF), intracellular membrane (ICM), tonoplast (TON), and plasma membrane (PM) fractions from root tips of *T. aestivum* grown in the presence (+Al) and absence (−Al) of Al. Three-day-old plants were treated with 100 μM $AlCl_3$ for 72 h. The plasma membrane and intracellular membranes were isolated using an aqueous two-phase system (6.5% Dextran T500 and 6.5% PEG). The tonoplast was isolated from the ICM using a two-step gradient (2 and 10%) of Dextran T70. SDS-PAGE was performed as described in "Materials and Methods." Polypeptide bands were visualized by silver-staining. The molecular masses of marker proteins are given in kilodaltons.

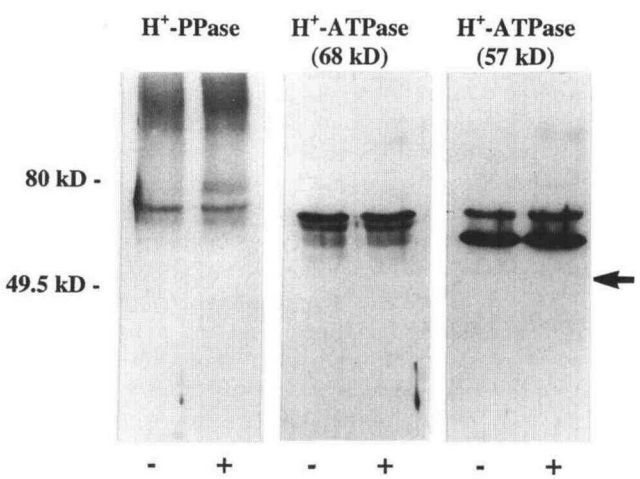


**Figure 8.** Western blots of microsomal membrane proteins from roots of *T. aestivum* cv PT741 grown in the presence (+) or absence (−) of Al. Microsomal proteins (30 μg) were separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. Proteins were probed with rabbit anti-tonoplast $H^+$-PPase, anti-tonoplast $H^+$-ATPase (68-kD subunit), and anti-tonoplast $H^+$-ATPase (57-kD subunit), followed by goat anti-rabbit IgG conjugated horseradish peroxidase. Blots were developed with a chemiluminescent detection reagent. The molecular masses of marker proteins are given and the position of the 51-kD band is indicated by an arrow.

range of concentrations (Fig. 5) and times of exposure (Fig. 6). Although enhanced expression of the 51-kD band has only been detected after 6 h of exposure, the possibility remains that more sensitive techniques for detection would show more rapid induction. Furthermore, reports of induced resistance to Al (Aniol, 1984; Cumming et al., 1992) suggest that a period of acclimation during which growth rate is reduced may be required before full resistance is achieved. The fact that the 51-kD band is specifically labeled with short-term exposure of plants to [$^{35}$S]Met (Fig. 1), and the observation that cycloheximide caused the disappearance of the 51-kD band under conditions of continuous Al exposure (Basu et al., 1994a), indicates that protein accumulation reflects de novo synthesis rather than Al-induced degradation. This enhanced synthesis of the 51-kD protein (Fig. 1) occurs despite a general decline in total membrane protein (Fig. 2).

As a first step in identifying a possible function for these proteins, we have demonstrated that the 51-kD band is most clearly associated with membranes enriched in the tonoplast. Although our tonoplast preparations show an 1.9-fold enrichment of $NO_3^-$-sensitive ATPase activity (tonoplast marker) and a 90% loss of Cyt *c* oxidase activity (mitochondria marker), the activities of the $K^+$-stimulated, $Mg^{2+}$-dependent ATPase (plasma membrane marker), and NADH-Cyt *c* reductase (ER marker) were similar to microsomal preparations (Table I). The enrichment of the 51-kD band in tonoplast preparations, coupled with the loss of Cyt *c* oxidase activity, suggest that these proteins are not located in mitochondrial membranes. Furthermore, the lack of a clearly identifiable 51-kD band and the strong enrichment of $K^+$-stimulated, $Mg^{2+}$-dependent ATPase activity in plasma membrane preparations suggests that these proteins are not located at the plasma membrane. Although the greatest enrichment of the 51-kD band and the unique enrichment of $NO_3^-$-sensitive ATPase activity in tonoplast preparations suggest that these proteins are most likely located at the tonoplast, it remains possible that one or more of these proteins is associated with endomembranes other than the tonoplast.

The possibility that one or both of our 51-kD proteins might be localized at the tonoplast is interesting in light of several recent reports. Matsumoto (1991) and Kasai et al. (1992, 1993) demonstrated that treatment of intact plants with Al increased the ATP-dependent and pyrophosphate-dependent proton-pumping activity of microsomal membrane and tonoplast-enriched vesicles isolated from roots of *Hordeum vulgare*. The inward-directed proton-pumping activity of the tonoplast is mediated by a proton-translocating ATPase ($H^+$-ATPase) and a proton-translocating inorganic pyrophosphatase ($H^+$-PPase; Rea and Sanders, 1987; Taiz, 1992). Thus, these data suggest that the activities of these transport proteins are increased by treatment with Al in vivo (although a supply of Al in vitro inhibits proton pumping activity; Matsumoto, 1988, 1991). Matsumoto (1991) and Kasai et al. (1992, 1993) speculated that enhanced proton pumping activity of the tonoplast may be an adaptive response designed to (a) maintain pH in the cytoplasm, which could decline with an Al-induced decrease in proton pumping across the plasma membrane, or (b) sequester Al within the vacuole via an unspecified $H^+$/Al-exchange mechanism.

Sequestration of metals in the vacuole as complexes with malate, citrate, and phosphate has been proposed as a mechanism of resistance to a number of different metals, although evidence that Al may be immobilized in the vacuole is still limited (Taylor, 1988, 1991). Because uptake of ions across the tonoplast is driven by the electrochemical gradient established by the vacuolar $H^+$-ATPase and $H^+$-PPase (Taiz, 1992), the link between Al exposure, increased proton-pumping activity at the tonoplast, and induction of a membrane-bound protein at the tonoplast provides conceptual support for the possible role of the vacuole as a sink for symplastic Al. This possible role, however, is not supported by the results of our immunoblotting analysis. Although the subunit composition of the purified enzyme from different plant materials shows some variation (Sze et al., 1992), the $H^+$-ATPase is now believed to consist of 10 different polypeptides with molecular weights ranging from 12 to 70 kD (Ward and Sze, 1992). Antibodies raised against the 57- and 68-kD subunits of the tonoplast $H^+$-ATPase from *V. radiata* failed to cross-react with the 51-kD band. These data suggest that our Al-induced polypeptides are not a component of this multimeric enzyme, although this is still a possibility. In contrast to the $H^+$-ATPase, the $H^+$-PPase appears to be a homomultimer of three to five identical subunits of approximately 66 kD (Britten et al., 1989; Maeshima, 1990; Rea and Poole, 1993), considerably higher than the 51-kD Al-induced proteins reported here. Perhaps not surprisingly, antibodies raised against the $H^+$-PPase from *V. radiata* also failed to cross-react with the 51-kD band, suggesting that our Al-induced proteins are not a component of the $H^+$-PPase.

Snowden and Gardner (1993) and Richards et al. (1994) identified a total of seven different cDNA clones (*wali*1–*wali*7) with transcript levels induced by Al in an Al-resistant and an Al-sensitive cultivar of *T. aestivum*. Sequence analysis of these cDNAs demonstrated sequence homology to genes encoding for a plant metallothionein-like protein (*wali*1), Phe ammonium lyase (*wali*4), and a class of proteinase inhibitors (*wali*3, *wali*5, and *wali*6). The two remaining cDNAs (*wali*2 and *wali*7) showed little homology to any known sequences. At this point, it may be premature to speculate whether any of the *wali* genes might encode our 51-kD proteins; however, the fact that the *wali* genes are expressed in both resistant and sensitive genotypes argues against this point. Snowden et al. (1995) also demonstrated that transcript levels of *wali*1, *wali*3, *wali*4, and *wali*5 increased in root tips after treatment with excess Cd, Fe, Zn, Cu, Ga, In, and La. Low levels of Ca and wounding also increased transcript levels of *wali*3, *wali*4, and *wali*5. Thus, Snowden et al. (1995) envisioned that the *wali* genes might encode a series of general stress response genes. We previously reported that excess Cd and Ni were mildly effective in increasing levels of our 51-kD proteins; however, excess Cu, Mn, and Zn, along with heat shock and cold stress, were ineffective elicitors (Basu et al., 1994a). Thus, at first sight, our proteins appear to be distinct from those encoded for by the *wali* genes.

## ACKNOWLEDGMENT

Antibodies for the tonoplast $H^+$-ATPase and $H^+$-PPase were kindly provided by Dr. M. Maeshima (Laboratory of Biochemistry, School of Agricultural Sciences, Nagoya University, Japan).

Received October 15, 1996; accepted January 29, 1997.
Copyright Clearance Center: 0032–0889/97/114/0363/10.

## LITERATURE CITED

**Aniol A** (1984) Introduction of aluminium tolerance into aluminium-sensitive wheat cultivars. Z Pflanzenzeucht **93:** 331–339

**Aniol A** (1990) Genetics of tolerance to aluminium in wheat (*Triticum aestivum* L. Thell). Plant Soil **123:** 223–227

**Aniol A, Gustafson JP** (1984) Chromosome location of genes controlling aluminum tolerance in wheat, rye, and triticale. Can J Genet Cytol **26:** 701–705

**Basu A, Basu U, Taylor GJ** (1994a) Induction of microsomal membrane proteins in roots of an aluminum-resistant cultivar of *Triticum aestivum* L. under conditions of aluminum stress. Plant Physiol **104:** 1007–1013

**Basu U, Basu A, Taylor GJ** (1994b) Differential exudation of polypeptides by roots of aluminum-resistant and aluminum-sensitive cultivars of *Triticum aestivum* L. in response to aluminum stress. Plant Physiol **106:** 151–158

**Basu U, Godbold D, Taylor GJ** (1994c) Aluminum resistance in *Triticum aestivum* associated with enhanced exudation of malate. J Plant Physiol **144:** 747–753

**Berzonsky WA** (1992) The genomic inheritance of aluminum tolerance in Atlas 66 wheat. Genome **35:** 689–693

**Bona L, Carver BF, Wright RJ, Baligar VC** (1994) Aluminum tolerance of segregating wheat populations in acidic soil and nutrient solutions. Commun Soil Sci Plant Anal **25:** 327–339

**Briskin DP, Leonard RT, Hodges TK** (1987) Isolation of the plasma membrane: membrane markers and general principals. *In* L Packer, R Douce, eds, Methods in Enzymology, Vol 148. Academic Press, San Diego, CA, pp 542–558

**Britten CJ, Turner JC, Rea PA** (1989) Identification and purification of substrate-binding subunit of higher plant $H^+$-translocating inorganic pyrophosphatase. FEBS Lett **256:** 200–206

**Camargo CE** (1981) Wheat improvement: the heritability of tolerance to aluminum toxicity. Bragantia **40:** 33–45

**Campbell LG, Lafever HN** (1981) Heritability of aluminum tolerance in wheat. Cereal Res Commun **9:** 281–287

**Campbell TA, Jackson PR, Xia ZL** (1994) Effects of aluminum stress on alfalfa root proteins. J Plant Nutr **17:** 461–471

**Cruz-Ortega R, Ownby JD** (1993) A protein similar to PR (pathogenesis-related) proteins is elicited by metal toxicity in wheat roots. Physiol Plant **89:** 211–219

**Cumming JR, Buckelew Cumming A, Taylor GJ** (1992) Patterns of root respiration associated with the induction of aluminium tolerance in *Phaseolus vulgaris* L. J Exp Bot **43:** 1075–1081

**Delhaize E, Craig S, Beaton CD, Bennet RJ, Jagadish VC, Randall PJ** (1993a) Aluminum tolerance in wheat (*Triticum aestivum* L.). 1. Uptake and distribution of aluminum in root apices. Plant Physiol **103:** 685–693

**Delhaize E, Higgins TJV, Randall PJ** (1991) Aluminum tolerance in wheat: analysis of polypeptides in the root apices of tolerant and sensitive genotypes. *In* RJ Wright, VC Baligar, RP Murrman, eds, Plant-Soil Interactions at Low pH. Proceedings of the Second International Symposium on Plant-Soil Interactions at Low pH, June 24–29, 1990, Beckley, WV. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1071–1079

**Delhaize E, Ryan PR, Randall PJ** (1993b) Aluminum tolerance in wheat (*Triticum aestivum* L.). II. Aluminum-stimulated excretion of malic acid from root apices. Plant Physiol **103:** 695–702

**Kasai M, Sasaki M, Tanakamaru S, Yamamoto Y, Matsumoto H** (1993) Possible involvement of abscisic acid in increases in activities of two vacuolar $H^+$-pumps in barley roots under aluminum stress. Plant Cell Physiol **34:** 1335–1338

**Kasai M, Sasaki M, Yamamoto Y, Matsumoto H** (1992) Aluminum stress increases $K^+$ efflux and activities of ATP- and PPi-dependent $H^+$ pumps of tonoplast-enriched membrane vesicles from barley roots. Plant Cell Physiol **33:** 1035–1039

**Kerridge PC, Kronstad WE** (1968) Evidence of genetic resistance to aluminum toxicity in wheat (*Triticum aestivum* Vill., Host). Agron J **60:** 710–711

**Laemmli UK** (1970) Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

**Larsson C, Sommarin M, Widell S** (1994) Isolation of highly purified plant plasma membranes and separation of inside-out and right-side-out vesicles. *In* H Walter, G Johansson, eds, Methods in Enzymology, Vol 228. Academic Press, San Diego, CA, pp 451–469

**Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ** (1951) Protein measurement with the Folin reagent. J Biol Chem **193:** 265–275

**Maeshima M** (1990) Oligomeric structure of $H^+$-translocating inorganic pyrophosphatase of plant vacuoles. Biochem Biophys Res Commun **168:** 1157–1162

**Maeshima M, Yoshida S** (1989) Purification and properties of vacuolar membrane proton-translocating inorganic pyrophosphatase from mung bean. J Biol Chem **264:** 20068–20073

**Matsumoto H** (1988) Inhibition of proton transport activity of microsomal membrane vesicles of barley roots by aluminium. Soil Sci Plant Nutr **34:** 499–526

**Matsumoto H** (1991) Biochemical mechanism of the toxicity of aluminium and the sequestration of aluminium in plant cells. *In* RJ Wright, VC Baligar, RP Murrmann, eds, Plant-Soil Interactions at Low pH. Proceedings of the Second International Symposium on Plant-Soil Interactions at Low pH, June 24–29, 1990, Beckley, WV, Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 825–838

**Matsuura-Endo C, Maeshima M, Yoshida S** (1992) Mechanism of the decline in vacuolar $H^+$-ATPase activity in mung bean hypocotyls during chilling. Plant Physiol **100:** 718–722

**Moroni JS, Briggs KG, Taylor GJ** (1991) Pedigree analysis of the origin of manganese tolerance in Canadian spring wheat (*Triticum aestivum* L.) cultivars. Euphytica **56:** 107–120

**Ownby JD, Hruschka WR** (1991) Quantitative changes in cytoplasmic and microsomal proteins associated with aluminum toxicity in two cultivars of winter wheat. Plant Cell Environ **14:** 303–309

**Picton SJ, Richards KD, Gardner RC** (1991) Protein profiles in root-tips of two wheat (*Triticum aestivum* L.) cultivars with differential tolerance to aluminium. *In* RJ Wright, VC Baligar, RP Murrmann, eds, Plant-Soil Interactions at Low pH. Proceedings of the Second International Symposium on Plant-Soil Interactions at Low pH, June 24–29, 1990, Beckley, WV. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1063–1070

**Rea PA, Poole RJ** (1993) Vacuolar $H^+$-translocating pyrophosphatase. Annu Rev Plant Physiol Plant Mol Biol **44:** 157–180

**Rea PA, Sanders D** (1987) Tonoplast energization: two $H^+$ pumps, one membrane. Physiol Plant **71:** 131–141

**Richards KD, Snowden KC, Gardner RC** (1994) *Wali6* and *wali7*. Genes induced by aluminum in wheat (*Triticum aestivum* L.) roots. Plant Physiol **105:** 1455–1456

**Rincon M, Gonzales RA** (1991) Induction of protein synthesis by aluminium in wheat (*Triticum aestivum* L.) root tips. *In* RJ Wright, VC Baligar, RP Murrmann, eds, Plant-Soil Interactions at Low pH. Proceedings of the Second International Symposium on Plant-Soil Interactions at Low pH, June 24–29, 1990, Beckley, WV. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 851–858

**Ryan PR, DiTomaso JM, Kochian LV** (1993) Aluminium toxicity in roots: an investigation of spatial sensitivity and the role of the root cap. J Exp Bot **44:** 437–446

**Ślaski JJ** (1995) $NAD^+$ kinase activity in root tips of nearly isogenic lines of wheat (*Triticum aestivum* L.) that differ in their tolerance to aluminium. J Plant Physiol **145:** 143–147

**Snowden KC, Gardner RC** (1993) Five genes induced by aluminum in wheat (*Triticum aestivum* L.) roots. Plant Physiol **103:** 855–861

**Snowden KC, Richards KD, Gardner RC** (1995) Aluminum-induced genes. Induction by toxic metals, low calcium, and wounding and pattern of expression in root tips. Plant Physiol **107:** 341–348

**Sze H, Ward JM, Lai S, Perera I** (1992) Vacuolar-type $H^+$-translocating ATPases in plant endomembranes: subunit organization and multigene families. J Exp Biol **172:** 123–135

**Taiz, L** (1992) The plant vacuole. J Exp Biol **172:** 113–122

**Taylor GJ** (1988) The physiology of aluminum tolerance. *In* H Sigel, ed, Metal Ions in Biological Systems, Vol 24. Aluminum and Its Role in Biology. Marcel Dekker, New York, pp 165–198

**Taylor GJ** (1991) Current views of the aluminum stress response: the physiological basis of tolerance. *In* DD Randall, DG Blevins, CD Miles, eds, Current Topics in Plant Biochemistry and Physiology, 1991. Ultraviolet-B Radiation Stress, Aluminum Stress, Toxicity and Tolerance, Boron Requirements, Stress and Toxicity, Vol 10. Interdisciplinary Plant Biochemistry and Physiology Program, University of Missouri, Columbia, pp 57–93

**Taylor GJ** (1995) Overcoming barriers to understanding the cellular basis of aluminium resistance. Plant Soil **171:** 89–103

**Taylor GJ, Foy CD** (1985a) Mechanisms of aluminum tolerance in *Triticum aestivum* L. (wheat) I. Differential pH induced by winter cultivars in nutrient solutions. Am J Bot **72:** 695–701

**Taylor GJ, Foy CD** (1985b) Mechanisms of aluminum tolerance in *Triticum aestivum* L. (wheat) II. Differential pH induced by spring cultivars in nutrient solutions. Am J Bot **72:** 702–706

**Ward JM, Sze H** (1992) Subunit composition and organization of the vacuolar $H^+$-ATPase from oat roots. Plant Physiol **99:** 170–179

**Zhang G, Archambault DJ, Ślaski JJ, Taylor GJ** (1995) Effects of protein synthesis inhibitors on uptake of aluminum in aluminum-resistant and aluminum-sensitive cultivars of wheat. J Plant Physiol **147:** 457–462

**Zhang G, Hoddinott J, Taylor GJ** (1994) Characterization of 1,3-β-D-glucan (callose) synthesis in roots of *Triticum aestivum* in response to aluminum toxicity. J Plant Physiol **144:** 229–234