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A 23-kDa, root exudate polypeptide co-segregates with aluminum resistance in *Triticum aestivum*

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We previously reported that treatment with aluminum (Al) leads to the accumulation of several polypeptides (12-, 23-, and 43.5-kDa) in root exudates of an Al-resistant cultivar of *Triticum aestivum*. In this report, we examine the segregation of the 23-kDa, Al-induced polypeptide and the Al-resistant phenotype in single F_2 plants arising from a cross between Al-resistant and Al-sensitive doubled-haploid (DH) lines. Single plants and plant populations were screened for sensitivity/resistance to Al using synthesis of 1,3-β-glucans (callose) as a sensitive marker for Al injury. Callose production in the Al-sensitive cv. Katepwa was approximately 3-fold higher than observed in the Al-resistant cv. Maringa, or a near-isogenic line derived from Katepwa and Maringa (Alikat), over a broad range of Al concentrations (0–100 μM). Similar results were observed with DH lines developed from cv. Katepwa, which produced two–four times more callose than DH lines developed from cv. Alikat. When single plants from F_1 and F_2 populations derived from a cross between DH Katepwa and DH Alikat were scored for Al-induced callose production after 4 days exposure to 100 μM Al, all F_1 plants were Al-resistant and F_2 plants segregated approximately 3:1 for Al-resistance/sensitivity. A backcross population derived from crossing Al-resistant F_1 with Al-sensitive Katepwa, segregated 1:1 for Al-resistance/sensitivity. Thus, the Al-resistant phenotype is inherited in a monogenic, dominant fashion in our DH lines. Enhanced accumulation of the Al-induced, 23-kDa polypeptide in root exudates was a trait which co-segregated with the Al-resistant phenotype in F_2 populations. This polypeptide was strongly labeled with ^{35}S-methionine after 3 days of Al exposure and 6 h labeling. When root exudate polypeptides were separated by immobilized metal ion affinity chromatography, the 23-kDa polypeptide demonstrated significant Al-binding capacity. This polypeptide has been purified to near-homogeneity, providing an opportunity to isolate the gene(s) encoding this polypeptide.

Introduction

Genetically based variability in resistance to aluminum (Al) within *Triticum aestivum* has been widely reported and used by plant breeders to develop Al-resistant cultivars. While several studies have suggested that a single dominant gene controls Al resistance (Delhaize et al. 1993a,b), others have implicated genes from multiple sites on multiple chromosomes (Aniol 1990, Berzonsky 1992, Bona et al. 1994). Carver and Ownby (1995) argued that the wide range of Al tolerance observed in wheat cannot be explained on the basis of a single-gene theory. Nonetheless, they acknowledged that single-cross populations may segregate for a single-gene pair. As we begin to appreciate the complexity of Al resistance, the importance of developing genetic systems in which differences between genotypes are controlled at a single locus has become apparent. Development of near-isogenic systems provide a powerful tool for the study of Al resistance mechanisms (see, for example, Aniol 1995, Basu et al. 1997, Delhaize and Ryan 1995, Delhaize et al. 1993a,b, Kochian 1995, Pellet et al. 1995, 1996, Slaski 1995, Somers and Gustafson 1995, Somers et al. 1996, Taylor et al. 1997a,b).

Recent studies have focused on the potential role of root exudates in resistance to Al (Delhaize and Ryan 1995, Kochian 1995, Taylor 1988, 1991). In several studies, Al-in-

Abbreviations – DH, doubled-haploid; PE, pachyman equivalents.

duced release of organic anions from roots of Al-resistant genotypes has been demonstrated (Basu et al. 1994b, Delhaize et al. 1993b, Miyasaka et al. 1991, Pellet et al. 1995, 1996, Ryan et al. 1995). For example, Delhaize et al. (1993b) demonstrated that Al-induced exudation of malate is a trait that co-segregates with the Al-resistant phenotype in near-isogenic wheat lines that differed in Al tolerance at a single dominant locus. This increased exudation appears to reflect both increased synthesis and export of malate to the apoplasm, since malate present in root exudates is rapidly labeled with ^{14}C (Basu et al. 1994b). Recently, Pellet et al. (1996) suggested that exudation of phosphate from the root apex may be another mechanism of Al resistance that may be conditioned by different genes than those controlling Al-induced malate efflux. A variety of macromolecules are also exuded by roots, including isoflavanoids (Dakora et al. 1993), cytokinins (Grayling and Hanke 1992), and polypeptides (Brigham et al. 1995, Jun et al. 1996, Vancura 1964). Exogenous supply of acidic polypeptides, such as poly-L-aspartic acid and poly-L-glutamic acid, can alleviate the toxic effects of Al (Konishi et al. 1988, Putterill and Gardner 1988), although a role of root exudate polypeptides in Al resistance has not yet been established. In a previous study, we analyzed the suite of polypeptides exuded from roots of Al-resistant and Al-sensitive cultivars of *Triticum aestivum* grown in the presence and absence of Al (Basu et al. 1994a). We observed differences between cultivars in the amount of polypeptide exuded in response to Al stress, and showed enhanced accumulation of 12-, 23-, and 43.5-kDa polypeptides in the Al-resistant cv. Maringa. A greater association of Al with high molecular mass fractions (including the Al-induced polypeptides) from Al-resistant cultivars suggested that these polypeptides may have significant Al-binding capacity (Basu et al. 1994a).

In the present study, we developed DH, embryo-derived lines from the Al-sensitive cultivar, Katepwa, and the Al-resistant backcross isoline, Alikat. We then used these genetically homogeneous lines to explore the potential role of the 23-kDa, Al-induced polypeptide in mediating resistance to Al.

Materials and methods

Development of doubled-haploid lines

Two hexaploid wheat cultivars (*Triticum aestivum* L.) form the basis of work reported here; Maringa (Al-resistant) is a spring wheat derived from Frontana//Kenya 58/Ponta Grossa, and Katepwa (Al-sensitive) is a hard, red spring wheat derived from Neepawa6*/RL2938/3/Neepawa6*//C18154/2*Frocor. Near-isogenic lines were produced by crossing Maringa (male) with Katepwa (female) and backcrossing Al-resistant offspring to Katepwa for three generations (Katepwa3*/Maringa) resulting in Alikat.

Single plants of Katepwa, Maringa, and Alikat were grown in 7.5-cm pots until the 3–4 leaf stage, then transferred to 20-cm pots in soil mixture. Small and late tillers were trimmed in order to produce plants with approximately ten vigorous tillers. Spikes were emasculated 1 day before anthesis, and florets pollinated with freshly gathered pollen from maize. Pollinated florets were sprayed three times over 72 h with 2,4-D (75 mg l^{-1}) and two times over the following 48 h with GA_3 (1 g l^{-1}). Caryopses were harvested from the fertilized florets 14–16 days after pollination and were surface-sterilized with 70% (v/v) ethanol for 40 s, and 2.5% sodium hypochlorite (v/v) for 120 s. Immature embryos were dissected and removed from the caryopses, transferred into 30-ml square glass bottles with Gamborg 5 tissue culture medium. The embryos on culture media were subjected to cold treatment (5°C) in the dark for 3 days and placed under moderate light intensity for 14 h at 22°C. Approximately 70% of cultured embryos developed into small plantlets and 95% of these plantlets developed into haploid plants with half the chromosome number ($n = 21$) of the hexaploid wheat genome. Small plantlets were removed from the culture medium and transplanted into soil mixture. Plants were removed from soil at 3–4 tiller stage, and the base of each plant was washed with distilled water, treated with 0.2% (v/v) colchicine for 4 h, washed with running water at 20°C for 3 h, and transferred to soil. About 75% of the treated haploid plants gave rise to mixoploid plants having both haploid and diploid tillers. Diploid tillers resulting from chromosome doubling in haploid meristematic cells gave rise to fertile plants with a normal hexaploid chromosome complement. Homozygous, DH lines were then isolated from a single selfed DH plant. These lines were evaluated for Al resistance using 1,3-β-glucan synthase activity as a marker for Al injury to confirm that each line showed the same Al resistance or sensitivity as its parent (Taylor et al. 1997b). We previously showed that callose is an accurate and reliable measure of Al sensitivity/resistance in a variety of wheat cultivars (Slaski et al. 1996, Taylor et al. 1997a, Zhang et al. 1994). Single Al-resistant (RR) male DH plants derived from Alikat were crossed to Al-sensitive (rr) DH Katepwa females to produce F_1 seeds (Rr). These plants were backcrossed to Katepwa to produce populations segregating for Al resistance/sensitivity and allowed to self produce F_2 populations.

Sterile plant culture

Seeds were surface-sterilized in 1% sodium hypochlorite for 20 min, imbibed in sterile deionized water for 4–6 h, and grown in Petri dishes containing Murashige and Skoog medium solidified with 8 g l^{-1} agar (Murashige and Skoog 1962). After 48 h, healthy, sterile seedlings were selected and transferred to Magenta (Sigma, Oakville, ON, Canada) vessels (five plants per vessel) containing 60 ml of sterile liquid nutrient medium (pH 4.5) as described by Basu et al. (1994a). Plants were grown in the presence and absence of Al (100 μM) in a controlled-environment chamber with 16 h of light (20°C, 68% RH) and 8 h of darkness (16°C, 85% RH) for 5 days. At the termination of Al exposure, plants were removed, an aliquot of the growth media was plated onto solid MS media to ensure sterility, and the remainder stored at -20°C for further processing. In experiments with F_2 populations, single seedlings were transferred to each Magenta vessel containing an Al-free liquid nutrient solu-

tion (pH 4.5) for 3 days. Plants were then transferred to liquid nutrient solution (pH 4.5, ± 100 μ*M* Al) and grown for another 9 days. To maintain solution pH below 4.5, aliquots of ammonium chloride were added after 3 days of growth in treatment solutions to give a final concentration of 400 μ*M* in each vessel. At the end of the growth period, growth media were collected and stored at −20°C. Prior to analysis, growth media containing root exudates were thawed and concentrated using an Amicon pressure ultrafiltration system with Amicon YM 10 membranes (cutoff 10-kDa) at 4°C. Sample volumes were further reduced in a Savant SpeedVac concentrator (Savant Instruments Inc., Farmingdale, NY, USA).

SDS-PAGE analysis

SDS-PAGE analysis of concentrated root exudates was performed according to the method of Laemmli (1970) using a Mini-Protean II electrophoresis apparatus (Bio-Rad, Mississauga, Canada). Polypeptide samples were mixed with sample buffer (0.125 *M* Tris-HCl, 4% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] 2-mercaptoethanol) and incubated at 70°C for 10–12 min. Polypeptides were separated 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 (12.5% or 15% total monomer concentration, 2.7% cross-linking monomer solution). Polypeptide bands were visualized by silver staining (Basu et al. 1994a) and analyzed with an AlphaImager 2000 documentation and analysis system using AlphaEase 3.3b software (Alpha Innotech Corp., San Leandro, CA, USA). Polypeptide concentrations were determined by Coomassie Blue dye-binding with BSA as a standard (Bradford 1976).

Spectrofluorometric estimation of callose

Synthesis of 1,3-β-glucans (callose) in 5 mm root tips was employed as a marker for Al-induced injury using the spectrofluorometric procedure described by Zhang et al. (1994). Pachyman (Calbiochem, La Jolla, CA, USA) was used as an external standard and callose content was expressed as mg pachyman equivalents (PE) g^{-1} fresh weight.

Separation of the 23-kDa polypeptide by immobilized metal ion affinity chromatography

The ability of the 23-kDa polypeptide to bind Al was demonstrated by metal chelate ion affinity chromatography using Al-saturated iminodiacetic acid (insolubilized on Sepharose 6 B Fast Flow, activation: epoxy) column as the chromatographic support. The gel was packed to 4-cm height in 10-ml plastic columns and washed thoroughly with 10 ml of deionized water (18 mΩ) which had been further purified by passing through Chelex 100 resin (50–100 mesh, sodium form, Bio-Rad, Mississauga, Canada). The Al-affinity column was charged by incubating the resin in 5 ml of Al reference solution (1 mg ml^{-1}, Fisher) for 2 h and washed with 20 ml of Chelex-purified water to remove unbound Al (Al was not detectable in the last ten 1-ml fractions). Both Al-saturated and Al-unsaturated columns were loaded with 100 μg of root exudate polypeptide and eluted sequentially with 5 ml of Chelex-purified water, 5 ml of 100 m*M* potassium phthalate buffer (pH 4.0), 5 ml of 20 m*M* EDTA, and 5 ml of 100 m*M* EDTA at a flow rate of 15 ml h^{-1}. The EDTA fractions were dialyzed overnight against water at 4°C. All fractions were reduced to 50 μl and analyzed by SDS-PAGE. Affinity columns prepared without Al activation did not retain any exudate polypeptides.

Purification of the 23-kDa, Al-induced polypeptide

Concentrated root exudates were filtered through a 0.22-μm filter, loaded onto a Mono-Q ion exchange column (Type HR 5/5, 0.5 × 5 cm, 1 ml bed volume, Pharmacia, Uppsala, Sweden) and separated using a Pharmacia fast protein liquid chromatography system with 25 m*M* Tris-HCl, pH 7.5, as the running buffer (buffers were filtered through a 0.22-μm filter and deaerated overnight before use). Samples (1 mg protein [500 μl]$^{-1}$) were loaded on the column, washed with 4 ml of additional buffer, and the polypeptides were eluted using a 30-ml step gradient, 0, 20–40, 40–100% 25 m*M* Tris-HCl with 0.5 *M* NaCl, pH 7.5, at a flow rate of 0.5 ml min^{-1}. Eluted fractions were monitored using Pharmacia UV detector (280 nm), analyzed for their protein content, and subjected to SDS-PAGE and silver staining.

Further separation of the 23-kDa polypeptide from other polypeptides could not be achieved by gel filtration on Superose 12 or Sephadex G-50 (superfine, 2–30-kDa), Mono-P or Phenyl Sepharose CL-4B, or Hydroxylapatite (BioGel HT). Thus, Mono-Q eluted fractions showing the 23-kDa band were pooled from several runs and separated by SDS-PAGE. Gels were stained with 0.1% (w/v) Coomassie Blue R-250 for 15 min, followed by rapid destaining in 50% methanol/10% acetic acid until polypeptide bands were visible. The 23-kDa bands were excised and electroeluted using 10-kDa membrane cups in a 50 m*M* ammonium bicarbonate, 0.1% SDS as elution buffer for 5 h at 10 mA in a Bio-Rad electroelution apparatus. The electroeluted polypeptide was dried in Speed Vac and an aliquot was analyzed by SDS-PAGE to verify purity.

Results

In an effort to clarify a potential role for the 23-kDa, Al-induced, root exudate polypeptide in mediating resistance to Al, we studied the segregation of the polypeptide in an F_2 population derived from a cross between DH, Al-resistant Alikat (Katepwa3*/Maringa), and DH, Al-sensitive Katepwa. Single plants were screened for Al resistance/sensitivity using synthesis of 1,3-β-glucans (callose) as a sensitive, short-term marker of Al injury. Callose production in root tips of the Al-sensitive cv. Katepwa was 2–3-fold higher than in the Al-resistant cvs. Maringa and Alikat over a broad range of Al concentrations (0–100 μ*M*; Fig. 1). Similarly, root tips of single DH plants derived from cv. Katepwa showed 2–4-fold higher callose levels than root tips of DH Alikat and DH Maringa after 4 days of exposure to 100 μ*M* Al (Fig. 2A). Root tips from an F_1 population,

derived from a cross between Al-resistant (Alikat) and Al-sensitive (Katepwa) DH lines, all showed callose levels similar to the Al-resistant parent (≤ 1.3 mg PE g^{-1} fresh weight; Fig. 2B). The backcross population ($F_1 \times$ DH Katepwa) showed a 1:1 segregation ratio ($\chi^2 = 0.675$, $P > 0.05$; Fig. 2C). Thirty-one of 40 F_2 plants produced between 0.1–1.0 mg PE g^{-1} fresh weight and the remaining nine plants produced between 1.3–3.6 mg PE g^{-1} fresh weight (Fig. 2D). Thus, the callose content of F_2 plants showed a 3:1 ratio (resistant:sensitive; $\chi^2 = 0.133$, $P > 0.05$). In a second segregating F_2 population, an association between low callose levels and accumulation of the Al-induced 23-kDa polypeptide was observed. In this population, 27 F_2 plants showed callose content below 1.0 mg PE g^{-1} fresh weight after exposure to Al (indicating resistance to Al). In these plants, the 23-kDa band was clearly expressed. In the remaining 13 plants, callose contents were higher than 1.2 mg PE g^{-1} fresh weight (indicating sensitivity to Al), and all but one of the plants showed no detectable 23-kDa band (Fig. 3). The level of expression of the 23-kDa polypeptide in this single plant was lower than all 23 resistant plants. Thus, enhanced expression of the 23-kDa polypeptide consistently co-segregated with the Al-resistant phenotype.

The total amount of all proteins exuded by single plants ranged from 17.5 to 22.8 μg plant^{-1} for sensitive plants and 25.7 to 29.1 μg plant^{-1} for resistant plants (100 μ*M*, Fig. 4), consistent with previous observations that Al treatment reduces exudation of polypeptides to a greater extent in Al-sensitive than in Al-resistant plants (Basu et al. 1994a). This raises the possibility that the 23-kDa polypeptide may not have been identified in Al-sensitive plants because of greater perceived stress. Synthesis of a putative, Al-induced, 23-kDa polypeptide may have been inhibited by Al at higher concentrations. To test this hypothesis, cvs. Maringa and Katepwa were grown on media containing varying concentrations of Al (insufficient seeds for DH lines dictated the use of parental cultivars). Enhanced accumulation of the 23-kDa, Al-induced polypeptide was observed in cv. Maringa over a broad range of concentrations (50–200 μ*M*), after 120 h of Al exposure. The greatest accumulation

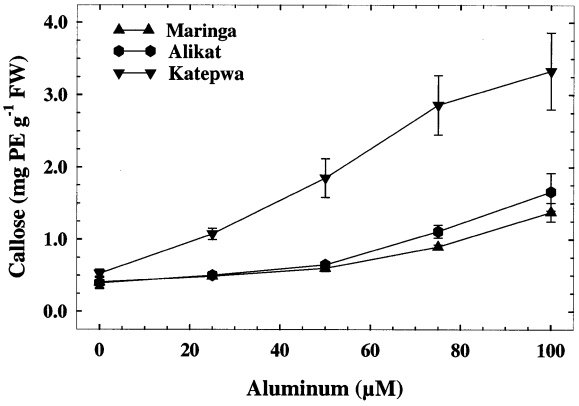

Fig. 1. The effect of Al on callose content of 5-mm root tips of cvs. Maringa (Al-resistant), Alikat (Al-resistant), and Katepwa (Al-sensitive). Plants were grown for 5 days in nutrient solution containing different concentrations of Al under aseptic conditions. Values for callose content are means ± SE of three replicates.

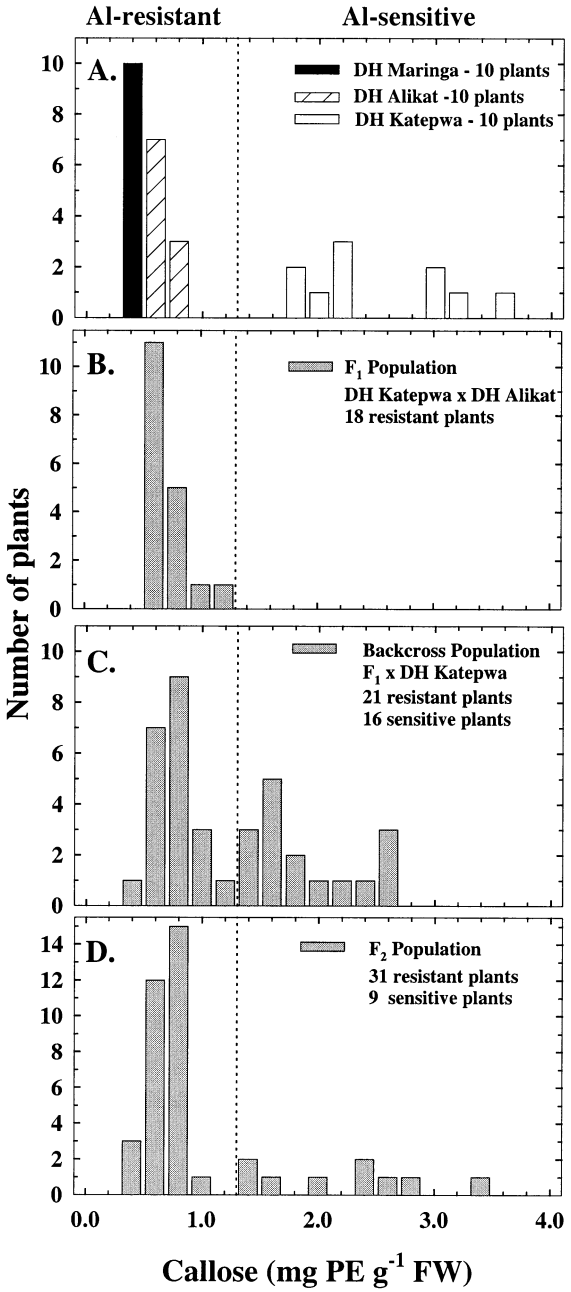

Fig. 2. Phenotypic distribution of callose content values in DH lines derived from Alikat (Al-resistant), Maringa (Al-resistant), and Katepwa (Al-sensitive) (panel A), F_1 (panel B), and F_2 (panel D) populations derived from a cross between DH Alikat and DH Katepwa, and a backcross population ($F_1 \times$ DH Katepwa; panel C). Plants were grown for 4 days in the absence of Al and then exposed to 100 μ*M* Al for 5 days. The distinction between Al-resistant and Al-sensitive plants (dotted line at 1.3 mg PE [g FW]$^{-1}$) was defined as the mid-point between the highest callose value for the Al-resistant, DH parent (Alikat) and the lowest value for the Al-sensitive, DH parent (Katepwa).

was observed at 100 μ*M* Al. In contrast, accumulation of 23-kDa polypeptide was not observed in cv. Katepwa, irrespective of the concentration of Al (Fig. 5). Based upon the callose dose-response data presented in Fig. 1, plants of cv. Katepwa grown at 5–45 μ*M* Al experience the same level of

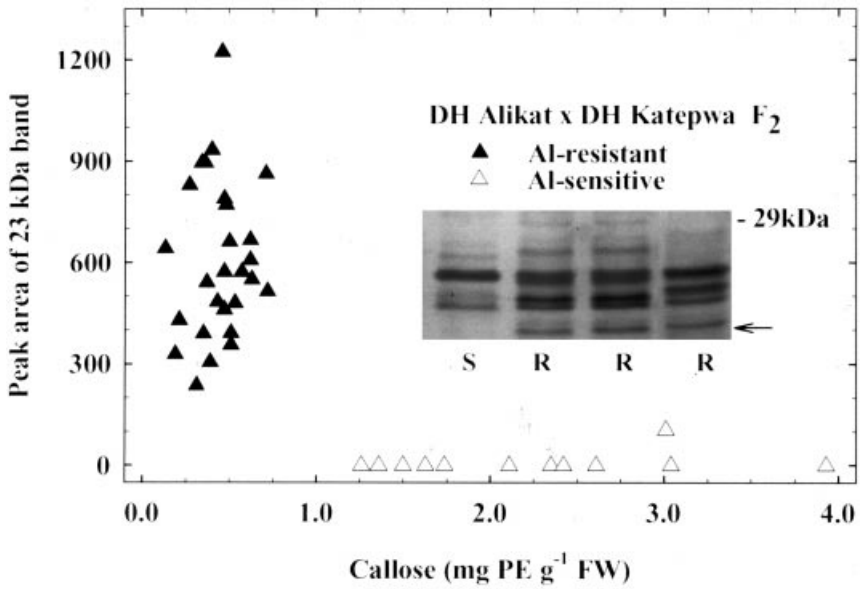

Fig. 3. Segregation analysis of the Al-induced, 23-kDa polypeptide and callose content in root tips (5 mm) of single F_2 plants arising from a cross between the DH Alikat and DH Katepwa. Seedlings were grown for 3 days in a complete nutrient solution without Al and then transferred to treatment solution containing 100 μ*M* Al. After 9 days of Al exposure, five root tips were harvested for callose estimation and root exudates were collected, concentrated, and analyzed on 12.5% SDS-gels (silver staining). Closed triangles indicate plants designated Al-resistant and the open triangles indicate plants designated Al-sensitive. Representative gel lanes with (R, Al-resistant) and without (S, Al-sensitive) the 23-kDa polypeptide are presented in the insert. Note, overlapping symbols show as a single triangle.

stress as plants of cv. Maringa and Alikat grown at 50–100 μ*M* Al. Nonetheless, the 23-kDa polypeptide was not detected in cv. Katepwa at 5, 10, 25, or 50 μ*M* Al. This indicates that the polypeptide is not expressed, or only weakly expressed, in cv. Katepwa under mild stress conditions.

We also investigated the effect of Al on synthesis of root exudate polypeptides by labeling roots with ^{35}S. When seedlings of cv. Maringa were grown in the presence or absence of Al for 3 days, labeled with [^{35}S]-methionine for 6 h, and grown for another 3 days in the fresh nutrient media ($\pm$ Al), the 23-kDa polypeptide was strongly labeled in the presence of Al, indicating de novo synthesis (Fig. 6). Interestingly, the polypeptide was also detected in control plants using the more sensitive ^{35}S assay (the 23-kDa band was not detected under control conditions using silver stain, Figs. 5 and 7). Several other polypeptides showed enhanced labeling after exposure to 100 μ*M* Al, including the 43.5-kDa Al-induced polypeptide (Basu et al. 1994a) and also polypeptides at 19-, 47-, 50-, and 52-kDa. Reduced labeling of 32- and 36-kDa polypeptides was clearly apparent (Fig. 6).

The ability of the Al-induced, 23-kDa polypeptide to bind Al was demonstrated by immobilized metal ion affinity chromatography. Root exudate polypeptides were passed through Al-saturated and unsaturated iminodiacetic acid columns. The columns were subsequently washed with water and eluted sequentially with 100 m*M* potassium phthalate buffer (pH 4.0), 20 m*M* EDTA, and 100 m*M* EDTA. When samples were loaded on columns without prior Al activation, all polypeptides eluted in the initial two washes. When samples were loaded onto Al-saturated columns, the 23-kDa polypeptide was not present in the water or the phthalate buffer washes, but was enriched in the 20 m*M* EDTA fraction (Fig. 7), demonstrating significant Al-binding capacity.

We have successfully purified the 23-kDa polypeptide to near homogeneity by combining Mono-Q ion exhange FPLC and electroelution. Root exudates were loaded onto a Mono-Q ion exchange column and the 23-kDa polypeptide was eluted from the column as a relatively broad peak between 0.1 and 0.15 *M* in the NaCl gradient. This elution pattern was reproducible through many preparations; however, a number of major polypeptides co-eluted in this peak (Fig. 8, compare lanes 1 and 2). Subsequent efforts to purify the 23-kDa polypeptide by gel filtration on Superose 12 or Sephadex G-50 (Superfine, 2–30-kDa), Mono-P, Phenyl Sepharose CL-4B, or hydroxylapatite (BioGel HT) were unsuccessful. As a result, we passed the Mono-Q immobilized polypeptides through a second Mono-Q column, separated the immobilized polypeptides on SDS-PAGE gels, and eluted the 23-kDa polypeptide from these gels (Fig. 8, lanes 3 and 4). The 23-kDa band comprised approximately 4% of the total polypeptide in the crude extract fraction, 14% of

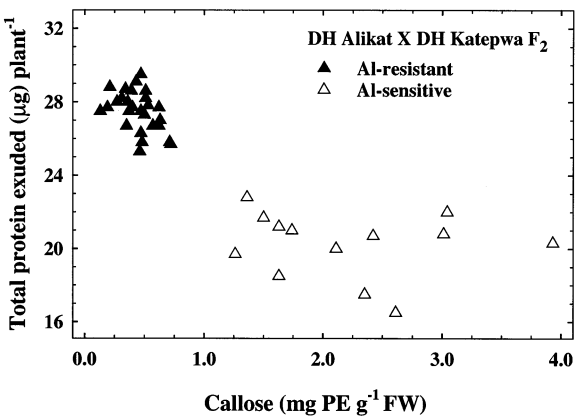

Fig. 4. Relationship between total protein exuded and callose content of root tips (5 mm) by single F_2 plants arising from a cross between DH Alikat and DH Katepwa. Seedlings were grown for 3 days in a complete nutrient solution without Al and then transferred to treatment solution containing 100 μ*M* Al. After 9 days of Al exposure, five root tips were harvested for callose estimation and root exudates were collected, concentrated, and their protein content analyzed. Closed triangles indicate plants designated Al-resistant and the open triangles indicate plants designated Al-sensitive.

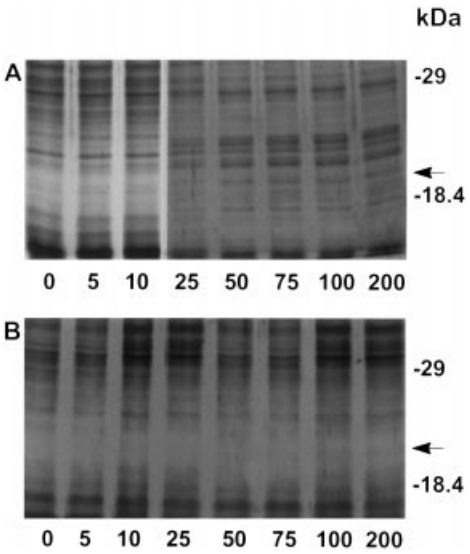

Fig. 5. The effect of varying concentrations of Al on polypeptide profiles of Maringa (A) and Katepwa (B). Plants were grown for 5 days in nutrient solution containing 0–200 μ*M* Al under aseptic conditions. Root exudates were collected, concentrated, and aliquots analyzed on 15% SDS gels (silver staining). The molecular masses of marker proteins are indicated in kDa. Arrows indicate the presence (Maringa) or absence (Katepwa) of the 23-kDa Al-induced polypeptide.

the total polypeptide after Mono-Q purification (Table 1), and 75% after electroelution as determined by the Bradford (1976) assay and densitometry of SDS-PAGE gels (Table 1 and Fig. 8).

Discussion

In an effort to explore the role of Al-induced root exudate polypeptides in mediating Al resistance, we developed near-isogenic backcross isolines from a cross between the Al-resistant cv. Maringa and the Al-sensitive cv. Katepwa (Katepwa3*/Maringa = Alikat) (Basu et al. 1997). Somers and Gustafson (1995) completed a genetic analysis of our germplasm and suggested that Al resistance had been successfully transferred from Maringa to Alikat, with one major gene controlling root growth in the presence of Al. Our own analysis of this material confirmed that resistance is indeed controlled by a single dominant gene, but transfer of resistance from Maringa to Alikat was incomplete (Taylor et al. 1997b). These conclusions are supported by our current data. Parental DH lines and F_1, backcross and F_2 populations derived from a cross between DH Katepwa and DH Alikat were scored for Al resistance/sensitivity using the callose assay. All single F_1 plants showed callose levels similar to the Al-resistant parent, the backcross population segregated for 1:1 for resistance/sensitivity, and the F_2 population segregated approximately 3:1 for Al resistance/sensitivity (Fig. 2), a pattern consistent with a single dominant gene inheritance. The callose assay also provided evidence for incomplete transfer of Al resistance from Maringa to Alikat. Accumulation of callose in root tips of DH Maringa (0.34 ± 0.01 mg PE g^{-1} fresh weight) was significantly less ($P < 0.001$) than in DH Alikat (0.53 ± 0.06 mg PE g^{-1} fresh weight) at 100 μ*M* Al (Fig. 2A). While quantitative differences in callose accumulation between Maringa and Alikat were relatively small and restricted to high levels of stress (Fig. 1), they were consistent with visual observations of foliar symptoms, which also suggest incomplete transfer of resistance from Maringa to Alikat (Taylor et al. 1997a).

The data presented here are consistent with the hypothesis that the 23-kDa, root exudate polypeptide plays a role in mediating Al resistance, but the role it plays in resistance is still unclear. When we measured accumulation of this polypeptide in single F_2 plants using silver staining, all resistant genotypes showed significant levels of the polypeptide, while only 1 of 13 sensitive plants showed detectable (trace) amounts. The polypeptide was detected in control plants using the more sensitive ^{35}S assay. Perhaps the polypeptide is required for normal plant function under control and Al stress conditions, but the quantitative requirement for the polypeptide increases under conditions of Al stress. Increased expression could reflect an increased requirement for that function under conditions of stress, or enhanced exudation required to counteract disruption of

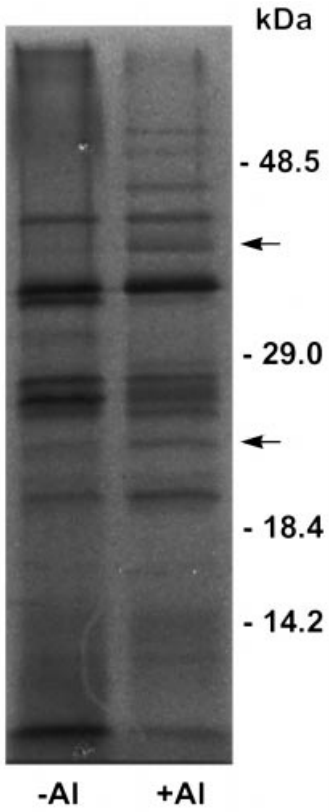

Fig. 6. SDS-PAGE analysis of ^{35}S-methionine-labeled root exudate polypeptides from cv. Maringa. Seedlings were grown in the presence (+ Al) and absence (− Al) of 100 μ*M* Al for 3 days, labeled with, L-[^{35}S]-methionine for 6 h, and grown for another 3 days in a fresh nutrient media (± Al). Root exudates were collected, polypeptides were concentrated, and equal cpm were loaded on 15% polyacrylamide gels. The molecular masses of marker proteins are indicated in kDa. The Al-induced polypeptides (23- and 43.5-kDa) identified by Basu et al. (1994a) are indicated by arrows. Each is prominently labeled under conditions of Al stress.

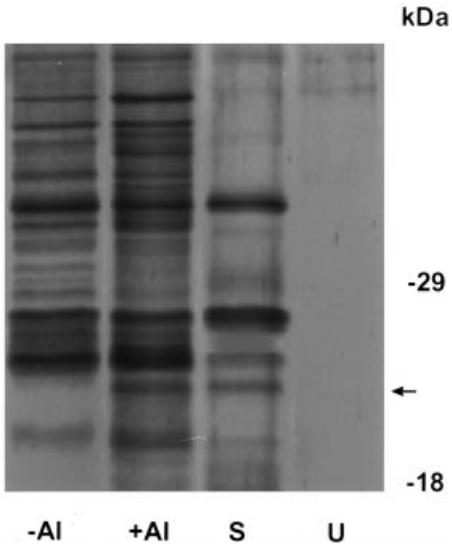

Fig. 7. SDS-PAGE analysis of root exudate fractions separated by immobilized metal ion affinity chromatography. Differences between plants grown in the presence (+ Al) and absence (− Al) of Al are shown for reference. Root exudate polypeptides from cv. Maringa grown in the presence of 100 μ*M* Al were loaded onto an Al-saturated (S) and Al-unsaturated (U) iminodiacetic acid column and eluted with 20 m*M* EDTA. Molecular mass markers are indicated in kDa on the right and the 23-kDa polypeptide is indicated by an arrow.

function, which might occur when Al forms a stable complex with the polypeptide. Differences in polypeptide profiles between resistant and sensitive lines (Fig. 5) were observed both at a specific Al concentration and at different concentrations producing a similar biological response (Fig. 1). Thus, differences in polypeptide abundance cannot be accounted for by differences in perceived stress. Thus, our data suggest that enhanced production of the 23-kDa polypeptide co-segregates with the Al-resistant phenotype. Our observation that the 23-kDa band is specifically labeled after short-term exposure of plants to ^{35}S-methionine indicates that de novo synthesis of polypeptide plays a role in accumulation of the polypeptide. This enhanced synthesis occurred despite a general decline in polypeptide exudation from Maringa under conditions of Al stress (Basu et al. 1994a).

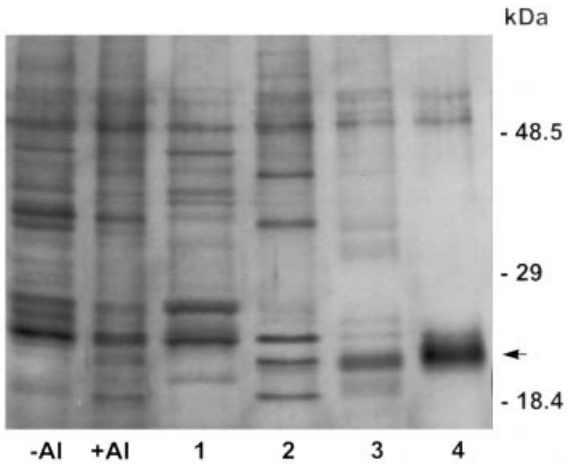

Fig. 8. Polypeptide profiles from various steps of the purification protocol resolved by 12.5% SDS-PAGE and stained with silver. Root exudates from plants of cv. Maringa grown in the absence (− Al) and presence (+ Al) of Al (5 μg protein lane^{-1}) are shown for reference. Lane 1 represents 5 μg of protein eluted in wash step of Mono-Q column (25 m*M* Tris HCl, pH 7.5), lane 2 represents 3 μg of protein eluted between 0.1 and 0.15 *M* NaCl gradient, lane 3 represents 2 μg of Mono-Q eluted proteins re-loaded onto Mono-Q and re-eluted with the same gradient as in the first Mono-Q elution, lane 4 represents 1.5 μg of electroeluted 23-kDa polypeptide. Molecular mass markers are indicated in kDa on the right and the 23-kDa polypeptide is indicated by an arrow.

Aluminum can bind reversibly to a number of macromolecules, including proteins, polynucleotides, and glycosides (MacDonald and Martin 1988). Several studies have demonstrated binding of Al to protein. Aluminum has been shown to induce tubulin assembly into microtubules and inhibit subsequent Ca^{2+}-induced depolymerization of the microtubules (MacDonald et al. 1987). It has also been shown to form a complex with the Ca^{2+}-binding protein, calmodulin (Siegel et al. 1983). Formation of Al complexes with acidic polypeptides such as poly-L-Asp, poly-L-Glu, and the serum protein transferrin have also been demonstrated (Konishi et al. 1988, Putterill and Gardner 1988). These polypeptides bind Al at very low concentrations and are able to relieve an Al-induced inhibition of calmodulin-activated cAMP phosphodiesterase activity (Putterill and Gardner 1988). In our previous study, root exudates were separated by ultrafiltration and the Al content was estimated in both protein and ultrafiltrate fractions. Approximately two times more Al was detected in polypeptide fractions from Al-resistant cultivars than similar fractions from the Al-sensitive cultivars (Basu et al. 1994a). This Al was not removed by dialysis against water; however, 70% of the total Al was released after protease digestion, indicating that differential binding reflected binding to polypeptides in root exudates (Basu et al. 1994a). The present work has confirmed the ability of the 23-kDa Al-induced polypeptide to bind Al using immobilized metal ion affinity chromatography.

While our data establish that the 23-kDa polypeptide can bind Al, it is important to recognize that direct evidence linking the actual binding of Al to this polypeptide and subsequent expression of Al resistance is lacking. Thus, these data cannot be interpreted as evidence that the 23-kDa polypeptide functions to chelate and detoxify Al within apoplasm. In fact, alternative hypotheses may provide a more attractive explanation for our data. Incomplete information about the amount of polypeptide excreted, the spatial distribution of the polypeptide in the apoplasm, and the stoichiometry and pH dependence of Al binding preclude quantitative analysis of the potential for Al binding. Nonetheless, the relatively small amount of polypeptide detected in the growth solution raises questions about the degree of protection that Al binding could provide. It remains possible that the capacity of the polypeptide to bind Al is incidental to its function in the apoplasm. Increased synthesis and export could provide enhanced enzymatic activity, which carries adaptive significance or may simply serve to counteract loss of function that accompanies formation of a stable Al-polypeptide complex.

Table 1. Purification of the 23-kDa Al-induced polypeptide from root exudates of cv. Maringa (Al-resistant). Concentrated root exudates grown in the presence of Al were loaded onto Mono-Q ion exchange column and separated using a 30-ml step gradient. Twenty-three kilodalton bands were excised from Coomassie stained gels and electroeluted. Total protein was estimated using the Bradford assay and values for percent total protein were estimated by densitometric analysis.

Fraction	Total protein (μg)	Total protein (%)	Yield (μg)
Crude fraction	1 000	4	40
Mono-Q fraction	40	14	5.6
Electroeluted protein	3	75	2.25

As a first step in identifying the gene encoding the 23-kDa, root exudate polypeptide, we have purified the polypeptide to near-homogeneity by Mono Q-FPLC and electroelution. We hope that comparison of amino acid sequence data to databank sequences might provide information about the homology of this polypeptide to other Al-induced or stress-related polypeptides. Cruz-Ortega and Ownby (1993) identified an Al-induced, acidic polypeptide from Al-stressed roots of wheat. Strong homology to pathogenesis-related proteins PR2 from parsley cultures was reported (Cruz-Ortega and Ownby 1993). Richards et al. (1994) and Snowden and Gardner (1993) identified seven cDNA clones coding for Al-induced proteins in *Triticum aestivum*. Six of these genes showed an Al-induced increase in transcript levels in both Al-resistant and Al-sensitive cultivars. The proteins encoded by these cDNAs showed homology to the metallothionein-like proteins (*wali* 1), phenylalanine ammonia-lyase (*wali* 4), proteinase inhibitors (*wali* 3, *wali* 5 and *wali* 6), and part of plant Asn synthetases (*wali* 7) (Snowden et al. 1995). Cruz-Ortega et al. (1997) and Ezaki et al. (1995, 1996) also identified several cDNA clones whose expression is induced by Al toxicity. These clones showed homology to the *par*A and *par*B genes of *Nicotiana tabacum*, which encode an auxin-regulated gene and glutathione S-transferase (Ezaki et al. 1995). Al-induced genes encoding an anionic peroxidase (Ezaki et al. 1996), a 1,3-β-glucanase, and a fimbrin-like cytoskeletal protein (Cruz-Ortega et al. 1997) have also been reported.

While it is not yet possible to infer function, the segregation of the 23-kDa polypeptide with the Al-resistant phenotype in F_1 and F_2 populations derived from crosses between Al-resistant and Al-sensitive DH plants is consistent with a role in mediating Al resistance. Further studies aimed at identifying and characterizing the gene for this polypeptide and studies evaluating enhanced exudation of the polypeptide and other root exudates in this unique genetic system could provide more substantive evidence concerning the precise role the polypeptide plays in Al resistance.

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