Five Genes Induced by Aluminum in Wheat (Triticum aestivum L.) Roots

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Aluminum in the earth's crust is the most abundant metal, comprising approximately 7.5% by weight (Haug, 1984). In soils with a pH of ≥5, Al is predominantly bound as insoluble oxides and complex aluminosilicates. However, as soil pH drops there is a release of ionic Al³⁺ and a reduction in the availability of exchangeable cations such as Ca²⁺, Mg²⁺, and K⁺. Ionic Al can be toxic to plants at micromolar concentrations (Kinraide and Parker, 1989).

Low pH values (below 5) occur naturally in many volcanic and tropical soils. In addition, the amount of acidity introduced by fertilizers and "acid rain" has outstripped the buffering capacities of soils in many areas, leading to toxic levels of Al (van Breemen, 1985). There is a need to improve agricultural output on these infertile soils and also to understand the causes of forest dieback on acid soils. This situation has spurred a large body of research into the mechanisms and physiology of Al toxicity in plants and resulted in breeding programs aimed at producing Al-tolerant varieties (see reviews by Foy et al., 1978; Taylor, 1991).

The phenotypic effect of Al toxicity is an inhibition of root elongation and subsequent inhibition of plant growth (Clarkson, 1965). However, in spite of the large amount of research on Al toxicity, there is no consensus on the physiological mechanism(s) of Al toxicity or tolerance in plants (Taylor, 1981). Hypotheses on the target of Al toxicity range from the root cap of the plant (Bennet et al., 1987), to the plasma membrane (Kochian et al., 1991), or to direct binding of Al to the plant's DNA (Matsumoto et al., 1977). Moreover, little is known about the molecular response of plant roots to Al. Attempts have been made using two-dimensional gel electrophoresis to identify proteins that are induced by Al treatment in wheat (Triticum aestivum L.) roots (Delhaize et al., 1991; Picton et al., 1991; Rincón and Gonzales, 1991). Several protein "spots" have been identified that are induced by Al, but none have been further characterized. Czarnecka et al. (1984) found that the expression of the soybean heat-shock clone pCES4 was increased in hypocotyls in response to Al as well as to Cd, Ni, and K. However, in these experiments Al was added to the hypocotyls at pH 6.0. At this pH the level of phytotoxic Al would have been low, and the induction may have been due to factors other than Al toxicity.

In this paper we report the isolation and characterization of five genes whose expression is induced in wheat root tips by treatment with Al. We monitor the changes in expression of these genes in response to Al stress using two related wheat lines that differ by a single dominant gene for Al tolerance.

MATERIALS AND METHODS

Plant Material

Wheat (Triticum aestivum L.) cultivars Warigal (Al sensitive) and Waalt (Al tolerant) were used in this study. Waalt is an Al-tolerant segregant derived from Warigal (Larkin, 1987). The growth of Warigal roots is almost completely inhibited by 10 μM Al, whereas this concentration of Al does not inhibit Waalt root elongation significantly (Picton et al., 1991). Waalt root growth is inhibited by 100 μM Al.

Growth Conditions

Wheat seeds were surface sterilized in 1.5% hypochlorite for 10 min, then rinsed three times with sterile distilled water. The seeds were spread over stainless-steel grids (approximately 100 seeds per grid), placed on damp filter paper in a

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Abbreviations: MLP, metallothionein-like protein; PAL, phenylalanine ammonia-lyase; pI, isoelectric point.
sealed container, and germinated in the dark in a controlled environment chamber on a cycle of 16 h at 22°C, 8 h at 18°C. After 48 h, the grids covered with the germinated seeds were placed over plastic pots containing 300 mL of Ruakura growth medium (0.1 mM MgSO₄, 0.3 mM KNO₃, 0.2 mM NaCl, 0.15 mM NH₄NO₃, 5 μM FeCl₃, 5 μM NH₄H₂PO₄, 5 μM H₂BO₃, 1 μM ZnSO₄, 1 μM MnSO₄, 0.2 μM CuSO₄, 0.2 μM CoCl₂, 0.5 mM CaSO₄, adjusted to pH 4.3 with HCl). This low-ionic strength medium approximates soil solution composition (Edmeades et al., 1985). The seedlings were replaced in the same chamber maintained on a cycle of 16-h days (190 μmol m⁻² s⁻¹ light, 65% RH, 22°C) and 8-h nights (18°C, 85% RH). After 2 d the seeds were transferred to larger pots containing 1 L of medium, which was changed daily thereafter. The growth medium was continuously aerated using an aquarium pump connected via plastic tubing to 22-gauge needles inserted into the sides of the pots.

Experimental treatments were started approximately 7 d after germination. For Al treatments, aliquots of a 20-mM stock solution of Al₂(SO₄)₃ were added to Ruakura medium to give final concentrations of 5 or 50 μM Al₂(SO₄)₃. These conditions will be referred to as the 10- and 100-μM Al treatments, respectively. The seedlings remained in the Al medium for 0.5 to 96 h. To simulate recovery from Al, seedlings were grown in medium containing Al for 24 h; the roots were gently rinsed in Ruakura medium without Al, and they were grown for a further 4 to 96 h in Al-free medium. Cd treatments were carried out by adding Cd to the medium to a final concentration of 250 μM from a 250-mM stock solution of CdCl₂. Plants were then grown with Cd in the medium for a further 4 h. For heat-shock treatments, grids of seedlings were transferred to pots containing medium heated to 42°C. These plants were then grown for an additional 4 h while the medium was maintained at 42°C.

To harvest samples, the seedlings were removed from the medium and gently blotted dry and root tips (3-5 mm from the apex) and leaf tissue were cut off and immediately frozen in liquid nitrogen. To reduce the effect of pot-to-pot variation in the time-course experiment (Fig. 1), approximately 30 seedlings were collected from each of three different pots for each time point. Tissue taken from seedlings in the other treatments were collected from one pot only. Tissue was stored at −80°C until required for RNA isolation.

cDNA Library Construction

Total RNA was isolated from the root tips of wheat cv Warigal treated for 2 d with 10 μM Al using a phenol/SDS method (Ausubel et al., 1987). Poly(A)⁺ RNA was isolated on an oligo(dT) cellulose column using an mRNA purification kit from Pharmacia. A cDNA library was constructed using a ZAP-cDNA synthesis kit from Stratagene in the vector λ ZAPII. A second library was made in the pSPORT 1 vector using the Superscript Plasmid System from GIBCO BRL.

Isolation of cDNA Clones

The primary amplified λ ZAPII library was screened by differential hybridization with cDNA probes synthesized from poly(A)⁺ RNA from control Warigal roots (no Al) and from Warigal roots treated for 2 d with 10 μM Al. The cDNA was ³²P-labeled using a Random Primers DNA Labeling System from GIBCO BRL. Duplicate plaque lifts were performed on Colony/Plaque Screen membranes from DuPont-New England Nuclear. The membranes were prehybridized at 65°C for 2 to 4 h in 1% SDS, 1 m NaCl, 10% dextran sulfate. Denatured salmon sperm DNA (100 μg mL⁻¹) and the denatured cDNA probes were then added, and the membranes were hybridized for 16 to 18 h at 65°C. The membranes were washed twice with 2X SSC at room temperature for 5 min, twice with 2X SSC, 1% SDS at 65°C for 30 min, then finally with 0.1X SSC at room temperature for 20 min. Putative differential clones (150 from a screen of 55,000 plaques) were rescreened using a large plaque assay (Meeks-Wagner et al., 1989). Clones that appeared differential after rescreening (43 of 150) were then replated at low density and again rescreened. Plasmids were rescued from candidate phage by superinfection with the R408 helper phage as recommended by Stratagene. Plasmid DNA was purified from the clones using standard procedures (Sambrook et al., 1989), dot blotted onto Hybond-N+ membranes (Amersham), and rescreened. A total of 11 clones were selected for further analysis.

Northern Analysis

Total RNA (10 μg) samples were denatured with glyoxal and DMSO and electrophoresed through sodium phosphate agarose gels as described by Sambrook et al. (1989), except that sodium iodoacetate was omitted from the gel. After electrophoresis, the RNA was transferred to Hybond-N+ membranes (Amersham) by capillary transfer using 0.05 m NaOH as the transfer buffer. Probes were prepared from cDNA inserts isolated from agarose gels and labeled as described above. Hybridizations were performed as described in Virca et al. (1990). The filters were washed twice with 2X SSC for 15 min and twice with 2X SSC, 0.1% SDS for 20 min at 85°C. Filters were stripped by immersion in boiling 0.1% SDS and reprobed up to seven times. To estimate the relative loading of RNA in each lane of the blots, the membranes were also probed with the 1.2-kb SalII to BamHI fragment from the plasmid pTA250.2, containing a portion of the wheat 26S rRNA gene (Appels and Dvorák, 1982).

DNA Sequencing

Subclones were generated using standard techniques (Sambrook et al., 1989) and random deletion subclones were also made using the procedure of Lin et al. (1985). Plasmid DNA for sequencing was prepared using the Magic MiniPreps DNA Purification System (Promega). DNA was sequenced by the dideoxynucleotide chain-termination method (Sanger et al., 1977) using an Applied Biosystems Catalyst robotic workstation and 373A DNA sequencer. The computer programs of Devereux et al. (1984), comprising the Genetics Computer Group (GCG) package version 7.1.1-Unix installed on a Silicon Graphics 4D-30 workstation, were used for the alignment and analysis of sequence data and the searching of sequence data banks.
RESULTS

Isolation of Al-Induced Clones

A cDNA library was constructed in λ ZAP II using mRNA isolated from root tips of the Al-sensitive wheat cultivar Warigal that had been grown in the presence of 10 μM Al for 2 d. This library was differentially screened for clones whose expression was increased or induced by Al treatment. A total of 11 clones were selected for further characterization by northern hybridization. Three of these proved to be false positives and one was a complex clone; these were not pursued further. The remaining seven clones all showed induction by Al and were initially characterized by sequencing of their 5′ and 3′ ends. Five different genes were represented by the seven clones; two of the genes (wali2 and wali3) were each represented by two highly homologous, although not identical, clones. The initial sequencing and northern analysis revealed that none of the clones isolated were full length and that all contained multiple cloning adaptors at their 5′ ends. Hence, a new root tip cDNA library from Al-treated Warigal plants was constructed in the vector pSPORT 1. For each of the five induced genes, homologous clones were isolated from the pSPORT library and the longest representative of each was analyzed further. These clones were named wali1 to wali5 (for wheat aluminum induced). Most of these clones appeared to be nearly full length (as judged by comparison with the size of the induced mRNA in the northern analyses), with the exception of wali4. Longer “wali4-like” clones that hybridized to the original λ isolate were identified, but these showed lower homology to the original clone, and the shorter, more homologous clone was chosen for sequencing. The sizes of the five wali clones are summarized in Table I.

Al Induction of the cDNA Clones

Figure 1 shows transcript levels of the wali genes in the root tips of Warigal plants treated for varying lengths of time with Al. The comparison includes root tips from plants that were treated with Al for 24 h and then allowed to recover in medium without Al for varying lengths of time.

The expression of wali1 increased dramatically after 24 h of Al treatment and continued to rise up to 96 h, when treatments stopped (Fig. 1). During recovery from the Al treatment (for up to 96 h), the expression of wali1 decreased to nearly basal levels. The expression patterns of wali3 and wali5 were similar to those of wali1 except that the expression of these two genes reached a maximum after 48 h of Al treatment. wali4 had a lower level of induction, with the maximum occurring after 24 h. In contrast to these four genes, wali2 showed a complex pattern of induction, with a transient peak of expression after 0.5 h of Al treatment and a second increase after 24 h. The expression of wali2 mRNA did not return to pretreatment levels even after a recovery period of 96 h. The time course patterns above, including the bimodal induction of wali2, were seen using RNA isolated independently from two lots of plants grown on separate occasions.

Specificity of the Al Response

A second series of northern hybridizations was performed to determine whether higher levels of Al are required to induce the wali genes in the more tolerant wheat cultivar, Waalt (Fig. 2). In roots of the sensitive cultivar, Warigal, all of the genes except wali2 showed increased expression levels with increasing concentrations of Al. However, in Waalt, expression of wali1, -3, -4, and -5 was induced only at the inhibitory concentration of Al (100 μM). A reduction in transcript abundance of these four genes was observed in Waalt at the lower concentration of Al (10 μM); at this level of Al, Waalt seedlings showed no reduction of root growth (Picton et al., 1991). We do not know whether the reduction seen in this experiment occurs consistently.

wali2 showed a different pattern of induction from the other four genes. The level of expression of wali2 was increased in Warigal after 2 d of treatment with 10 μM Al, but reduced after 2 d in 100 μM Al. In addition, the expression of wali2 was not significantly affected in Waalt at 10 μM Al (in contrast to the reduction in expression found for the other four wali clones) and was reduced at the higher Al

![Figure 1. Northern hybridizations showing a time course of wali gene induction by Al. Lanes contained 10 μg of total RNA extracted from the roots of wheat seedlings that were either untreated (0), treated with 10 μM Al for 0.5, 2, 4, 24, 48, or 96 h, or Al treated for 24 h, followed by recovery in Al-free media for 4 (r4), 24 (r24), or 96 h (r96). The membrane was successively hybridized with 32P-labeled cDNA isolated from each of the wali clones and a 26S wheat rRNA probe (from pTA250.2). The RNA probe served to estimate relative loadings of RNA in each lane.](https://www.plantphysiol.org)
Snowden and Gardner

had a dialogue between the two wheat cultivars (Fig. 2).

was the only gene of the five in other tissues (Fig. 2).

induced by the heat-shock treatment but remained at basal levels in Waalt.

levels in Waalt relative to Warigal,

wali2 encoded a 37.5-kD protein with a calculated pI of 5.3 and has no significant homology to any other sequences in the data base. It has no obvious leader sequence. The putative protein contains 24 Cys residues (of 347 amino acids), 16 of which are aligned into four Cys-X-Cys motifs. (Fig. 4).

wali3 and wali5 have 62% nucleotide identity to each other, but show no close relationship to any other sequences in the data base. The sequence of wali5 contains two open reading frames that could encode proteins of 9.5 or >14 kD. However, wali3 has a stop codon in the larger of these two frames. On this basis we have identified the 9.5-kD open reading frame as the gene product of both genes. The homology between the two genes extends throughout this reading frame. The

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Wheat roots were also treated with toxic levels of Cd or heat shocked to determine if the wali genes were induced in response to these stresses. For all of the wali genes, expression levels were down-regulated by both Cd stress and heat-shock treatment (Fig. 2). A control hsp70 heat-shock probe was induced by the heat-shock treatment but remained at basal levels after the Cd treatment (data not shown).

Leaf tissue from plants whose roots had been stressed with Al was also examined to see if the wali genes are expressed in other tissues (Fig. 2). wali1 was the only gene of the five that had detectable expression in leaves. There was no induction of wali1 in leaves by Al; transcript levels appeared to decrease slightly in response to the Al treatment.

Nucleotide Sequences of the wali Genes

The nucleotide sequences of the five wali clones was completed over both strands.

Figure 2. Northern hybridization of the wali clones to total RNA isolated from wheat plants after different treatments. Lanes contained 10 µg of total RNA extracted from either the roots or leaves of the sensitive wheat cultivar Warigal (Wg) and the tolerant cultivar Waalt (Wt). Plants were treated with 0, 10, or 100 µM Al for 48 h, or with 250 µM Cd for 4 h, or heat shocked (h/s) at 42°C for 4 h. Two separate gels (A and B) were electrophoresed and alkali blotted, and the membranes were successively hybridized with 32P-labeled cDNA isolated from the wali clones indicated and a 26S rRNA probe (from pTA250.2). The rRNA probe served as a control to estimate the relative lane loadings of RNA on each membrane.

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Figure 4. Nucleotide and deduced amino acid sequence of the wali2 cDNA clone. Cys residues that are arranged in the Cys-X2-Cys-X10+Cys-X2-Cys motif are highlighted in gray, and the motif is underlined.

The putative proteins these genes encode have 66% amino acid identity (Fig. 5), are slightly basic (pI = 8.2 for wali3 and 7.9 for wali5), and include a hydrophobic string of 20 residues at the N terminus. Both are relatively rich in Cys (10 residues out of 89 or 90), all of which are conserved between the two proteins.

The putative protein encoded by the wali4 gene is homologous to the enzyme PAL. The wali4 clone contains sequences that correspond to the C-terminal 129 amino acids of the PAL protein. Over this region the wheat PAL protein is homologous to other plant PAL genes currently in the database, with amino acid identities ranging from 63% (e.g. tomato [Lycopersicon esculentum], accession number M90692, Lee et al., 1992) to 68% (e.g. sweet potato [Ipomoea batatas], accession number M29232, Tanaka et al., 1989).

The bias toward late genes probably arises from our use of a cDNA library constructed from root tips treated for 2 d with Al.

Somewhat surprisingly, all four wali genes were induced to higher levels by 100 μM Al in the sensitive cultivar Warigal than they were at 10 μM Al. The lower concentration of Al is sufficient to halt root elongation almost completely in Warigal (see Picton et al., 1991). Therefore, it appears likely that the induction by Al stress in wheat roots. Previous protein analysis of Waalt and Warigal identified a single protein (Q/q, molecular mass of 26 kD, pl of 6.5; Picton et al., 1991) that was consistently induced following Al treatment. None of the proteins encoded by the wali genes correspond in size to this protein. The three wali genes whose mRNA accumulated at the highest levels, wali1, -3, and -5 (Figs. 1 and 2), encode proteins too small to have been observed in the protein work (<20 kD; see Picton et al., 1991).

Four of the five wali genes had similar patterns of induction by Al (wali1, -3, -4, and -5). All four showed simple induction, returned to near basal levels when the roots were removed from medium containing Al, and were induced in both cultivars tested (Waalt and Warigal). The response to Al treatment of the fifth gene, wali2, was notably different. wali2 had a complex bimodal induction pattern and its expression did not return to basal levels after removal of Al. Induction was seen in only one of the two cultivars tested (Warigal) and then at only the lower of two Al concentrations tested. For these reasons we consider primarily the other four wali genes in the discussion below.

Higher levels of Al were required for induction of the four wali genes in the tolerant cultivar compared with the sensitive cultivar (Fig. 2). This result supports the conclusion that induction of these wali genes is a specific response by the roots to the inhibition of growth by Al. It also suggests that these four genes may be induced in response to Al in other wheat cultivars. Homologs of wali1 (de Miranda et al., 1990; Evans et al., 1990) and wali4 (Ohl et al., 1990) have been shown to be induced (or repressed) in response to varying concentrations of metal ions and other environmental stresses in other plants; thus, it seems likely that they might also be induced by Al treatment in different species.

The genes wali1, -3, -4, and -5 did not show induction within 4 h of the introduction of Al stress, but were induced by 24 h (Fig. 1). Thus, they fit the category of "late" induction and probably are not induced as part of the initial, primary response of the root to Al, but rather are downstream effects. The bias toward late genes probably arises from our use of a cDNA library constructed from root tips treated for 2 d with Al.
four wali genes are not responding to the cessation of root growth per se, but to some other effect of the Al treatment. For example, the more severe treatment with 100 μM Al affects the ability of the root tip to recover and continue growing if Al is removed (S.J. Picton and K.D. Richards, unpublished data) and also has a more drastic effect on protein synthesis (Picton et al., 1991).

Expression levels of all five wali genes were reduced after 4 h of exposure to Cd stress or to heat shock (relative to untreated controls). The pattern of Al-induced changes in protein synthesis (Picton et al., 1991), combined with the fact that none of the wali clones corresponded to known heat-shock genes, suggests that the plant’s reaction to Al stress is different from that to heat shock. Recently, it has been shown that heat-shock genes corresponding to hsp70 and hsp17 are not induced by Al stress (K.D. Richards, personal communication). It remains possible, however, that the wali genes may be induced by other environmental or nutrient stresses in plants.

The only function attributable to any of the Al-induced genes at this stage is to wali4, which encodes a protein with high homology to PAL. PAL expression in plants has been shown to be regulated developmentally and by various environmental stresses such as wounding, HgCl₂, UV light, and fungal elicitors (see Ohl et al., 1990, and refs. therein). Many of the plant metabolites synthesized via pathways downstream from PAL, including flavonoids and anthocyanins, have high affinity for Al (Taylor, 1991). A reduction in the effective cellular concentration of one or more of these compounds due to sequestering by Al may lead to the induction of PAL. Another possibility is that the root thickening or the “browning” that occurs in the presence of Al requires the biosynthesis of lignin, tannins, or other downstream products of the PAL enzyme.

wali2, -3, and -5 are novel genes with no significant homology to any sequences found in the data base. All three genes are relatively rich in Cys amino acids. The Cys amino acids in wali3 and -5 display no obvious pattern. However, the periodicity of the Cys’s in the protein encoded by wali2 (Fig. 4) is reminiscent of Cys2Cys2 Zn fingers, although conserved amino acids present at other locations in the Cys2Cys2 Zn finger motifs are not present in wali2, and the precise spacing of the Cys pairs differs (Glover, 1989).

wali1 is homologous to a group of Cys-rich plant proteins that are similar to animal metallothioneins and that are termed MLPs. It is highly unlikely that MLPs bind Al, since Al coordination by proteins involves carboxyl groups rather than sulfhydryl groups, and it has been shown that animal metallothionein does not bind Al (Putter and Gardner, 1988). Since the barley MLP gene is induced in Fe-deficient growth conditions (Okumura et al., 1991), and since the Fe²⁺ and Al³⁺ ions are very similar (e.g. Martin et al., 1987), one possibility is that Al may induce Fe deficiency in wheat roots. However, lowered Fe concentrations have not been observed consistently in wheat roots or leaves after long-term treatment with Al (Wheeler et al., 1992). Another possibility is that Al might induce wali1 by interfering with the plant’s normal pathways for uptake or homeostasis of other metal ions. For example, it is known that the MLP gene from Mimulus decreases in abundance in response to Cu, Cd, and Zn (de Miranda et al., 1990), and in vivo experiments suggest the MLP may bind to these ions (Tommey et al., 1991; Evans et al., 1992). However, the only ion consistently altered by Al treatment of wheat roots is Mg (Wheeler et al., 1992), and a possible role for Mg in regulation of MLPs has not been investigated.

This paper describes the first characterization of genes induced in plants by Al. In the future we plan to identify other genes that are induced earlier in response to Al toxicity, because the expression of these genes is likely to represent a primary response to the Al stress. We also aim to establish the function of the wali gene products, and experiments are in progress to determine the site of expression of these genes in the plant. Understanding the role of the wali genes in plants may aid our understanding of the mechanism of toxicity of the Al³⁺ ion in acid soils.

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The GenBank accession numbers for the sequences reported in this article are L11879 for wali1, L11880 for wali2, L11881 for wali3, L11883 for wali4, and L11882 for wali5.

LITERATURE CITED


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