

# Five Genes Induced by Aluminum in Wheat (*Triticum aestivum* L.) Roots<sup>1</sup>

Kimberley C. Snowden and Richard C. Gardner\*

Centre for Gene Technology, School of Biological Sciences, University of Auckland,  
Private Bag 92019, Auckland, New Zealand

Five different cDNAs (termed *wali1* to *wali5* for wheat aluminum induced) whose expression was induced by Al stress have been isolated from the root tips of Al-treated wheat (*Triticum aestivum* L.) plants. Four of these genes were induced 24 to 96 h after Al treatment, and their expression is reduced when the Al is removed. Each of these four genes was induced by inhibitory levels of Al in two wheat cultivars—Warigal, an Al-sensitive cultivar, and Waalt, an Al-tolerant cultivar. The fifth gene (*wali2*) showed a complex bimodal pattern of induction and was induced by Al only in the sensitive cultivar. Comparison of the nucleotide sequences of these clones to those in the sequence data bases showed that *wali4* is homologous to phenylalanine ammonia-lyase and *wali1* is homologous to a group of plant proteins that are cysteine-rich and have homology to metallothioneins. *wali2* encodes a novel protein with a repeating motif of cysteine amino acids. The remaining two *wali* clones (*wali3* and *wali5*) encode related, cysteine-rich proteins that show no significant homology to any known sequences.

Al is the most abundant metal in the earth's crust, comprising approximately 7.5% by weight (Haug, 1984). In soils with a pH of  $\geq 5$ , Al is predominantly bound as insoluble oxides and complex aluminosilicates. However, as soil pH drops there is a release of ionic  $\text{Al}^{3+}$  and a reduction in the availability of exchangeable cations such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{K}^+$ . 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 (Clark-

son, 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, 1991). 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 pCE54 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  $10 \mu\text{M}$  Al, whereas this concentration of Al does not inhibit Waalt root elongation significantly (Picton et al., 1991). Waalt root growth is inhibited by  $100 \mu\text{M}$  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

Abbreviations: MLP, metallothionein-like protein; PAL, phenylalanine ammonia-lyase; pI, isoelectric point.

<sup>1</sup> This work was supported by a grant from the Foundation for Research Science and Technology (AGR-06-052) to the New Zealand Pastoral Agriculture Research Institute Ltd. (formerly Ministry of Agriculture and Fisheries). K.C.S. was the recipient of a University of Auckland Doctoral Scholarship.

\* Corresponding author; fax 64-9-3737414.

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 MgSO<sub>4</sub>, 0.3 mM KNO<sub>3</sub>, 0.2 mM NaCl, 0.15 mM NH<sub>4</sub>NO<sub>3</sub>, 5 μM FeCl<sub>3</sub>, 5 μM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 5 μM H<sub>3</sub>BO<sub>3</sub>, 1 μM ZnSO<sub>4</sub>, 1 μM MnSO<sub>4</sub>, 0.2 μM CuSO<sub>4</sub>, 0.2 μM CoCl<sub>2</sub>, 0.5 mM CaSO<sub>4</sub>, 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<sup>-2</sup> s<sup>-1</sup> 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 Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> were added to Ruakura medium to give final concentrations of 5 or 50 μM Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>. 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 CdCl<sub>2</sub>. 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)<sup>+</sup> 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)<sup>+</sup> RNA from control Warigal roots (no Al) and

from Warigal roots treated for 2 d with 10 μM Al. The cDNA was <sup>32</sup>P-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<sup>-1</sup>) 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 2× SSC at room temperature for 5 min, twice with 2× SSC, 1% SDS at 65°C for 30 min, then finally with 0.1× 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 2× SSC for 15 min and twice with 2× 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 *SalI* to *Bam*HI 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  $\lambda$  ZAP II using mRNA isolated from root tips of the Al-sensitive wheat cultivar Warigal that had been grown in the presence of  $10 \mu\text{M}$  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  $\lambda$  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

Table I. Characteristics of the *wali* clones

Sizes (in bp) of cDNA inserts are taken from the nucleotide sequences [excluding poly(A) residues]. Sizes of mRNAs have been estimated from northern hybridizations.

| Clone        | Insert Size | mRNA Size | Homology                           |
|--------------|-------------|-----------|------------------------------------|
| <i>wali1</i> | 558         | 700       | Cys-rich, metal-regulated proteins |
| <i>wali2</i> | 1163        | 1300      |                                    |
| <i>wali3</i> | 564         | 700       | <i>wali5</i>                       |
| <i>wali4</i> | 634         | 2700      | PAL                                |
| <i>wali5</i> | 520         | 700       | <i>wali3</i>                       |

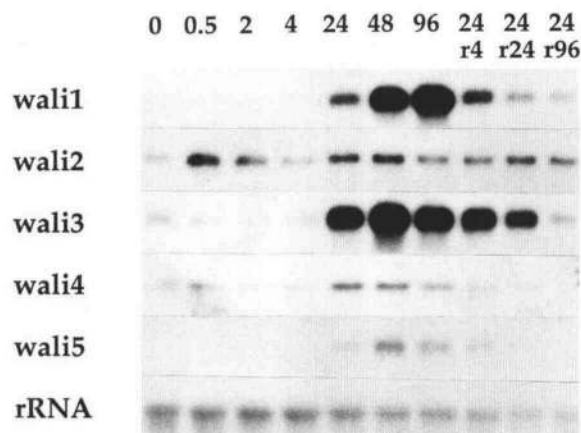


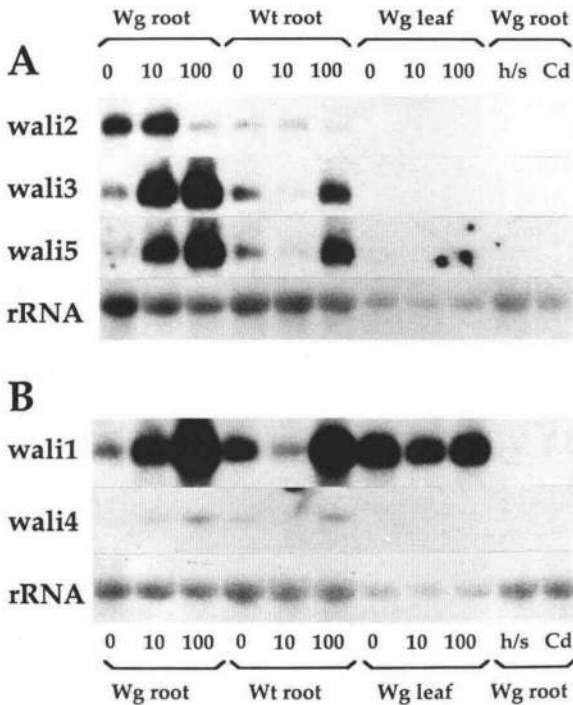
Figure 1. Northern hybridizations showing a time course of *wali* gene induction by Al. Lanes contained  $10 \mu\text{g}$  of total RNA extracted from the roots of wheat seedlings that were either untreated (0) or treated with  $10 \mu\text{M}$  Al for 0.5, 2, 4, 24, 48, or 96 h, or Al treated for 24 h, followed by recovery in Al-free media for 4 (r4), 24 (r24), or 96 h (r96). The membrane was successively hybridized with  $^{32}\text{P}$ -labeled cDNA isolated from each of the *wali* clones and a 26S wheat rRNA probe (from pTA250.2). The rRNA probe served to estimate relative loadings of RNA in each lane.

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* had 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 ( $100 \mu\text{M}$ ). A reduction in transcript abundance of these four genes was observed in Waalt at the lower concentration of Al ( $10 \mu\text{M}$ ); at this level of Al, Waalt seedlings showed no reduction of root growth (Pictor 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  $10 \mu\text{M}$  Al, but reduced after 2 d in  $100 \mu\text{M}$  Al. In addition, the expression of *wali2* was not significantly affected in Waalt at  $10 \mu\text{M}$  Al (in contrast to the reduction in expression found for the other four *wali* clones) and was reduced at the higher Al concentration.



**Figure 2.** Northern hybridization of the *wali* clones to total RNA isolated from wheat plants after different treatments. Lanes contained 10  $\mu\text{g}$  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  $\mu\text{M}$  Al for 48 h, or with 250  $\mu\text{M}$  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  $^{32}\text{P}$ -labeled cDNA isolated from the *wali* clones indicated and a 26S wheat rRNA probe (from pTA250.2). The rRNA probe served as a control to estimate the relative lane loadings of RNA on each membrane.

The *wali* genes differed in their basal levels of expression between the two wheat cultivars (Fig. 2). *wali2* had a decreased level of expression in Waalt relative to Warigal, whereas the other four genes had slightly higher expression levels in Waalt.

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.

*wali1* encodes a 7.4-kD protein with a calculated pI of 4.3. The *wali1* sequence has homology to six plant genes that have been referred to as "metallothionein-like," isolated previously from *Mimulus guttatus* (de Miranda et al., 1990), *Pisum sativum* (Evans et al., 1990), *Hordeum vulgare* (Okumura et al., 1991), *Arabidopsis thaliana* (K. Takahashi, unpublished; GenBank data base), *Glycine max* (Kawashima et al., 1991), and *Zea mays* (de Framond, 1991). Figure 3 shows a comparison of predicted protein sequences of the wheat *wali1* gene with these six genes. The proteins all contain a central hydrophobic region separating two Cys-rich domains, each containing three Cys-X-Cys motifs. (One of the motifs in the *Arabidopsis* and soybean genes has the sequence Cys-Gly-Gly-Cys.) These regions also show similarity to metallothionein genes from other eukaryotes (de Miranda et al., 1990; Evans et al., 1990).

*wali2* encodes 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<sub>3</sub>-Cys-X<sub>10-14</sub>-Cys-X<sub>3</sub>-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

|             |          |          |         |          |                  |
|-------------|----------|----------|---------|----------|------------------|
| arabidopsis | MSCCGGNC | CGSSCK   | CGNGCG  | CKMYPDL  | .GFSGETTTTTF     |
| soybean     | MSCCGGNC | CGSSCK   | CGNGCG  | CKMYPDL  | .SY.TESTTTELT    |
| pea         | MS...    | CGCGSSCK | CGNDCK  | CKNRSSGL | .SYS.EMETTETV    |
| mimulus     | MS...    | SGCGSSCK | CGNDCK  | CKMYPDL  | .MYPDM...ETNTTVM |
| maize       | MS...    | CGCGSSCK | CGSSCK  | CGKYPDL  | .EETSTAAQ.PTV    |
| barley      | MS...    | CGCGSSCK | CGSSCK  | CGKYPDL  | .EEKSGATMQVTVI   |
| wheat       | MS...    | CGCGSSCK | CGSSCK  | CGKYPDL  | .TEEQSGAAAQVA    |
| Consensus   | MS----   | C-CGSSCK | CGS-CCK | -KMYPDLE | -E-YS-ET-TTETV   |

|             |                      |                  |               |                    |
|-------------|----------------------|------------------|---------------|--------------------|
| arabidopsis | VLGVAPAMKNQYEASGESNN | .AESD            | .ACKCGSSDCK   | CDPCTCK            |
| soybean     | VMGVAP               | .VKAQFEGAEMGVP   | .AEND         | .GCKCGSPNCSNCPCTCK |
| pea         | ILGVGPA              | .KIQFEGAEMSA     | .SEDG         | .GCKCGDNCTCDPCTCK  |
| mimulus     | IEGVAP               | .LKMYSSEGSEKSF   | .AEGNGCGSSNCK | CDPCTCK            |
| maize       | VLGVAP               | EEKAAPEFVEAAAESG | AAHGCSSCGSSCK | CDPCTCK            |
| barley      | VLGVGSAKQ            | ...FEAAEFGEAAHGC | SEANCK        | CDPCTCK            |
| wheat       | VLGVAPENKAGQ         | ...FEVAA         | ...GQSGEGCS   | CGDNCK             |
| Consensus   | VLGVAPA-KAQFEG-E     | -A---E-G-        | CCKCGSSNCK    | CDPCTCK-           |

**Figure 3.** Alignment of the deduced amino acid sequences of the wheat *wali1* clone and the other known plant MLPs. The conserved Cys residues are highlighted by black boxes. Gaps (represented by dots) have been introduced into the sequences to maximize the alignment. The consensus sequence was generated using the PRETTY program. The plant sequences were obtained from the GenBank and SwissProt data bases as follows: barley (*Hordeum vulgare*), accession number X58540 (Okumura et al., 1991); *Arabidopsis thaliana*, X62818 (K. Takahashi, unpublished); pea (*Pisum sativum*), P20830 (Evans et al., 1990); *Mimulus guttatus*, P20238 (de Miranda et al., 1990). Also included are the maize (*Zea mays*; de Framond, 1991) and soybean (*Glycine max*; Kawashima et al., 1991) sequences.



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  $\mu\text{M}$  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,  $\text{HgCl}_2$ , 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  $\text{Cys}_2\text{Cys}_2$  Zn fingers, although conserved amino acids present at other locations in the  $\text{Cys}_2\text{Cys}_2$  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 (Putterill and Gardner, 1988). Since the barley MLP gene is induced in Fe-deficient growth conditions (Okumura et al., 1991), and since the  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$  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  $\text{Al}^{3+}$  ion in acid soils.

#### ACKNOWLEDGMENTS

We wish to thank Tom Richardson, Andreas Königstorfer, and Keith Richards for helpful discussions during the course of this work and for assistance with construction of the  $\lambda$  ZAPII library; Jim Crush, John Caradus, Bart Janssen, and Jeannette Keeling for comments on the manuscript; and Rudi Appels for kindly providing the pTA250.2 vector.

Received April 13, 1993; accepted July 5, 1993.

Copyright Clearance Center: 0032-0889/93/103/0855/07.

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

- Appels R, Dvorák J (1982) Relative rates of divergence of spacer and gene sequences within the rDNA region of species in the Triticeae: implications for the maintenance of homogeneity of a repeated gene family. *Theor Appl Genet* **63**: 361–365
- Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987) *Current Protocols in Molecular Biology*. John Wiley & Sons, New York
- Bennet RJ, Breen CM, Fey MV (1987) The effects of aluminum on root cap function and root development in *Zea mays* L. *Environ Exp Bot* **27**: 91–104
- Clarkson DT (1965) The effect of aluminum and some other trivalent metal cations on cell division in the root apices of *Allium cepa*. *Ann Bot* **29**: 309–315
- Czarnecka E, Edelman L, Schöffl F, Key JL (1984) Comparative analysis of physical stress responses in soybean seedlings using cloned heat shock cDNAs. *Plant Mol Biol* **3**: 45–58
- de Framond AJ (1991) A metallothionein-like gene from maize (*Zea mays*): cloning and characterization. *FEBS Lett* **290**: 103–106
- 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 Murrmann, eds, *Plant-Soil Interactions at Low pH*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1071–1079
- de Miranda JR, Thomas MA, Thurman DA, Tomsett AB (1990) Metallothionein genes from the flowering plant *Mimulus guttatus*. *FEBS Lett* **260**: 277–280
- Devereux J, Haeblerli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**: 387–395
- Edmeades DC, Wheeler DM, Clinton DF (1985) The chemical composition and ionic strength of soil solutions from New Zealand topsoils. *Aust J Soil Res* **23**: 151–165
- Evans IM, Gatehouse LN, Gatehouse JA, Robinson NJ, Croy RRD

- (1990) A gene from pea (*Pisum sativum* L.) with homology to metallothionein genes. *FEBS Lett* **262**: 29–32
- Evans KM, Gatehouse JA, Lindsay WP, Shi J, Tommey AM, Robinson NJ** (1992) Expression of the pea metallothionein-like gene *PsMT<sub>A</sub>* in *Escherichia coli* and *Arabidopsis thaliana* and analysis of trace metal ion accumulation: implications for *PsMT<sub>A</sub>* function. *Plant Mol Biol* **20**: 1019–1028
- Foy CD, Chaney RL, White MC** (1978) The physiology of metal toxicity in plants. *Annu Rev Plant Physiol* **29**: 511–566
- Glover CVC** (1989) Sequence-specific protein-DNA recognition by transcriptional regulatory proteins. *Plant Mol Biol Rep* **7**: 183–208
- Haug A** (1984) Molecular aspects of aluminum toxicity. *CRC Crit Rev Plant Sci* **1**: 345–373
- Kawashima I, Inokuchi Y, Chino M, Kimura M, Shimizu N** (1991) Isolation of a gene for a metallothionein-like protein from soybean. *Plant Cell Physiol* **32**: 913–916
- Kinraide TB, Parker DR** (1989) Assessing the phytotoxicity of mononuclear hydroxy-aluminum. *Plant Cell Environ* **12**: 479–487
- Kochian LV, Shaff JE, Ryan PR** (1991) Microelectrode-based investigations into the relationship between Al toxicity and root-cell membrane transport processes. In DD Randall, DG Blevins, CD Miles, eds, *Current Topics in Plant Biochemistry and Physiology*, Vol 10. University of Missouri, Columbia, pp 117–133
- Larkin PJ** (1987) Calmodulin levels are not responsible for aluminum tolerance in wheat. *Aust J Plant Physiol* **14**: 377–385
- Lee S-W, Robb J, Nazar RN** (1992) Truncated phenylalanine ammonia-lyase expression in tomato (*Lycopersicon esculentum*). *J Biol Chem* **267**: 11824–11830
- Lin H-C, Lei S-P, Wilcox G** (1985) An improved DNA sequencing strategy. *Anal Biochem* **147**: 114–119
- Martin RB, Savory J, Brown S, Bertholf RL, Wills MR** (1987) Transferrin binding of Al<sup>3+</sup> and Fe<sup>3+</sup>. *Clin Chem* **33**: 405–407
- Matsumoto H, Morimura S, Takahashi E** (1977) Binding of aluminum to DNA in pea root nuclei. *Plant Cell Physiol* **18**: 987–993
- Meeks-Wagner DR, Dennis ES, Van KTT, Peacock WJ** (1989) Tobacco genes expressed during *in vitro* floral initiation and their expression during normal plant development. *Plant Cell* **1**: 25–35
- Ohl S, Hedrick SA, Chory J, Lamb CJ** (1990) Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. *Plant Cell* **2**: 837–848
- Okumura N, Nishizawa N-K, Umehara Y, Mori S** (1991) An iron deficiency-specific cDNA from barley roots having two homologous cysteine-rich MT domains. *Plant Mol Biol* **17**: 531–533
- Picton SJ, Richards KD, Gardner RC** (1991) Protein profiles in root-tips of two wheat (*Triticum aestivum* L.) cultivars with differential tolerance to aluminum. In RJ Wright, VC Baligar, RP Murrmann, eds, *Plant-Soil Interactions at Low pH*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1063–1070
- Putterill JJ, Gardner RC** (1988) Proteins with the potential to protect plants from Al<sup>3+</sup> toxicity. *Biochim Biophys Acta* **964**: 137–145
- Rincon M, Gonzales RA** (1991) Induction of protein synthesis by aluminum in wheat (*Triticum aestivum* L.) root tips. In RJ Wright, VC Baligar, RP Murrmann, eds, *Plant-Soil Interactions at Low pH*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 851–858
- Sambrook J, Fritsch EE, Maniatis T** (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Sanger F, Nicklen S, Coulson AR** (1977) DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* **74**: 5463–5467
- Tanaka Y, Matsuoka M, Yamamoto N, Ohashi Y, Kano-Murakami Y, Ozeki Y** (1989) Structure and characterization of a cDNA clone for phenylalanine ammonia-lyase from cut-injured roots of sweet potato. *Plant Physiol* **90**: 1403–1407
- 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*, Vol 10. University of Missouri, Columbia, pp 57–93
- Tommey AM, Shi J, Lindsay WP, Urwin PE, Robinson NJ** (1991) Expression of the pea gene *PsMT<sub>A</sub>* in *E. coli*: metal-binding properties of the expressed protein. *FEBS Lett* **292**: 48–52
- van Breemen N** (1985) Acidification and decline of Central European forests. *Nature* **315**: 16
- Virca GD, Northemann W, Shiels BR, Widera G, Broome S** (1990) Simplified northern blot hybridization using 5% sodium dodecyl sulfate. *Biotechniques* **8**: 370–371
- Wheeler DM, Edmeades DC, Christie RA** (1992) Effect of aluminum on relative yield and plant chemical concentrations of cereals grown in solution culture at low ionic strength. *J Plant Nutr* **15**: 403–418