Characterization of Oxalate Oxidase and Cell Death in Al-Sensitive and Tolerant Wheat Roots

Georges Delisle, Marie Champoux and Mario Houde
Département des sciences biologiques, Université du Québec à Montréal, C.P. 8888 Succursale “Centre-Ville”, Montréal, Québec, Canada, H3C 3P8

Several genes including oxalate oxidase (Oxo) are up-regulated in Triticum aestivum L. root tips exposed to Al. To better understand the function of Oxo during Al exposure, the protein level and enzyme activity were measured. The data indicate that both Oxo protein and activity are increased proportionally to the level of root growth inhibition (RGI). A high level of Oxo expression may result in excess H₂O₂ production which could become toxic and induce cell death. However, the timing of H₂O₂ production (observed after 24 h) indicates that it cannot be the primary cause of cell death first observed after 8 h. Moreover, at Al concentrations resulting in 50% RGI, we did not observe any cell death in the sensitive cultivar while a punctated pattern of death involving small groups of cells was found in the tolerant cultivar. This pattern was maintained for several days in the tolerant cultivar, suggesting the involvement of a cell death mechanism aimed at replacing epidermal cells intoxicated with Al while root growth is maintained. The accelerated epidermal cell turnover may represent a new detoxification mechanism helping to protect deeper cell layers of the meristematic and elongation zone essential for root growth.

Key words: Aluminum toxicity — Cell death — Detoxification — H₂O₂ — Oxalate oxidase — Wheat.

Abbreviations: Oxo, oxalate oxidase; PR, pathogenesis-related; RGI, root growth inhibition.

Introduction

Al is one of the important causes of crop losses in acid soils. Both pathogen and abiotic stresses are known to activate a diverse array of plant defense genes, including glutathione S-transferase, peroxidases, proteinase inhibitors, hydrolytic enzymes (e.g. β-1,3-glucanase and chitinases), lignin and salicylic acid biosynthetic enzymes, such as phenylalanine ammonia lyase, chalcone synthase, superoxide dismutase and oxalate oxidase (see Hamel et al. 1998). We have previously identified several wheat (Triticum aestivum L.) Al-regulated genes suggesting that Al acts as an elicitor of a pathogenesis response (Hamel et al. 1998). Thus, the induction of pathogenesis-related (PR) proteins seems to be a general defense response rather than a specific response towards various stresses.

One of the wheat Al-regulated genes that we isolated (War13.2) encodes an oxalate oxidase-like protein (Oxo: oxalate : oxygen oxidoreductase, EC 1.2.3.4). Oxo catalyzes the oxidation of oxalic acid (stored as calcium salt) by molecular oxygen, yielding CO₂, Ca²⁺ and H₂O₂ and was shown to maintain its homopentameric structure and activity on SDS-PAGE (Lane et al. 1993). Oxos were identified in a limited number of plant, fungal and bacterial species. Oxo was previously demonstrated to be a member of the germin protein family (Lane et al. 1993). Germins are expressed constitutively at a low basal level, however the expression of different germin isoforms can be observed during growth and development (Lane et al. 1992). The role of the various isoforms is still poorly defined with some isoforms possessing a H₂O₂ generating activity (named germin-like oxalate oxidase or Oxo) while other isoforms have not unequivocally been demonstrated to possess such enzyme activity (named Oxalate oxidase-like protein, Lane 1994, Berna and Bernier 1999). In wheat, both the level of germin mRNA and Oxo activity were found to be increased in response to biotic and abiotic stresses (Berna and Bernier 1997, Berna and Bernier 1999). Oxalate oxidase-like proteins or mRNA were also shown to accumulate in barley roots upon salt stress (Hurkman and Tanaka 1996) or in response to the powdery mildew fungus (Zhang et al. 1995). However, very few reports were able to demonstrate the presence of an active, bona fide, H₂O₂ generating Oxo (Requena and Bornemann 1999).

The goal of this study was to evaluate the kinetics of protein expression and to demonstrate that an active H₂O₂ generating Oxo isoform is accumulating during Al exposure. We were also interested to determine whether the kinetics of H₂O₂ production could be associated with cell death which was previously observed in wheat epidermal cells exposed to Al (DeLima and Copeland 1994). Our results demonstrate that Oxo activity increases upon Al treatment especially in the root tips. The timing of H₂O₂ production indicates that it cannot be the cause of early cell death. Furthermore, when evaluated at Al concentrations resulting in a comparable stress level (50% root growth inhibition (RGI), see Parker 1995), we did not observe any cell death in the sensitive cultivar while the timing and pattern of cell death in the tolerant cultivar suggest that this may provide another means for roots to detoxify Al accumulat-
ing in epidermal cells, along with other protective events such as the excretion of organic acids in the rhizosphere (Delhaize et al. 1993, Ma et al. 1997).

Materials and Methods

Plant material, growth and Al exposure conditions

In this study, two winter wheat cultivars possessing a high (T. aestivum L. cv. Atlas-66) and low (T. aestivum L. cv. Fredrick) tolerance to Al were used and grown as previously described under conditions where Al remains mostly under the Al$^{3+}$ form (Hamel et al. 1998). Al concentrations ranging from 0 to 250 μM were used as described for each experiment. RGI was measured in all experiments and is expressed as 100 × [1 – (root growth of Al-treated seedlings divided by the root growth of control seedlings)]. To ensure that Al speciation was stable throughout the experiment, at least 100 mL of solution was used for each plant and the pH was verified and adjusted every day when needed. Under these conditions, the RGI measured after the first day was comparable to the RGI measured on day 2 and 3 in all experiments. All experiments were repeated at least three times and representative results are shown.

Axenic cultures of wheat were prepared by treating seeds with 1% hypochloric acid for 30 min in the presence of 0.1% Tween-20 with constant shaking. The seeds were then rinsed several times with sterile water and individually transferred to sterile tubes containing 1 mM CaCl$_2$ in sterile water for imbibition. The level of liquid was gradually increased as the leaf blade expanded. Once roots reached 2 cm in length, they were used for Al exposure experiments under sterile conditions in the same tubes (10 mL of filter sterilized 50 mM Al solution in 1 mM CaCl$_2$, pH 4.15).

Screening of the cDNA library

Library construction was described in Hamel et al. (1998). The cDNA library was screened with the $^{32}$P-labeled cDNA War13.2 from the first screening to isolate a full length cDNA.

Antibody production and purification

In order to obtain a specific polyclonal antibody, we identified a peptide region with a high antigenic potential and specific to oxalate oxidase. The antigenic profile of the WAR13.2 protein was deduced using the PEPTIDESTRUCTURE program by GCG. The most antigenic and specific region to Oxo (93% identity with GER3 Oxo from rice and 71% identity with Bh6-903 Oxo from barley) is the region between positions 58 and 74. This peptide (cAMLDQPRDTKNDP-AQHHSLLG) was the region specific to Oxo (93% identity with GER3 Oxo from barley) and 71% identity with Bh6-903 Oxo from barley. It is the region where Al remains mostly under the Al$^{3+}$ form (Hamel et al. 1998). Al concentrations ranging from 0 to 250 μM were used as described for each experiment. RGI was measured in all experiments and is expressed as 100 × [1 – (root growth of Al-treated seedlings divided by the root growth of control seedlings)].

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The second fraction from the supernatant described above, without heat denaturation or β-mercaptoethanol, was adjusted to 1 mM EDTA and loaded onto an SDS-PAGE (9%) to reduce enzyme agglomeration. In order to assess the Oxo-dependent production of H$_2$O$_2$, we used the oxalate-dependent and peroxidase-linked staining method, modified from Lane et al. (1993). Briefly, the reactions were performed in 50 mM succinate/NaOH, pH 4.0, containing 2 mM oxalic acid, 1 mg per 100 ml horseradish peroxidase (Sigma # P-6782), 30 μl per 100 ml N,N-dimethylamine and 8 mg per 100 ml 4-aminophenylamine. After transfer, the nitrocellulose filter was immersed in 5 min in 25 mM succinate (pH 4.0) and then in the staining solution for 18 h. No staining appeared when the substrate (oxalic acid) was omitted in the staining solution, demonstrating that the reaction specifically detected Oxo activity.

Histochemical evaluation of Oxo enzyme activity at the root surface

To evaluate Oxo activity at the root surface, the roots were immersed in the same staining solution described by Lane et al. (1993). The purple color started to appear only one min after putting roots in the staining solution. All pictures of wheat root tips were taken after staining for 5 min. Staining without oxalic acid appeared after 1 h and never reached the level observed after staining for 5 min with oxalic acid.

Evaluation of cell death using Evans blue staining

Cell death was evaluated by Evans blue staining, as described by Baker and Mock (1994). Wheat roots were stained in 0.25% aqueous Evans blue for 15 min at room temperature. Roots were then washed twice for 15 min with distilled water and directly photographed. For quantitative assessment, the last 5 mm of root tips were excised from 15 roots and the Evans blue was efficiently extracted without grinding using 100% N,N-dimethylformamide. Root tips were allowed to soak in N,N-dimethylformamide for 24 h at 4°C. Optical density was measured at 600 nm and the background of cell death observed in the control, without Al, was subtracted for each time point.
Results

Analysis of DNA sequences

A near-full-length insert of 860 bp was isolated upon re-screening of the cDNA library. Comparison with many published oxalate oxidase-like sequences is shown in Fig. 1. DNA and protein identity are shown in Table 1. The War13.2 cDNA sequence (Table 1A) has 76.2% identity and the deduced amino acid sequence (Table 1B) has 73.5% identity to the Bh6-903 oxalate oxidase-like cDNA and predicted amino-acid sequences of barley respectively. The War13.2 protein sequence is much less homologous (ca. 40%) to two other wheat germins (gf-2.8 and gf-3.8) than to the barley Bh6-903 oxalate oxidase-like protein (73.5%). However, three other barley sequences (OXOX, HvOxOa, and HvOxO) have a high homology to the gf-2.8 and gf-3.8 wheat proteins (greater than 88.3%). The War13.2 and the Bh6-903 proteins may thus have evolved in response to stress rather than to growth and development per se and probably belong to a different subfamily of germin-like proteins. The War13.2 wheat oxalate oxidase-like sequence contains a putative N-glycosylation site (N^79) and has an N-terminal stretch of hydrophobic amino acids (Fig. 1) suggesting the presence of a signal peptide. Computer-assisted analysis (GCG/Sigcleave) and alignment to N-terminal amino acid sequences of related barley proteins (Dumas et al. 1993, Lane et al. 1993, Hurkman and Tanaka 1996) indicate the presence of a signal peptide of 21 amino acids targeting the protein for extracellular localization. The proteins in Fig. 1 contain two conserved regions (A and B) and the longest region (B) is the rare HI/THPRA TEI found only in germin and spherulins 1a/1b (Lane 1994). After cleavage of the targeting leader peptide, the mature War13.2 protein would have 205 residues with a molecular mass of 22.3 kDa and a pI of 6.67.

Increased Oxo protein expression in response to Al exposure

To demonstrate that the antibody directed against the antigenic region of War13.2 is an anti-Oxo antibody, we tested its reactivity against a commercial preparation of barley Oxo (Roche Molecular Biochemical). Coomassie blue staining reveals the 23 kDa protein monomer (Fig. 2A). The purified antibody allowed a clear detection of commercial Oxo as shown in Fig. 2B thus validating our antibody preparation as an
Oxalate oxidase, cell death, and Al-toxicity

Anti-Oxo antibody. The WAR13.2 kinetics of expression was investigated in Atlas-66 and Fredrick wheat plants exposed to Al concentrations giving equivalent RGIs (50 μM Al for Atlas-66 and 5 μM for Fredrick, see Hamel et al. 1998). The immunoblot in Fig. 3B shows that at day 0, the WAR13.2 antibody reacts with two protein bands of 27 and 30 kDa in both cultivars. These proteins may represent different germin-like Oxo isoforms that do not accumulate under Al exposure but rather appear to decrease in abundance at high Al concentrations. The WAR13.2 antibody also detects a band of approximately 23 kDa which is

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Absence of cell death in both cultivars (Fig. 3B). However, it accumulates faster in the tolerant cultivar Atlas-66. The size of the protein induced by Al is in agreement with the molecular mass of commercial Oxo (Fig 2) and with the theoretical value deduced from the mature WAR13.2 amino acid sequence shown in Fig. 1.

Since Oxo protein expression was increased upon Al exposure, we determined whether this increase was accompanied by an increase in Oxo activity. The time-course is shown in Fig. 3C. In control plants (day 0), no Oxo activity was found suggesting that the 27 and 30 kDa proteins identified by the Oxo antibody are germin-like proteins devoid of Oxo activity that may correspond to previously identified germin-like proteins or to different glycosylation states (Lane et al. 1992). In plants treated with Al, Oxo activity was observed in both cultivars after 1 d of Al exposure. Oxo activity was observed at 125 kDa which is compatible with a pentameric form of active Oxo as previously suggested (Lane et al. 1993). Interestingly, we observed that the quantity of 23 kDa monomer of Oxo isoform continued to increase after 1 d of Al exposure without a corresponding increase in enzyme activity (compare Fig. 3B and C). These results suggest that the level of accumulation of this monomer isoform does not always correspond to the level of enzyme activity.

Oxo protein accumulation and activity in Atlas-66 and Fredrick exposed to different Al concentrations were evaluated after 3 d of exposure to achieve maximal accumulation (Fig. 4). Increasing Al concentrations lead to a higher accumulation of the 23 kDa Oxo isoform (Fig. 4B) reaching a maximum at 50 μM Al in both cultivars. However, this Oxo isoform accumulates at a lower Al concentration (5 μM, Fig. 4B) in the sensitive cultivar Fredrick compared to the cultivar Atlas-66 where it starts to accumulate only at 50 μM Al. Similarly, when we examined Oxo activity in the same samples (Fig. 4C), activity was detectable in Fredrick exposed to 5 μM Al and reached a maximum at 50 μM Al while in the tolerant cultivar, Atlas-66, Oxo activity was undetectable at 5 μM Al but observed at 50 μM Al. The appearance of Oxo activity is thus well correlated with Al concentrations resulting in 50% RGI. However, at higher Al concentrations, there is a discrepancy between the amount of 23 kDa Oxo isoform and the level of enzyme activity. The greatest discrepancy is in the sensitive cultivar Fredrick where the level of the 23 kDa isoform at 250 μM Al of exposure is equivalent to the level found at 50 μM Al (Fig. 4B) while the enzyme activity decreases at the higher Al concentration (Fig. 4C). These discrepancies may indicate that the Oxo enzyme is activated post-translationally through different steps including transport, cleavage of the leader peptide, assembly of the pentameric mature Oxo, and insolubilization in the cell wall. Furthermore, an increase in cell death may affect these post-translational events and cause an imbalance between all the forms of Oxo. A direct measurement of cell death and of Oxo activity in vivo was thus needed to clarify this issue.

**Cell death monitoring and histochemical staining of Oxo activity**

A time course of cell death was monitored with Evans blue staining as a measure of membrane integrity (Fig. 5B, D). Limited cell death can be observed in the root tips of control plants as previously reported (Wang et al. 1996). Interestingly, cell death is observed rapidly in other regions of the root apex (after only 8 h) when exposed to high Al concentrations. In Fredrick, cell death is seen at 50 μM Al and appears in the elongation zone at first and progresses gradually towards the root tip during longer exposures (Fig. 5D, 1–3 d). When Fredrick is exposed to 250 μM Al, cell death appears first at the root tip at 8 h and progresses in the elongation zone (Fig. 5D). When we examine closely the tolerant cultivar Atlas-66, we find that the pattern of cell death (large regions of the epidermis) at 250 μM is fairly similar to the pattern observed in the sensitive cultivar at the same Al concentration but cell death may be occurring at a slower rate or is slightly less extensive. If we analyze the data in the sensitive and tolerant cultivars at Al...
concentrations giving comparable RGIs, a strikingly different picture emerges. Cell death is not observed at any times of exposure in the sensitive cultivar Fredrick exposed to 5 μM Al which inhibits growth by 50% (Fig. 5D). On the contrary, cell death is rapidly seen in the tolerant cultivar exposed to 50 μM Al (Fig. 5B, 8 h) resulting in a similar RGI. Furthermore, a most interesting pattern of cell death is observed when the roots are closely examined. We see that only a few cells are dying in the root tips giving a punctated pattern (see close-up in Fig 5E, representing the pattern observed after 2 and 3 d). N,N-Dimethylformamide extraction of Evans blue confirmed that cell death was 52±5% as abundant on day 2 at 50 μM Al compared to the last day of exposure to 250 μM Al were maximal cell death was observed (Evans blue staining set at 100%). The amount of cell death on day 3 at 50 μM Al was still at a similar level with a value of 56±6%. A punctated pattern is also seen to some extent in the sensitive cultivar at high Al concentrations but in Atlas-66, this pattern is stable with long exposure periods at the physiologically relevant concentration of Al resulting in 50% RGI (Fig. 5B, 50 μM, from 1 to 3 d). This may suggest that limited cell death could contribute more efficiently to Al detoxification in the tolerant cultivar, especially
since there is no cell death observed in the sensitive cultivar exposed to an Al concentration (5 μM) giving an equivalent RGI.

Oxo-dependent H$_2$O$_2$ production on the root surface was investigated in Atlas-66 and Fredrick wheat plants exposed to different Al concentrations. Increasing Al concentrations leads to higher H$_2$O$_2$ production suggesting that Oxo may participate in the observed H$_2$O$_2$ increase (Fig. 5A, C). It indicates that in vivo, the enzyme is in excess of its substrate allowing a rapid use of available oxalic acid. When we examined Oxo activity during Al exposure, we found that Oxo staining is not observed (or is below the detection level) after 8 h of exposure and is thus appearing after cell death. Oxo staining gradually increases with time of exposure in root tips and is reproducibly detectable after 1 d of exposure at 50 or 250 μM Al in both Fredrick and Atlas-66 (Fig. 5A, C). In contrast to cell death patterns, which did not correlate with RGI in both cultivars, Oxo staining is observed at Al concentrations resulting in 50% RGI in both cultivars. In Fredrick, Oxo accumulation is detected after 2 d at 5 μM Al (Fig. 5D) while it is detectable after 1 d of exposure at 50 μM Al (but not 5 μM) in the tolerant cultivar Atlas-66. These results are in agreement with our previous data demonstrating that Oxo mRNA accumulates in response to Al stress as evaluated with RGI. Oxo staining always appears stronger first in the elongation zone and then becomes more extensive towards the root tips at all Al concentrations with longer exposure periods. This pattern is thus different from the one found for cell death.

Oxo also accumulates upon Al exposure in axenic cultures

In order to determine whether Al alone is able to induce Oxo activity or whether Al may act indirectly by increasing sensitivity to pathogens, we established wheat axenic cultures and evaluated the effect of Al on Oxo activity by the histochemical staining method for Oxo described above. The results (not shown) clearly demonstrated that Al can trigger by itself the accumulation of an active Oxo enzyme and is not due to an undetected interaction with pathogens.

Discussion

Oxo sequence analysis

The plant germin family includes protein members from monocotyledons, dicotyledons, and gymnosperms. Pairwise comparisons reveal DNA identities ranging from 43.2% to 97.8% (Table 1A) and amino acid identities ranging from 29.7% to 98.5% (Table 1B). All of these proteins share a number of constant features: a signal peptide, one or two putative N-glycosylation site(s), and two conserved domains of unknown function (called A and B in Fig. 1). Domain B (HXH(X)E) may be involved in the binding of the auxin carboxylic acid group because of its similarity with box 1 (Brown and Jones 1994) and D16 (Venis and Napier 1995). Auxin is known to play a role in root development and to regulate the expression of several genes. The wheat germin promoter was found to contain several auxin-response elements (Lane et al. 1991, Berna and Bernier 1997). It is therefore speculated that germin has a role in auxin-mediated cellular expansion during germination by altering the properties of the cell wall. However, since there is 10–15 germin-like genes in wheat and other species with very few cases where Oxo activity could be demonstrated, it remains unclear whether all the germin genes carry out the same function (Caliskan and Cuming 1998, Berna and Bernier 1999). We have identified three putative Oxo monomer isoforms; two of them (27 and 30 kDa) are expressed during normal growth conditions and may correspond to previously identified germins (Lane 1994). These two isoforms were not associated with any detectable Oxo activity (see Fig. 3C, lane 1) indicating that they may play a distinct role in promoting cellular proliferation during germination. Furthermore, Ohmiya et al. (1998) found a gene encoding auxin-binding proteins from peach that has significant peptide sequence similarity with germin proteins. They demonstrated that their auxin binding protein is associated with the cell wall like all other auxin-binding proteins. Unfortunately, the presence of domain B does not allow the distinction between germin-like Oxo and oxidase-like proteins (lacking Oxo activity). Criteria allowing a distinction between the two types of germins and their association with different physiological states needs to be established since Oxo activity may not be compatible with cellular proliferation (see below).

Oxo protein accumulation and activity in cereals

The accumulation kinetics of the 23 kDa Oxo monomer and Oxo activity was positively correlated with RGI in the tolerant and the sensitive cultivars (Fig. 3, 4). Oxalate oxidase-like proteins were also shown to accumulate under several different physiological conditions including biotic and abiotic stresses (Zhang et al. 1995, Hurkman and Tanaka 1996, Berna and Bernier 1997, Berna and Bernier 1999, Nowakowska et al. 1998). To demonstrate that the 23 kDa isoform of Oxo protein accumulation is correlated with an increase in Oxo activity in Al-exposed wheat, we measured the activity of Oxo in root tip extracts by an in vitro assay. An increase in Oxo enzyme activity appears in a 125 kDa band after the first day of Al exposure in the two cultivars (Fig. 3C). Oxo activity was also shown to increase in both cultivars when Al concentration was raised (Fig. 4C). However, in the sensitive cultivar Fredrick exposed to 250 μM Al (Fig. 4C), we found that soluble Oxo activity was decreasing. In order to evaluate the level of Oxo activity in vivo, the roots were directly immersed in the staining solution used for Oxo activity. Our results confirmed that Oxo is responsible for a sustained H$_2$O$_2$ production in wheat exposed to Al (Fig. 5A, C, note that the omission of oxalic acid from the staining solution did not allow the detection of Oxo activity). Although the results of histochemical staining in vivo demonstrated that Oxo activity increases with Al exposure, the kinetics of accumulation are different than for the soluble Oxo activ-
ity detected on blots (Fig. 3, 4). For example, there was no increase in soluble enzyme activity through time (Fig. 3) while it can be clearly seen that Oxo activity in vivo gradually increase between 1 and 3 d (Fig. 5A, C). Furthermore, the decrease in soluble enzyme activity detected in Fredrick exposed to 250 µM Al (Fig. 4C) did not result in a lower activity in vivo (Fig. 5C). The discrepancy between soluble and in vivo detected enzyme activity supports the postulate that soluble Oxo represents the fraction being actively synthesized and in transit to become insolubilized in the cell wall. This hypothesis would explain why the amount of soluble active Oxo is stable through time when analyzed at one concentration of Al (Fig. 3C) while in vivo activity increases with time by histo-chemical staining for the same concentration (Fig. 5A, C). The accumulation of a germin isoform in the cell wall which was 70% as abundant as the soluble form was previously reported and supports our finding (Lane et al. 1992). Similarly, a germin-like protein was found to be insolubilized during oxidative stress in barley (Vallelian-Bindschedler et al. 1998).

Possible role of H$_2$O$_2$ production and cell death in Al detoxification

A role for Oxo in cell wall restructuring was previously postulated (Lane et al. 1993). H$_2$O$_2$ can lead to rapid cell wall reinforcement because it is involved in oxidative cross-linking (Brisson et al. 1994) and insolubilization of hydroxyproline-rich proteins (Otte and Barz 1996). H$_2$O$_2$ is required to catalyze crucial peroxidase cross-linking reactions which occur in lignification, in particular the formation of diferulate and ferulate-poly saccharide esters. We have identified a distinct Oxo isoform possessing a H$_2$O$_2$-generating activity (see Fig. 3B, C). Caliskan and Cuming (1998) demonstrated that the germination-associated accumulation of Oxo activity occurs specifically in cells and tissues in which cellular expansion is restricted. An increase in peroxidase mRNA levels upon exposure to Al (Ezaki et al. 1996, Hamel et al. 1998) or after inoculation with fungus (Thordal-Christensen et al. 1992) was also previously demonstrated. The plant may use Oxo and peroxi- dase to restructure the cell wall and restrict Al entry by reducing cell wall porosity (lignification through isodityrosine linkages, glycoprotein cross-links) and by adding chelating groups (negatively charged ferulate and carboxyl groups of pectin) that can bind positively-charged Al$^{3+}$. An increase in pectin, hemicellulose and cellulose content was previously demonstrated upon Al exposure in squash root cell walls (Le Van et al. 1994) and lignin deposition was observed in wheat exposed to Al (Sasaki et al. 1996). These reactions could play a role in reducing Al entry rates which, in soybean, were shown to be rapid during the first h of Al exposure but greatly reduced when examined after 24 h (Lazof et al. 1994).

During Al exposure, we have found an interesting parallel with a hypersensitive reaction by detecting significant cell death after only 8 h of exposure to Al. This early cell death cannot be attributed to the overproduction of H$_2$O$_2$ since this product is hard to detect before 24 h of Al exposure. However, since cell death occurs much after the beginning of root growth inhibition (Matsumoto 2000), the arrest of root elongation may trigger a signal leading to cell death. Interestingly, early cell death is detected in the tolerant cultivar Atlas-66 exposed to an Al concentration resulting in 50% RGI (50 µM). When the sensitive cultivar Fredrick was analyzed at an Al concentration resulting in a similar RGI (5 µM) we did not find any cell death (Fig. 5), demonstrating that cell death is not related to RGI in the sensitive cultivar and that cell death is controlled by a different signaling pathway to stress gene induction. We chose to use RGI as a measure of Al stress as suggested by Parker (1995), because RGI was also correlated with similar molecular responses in Al tolerant and sensitive cultivars (Hamel et al. 1998). When the tolerant cultivar is observed more closely, we realize that cell death is limited to a few cells at a time, especially in the elongation zone, and the number of cells involved does not increase throughout the exposure period (the punctuated pattern of cell death at day 2 and 3 is very similar, Fig. 5E). The region shown in Fig. 5E is approximately 5 mm in length and represents the average growth in a 24-h period under Al stress (growth in the control is approximately 1 cm d$^{-1}$ and a growth of 5 mm is equivalent to 50% RGI). The fact that we can see individual cells dying (last 3–4 mm) even after 3 d of Al exposure demonstrates that an equilibrium between growth and cell in this region has been reached. This response may be useful to maintain the activity of the elongation zone and of meristematic cells by allowing the replacement of epi- dermal cells once they have accumulated a high amount of Al in a bound form. Such a cellular turnover would reduce the amount of Al detected in epidermal cells especially at long exposure times. This phenomenon must be considered when sensitive and tolerant cultivars are compared and is another parameter that could contribute to the greater exclusion of Al found in tolerant cultivars. Lazof et al. (1994) previously demonstrated that the rate of Al accumulation is rapid during the first 30 min of exposure but decreases after 24 h. The reduc- tion in Al entry rates could be explained by various mecha- nisms and the extent to which cellular turnover could contrib- ute to overall exclusion remains to be established. The precise mechanism leading to cell death is unknown but it shares some similarities with a hypersensitive reaction (Levine et al. 1994). The timing of events indicates that H$_2$O$_2$ is accumulating as a second wave of responses (with the up-regulation of other War genes) which appears much after cell death and is thus reminis- cent of a hypersensitive response where limited cell death is followed by a secondary response increasing plant resistance to pathogens (Strobel et al. 1996). In this paradigm, Oxo activity may be viewed as another means for root tip cells to help trap Al in the cell since it is well known that over 90% of Al accumu- lates in cell walls. Oxo may thus be considered as playing a supporting role in reducing Al toxicity.

Further studies are necessary to determine the impact of cell death and Oxo activity on Al toxicity. The War13,2 cDNA...
can be used to overexpress or reduce Oxo activity (by translational inhibition) in transgenic plants to evaluate the relative importance of Oxo in detoxifying Al or in causing further cell death at low exposure times with high Al concentrations. The overexpression of a peroxidase gene in Arabidopsis resulted in an increased resistance to Al and oxidative stresses (Ezaki et al. 2000) supporting the idea that H₂O₂ does accumulate to toxic levels during Al exposure. During the course of this study, we uncovered a very interesting mechanism of early cell death that may play an important role in Al detoxification since it occurs at an Al concentration that limits root growth by 50% only in the tolerant cultivar. However, as for organic acid excretion (Ishikawa et al. 2000), it is difficult to determine the extent to which this cell death detoxification mechanism could contribute to Al tolerance. Interestingly, while plants such as buckwheat use oxalate as an Al-chelating agent (Ma et al. 1997), wheat, which is known to excrete mostly malate in the rhizosphere (Delhaize et al. 1993), uses oxalate to produce H₂O₂ thus reducing the amount of available oxalate.

We are currently trying to distinguish the signalling pathways that regulate the induction of cell death from the one that leads to the induction of War genes. A better understanding of these pathways will allow us to modulate their activities and to evaluate the importance of controlled cell death as a detoxification mechanism.

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