Genotypic Differences in Aluminium Resistance in Maize: Inheritance, the Role of Cell-Wall Properties, and Al Localization in the Root Apex

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Dedicated to

my mother, Dawi Gemechu

and

my late father, Eticha Safo

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ABSTRACT

Aluminium (Al) toxicity is a major limiting factor for maize production on acid mineral-soils. A specific symptom of Al injury is the inhibition of root growth which limits the uptake of water and mineral nutrients. There is wide genotypic variation in Al resistance in maize. A well-known mechanism of Al resistance in maize is the release of organic acid anions which detoxify Al. However, this can only partly explain the genetic variations. Therefore, the presence of additional Al-resistance mechanisms has been suggested. These additional mechanisms could be based on differences in cell-wall composition.

The objectives of this work were to study:

1. the inheritance of Al resistance in maize
2. the role of cell-wall properties in Al resistance
3. the cellular distribution of Al in the root apex

The inheritance of Al resistance in 15X15 diallel crosses of maize cultivars was studied in hydroponic culture using callose induction as a physiological marker of Al sensitivity. The diallel analysis indicated that Al resistance in maize is controlled by additive genes, i.e., polygenic inheritance, in agreement with the adaptation of the same maize cultivars to tropical acid soil environments. Additive genes can be exploited through recurrent selection, a breeding strategy which increases the frequency of favourable genes in a population. Moreover, Al-resistant cultivars with good general combining abilities were identified to be used for the breeding of maize with high yielding capacity on acid soils.

Al mainly accumulates in the cell wall, particularly bound to the negative charges of the pectic matrix. Al-resistant maize cultivars accumulate less Al than Al-sensitive cultivars, suggesting an important role of Al exclusion for Al resistance. The cell-wall pectin-content and degree of methylation of pectin was studied using maize cultivars contrasting in their Al resistance. The Al-sensitive cultivar compared to the Al resistant cultivar had a higher pectin content particularly in the most Al-sensitive apical root zone. In addition immunofluorescence staining of apical root cross sections using monoclonal antibodies specific for pectin with different degrees of methylation revealed that the Al-sensitive cultivar has a higher proportion of low-methylated pectin and thus a higher negativity of the cell wall which is in agreement with its higher Al content and Al sensitivity.

Using the fluorochrome morin, Al was mainly localized in the cytosol but not in the cell wall of the maize root apex. This is in contrast to the results showing a higher accumulation of Al in the cell wall. However, in vitro assays clearly showed that morin cannot form a fluorescent complex with pectin-bound Al. Thus, in spite of its higher accumulation in the cell wall, pectin-bound Al cannot be detected by morin. This investigation clarifies contradicting results in the literature about the cellular distribution of Al and leads to a reconsideration of conclusions based on the morin staining technique.

In conclusion, Al resistance in maize is polygenically inherited which is in agreement with the involvement of multiple mechanisms, release of organic acid anions and lower negativity of the cell wall, conferring Al resistance in maize.

Key Words: Al resistance, Cell wall, Inheritance

ZUSAMMENFASSUNG

Aluminium (Al) ist der Faktor, der die Produktion von Mais auf sauren Böden am stärksten begrenzt. Ein spezifisches Symptom für eine Schädigung durch Al ist die Hemmung des Wurzelwachstums. Dies führt zu einer Hemmung der Wasser- und Nährstoffaufnahme. Bei Mais findet man eine große genotypische Variation in Bezug auf Al-Resistenz. Ein gut dokumentierter Mechanismus der Al-Resistenz ist die Ausscheidung organischer Säuren, diese überführen Al in eine nicht-phytotoxische Form. Dies kann aber nur einen Teil großen genetischen Variation erklären. Es wird daher angenommen, dass noch weitere Resistenzmechanismen existieren. Eine besondere Bedeutung wird dabei der Zellwandzusammensetzung zugeschrieben.

Das Ziel der vorliegenden Arbeit war daher die Vererbung von Al-Resistenz in Mais, die Rolle von Zellwandeigenschaften in der Al-Resistenz und die zelluläre Verteilung von Al in der Wurzelspitze zu untersuchen.

Die Vererbung der Al-Resistenz wurde an 15X15 diallelen Kreuzungen in Hydrokultur untersucht. Dabei wurde die Bildung von Callose als physiologischer Marker für Al-Sensitivität benutzt. Diese Untersuchungen zeigten, dass Al-Resistenz durch additive Gene kontrolliert wird, d. h. sie wird polygenisch vererbt. Additive Gene können durch rückgreifende Selektion dazu genutzt werden die Al-Resistenz zu erhöhen. Außerdem wurden durch diese Untersuchungen Sorten charakterisiert, die eine gute allgemeine Kombinationseignung besitzen und daher in der Züchtung Al-resistenter Maissorten mit einem hohen Ertragspotential auf sauren Böden eingesetzt werden können.

Al akkumuliert vor allem in der Zellwand und bindet dort an die negativen Ladungsstellen der Pektinmatrix. Al-resistente Maissorten akkumulieren im Allgemeinen weniger Al als Al-sensitive Sorten. Dies weist auf eine große Bedeutung von Ausschlussmechanismen für die Al-Resistenz hin. An zwei unterschiedlich Al-resistenten Maissorten wurde der Pektingehalt der Zellwand und der Methylierungsgrad des Pektins untersucht. Es konnte gezeigt werden, dass die sensitive Sorte einen höheren Pektingehalt und einen geringeren Methylierungsgrad als die resistente Sorte in der Al-sensitiven apikalen Wurzelzone besitzt. Auch Immunofluoreszenzfärbungen mit Antikörpern, die spezifisch für Pektine mit unterschiedlichem Methylierungsgrad sind, zeigten, das die sensitive Sorte einen höheren Anteil an schwach methyliertem Pektin besitzt und damit eine höhere Dichte negativer Ladung als die resistente Sorte. Die höhere Dichte negativer Ladung in der Zellwand ist in Übereinstimmung mit dem höheren Al-Gehalt und der höheren Empfindlichkeit des sensitiven Genotyps.

Mit dem Fluorochrom Morin konnte Al im Cytosol, nicht aber in der Zellwand lokalisiert werden. Dies steht im Widerspruch zu den Untersuchungen, die den negativen Bindungsstellen in der Zellwand eine wesentliche Rolle in der Al-Resistenz zuweisen. Mit in vitro Assays konnte aber gezeigt werden, das Morin an Pektin gebundenes Al nicht komplexieren kann. Daher kann pektin-gebundenes Al trotz einer starken Akkumulation in der Zellwand durch Morin nicht nachgewiesen werden. Die hier dargestellten Ergebnisse klären widersprüchliche Ergebnisse zur Al-Akkumulation und führten zu einer Neubewertung der mit Hilfe von Morin erzielten Ergebnisse.

Zusammenfassend konnte gezeigt werden, dass Al-Resistenz in Mais polygenisch vererbt wird und dass mehrere Mechanismen wie die Ausscheidung organischer Säuren und eine geringere Negativität der Zellwand, an der Ausprägung der Al-Resistenz in Mais beteiligt sind.

Schlagworte: Al-Resistenz, Vererbung, Zellwand

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ABBREVIATIONS

Al	aluminium
Al_{mono}	monomeric aluminium
ALMT1	aluminium-activated malate transporter
ANOVA	analysis of variance
℃	degree Celsius
ca	circa (about)
cm	centimetre
cv	cultivar
CW	cell wall
EDTA	Ethylendiamine tetra acetate
g	gram
µg	microgram
GaE	galacturonic acid equivalents
GAX	glucuronoarabinoxylan
GCA	general combining ability
GFAAS	Graphite furnace atomic absorption spectrometer
h	hour
ha	hectare
HG	homogalacturonan
i.e.	id est (that is)
l	litre
M	molar concentration
mg	milligram
min	minute
ml	millilitre
mm	millimetre
µm	micrometer
mM	millimolar
µM	micromolar
N	normal concentration

n	number of observations
nm	nanometre
nM	nanomolar
ns	nonsignificant
P	probability
PE	pachyman equivalents
QTL	quantitative trait loci
SCA	specific combining ability
SD	standard deviation
x g	gravity

GENERAL INTRODUCTION

Acidic Soils and Al toxicity

Acid soils with pH below 5.5 cover 30 – 40% of the world's arable land and limit crop production worldwide. They mainly occur in two global belts: the northern belt of organic acid-soils in the humid temperate zone, and the southern belt of mineral acid-soils in the humid tropics (von Uexküll and Mutert, 1995). Crop production on acid soils is constrained by a combination of several factors such as proton toxicity, mineral toxicity (Al and Mn), and nutrient deficiencies (P, Ca, Mg, and Mo) (Marschner, 1995). However, Al toxicity is the single most important factor, being a major constraint for crop production on 67% of the total acid soil area (Eswaran et al., 1997).

Al is the most abundant metal constituting 8% of the earth's crust and on average about 7% in soils (Lindsay, 1979). Most of the Al in the soil is in alumino-silicate compounds and normally exists as insoluble and non-toxic form. However, in acid soils it is readily solublised and becomes toxic to plants and other biological organisms at micro molar concentrations. Martin (1988) elaborated the details of bioinorganic chemistry of Al. In solutions more acidic than pH 5.0, Al exists as octahedral hexahydrate ($Al(H_2O)_6)^{3+}$) usually denoted as Al^{3+}. As the pH rises, $Al(H_2O)_6)^{3+}$ undergoes successive deprotonation to yield $Al(OH)^{2+}$ and $Al(OH)_2^{+}$. Above pH 5.0 it precipitates as $Al(OH)_3$ which redissolves in basic solutions (above pH 8.0) due to the formation of tetrahedral $Al(OH)_4^{-}$ (aluminate ion). Despite its ubiquity, Al is not a nutrient element for plants. In contrast, its phytotoxicity is well documented (Horst, 1995; Matsumoto, 2000, Kochian et al., 2004). Among the

monomeric Al forms present at acidic pH [Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_2^{+}$], Al^{3+} is considered to be the most phytotoxic Al species (Kinraide, 1991; Taylor, 1995; Kochian et al., 2004). The polymeric Al species, tridecamer polycation [$AlO_4Al_{12}(OH)_{24}(H_2O)_{12}^{7+}$] abbreviated as Al_{13} was shown to be even highly toxic in a simple solution culture (Parker et al., 1989). However, it is doubtful whether this highly-charged cation exists in soil solution given the inhibition of its formation by silicate (SiO_3^{2-}) (Larsen et al., 1995) and sulphate (SO_4^{2-}) (Kerven et al., 1995).

Mechanisms and effects of Al toxicity

Al toxicity was known since the beginning of the 20th century as the cause of inhibition of root growth of barley and rye in acid soils (Hartwell and Pember, 1918). Despite concentrated research efforts since then, the physiological mechanism of Al-induced inhibition of root growth is still not well elucidated. However, a significant understanding of the effect of Al on the physiology and molecular biology of the plants was gained during the last two decades, as several reviews indicate (Horst, 1995; Kochian, 1995; Delhaize and Ryan, 1995; Taylor, 1995; Matsumoto, 2000; Rengel and Zhang, 2003; Kochian et al., 2004). Al rapidly affects a number of cellular functions and thus it has been difficult to identify the primary effect of Al on plant cells and to distinguish the causes from the consequences of Al toxicity. Since Al interacts with a number of extra cellular and intracellular structures, several different mechanisms of Al toxicity have been hypothesised. These include:

i. interaction of Al with apoplastic constituents (Klimaschevskii and Dedov, 1975; Horst, 1995; Blamey, 2001),
ii. disruption of plasma membrane and plasma-membrane transport-processes (Huang et al., 1992; Gassmann and Schroeder, 1994), and

iii. interaction of Al with symplastic constituents (Martin, 1988; Kochian, 1995).

There is still no general consensus on any of these hypotheses. However, there is a unanimous agreement that the primary target of Al toxicity is the root apex (Ryan et al., 1993; Sivaguru and Horst, 1998), the common symptom being the rapid inhibition of root elongation. There is also no doubt that Al strongly and rapidly binds to the cell wall. Al bound to the cell wall may cause cell-wall rigidity, which is responsible for the rapid cessation of root elongation (Blamey, 2001). In addition, the resumption of root growth after desorption of Al by citrate (Ownby and Popham, 1989) suggests that Al-induced inhibition of root elongation is primarily due to apoplastic rather than symplastic lesions. Thus the current work focuses on the apoplastic mechanisms of Al toxicity.

Mechanisms of Al resistance

The physiological mechanisms of Al resistance are well documented in recent reviews (Matsumoto, 2000, Ma et al., 2001; Ryan at al., 2001; Samac and Tesfaye, 2003; Kochian et al., 2004) and can generally be divided into two: a) internal detoxification mechanisms, and b) exclusion mechanisms.

Some plant species accumulate large quantities of Al in the roots and shoots in non-toxic forms and also readily distribute Al within the plant tissues. The complexing role of organic acids was demonstrated to contribute to the internal detoxification of Al by complexation to oxalate in buckwheat and melastoma (Ma et al., 1998; Watanabe et al., 1998) and to citrate in hydrangea, (Ma et al., 1997; Naumann and Horst, 2003).

In most crop species, Al-resistant genotypes contain less Al in their root tissue than Al-sensitive genotypes indicating that the resistance is due to Al exclusion. A number of Al-exclusion mechanisms were observed in different crop species. These include:

i) Root exudation of organic acids anions that complex Al in the apoplast and in the rhizosphere (Ma et al., 2001; Ryan et al., 2001)

ii) Root exudation of phosphate which precipitates Al (Pellet et al., 1995, 1996 and 1997)

iii) Root exudation of phenolic compounds that detoxify Al in the rhizosphere (Kidd et al., 2001)

iv) Root-mediated increase in the rhizosphere pH which precipitates Al or may shift the equilibrium to less toxic Al ions (Degenhardt et al., 1998)

v) Root secretion of mucilage which binds Al (Horst et al., 1982; Henderson and Ownby, 1991; Archambault et al., 1996a; Feng Li et al., 2000)

vi) Reduction in root cation exchange capacity (Blamey et al., 1990)

vii) Exclusion at the plasma membrane (Archambault et al., 1997)

viii) Accelerated turnover of root epidermal cells (Delisle et al., 2001)

Most of these physiological processes and modifications are concentrated at the root apex, the region which is very Al-sensitive. Among these mechanisms, Al-induced release of organic acid anions is the most effective Al-resistance mechanism in many crop species and has been the subject of intensive studies in many research groups. For example, Al-resistant wheat genotypes release malate whereas citrate is released by Al-resistant maize and soybean genotypes (Delhaize et al., 1993a; Pellet et al., 1995 and 1996; Yang et al., 2000; Kollmeier et al., 2001; Silva et al., 2001). The release of organic acid anions is mediated by Al-activated opening of anion

channels (Ryan et al., 1997; Zhang et al., 2001; Kollmeier et al., 2001; Piñeros and Kochian, 2001).

Besides these well known mechanisms, there are still unknown mechanisms of Al resistance in some plant species. A peculiar example is the signalgrass (*Bracchiaria ducumbens*) which is extremely resistant against Al, but the mechanisms of resistance are not yet known (Wenzl et al., 2001 and 2002).

Genetics of Al resistance

A range of studies have been made to understand the inheritance of Al resistance. These studies were mainly concentrated on cereals which are the priority crops for food production (see recent reviews, Hede et al., 2001; Garvin and Carver 2003). In crop species such as wheat, rye, barley and sorghum, Al resistance is known to be a qualitative trait. Intensive studies on wheat indicate that in many crosses between Al-resistant and Al-sensitive wheat cultivars, Al resistance segregates as a single, dominant locus (Kochian et al., 2004 and references therein). Moreover, a survey of 36 wheat cultivars by Ryan et al. (1995) showed that 84% of the variation in Al resistance is explained by the quantity of Al-activated malate released from root apices. This strongly suggests that the genetic variation observed in wheat exists within a single physiological mechanism.

In contrast, quantitative inheritance of Al resistance operates in plant species such as maize, rice and *Arabidopsis* (Kochian et al., 2004). Understanding the genetic basis of Al resistance has been the subject of many breeding programs seeking to increase Al resistance in maize (Magnavaca et al., 1987a). Some authors have concluded that Al resistance in maize is a qualitative trait (Ruhe et al., 1978; Sibov et al., 1999) but most investigations show that it is a quantitative trait (Magnavaca et al., 1987a; Lima

et al., 1992; Giaveno et al., 2001; Ninamango et al., 2003). In line with this, physiological studies suggest the presence of multiple Al-resistance mechanisms in maize (Kollmeier et al., 2001; Piñeros et al., 2002) which are possibly controlled by different genes.

Rectifying Al toxicity

Several agronomic practices are used to correct Al toxicity and improve crop growth. Practices such as liming, phosphate fertilization, organic manuring are essential to reduce acidity-related problems and increase the productivity of acid soils (Baligar et al., 1997). However, the use of such practices is beyond the capacity of many small farmers in developing countries. Moreover, the remedial effects of these practices are restricted only to the top soil while the phytotoxicity still remains in the sub soil. Therefore, the use of resistant crop cultivars is suggested along with sound agronomic managements (Granados et al., 1993; Bellon, 2001; Delhaize et al., 2004). Increasing the Al resistance and acid-soil tolerance of major crop plants has been the priority of many breeding programmes, and in fact resulted in remarkable achievements. The best example of such success is the conversion of the low-productivity land of the Brazilian *Cerrados* to an agricultural industry (Borlaug and Rowswell 1997). There is still great potential to increase the productivity of acid and Al-toxic soils.

Maize production on acid soils

Among the worlds cereal crops, maize (*Zea mays* L.) stands first in terms of production and ranks third (next to wheat and rice) in terms of area coverage world wide (FAO 2004). It is grown on more than 140 million ha, out of which 20% is

located in acid-soil environments (von Uexküll and Mutert, 1995). Thus, increasing the Al resistance of maize could significantly increase the world's food production.

Objectives of the study

The aim of the present study was to answer the following questions:

- What is the mode of inheritance of Al resistance in maize?
- Can Al-induced callose formation be used as a physiological marker to characterise the Al resistance of maize cultivars?
- What is the relationship between laboratory results and field performance in studying the inheritance of Al resistance?
- Can differences in cell wall properties contribute to explain the genetic variation in Al resistance?
- Is it possible to clearly show the cellular distribution of Al in the maize root-apex using morin staining?

The answers to these questions are presented and discussed in detail in the following three chapters.

CHAPTER 1

ALUMINIUM-INDUCED CALLOSE FORMATION IN ROOT APICES: INHERITANCE AND SELECTION TRAIT FOR ADAPTATION OF TROPICAL MAIZE TO ACID SOILS

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ABSTRACT

Aluminium (Al) toxicity limits maize production on acid soils of the tropics. However, wide genetic variation exists in maize for Al resistance. The objective of this study was to assess the Al resistance of open pollinated tropical maize cultivars, from widely differing origin, and their diallel crosses based on callose formation as a physiological marker, and to study the inheritance and combining ability for Al resistance. Fifteen maize cultivars from four maize breeding programmes and their 105 crosses were grown under controlled environmental conditions in a growth chamber and treated without or with 25 µM Al at pH = 4.3. After 12 hours of Al treatment, callose contents of 1 cm root apices were determined. There was a significant genotypic variation in callose formation under Al stress. Furthermore, diallel analysis indicated a significant general combining ability (GCA) but not specific combining ability (SCA), indicating that Al resistance is mainly controlled by additive genes. In general, Al-resistant cultivars showed favourable GCA effects while the sensitive cultivars had unfavourable GCA effects clearly indicating the dominant role of Al-resistant cultivars in the development or improvement of Al-resistant maize varieties. Moreover, a relatively high heritability (h^2 = 0.7) was obtained for Al resistance in nutrient solution. Aluminium resistance as revealed by callose content in Al-treated root apices was positively correlated to the relative grain yield of the same crosses evaluated across 5 tropical environments. In addition, strong genetic correlation was observed as GCA of callose formation in nutrient solution closely correlated with GCA of yield on acid soils. These findings suggest that Al-induced callose formation is a powerful tool to enhance the breeding of maize cultivars with superior adaptation to acid and Al-toxic soils.

Key words: Aluminium toxicity; Callose; Diallel cross; General combining ability (GCA); Maize (*Zea mays* L.); Soil acidity

INTRODUCTION

Maize (*Zea mays* L.) is the third most important cereal crop covering worldwide 140 million hectares (Aquino et al., 2001) out of which 26 million hectares are located in acid soil environments (Von Uexküll and Mutert, 1995). The crop yield on acid soils is mainly limited by aluminium toxicity. In addition, other acidity-related stresses, such as proton toxicity, Mn toxicity, and nutrient deficiencies particularly of P, Mg, Ca, and Mo are also important constraints (Marschner, 1995).

Though abundant in the earth's crust, Al occurs as insoluble and non-toxic forms in neutral soils. However, as the soil pH (H_2O) drops below 5.5, Al enters into the soil solution and affects plant growth. The first visible Al injury is the inhibition of root elongation. This has been widely used as a trait for the screening of cultivars for Al resistance (Foy, 1976; Foy et al., 1993). Al-induced callose formation particularly in root apices has been reported to be an even more sensitive physiological marker of Al injury (Wissemeier et al., 1987; Wissemeier and Horst, 1995) and an indicator of genotypic differences in Al sensitivity in maize (Horst et al., 1997; Collet et al., 2002), wheat (Zhang et al., 1994), and soybean (Wissemeier et al., 1992).

To overcome the problem of Al toxicity, breeding of resistant cultivars was suggested as the best option (Bellon, 2001). This is particularly true since liming, the most common practice to alleviate the problem of soil acidity, is too expensive for small farmers, and also not effective to correct soil acidity in deep soil layers. Developing Al-resistant and acid-soil tolerant cultivars could offer a less

expensive, ecologically friendly and permanent solution (Granados et al., 1993) if combined with measures to avoid further soil acidification.

Considerable genetic variations in maize for Al resistance and adaptation to acid and Al-toxic soils have been reported with both qualitative inheritance (Rhue et al., 1978; Miranda et al., 1984) and quantitative inheritance (Lima et al., 1992; Duque-Vergas et al., 1994; Pandey et al., 1994; Borrero et al., 1995; Salazar et al., 1997). Thus, the potential of improving adaptation to acid and Al-toxic soils in maize is promising. We used diallel crosses among 15 maize cultivars selected for their adaptation to varying tropical environments to study their performance, breeding value, as well as gene effects across 5 tropical acid soil environments. In the present study, the same set of cultivars was tested for Al resistance under controlled climatic conditions, using callose formation as a physiological marker. The objectives were to assess the Al resistance of tropical maize cultivars and to study the inheritance of Al resistance based on callose formation in Al-enriched nutrient solution, and to relate Al-induced callose formation to grain yields on acid soils.

MATERIALS AND METHODS

The plant materials consisted of seeds of 15 open-pollinated maize cultivars (Table 1) and 105 F1 crosses obtained among them in a diallel mating design. Two contrasting checks, Al-resistant cv ATP-Y and Al-sensitive cv Lixis were also included. The F1 was developed by using bulk pollen of 100 plants of each cultivar to pollinate at least 25 female plants of each of the 14 other parents. Seeds from the 25 ears obtained per cross (both direct and reciprocal crosses) were bulked, shelled, and random samples of the bulk seed for each cross were used in this study. Crosses were made by IRAD, Cameroon, and testing materials were transferred to INRA-Guadeloupe for distribution to the other partners.

The seeds were surface sterilized for 1 min in diluted sodium hypochlorite with 3-7% active chloride and germinated between wet filter papers soaked in 1 mM $CaSO_4$ solution. After four to five days, seedlings were transplanted to 22-liter pots containing nutrient solution of the following composition [µM]: KNO_3 400, NH_4NO_3 200, KH_2PO_4 10, $MgSO_4$ 100, H_3BO_3 8, $CuSO_4$ 0.2, $ZnSO_4$ 0.2, $MnSO_4$ 1, $(NH_4)_6Mo_7O_{24}$ 0.1, Fe-EDTA 20, $CaSO_4$ 250.

Table 1. Description and origin of the maize cultivars used in the diallel crossing (Y = yellow, W = white, R = red seeded).

Cultivar name	Abbreviation	Grain colour	Origin	Characteristic
ATP.S4.Syn.Y	ATPSY	Y	IRAD, Cameroon	Al-resistant
Tuxpeno Sequia	Tuxp	W	CIMMYT, Columbia	Acid-soil sensitive
96 SA 7	SA7	W	CIMMYT, Columbia	Acid-soil tolerant
96 SA 6	SA6	W	CIMMYT, Columbia	Acid-soil tolerant
96 SA 4	SA4	Y	CIMMYT, Columbia	Acid-soil tolerant
96 SA 3	SA3	Y	CIMMYT, Columbia	Acid-soil tolerant
BR 106+	BR106	Y	EMBRAPA, Brazil	Acid-soil sensitive
Spectral	Spec	W-Y-R	INRA, Guadeloupe	Acid-soil mod. tolerant
Kristal 27	Kris	Y	INRA, Guadeloupe	Acid-soil sensitive
CMS 9213	CM92	W	IRAD, Cameroon	Acid-soil sensitive
ATP.Syn.I.W	ATPW	W	IRAD, Cameroon	Al-resistant
CMS 36	CM36	Y	EMBRAPA, Brazil	Al-resistant
CMS 14C	CM14	Y	EMBRAPA, Brazil	Al-resistant
Antigua GPo2	Antg	Y	CIMMYT, Mexico	Unknown
Natal	Natal	Y	INRA, Guadeloupe	Acid-soil sensitive
Checks				
ATP-SR-Y	ATP-Y	Y	IRAD, Cameroon	Al-resistant
Lixis	Lixis	Y	Germany	Al-sensitive

Source: Welcker (2000)

During 2 days of preculturing, the pH of the nutrient solution was lowered gradually to 4.3 and kept constant during the experiment using an auto-titration device with 0.1 M HCl/KOH. Half of the pots were treated with 25 µM Al (as $AlCl_3$) and the remaining half were kept without Al. The number of replications per cultivar was four. After 12 h Al treatment, 1 cm root tips were cut from the primary or longest seminal root of each plant and immediately transferred to 96% ethanol or shock frozen in liquid nitrogen for callose analysis. Three root tips were rinsed with distilled water and transferred to Eppendorf reaction vials. The root tips were homogenized with a Mixer mill (MM 200, Retsch GmbH & Co. KG, Haan,

Germany). Callose was extracted with 1 ml of 1 M NaOH solution by heating the sample at 80°C in a water bath for 20 min and measured fluorometrically after adding aniline blue according to Kauss (1989). Pachyman (1,3-ß-D-glucan) solution was used as a standard, and thus, root callose-content was expressed as pachyman equivalents (PE) per cm root tip.

The experiment was divided into four sets because of space limitation. Two well-known checks, Al-sensitive cv Lixis and Al-resistant cv ATP-Y (Kollmeier et al., 2000), were included in each set. To allow comparison of cultivars used in separate sets, the data were expressed as a percentage of the Al-sensitive check (Lixis = 100%) in the respective set. In the first set, parental cultivars were evaluated for both callose formation and root elongation, while the crosses were tested only for callose formation in the subsequent runs. Root elongation was determined using neutral red pre-staining procedure according to Schumacher et al. (1983).

Before the statistical analysis of the diallel on the basis of the relative callose contents, a separate set of diallel crosses (8×8 maize inbred-lines diallel) was analysed using both relative callose (Lixis = 100%) and absolute callose content as parameters. The purpose was to check whether both procedures lead to the same results and conclusions regarding the inheritance of Al resistance. Analysis of variance of the 8×8 diallel cross indicated that both relative and absolute callose contents revealed similar gene effects (Table 2). In addition, both parameters gave exactly the same ranking of the GCA effects of the parents thus leading to the same conclusion (Fig. 1). Hence, data on relative callose content (Lixis = 100%) was used as input for the analysis of variance in this study.

Table 2. Diallel analysis of variance for an 8 x 8 diallel of maize lines based on absolute or relative callose content in root apices of plants treated with 25 µM Al for 12 h.

		Absolute callose content [µg PE cm^{-1} root tip]		Relative callose content [Lixis = 100%]	
Source	DF	Mean square	F-value	Mean square	F-value
Crosses	27	0.0709		229.818	
GCA	7	0.1459	3.27*	473.104	3.27*
SCA	20	0.0446	0.92^{ns}	144.668	0.92^{ns}
Error	52	0.0485		157.525	

* = significant at $\alpha = 0.05$; ns = non significant

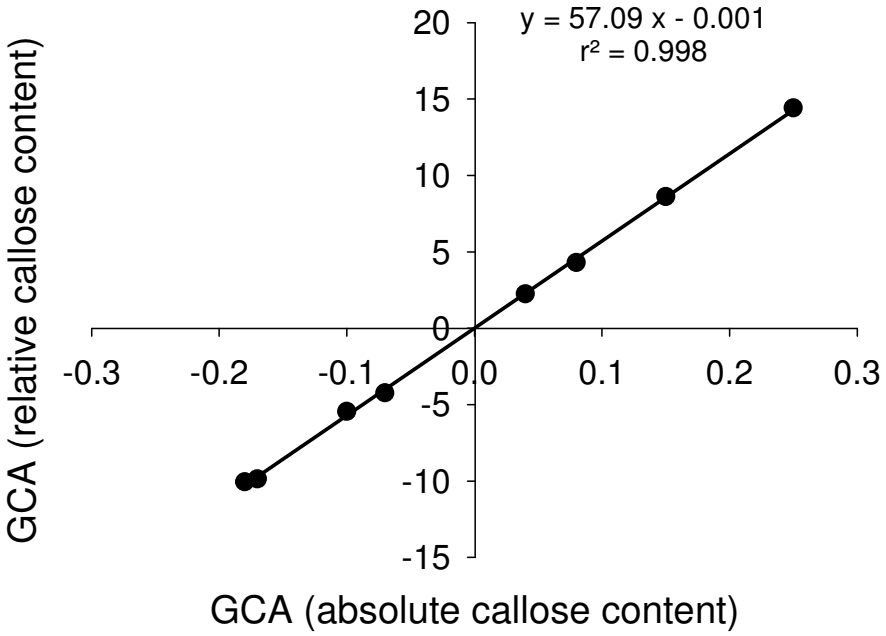

Figure 1. Relationship between GCA effects calculated from absolute callose contents (µg PE cm^{-1} root tip) and relative callose contents (Lixis = 100%) in root apices of plants treated with 25 µM Al for 12 h for a 8x8 diallel crosses of maize inbred lines.

The 15 parents as well as the same 105 F1 crosses used in this experiment were also evaluated in field experiments at two locations (Cameroon and Guadeloupe) for two years and in Colombia for one year, both on acid and non-acid soils. Relative grain yield (%) was calculated as:

$$RGY = \frac{GYa}{GYn} * 100$$

Where, RGY = relative grain yield (%); GYa = grain yield on acid soil; GYn = grain yield on non-acid soil.

Analysis of variance (ANOVA) for callose content was performed using SAS version 8.1 GLM procedure (SAS, 2001). The parental cultivars were compared with the Al-sensitive check, cv Lixis, using many-to-one comparisons (Dunnett test). The diallel data was analysed according to Griffing's method 4, model-I; which considers parents as a fixed effect and involves only direct crosses, without parents and reciprocal crosses (Griffing, 1956). For this purpose the statistical software package PZ14 (Utz, 2002) was used. The error MS used in PZ14 was obtained from ANOVA results. Since there were missing crosses specifically those involving two parents, Antigua and Natal, these two parents were excluded and 13×13 diallel data was used for combining-ability analysis.

RESULTS

Cultivar evaluation and improvement of the screening procedure

Twelve hours of Al treatment (25 µM) resulted in about ten times increase in callose contents of the root apices of maize plants. In addition, root elongation was significantly reduced with an average inhibition of 34% compared to the control without Al. A great genotypic variation among the parental cultivars was observed in terms of both callose formation and inhibition of root elongation, (Fig. 2). However, there was no significant relationship between Al-induced callose formation and inhibition of root elongation after 12 h of Al treatment. Induction of callose formation in cultivars that have been previously classified as Al-resistant on the basis of root-growth inhibition by longer-term Al treatment (Table 1) such as ATP-Y, ATPSY, ATPW, CMS36, CMS14 fell clearly below the mean while callose formation of the Al-sensitive cultivars such as Lixis, Tuxpeno and BR106 was higher. On the other hand, characterization based on inhibition of root elongation after short-term Al treatment did not show such a clear distinction between these groups of cultivars. This result suggested that cultivar differences in response to short-term Al treatment were better assessed by callose formation than by inhibition of root elongation.

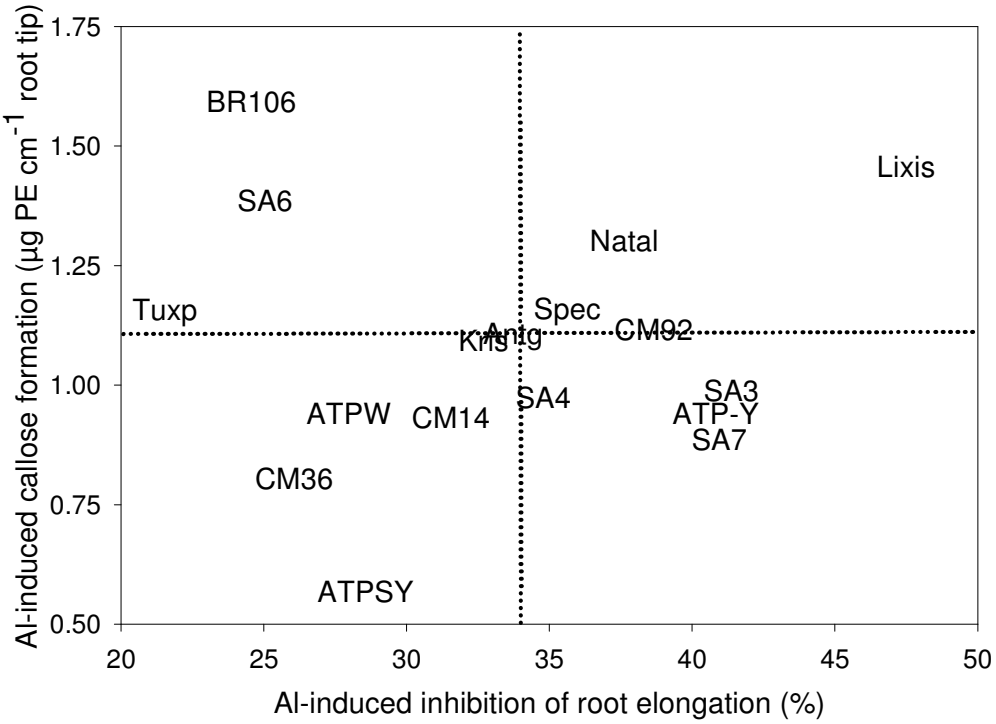

Figure 2. Relationship between Al-induced inhibition of root elongation and callose formation in root apices of maize cultivars (parents) treated with 25 µM Al for 12 h. Dotted lines indicate the average of all cultivars. For designation of cultivars, see Table 1.

There was a significant relationship between callose formation in the root apices of Al-treated plants and the controls (Fig. 3) indicating that the cultivars differed in their inherent capacity to produce callose. However, there was a much closer positive correlation between Al-induced callose formation and total callose content in Al-treated root apices (Fig. 4). This was mainly due to a low level of callose in the root apices of the control plants, which was only 13% of the Al-treated plants. Consequently, ranking of the cultivars based on Al-induced callose formation and total callose formation was similar. Thus, the latter was subsequently used to simplify the screening procedure.

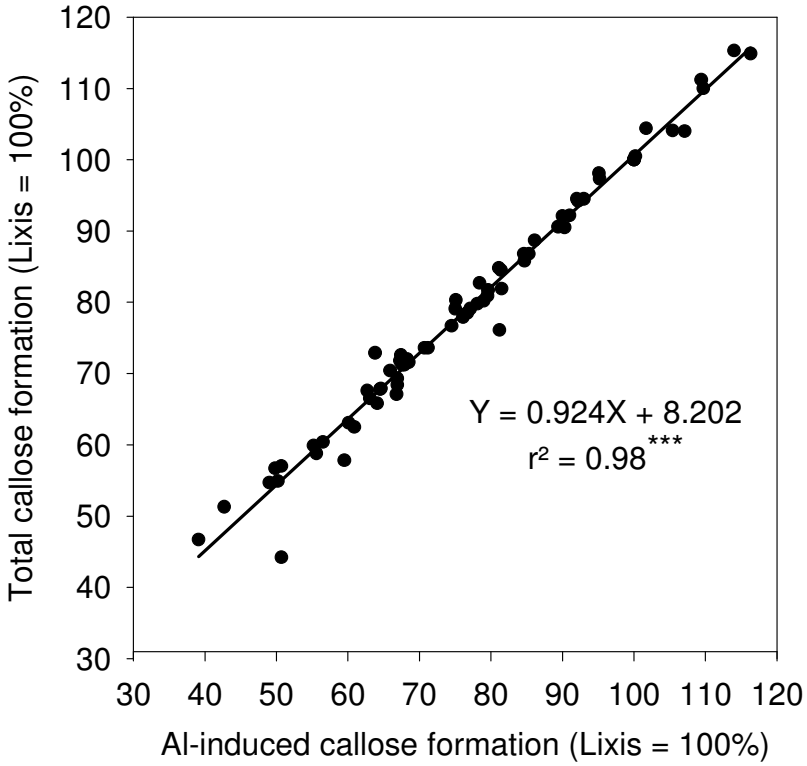

Figure 4. Relationship between relative (cv Lixis = 100%) Al-induced callose formation (callose content with Al minus without Al treatment) and relative total callose contents in root apices of maize cultivars (parents and crosses used in the first three sets of experiment, n = 70) treated with 25 µM Al for 12 h. *** designates significant Pearson correlation coefficient (r = 0.991; P = 0.0001).

The parents included in the diallel cross significantly differed in Al resistance ($P < 0.0001$). Parental cultivars with a significantly ($P < 0.05$) lower callose content than the sensitive check, cv Lixis, were classified as Al-resistant while the remaining cultivars were grouped as Al-sensitive cultivars (Fig. 5). Except for SA6 and ATPW, this classification agrees with the previous information about the cultivars (see Table 1).

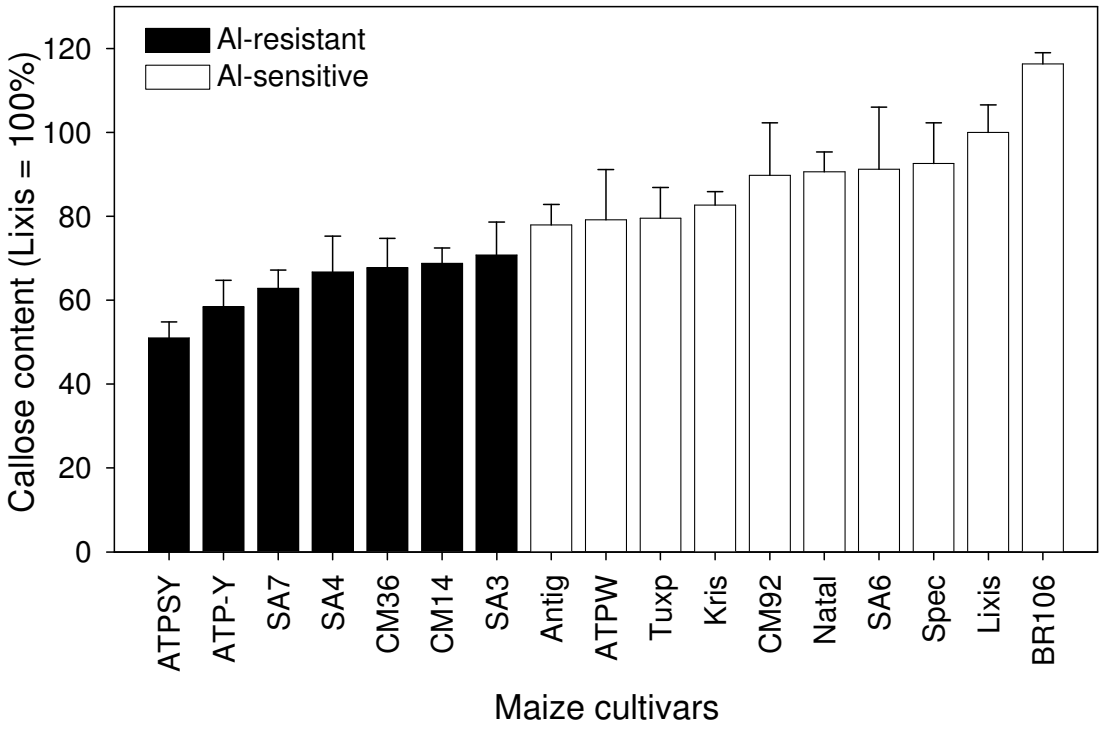

Figure 5. Callose contents in root apices (mean ± SE) of 15 open pollinated maize cultivars (parents) and two contrasting checks, cv ATP-Y and cv Lixis. Plants were treated with 25 µM Al for 12 h. Solid bars represent cultivars with callose contents significantly lower than cv Lixis and open bars represent cultivars with callose contents not significantly different from cv Lixis (Dunnett test, $\alpha = 0.05$).

Variation among the diallel crosses was also significant ($P < 0.0001$) with regard to callose formation in root apices at 25 µM Al (Fig. 6). About 25 crosses showed Al resistance as good as or better than the Al-resistant check cv ATP-Y. Among the 50 % best crosses tested (Fig 6 upper panel), 15 (38 %), 22 (56 %), and only 2 (5 %) originated from resistant × resistant, resistant × sensitive, and sensitive × sensitive parents, respectively. On the other hand, among the 50 % worst crosses (Fig 6, lower panel), 19 (49 %), 19 (49 %), and 1 (2 %) originated from sensitive × sensitive, resistant × sensitive, and resistant × resistant parents, respectively.

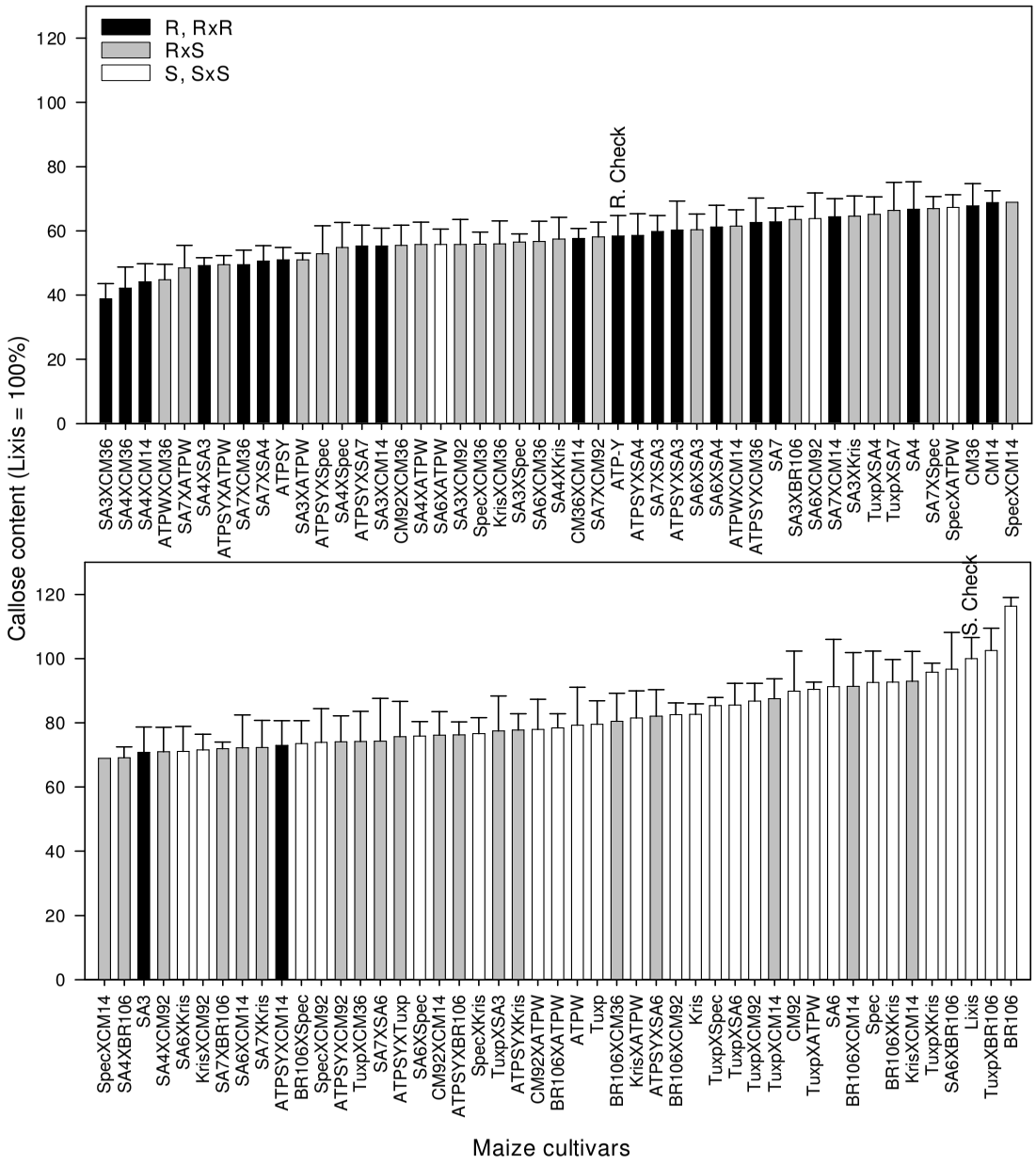

Figure 6. Callose contents (mean ± SE) in root apices of maize plants (13×13 diallel plus the parents) and checks (R = Al-resistant, S = Al-sensitive). Plants were treated with 25 µM Al for 12 h. Lower panel is upper panel continued.

Diallel Analysis

Since the variation among the crosses was significant ($P < 0.0001$) a diallel analysis of variance was performed to identify the mode of gene action (Table 3). The general combining ability (GCA) accounted for 78.5% of the sum of squares and was significant ($P < 0.01$) indicating the predominance of additive genes in

controlling Al resistance in these maize cultivars. The specific combining ability (SCA), though accounting for one-fifth of the sum of squares, was not significant.

Table 3. Diallel analysis of variance for relative callose contents (Lixis = 100%) in root apices of plants treated with 25 µM Al for 12 h for a 13×13 diallel of open-pollinated maize cultivars. GCA = general combining ability; SCA = specific combining ability.

Source	DF	SS	F
Crosses	77	15468.79	
GCA	12	12136.52	19.73**
SCA	65	3332.27	0.29 ns
Error	231	41206.75	

** = significant at $\alpha = 0.01$; ns = non significant

The GCA effect, which determines the average performance of a parent in a series of crosses, was calculated for each parent (Fig. 7). Since higher callose formation is an index of sensitivity to Al stress, a negative GCA effect shows the cultivar's positive contribution to the Al resistance of the crosses. Parents such as cvs CMS36, SA4, SA3, SA7, ATPW, and ATPSY had favourable GCA effects, and thus were good combiners for Al resistance. Among the hybrids that were more Al-resistant than ATP-Y, the resistant check, CMS36, SA4, SA3, ATP-W and SA7 were involved in 9, 7, 6 and 5 crosses, respectively. Except ATPW, all parents that showed a more favourable GCA effect had been classified as Al-resistant. However, ATPSY, the most Al-resistant parent (Fig. 5) was not the best combiner (Fig. 7).

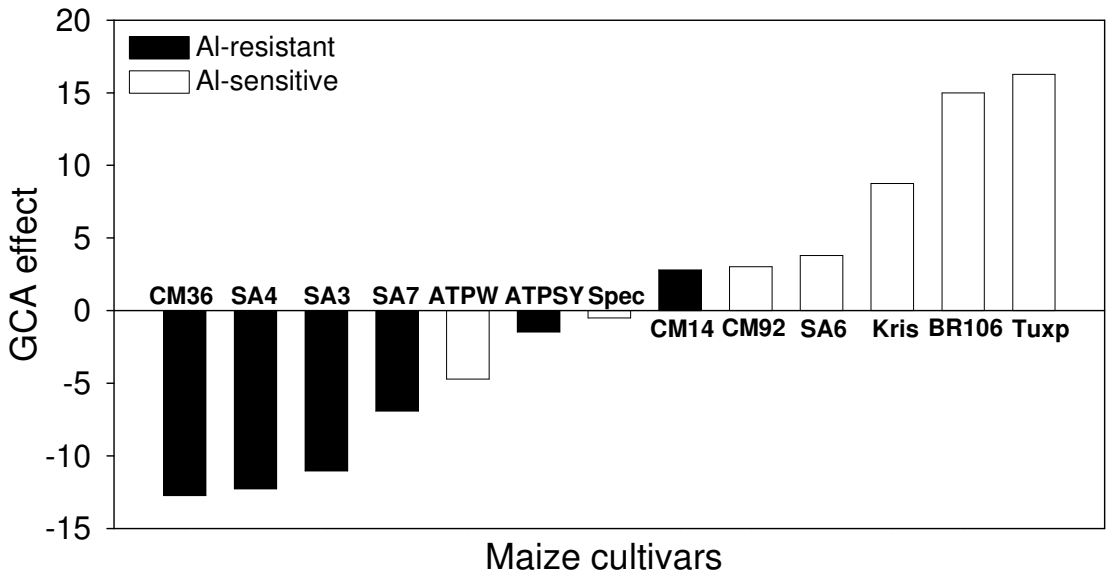

Figure 7. General combining ability (GCA) effects of 13 open-pollinated maize cultivars (parents) based on relative callose contents in root apices. Plants were treated with 25 µM Al for 12 h.

The SCA was non-significant (Table 3) and thus the role of non-additive gene effects appears to be small in the inheritance of Al resistance of these genotypes. Figure 8 shows the relationship between observed and expected performances of the crosses. The SCA effects of the crosses are visualized by the deviation from the 1:1 line (line of expectation). The observed callose contents of the crosses were close to the line of expectation indicating that the performance of the offspring could be well predicted from the sum of the GCAs of the parents.

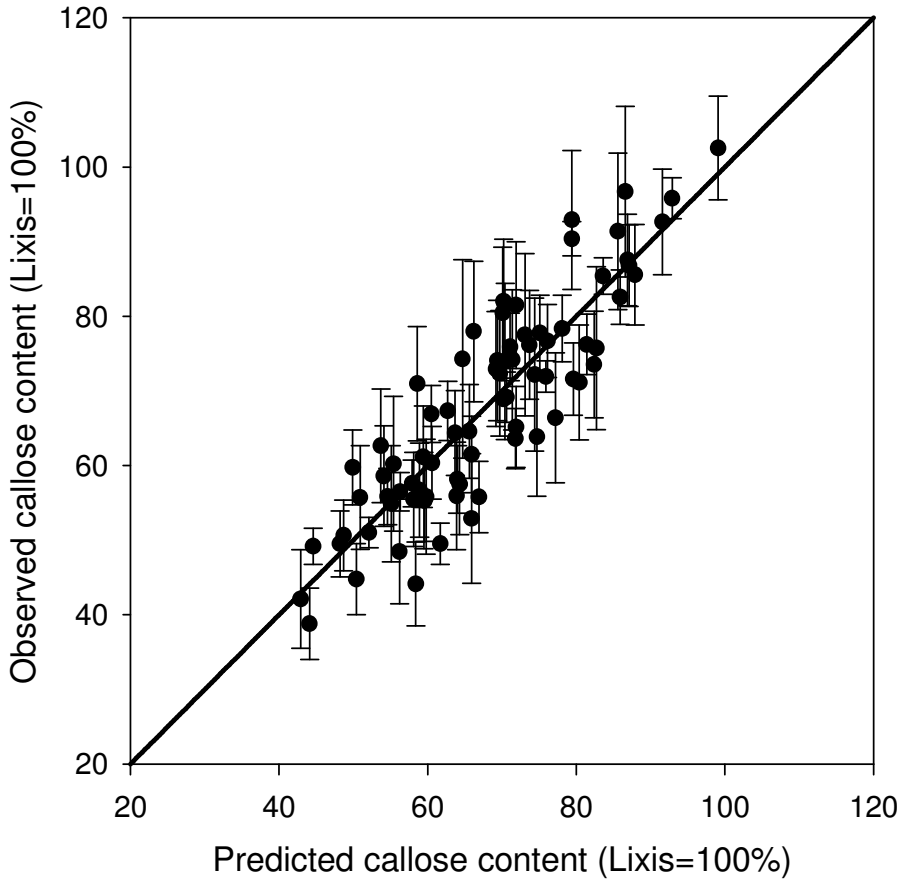

Figure 8. Observed relative callose contents in root apices (Lixis = 100 %) of maize plants (13×13 diallel) treated with 25 µM Al for 12 h, and predicted callose contents calculated for each cross from the GCA effects of its two parents and the over-all mean. Data points are the observed mean callose content ± SE. Note: The deviation of the points from the 1:1-line shows the specific combining ability (SCA) effects.

The heritability of Al-induced callose formation was calculated as the coefficient of offspring- parent regression according to Hallauer and Miranda (1988). Narrow sense heritability (h^2) is the proportion of the total phenotypic variance that can be attributed to additive genotypic variance. Its value ranges between 0 and 1. In the tested maize cultivars, high heritability (h^2 = 0.71) was observed for Al-induced callose formation (Fig. 9) indicating that the genotypic variation observed among the parents was very well reflected in the variation among the progenies.

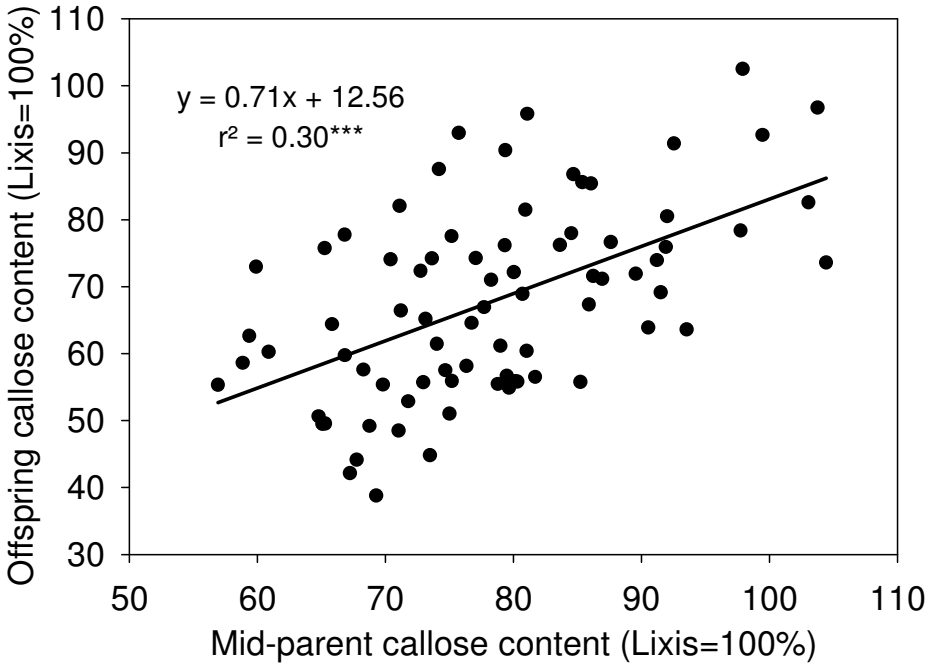

Figure 9. Heritability (h^2) estimate of a 13×13 maize diallel for callose formation in root apices of plants treated with 25 µM Al for 12 h. The heritability of the trait is equal to the value of the offspring-parent regression coefficient ($ß_1$). *** designates significant slope ($ß_1$ = 0.71; P = 0.0001).

Callose formation vs. field performance

The parents as well as the crosses used in this study were also tested in the field at two locations (Cameroon and Guadeloupe) for two years and in Colombia for one year both on acid and non-acid soils. The correlation between the callose contents in root apices after 12 h Al treatment in nutrient solution and the average relative grain yield in the field was not significant for the parents (Fig. 10A) but highly significant for the crosses (Fig. 10B), though the coefficient of correlation was low. But there was a highly significant negative correlation between the GCA effect for absolute grain yield on 5 acid soil environments and the GCA effect for callose formation (Fig. 11).

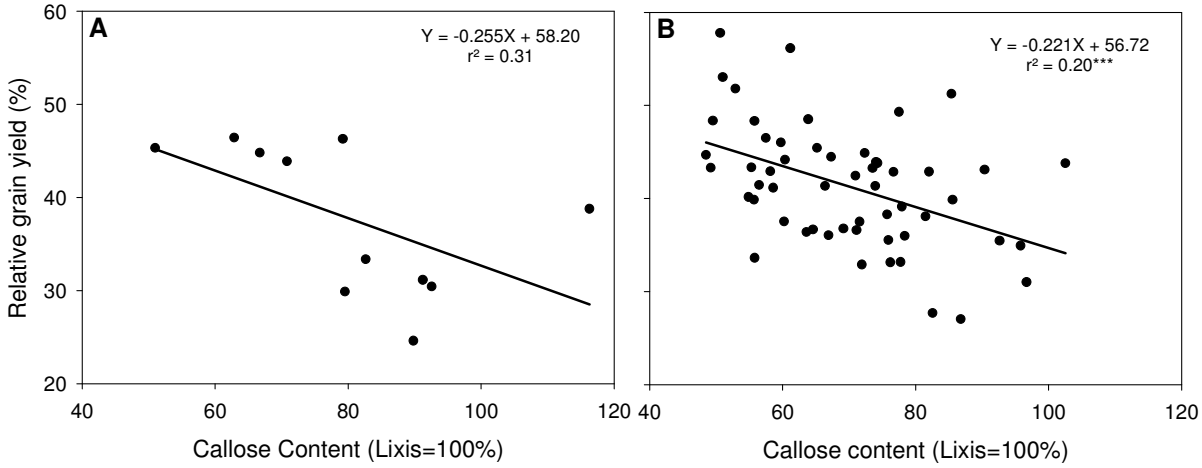

Figure 10. Relationship between relative callose contents in root apices of plants treated with 25 µM Al for 12 h (Lixis = 100 %) and relative grain yields of 11 open-pollinated maize cultivars (A) and their diallel crosses (B). Mean relative grain yields of the cultivars were calculated from field experiments at five environments (one year in Colombia and two years in Cameroon and Guadeloupe) on acid soil relative to the yields on non-acid soils. *** designates significant Pearson correlation coefficient (r = -0.45; P = 0.0005).

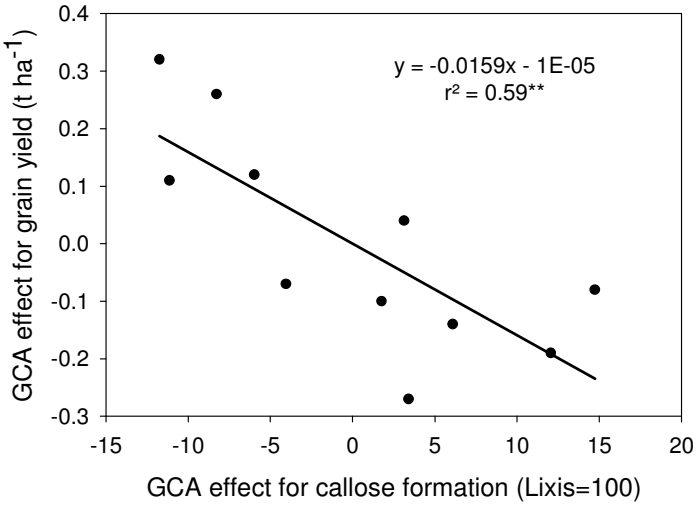

Figure 11. Relationship between general combining ability (GCA) effects for grain yields on acid soils (field experiments) and for relative Al-induced callose formation (nutrient solution experiments) of 11 open-pollinated maize cultivars. ** designates significant Pearson correlation coefficient (r = -0.77; P = 0.006).

DISCUSSION

Aluminium treatment induced a ten-fold increase in callose formation in maize genotypes, in agreement with previous studies (Horst et al., 1997; Collet et al., 2002). Al-resistant cultivars produced less callose compared to Al-sensitive cultivars. After short-term Al treatment, characterization of the cultivars based on Al-induced callose contents was better than characterization based on inhibition of root elongation (Fig. 2). Although Al-induced inhibition of root growth can be detected after short-term Al treatment (Llugany et al., 1994), it is not a suitable and reliable parameter for the classification of cultivars for Al-resistance because of high standard error associated with the root measurement. Al-induced callose contents, however, proved to be a more consistent parameter and more reliably characterized the cultivars for Al sensitivity in our experiments.

Al-induced callose formation (i.e., callose content with Al minus without Al treatment) has been described as a suitable indicator of sensitivity to Al stress in maize (Horst et al., 1997, Collet et al., 2002). The current study suggests that total callose content in Al-treated root apices is equally suited (Fig. 4). This can be attributed to the low constitutive callose contents in the root apices of the maize cultivars grown without Al treatment (control). There was only a small, though significant, variation among cultivars with regard to the callose content in the control treatment. Consequently, ranking of the cultivars based on both total callose contents in Al-treated root apices and Al-induced callose contents was similar (Fig. 4). The screening for only total callose content greatly simplifies the work because including the control treatment in the screening procedure will not be necessary.

The complete set of diallel material was divided into several runs (four sets) since handling of all genotypes in one experiment was not possible. Two well-characterized, contrasting checks (cv ATP-Y and cv Lixis) were incorporated in each run. In all cases, the differences between the checks were clear and consistent. The Al-sensitive check, Lixis, produced more callose than the Al-resistant check, ATP-Y. However, the general levels of absolute and Al-induced callose contents were too variable among the sets to allow a meaningful combined statistical analysis. Thus, for the comparison of genotypes used in different sets, the relative callose content was normalized by setting the Al-sensitive check cv Lixis in the same set as 100 %. The reliability of the relative callose content for statistical analysis, particularly of the diallel analysis, was checked using both relative and absolute callose contents of a line-diallel data-set from a separate experiment. The output of both relative and absolute values led to the same conclusion.

The problem of Al toxicity and soil acidity is traditionally alleviated through liming and fertilization. However, the effect of this practice is restricted only to the top soil layer while the subsoil still remains problematic. Thus, there are growing efforts to develop resistant varieties through breeding. Systematic breeding of maize for adaptation to soil acidity was started in the mid 70's particularly by EMBRAPA, Brazil and CIMMYT, Columbia (Borlaug and Rowswell, 1997). Acid-soil tolerant breeding materials were obtained through selection from exotic maize germplasm (materials which have no immediate use without selection for adaptation) grown on acid soils. Further breeding activities resulted in development of acid-soil tolerant source populations. Development of open pollinated varieties from the

acid-soil tolerant base populations was successful not only in EMBRAPA and in CIMMYT, but also in IRAD-Cameroon and in INRA-Guadeloupe. The parental cultivars used in the current study were obtained from these research institutions. There was a close relationship between field performance in acid soils and resistance against Al in nutrient solution. Most of the acid-soil tolerant cultivars were found to be Al-resistant using the callose test, and all acid-soil sensitive cultivars were Al-sensitive indicating that in many acid soils Al toxicity is the most important factor limiting maize yields. This holds true particularly for environments with moisture stress during the growing period. Under such conditions Al-inhibited root growth will limit water use from the subsoil thus leading to drought stress (Goldman et al. 1989). However, in many acid soils, low P availability, low supply of Mg, and Mn toxicity may be equally or even more yield-limiting and will confound the relationship between acid soil tolerance and Al resistance (Marschner, 1995), independent of the method of its assessment, i.e., root growth after long-term or callose formation after short-term Al treatment.

Studies made so far about the inheritance of soil acidity tolerance and Al resistance indicate that there is no cytoplasmic inheritance. Salazar et al. (1997) conducted a diallel study involving 8 segregating maize populations and their 56 crosses (i.e., direct and reciprocal crosses) in five acid soil environments to determine the relative importance of nuclear and cytoplasmic factors for yield, days to silk, ear height, ear per plant and ear rot. There was no difference between the direct and reciprocal crosses for all traits studied indicating that resistance to soil acidity was controlled by nuclear genes. Similarly, Lopes et al. (1987) evaluated maize populations, *per se,* and their F1s - both direct and reciprocal

crosses - for Al resistance in nutrient solution. However, cytoplasmic inheritance was not detected. For this reason, the diallel crosses used in the current experiment were composed of bulked seeds of direct and reciprocal crosses.

The evaluation of the performance of the diallel indicated that crosses among Al-resistant parents showed a better Al resistance compared to crosses between resistant and sensitive or among sensitive parents. Pandey et al. (1994) reported similar observations after evaluating a diallel derived from 6 acid-soil tolerant (T) and 2 sensitive (S) parents. Yield on acid soil of TxT (3.0 t ha^{-1}) was greater than TxS (2.4 t ha^{-1}) and SxS (2.0 t ha^{-1}) suggesting polygenic inheritance (Falconer, 1981) of Al resistance.

Analysis of variance of the diallel crosses showed significant GCA effects while SCA effects were not significant indicating that additive genes have a more prominent role for Al resistance (Table 3). The predominance of GCA effects for most characters of maize populations tested in the field under both acid and non-acid soil conditions has been reported (Hallauer and Miranda, 1988; Welcker, 2004, personal communication). Salazar et al. (1997) evaluated a diallel derived from acid-soil tolerant and sensitive populations in five acid soil environments and reported that non-additive gene effects were unimportant in the performance of the crosses. Similarly, Lopes et al. (1987) studied Al resistance of maize population and their F1 crosses in nutrient solution and observed that GCA variance explained most of the variation in relative root growth. In summary, the available results strongly suggest that additive gene effects are more important than non-additive gene effects in controlling the inheritance of Al resistance and soil-acidity

tolerance. Since grain yield formation on acid soils is a much more complex trait, it is more likely that SCA effects may still be important for specific environments.

Additive genes can be exploited through recurrent selection, a breeding method that increases the frequency of favourable genes. Several studies indicate that selection of maize for tolerance of soil acidity and Al toxicity has been effective to increase grain yield and other agronomic traits. Stockmeyer et al. (1978) reported that selection was very effective for Al resistance using the nutrient solution technique. Similarly, Magnavaca et al. (1987b) obtained gains in grain yield after four cycles of full-sib selection from a CompostoAmpolo population on a soil with 45% Al saturation. Remarkable improvements for both Al resistance (Lima et al., 1992; Giaveno and Miranda Filho, 2002) and soil-acidity tolerance (Granados et al., 1993; Ceballos, et al., 1995) were obtained through recurrent selection. Advanced cycles of selection showed better performance in grain yield and other agronomic traits indicating the valuable effect of recurrent selection.

Parental cultivars such as CMS36, SA4, SA3 and SA7 had favourable GCA effects indicating that they are good combiners for Al resistance. Moreover, there was a close association between the reaction to Al stress and the GCA of the parents, i.e., Al-resistant cultivars had better GCA than Al-sensitive cultivars. These results are in agreement with field studies using the same maize cultivars (Welcker, 2004, personal communication). Crosses involving the above parents generally exhibited better resistance to Al. In view of the preponderance of additive genes controlling Al resistance, these maize cultivars could be further improved through recurrent selection. Furthermore, Al-resistant lines could be extracted from these cultivars and used to produce Al-resistant hybrids.

The relationship between Al-induced callose content and relative grain yield of maize cultivars was loose for the environment average or even absent in some environments (data not shown). This might be due to the high variability of cultivar performance across locations and years as indicated by the significant interaction of cultivar × location × year (Welcker, 2004, personal communication). The interaction suggests that Al was not the main growth-limiting factor on the acid soils across all environments. It can be expected that the soil moisture regime affects the relative importance of Al toxicity. Al-induced inhibition of root growth leads to a shallow root system and thus less water uptake and nutrient acquisition from the subsoil. Under limiting moisture condition, this effect of Al results in severe drought stress and N/Mg deficiencies since the root cannot reach the subsoil (Goldman et al. 1989; Tang et al., 2003). In contrast, under excess moisture condition, Mn toxicity could be more important (Poter et al., 2004). Since the cultivars respond differently to different environmental stresses, identifying the best breeding material for a specific stress under field conditions is difficult. Thus, coupling of field to laboratory studies (controlled environment) appears to be necessary. A simple screening for Al-induced callose formation in root apices offers an attractive tool for a quick and non-destructive (Collet et al., 2002) screening for Al resistance, a prerequisite for the adaptation of cultivars to acid and Al-toxic soils.

In summary, we conclude from this study that the inheritance of Al resistance is mainly controlled by additive genes. Thus, the Al resistance of these cultivars can be further improved through crossing among the good combiners followed by recurrent selection.

CHAPTER 2

CELL-WALL PECTIN AND ITS DEGREE OF METHYLATION IN THE MAIZE ROOT-APEX: SIGNIFICANCE FOR GENOTYPIC DIFFERENCES IN ALUMINIUM RESISTANCE

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ABSTRACT

Previous studies indicate that a higher content and a lower degree of methylation of cell-wall pectin in the root apex are positively related to Al accumulation and Al sensitivity. The current study aimed at investigating genotypic differences in cell-wall pectin-content and its degree of methylation in root apices of selected maize cultivars differing in Al resistance. Four maize cultivars were grown in nutrient solution and treated without or with 25 µM Al for 12 h at pH 4.3. Control plants did not differ in pectin content in the 5 mm root apex. Al treatment increased the pectin content of the root apex in all cultivars. Al-sensitive cultivars had higher pectin content than Al-resistant cultivars. Pectin and Al contents in 1 mm root sections decreased from the apex to the 3 – 4 mm zone. The pectin contents of the apical root sections were consistently higher although significantly different only in the 1 – 2 mm zone in Al-sensitive cv Lixis. Al contents in most root sections were significantly higher in cv Lixis than in Al-resistant cv ATP-Y.

Since a quantitative determination of its degree of methylation was not possible cell-wall pectin was stained in fresh apical root cross-sections by immunofluorescence using monoclonal antibodies specific for pectin with different degrees of methylation. Fluorescence intensity of cell wall of cortical root cells treated with JIM5 antibody staining specifically low-methylated pectin and with JIM7 specific for high-methylated pectin was higher and lower for cv Lixis than for cv ATP-Y, respectively. This indicates that Al-sensitive cv. Lixis has a higher proportion of low-methylated pectin and thus a higher negativity of the cell wall which is in agreement with its higher Al content and Al sensitivity. We conclude that differences in cell wall pectin and its degree of methylation contribute to the genotypic differences in Al resistance in maize.

Key words: aluminium, cell wall, degree of methylation, immunofluorescence, pectin

INTRODUCTION

Al toxicity is a major limiting factor for crop productivity on acid soils, which account for about 40% of the world's cultivable land (von Uexküll and Mutert, 1995). The most common symptom of Al toxicity is the inhibition of root growth that can be observed within a short time after Al treatment (Llugany et al., 1995). Root growth is basically a function of cell division and cell elongation. Although Al can affect both cell division and cell elongation, the inhibition of cell elongation rather than of cell division is the primary effect of Al toxicity (Horst and Klotz, 1990) which leads to the rapid reduction in root growth. Besides inhibition of root elongation, induction of callose formation is also a sensitive indicator of Al injury (Zhang et al., 1994; Horst et al., 1997).

The target site of Al toxicity is the root apex, particularly the distal part of the transition zone is most sensitive to Al (Ryan et al., 1993; Sivaguru and Horst, 1998). Al-resistant genotypes generally accumulate less Al in the root apex than the sensitive genotypes. Thus, exclusion mechanisms play a major role in Al resistance.

Mechanisms of Al resistance in plants were extensively discussed in recent reviews (Matsumoto, 2000, Ma et al., 2001; Ryan at al., 2001; Samac and Tesfaye, 2003; Kochian et al., 2004) The release of organic acid anions is believed to play a major role. In wheat, the release of malate can fully explain Al resistance (Delhaize et al., 1993a; Pellet et al., 1996; Ryan et al., 2001; Sasaki et al., 2004). Also in maize, citrate exudation appears to be associated with Al resistance (Pellet et al., 1995; Kollmeier et al., 2001). Kollmeier et al. (2001) compared organic acid anion exudation of two contrasting maize cultivars (Al-

resistant ATP-Y, and Al-sensitive Lixis,) and observed that Al activated the release of both citrate and malate at a similar rate. The Al-resistant cv ATP-Y released more organic acid anions than the Al-sensitive cv Lixis. Nevertheless, the differential release of organic acid anions did not fully explain the genotypic difference in Al resistance. Thus they speculated that additional physiological mechanisms contribute to Al resistance in maize. Similarly, Piñeros et al. (2002) described Al resistance in maize as a complex trait that involves several different mechanisms of resistance.

Al^{3+} is a polyvalent cation which rapidly and strongly binds to the negatively charged binding sites in the root (Zhang and Taylor, 1989; Blamey et al. 1990). Several reports indicate that Al accumulates mainly in the cell wall, specifically binding to the pectic matrix (Chang et al., 1999; Taylor et al., 2000; Wang et al., 2004). Schmohl and Horst (2000) observed a close positive relationship between cell-wall pectin-content and Al accumulation. Pectin is a complex polysaccharide which is mainly composed of galacturonic acid chains. Methyl esterification of the carboxylic group of pectin determines the negative charge it carries and ultimately the quantity of Al it can bind. Schmohl et al. (2000) found a close negative correlation between the degree of methylation of pectin and Al accumulation. Thus, it was hypothesized that a low pectin content and/or a high degree of methylation of pectin contributes to Al resistance. Cell-wall pectin of Al-resistant genotypes may have a higher degree of methylation, and thus lower Al accumulation than Al-sensitive cultivars. Therefore, the objective of the current study was to investigate the significance of pectin content and its degree of methylation for genotypic differences in Al resistance of maize cultivars.

MATERIALS AND METHODS

Plant growth and Al treatment

Maize cultivars with differential Al resistance were used for this study. Seeds of Al-resistant (ATP-Y and Sikuani) and Al-sensitive (Lixis and ICA-V-109) cultivars were surface-sterilized for 1 min in diluted sodium hypochlorite (3-7% active chloride) and germinated between wet filter papers soaked in 1 mM $CaSO_4$ solution. Four day-old seedlings were transplanted to 22-liter pots containing nutrient solution of the following composition [µM]: KNO_3 400, NH_4NO_3 200, KH_2PO_4 10, $MgSO_4$ 100, H_3BO_3 8, $CuSO_4$ 0.2, $ZnSO_4$ 0.2, $MnSO_4$ 1, $(NH_4)_6Mo_7O_{24}$ 0.1, Fe-EDTA 20, $CaSO_4$ 250. Plants were cultured in a growth chamber with controlled environmental conditions of a 16/8 h day/night cycle, 30/27 °C day/night temperature, 75% relative air humidity, and a photon flux density of 230 µmol m^{-2} s^{-1} photosynthetic active radiation at plant height. During the first two days of cultivation, the pH of the nutrient solution was lowered gradually to 4.3 and kept constant throughout the treatment period using an auto-titration device with 0.1 M HCl/KOH. Plants were treated with 0 or 25 µM Al (as $AlCl_3$) for 12 h. Then root tips were harvested for callose, pectin, and Al determination. After harvest, the culture solutions were filtered immediately through 0.025 µm nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) to determine Al in solution. Monomeric Al (Al_{mono}) concentration was measured colorimetrically using the aluminon method according to Kerven et al. (1989). The Al_{mono} concentration of the nominal 25 µM Al treatment solution was 17 – 21 µM after the 12 h Al treatment period.

Callose determination

Root tips of 1 cm length were excised from primary roots of three plants, collected in Eppendorf reaction vials and instantly frozen in liquid N_2. The root tips were homogenized in 500 µl of 1 M NaOH with a mixer mill (MM 200, Retsch GmbH & Co. KG, Haan, Germany) at a speed of 20 cycles s^{-1} for 2 min. After homogenization, another 500 µl of 1 M NaOH was added, and callose was solubilized by heating in a water bath at 80 °C for 20 min. Callose was measured according to Kauss (1989), after addition of aniline blue reagent using a fluorescence spectrophotometer (F 2000, Hitachi Ltd., Tokyo, Japan) at excitation and emission wavelengths of 339 and 484 nm, respectively. Pachyman (1,3-ß-D-glucan) solution was used as a calibration standard, and thus, root callose-content was expressed as pachyman equivalents (PE) per cm root tip.

Pectin determination

Since large quantities of root tips are required for pectin determination, one primary and two seminal roots were harvested per plant. Either the 5 mm root apex or individual one mm segments of the apex were excised and collected in 96% ethanol in Eppendorf reaction vials. The number of root sections per sample ranged from 12 to 156. Root samples were thoroughly homogenised in ethanol using a mixer mill at a speed of 30 cycles s^{-1} for 3 min. The homogenisation was repeated 2 – 5 times. Cell-wall material was prepared as alcohol-insoluble residue after repeated washing with ethanol, modified after Schmohl and Horst (2000). After every ethanol addition, the samples were centrifuged at 23,000 x g for 10 min and the supernatant was discarded. The remaining cell-wall material was dried using a centrifugal evaporator (RC10-22T, Jouan SA, France), weighed, and

hydrolysed according to Ahmed and Labavitch (1977) extending the incubation time to 10 min in concentrated H_2SO_4 and 2 h after each step of water addition. The uronic acid content was determined colorimetrically according to Blumenkrantz and Asboe-Hansen (1973) using a microplate spectrophotometer (µQuant™, Bio-Tek Instruments, USA). Galacturonic acid was used as a calibration standard, thus the root pectin content was expressed as galacturonic acid equivalents (GaE).

Al determination

Root tips of Al-treated maize plants were cut into 0 – 1 mm, 1 – 2 mm, 2 – 3 mm, 3 – 4 mm, 4 – 5 mm, and 9 – 10 mm segments. Ten root segments from each mm zone were placed in separate Eppendorf reaction vials and digested in 500 µl ultra pure HNO_3 (65%) by overnight shaking on a rotary shaker. The digestion was completed by heating the samples in a water bath at 80°C for 20 min. Then 1.5 ml ultra pure water (18.2 megohm-cm; E-pure, D4642, Barnstead, Dubuque, IA,) was added after cooling the samples in an ice-water bath. The samples were diluted by a factor of four and measured with a GFAAS (Unicam 939 QZ graphite furnace atomic absorption spectrophotometer, Analytical Technologies Inc., Cambridge, UK) at a wavelength of 308.2 nm, and an injection volume of 20 µl.

Degree of methylation of pectin

Cell-wall material from maize root apices was prepared in the same way as for pectin determination. Methanol was released from the cell-wall material by saponification according to Fry (1988), modified after Wojciechowski and Fall (1996). After addition of 2 units alcohol oxidase (EC 1.1.3.13 from *Piccia pastoris*

Sigma, Deisnhofen Germany) the complex of formaldehyde with Fluoral-P (15 mg ml^{-1}) (Molecular Probes, Leiden, The Netherlands) was measured fluorometrically,.

Localization of pectin by immunofluorescence

Indirect localization of cell-wall pectin by immunofluorescence was performed using monoclonal antibodies which are specific for pectin with different degrees of methylation. For this purpose, fresh roots were hand sectioned from 3 to 4 day-old seedlings of maize (Al-resistant cv ATP-Y and Al-sensitive cv Lixis) not treated with Al. Thin root cross-sections of about 100 to 300 µm thickness were sectioned from the root zone 1 – 3 mm behind the apex and directly collected into a fixative solution containing 4% Paraformaldehyde in 50 mM PIPES (1,4-piperazine-diethanesulphonic acid), 5 mM $MgSO_4$, and 5 mM EGTA (ethylene glycol bis(ß-amino-ethylether)-N,N,N',N'-tetraacetic acid) pH 6.9. After 1 – 2 hours of fixation at room temperature, the samples were washed repeatedly with phosphate-buffered saline (PBS: 0.14 M NaCl, 2.7 mM KCl, 10.1 mM $Na_2HPO_4.2H_2O$, 1.8 mM KH_2PO_4, pH 7.4) and blocked with 0.2% bovine serum albumin in PBS for 30 min. Then the samples were incubated in presence of the diluted primary antibody (Table 1) for 2 hours at room temperature. The antibody dilution was made with PBS containing 0.2 % bovine serum albumin. The primary antibody was thoroughly washed off the samples with PBS three times for 5 min, each. Next, they were incubated for 2 hours in presence of 50-fold diluted solution of secondary antibody, antirat-IgG coupled with FITC (fluorescein isothiocyanate). The samples were washed as mentioned above, mounted on glass slides and examined under a confocal laser scanning microscope (Leica TCS SP2, Leica

Microsystems, Heidelberg, Germany). Images were captured with the Leica Confocal Software.

Table 1. Rat monoclonal antibodies and their specific pectic epitopes.

Antibody	Class	Epitope♀	Dilution♣	Reference
JIM5	IgG	low-methyl-ester HG*	1:10	Knox *et al*. (1990), Willats *et al*. (2000), Clausen *et al*. (2003)
JIM7	IgA	high- methyl-ester HG	1:10	Knox *et al*. (1990), Willats *et al*. (2000), Clausen *et al*. (2003)
LM7	IgM	randomly de-esterified HG	1:3	Willats *et al*. (2001a), Clausen *et al*. (2003)

♀ Epitope is the part of the antigen molecule (pectin) to which the antibody binds.

♣ Dilution factor of the hybridoma supernatant used in this study.

* HG, Homogalacturonan is a polymer of galacturonic acid, the backbone of the pectin molecule.

Control samples (not treated with the primary antibody but only with the secondary antibody) were also examined but no fluorescence was observed and thus images are not shown. However, fluorescence was detected in the cell wall of the control samples when an antifade mounting agent (Citifluor, AF1) was used (Fig. 1). Since this phenomenon was observed at the preliminary stage, antifade agent was not used for the experiment presented here. Only distilled water was used to mount the specimens on glass slides for microscopy. Autofluorescence was not observed for specimen mounted in water. However, fading of fluorescence was experienced upon prolonged exposure to the high intensity laser.

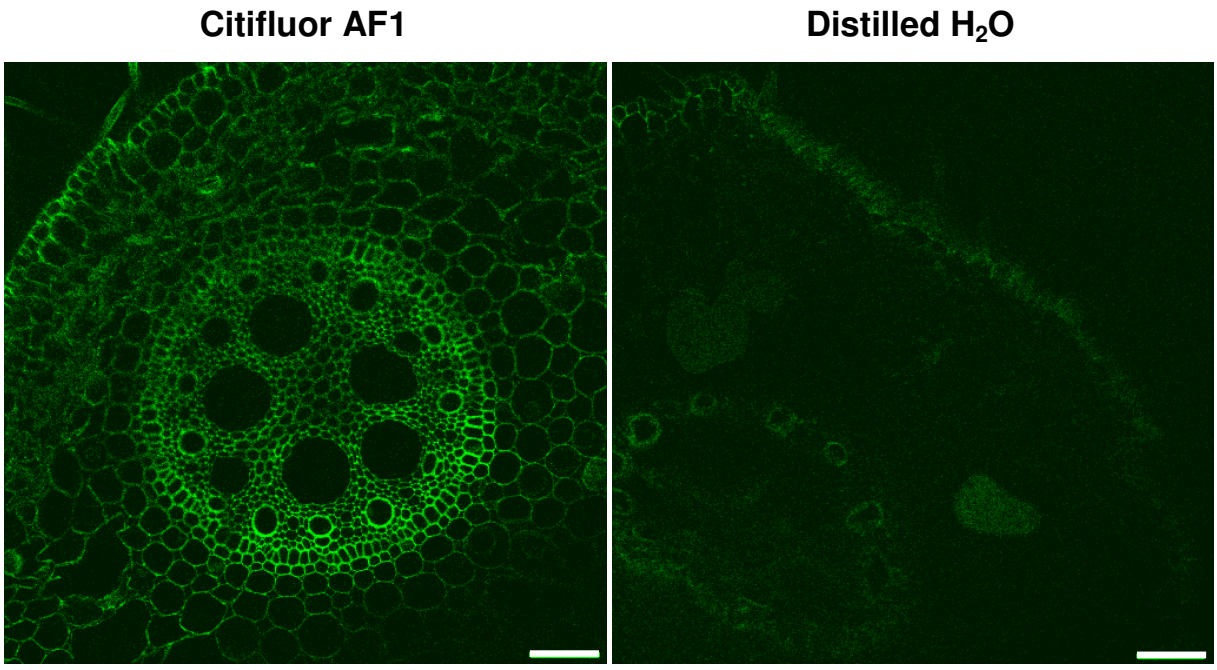

Figure 1. Effect of the mounting medium (Citifluor and water) on the autofluorescence of maize root sections not treated with antibody. Root sections were taken from 5 – 10 mm behind the root apex. Scale bars = 80 µm.

Experimental design and statistical analysis

A randomized complete block design was used, with 4 – 5 replications per treatment. Statistical analysis was carried out using SAS version 8.1 (SAS, 2001).

RESULTS

The Al resistance of the maize cultivars was reflected in differential Al-induced callose formation (Fig. 2). Al treatment induced callose formation of the cultivars about seven times higher than in the controls. The Al-resistant cultivars ATP-Y and Sikuani produced less callose than the Al-sensitive cultivars Lixis and ICA-V-109. However, such a clear difference was not observed in pectin contents (Fig. 3). The cultivars did not differ in pectin contents of the root apex in the control (without Al). Al treatment increased the pectin content, particularly in the Al-sensitive cultivars.

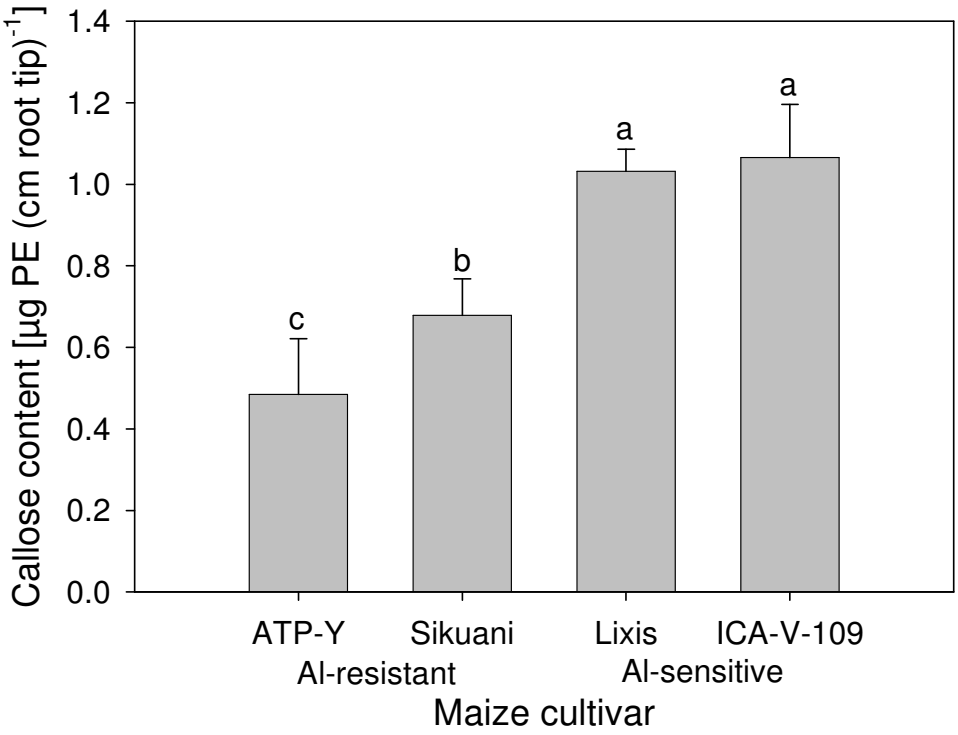

Figure 2. Callose contents (mean ± SD) in 10 mm root apices of four maize cultivars treated with 25 µM Al for 12 h. Bars with different letters are significantly different, $p < 0.05$ (Tukey test).

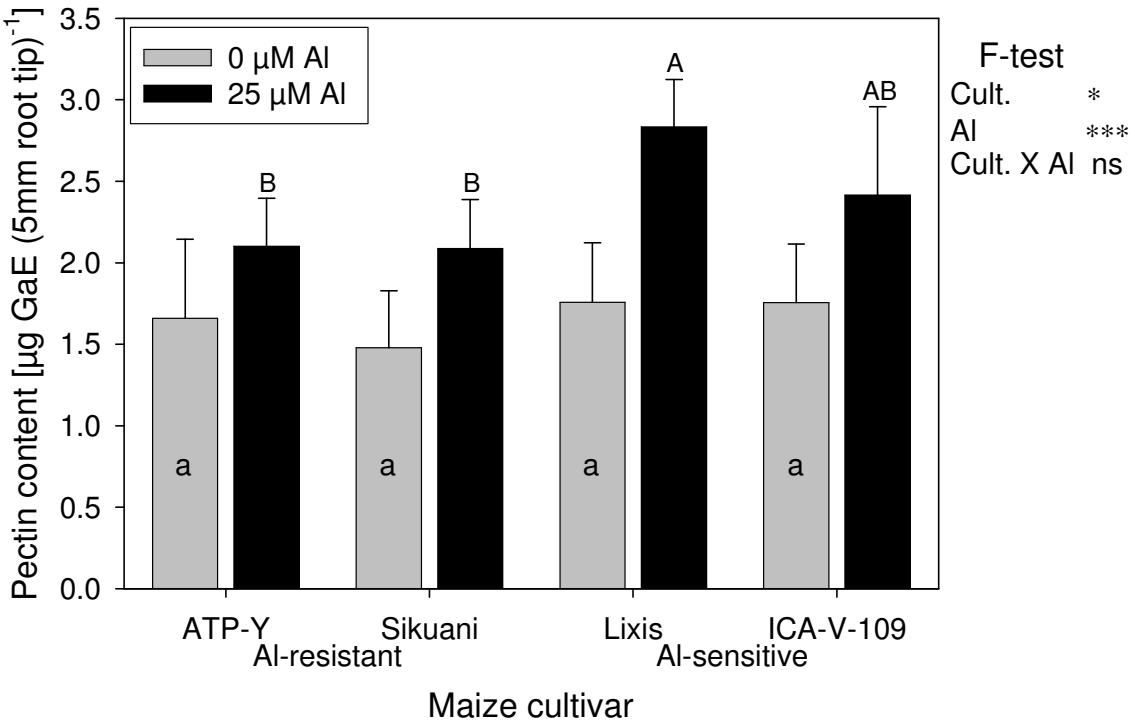

Figure 3. Pectin contents of root apices of four maize cultivars treated with or without 25 µM Al for 12 h. Bars are means ± SD of four replications.12 sections of 5 mm root apices were sampled per replicate. Letter designations stand for cultivar comparison at 0 µM Al (small letters) and 25 µM Al (capital letters). Bars with different letters are significantly different, $p < 0.05$ (multiple t-test). * and ***, significant at $\alpha = 0.05$ and $\alpha = 0.001$, respectively (F-test). ns = non significant.

Since Al injury is differentially expressed along the root axis, the distribution of pectin in different root zones was studied using two of the above maize cultivars (ATP-Y and Lixis) which were the most contrasting in Al resistance. For this purpose root tips were cut into mm segments starting from the root apex to the mature zones and the distribution of pectin and Al along the root axis was determined.

The cell-wall dry-weight per root segment decreased basipetally from the root apex to the 3 – 4 mm zone and remained constant thereafter (Fig. 4). The highest cell wall dry weight was found in the zones 0 – 1 and 1 – 2 mm. There was no

significant difference between the two cultivars for all root zones although the dry weight of the apical sections tended to be consistently higher in cv Lixis. Pectin and Al contents showed a similar pattern along the root axis with decreasing contents from the apex to the 3 – 4 mm zone in both cultivars (Fig. 5). The pectin contents of the apical sections were consistently higher in cv Lixis although significantly different only in the 1 – 2 mm root zone. The Al contents in all root sections with the exception of the 2 – 3 mm and 3 – 4 mm zones were higher in cv Lixis than in cv ATP-Y.

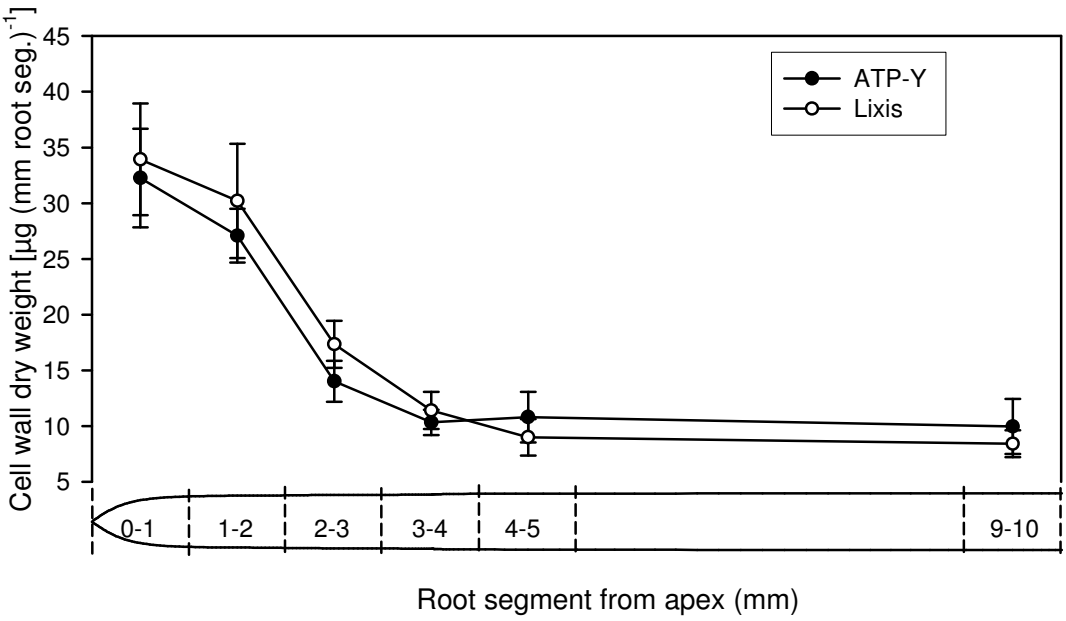

Figure 4. Cell wall dry weight of different apical root zones of two maize cultivars (Al-resistant ATP-Y and Al-sensitive Lixis) not treated with Al. Data points are mean ± SD of five replications.

The Al content of the cell wall should be related to the density of its negative charge. Since the degree of methylation of pectin defines the negativity of the cell wall, it was attempted to determine the degree of methylation of cell-wall pectin. However, chemical analysis based on the amount of methanol released from the

cell-wall pectin of maize root apices was not successful, because the concentrations were always below the detection limit even when a large number of root tips (156 root tips per sample) were used. Therefore, different monoclonal antibodies (JIM5, JIM7 and LM7) which are specific for cell-wall pectin differing in the degree of methylation were used for immunofluorescence localization of cell-wall pectins.

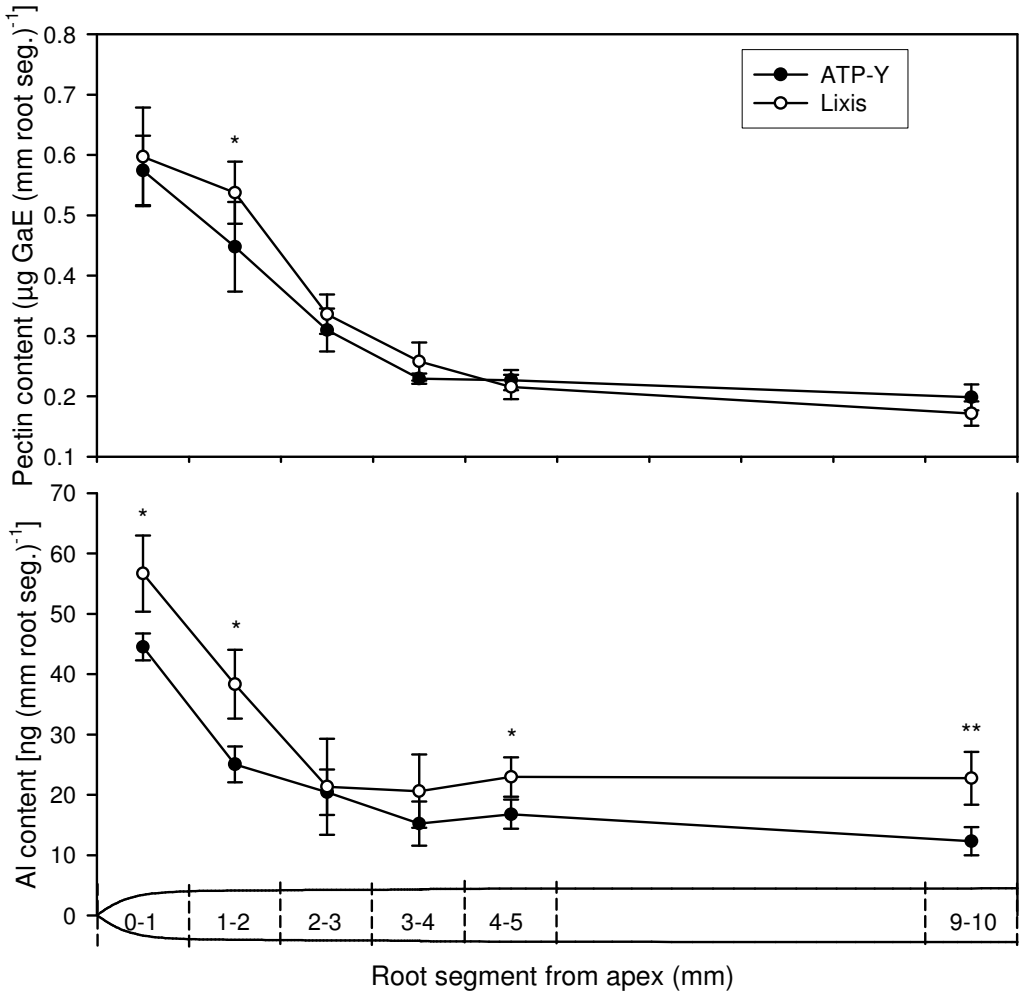

Figure 5. Pectin contents (A) and Al contents (B) of different apical root zones of two maize cultivars (Al-resistant ATP-Y and Al-sensitive Lixis). Data points are mean ± SD of five replications. *, ** denote differences between the cultivars at $p < 0.05$ and 0.01, respectively (t-test). The pectin content was determined from control samples (without Al).

JIM7, staining high-methyl-ester pectin, localized pectin in the cell wall of cortical cells in both cultivars as indicated by bright fluorescence, whereas in the epidermis and the stele, there is nearly no fluorescence (Fig. 6). The fluorescence appears brighter in cv ATP-Y than in cv Lixis, which implies that the content of high-methyl-ester pectin is higher in cv ATP-Y than in cv Lixis. Also, the distribution pattern differed between the cultivars (Fig. 7). In cv ATP-Y (Fig 7 A, C), the whole intercellular space shows high fluorescence whereas in cv Lixis (Fig. 7 B, D) the regions of maximum fluorescence are the cell junction points.

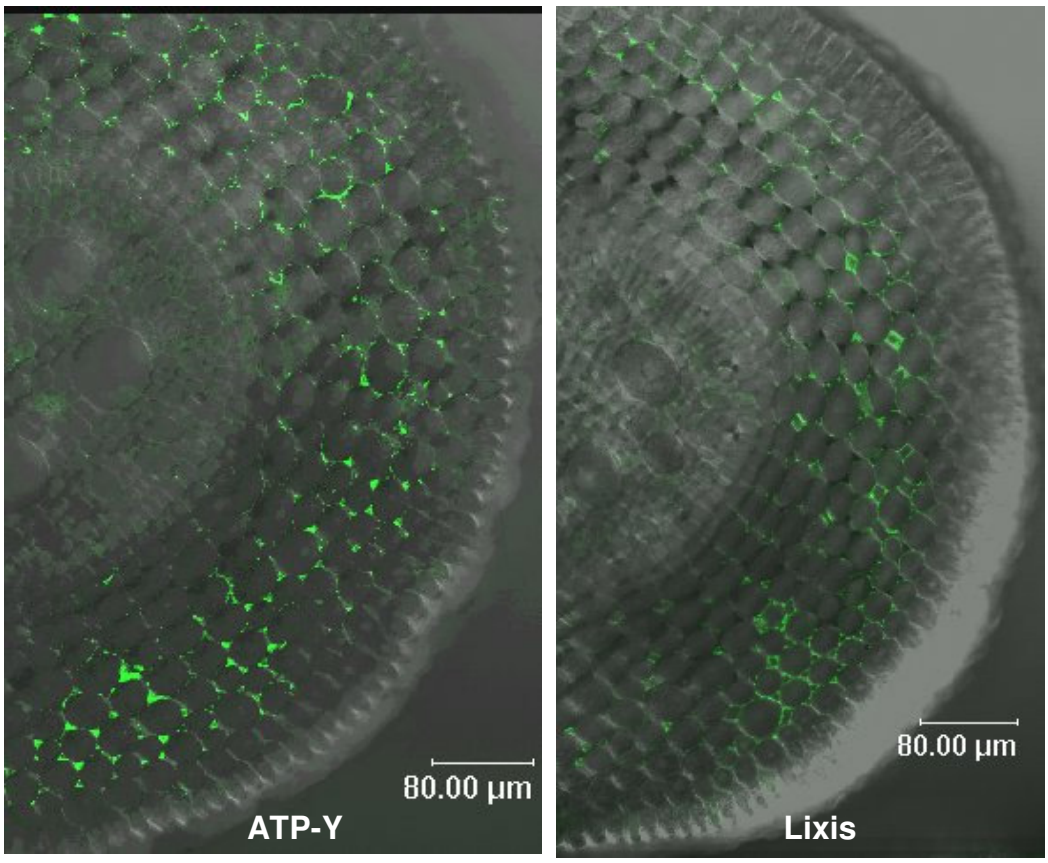

Figure 6. Distribution of high-methyl-ester pectin (JIM7 epitope) in root cross-sections of two maize cultivars (Al-resistant ATP-Y and Al-sensitive Lixis) 1 – 3 mm behind the apex.

Similar to JIM7, the pectic epitope of JIM5 specifically staining low-methyl-ester pectin mainly stained pectin in the cortex in both cultivars (Fig. 8). There is

generally brighter fluorescence in Lixis (Fig. 8 B) than in ATP-Y (Fig. 8 A), suggesting that cv Lixis has a higher content of low-methyl-ester pectin compared to cv ATP-Y. The regions of maximum fluorescence were the junction points for ATP-Y (Fig. 8 C), but the junction points as well as the wall facing the intercellular space for Lixis (Fig. 8 D). LM7, a monoclonal antibody which binds to a randomly (nonblock-wise) de-esterified pectin also mainly localized pectin in the cortical cells. However, fluorescence was faint in most of the images. So, it was difficult to make an appropriate comparison of the two cultivars but in general it appears that Lixis had a brighter fluorescence than ATP-Y (Fig. 9).

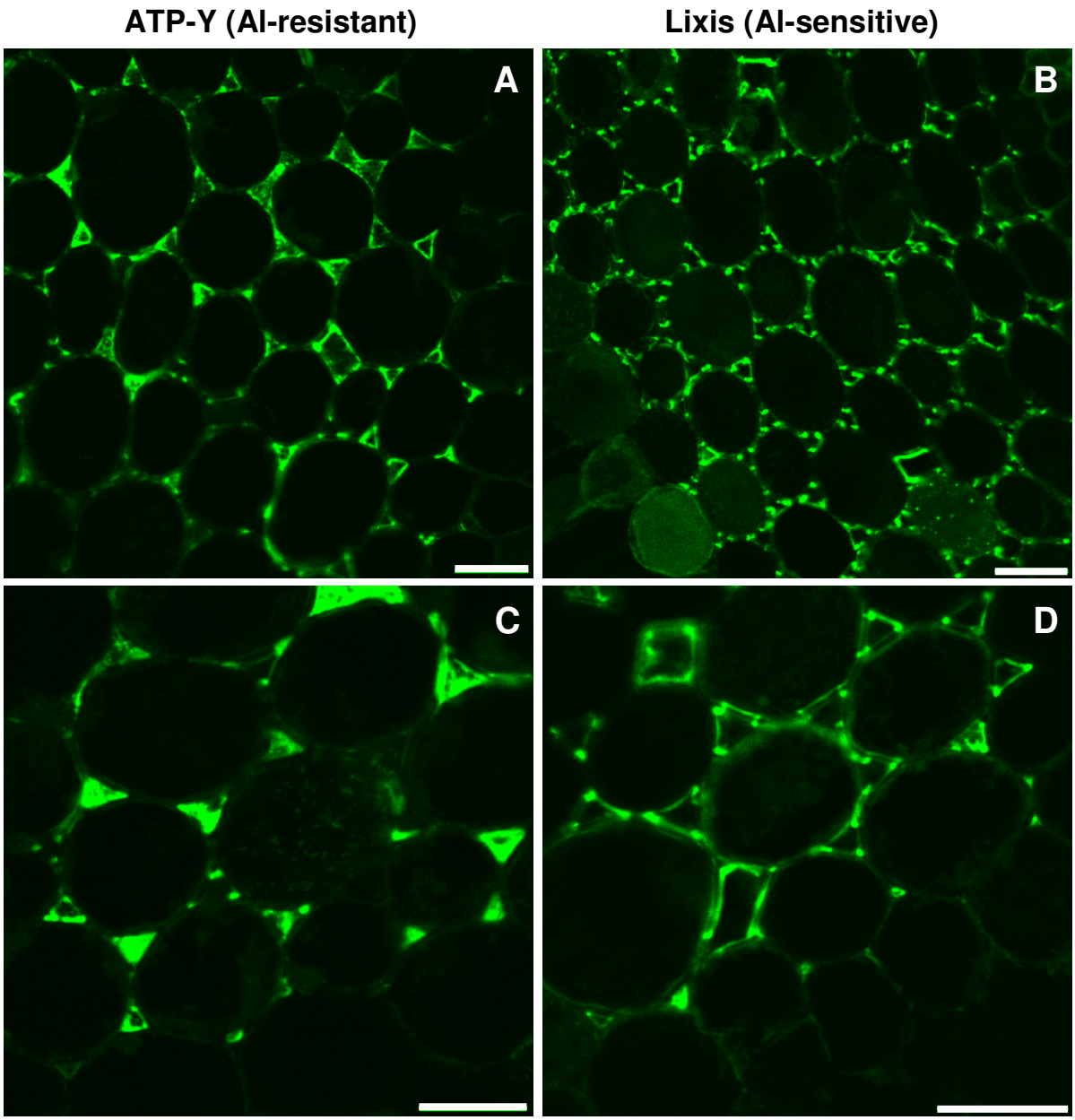

Figure 7. Immunolocalization of high-methyl-ester pectin (JIM7 epitope) in root cross-sections of two maize cultivars (Al-resistant, ATP-Y, and Al-sensitive, Lixis). A and B – focused to the cortex; C and D – closer view. Root sections were taken from 1 – 3 mm behind the apex. Scale bars = 20 µm.

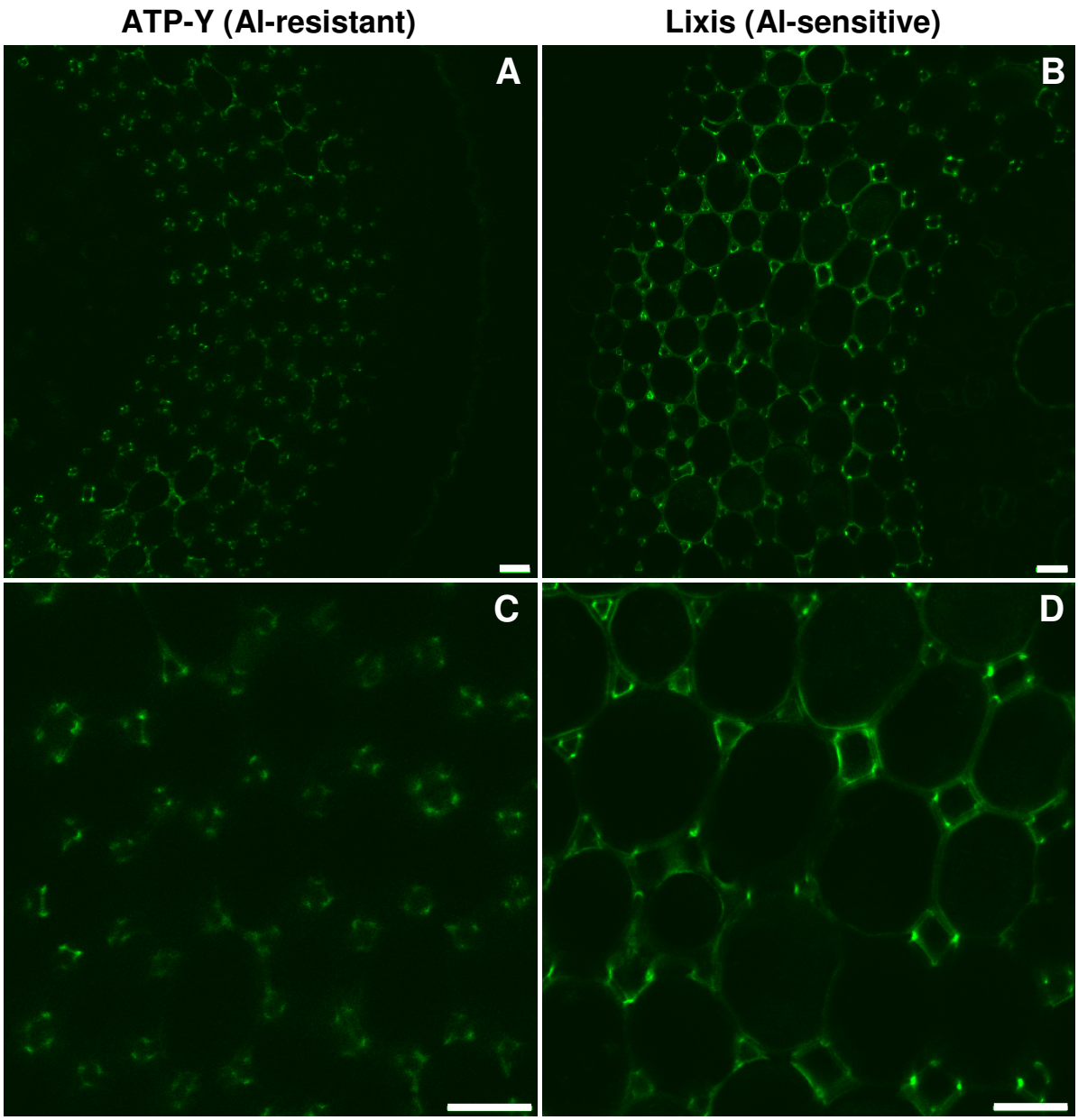

Figure 8. Immunolocalization of low-methyl-ester pectin (JIM5 epitope) in root cross sections of two maize cultivars (Al-resistant, ATP-Y, and Al-sensitive, Lixis). A and B – overview; C and D – closer view. Root sections were taken from 1 – 3 mm behind the apex. Scale bars = 20 µm.

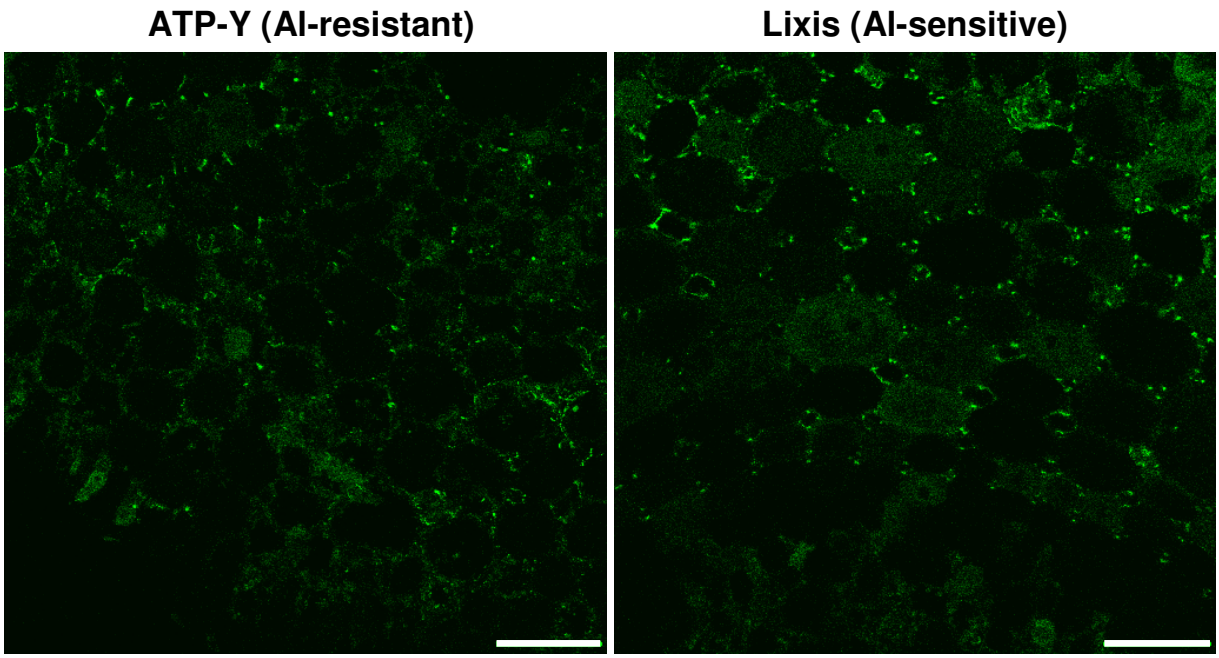

Figure 9. Immunolocalization of randomly methyl-de-esterified pectin (LM7 epitope) in root cross sections of two maize cultivars (Al-resistant, ATP-Y, and Al-sensitive, Lixis). Root sections were taken from 1 – 3 mm behind the apex. Scale bars = 40 µm.

DISCUSSION

The root apoplast plays a major role both in recognition of Al toxicity and expression of resistance mechanisms (Horst, 1995), since the bulk of Al in the root is found in the apoplast, particularly in the cell wall. Ma et al. (2004) found more than 77% of the total Al in the cell wall of wheat root-apices. Similarly, Wang et al. (2004) reported that 85% of the Al taken up by maize roots accumulated in the cell wall. With a physical separation method, even more than 99% of the total Al taken up was found in the cell wall of *Chara corallina* (Taylor et al. 2000).

Al^{3+} has a high binding affinity to anions such as F^-, O^{2-}, OH^-, SO_4^{2-}, NO_3^-, and $C{=}O{-}O^-$ (Nieboer and Richardson, 1980). Among the cell-wall polymers, pectin with its $C{=}O{-}O^-$ groups is the most likely candidate which binds Al^{n+}. Pectin is one of the major components of primary cell walls of all terrestrial plants, and generally accounts for about one third of the total cell-wall weight, although lower levels occur in some families belonging to the poales (Carpita and Gibeaut, 1993; Smith and Harris, 1999). It is mainly composed of poly-α-(1$\rightarrow$4)-D-galacturonic acid, which is highly methyl-esterified during synthesis but partially de-esterified (de-methylated) upon assembly in the primary cell wall (Carpita and McCann, 2000). The non-methylated carboxylic group of pectin provides the negative charge of the cell wall where Al^{n+} binds.

There is ample experimental evidence which shows that Al strongly binds to the pectin of the cell wall. According to Change et al (1999), about 72 – 82% of the total cellular Al was found associated with pectin in cultured tobacco cells. In addition, an increase in pectin content resulted in a higher Al accumulation and consequent loss of viability in maize cells grown in suspension culture, indicating

that cells with higher pectin content were more Al-sensitive (Schmohl and Horst, 2000). Not only cultured cells but also maize plants with higher pectin content (induced by NaCl treatment) accumulated more Al in the root apices and were found to be more sensitive to Al (Horst et al. 1999).

The current study aimed at examining whether the cell-wall pectin-content and its degree of methylation are related to genotypic differences in Al resistance of maize cultivars. The differential response of the cultivars to Al treatment was reflected by differential induction of callose formation. The higher Al-induced callose contents (Fig. 1) are in agreement with reported differences in Al-induced inhibition of root elongation (Collet et al., 2002). Thus cv Lixis and cv ICA-V-109 proved to be Al-sensitive. However the differential Al resistance was not associated with the pectin content of the root apex since the cultivars did not differ in constitutive pectin content (Fig. 2). Al treatment increased the pectin content in all cultivars. Similarly, Chang et al. (1999) observed an increase in cell-wall pectin content of cultured tobacco cells upon exposure to Al. Van et al. (1994) also reported that 3 h of Al treatment increased pectin, hemi-cellulose and cellulose content of squash roots. In the same way, the hemi-cellulose, ferulic acid and diferulic acid contents of the root cell-wall of Al-sensitive wheat increased with Al treatment, indicating that Al alters the metabolism of cell-wall constituents (Tabuchi and Matsumoto, 2001). However, since Al treatment increases the dry weight of the root apex (Blancaflor et al., 1998) it cannot be excluded that the higher contents of pectin and other cell-wall constituents is due to the fact that their synthesis and release into the apoplast is less inhibited than cell elongation.

The primary site of Al injury is the root apex. Particularly the distal part of the transition zone (1 – 2 mm apical root zone) is the most Al-sensitive root zone in maize (Sivaguru and Horst 1998). Al-resistance mechanisms are also expressed in the root apex in order to protect the Al-sensitive sites (Delhaize et al., 1993a; Pellet et al., 1996; Kollmeier et al., 2001). For this reason the distribution of pectin content and Al accumulation along the root axis was studied using two maize cultivars with contrasting Al resistance. Cultivar Lixis tended to have a higher pectin content than cv ATP-Y which, however, was only significant in the 1-2 mm apical root zone. But the Al contents particularly of the most apical root zones were significantly higher in the Al-sensitive cv Lixis compared to the Al-resistant cv ATP-Y. This is in agreement with previous studies made using these cultivars (Kollmeier et al., 2000; Collet et al., 2002). Al-sensitive cultivars generally accumulate more Al in the root than Al-resistant cultivars. This is true not only for maize but also for other plant species such as wheat (Tice et al., 1992), soybean (Silva et al., 2000) and Arabidopsis (Larsen et al., 1998).

Binding of Al to the cell wall may largely depend on the density of the negative charge carried by the cell wall, which is governed by the degree of methylation of pectin. Schmohl et al. (2000) found a close negative correlation between the degree of methylation of cell-wall pectin and Al accumulation in suspension-cultured maize cells. A higher degree of methylation was associated with a lower Al accumulation and reduced Al injury. Based on this observation, a comparative assessment of the degree of methylation of cell-wall pectin was made in this study using two maize cultivars contrasting in Al resistance. For this purpose, we used antibodies which are specific for high-methyl-ester pectin (JIM7) and low-methyl-

ester pectin (JIM5 and LM7) (Table 1). The antibodies localized pectin particularly in the root cortex rather than in the epidermis and stele (Fig. 6-9) similar to the observation by Knox et al. (1990). Since the fluorescence intensity is proportional to the quantity of the specific pectic epitope present in the cell wall the two cultivars proved to differ in the contents of low- and high-methyl-ester pectin (Fig. 6 and 8). This indicates that in addition to a slightly higher pectin content in the most Al-sensitive apical root section (Fig. 5) Al-sensitive cv Lixis was also characterised by a lower degree of methylation of the pectin and thus higher negativity of the cell walls.

There was an apparent difference between ATP-Y and Lixis in the pattern of distribution of the JIM7 epitope (high-methyl-ester pectin). This epitope was localized in the whole intercellular space in cv ATP-Y but restricted to the cell corners and the wall lining the intercellular space in cv Lixis (Fig. 7). Whether this has any implication for Al resistance is not known. The JIM5 epitope (low-methyl-ester pectin) was generally abundant at the cell corners, where it is supposed to contribute to cell adhesion through Ca^{2+} cross-linking (Carpita and McCann, 2000). As junction points, the cell corners bear greater tension and the conductivity of mechanical stresses throughout the plant tissue greatly depends on the cell corners and intervening edges (Ryden et al., 2003).

Al^{3+} has a high charge to ionic size ratio, and rapidly and strongly binds to pectin of low degree of methylation (Blamey, 2001). Thus the higher content of low-methyl-ester pectin in cv Lixis than in cv ATP-Y could explain the differential accumulation of Al and the resulting differential responses to Al stress. However, it also has to be considered that these cultivars also differ in the exudation of

organic acid (Kollmeier et al., 2001) one of the well-known mechanisms of Al resistance (see Introduction).

Pectin methylation (methyl esterification) determines the cation exchange-capacity (CEC) of the root. Knight et al. (1961) and Haynes (1980) reported that increased methylation of uronic acid (pectin) in the root cell-wall decreased root CEC. A decrease in root CEC reduces the binding of Al in the cell wall. Indeed this was illustrated in two *Lotus* species, where Al-resistant cv Maku had lower CEC and accumulated less Al than the sensitive cv Maitland (Blamey et al., 1990). In contrast, Ishikawa et al. (2001) did not find a close relationship between root CEC and Al resistance among five plant species (maize, rice, wheat, sorghum and pea). This indicates that root CEC is not a good indicator of Al resistance across plant species.

Pectins are thought to govern a wide range of wall functions: i) determine wall porosity; ii) provide charged surfaces that modulate cell wall pH and ion balance; iii) regulate cell to cell adhesion; iv) serve as recognition molecules alerting the plant cells to the presence of symbiotic organisms, pathogens, and pests; V) regulate the access of wall-loosening enzymes to their glucan substrates (Carpita and McCann, 2000). It is likely that the binding of Al to the cell-wall pectic matrix affects these functions. Using artificial pectin membranes, Blamey et al. (1993) presented *in-vitro* evidence that Al reduced water movement through root cell-walls. Horst et al. (2004), and Sivaguru et al., (2004) showed that Al inhibits the apoplastic bypass flow of solutes (HPTS and dextrans) but not of water in the maize root apex. The reduced cell-wall permeability may limit the movement of wall-loosening enzymes and the access to their substrates, which could

consequently result in reduced cell expansion. This is corroborated by the observation of decreased mechanical extensibility of the cell wall upon Al treatment (Tabuchi and Matsumoto, 2001).

In conclusion, our results suggest that differences in cell-wall pectin-content and its degree of methylation contribute to genotypic differences in Al resistance in maize.

CHAPTER 3

LOCALIZATION OF ALUMINIUM IN THE MAIZE ROOT APEX: CAN MORIN DETECT CELL WALL-BOUND ALUMINIUM?

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ABSTRACT

Morin is a fluorochrome, which forms a fluorescent complex with Aluminium (Al) and is thus used to localize Al in plant tissues. However, reports about the cellular distribution of Al – apoplastic vs. symplastic – based on morin staining are often conflicting. The objective of this work was to investigate whether Al localization with morin staining can show the proper cellular distribution of Al. Fresh root cross-sections were made from root apices of maize (cv Lixis) treated with 25 µM Al for 6 h and stained with morin. Fluorescence microscopic investigation showed Al-morin fluorescence in the cytosol but not in the cell wall. This is in contrast to the growing evidence which shows that Al mainly accumulates in the cell wall, especially bound to the pectic matrix. Therefore, *in vitro* analyses were made to study whether morin can form a fluorescent complex with Al, which is bound to pectin, cell wall and other Al-binding ligands such as phosphate and galacturonate. Compared to the control treatment without Al-binding ligands, fluorescence intensity was reduced by about ten-fold in the presence of pectin and isolated cell walls, but fairly unaffected in the presence of phosphate and galacturonate. This implies that although Al is mainly accumulated in the cell wall, it cannot be detected with morin as it is tightly bound to cell-wall pectin. Thus, conclusions about the cellular distribution of Al based on morin staining should be cautiously reconsidered.

Key words: Al localization, cell wall, cytosol, fresh root cross section, morin,

INTRODUCTION

Aluminium (Al) phytotoxicity is a major threat to plant growth on acid soils (Taylor, 1988). The most commonly observable symptom of Al injury is inhibition of root elongation, which can be recognized within an hour of exposure to Al (Llugany et al., 1995). There is wide genetic diversity between plant species for Al resistance. Al-sensitive genotypes usually accumulate more Al in the root tissue than Al-resistant ones. For example, Al-sensitive maize cultivars had higher Al contents in the root tip than Al-resistant cultivars (Collet et al., 2002). Similar observations were obtained for wheat (Tice et al., 1992), soybean (Silva et al., 2000), and Arabidopsis (Larsen et al., 1998).

The uptake and accumulation of Al in the root tissue can be assessed with several methods one of which is staining using Al-specific dyes. Staining techniques are relatively simple and rapid tools for examining Al accumulation in plant roots. Cancado et al. (1999) used haematoxylin staining as a phenotypic index of selection for Al resistance in maize. Aniol (1983) used eriochrome cyanine R to assess Al uptake of winter wheat varieties. Other chromophores such as aluminon and solochrome azurin were also used to detect Al distribution in biological samples (Denton et al., 1984). However, the low sensitivity and poor spatial resolution of these staining techniques did not allow using them as tools for studying cellular distribution of Al. Fluorophores such as morin and lumogallion are highly sensitive and can detect very low concentrations of Al (Eggert, 1970; Kataoka et al., 1997).

Morin is a pentaprotic acid that forms a highly fluorescent complex with Al. The Al-morin complex has excitation and emission wavelengths of 420 nm and 515 nm,

respectively (Browne et al., 1990). Its fluorescence detection limit is as low as 2×10^{-9} M (Lian et al., 2003b) and thus morin is used along with fluorescence microscopy to sensitively localize Al in plant cells. Several authors used morin to study the cellular distribution of Al. However, results are conflicting with regard to the major cellular site of Al accumulation. Ahn et al. (2002) observed Al-morin fluorescence in the cell wall of squash root apices after three hours of Al treatment whereas Vitorello and Haug (1996) did not see any fluorescence in the cell wall of cultured tobacco cells. They observed Al-morin fluorescence in the cytoplasm, at a discrete zone of the cell periphery. Similarly, Tice et al. (1992) observed Al-morin fluorescence particularly in the cytoplasm and the nucleus and less in the cell wall of wheat root tips. They concluded that the symplastic Al fraction accounted for 60-70 % of total cellular Al while the remaining 30-40% represented apoplastic Al. However, this is in clear contrast to the growing evidence which shows that symplastic Al is many folds lower than apoplastic Al. Marienfeld et al. (2000) measured a higher concentration of Al in the cell wall of maize and bean root tips using laser-microprobe mass-analysis. They attributed the differences in cellular localization and tissue distribution of Al to differences in cell-wall pectin-content of the plant species. In agreement with this, an increase in cell-wall pectin-content resulted in a higher accumulation of Al in maize suspension cells (Schmohl and Horst, 2000). Furthermore, a decrease in the degree of esterification of cell-wall pectin enhanced Al accumulation (Schmohl et al., 2000). Using fractionated extraction, Wang et al. (2004) measured about 85% of the total Al in the cell wall of maize root tips. Taylor, et al. (2000) determined even much higher (>99%) accumulation of Al in the cell wall of the giant algae *Chara corallina* after physically

separating the cell wall from the protoplast. The objectives of the present study were to investigate the cellular localization of Al in maize root apex using morin staining and to determine whether morin can form a fluorescent complex with pectin-bound Al.

MATERIALS AND METHODS

Root sectioning, Staining and microscopy

Maize (cv Lixis) seeds were germinated in rolls of wet filter paper. Several rolls, each having about 10 seeds, were placed in a glass beaker containing a small amount of tap water and placed in a dark chamber at a temperature of 30°C. After germination, the seedlings were exposed to light for one day before the treatment. Either the whole root system or only root cross sections were treated with Al. To treat intact root system, the seedlings were transplanted into a continuously aerated solution containing 0.5 mM $CaCl_2$ with or without 25 µM Al as $AlCl_3$ at pH 4.3 for six hours. Root tips were excised, inserted in wet styrofoam and thin cross sections (a few cell layers) were made from the 1-3 mm zone of the root apex. Free hand sectioning, without fixation and embedding was employed in order to avoid artefacts related to cellular redistribution of Al. The sections were made with sharp razor blades (Wilkinson Sword GmbH, Solingen, Germany). The blade was dipped in water before cutting so that the sections would remain on the blade after sectioning. The sections were carefully removed with a paintbrush and collected in Petri dishes containing 0.5 mM $CaCl_2$ solution. After collecting sufficient root sections, they were transferred to staining tubes having a nylon mesh at the base, which facilitated washing. The sections were rinsed with double deionised water and stained with a 100 µM aqueous solution of morin ($C_{15}H_{10}O_7$) for 30 min. Then they were washed twice with double deionised water for 5 min each. Slides were prepared, mounting the sections in distilled water, and examined with an Axioscope microscope (Zeiss, Axioscope, Jena Germany) equipped with epifluorescence illumination (Mercury lamp, HBO 50 W). The filter set used to

observe morin-Al fluorescence consisted of a band pass filter BP 395-440 nm (exciter), a beam splitter FT 510 nm, and a long-wave pass filter LP 515 nm (emitter) since morin-Al has excitation and emission wavelengths of 420 nm and 515 nm, respectively (Browne et al., 1990). Pictures were taken with a digital camera (Sony Cyber-Shot, DSC-S85, Japan) mounted on the microscope.

Treating root cross sections with Al

Free hand-sections of maize cv. Lixis root tips were made as described above but from plants not treated with Al. The sections were treated with 0 nM, 10 nM 100 nM and 37 µM Al for five minutes in order to study the sensitivity of morin staining. In another experiment, the effect of membrane damage on Al-associated but unspecific fluorescence was studied. For this purpose, root sections were treated with either Al (0 nM, 10 nM 100 nM, and 10 µM), or digitonin (10 and 100 µM) for 30 min. Staining, and microscopy was done as explained above.

Fluorometry

The fluorescence of Al bound to pectin, plant cell walls and other Al-binding ligands was investigated using morin reagent according to Browne et al. (1990). Two citrus pectins differing in degree of methyl esterification were purchased from Sigma, Steinheim, Germany. The degrees of esterification were 92% and 28.5% while galacturonic acid contents were 82% and 65% respectively for the two pectins. A solution resulting in 100 mg l^{-1} galacturonic acid was prepared from both pectins. In addition, solutions of pure galacturonic acid (100 mg l^{-1}), KH_2PO_4 (30µM) and control (only double deionised water) were prepared. To each of the above solutions, Al was added to a final concentration of 1 µM in the assay. The

pH of the solutions was adjusted to 4.8 using 0.1 N HCl/NaOH and left to equilibrate for one hour at room temperature. Samples were taken from the solutions and filtered through 0.025 µm membrane filters (Schleicher & Schuell, Dassel, Germany) on a Millipore filtration unit (Millipore GmbH, Germany). Samples of 25 ml were taken from both the filtered and the unfiltered solutions. Then 7.5 µl of 33.3 mM morin dissolved in dimethyl sulfoxide (DMSO) was added to make up 10 µM morin in the assay. The samples were vortexed and kept dark for 15 min. Finally, 2 ml sample was transferred to microcuvettes and Al-morin fluorescence was measured with a fluorescence spectrophotometer (F 2000, Hitachi Ltd., Tokyo, Japan) at excitation and emission wavelengths of 418 and 502 nm, respectively.

A similar assay was performed using cell-wall material extracted from the maize root apex. Root tips (1 cm) were excised from maize (cv Lixis) seedlings treated with or without 25 µM Al for 12 h and cell-wall material was prepared according to Schmohl and Horst (2000). Dried cell-wall material (4 mg) was suspended in 2 ml of 1 µM Al solution and was shaken on a rotary shaker (Landgraf Laborsysteme, Germany) for 1 h. The pH was adjusted to 4.8, and 4 µl of 5 mM morin in DMSO solution was added to the suspension to make up 10 µM morin in the assay. Next, the samples were shaken for 15 min, centrifuged at 23000 x g, the supernatant was collected and the fluorescence was determined as indicated above. Interaction of Al with DNA and possible formation of Al-morin fluorescence was also tested using Herring Sperm DNA (Sigma, Steinheim, Germany).

RESULTS

The cellular distribution of Al in maize root tissue as revealed by Al-morin fluorescence is shown in Fig. 1. There was bright green fluorescence in the root sections of Al-treated plants but there was hardly any fluorescence in the control (-Al). During the six hours treatment, Al reached the endodermis but was not detected in the stele, indicating that radial transport of Al was greatly restricted by the endodermis. Epidermal, cortical and endodermal cells were heavily stained (Fig. 1B). The absence of fluorescence in the stele, showed that cross contamination during root sectioning and staining operations was minimal.

In thin root sections, more intense fluorescence was observed in the cytosol than in the cell wall (Fig. 1). The brightest fluorescence in the cytosol appeared in the nucleus. In cells where the cytoplasmic contents were lost through cutting, there was apparently no fluorescence (see the arrows in Fig. 1C and compare with the fluorescent image in Fig. 1B). This can be visualized from ultra-thin longitudinal section of the epidermis (Fig. 2). From the bright light image, the cell wall was clearly seen; however, there is virtually no fluorescence in the cell wall. Fluorescence can be observed only in the cells with cytoplasm.

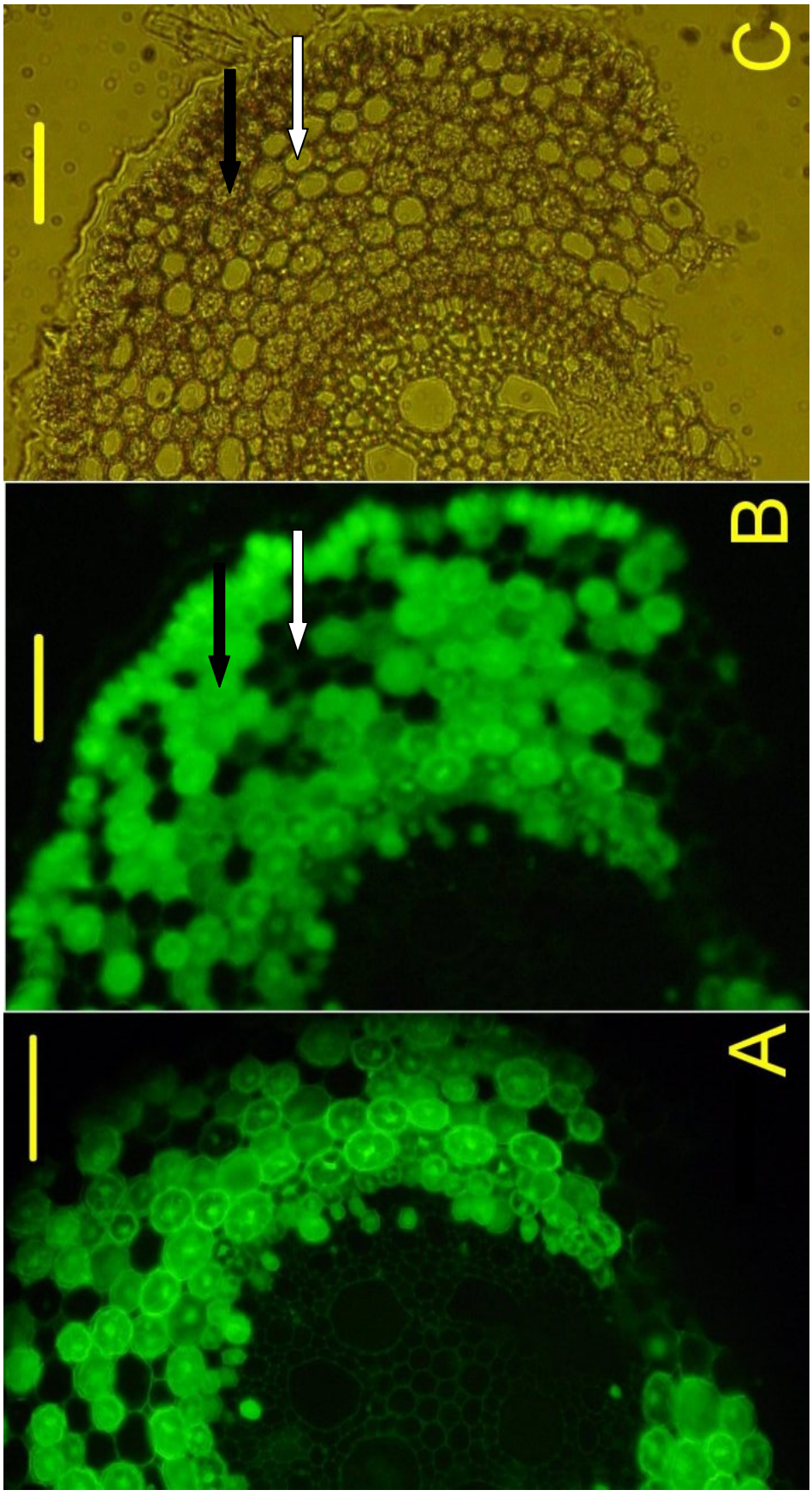

Figure 1. Al distribution in maize root sections localized by morin staining. *A*, *B*, fluorescence images focused on the cortex and the stele; *C*, bright light image of B. Black and white arrows indicate a cell with intact cytoplasm and a cell that lost the cytoplasmic content during sectioning, respectively. The sections were taken from the root zone between 1 – 3 mm behind the root apex of maize seedlings treated with 25 µM Al for 6 h. Scale bars = 100 µm.

In order to test the sensitivity of morin for Al staining, root cross sections from plants not treated with Al were exposed to Al from nanomolar to micromolar concentrations for 5 min only and then stained with morin. Fluorescence was observed again only in the cytosol of the root cells exposed to Al for a short time (Fig. 3D) similar to Fig. 1, where the whole root was treated with Al for a much longer time. There was low but distinct fluorescence in sections exposed to nanomolar concentrations of Al (Fig. 3B and C) vs. the control (Fig. 3A).

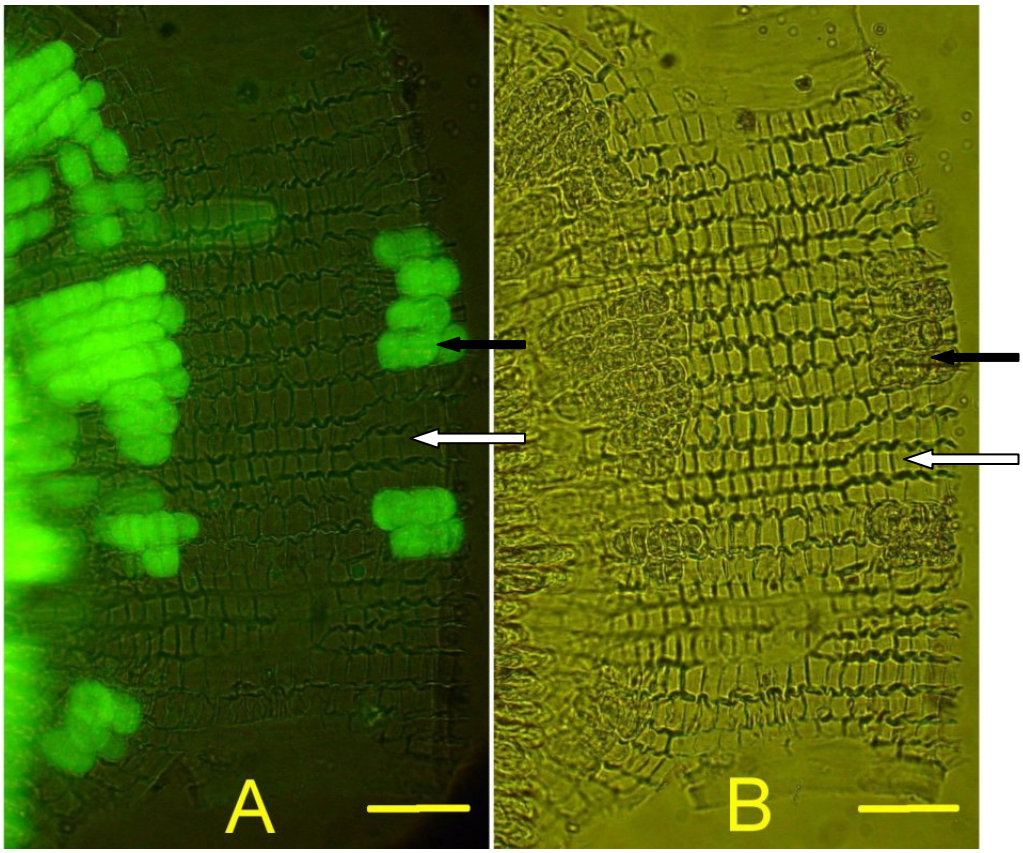

Figure 2. Al localization in the cytosol of epidermal cells of the maize root apex. A, overlay of fluorescence and bright light images; B, bright light image of A. Black and white arrows indicate a cell with intact cytoplasm and a cell that lost the cytoplasmic content during sectioning, respectively. The sections were taken from the root zone between 1 – 3 mm behind the root apex of maize seedlings treated with 25µM Al for 6 h. Scale bars = 100 µm

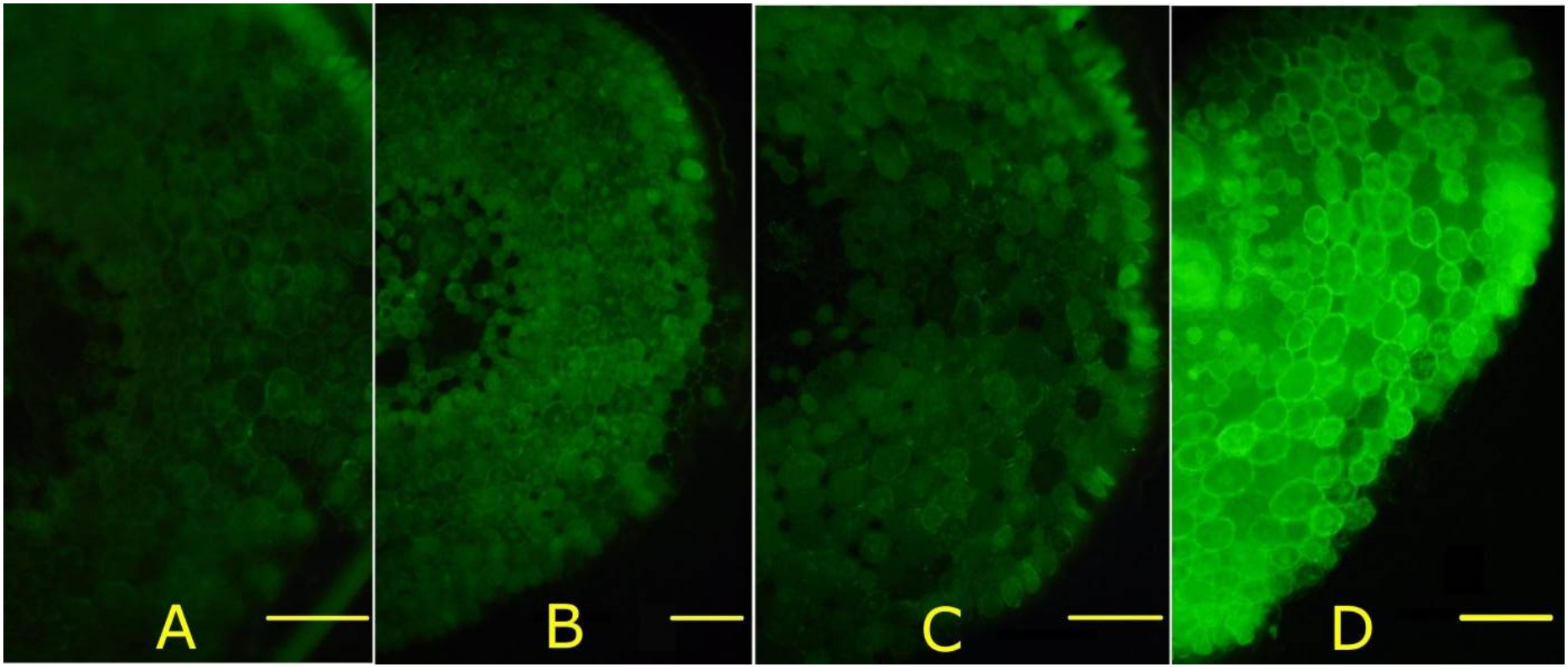

Figure 3. Al localization in maize root cross sections after 5 min of Al treatment of the cross sections. A, control; B, 10 nM Al; C, 100 nM Al; D, 37 µM Al. Fresh root cross sections were taken from the root zone between 1 – 3 mm behind the root apex of maize seedlings not treated with Al. They were treated with different levels of Al for 5 min. Scale bars = 100 µm.

There is hardly any doubt that fluorescence was associated with Al treatment (Fig. 1, 2, 3) but there is uncertainty whether the fluorescence in the cytosol originated from Al-morin or other cation-morin complexes since some metallic cations such as Br, Mg and Zn may also form fluorescent complexes with morin (Lian et al., 2003a). This led to doubts that the fluorescence in the cytosol might come not from Al-morin but from complexes of morin with other cations, and that Al may merely disrupt the plasma membrane and open a gateway for high morin permeation into the cytosol. In order to clarify this, thin cross-sections of the maize root apex were treated with digitonin which permeablizes the plasma membrane (Tsay et al., 1999), and then stained with morin in comparison with sections treated with or without Al.

Membrane disruption through digitonin treatment did not result in fluorescence different form the control (Data not shown). Distinct fluorescence was observed only in Al-treated sections. Thus, the fluorescence resulted specifically from the Al-morin complex. Similar to the above observations (Fig. 1, 2, 3), bright Al-morin fluorescence was mainly localized in the cytosol. This observation leads to the formulation of two possible hypotheses: i) Al is mainly accumulated in the cytosol but not in the cell wall, ii) Al may accumulate in the cell wall but cannot be detected with morin. Further elucidation of these hypotheses is presented below.

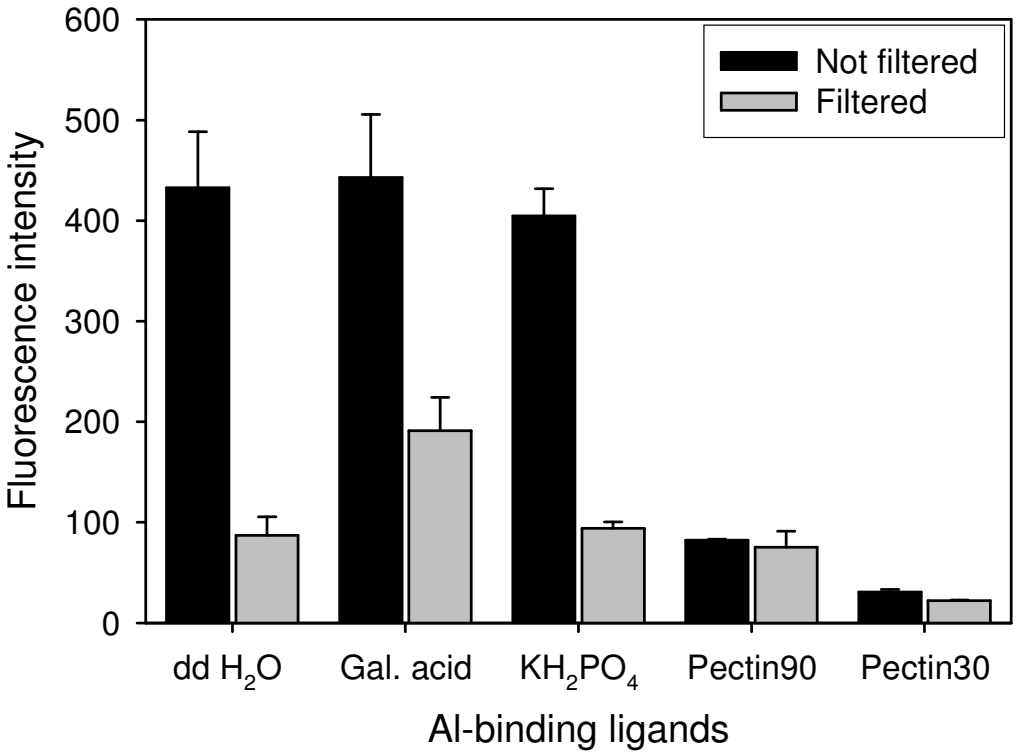

Figure 4. Effect of Al-binding ligands on the formation of the fluorescent Al-morin complex in double deionised H_2O (dd H_2O). Pectin30 and Pectin90 are citrus pectins with 30% and 90% degree of methylation, respectively. Each solution contains 1 µM Al. Solutions were filtered or not prior to the fluorescence measurement. Bars represent means ± SD of 4 replicates.

In the cell wall and particularly the cytosol the presence of free Al is rather unlikely. It can be assumed that Al is bound to negatively charged ligands. Thus the formation of the Al-morin complex was studied *in vitro* in the presence of Al-binding ligands such as DNA, galacturonate, phosphate and pectin, the most likely Al-binding compounds in the cell (Crowford et al., 1998; Chang et al., 1999; Schmohl and Horst, 2000; Zhang et al., 2002). Al-morin fluorescence was detected in DNA even without adding Al, showing that the commercial DNA contained trace level of Al. Addition of Al to the DNA sample increased the fluorescence intensity (data not shown) indicating that Al bound to DNA can be

detected with morin. Phosphate and galacturonate did not have a significant influence on Al-morin fluorescence, in non-filtered samples (Fig. 4). Filtered samples generally had lower fluorescence compared to non-filtered samples indicating that a larger proportion of the added Al was precipitated even in the control (deionised distilled water) samples at pH 4.8 used in this experiment. It was evident that morin could make complexes with freshly precipitated Al ($Al(OH)_3$, $AlPO_4$, Al-galacturonate) but not with pectin-bound Al. It appeared that Al-pectin is more stable than the Al-morin complex. The formation of the fluorescent Al-morin complex was greatly reduced in the presence of pectin, particularly of the pectin with a low degree of esterification (DE 30%) compared to the pectin with a high degree of esterification (DE 90%) regardless of the filtration of the samples.

The sorption of Al to cell-wall materials derived from maize root tips was investigated by applying 1 µM Al followed by testing with morin. Al was strongly sorbed to the cell-wall material, causing a great reduction in the fluorescence intensity of Al-morin (Fig. 5). The origin of the cell-wall material had significant influence on the amount of Al sorbed, as reflected by a decrease in fluorescence intensity. Al was more strongly bound to cell-wall material derived from control plants (which were not treated with Al, i.e., Al-0), possibly due to the availability of more free Al-binding sites. Morin did not desorb Al from the cell-wall material showing that Al has a higher affinity to the cell wall than to morin.

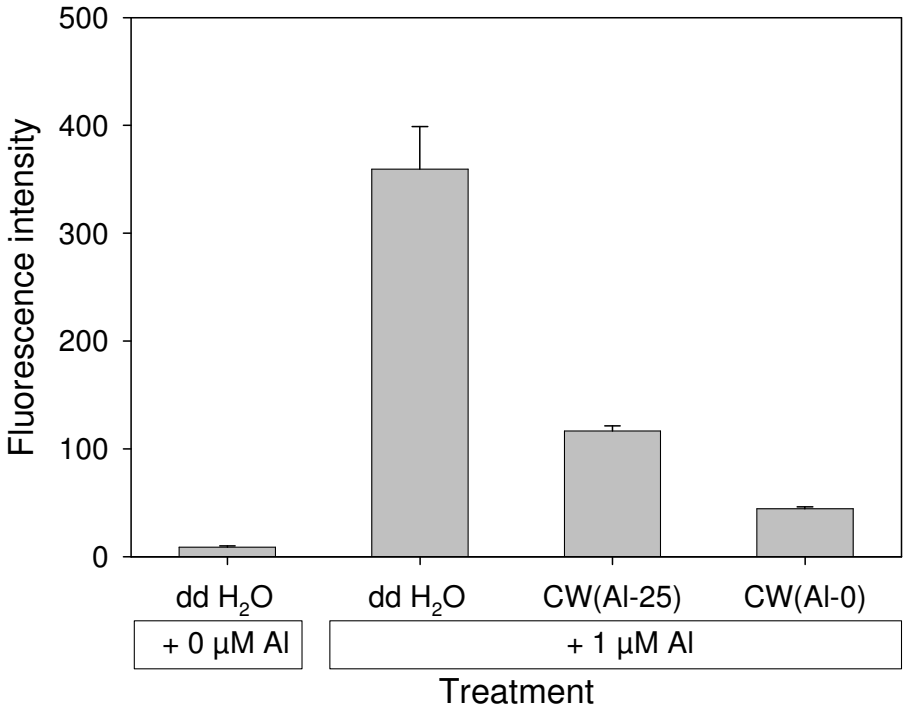

Figure 5. Effect of cell-wall material (CW) on the formation of the fluorescent Al-morin complex in double deionised H_2O (dd H_20) with no or 1 µM Al added. Cell-wall material was derived from root apices of maize plants treated without (Al-0) or with 25 µM Al (Al-25) for 12 h. Bars represent means ± SD of 6 replicates.

DISCUSSION

The cellular distribution of Al is debated among two main groups of observations. One group of observation indicates that Al is mainly accumulated in the apoplast while the other supports symplastic accumulation. Several methods have been employed by different authors to investigate Al uptake and distribution. Zhang and Taylor (1989, 1990, and 1991) used a kinetic approach and observed a bi-phasic pattern of Al uptake in wheat. The bi-phasic pattern was characterized by an initial rapid, nonlinear phase followed by a slower linear phase, which was traditionally interpreted as rapid apoplastic binding followed by slow uptake across the plasma membrane (Zhang and Taylor, 1989). Electron diffraction x-ray microanalysis (EDXMA) showed that Al is predominantly localized in the cell wall of Al-treated *Avena sativa* roots (Marienfeld and Stelzer (1993). In addition, laser microprobe mass analysis (LAMMA) indicated higher accumulation of Al in the cell wall of maize roots after a short-term (1 – 3 h) Al treatment (Marienfeld et al., 2000). On the other hand, Tice et al. (1992) combined a kinetic study with fluorescence spectroscopic methods to operationally define symplastic and apoplastic Al fractions in root tips of Al-treated wheat. According to this study, only 30 – 40% of Al belonged to the apoplastic fraction while the remaining was allocated to the symplastic fraction. Lazof et al. (1994), used secondary ion mass spectrometry (SIMS), a rather sensitive method, and detected symplastic accumulation of Al in intact soybean root tips after a relatively short time (30 min) of Al exposure and washing of the roots with citrate to remove cell-wall Al.

All of the above approaches face specific methodological limitations to unequivocally give the precise cellular distribution of Al. The first, unambiguous

and direct measurement of Al uptake and distribution was achieved by Taylor et al. (2000), who used the rare ^{26}Al isotope, accelerator mass spectroscopy, and a surgical technique to physically separate the cell wall from the cytosol in single cells of giant algae, *Chara corallina*. They observed that Al accumulation in the cell wall dominated total uptake (up to 99.99%), but transport across the plasma membrane was also detected within 30 min of exposure to Al. *Chara* showed a growth response to Al similar to that of wheat (Reid et al., 1995). Moreover, the electrical properties of *Chara* and wheat-root cell-walls were similar (Reid et al., 1996). In agreement with the observation in *Chara*, Chang et al. (1999) found that the cell wall isolated from Al-treated tobacco cells contained as much Al as the intact cells. Thus, there is little doubt that the majority of the cellular Al is located in the cell wall. Accordingly, genuine Al-localization methods have to reflect similar observation. However, reports are usually conflicting in this regard.

One of the easiest and most commonly used Al localization methods is the use of the fluorophore morin (Eggert, 1970; Tice et al., 1992; Larsen et al., 1996 and 1998; Vitorello and Haug, 1996 and 1997; Ezaki et al., 2000; Ahn et al., 2001 and 2002). Morin (2,3,4,5,7-pentahydroxy flavone) makes a highly fluorescent complex with Al. It is specific to Al, especially at low pH (Browne et al., 1990) and highly sensitive, with an *in vitro* detection limit of 2 nM (Lian et al., 2003b). This makes it very attractive for Al studies. However, Tice et al. (1992) and Vitorello and Haug (1996) who used morin staining to localize Al, appeared to have greatly underestimated the proportion of Al found in the cell wall. Therefore, Archambault et al. (1996b) questioned whether morin can detect cell wall-bound Al.

In an attempt to clarify the prospects and limitations of morin as a stain for *in vivo* cellular distribution of Al, thin hand-sections (1-3 cell layers) of maize root tips were used in our study. The advantage of thin cross sections is that free apoplastic Al and Al from the symplast of damaged cells could be easily removed by simple washing with double deionised water. Moreover, doubts related to desorption of Al from the cell wall during staining and washing procedures were mitigated by using an aqueous solution of morin. In the conventional method of morin staining, acetate or MES buffers, which readily complex Al, were used as a solvent for morin and also as a washing solution before and after staining (Tice et al., 1992; Larsen et al., 1996). These buffering chemicals may desorb Al from the cell wall.

Al localization using morin staining detected the presence of Al in the cytosol but not in the cell wall. The result was consistent throughout our experiments (Fig.1 – 3) and also similar to the observations of Tice et al. (1992) and Vitorello and Haug (1996). This does not necessarily show that Al is more abundantly found in the cytosol than in the cell wall. It may indicate that morin cannot detect cell wall-bound Al.

Chang et al. (1999) reported that about 71 – 82% of the total cellular Al was found associated with pectin of the cell wall. Hence we tested the interaction between morin and pectin-bound Al. The results clearly indicated that morin could not form a fluorescent complex with pectin-bound Al (Fig. 4). Experiments with isolated cell-wall material also reflected similar phenomena (Fig. 5). Even using the common and strong metal-chelating agent, EDTA (Al-EDTA binding affinity constant Log K = 16.5; Orvig, 1993), Chang et al. (1999) were able to desorb only 17% of the cell

wall-bound Al. Therefore, it can be speculated that Al has a higher affinity to the cell wall than to EDTA. Thus, morin (Al-morin binding affinity constant Log K = 6.5; Katyal and Prakash, 1997) could not form a fluorescent complex with cell wall-bound Al.

Intracellular Al may exist in association with cytosolic ligands with smaller binding affinity and thus can form fluorescent complexes with morin. Intense fluorescence was observed in the nucleus as was previously reported (Tice et al., 1992; Vitorello and Haug, 1996). Al binds to DNA in the nucleus. The binding site of Al on DNA was shown to be the phosphate backbone but not the bases (Zhang et al., 2002). Similarly, Crowford et al. (1998) reported that Al appeared to be co-localized with P in the nuclei of root cap and meristematic cells. Al associated with phosphate can be detected with morin (Fig. 4) that is why Al in the nuclei gives a bright fluorescence when stained with morin.

In conclusion, the results clearly show that morin is not able to detect Al tightly bound to the cell-wall pectin. Therefore, any interpretation of results regarding cellular Al distribution based on morin staining should be cautiously reconsidered.

GENERAL DISCUSSION

Soil amendments such as liming, fertilization and organic manuring are commonly used to tackle the problem of soil acidity and Al toxicity. In countries like Brazil and the US, liming is a widely used management practice for acid soils. Lime application has to be repeated every few years to ensure better crop growth. However, this is beyond the economic reach of the resource-poor farmers of the developing countries. Moreover, the ameliorative effect of liming is restricted only to the topsoil since liming the subsoil is difficult. Therefore the development of Al-resistant and acid-soil tolerant cultivars was considered to provide a cheap and permanent solution (Bellon, 2001). Unlike the resistance against plant diseases which can be overcome through mutation of the pathogen, the resistance to abiotic stresses such as Al toxicity is not prone to break down. However, continuous uses of Al-resistant cultivars on Al-toxic soils without soil amendments could aggravate the problem due to the continued removal of neutralizing substances (Delhaize et al., 2004). Sustainable crop production on Al-toxic soils can thus be achieved by using Al-resistant cultivars along with sound agronomic practices.

The development of Al-resistant cultivars primarily depends on the effectiveness of selection methods. Selection of Al-resistant maize cultivars under field condition is complicated due to soil variability and interaction of different stress factors related to soil acidity. Therefore, cultivar assessment is usually done under controlled condition in hydroponic cultures. Since Al toxicity inhibits root elongation, the basis of assessment for Al resistance was root growth (Foy, 1976; Foy et al., 1993). However, measurement of root growth is relatively tedious and not suited to

evaluate large number of genotypes at a time. On the other hand, Al-induced callose formation was reported to be a more sensitive marker of Al injury (Wissemeier et al., 1987; Wissemeier and Horst, 1995) and could indicate genotypic differences in Al resistance in maize (Horst et al., 1997; Collet et al., 2002), wheat (Zhang et al., 1994), and soybean (Wissemeier et al., 1992). Furthermore, Collet et al. (2002) found a positive correlation between Al-induced callose formation and inhibition of root elongation in maize. In the present study Al-induced callose was used as a physiological marker for Al injury to study the inheritance of Al resistance in maize cultivars. A close negative correlation was observed between the GCA effect of callose formation and the GCA effect of grain yield on acid soils. Thus, screening for Al-induced callose formation in the root apices is an attractive tool for rapid and non-destructive assessment of Al resistance on a single plant level. In spite of this, callose formation appears not to be a suitable tool for screening bean (*Phaseolus vulgaris*) cultivars for Al resistance (Rangel et al., 2004). This could be mainly due to the relatively longer lag period until the resistance mechanism is switched on, in this case the release of organic acids. Before the resistance mechanism starts, the Al-resistant cultivars produce as much callose as the sensitive cultivars. Extending the duration of screening on the other hand results in the breakdown of the synthesized callose. This implies that callose formation is not a good selection parameter for plants exhibiting slowly induced-resistance mechanisms.

Considerable genetic variation was observed in maize cultivars for Al resistance. This variation is heritable as indicated by a relatively high heritability estimate (h^2 = 0.7). The diallel analysis of variance showed a significant GCA effect while the

SCA effect was non-significant. This indicates that Al resistance in these maize cultivars is mainly controlled by additive genes, i.e., polygenic inheritance. Several studies (Lima et al., 1992; Duque-Vergas et al., 1994; Pandey et al., 1994; Borrero et al., 1995; Salazar et al., 1997) also showed that Al resistance and soil-acidity tolerance are quantitative traits, which are controlled by many genes having minor individual effects but define the trait as a sum of their individual effects. The polygenes responsible for Al resistance in maize were not yet characterized. Moreover, it is not known whether the polygenes stand for multiple Al-resistance mechanisms.

Although early studies (Rhue et al., 1978; Miranda et al., 1984) indicated qualitative inheritance of Al resistance in maize, recent works generally agree that it is a quantitative trait (Lima et al., 1992; Duque-Vergas et al., 1994; Pandey et al., 1994; Borrero et al., 1995; Salazar et al., 1997). Moreover, Piñeros et al. (2002) described Al resistance of maize as a “genetically complex trait” due to the presence of multiple resistance mechanism and/or the involvement of many genes controlling a single resistance mechanism. A further investigation is needed to find out possible Al-resistance mechanisms operating in maize.

In contrast to maize, Al resistance in wheat is controlled by a single major gene although some other genes have also been implicated in conditioning the degree of Al resistance (Delhaize, 1993a, b; Carver and Ownby, 1995) which can easily be utilized both in classical breeding and for genetic engineering. Recently, the gene which codes for the Al-activated malate transporter, *ALMT1*, has been successfully cloned (Sasaki et al., 2004). This gene was used to transform barley; a plant species which is very sensitive to Al. Expression of the *ALMT1* gene in

barley transgenes conferred an Al-activated efflux of malate similar to that of Al-resistant wheat. Moreover, the transgenic barley showed a high level of Al resistance when grown in both hydroponic culture and on acid soils (Delhaize et al., 2004) indicating the potential of utilizing this gene both in classical breeding and for genetic engineering. The media release of CSIRO (2004) reported that the gene is being used as a molecular marker for selecting Al-resistant wheat in conventional breeding. However, marker assisted selection of maize using this gene may be of little value since Al resistance in maize is a polygenic trait. Moreover, the physiological mechanisms of Al resistance in maize are different from that of wheat.

Mechanisms of Al resistance in plants are discussed in several reviews (Matsumoto, 2000, Ma et al., 2001; Ryan at al., 2001; Samac and Tesfaye, 2003; Kochian et al., 2004). In wheat, the mechanism of Al resistance is the release of malate by the root apex (Delhaize et al., 1993a; Ryan et al., 1995) whereas in maize, Al-activated citrate release by the root apex correlated with Al resistance (Pellet et al., 1995; Kollmeier et al., 2001). However, recent studies indicate the presence of multiple resistance mechanism in maize (Piñeros et al., 2002).

Kollmeier et al. (2001) observed that the Al-resistant cv ATP-Y released more organic acid anions (mainly citrate but also malate) than the Al-sensitive cv Lixis. However, the difference in organic acid anion release alone did not explain the difference in Al resistance between these cultivars. Thus, the presence of other mechanisms was suggested. The search for additional Al-resistance mechanisms was focused mainly on the cell wall since the apoplast plays a major role in Al toxicity and resistance (Horst, 1995). According to Taylor et al. (2000) more than

99% of the cellular Al is found in the cell wall bound to pectic matrix (Chang et al., 1999). In addition, Schmohl and Horst (2000) and Schmohl et al. (2000) demonstrated that the cell-wall pectin-content and its degree of methylation modulate Al toxicity in solution-cultured maize cells. The current study on the cell-wall characteristics was undertaken based on this background.

Comparative assessment of the cell-wall characteristics of contrasting maize cultivars, ATP-Y and Lixis, indicated that they differed in the degree of methylation of pectin, which in turn determines the charge density on the cell wall. The cell-wall pectin of the Al-sensitive cv Lixis had a low degree of methylation, i.e., high negative charge, compared to the Al-resistant cv ATP-Y. The high negative charge could provide more Al binding sites in the cell wall. Indeed root-tip Al content was higher in Lixis than in ATP-Y, in agreement with the common observation that sensitive cultivars accumulate more Al than resistant ones. Using methylene blue staining, which has a high affinity to anionic surfaces, Schildknecht and Vidal (2002) studied the cell-wall negativity of two maize cultivars differing in Al resistance. In the control plants, they observed that the cell wall of the Al-resistant cultivar had a lower negative charge density than the Al-sensitive cultivar, but the difference in cell-wall negativity disappeared after Al treatment. This is in line with the present study and together suggests that cultivar differences in cell-wall negativity are related to differences in Al resistance. However, this requires further studies of the genetic variability of maize for cell-wall negativity.

The cell-wall charge-density of grasses and dicots is differentially regulated. In dicots (Type-I walls) the α-D-galacturonic acid unit of the pectic homogalacturonan (HG) defines the charge density of the cell wall. In contrast, cell walls of grasses

(Type-II walls) are poor in pectin but contain glucuronoarabinoxylan (GAX). Thus, additional contribution of the charge density of the wall is provided by the α-L-glucuronic acid unit on the GAX (Carpita, 1996; Carpita and McCann, 2000).

There are serious methodological limitations to study the cell-wall properties and Al distribution in the plant cell, which could reflect *in planta* phenomena. Chemical determination of the degree of methylation of pectin was not successful because the amount of methanol released from the cell wall of maize roots was below detection limit. Methods of Al localization in the cell wall also suffer from specific limitations as discussed in chapter III. However, the development of cell-wall antibodies helped to study cell-wall pectin and its degree of methylation through an imunofluorescence method. This method could contribute to a better understanding of the pattern of pectin distribution in plant tissue (Knox et al., 1990; McCartney and Knox, 2002). Moreover, it helped to place the structural complexity of pectin in cell biological and developmental context (Willats et al., 2001b).

Immunolocalization of pectin in the root cross-sections of two maize cultivars showed that they differ in the degree of methylation of cell-wall pectin, in agreement with their Al content and Al resistance characteristics. The pectin with low degree of methylation is mainly localized in the root cortex particularly at the cell corners. High Al accumulation is also believed to occur in the same region since pectin with a low degree of methylation offers more Al-binding sites. Fluorescence localization of Al using morin, however, did not detect the presence of Al in the cell wall but in the cytosol. Using the same method, Tice et al. (1992) and Vitorello and Haug (1996) reported similar observation and concluded that Al mainly accumulates in the cytosol rather than in the cell wall. However, recent

studies using more reliable methods indicated that more than 99% of the total Al taken up accumulates in the cell wall (Taylor, 2000) particularly bound to pectin (Chang et al., 1999, Schmohl and Horst, 2000). In vitro analysis of the interaction between Al and morin in the presence and absence of pectin revealed that morin could not complex pectin-bound Al. This was further confirmed by making a similar assay using isolated cell-wall material. Thus, despite the presence of large proportion of Al in the cell wall, morin cannot detect Al which is strongly bound to the pectic matrix of the cell wall. This finding has contributed to clarify the confusions regarding the cellular distribution of Al.

There is a long standing debate as to whether Al toxicity is an apoplastic or symplastic phenomena (see reviews by Horst, 1995; Kochian, 1995). Although many investigators report that the majority of Al is found in the apoplast (Marienfeld and Stelzer, 1993; Marienfeld et al., 1995; Rengel and Reid, 1997; Chang et al., 1999; Marienfeld et al., 2000; Taylor et al., 2000; Ma et al., 2004; Wang et al., 2004), the studies of Tice et al. (1992) and Lazof et al. (1994) indicate the presence of a large quantity of Al in the symplast. The symplastic-lesion hypothesis was proposed basically depending on these observations. However, as indicated above, Tice et al. (1992) obviously underestimated the apoplastic Al since they used morin staining which can not detect cell-wall-bound Al. Lazof et al. (1994) used citrate washing to remove the cell wall Al before determining the symplastic Al. Hence, redistribution of Al during the washing procedure might have contributed to the observed high concentration of symplastic Al. Moreover, it is less likely to find free Al in the cytosol given the high pH (ca 7.5) of the cytoplasm and the abundance of potential ligands which can readily complex Al (Martin,

1988). Thus the current study strongly agrees with the idea that Al toxicity is primarily an apoplastic phenomenon.

SUMMARY AND OUTLOOK

About 20% of the world's maize producing area lies in acid soil environments where Al toxicity is the single main yield-limiting factor among all other problems of soil acidity. The use of Al-resistant cultivars is usually recommended together with suitable agronomic management for sustainable productivity of acid soils. Successful development of Al-resistant cultivars relies on a better understanding of the physiological mechanism of Al resistance and the inheritance of the trait. In this study, diallel crosses from 15 open-pollinated maize cultivars were used to study the inheritance and combining ability for Al resistance using Al-induced callose formation as a marker for Al injury. The diallel analysis showed significant GCA but not SCA effect indicating that Al resistance in maize is a polygenic trait which is mainly (ca 80%) controlled by additive genes. Al-resistant cultivars such as CMS36, SA4, SA3 and SA7 had better GCA effects. This result agrees well with the results of the field trial, indicating that assessment of Al-induced callose formation is a suitable tool to screen maize cultivars for Al resistance.

Mechanisms of Al resistance in maize are known to involve the release of organic acid anions, (mainly citrate and to some extent malate) by the root apex. Released organic acid anions can detoxify Al in the apoplast and in the rhizosphere, thus Al-resistant cultivars accumulate less Al in their root tissue than Al sensitive cultivars. Al accumulates mainly in the cell wall of the root where the negatively charged pectin molecules provide the binding sites. Binding of Al to the cell wall limits cell wall extensibility which explains the rapid reduction in root elongation, indicating that the apoplast plays a major role in Al toxicity and resistance. Some studies

show that the release of organic acid anion alone cannot account for the wider genotypic differences in Al resistance in maize and suggest that additional mechanisms are involved. In the experiments presented here the role of the root cell-wall characteristics (particularly pectin content and the degree of methylation of pectin) in Al resistance was investigated using maize cultivars differing in Al resistance. The Al-resistant cultivar had a lower pectin content in the cell wall of the root apex compared to the Al-sensitive cultivar. In addition, the cell-wall pectin of the Al-sensitive cultivar had a low degree of methylation, i.e., a higher negative charge, than that of the Al-resistant cultivar. The high pectin content and low degree of methylation of pectin contributed to the high Al content and the greater Al injury in the Al- sensitive cultivar.

The localization of Al in cross sections of the maize root apex using morin staining did not show the presence of Al in the cell wall, which contradicts with the evidences showing that the majority of the cellular Al resides in the cell wall. However, results of *in vitro* analysis proved that morin cannot form a fluorescent complex with pectin-bound Al. Thus, although a large quantity of Al accumulates in the cell wall it cannot be detected with morin. Some conclusions made in previous studies regarding the cellular distribution and mode of action of Al based on this morin staining technique have to be reconsidered.

To continue and round up the results presented here the following experiments will be necessary:

- This study clearly indicates that Al-resistance is a polygenic trait. However, the number of genes and the genomic locations associated with Al

resistance are not yet known. Therefore, a QTL analysis for further understanding of the genetics of Al resistance in maize is needed.

- The comparative study of cell-wall pectin and its degree of methylation in two maize cultivars differing in Al resistance indicated that cell-wall negativity plays a role in Al resistance. Further study involving more maize genotypes is required to relate the genetic variability of this trait with genetic variability of Al resistance.

- In conjunction with previous observations (Kollmeier et al., 2001; Piñeros et al., 2002) this study asserts the presence of multiple Al-resistance mechanisms such as organic acid anion release, internal detoxification and reduced cell-wall negativity. It is thus necessary to study the relative importance of these mechanisms in order to define the major physiological mechanism of Al resistance in maize.

- The recent success in cloning the gene for Al resistance (*ALMT1*) in wheat is a major breakthrough for increasing the Al resistance of crops. To assess the importance of this gene for Al-resistance in maize the transformation of maize with this gene is necessary.

- The Al-localization study showed that morin is unsuitable to detect pectin-bound Al. For a detailed study of the localisation of Al within the root apex an alternative method has to be developed.

ZUSAMMENFASSUNG UND AUSBLICK

Weltweit sind ca 20 % der zur Maisproduktion verwendeten landwirtschaftlichen Nutzfläche von Bodenversauerung betroffen. Auf diesen Böden ist Al der Faktor, der, ungeachtet aller anderen auf sauren Böden auftretenden Probleme, den Ertrag am stärksten limitiert. Für eine nachhaltige Bewirtschaftung saurer Böden wird daher neben einer angemessenen Bodenbearbeitung, die Verwendung Al-resistenter Sorten empfohlen. Die erfolgreiche Entwicklung Al-resistenter Sorten ist aber von einem besseren Verständnis der physiologischen Mechanismen, die zur Al-Resistenz führen, und der Vererbung dieser Eigenschaften abhängig. In der vorliegenden Arbeit wurden Diallelkreuzungen von 15 offen bestäubten Maissorten verwendet, um die Vererbung und die Kombinationseigenschaften von Al-Resistenz zu untersuchen. Die Al-induzierte Callosebildung wurde dabei als Marker für Al-Sensitivität benutzt. Die Analyse der Diallelkreuzungen zeigte einen signifikante GCA, aber keinen SCA Effekt. Dies zeigt, das Al-Resistenz eine polygenische Eigenschaft ist, die hauptsächlich (ca 80 %) durch additiv wirkende Gene kontrolliert wird. Die Al-resistenten Genotypen CMS36, SA4, SA3 und SA7 zeigten daher auch einen besseren GCA Effekt als die sensitiven Genotypen. Die hier dargestellten Ergebnisse zeigen auch eine gute Übereinstimmung mit Ergebnissen aus Feldversuchen. Dies ist ein Beleg dafür, dass die Al-induzierte Callosebildung ein geeigneter Parameter für die Selektion Al-resistenter Maissorten ist.

Einer der Al-Resistenzmechanismen bei Mais ist die Ausscheidung organischer Säuren (v.a. Citrat, aber auch Malat) aus der Wurzelspitze. Die organischen

Säuren komplexieren Al im Apoplasten und der Rhizosphäre. Al wird dadurch in eine nicht phytotoxische Form überführt und die Al resistenten Sorten akkumulieren daher weniger Al in der Wurzel als sensitive Sorten. Al akkumuliert hauptsächlich in der Zellwand der Wurzel, hier bindet es an Pektin, welches den größten Teil der negativen Bindungsstellen der Zellwand stellt. Die Bindung von Al an die Zellwand vermindert deren Dehnbarkeit und dies führt zu einer schnellen Reduktion des Wurzellängenwachstums. Dies legt nahe, dass der Apoplast eine wichtige Rolle in der Ausprägung der Al-Toxizitätssymptome und damit auch in der Al-Resistenz spielt. Einige Untersuchungen zeigen, das Ausscheidung organischer Säuren alleine nicht die großen genotypischen Unterschiede in der Al-Resistenz bei Mais erklären kann, und nehmen daher an, das noch weitere Mechanismen an der Ausprägung der Al-Resistenz beteiligt sind. Aus diesem Grund wurden in der vorliegenden Arbeit die Charakteristika der Zellwand (v. a. Pektingehalt und der Methylierungsgrad von Pektin) an zwei Maissorten untersucht, die sich stark in ihrer Al-Resistenz unterscheiden. Die resistente Sorte hat einen geringeren Pektingehalt in der Wurzelspitze als die sensitive Sorte. Außerdem war das Pektin der sensitiven Sorte zu einem geringeren Grad methyliert, d. h. es trägt mehr negative Ladung, als das Pektin der resistenten Sorte. Der hohe Pektingehalt und der geringe Methylierungsgrad des Pektins führen zu einem hohen Al-Gehalt und stärkerer Schädigung durch Al in der sensitiven Sorte.

Die Lokalisation von Al in Wurzelquerschnitten der Wurzelspitze mit dem Fluoreszenzfarbstoff Morin zeigte, das Al nicht in der Zellwand vorkommt. Dies steht im Widerspruch zu Ergebnissen, die zeigen, dass der größte Teil des Al in

der Zellwand gebunden ist. Experimente in-vitro zeigten, das Morin keinen Komplex mit an Pektin gebundenem Al bilden kann. Daher kann Al, auch wenn es in hoher Konzentration an der Zellwand gebunden vorliegt nicht mit Morin detektiert werden.

Um die hier dargestellten Untersuchungen weiterzuführen sind die im Folgenden aufgeführten Untersuchungen notwendig:

Es konnte gezeigt werden, das Al-Resistenz eine polygenische Eigenschaft ist. Allerdings sind die Anzahl der Gene die die Al-Resistenz bestimmen und ihre Lokalisation im Genom noch nicht bekannt. Daher ist eine QTL Analyse für ein besseres Verständnis der Al-Resistenz in Mais notwendig

Die vergleichenden Untersuchungen von Pektin und dessen Methylierungsgrad an zwei Maissorten, die sich in ihrer Al-Resistenz unterscheiden zeigen, das die Zellwandnegativität eine Rolle in der Al-Resistenz hat. Um aber die genetische Variabilität dieses Parameters mit der Ausprägung von Al-Resistenz zu korrelieren sind Untersuchungen an einer größeren Anzahl von Maissorten notwendig.

Die eigenen Untersuchungen belegen im Zusammenhang mit früheren Beobachtungen (Kollmeier et al. 2001, Pineros et al. 2002), das die Ausprägung von Al-Resistenz auf mehreren Mechanismen beruht, wie die Ausscheidung organischer Säuren, interne Detoxifizierung und Zellwandnegativität. Es ist notwendig die relative Bedeutung dieser Mechanismen zu untersuchen, um ihren Beitrag an der Al-Resistenz abschätzen zu können.

Die gelungene Klonierung des Al-Resistenzgens (ALMT1) aus Weizen ist ein bedeutender Fortschritt um die Al-Resistenz von Getreide zu erhöhen. Welche

Bedeutung dieses Gen für die Al-Resistenz von Mais hat sollte durch eine entsprechende Transformation von Mais untersucht werden.

Die Untersuchungen zur Al-Lokalisation zeigten, das Morin nicht geeignet ist an Pektin gebundenes Al zu detektieren. Für eine detaillierte Untersuchung der Al-Lokalisation in der Wurzelspitze ist daher die Entwicklung einer alternativen Methode notwendig.

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IV. Publications

Articles

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Posters

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