ALUMINUM TOLERANCE IN MEDICAGO TRUNCATULA GAERTN.

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI‘I AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

TROPICAL PLANT AND SOIL SCIENCES

MAY 2012

By

Yawadee Srimake

Dissertation Committee:
Susan Miyasaka, Chairperson
Robert Paull
Richard Manshardt
Gernot Presting
Douglas Cook

Keywords: aluminum toxicity, Medicago truncatula, acid soil, root growth
ACKNOWLEDGEMENT

Years ago when I was accepted into the Ph. D. program in TPSS, I felt overwhelmingly excited and fortunate, although I knew it would be a big challenge. It was, but it has also been a spectacular journey of learning, trouble-shooting, persistence, and patience. I would like to use this opportunity to thank many people who helped me to complete this dissertation, finally.

I greatly appreciate my academic adviser, Dr. Susan Miyasaka for giving me the opportunity to enter into her program, and for the advice and support, both mentally and financially. I am grateful for her patience throughout these years.

I am deeply grateful to Dr. Robert Paull and Dr. Nancy Chen, who have been so kind and supportive. Half of the experiments in this dissertation have been conducted in their laboratory. This dissertation would not have been finished without their help. I would like to thank Dr. Douglas Cook, who has given me crucial advice, generously supported the experiments in genetics research section, and allowed me to work in his lab at University of California at Davis. Also, I would like to thank my other committee members, Dr. Gernot Presting, and Richard Manshardt for the advice on the direction of my work.

I am thankful to Dr. R Varma Penmetsa for his advice and help with the experiments conducted during my time at the University of California at Davis. In addition, I would like to thank Nolia Carrasquilla, and Dr. Jongmin Baek, who helped me with the DNA library construction and genetic analysis. Also, I would like to thank everyone at Dr. Paull’s Lab, including Helen Turano, Siwaporn Thumdee, Abby Wu, Jenifer, and Bronson Yadao. I appreciate the faculty and friends in the department of Tropical Plant and Soil Sciences (TPSS), Dr. Cook’s laboratory, and all others who have lent me a helping hand and given me warm support.

Finally, I am forever grateful to my family back in Thailand, Mom and Dad, who have been dedicating their time, money, best wishes, and whole-hearted support to me throughout this journey. With their love and understanding, I kept going. They are the reason behind my accomplishments.
ABSTRACT

Aluminum (Al) toxicity in acid soils is one of the major problems in agricultural production worldwide. In this study, we investigated Al tolerance in a legume crop, *Medicago truncatula*. Ninety-one ecotypes and 62 ethylmethylsulfonate (EMS) -generated mutants were screened for their Al-sensitivity using three different methods: 1) Al-containing agarose media; 2) hematoxylin staining; and 3) Al-toxic soil. Root lengths and Relative Root Growth (RRG, i.e. ratio of root elongation of seedlings grown with Al at a particular level to that of seedlings grown without Al) were measured. We found significant differences in response to Al-toxicity among the ecotypes and EMS mutant lines, and the results correlated positively between each method. We identified 3 Al-tolerant, 3 sensitive ecotypes, and 1 sensitive mutant. The Al-tolerant and sensitive lines were cross-pollinated to generate segregating populations. The F₂ population of PI 577633 (Al-tolerant ecotype) and S69 (sensitive mutant) were screened using the Al-containing agarose assay. Genomic DNA of extremely tolerant and sensitive seedlings was extracted for Restriction site-associated DNA (RAD) analysis. We identified 11 candidate alleles that might be associated with Al tolerance. This information could be useful in breeding or selecting for improved Al tolerance in the model legume *M. truncatula*, as well as other crop legume species.
# TABLE OF CONTENTS

Acknowledgement........................................................................................................iii
Abstract......................................................................................................................iv
List of Tables..............................................................................................................vi
List of Figures.............................................................................................................vii
List of Abbreviations..................................................................................................ix

## CHAPTER 1 Literature review.................................................................1

## CHAPTER 2 Isolation of an aluminum sensitive mutant in *Medicago truncatula*...20
   Introduction...............................................................................................................20
   Materials and methods..........................................................................................22
   Results....................................................................................................................25
   Discussion...............................................................................................................25
   References.............................................................................................................31

## CHAPTER 3 Responses to aluminum in *Medicago truncatula* germplasm......36
   Introduction...............................................................................................................36
   Materials and methods..........................................................................................38
   Results....................................................................................................................41
   Discussion...............................................................................................................50
   References.............................................................................................................53

## CHAPTER 4 Genetic study of aluminum tolerance in *Medicago truncatula*.......57
   Introduction...............................................................................................................57
   Materials and methods..........................................................................................60
   Results....................................................................................................................61
   Discussion...............................................................................................................61
   References.............................................................................................................70

## CHAPTER 5 Conclusion and future research...........................................74

Appendix A...............................................................................................................75
Appendix B...............................................................................................................78
REFERENCES.........................................................................................................79
LIST OF TABLES

Table 1.1 Genes up-regulated in response to Al excess in some plant species........15

Table 2.1 Soil analysis of unlimed and limed Leilehua soil............................25

Table 3.1 Soil analysis of unlimed and limed Leilehua soil............................40

Table 3.2 The geographic origin, seed weight (g/100 seeds), and relative root growth of seedlings grown under different Al-containing media.................................42

Table 4.1 Results of cross pollination between Al-tolerant and sensitive ecotypes or lines...........................................................................................................62

Table 4.2 Number of sequence reads and loci mapped of RAD library generated from Bam HI and Hind III.................................................................66

Table 4.3 Possible alleles associated with Al-tolerance in M. truncatula F2 population..68

Table 5.1 Microsatellite markers and BAC associations used for progeny validation (Mun et al., 2006)..............................................................75

Table 5.2 Direct amplification of length polymorphism (length) markers used for progeny validation (Choi et al., 2004).......................................................75

Table 5.3 Barcode adaptors used for RAD library construction (Hind III)..........76

Table 5.4 Barcode adaptors used for RAD library construction (Bam HI)...........77
LIST OF FIGURES

Figure 2.1 Root length of *M. truncatula* genotype A17 grown at various pH levels and Al levels in agarose at 72 h after transplanting.................................................................25

Figure 2.2 Relative root growth (RRG, ratio of root length of plants grown at 0 Al compared to that of plants grown at varying Al levels) of 62 mutants relative to wild-type genotype A-17 grown in agarose-containing media......................................................27

Figure 2.3 Relative root growth (RRG) of putative mutants and genotype A17 grown in agarose-containing media.................................................................29

Figure 2.4 Root length of putative mutants and genotype A17 grown in limed (pH5.5) and no-limed soil (pH 4.7) and relative root growth (RRG)..........................30

Figure 3.1 Distribution of ecotypes at different RRG in different level Al containing-media.................................................................46

Figure 3.2 Hematoxylin staining between a) W6 6037 (tolerant), b) PI 535622 (sensitive), c) A 17 (intermediate), and d) Intensity score.................................47

Figure 3.3 Correlation between RRG and the visual score from hematoxylin staining (n=30, P< 0.05).................................................................48

Figure 3.4 Root length and RRG of selected ecotypes grown in limed and unlimed acid soil .................................................................49

Figure 3.5 Root dry weight of selected ecotypes grown in limed and unlimed acid soil.................................................................49
Figure 3.6 Correlation between RRG from soil experiment and agarose experiment (n=12, P< 0.05) .................................................................50

Figure 3.7 Root length of a) sensitive lines PI 566889 and PI 535622, and b) tolerant lines PI 577633 and W6 6037, grown in different Al-containing media (0, 50, 100, and 200 µM) .................................................................50

Figure 4.1 Left, F1 seedlings from PI 566889 (Female recipient, R) and W6 6037 (Male donor, D) grew poorly; Right, parent PI 566889 .................................................................63

Figure 4.2 Polymorphisms of markers DK296L, (top) MU10, PPDK and DK049R (Choi et al., 2004) and MU10 (bottom) among ecotypes selected as on parents ......................64

Figure 4.3 Root length individual seedlings of PI 577633, S69, and F2 populations were significantly different when grown in a) 0 µM, b) 50 µM Al containing media ..........65

Figure 4.4 Manhattan plot of 1,729 alleles with valid SNP on 8 chromosome of M. truncatula .................................................................67

Figure 4.5 Genes associated with Al tolerance that had been reported and candidate alleles of Al tolerance from PI 56677633 x S69 population ........................................69

Figure 5.1 Correlation between relative root growth (RRG) and seed weight (g/100 seeds) of the evaluated germplasm (n=91, P< 0.05) .................................................................78

Figure 5.2 Correlation between relative root growth (RRG) grown in different Al concentration (n=91, P< 0.05) .................................................................78
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFLP</td>
<td>Amplified fragment length polymorphism</td>
</tr>
<tr>
<td>Al</td>
<td>Aluminum</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacteria artificial chromosome</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTZ</td>
<td>Distal transition zone</td>
</tr>
<tr>
<td>EMS</td>
<td>Ethylmethylsulfonate</td>
</tr>
<tr>
<td>EZ</td>
<td>Elongation zone</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole acetic acid</td>
</tr>
<tr>
<td>ICP</td>
<td>Coupled plasma spectrometer</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NPA</td>
<td>Naphthylphthalamic acid</td>
</tr>
<tr>
<td>MATE</td>
<td>Multidrug and toxin efflux</td>
</tr>
<tr>
<td>MFs</td>
<td>Microfilaments</td>
</tr>
<tr>
<td>MTs</td>
<td>Microtubules</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait loci</td>
</tr>
<tr>
<td>RAD</td>
<td>Restriction site-associated DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RIL</td>
<td>Recombinant inbred lines</td>
</tr>
<tr>
<td>RRG</td>
<td>Relative root growth</td>
</tr>
<tr>
<td>RRL</td>
<td>Relative root length</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SSR</td>
<td>Simple sequence repeat</td>
</tr>
</tbody>
</table>
CHAPTER 1
Literature review

Introduction

Aluminum (Al) makes up approximately 7% of earth’s crust. Aluminum toxicity is one of the most widespread problems of ion toxicity stress in plants. It is a major factor limiting crop productivity in acid soils that constitute 40% of the world’s arable land (Von Uexkull and Mutert, 1995). Most Al forms are non-toxic, such as aluminum oxides, gibbsite and aluminum silicates. However, under acidic conditions, Al is solubilized into the soil solution where it rapidly inhibits root growth. The trivalent cationic Al\(^{3+}\) present as Al(H\(_2\)O)\(_6\)\(^{3+}\) in acid environments is the most toxic, inorganic species to plants. Many organic Al-complexes have low toxicity (Kochian, 1995, Miyasaka et al., 2006).

The primary target of Al toxicity is the root apex. Aluminum can react with many oxygen- and phosphate-containing compounds. It can affect multiple sites in root apical cells, such as cell walls, plasma membranes, cytoskeletons, and nuclei. It also disrupts the function of potassium and calcium ion channels on the root membrane surface. Aluminum inhibits both cell elongation and cell division in roots. As a result, an Al-damaged root system has limited water and mineral nutrient uptake (Barceló and Poschenrieder, 2002; Kochian, 1995; Kochian et al., 2005; Ma et al., 2001; Miyasaka et al., 2006; Samac and Tesfaye, 2003).

Effect of aluminum toxicity on root growth

The root tip is the most Al-sensitive plant part. Ryan et al. (1993) found that Al inhibited root growth when applied to the terminal 2.0-3.0 mm of maize (Zea mays L.) roots. The most Al-sensitive area based on inhibition of root elongation or callose formation is the distal transition zone (DTZ). The DTZ is characterized by cells that are changing from a mitotic mode and preparing for rapid elongation (Sivaguru and Horst, 1998).

Kollmeier et al. (2000) hypothesized that Al application to the DTZ inhibited basipetal auxin transport that mediates the Al signal between the DTZ and the elongation zone (EZ). They found that application of the auxin indole acetic acid (IAA) to the EZ significantly alleviated the inhibition of root elongation induced by application of Al to
the DTZ. This hypothesis was supported by Doncheva et al. (2005) who found that individually either Al or NPA (Naphthylphthalamic acid), an auxin transport inhibitor, could inhibit cell division in root tip meristems after a few minutes of exposure. Aluminum toxicity disoriented cell expansion, and caused abnormalities in tissue maturation, and lateral root formation in the meristematic zone.

Using the fluorescent stain lumogallion, Silva et al. (2000) showed that Al entered root tip cells of an Al-sensitive soybean (*Glycine max* L.) genotype and accumulated in the nuclei. Aluminum was present also in cell walls at the cell periphery (Silva et al., 2000).

*Cell walls*

Aluminum accumulated in the epidermal layer and cortical layers immediately below the root epidermis in Al-sensitive wheat (*Triticum aestivum* L.) derived from a segregating population of Carazinho X Egret (Delhaize et al., 1993a). Aluminum can alter the structure and properties of cell walls by binding strongly and rapidly to negatively charged sites. This binding leads to a decrease in the mechanical extensibility of the cell wall required for normal cell expansion (Samac and Tesfaye, 2003; Kochian et al., 2005; Miyasaka et al., 2006).

Among the many components of the cell wall network, pectins have been proposed to be a critical site for Al-cell wall interactions. Godbold and Jentschke (1998) reported that Al accumulated in the apoplast of root cortical cells of Norway spruce (*Picea abies* (L.) Karst), displacing other cations (e.g. Ca and Mg). Zheng et al. (2004) reported that Al adsorption and desorption kinetics on the root cell walls from wheat genotypes that differed in Al sensitivity could explain partly their differential Al-stress response. Most Al ions in wheat roots were electrostatically bound to cell wall components, especially pectin (Zheng et al., 2004). Localization of pectin by immunofluorescence revealed that Al-sensitive maize cv. Lixis had a higher proportion of low-methylated pectin and a higher negativity of the cell wall than Al-resistant cv. ATP-Y (Eticha et al., 2005). They concluded that differences in cell wall pectin and its degree of methylation contributed to genotypic differences of Al resistance observed in maize.

Research by Sasaki et al. (1996) indicated that the extent of growth inhibition is closely correlated with the extent of lignin deposition in both Al-tolerant and Al-sensitive
wheat varieties, and Al-sensitive varieties accumulated more lignin in roots than Al-tolerant varieties. Lignin is a principal structural component of plant secondary cell walls. Various stress factors, including ion deficiency, invasion by fungal pathogens and wounding, can induce the deposition of lignin in cell walls.

Tabuchi and Matsumoto (2001) suggested that Al modified the metabolism of cell wall components, increasing the thickness and rigidity of cell walls. Deposition in the cell walls of lignin, hemicellulose, glycoprotein, and other secondary metabolites could prevent Al from reaching the plasma membrane. However, this deposition could result also in the arrest of root growth and cellular elongation. Van et al. (1994) reported that Al toxicity increased cellulose content in the non-elongating zones of squash (Cucurbita maxima Duch.), whereas it increased hemicellulose content in all of the axial regions along the root. They hypothesized that Al rapidly reduced squash root growth by inhibiting cell elongation and altering the metabolism of cell-wall polysaccharides in the non-elongating zone as well as in the elongating zone.

Mao et al. (2004) investigated the molecular mechanisms of Al toxicity and Al tolerance of rice (Oryza sativa L.), using cDNA amplified fragment length polymorphism (AFLP). Nineteen function-known genes were up-regulated by Al stress. Of these 19 genes, seven were possibly involved in cell wall metabolism, indicating that Al stress could induce the biosynthesis of lignin and other cell wall components in rice roots.

**Plasma membrane**

The trivalent Al$^{3+}$ can interact strongly with the negatively charged plasma membrane surface (Kinraide et al., 1998). Divalent cations such as Ca$^{2+}$ form bridges between the phospholipid head groups of the membrane bilayer. Aluminum has a greater affinity than divalent cations for the phospholipid head groups and can displace them (Akeson et al., 1989; Akeson and Munns, 1989). As a consequence, Al can alter the phospholipid packing and fluidity of the plasma membrane. In addition, interactions of Al with the plasma membrane could disrupt the stability of local charges at the surface of the plasma membrane, altering the ionic environment. Both types of Al interactions could lead to disturbances of ion-transport processes, which could then perturb cellular homeostasis (Kochian et al., 2005).
One of the most noticeable consequences of Al exposure to roots is an almost instantaneous depolarization of the plasma membrane (Papernik and Kochian, 1997). Membrane potential and ion fluxes at the root cell plasma membrane were disrupted less in Al-tolerant wheat. The change in the plasma membrane electrochemical potential may be due to both direct and indirect interactions of Al with a number of different ion transport pathways (Miyasaka et al., 1989). In soybean, an Al-sensitive cultivar showed more plasma membrane permeability than an Al-tolerant cultivar when treated with Al in nutrient solutions. The number of mitochondria had proliferated significantly in the root tip cells; plasmolysis was observed. Both cultivars showed ruptured cell walls when Al concentration increased to 90 mg L\(^{-1}\) (Yu et al., 2011).

Ahn et al. (2001; 2002) reported that the spatial pattern of root growth inhibition induced by Al is closely associated with the inhibition of H\(^+\)-ATPase activity and decreasing surface negativity of plasma membrane vesicles isolated from 5-mm apical root segments of squash (\textit{Cucurbita pepo} L. cv. Tetsukabuto) plants. They found that the decrease in H\(^+\)-ATPase activity correlated with increasing Al concentration and treatment duration.

\textit{Cytoskeleton}

Aluminum disrupts the orientation of the cytoskeleton that provides a template both for cell division and cell-wall biosynthesis. Aluminum either could interact directly with cytoskeletal elements or indirectly, via alteration of signaling cascades that are involved in cytoskeletal stabilization (Kochian et al., 2005; Miyasaka et al. 2006).

Aluminum was shown to affect the orientation of microtubules (MTs) in a population of maize cells located in the protoderm and the two outer layers of cortex. The interphase cortical MT arrays lost their normal transverse organization and became random or longitudinal; the preprophase band of MTs, mitotic spindle, and phragmoplast developed at planes that were 90\(^{\circ}\) rotated compared to their counterparts in control cells (Seju and Lee, 1998). Aluminum was also shown to affect actin microfilaments (MFs) of the distal transition zone of roots of an Al-sensitive maize cultivar (Sivaguru et al., 1999). They found dramatic lesions in the MT cytoskeleton of the epidermis and outer two to three cortical cells of the DTZ. Frantzios et al. (2000) found similar results in \textit{Triticum turgidum}, with Al-induced abnormal assembly and disassembly of MTs in each stage of
cell mitosis, movement disorder of chromosomes, and inhibition of chromatin condensation. Similarly, Verma (2001) showed that Al caused abnormal planes of cell division due to interference with cortical actin filaments. Actin filaments are thought to play an early role in fixing the site of the preprophase band that is involved in the correct orientation of the cell plate.

Callose formation

Callose formation can serve as a sensitive indicator of Al injury in roots of maize (Sivaguru et al., 1999) and bean (Phaseolus vulgaris L.) (Massot et al., 1999). The induction of callose production by Al also appears related to the alteration of the plasma membrane function, since D-1, 3-glucan synthase (callose synthase) is located at the inner surface of the plasma membrane and is activated by an increase in intracellular Ca\textsuperscript{2+} concentration. This increase in influx of Ca\textsuperscript{2+} could occur through an Al-damaged plasma membrane (Yamamoto et al., 2001)

Under Al stress, callose accumulation may lead to further cellular damage by inhibiting intercellular transport through plasmodesmatal connections (Sivaguru et al., 2000). However, two Al-sensitive Arabidopsis (Arabidopsis thaliana) mutants did not show significant accumulation of callose when exposed to Al, indicating that the relationship between callose deposition and Al-induced inhibition of root growth might be complex (Larsen et al., 1996).

Tolerance mechanisms to aluminum stress

Mechanisms of Al resistance can be classified into 2 groups: exclusion or internal detoxification. Exclusion is a mechanism by which plant roots avoid direct Al contact. Alkalinization of the rhizosphere or release of chelating exudates, such as organic anions or mucilages that bind to Al, are mechanisms that could be involved in exclusion of Al through formation of non-phytotoxic complexes. Formation of less toxic organic Al-complexes is an internal resistance mechanism that has been observed in Al-tolerant plants, such as tea (Camellia sinensis), buckwheat (Fagopyrum esculentum Moench.) and Hydrangea sp. (Barceló and Poschenrieder, 2002; Ma and Furukawa, 2003).

Exclusion of aluminum

Exclusion of Al can be accomplished via modification of the rhizospheric environment by formation of a pH gradient, exudation of Al-chelating ligands, or
immobilization of Al in the cell wall itself. Since the solubility of Al is strongly pH dependent, developing a high rhizospheric pH may help the plant to tolerate Al (Panda and Matsumoto, 2007).

Binding of the phytotoxic form of Al with organic anions around root apices has been identified in several crop species and cultivars as an important Al-resistance mechanism (Horst et al., 2010). Organic anions include: a) citrate in snapbean (Miyasaka et al. 1991), Cassia tora L. (Yang et al., 2004), maize (Piñeros et al. 2002), and soybean (Shen et al., 2005; Liao et al. 2006); b) malate in wheat (Delhaize et al., 1993b); c) oxalate in buckwheat (Zheng et al. 1998) and taro (Colocasia esculenta) (Ma and Miyasaka, 1998; and d) both citrate and malate in rye (Secale cereale L.) (Li et al. 2000b).

**Root exudation of chelating organic anions: citrate**

The first evidence of Al-induced root exudation of an organic anion came from Miyasaka et al. (1991), who showed that an Al-resistant cultivar of snapbean (Phaseolus vulgaris L.) excreted eight-fold more citrate from its roots in the presence of Al than did an Al-sensitive genotype. Citrate is a chelator of Al, and the Al-citrate 1:1 complex is non-phytotoxic and its transport through the plasmalemma appears to be very slow, retarding the entry of Al into the plant cell (Kochian, 1995). In soybean, a higher activity of plasma membrane H⁺-ATPase coincided with more citrate secretion in Al-resistant than Al-sensitive cultivars (Shen et al., 2005).

An Al-resistant cultivar of barley (Hordeum vulgare L. cv. Sigurgkorn) secreted citrate from roots in response to Al (Zhao et al., 2003). The secretion of citrate was characterized by a rapid, non-dose-responsive, and temperature-dependent pattern. It was inhibited by anion channel inhibitors, suggesting that citrate secretion is probably mediated through an anion channel on the plasma membrane. A positive correlation between citrate secretion and Al resistance was observed in 21 barley cultivars differing in Al sensitivity (Ma et al., 2004).

Yang et al. (2004) also reported that activity of citrate synthase (CS) in Cassia tora L. increased when Al was applied. In contrast, aconitase activity was decreased in Al-treated root tips, which could contribute to the accumulation of citrate in root tips, because this enzyme catalyzes the isomerization of citrate to isocitrate.
Recently, Rangel et al. (2010) reported that kinetics of citrate exudation from root tips explained differential responses in root elongation and Al uptake of both resistant (cv. Quimbaya) and sensitive (cv. VAX-1) genotypes of common bean to Al treatment. Aluminum-resistant cv. Quimbaya was able to restore the internal citrate pool after 24 h Al treatment and had a constitutive high activity of citrate synthase. Some plants such as citrus, *Citrus grandis*, and *Citrus sinensis* has been reported to secrete both citrate and malate (Yang et al., 2011). Citrate secretion was found also as a secondary tolerance mechanism in wheat, with the first mechanism of malate secretion in response to Al (Ryan et al., 2009).

*Rhot exudation of chelating organic anions: malate*

Aluminum-induced malate secretion was observed in wheat (Delhaize et al., 1993b). Aluminum-resistant genotypes released malate and accumulated significantly less Al in the root apex compared with Al-sensitive genotypes (Delhaize et al., 1993b; Pellet et al., 1996; Tang et al., 2002). There was no significant difference in concentration of endogenous malic acid in the Al-tolerant and sensitive cultivars, suggesting that transport of malic acid out of root apices and not the ability to accumulate malic acid within apices, was the important factor (Pellet et al., 1996). Pre-incubation of cell walls in exogenously applied malate decreased the total amount of Al adsorbed in the root wheat, suggesting that the malate secreted in response to Al stress reduced the capacity of the cell walls to bind Al (Zheng et al., 2004).

Sasaki et al. (2004) reported on cloning a wheat gene *ALMT1* (*Al*-activated malate transporter) derived from crosses between near-isogenic lines that differed in Al tolerance. The *ALMT1* gene encodes a membrane protein that is constitutively expressed in root apices of an Al-tolerant wheat line at a greater level than in an Al-sensitive line. Expression of *ALMT1* in cultured tobacco cells increased the tolerance to Al treatment (Sasaki et al., 2004).

*Mucilage and border cells*

Mucilage and border cells have been reported to function in Al-resistance mechanisms (Hawes et al., 2000). Horst et al. (1982) reported that 50% of the total Al of root apices was located in the mucilage of cowpea (*Vigna unguiculata*), and that removal
of mucilage prior to treatment with Al, increased accumulation of Al in root apices and enhanced Al rhizotoxicity.

Maize mucilage binds Al strongly and the mucilage-Al complex is non-phytotoxic (Li et al., 2000a). Surprisingly, the inhibition of root elongation by Al wasn’t increased when the mucilage was removed from the root tip prior to Al exposure, perhaps because the total binding capacity of the mucilage was too small to confer effective protection from Al-induced root inhibition in hydroponic culture (Li et al., 2000a).

In a study using snapbean, a thicker mucilage layer was found around detached border cells of both cultivars when exposed to excess Al. However, border cells of the Al-sensitive cultivar had a thinner mucilage layer and reduced viability compared with those of an Al-resistant cultivar, suggesting that mucilage from border cells could bind to Al, protecting root tips from Al toxicity (Miyasaka and Hawes, 2001). Similarly, Zhu et al. (2003) found that Al treatment induced the death of root border cells in wheat and removal of root border cells from root tips increased Al inhibition of root growth.

In contrast, production of root border cells in barley roots was strongly inhibited by Al, indicating that their role in protecting root tips against excess Al was limited (Pan et al., 2004). Tamas et al. (2005) reported that removal of root border cells from Al-treated roots of barley increased inhibition of root growth, Al uptake, inhibition of root border cell production, number of dead root border cells, activities of peroxidase and oxalate oxidase, and production of H$_2$O$_2$ compared to Al-treated roots with root border cells. They suggested that the presence of root border cells around the root tip of barley effectively protects the barley root tip from Al.

Application of Al led to higher Al concentrations in border cells than in root apices of common bean (Yu et al., 2009). The analysis of cell-wall pectin indicated that the concentrations of several polysaccarides that typically bind to Al, were higher in border cells than in root apices, especially when exposed to Al. These results suggested that root border cells enhanced the Al resistance of root apices by immobilizing Al in their cell-wall pectin, thus protecting the root apex (Yu et al., 2009).

The variable results regarding the role of root border cells suggest that diverse Al-tolerance mechanisms exist among plant species. Some plant species may have more than one mechanism in order to tolerate Al toxicity.
**Internal detoxification**

Exclusion from root tips and restriction of Al transport to upper plant parts are the most important mechanisms that allow non-Al-accumulating plant species to grow on acid soils with high Al availability. However, numerous Al-accumulating plant species can tolerate relatively high Al concentrations in roots and upper plant parts by internal immobilization of Al with organic acids, phenolic compounds, or phosphate (Barceló and Poschenrieder, 2002; Ma and Furukawa, 2003).

Hydrangea (*Hydrangea macrophylla* Ser.) has blue-colored sepals when the plant is grown in acidic soils and red-colored sepals when grown in alkaline soils. The blue color of hydrangea sepals is due to Al complexing with the anthocyanin, delphinidin 3-glucoside, and the copigment, 3-caffeoylquinic acid (Takeda et al., 1985). Hydrangea can accumulate Al to levels greater than 3000 mg kg\(^{-1}\) in both leaves and sepals. Aluminum exists in leaves primarily as a 1:1 Al-citrate complex. The strong chelation of Al by citrate reduced its activity in the cytosol and prevents the complexation of Al with other cellular components (Ma et al., 1997a).

Buckwheat (*Fagopyrum esculentum* Moench. Cv. Jianxi) accumulated Al in concentrations as high as 15,000 mg kg\(^{-1}\) in leaves when grown on acid soils. In the roots and leaves, most Al is complexed with oxalate in a 1:3 Al:oxalate complex (Ma et al., 1998). In addition, Al-activated oxalate exudation from the root apex occurred in response to Al stress (Zheng et al., 1998).

Wenzl et al. (2001) reported that signalgrass (*Brachiaria decumbens* Stapf cv. Basilisk), which is considered a highly Al-resistant plant, secreted only moderately greater quantities of organic acids than the Al-sensitive relative species ruzigrass (*Brachiaria ruziziensis* Germain and Evrard cv. Common). Root apices of signalgrass accumulated less Al than those of ruzigrass. There was no significant difference in P release from root apices of Al-resistant ruzigrass and signalgrass. Alkalinization was found not to contribute to the difference between these species. Wenzl et al. (2001), indicating that the main mechanism of Al resistance in signalgrass involved internal detoxification of Al.

In common sorrel (*Rumex acetosa* L.), Al treatment enhanced internal root citrate concentrations but decreased shoot organic acid levels. In addition, high levels of many
phenolic compounds were induced in shoots suggesting that citrate in roots and phenolics in shoots could bind to Al, resulting in non-phytotoxic complexes (Tolrá et al., 2005).

**Development of Al-tolerant plants for growth in acid soil**

Surface application of calcium carbonate (lime) or phosphate fertilizer in the top soil layer is one management practice to increase soil pH or to precipitate soluble Al, maintaining crop production in acid soils. However, this approach may not be the ideal solution, because of its high cost and greater energy consumption. Often fertilizers are not affordable in many parts of the world (Ishitani et al., 2004). Moreover, lime applied to the surface of a soil is generally ineffective in ameliorating subsurface acidity. As a result, acid-resistant plants and cultivars are needed to maintain production on acid soils (Conyers et al., 2003). Therefore, development of Al-tolerant crop cultivars are a more practical solution to reducing inputs of agricultural amendments while maintaining crop production in acid soils.

Plant species and genotypes within species vary widely in resistance to Al, indicating that Al-resistant species or genotypes possess several mechanisms to avoid Al toxicity (Taylor, 1991; Li et al., 2009). Along with diverse germplasm and an appropriate breeding program, a reliable screening procedure for Al stress is one of the most important tools required to effectively develop Al-tolerant cultivars. The outcome of such evaluations depends on multiple factors, such as pH, temperature, interaction of Al with other nutrients in solution, and the biological sensitivity of particular crops (Narasimhamoorthy et al., 2007a). Due to the complexities involved in each method, the use of a single method to identify Al-tolerant genotypes of a particular species may lead to misleading results.

A combination of surface liming and Al-resistant genotypes could be required for optimum production. For example, wheat shows a large intraspecific variation in Al resistance (Stodart et al., 2007; Raman et al., 2008). In wheat and barley, although Al-resistant lines could maintain fine root lengths under these Al-toxic conditions, they were unable to maintain rhizosheath mass. There is a metabolic cost of synthesizing and exuding organic anions (Haling et al., 2010). This finding helps to explain why Al-resistant wheat lines which yield relatively well in deep acid soils, may also benefit from application of lime to the surface layers of soil.
Evaluation of aluminum tolerance

A number of techniques have been used for identifying Al-tolerant plants. These include assays based on measurement of root growth or alternative staining procedures in solution culture, cell culture, or soil culture (Samac and Tesfaye, 2003).

Soil-based screening is considered the best evaluation method for determining Al tolerance in plants. However, it is time- and space-consuming as well as expensive to study a large number of genotypes. Moreover, interactions between soil and genotypes could occur, confounding the effects of Al toxicity (Narasimhamoorthy et al., 2007a; Villagarcia et al., 2001). Soils with a similar pH could vary in the amount of Al saturation, as well as other minerals such as Ca and Mg that could affect Al-phytotoxicity. Such variation in acid soils is common and is a disadvantage with regards to reproducibility in field studies (De Sousa, 1998).

Evaluation of plants in hydroponic solutions is relatively rapid to perform in a short time and a small space (Ma et al., 1997b). Aluminum levels can be carefully controlled, keeping all other nutrients equal (Sledge et al., 2005). However, transfer of seedlings into a hydroponic system could cause stress that reduces subsequent growth, and it is not suitable for plants that are sensitive to hypoxia (Tamas et al., 2006).

Aluminum also may form complexes with other compounds in nutrient solution. For example, rice seedlings grown in the presence of ammonium accumulated less Al than those grown in nitrate-containing solution, possibly through pH changes in the medium and ionic competitive effects (Zhao et al., 2009). Screening barley cultivars for Al tolerance revealed no differences in growth between hydroponics and a filter paper cultivation system (Tamas et al., 2006).

Hematoxylin is a natural dye extracted from the heartwood of a logwood tree (Hematoxylin campechianum L.). Hematoxylin itself does not stain tissue but is an excellent bright-field stain for nucleic acids when oxidized by a suitable mordant, commonly iron or Al salt (Ruzin, 1999). Hematoxylin is the most widely used staining method to measure Al tolerance. It has been reported as a sensitive method for evaluation of Al accumulation in a number of plants. This dye has the property of turning blue when it forms a complex with Al so that the penetration and retention of this ion in the roots can be assessed (Delhaize et al., 1993°). Maize seedling roots from contrasting genotypes
for Al-sensitivity stained differently after 24-h, 48-h, and 7-day exposures to an Al-containing nutrient solution. The Al-dye complex was detected in both the epidermis and the cortex of roots of the Al-sensitive maize cultivar. In contrast, the Al-tolerant line did not display staining or signs of physical damage in the root epidermis. The intensity of hematoxylin staining also correlated negatively with two other parameters used in maize breeding programs: a) relative seminal-root length (the difference between initial root length and final root in Al treatment); and b) net seminal-root length (net seminal-root length divided by initial root length) (Cançado et al., 1999). These results indicate that this region is a primary site for the deleterious action of Al.

Hematoxylin staining appears to be the best procedure to determine Al tolerance in barley cultivars, because its complex root system makes it difficult to measure root regrowth in nutrient solution (Echart et al., 2002). Hede et al. (2002) evaluated Al tolerance of 63 rye accessions from a world spring rye collection, using the hematoxylin method and the root growth method. This hematoxylin method is based on the ability of Al-tolerant seedlings to continue root growth following a short pulse treatment with a high Al concentration. Hematoxylin is used to stain Al-treated roots, allowing easy measurement of subsequent root regrowth. The root regrowth method measures root elongation and then calculates the root tolerance index (root length in Al divided by root length at 0 Al) to evaluate Al tolerance. They reported that both the hematoxylin method and the root growth parameter identified genotypes with greater root growth under Al stress, but failed to detect Al-tolerance in genotypes with poor root vigor.

It is noteworthy that in a previous investigation with maize seedlings, no significant differences in the staining pattern with hematoxylin was observed between sensitive and tolerant cultivars (Ryan et al., 1993). However, one possible explanation is that the cultivars used in those studies did not represent extremes of tolerance or susceptibility to toxic Al (Ryan et al. 1993).

Narasimhamoorthy et al. (2007a) compared three methods (hydroponic culture, soil culture, and root staining with lumagallion) for evaluation of Al tolerance in *Medicago truncatula*, and found significant differences among these methods. Genotypes that appeared Al-tolerant in the hydroponic system were not always Al-tolerant in the soil assay. The soil assay demonstrated a higher capacity for discriminating Al response
among genotypes and a higher experimental reproducibility than either hydroponic culture or lumogallion staining. Lumogallion root staining is a time-consuming procedure, because roots of multiple seedlings per accession must be sectioned, limiting its usefulness. Villagarcia et al. (2001) reported a similar result when comparing the Al-response of 10 soybean genotypes in sand culture to a standard hydroponics-based seedling culture. They found that hydroponic-based screening altered rankings in comparison with sand culture. Some genotypes that were tolerant to Al in sand culture, were remarkably sensitive to Al in hydroponics.

Eriochrome cyanine R staining, which forms complexes with nuclei in the presence of Al, was used for screening root regrowth after Al treatment in wheat (Aniol, 1995). If the Al treatment did not destroy the root apical meristem, then root regrowth after Al treatment was white (unstained).

However, Hossain et al. (2005) reported that eriochrome cyanine R staining was difficult to score visually in barley and that he was not able to duplicate results of Raman et al. (2002). Root regrowth was very small and no significant differences in Al tolerance were found among barley cultivars. In contrast, Hossain et al. (2005) suggested that relative root length of barley grown in a short term hydrophonic culture showed significant differences between Al-tolerant and Al-sensitive cultivars, and also correlated well with results from soil-based screening.

Relative root length, callose formation or reactive oxygen species are suitable for estimating Al tolerance of *Arabidopsis thaliana* accessions (Kobayashi et al., 2005). However, care must be taken to avoid confounding effects of other biotic stresses on root growth while evaluating Al response.

In the case of *Medicago truncatula*, the relationship between RRL (ratio of seedling root length grown with Al at a particular level divided by seedling root length grown without Al) determined in Al-toxic hydroponic culture with that in Al-toxic soils requires further investigation, because the results from hydroponic culture were different from soil culture (Sledge et al., 2005). Also, slower-growing plants may appear to be more Al tolerant than in actuality, because their relative reduction in root growth often is much less than that of the faster growing plants (Sledge et al., 2002).
If multiple Al tolerance mechanisms exist in plants, then they would presumably be encoded by different genes. Thus, not all sources of Al tolerance would be identified with one screening method and using a single method to identify Al-tolerant accessions could be misleading (Narasimhamoorthy et al., 2007a).

**Effect of proton toxicity on Al tolerance evaluation**

Proton (H⁺) toxicity is an important factor in determination of actual response to Al-toxicity. In *Arabidopsis thaliana*, low pH irreversibly damaged growing roots after 1 h of exposure (Koyama et al., 2001). Some Arabidopsis genotypes are sensitive to proton toxicity, making identification of Al-resistance mechanisms at the molecular level difficult (Koyama et al., 1995). Proton toxicity interference during the evaluation of Al response was reported in common bean (*Phaseolus vulgaris* L.) (Rangel et al., 2005) and spinach (*Spinacia oleracea* L. cv. Quanneng) (Yang et al., 2005). Root elongation in both species was significantly inhibited under low pH conditions alone and could mask resistance to Al-toxicity.

A screening method in soybean indicated that Al resistance could be separated from proton resistance (Lazof and Holland, 1999). Similarly, Ikka et al. (2007) used a hydroponic solution at pH 4.7 to screen *A. thaliana* for proton sensitivity and a hydroponic solution containing Al (4 µM Al) at pH 5.0 to screen for Al resistance. They found that the QTLs (quantitative trait loci) mapping for proton resistance did not overlap with previously identified Al-resistant QTLs, indicating that different genetic factors regulate mechanisms of resistance to each stress (Ikka et al., 2007). No correlation was observed between proton resistance and Al resistance among 260 accessions of *A. thaliana*, indicating that there is no simple relationship between the genetic factors controlling each trait.

**Genetics of aluminum tolerance**

Advances in DNA technology have expanded the study of genetic traits during the past decade. Genome-based technologies, such as microarrays, are used to analyze large numbers of genes in parallel. A functional genomic approach provides an overall picture of how genes are expressed under a particular condition. A structural genomic approach, such as genetic mapping, is used to locate genes, based on the desired phenotype (Ishitani et al., 2004). Identification of genetic linkages using molecular markers has led to a
greater understanding of the structure and behavior of plant genomes. Molecular markers have been used to monitor DNA sequence variation in and among species and to aid in introduction of useful traits in plant breeding programs (Landjeva et al., 2007; Poschenrieder et al., 2008).

Table 1.1. Genes up-regulated in response to Al excess in some plant species

<table>
<thead>
<tr>
<th>Genes and loci</th>
<th>Plant species</th>
<th>Note</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>TaALMT1</em></td>
<td>Wheat</td>
<td>The first cloned aluminum-tolerance gene, response for malate efflux</td>
<td>Sasaki et al., 2004; Ma et al., 2005</td>
</tr>
<tr>
<td>QTL on chromosome 1, 3 and 4</td>
<td>Arabidopsis</td>
<td>Al tolerance in recombinant inbred lines (RIL) population</td>
<td>Kobayashi and Koyama, 2002; Kobayashi et al., 2005</td>
</tr>
<tr>
<td><em>ALS3</em></td>
<td></td>
<td>ABC-transporter-like protein</td>
<td>Larsen et al., 2005; Gabrielson et al., 2006</td>
</tr>
<tr>
<td><em>AtALMT1</em></td>
<td></td>
<td><em>TaALMT1</em> Homolog</td>
<td>Hoekenga et al., 2006; Kobayashi et al., 2007</td>
</tr>
<tr>
<td><em>Stop1</em></td>
<td></td>
<td>Zinc finger transcriptional regulator</td>
<td>Iuchi et al., 2007</td>
</tr>
<tr>
<td><em>SbMATE</em></td>
<td>Sorghum</td>
<td>an aluminum-activated citrate efflux transporter, a major Al-tolerant locus</td>
<td>Magalhaes et al., 2004; 2007</td>
</tr>
<tr>
<td><em>Alp</em></td>
<td>Barley</td>
<td>Response for Al-activated citrate secretion</td>
<td>Tang et al., 2000; Raman et al., 2002; Ma et al., 2004</td>
</tr>
<tr>
<td><em>HvMATE</em></td>
<td></td>
<td>Correlated with Al-activated citrate efflux</td>
<td>Wang et al., 2007</td>
</tr>
<tr>
<td><em>MtMATE</em></td>
<td><em>M. truncatula</em></td>
<td>Show 77% similarity to <em>SbMATE</em></td>
<td>Chandran et al., 2008a</td>
</tr>
</tbody>
</table>
The interest in the molecular genetics and physiological mechanisms of Al resistance in plants around the world has resulted in an increasing number of studies. Many Al-resistant plants had been studied for their genetic response to Al, as shown in Table 1.

**Aluminum tolerance in Legumes**

The Leguminosae is the third largest family of higher plants, with approximately 20,000 species. Legumes are second to cereal crops in agricultural importance based on area harvested and total production. Grain legumes provide about one third of all dietary protein nitrogen and one-third of processed vegetable oil for human consumption (Gepts et al., 2005).

Many legumes are among the plant species that are least adapted to soil acidity in general (Rao et al., 2001). Attempts to characterize genotypic differences in Al resistance in beans dated from the mid 1970s. Since then, considerable progress has been attained in improving germplasm better adapted to acid soils (Rangel et al., 2010).

The enhanced exudation of citrate in response to Al stress has been reported in snapbean (Miyasaka et al., 1991) and soybean (Yang et al., 2001; Shen et al., 2005). QTLs associated with Al tolerance in soybean have been reported (Bianchi-Hall et al., 2000). Root characteristics of common bean under Al treatment were found to be under polygenic control, and some QTLs were identified at the same location as QTLs for tolerance to low phosphorus stress, thus, suggesting cross-links in genetic control of adaptation of common bean to abiotic stresses. (Lopaz-Marin et al., 2009)

**Aluminum tolerance in Medicago species**

Alfalfa (*Medicago sativa*) is the most important and widely grown forage legume in the world. Its global production is seriously affected by Al toxicity (Rechcigl et al. 1998). Several screening procedures have been used to select for resistant cultivars. However, neither cultivated alfalfa varieties nor plant introductions varied greatly in resistance when screened in acid soil (Bouton, 1996). This finding indicated that selection for Al-tolerance in alfalfa is complex due to tetraploid inheritance, obligate outcrossing and inbreeding depression, which can mask Al tolerance (Bouton, 1996; Dall’Agnol et al., 1996). Recently, two Al tolerant QTLs on linkage groups 1 and 8 were identified in diploid alfalfa (*Medicago sativa* subsp. Coerulea) using RFLP (Restriction fragment
length polymorphism) analysis of F2 and backcross populations (Sledge et al., 2002). Narasimhamoorthy et al. (2007b) studied additional Al-tolerant QTLs by using simple sequence repeat (SSR) markers that flanked the previously identified QTLs.

In addition, Narasimhamoorthy et al. (2007b) used genes from *M. truncatula* with high homology to Al-tolerance genes in sugarcane (*Saccharum spp.*) as candidate genes to map the alfalfa genome. Three putative QTLs were mapped on linkage groups 1, 2 and 3, explaining 38, 16 and 27% of the phenotypic variation, respectively. A marker designed from a marginally significant candidate gene (malate dehydrogenase) involved in malic acid release mapped near a marginally significant QTL on linkage group 1.

*Medicago truncatula* Gaetn. is a legume species closely related to alfalfa. It is also an important forage in the integral farming system in Australia and is comparable to alfalfa in forage quality. Both *M. sativa* and *M. truncatula* are sensitive to soil acidity and Al toxicity (Rechcigl et al., 1998). Alfalfa is a heterozygous, out-crossing species with a complex autopolyploid genome, making it a difficult subject for genomic studies. In recent years, *M. truncatula* has been chosen as a model legume for genomic studies, because of its small diploid genome, fast generation time (from seed-to-seed), self pollination, and high transformation efficiency (Cook, 1999). An efficient mutagenesis protocol using ethyl-methyl sulfonate (EMS) and a polymorphic ecotype with properties appropriate for use as a mapping parent are established (Penmetsa and Cook, 2000).

Aluminum tolerance in the *M. truncatula* germplasm has been reported based on a hydroponic screening method (Sledge et al., 2005), Al-toxic soil assay, and root staining method (Narasimhamoorthy et al., 2007a). The result from hydroponic screening indicated that sufficient variation of Al tolerance exists within this collection of *M. truncatula* accessions to select sensitive and tolerant accessions, which could be used to identify QTLs for Al tolerance, the first step in a map-based cloning approach to discover Al tolerance genes (Sledge et al., 2005). However, the results from these three screening methods differed from each other, with altered rankings for genotypes depending on the method (Narasimhamoorthy et al., 2007a). The physiological mechanisms that are responsible for these reported differences are unknown.

Thoquet et al. (2002) established a genetic map of *M. truncatula* using two parental homozygous lines from cv. Jemalong (A17) and from the Algerian natural
population (DZA315) as the basis of their molecular and phenotypic polymorphism. The mapping population was analyzed with 313 markers with 292 dominant anonymous markers (72 RAPD and 220 AFLP), 19 genes with known functions and 2 codominant isoenzyme markers. This map spanned 1225 cM (average 470 kb/cM) and comprised 289 markers in 8 linkage groups (2n = 16). Markers are uniformly distributed throughout the map. By mapping a number of common markers, the eight linkage groups were shown to be homologous to those of diploid alfalfa, implying a good macrosynteny between genomes.

Choi et al. (2004) developed a sequence-based genetic map using 288 sequence-based markers, covering 513 cM on the genetic map of *M. truncatula*. Using a mapping population of genotype A-17 crossed with genotype A-20, Mun et al. (2006) analyzed a total of 1,236 microsatellite markers for polymorphism in gene-rich BAC (Bacteria Artificial Chromosome) sequences, 27 Mbp of nonredundant transcript sequences, 20 Mbp of random whole genome shotgun sequences, and 49 Mbp of BAC-end sequences.

Chandran et al. (2008a, b) studied gene expression in *M. truncatula* A-17, and reported that genes involved in cell-wall modification, abiotic and biotic stress responses were up-regulated in response to Al. In contrast, genes involved in secondary metabolism, protein synthesis, and cell cycle processing were down-regulated in response to Al. Transcriptional profiling using DNA microarray revealed three genes that encode for an ABC transporter, a putative multidrug and toxin efflux (MATE) protein, and a putative metal-binding isoprenylated protein), which have potential roles in binding and/or sequestration of Al in *M. truncatula*.

To date, numerous databases of molecular markers, expression profiles, functional and structural genomics are available (http://www.medicago.org). Thus, *M. truncatula* is the ideal species for studying genes that are involved in Al tolerance.
Objectives

The overall objective is to improve Al tolerance in *M. truncatula*. Specific objectives are as follows:

1. To identify EMS-generated mutant lines and ecotypes that differ in response to Al.
2. To evaluate the best methods for screening Al sensitivity in *M. truncatula*.
3. To generate a segregating population from Al-tolerant and sensitive lines for studying genes involved in Al tolerance in *M. truncatula*.

We hypothesize that there are EMS-generated mutant lines or ecotypes that are highly sensitive or highly tolerant to Al. The Al-tolerant lines may possess physiological mechanisms, such as enhanced organic acid exudation or mucilage production of root border cells compared to Al-sensitive lines. Finally, we will use these lines to generate a population that segregates for Al-tolerance in order to conduct genetic analysis.
CHAPTER 2

Isolation of an aluminum sensitive mutant in *Medicago truncatula*

Introduction

Soil acidity is a major constraint to global agricultural production, affecting 30% of the world’s land area (von Uexkull and Mutert, 1995). Aluminum (Al) species that are commonly found in the soil, such as Al oxides, gibbsite, Al silicates and organic-Al complexes are non-phytotoxic. However, under acidic conditions, Al is solubilized into the soil solution where it rapidly inhibits root growth (Kochian, 1995; Miyasaka et al., 2006). Soil acidity problems, particularly those that occur in the subsoil, are often difficult to correct economically with conventional fertilization and liming practices. The use of Al tolerant cultivars provides an effective strategy for the production of important crops in acid soils (Echart et al., 2002).

To develop Al-tolerant cultivars, an efficient screening method is critical. Evaluation of plants in hydroponic solutions is relatively rapid and requires only a small area (Ma et al., 1997). Aluminum levels can be carefully controlled, keeping all other factors equal (Sledge et al., 2005). However, transfer of seedlings into a hydroponic system could result in abiotic stresses that reduce subsequent growth, particularly for plants that are sensitive to hypoxia (Tamas et al., 2006). Another problem is that complexes of Al could form with compounds in nutrient solution, interfering with Al phyto-toxicity (Zhao et al., 2009).

Field evaluation for Al tolerance can be laborious and costly. Pot studies using Al-toxic soil require large amounts of time, and space for studying a large number of genotypes (Wang et al., 2006). Most screening tests for selection of, and breeding for Al-tolerance have been performed in homogeneous Al-toxic root media. However, many acidic soil fields are slightly acidic in the top horizon while the subsoils are strongly acidic (Bushamuka and Zobel, 1998). In addition, soils with a similar pH could vary in the amount of Al-saturation, as well as other minerals such as Ca and Mg (De Sousa, 1998). However, soil-based screening is considered the most realistic method of evaluating plants for Al-tolerance. Short-term screening systems such as hydroponic and
sand cultures should be compared to soil-based experiments (Villagarcia et al., 2001; Voigt and Staley, 2004; Brauer and Staley, 2005; Hossain et al., 2005).

Agar and agarose gel have been used often in rhizosphere studies. In particular, agarose is a suitable substrate for studies of Al interaction in the rhizosphere, because it contains very low levels of phosphorus and other Al-complexing substances that could interfere with plant response to Al toxicity (Calba et al., 1996).

Aluminum-toxicity can be characterized quickly in hydroponic or agarose systems by measuring inhibition of root elongation. Relative root growth (RRG) has been shown to be suitable for estimating Al-tolerance of accessions of various plants such as rye (*Secale cereale* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), and Arabidopsis (*Arabidopsis thaliana*) (Hede et al., 2002; Llugany et al., 1995; Tang et al., 2002; Kobayashi et al., 2005). Aluminum-tolerant and Al-sensitive cultivars of barley (*Hordeum vulgare* L.) grown in a short term hydrophonic culture had significantly different RRG, and these differences correlated well with results from soil-based screening (Hossain et al., 2005). However, slower-growing plants may appear to be more Al-tolerant than in actuality, because their relative reduction in root growth is much less than that of faster growing plants (Dall’Agnol et al., 1996; Sledge et al., 2002).

In recent years, *Medicago truncatula* has been chosen as a model legume for genomic studies because of its small diploid genome, fast generation time (from seed-to-seed), self-pollination, and high transformation efficiency (Cook, 1999). Differences in Al tolerance in the *M. truncatula* germplasm have been reported based on a hydroponic screening method (Sledge et al., 2005), Al toxic soil assay (Narasimhamoorthy et al., 2007), and root staining method (Narasimhamoorthy et al., 2007). Results from three screening methods differed from each other, with altered rankings for genotypes depending on the method. Moreover, the physiological mechanisms of Al tolerance in *M. truncatula* are unknown (Narasimhamoorthy et al., 2007).

Numbers of novel genes and transcription factors have been identified in model plants such as Arabidopsis (Konieczny and Ansubel, 1993; Gabrielson et al., 2006) and *M. truncatula* (Mitra et al., 2004; Starker et al., 2006) from isolation of functional mutants. An efficient mutagenesis protocol using ethyl-methyl sulfonate (EMS) for *M.
truncatula has been developed and used for morphological, physical, and genetic studies (Penmetsa and Cook, 2000).

In this report, we describe the variation in response to Al by EMS mutants of M. truncatula, using both an Al-containing agarose assay and a soil-based assay. We identified an Al-sensitive mutant line that appears to be consistently more sensitive to Al than wild type in both assays.

**Materials and Methods**

Sixty-two putative Al-tolerant or Al-sensitive EMS-generated M₄ mutant lines of M. truncatula were provided by Drs. Miyasaka and Cook (Miyasaka and Cook, 2005). These putative Al-tolerant or Al-sensitive lines were based on earlier screening of individual M₃ mutants, using Al-containing agarose assay (200 μM AlCl₃ at pH 4.2). Based on root elongation over 24 hours, mutant lines that appeared to have much longer or shorter root elongation relative to wild-type A-17 were grown, allowed to self-pollinate, and seed collected for the M₄ population.

Two procedures were used for seed germination in this study. First, bulk seeds were soaked in 5 volumes of concentrated sulfuric acid (H₂SO₄) (Cook laboratory, University of California at Davis) for 5-12 minutes with intermittent gentle vortexing. Acid was decanted and approximately 40 ml of sterile water was added to rinse seeds four times. Second, for a small number of seeds, seed coats were individually nicked on the opposite side of radical. Five volumes of commercial grade bleach was added (~5% sodium hypochlorite). Seeds were immersed in bleach for 2 minutes, then rinsed with sterile water 5 times. Seeds for both procedures were soaked in sterile water overnight, then transferred to moistened filter paper (2 sheets thick) and placed in a petri-dish. Petri-dishes were sealed with parafilm and inverted. Alternatively, seeds that adhered to one side of 50 mL test tubes were oriented so that they were on the top side of a horizontally-placed test tube. Then, seeds were placed in the dark at 4°C for 36-48 hours to synchronize germination. Following this cold treatment, seeds were incubated at room temperature in the dark for 16-24 h. Seedlings with roots that were 1-1.5 cm in length were used in subsequent experiments.
Preliminary trial using agarose culture

The basic medium contained 1% agarose and 0.5 mM CaCl$_2$, with either 0.5 M HCl or 1M NaOH added to achieve the desired final pH. Filter sterilized AlCl$_3$ stock solution was added to the autoclaved media to achieve desired Al levels. Then, to determine the optimal levels of Al and pH for screening, seedlings of wild-type A17 were grown in agarose media that contained 0, 50, 100, or 200 µM AlCl$_3$ at pH 4.25, 4.5, 5.0, 5.5, or 6. Root lengths were measured at 24, 48 and 72 hour after transferal. The treatments resulting from combinations of pH and Al levels were statistically analyzed by analysis of variance (ANOVA), using SAS v. 9.1 (SAS Institute, 2003). A probability level (P-value) equal to or less than 0.05 was considered to be statistically significant.

First screening study in agarose culture

In the first screening study, five seedlings of each mutant line (62 M$_4$ mutant lines) were grown on agarose gel (1% agarose, 0.5 mM CaCl$_2$) that contained varying Al levels (0 µM, 50 µM, 100 µM, and 200 µM of AlCl$_3$ at pH 4.5). These Al and pH levels were selected based on results from the first preliminary study on genotype A17. Root elongation of seedlings was measured at 72 h after transfer, and RRG was calculated. The experiments were repeated 3 times. Significant differences among the means of all mutants compared to A17 were estimated using Student’s t-test, and one-way ANOVA (P-value ≤0.05).

Second screening study in agarose culture

In the second screening study, 15 putative mutants were chosen from the first screening trial and re-screened in agarose media that contained varying Al levels (0 µM, 50 µM, 100 µM and 200 µM of AlCl$_3$) at pH 4.5. The Al$^{3+}$ free activities were calculated to be 0, 24, 49, and 100 µM, respectively (Geochem-EZ, Shaff et al., 2010). Root elongation was measured at 72 h after transplanting and RRG (%) calculated. Results were statistically analyzed using one-way ANOVA (P-value ≤0.05).

Screening study in soil culture

Soil experiments were conducted with selected M$_4$ mutants, using Leilehua soil series (Order: Ultisols; Very-fine, ferruginous, isothermic Ustic Kanhaplohumults), pH 4.7. For the limed treatment, calcium hydroxide [Ca(OH)$_2$] was added to achieve pH 5.5. Soil was analyzed (Table 2.1) at Agricultural Diagnostic Service Center, College of
Tropical Agriculture and Human Resources, University of Hawai‘i at Manoa. Aluminum was extracted with 1M KCl solution, and analyzed with an inductively coupled plasma spectrometer (ICP; PerkinElmer, Optima 7000 DV).

Polyvinyl chloride (PVC) pipes were cut in half vertically, resealed with tape, and used as pots (10 cm diameter x 17.5 cm height). Soil equal to 900 g of oven-dry soil was added into the pots.

The following fertilizers were mixed into the surface soil to a depth of 2.5 cm: P and Ca, added as monocalcium phosphate Ca(H₂PO₄)₂, 0.33 g kg⁻¹ and 0.21 g kg⁻¹ soil respectively; K and N added as potassium nitrate KNO₃, 0.27 g kg⁻¹ and 0.1 g kg⁻¹ soil respectively; Mg added as magnesium sulfate MgSO₄, 0.05 g kg⁻¹; and micronutrient fertilizer (Granusol, NutriChem, Florida), 0.2 g kg⁻¹ soil (0.01 g kg⁻¹ Mg, 0.001 g kg⁻¹ B, 0.01 g kg⁻¹ Fe, 0.01 g kg⁻¹ Mn, 0.003 g kg⁻¹ Cu, 0.01 g kg⁻¹ Zn). Two seedlings were grown in each pot and arranged as a randomized complete block design with three replications per treatment. Experiments were conducted in the Pope Greenhouse facility, University of Hawaii at Manoa (21° 17' 47.8"N, 157° 49' 0.26"W). Average temperature was 23 °C (maximum 28 °C, minimum 20 °C), and average photoperiod was 12 hours.

At 30 days after transplanting, pots were cut open, and whole roots were carefully removed. Plants were rinsed in distilled water. Images of whole plants and roots were taken using a digital camera (Canon model A620, USA). Roots were separated from shoots and root lengths were measured using a ruler. Shoots and roots were weighed immediately for fresh weights, then dried to constant dry weight at 80°C for 48 hours. Relative root growth (RRG) was calculated as the ratio of root length in no-limed soil to that in limed soil.

Differences due to treatments for root lengths, RRG, fresh weights, and dry weights were estimated using one-way ANOVA (P-value < 0.05) (SAS Institute, 2003).
Table 2.1. Soil analysis of unlimed and limed Leilehua soil

<table>
<thead>
<tr>
<th>Soil</th>
<th>pH</th>
<th>Total N (%)</th>
<th>Soil analysis (mg Kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>Unlimed</td>
<td>4.7</td>
<td>0.34</td>
<td>4.2</td>
</tr>
<tr>
<td>Limed</td>
<td>5.5</td>
<td>0.34</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Results and Discussion

*Preliminary trial using agarose culture*

Increased acidity significantly reduced root elongation of wild-type A17 seedlings (P < 0.01), particularly at pH 4.25 compared to those grown at higher pH levels regardless of Al levels (Figure 2.1). There were no significant differences in root elongation of control plants (0µM Al) grown at a pH range from 4.5 to 6, suggesting that proton toxicity was not a problem within this pH range. At the lowest pH (4.25) level, the observed reduction of root growth might be a result of proton toxicity, as found in Arabidopsis (Koyama et al., 2001), and common bean (Rangel et al., 2005).

Figure 2.1. Root length of *M. truncatula* genotype A17 grown at various pH levels and Al levels in agarose at 72 h after transplanting.
Increasing levels of Al significantly reduced root elongation (P < 0.01), although significant interaction effects were found (P < 0.01), indicating different responses to Al-toxicity depending on pH level. At pH 4.5, 5.0 and 5.5, significant differences in root elongation were found between controls (0 Al) and Al treatments, especially at pH 4.5. At pH 6 there are no significant differences in root elongation of plants grown in different levels of Al, confirming earlier findings that Al forms non-phytotoxic species or complexes with other compounds at a pH level of 5.5 or higher (Kinraide et al., 1992).

Using too low a pH level in experiments could cause proton toxicity that masks the effect of Al toxicity (Koyama et al., 1995, Lazof and Holland, 1999). It is important to separate proton toxicity from Al toxicity, because resistances to Al toxicity and proton toxicity were reported in Arabidopsis to be controlled by different genetic factors (Ikka et al., 2007). Based on these results, a pH level of 4.5 was chosen for further studies. Also, Al concentrations of 50, 100, 200 µM were chosen, because they resulted in the greatest differences in root lengths between control and Al treatments under minimal proton toxicity conditions.

First screening of 62 EMS mutant lines in agarose

Root lengths of seedlings were significantly reduced in the presence of Al compared to those grown in control (0 Al) conditions (P < 0.01; between control and each Al level). There were significant differences of root lengths among 62 M₄ mutant lines grown in Al-containing agarose media (P < 0.01; within each level). Relative root growth of seedlings grown in 50 µM ranged from 0.28 to 1.15, with an average of 0.64 (Figure 2.2). Root growth of several mutants were observed to be greater at this low Al-concentration. This enhancement of root growth in low Al-containing medium was reported earlier in wheat, and maize (Kinraide, 1993; Llugany et al. 1995). At 100 µM, RRG ranged from 0.06 to 0.89 with an average of 0.28. At 200 µM, RRG ranged from 0.03 to 0.27 with an average of 0.11. Seedlings grown under high Al-toxic conditions showed extremely reduced root elongation, as has been reported in many studies (Barceló and Poschenrieder 2002). The differences in Al sensitivity among genotypes were highest at 50 µM, and decreased as the Al-concentration increased.
Figure 2.2. Relative root growth (RRG, ratio of root length of plants grown at 0 Al compared to that of plants grown at varying Al levels) of 62 mutants (putative Al-sensitive lines are labeled with S; putative Al-resistant lines are labeled with T) relative to wild-type genotype A-17 grown in agarose-containing media.
The ranking of Al sensitivities of mutant lines differed at different levels of Al concentration (Figure 2.2). For example, some mutants appeared to be Al-tolerant at 50 μM Al relative to genotype A17, but appeared Al-sensitive at higher Al levels. Such results are not surprising, since most Al-excluder species are able to resist Al-toxicity up to a certain level, but then as Al concentrations increase, they will exhibit phytotoxicity (Mariano and Keltjens; 2004; Miyasaka et al., 2006).

Due to these different responses at different Al levels, putative mutants were selected based on RRG from at least two Al concentrations. Eight mutants T10, T11, T16, T19, T22, T26, T28, and T34 showed consistently higher RRG compared to wild type A17 and seven mutants S39, S50, S57, S51, S62, S38, and S69 showed consistently lower RRG compared to A17 in at least 2 levels of Al concentration.

Second screening of 15 EMS mutant lines in agarose

Based on results of the first screening, 15 putative Al-tolerant or Al-sensitive mutant lines were selected for a second screening in agarose. Mutant lines exhibited significantly different RRG at different concentrations of Al (Figure 2.3). Putative Al-tolerant lines from the previous experiment were not significantly different in RRG compared to A17 except T19. Putative sensitive lines S38, S57, and S69 had significantly reduced RRG compared to A17. Based on the results of this study, three putative Al-tolerant mutants (T19, T28, and T34) and three putative Al-sensitive mutants (S38, S57, and S69) were selected for soil-based screening.
**Figure 2.3.** Relative root growth (RRG) of putative mutants and genotype A17 grown in agarose-containing media.

Screening of EMS mutant lines in soil

Root lengths of mutant lines and genotype A17 were reduced in Al-toxic soil at pH 4.7 compared to those grown in soil at pH 5.5 (Figure 2.4; $P \leq 0.05$). Line S69 had a significantly smaller RRG compared to A17 ($P \leq 0.01$). However, there was no significant difference between RRG of genotype A17 and other putative Al-sensitive or tolerant lines that had been observed in previous experiment ($P > 0.05$) (Figure 2.4). The results of the soil-based screening demonstrated the importance of screening for Al sensitivity in more than one medium.

The Al-sensitive line S69 was identified as Al-sensitive in both agarose and soil-based assays. Reductions in root lengths are considered the best indication of Al-tolerance or Al-sensitivity, and were consistently observed across two assay methods. Interestingly, these results indicated that agarose assays are better than hydroponic assays, because low correlations were observed between soil and hydroponic assays for *M. truncatula* (Narasimhamoorthy et al., 2007).

Isolation of mutants is an important tool for identifying genes involved in a specific process of interest. Several Al-sensitive and Al-resistant mutants have been
isolated from Arabidopsis (Larsen et al., 1998; Gabrielson et al., 2006; Inuchi et al., 2007), and rice (Ma et al., 2005). In *M. truncatula*, mutants with defective flowers (Penmetsa and Cook, 2000) or defective calcium oxalate crystal formation (Nakata and McConn, 2000) have been characterized. In this study, we have identified an Al-sensitive mutant line of *M. truncatula*, based on assays in both agarose and an Al-toxic soil.

**Figure 2.4.** Root length of putative mutants and genotype A17 grown in limed (pH5.5) and no-limed soil (pH 4.7) and relative root growth (RRG)

Sixty-two putative Al-sensitive or Al-tolerant mutant lines of *M. truncatula* were screened in both Al-containing agarose culture and an Al-toxic soil. We were able to identify one Al-sensitive mutant (S69), which exhibited reduced root growth in presence of Al in both assay methods. This line S69 is being used as an Al-sensitive parent in further genetic studies of the response to Al in *M. truncatula*.

**Acknowledgement**

This project was funded in part by the USDA - CSREES T-STAR program.
References


CHAPTER 3
Responses to Aluminum in *Medicago truncatula* germplasm

**Introduction**

Soil acidity is a major abiotic stress of plants, affecting one third of the world’s arable land area. In acid soils, crop productivity is affected by multiple stress factors, such as deficiencies of phosphorus, calcium and magnesium, and toxicities of manganese, iron, and aluminum. Aluminum (Al) toxicity has been considered the most important limiting factor of plant growth on acid soils (von Uexkull and Mutert, 1995). Aluminum species that are present in the environment, such as Al oxides, gibbsite, Al silicates and organic-Al complexes are non-toxic. However, under acidic conditions, Al is solubilized into the soil solution where it rapidly inhibits root growth (Kinraide, 1991; Kochian, 1995; Miyasaka et al., 2006). Although soil acidity problems can be overcome by fertilization and liming practices, it is often economically difficult to correct in many parts of the world (Pandey et al., 1994). The use of Al tolerant cultivars provides the most effective strategy for production of economically important crops in acid soils (Ishitani et al., 2004).

Aluminum tolerance has been reported in many plant species, and within plant species (Taylor 1991). Examples include wheat (Delhaize et al., 1993; Stodart et al., 2007), barley (Minella and Sorrells, 1992), maize (Kollmier et al., 2000; Mariano and Keltjens, 2004), and Arabidopsis (Kobayashi and Koyama, 2002; Hoekenga et al., 2003).

*Medicago truncatula* has been chosen as a model legume for genomic studies because of its small diploid genome, fast generation time, self pollination, and high transformation efficiency (Cook, 1999). Aluminum tolerance in the *M. truncatula* germplasm has been reported based on a hydroponic screening method (Sledge et al., 2005), an Al-toxic soil assay, and root staining method (Narasimhamoorthy et al., 2007). Results from the hydroponic screening indicated that sufficient variation of Al tolerance exists within this collection of *M. truncatula* accessions to select Al-sensitive or tolerant accessions, (Chandra et al., 2008; Sledge et al., 2005). However, the Al sensitivity of particular accessions also varied depend on the screening methods (Narasimhamoorthy et
al., 2007), and the physiological mechanisms that are responsible for this Al tolerance in *M. truncatula* need to be studied further.

A consistent, quick evaluation method is a key to selecting or breeding an Al-tolerant cultivar (Samac and Tesfaye, 2003). Agar and agarose gels have been used often in rhizosphere studies. In particular, agarose is a suitable substrate for studies of Al interaction in the rhizosphere, because it contains very low levels of phosphorus and other Al-complexing substances that could interfere with plant responses to Al toxicity (Calba et al., 1996). Agarose culture has an advantage over the hydroponic system, because solution culture can result in hypoxia particularly for plants that are sensitive to low oxygen conditions (Tamas et al., 2006).

Inhibition of root elongation can be used as an indicator for Al tolerance (Llugany et al., 1995). Relative root growth has been shown to be suitable for estimating Al tolerance of accessions of various plants such as rye, maize, wheat, and Arabidopsis (Godbold and Kettner, 1991; Hede et al., 2002; Llugany et al., 1995; Tang et al., 2002; Kobayashi et al., 2005).

Hematoxylin is a natural dye extracted from the heartwood of a logwood tree (*Hematoxylin campechianum* L.). It has been reported as a sensitive method for evaluation of Al-accumulation in a number of plants, because it has the property of turning blue when it forms a complex with Al (Delhaize et al. 1993a). The staining pattern of Al accumulation in roots was similar to lipid peroxidation and callose production, which are other symptoms of Al toxicity in pea (*Pisum sativum*) roots (Yamamoto et al. 2001). Both the hematoxylin method and RRG have been used for Al tolerance screening in many crop plants (Poschenrieder et al., 2008), such as barley (Echart et al., 2002), and wheat (Tang et al., 2002; Zhou et al., 2007).

Soil-based screening is considered the most realistic method of evaluating plants for Al tolerance; however, it is time-consuming and only a few ecotypes or genotypes can be evaluated using this method. Short-term screening systems such as hydroponic and sand cultures have been developed that correlated well to soil based assays (Bauer and Staley, 2005; Hossain et al., 2005; Villagarcia et al., 2001; Voigt and Staley, 2004). However, low correlations were observed between soil and hydroponic assays for *M. truncatula* (Narasimhamoorthy et al., 2007).
Objectives of our experiments are to: 1) identify Al-tolerant *M. truncatula* ecotypes; and 2) compare the best method(s) of screening *M. truncatula* for Al tolerance. The identification of Al-tolerant ecotypes in this model legume can be used in the future to determine molecular mechanisms underlying Al responses with the overall goal of improving Al tolerance in *M. truncatula* and other crop legume species.

**Materials and Methods**

**Plant materials**

Seeds of 91 ecotypes and cultivars were obtained from the USDA Germplasm Resources Information Network (GRIN), and the French National Institute for Agricultural Research (INRA). Seed weight, and geographic information are shown in Table 1. Most of these ecotypes have not been tested previously for Al tolerance.

**Seed germination**

Two procedures were used for seed germination in this study. First, bulk seeds were soaked in 5 volumes of concentrated sulfuric acid (H$_2$SO$_4$) (Cook lab, University of California at Davis) for 5-12 minutes with intermittent gentle vortexing. Acid was decanted and approximately 40 ml water was added to rinse seeds four times. Second, for a small number of seeds, seeds were individually nicked to slit open the seed coat on the opposite side of radical. Five volumes of commercial grade bleach was added (~5% sodium hypochlorite). Seeds were left in bleach for 2 minutes. Seeds for both procedures were soaked in sterile water overnight, then transferred to moist filter paper (2 sheets thick) and placed in a petri-dish. Petri-dishes were sealed with parafilm and inverted. Alternatively, seeds were left adhered along the top side of horizontally-placed 50 ml centrifuge tubes. Seeds were kept in the dark at 4°C for 36-48 hours to synchronize germination. Then, seeds were incubated at room temperature in the dark for 16-24 hours. Seedlings with roots 1-2 cm in length were used in the following experiments.

**Screening methods**

a. Inhibition of root elongation

Six seedlings of each ecotype were grown on agarose gel (1% agarose and 0.5 mM CaCl$_2$), that contained varying Al levels (0 μM, 50 μM, 100 μM, and 200 μM of AlCl$_3$ at pH 4.5). These Al and pH levels were selected based on results from a
preliminary study on genotype A17 (Chapter 2). Root elongation of seedlings was measured at 72 hours after transfer, and RRG was calculated. The experiments were repeated 3 times. Significant differences among the means of all lines compared to A17 were estimated using one-way ANOVA (P-value ≤ 0.05) and Student’s Test.

b. The accumulation of aluminum

Aluminum was detected with hematoxylin as described by Tamas et al. (2005). Briefly, the roots of seedlings cultivated for 72, and 120 hours, in various Al-containing agarose media were washed in distilled water for 15 minutes and then stained with a 0.2% hematoxylin and 0.02% potassium iodide (KIO₃) solution for 10 minutes at room temperature. After washing the entire root system with distilled water for 15 minutes, root tips (0.5 cm in length) were excised and washed with deionized water for 15 minutes.

Roots were observed under a stereomicroscope (Olympus model SZX16; Japan) and digital images were captured. To score the intensity of staining, we examined the darkest colored roots, determined its color balance as RGB 40/14/56 in Adobe Photoshop CS, and set that intensity as 5 in a scale of 0 to 5. Then, in Microsoft Office PowerPoint (2007), we increased the transparency level by 20% and set that intensity as 4; we continued to increase the transparency level by 20% for scales from 3 to 1. A total absence of color was assigned a zero (Figure 2d).

c. Screening in soil culture

Soil experiments were conducted with selected lines, using Leilehua soil series (Order: Ultisols, Very-fine, ferruginous, isothermic Ustic Kanhaplohumults), pH 4.6. For the limed treatment, calcium carbonate (CaCO₃) was added to achieve pH 5.5. Soil was analyzed (Table 3.1) at the Agricultural Diagnostic Service Center, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa. Aluminum was extracted with 1M KCl solution, and analyzed with an Inductively Coupled Plasma Emission spectrometer (ICP) (PerkinElmer, Optima 7000 DV).

Polyvinyl chloride (PVC) pipes were cut in half vertically, resealed with tape, and used as pots (10 cm diameter x 17.5 cm height). The pots were filled with 900 g dry weight soil. The following fertilizers were mixed into the surface soil to a depth of 2.5 cm: P and Ca, added as Ca(H₂PO₄)₂, 0.33 g kg⁻¹ and 0.21 g kg⁻¹ soil respectively; K and
N added as KNO₃, 0.27 g kg⁻¹ and 0.1 g kg⁻¹ soil respectively; and Mg added as MgSO₄, 0.05 g kg⁻¹, micronutrient fertilizer (Granusol, NutriChem, Florida), 0.2 g kg⁻¹ soil (0.01 g kg⁻¹ Mg, 0.001 g kg⁻¹ B, 0.01 g kg⁻¹ Fe, 0.01 g kg⁻¹ Mn, 0.003 g kg⁻¹ Cu, 0.01 g kg⁻¹ Zn.

Two seedlings of 12 lines were grown in each pot and arranged as a randomized complete block design with three replications per treatment. Experiments were conducted in the Magoon Greenhouse facility, University of Hawaii at Manoa (21° 17’ 47.8”N, 157° 49’ 0.26”W). Average temperature was 23 ºC (maximum 28 ºC, minimum 20 ºC), and average photoperiod was 12 hours.

At 28 d after transplanting, pots were cut open, and whole roots were carefully removed. Plants were rinsed in distilled water. Images of whole plants and roots were taken using a digital camera (Canon model A620, US). Roots were separated from shoots and root lengths were measured using a ruler. Shoots and roots were weighed immediately for fresh weights, then dried at 80°C for 48 h to constant dry weight. Relative root length (RRG) was calculated as the ratio of root length in unlimed soil to that in limed soil.

Differences due to treatments for root lengths, RRG, fresh weights, and dry weights were estimated using one-way Analysis of Variance (ANOVA) using PROC GLM in SAS v. 9.1 (SAS Institute, 2003).

Table 3.1. Soil analysis of unlimed and limed Leilehua soil

<table>
<thead>
<tr>
<th>Soil</th>
<th>pH</th>
<th>Total N (%)</th>
<th>Soil analysis (mg Kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>unlimed</td>
<td>4.6</td>
<td>0.34</td>
<td>4.2</td>
</tr>
<tr>
<td>limed</td>
<td>5.5</td>
<td>0.34</td>
<td>4.2</td>
</tr>
</tbody>
</table>
Results

a) Inhibition of root elongation

There were significant differences of root lengths among 91 lines grown in Al-containing agarose media (P < 0.01) (within each level) (Table 3.2). Root lengths of seedlings were significantly reduced in 100 µM and 200 µM Al-containing media compared to those grown in control conditions (0 Al) (P < 0.01).

Across all Al levels, relative root growth ranged from 0 to 148%, with an average of 70%. At 50 µM Al, RRG ranged from 14 to 148 %, the greatest range of RRG among the three Al levels. At 100 µM Al, RRG ranged from 5 to 81% with an average of 33%. At 200 µM Al, RRG ranged from 0 to 26% with an average of 8%. As Al levels increased, the range of RRG was reduced (Figure 3.1). No correlation between RRG and seed weights was found (Appendix B).

Ranking of Al sensitivities of ecotypes differed at different levels of Al concentration. Relative Root Growth at 50 µM Al was regressed against that at 100 µM Al; however, no significant relationship was found. Similarly, RRG at 100 µM Al was regressed against 200 µM Al; however, no significant relationship was found. For example, some ecotypes appeared to be Al-sensitive or Al-tolerant at 50 µM Al relative to genotype A17, but showed no significant difference relative to A17 at higher Al levels.
Table 3.2. The geographic origin, seed weight (g/100 seeds), and relative root growth of seedlings grown under different Al-containing media

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Origin</th>
<th>Seed weight</th>
<th>RRG of seedlings grown in different Al-containing media (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50 µM SE</td>
<td>100 µM SE</td>
</tr>
<tr>
<td>A 17</td>
<td>Australia</td>
<td>0.22 70.24 7.62</td>
<td>35.71 10.01 13.69</td>
</tr>
<tr>
<td>A 20</td>
<td>Australia</td>
<td>0.52 38.65 1.74</td>
<td>25.36 3.99 9.66</td>
</tr>
<tr>
<td>Caliph</td>
<td>Australia</td>
<td>0.37 74.42 3.68</td>
<td>29.07 6.04 6.40</td>
</tr>
<tr>
<td>Cyprus</td>
<td>Australia</td>
<td>0.31 56.82 5.17</td>
<td>21.69 1.03 5.17</td>
</tr>
<tr>
<td>DZA 012</td>
<td>Algeria</td>
<td>0.35 90.63 8.55</td>
<td>58.37 15.13 13.82</td>
</tr>
<tr>
<td>DZA 016</td>
<td>Algeria</td>
<td>0.27 69.09 11.78</td>
<td>79.25 12.42 6.13</td>
</tr>
<tr>
<td>DZA 022</td>
<td>Algeria</td>
<td>0.28 65.07 14.16</td>
<td>29.45 9.90 4.11</td>
</tr>
<tr>
<td>DZA 027</td>
<td>Algeria</td>
<td>0.29 68.33 21.67</td>
<td>66.67 3.33 8.33</td>
</tr>
<tr>
<td>DZA 033</td>
<td>Algeria</td>
<td>0.31 78.77 7.86</td>
<td>56.68 4.19 5.76</td>
</tr>
<tr>
<td>DZA 055</td>
<td>Algeria</td>
<td>0.29 100.00 18.52</td>
<td>80.74 1.85 3.70</td>
</tr>
<tr>
<td>DZA 058</td>
<td>Algeria</td>
<td>0.27 41.61 3.35</td>
<td>30.77 5.71 3.50</td>
</tr>
<tr>
<td>DZA 059</td>
<td>Algeria</td>
<td>0.33 42.86 7.14</td>
<td>17.86 5.95 3.57</td>
</tr>
<tr>
<td>DZA 061</td>
<td>Algeria</td>
<td>0.44 89.89 2.16</td>
<td>21.22 2.50 3.75</td>
</tr>
<tr>
<td>DZA 105</td>
<td>Algeria</td>
<td>0.32 34.47 10.22</td>
<td>30.22 8.07 8.97</td>
</tr>
<tr>
<td>DZA 202</td>
<td>Algeria</td>
<td>0.37 63.55 6.49</td>
<td>67.44 5.65 3.89</td>
</tr>
<tr>
<td>DZA 210</td>
<td>Algeria</td>
<td>0.27 93.65 26.98</td>
<td>15.87 0.00 4.76</td>
</tr>
<tr>
<td>DZA 213</td>
<td>Algeria</td>
<td>0.23 63.22 0.37</td>
<td>34.11 4.75 0.88</td>
</tr>
<tr>
<td>DZA 219</td>
<td>Algeria</td>
<td>0.24 54.87 4.80</td>
<td>11.96 0.62 2.09</td>
</tr>
<tr>
<td>DZA 220</td>
<td>Algeria</td>
<td>0.19 73.90 13.10</td>
<td>9.16 1.17 1.45</td>
</tr>
<tr>
<td>DZA 221</td>
<td>Algeria</td>
<td>0.19 79.52 9.64</td>
<td>14.67 0.00 3.31</td>
</tr>
<tr>
<td>DZA 230</td>
<td>Algeria</td>
<td>0.25 52.95 7.10</td>
<td>13.16 4.27 3.05</td>
</tr>
<tr>
<td>DZA 231</td>
<td>Algeria</td>
<td>0.23 69.61 3.32</td>
<td>35.28 2.73 2.12</td>
</tr>
<tr>
<td>DZA 233</td>
<td>Algeria</td>
<td>0.31 60.90 11.47</td>
<td>20.78 11.04 5.80</td>
</tr>
<tr>
<td>DZA 236</td>
<td>Algeria</td>
<td>0.23 75.39 27.03</td>
<td>19.91 7.21 7.11</td>
</tr>
<tr>
<td>DZA 241</td>
<td>Algeria</td>
<td>0.28 88.11 24.79</td>
<td>33.83 13.84 11.80</td>
</tr>
</tbody>
</table>
Table 3.2. (Continued) The geographic origin, seed weight (g/100 seeds), and relative root growth of seedlings grown under different Al-containing media

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Origin</th>
<th>Seed weight</th>
<th>50 µM SE</th>
<th>100 µM SE</th>
<th>200 µM SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DZA 242</td>
<td>Algeria</td>
<td>0.35</td>
<td>88.04</td>
<td>5.62</td>
<td>11.03</td>
</tr>
<tr>
<td>DZA 243</td>
<td>Algeria</td>
<td>0.25</td>
<td>95.18</td>
<td>5.01</td>
<td>9.52</td>
</tr>
<tr>
<td>DZA 244</td>
<td>Algeria</td>
<td>0.27</td>
<td>94.25</td>
<td>4.65</td>
<td>4.65</td>
</tr>
<tr>
<td>ESP 031</td>
<td>Spain</td>
<td>0.28</td>
<td>78.42</td>
<td>10.25</td>
<td>56.60</td>
</tr>
<tr>
<td>ESP 039</td>
<td>Spain</td>
<td>0.38</td>
<td>60.82</td>
<td>7.76</td>
<td>36.71</td>
</tr>
<tr>
<td>ESP 043</td>
<td>Spain</td>
<td>0.29</td>
<td>71.77</td>
<td>10.65</td>
<td>45.35</td>
</tr>
<tr>
<td>ESP 048</td>
<td>Spain</td>
<td>0.39</td>
<td>91.12</td>
<td>15.58</td>
<td>56.67</td>
</tr>
<tr>
<td>ESP 074</td>
<td>Spain</td>
<td>0.32</td>
<td>74.25</td>
<td>7.31</td>
<td>25.69</td>
</tr>
<tr>
<td>ESP 095</td>
<td>Spain</td>
<td>0.33</td>
<td>75.42</td>
<td>9.88</td>
<td>39.61</td>
</tr>
<tr>
<td>F 20009</td>
<td>France</td>
<td>0.30</td>
<td>61.83</td>
<td>2.69</td>
<td>40.32</td>
</tr>
<tr>
<td>F 20015</td>
<td>France</td>
<td>0.27</td>
<td>118.69</td>
<td>32.83</td>
<td>15.15</td>
</tr>
<tr>
<td>F 20025</td>
<td>France</td>
<td>0.24</td>
<td>122.55</td>
<td>19.33</td>
<td>22.88</td>
</tr>
<tr>
<td>F 20031</td>
<td>France</td>
<td>0.28</td>
<td>108.11</td>
<td>13.51</td>
<td>13.51</td>
</tr>
<tr>
<td>F 20047</td>
<td>France</td>
<td>0.25</td>
<td>147.64</td>
<td>23.66</td>
<td>35.00</td>
</tr>
<tr>
<td>F 20048</td>
<td>France</td>
<td>0.23</td>
<td>91.27</td>
<td>3.97</td>
<td>27.78</td>
</tr>
<tr>
<td>F 20058</td>
<td>France</td>
<td>0.21</td>
<td>61.51</td>
<td>3.71</td>
<td>28.27</td>
</tr>
<tr>
<td>F 20061</td>
<td>France</td>
<td>0.34</td>
<td>87.61</td>
<td>15.41</td>
<td>17.09</td>
</tr>
<tr>
<td>F 20069</td>
<td>France</td>
<td>0.23</td>
<td>73.62</td>
<td>9.10</td>
<td>14.10</td>
</tr>
<tr>
<td>F 20081</td>
<td>France</td>
<td>0.21</td>
<td>35.23</td>
<td>7.77</td>
<td>25.16</td>
</tr>
<tr>
<td>F 20089</td>
<td>France</td>
<td>0.31</td>
<td>57.03</td>
<td>24.84</td>
<td>16.48</td>
</tr>
<tr>
<td>F 34042</td>
<td>France</td>
<td>0.28</td>
<td>57.69</td>
<td>3.85</td>
<td>34.62</td>
</tr>
<tr>
<td>F 83005-5</td>
<td>France</td>
<td>0.25</td>
<td>62.23</td>
<td>9.45</td>
<td>39.51</td>
</tr>
<tr>
<td>Harbinger</td>
<td>Australia</td>
<td>0.31</td>
<td>98.00</td>
<td>6.63</td>
<td>49.00</td>
</tr>
<tr>
<td>Paraggio</td>
<td>Australia</td>
<td>0.38</td>
<td>50.04</td>
<td>1.57</td>
<td>24.48</td>
</tr>
<tr>
<td>PI 190089</td>
<td>Australia</td>
<td>0.37</td>
<td>68.27</td>
<td>4.62</td>
<td>26.10</td>
</tr>
</tbody>
</table>
Table 3.2. (Continued) The geographic origin, seed weight (g/100 seeds), and relative root growth of seedlings grown under different Al-containing media.

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Origin</th>
<th>Seed weight</th>
<th>50 μM</th>
<th>SE</th>
<th>100 μM</th>
<th>SE</th>
<th>200 μM</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI 190090</td>
<td>Australia</td>
<td>0.35</td>
<td>68.54</td>
<td>6.83</td>
<td>37.78</td>
<td>1.93</td>
<td>11.33</td>
<td>1.99</td>
</tr>
<tr>
<td>PI 190091</td>
<td>Australia</td>
<td>0.35</td>
<td>55.34</td>
<td>11.85</td>
<td>51.09</td>
<td>9.58</td>
<td>11.71</td>
<td>3.32</td>
</tr>
<tr>
<td>PI 197361</td>
<td>Australia</td>
<td>0.42</td>
<td>68.38</td>
<td>1.61</td>
<td>52.15</td>
<td>1.65</td>
<td>11.98</td>
<td>1.98</td>
</tr>
<tr>
<td>PI 319045</td>
<td>Spain</td>
<td>0.40</td>
<td>84.17</td>
<td>9.84</td>
<td>41.33</td>
<td>6.96</td>
<td>5.33</td>
<td>0.82</td>
</tr>
<tr>
<td>PI 384622</td>
<td>Morocco</td>
<td>0.36</td>
<td>81.16</td>
<td>5.73</td>
<td>56.04</td>
<td>3.20</td>
<td>16.67</td>
<td>3.09</td>
</tr>
<tr>
<td>PI 464815</td>
<td>Turkey</td>
<td>0.39</td>
<td>51.49</td>
<td>4.89</td>
<td>8.05</td>
<td>1.20</td>
<td>2.41</td>
<td>0.64</td>
</tr>
<tr>
<td>PI 469099</td>
<td>Australia</td>
<td>0.46</td>
<td>86.01</td>
<td>5.58</td>
<td>12.12</td>
<td>2.61</td>
<td>5.27</td>
<td>1.10</td>
</tr>
<tr>
<td>PI 469100</td>
<td>Australia</td>
<td>0.54</td>
<td>91.07</td>
<td>7.03</td>
<td>30.19</td>
<td>8.67</td>
<td>8.05</td>
<td>1.45</td>
</tr>
<tr>
<td>PI 469102</td>
<td>Australia</td>
<td>0.34</td>
<td>54.88</td>
<td>16.17</td>
<td>22.33</td>
<td>6.90</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PI 493295</td>
<td>Portugal</td>
<td>0.32</td>
<td>46.42</td>
<td>3.18</td>
<td>7.98</td>
<td>0.79</td>
<td>3.55</td>
<td>0.00</td>
</tr>
<tr>
<td>PI 535613</td>
<td>Tunisia</td>
<td>0.24</td>
<td>48.14</td>
<td>4.86</td>
<td>16.31</td>
<td>1.41</td>
<td>5.52</td>
<td>1.40</td>
</tr>
<tr>
<td>PI 535614</td>
<td>Tunisia</td>
<td>0.17</td>
<td>14.09</td>
<td>3.52</td>
<td>9.09</td>
<td>0.93</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PI 535622</td>
<td>Tunisia</td>
<td>0.36</td>
<td>18.52</td>
<td>4.75</td>
<td>7.87</td>
<td>2.29</td>
<td>0.46</td>
<td>0.49</td>
</tr>
<tr>
<td>PI 535648</td>
<td>Tunisia</td>
<td>0.30</td>
<td>91.52</td>
<td>7.14</td>
<td>55.06</td>
<td>7.94</td>
<td>11.90</td>
<td>3.17</td>
</tr>
<tr>
<td>PI 535739</td>
<td>Libya</td>
<td>0.35</td>
<td>58.43</td>
<td>5.96</td>
<td>29.21</td>
<td>5.62</td>
<td>5.62</td>
<td>0.81</td>
</tr>
<tr>
<td>PI 537168</td>
<td>Cyprus</td>
<td>0.26</td>
<td>38.15</td>
<td>1.20</td>
<td>4.86</td>
<td>1.60</td>
<td>2.62</td>
<td>0.87</td>
</tr>
<tr>
<td>PI 566887</td>
<td>Greece</td>
<td>0.38</td>
<td>86.10</td>
<td>16.18</td>
<td>13.15</td>
<td>2.85</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PI 566889</td>
<td>Turkey</td>
<td>0.21</td>
<td>21.94</td>
<td>3.49</td>
<td>12.14</td>
<td>2.06</td>
<td>7.24</td>
<td>2.47</td>
</tr>
<tr>
<td>PI 566890</td>
<td>Greece</td>
<td>0.25</td>
<td>28.95</td>
<td>8.59</td>
<td>12.50</td>
<td>4.19</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>PI 577605</td>
<td>Cyprus</td>
<td>0.23</td>
<td>28.11</td>
<td>4.59</td>
<td>9.54</td>
<td>0.41</td>
<td>3.11</td>
<td>0.62</td>
</tr>
<tr>
<td>PI 577609</td>
<td>Sweden</td>
<td>0.28</td>
<td>88.50</td>
<td>13.10</td>
<td>32.09</td>
<td>1.24</td>
<td>8.21</td>
<td>2.89</td>
</tr>
<tr>
<td>PI 577613</td>
<td>Italy</td>
<td>0.33</td>
<td>56.30</td>
<td>5.91</td>
<td>19.75</td>
<td>4.12</td>
<td>5.93</td>
<td>2.66</td>
</tr>
<tr>
<td>PI 577614</td>
<td>Malta</td>
<td>0.19</td>
<td>31.62</td>
<td>13.20</td>
<td>13.73</td>
<td>1.60</td>
<td>6.86</td>
<td>1.00</td>
</tr>
<tr>
<td>PI 577626</td>
<td>Algeria</td>
<td>0.57</td>
<td>82.24</td>
<td>12.90</td>
<td>46.70</td>
<td>10.00</td>
<td>12.15</td>
<td>4.66</td>
</tr>
<tr>
<td>PI 577628</td>
<td>Spain</td>
<td>0.38</td>
<td>72.09</td>
<td>11.63</td>
<td>44.19</td>
<td>5.81</td>
<td>4.07</td>
<td>2.18</td>
</tr>
</tbody>
</table>
Table 3.2. (Continued) The geographic origin, seed weight (g/100 seeds), and relative root growth of seedlings grown under different Al-containing media

<table>
<thead>
<tr>
<th>Ecotype</th>
<th>Origin</th>
<th>Seed weight</th>
<th>50 μM</th>
<th>SE</th>
<th>100 μM</th>
<th>SE</th>
<th>200 μM</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI 577633</td>
<td>Greece</td>
<td>0.38</td>
<td>130.37</td>
<td>6.98</td>
<td>65.19</td>
<td>5.16</td>
<td>26.14</td>
<td>4.92</td>
</tr>
<tr>
<td>PRT 176</td>
<td>Portugal</td>
<td>0.26</td>
<td>56.36</td>
<td>4.26</td>
<td>37.04</td>
<td>8.05</td>
<td>9.66</td>
<td>2.79</td>
</tr>
<tr>
<td>PRT 177</td>
<td>Portugal</td>
<td>0.36</td>
<td>50.67</td>
<td>2.67</td>
<td>34.67</td>
<td>8.00</td>
<td>10.67</td>
<td>0.00</td>
</tr>
<tr>
<td>PRT 178</td>
<td>Portugal</td>
<td>0.28</td>
<td>60.32</td>
<td>13.84</td>
<td>41.27</td>
<td>8.40</td>
<td>11.11</td>
<td>6.35</td>
</tr>
<tr>
<td>PRT 179</td>
<td>Portugal</td>
<td>0.31</td>
<td>104.76</td>
<td>9.91</td>
<td>61.90</td>
<td>14.29</td>
<td>14.29</td>
<td>2.75</td>
</tr>
<tr>
<td>PRT 180</td>
<td>Portugal</td>
<td>0.32</td>
<td>71.90</td>
<td>23.57</td>
<td>32.68</td>
<td>3.77</td>
<td>21.79</td>
<td>2.18</td>
</tr>
<tr>
<td>W6 6025</td>
<td>Italy</td>
<td>0.50</td>
<td>90.69</td>
<td>11.02</td>
<td>51.96</td>
<td>5.88</td>
<td>11.52</td>
<td>4.26</td>
</tr>
<tr>
<td>W6 6037</td>
<td>Spain</td>
<td>0.72</td>
<td>73.68</td>
<td>1.10</td>
<td>72.68</td>
<td>2.54</td>
<td>25.56</td>
<td>2.91</td>
</tr>
<tr>
<td>W6 6047</td>
<td>Tunisia</td>
<td>0.47</td>
<td>28.95</td>
<td>4.91</td>
<td>11.19</td>
<td>2.62</td>
<td>4.38</td>
<td>1.71</td>
</tr>
<tr>
<td>W6 6048</td>
<td>Tunisia</td>
<td>0.46</td>
<td>108.21</td>
<td>5.76</td>
<td>45.77</td>
<td>8.62</td>
<td>23.88</td>
<td>3.45</td>
</tr>
<tr>
<td>W6 6049</td>
<td>Tunisia</td>
<td>0.38</td>
<td>116.44</td>
<td>0.93</td>
<td>29.33</td>
<td>14.45</td>
<td>5.33</td>
<td>1.07</td>
</tr>
<tr>
<td>W6 6050</td>
<td>Tunisia</td>
<td>0.43</td>
<td>67.82</td>
<td>2.73</td>
<td>62.57</td>
<td>1.44</td>
<td>20.98</td>
<td>3.43</td>
</tr>
<tr>
<td>W6 6092</td>
<td>Tunisia</td>
<td>0.52</td>
<td>47.34</td>
<td>4.76</td>
<td>23.67</td>
<td>3.56</td>
<td>9.86</td>
<td>0.81</td>
</tr>
<tr>
<td>W6 6099</td>
<td>Portugal</td>
<td>0.41</td>
<td>65.60</td>
<td>16.19</td>
<td>38.40</td>
<td>15.28</td>
<td>12.80</td>
<td>4.71</td>
</tr>
<tr>
<td>W6 6108</td>
<td>Italy</td>
<td>0.31</td>
<td>42.10</td>
<td>4.55</td>
<td>16.84</td>
<td>1.98</td>
<td>2.27</td>
<td>1.35</td>
</tr>
<tr>
<td>W6 6110</td>
<td>Italy</td>
<td>0.45</td>
<td>46.72</td>
<td>4.92</td>
<td>24.13</td>
<td>0.78</td>
<td>4.98</td>
<td>0.41</td>
</tr>
</tbody>
</table>
**Figure 3.1.** Distribution of ecotypes in different categories of RRG at different levels of Al

b) The accumulation of aluminum

Selected lines from the agarose screening system were analyzed for Al accumulation in root tips using the hematoxylin dye. Thirty ecotypes, including putative Al-tolerant, intermediate, and Al-sensitive lines were tested, and visually scored. No significant difference in color intensity was found among all ecotypes grown in the control condition (0 score). All ecotypes except three (PI 535648, PI 353739, and W66037, 4.83 rating) had a dark blue color (5 rating) when grown in 200 µM. The accumulation is higher in the Al-sensitive ecotypes, which exhibited more intense color in 50 µM or 100 µM Al-containing media (Figure 2a, b, c). There was a significant, negative, linear relationship between RRG at 50 µM or 100 µM and the intensity of staining scores (Figure 3.3). The results indicated that substantial amounts of Al accumulated in root tips of seedlings exposed to Al.
**Figure 3.2.** Hematoxylin staining in: a) W6 6037 (putative Al tolerant); b) PI 535622 (putative Al sensitive); c) A 17 (intermediate); and d) visual intensity rating.
**Figure 3.3.** Correlation between RRG and the visual rating of intensity of hematoxylin staining (n=30, P< 0.05)

* significant different at P<0.05

c. Screening study in soil culture

Twelve ecotypes that represented putative Al tolerant lines (PI 577633, PRT 179, W6 6037), intermediate lines (A 17, A 20, DZA 058, PI 535648, PI 577609, W6 6092), and Al sensitive lines (PI 535614, PI 535622, PI 566889) were selected for the soil study. Root lengths of all ecotypes were reduced when grown in unlimed soil (P < 0.05) except W6 6037 (Figure 3.4). This ecotype (W6 6037) appeared to be exceptionally Al tolerant, because its root lengths were similar in limed and unlimed soil. No growth stimulation effects of Al were found in the soil experiment, in contrast to that observed in the 50 µM agarose-containing medium. No significant differences were found between root dry weights of ecotypes grown under limed and unlimed soil (P > 0.05), except for A20, PI 5535622 and PRT 179 (Figure 3.5). Root dry weights of every treatment and ecotypes were less than 0.06 g.

Soil-based RRG correlated best with agarose-based RRG at 100, and 200 µM. Although the range of RRG at 200 µM was small, rankings of ecotypes were similar to those in Al toxic soil (Figure 3.6).
**Figure 3.4.** Root length and RRG of selected ecotypes grown in limed and unlimed acid soil

**Figure 3.5.** Root dry weight of selected ecotypes grown in limed and unlimed acid soil
Figure 3.6. Correlation between RRG from soil experiment and agarose experiment (n=12, P< 0.05)

\[ R^2 = 0.3511 \]
\[ R^2 = 0.6292^* \]
\[ R^2 = 0.7189^* \]

* significantly different at P<0.05

Discussion

Variation in sensitivity to Al exists among *M. truncatula* germplasm. Relative Root Growth (RRG) appeared to be a reliable measure of Al tolerance or Al sensitivity for *M. truncatula* (Figure 3.7). Ranking of Al sensitivities of ecotypes differed at different levels of Al concentration. For example, several ecotypes appeared to be Al-sensitive or Al-tolerant at 50 µM Al relative to genotype A17, but showed no significant difference relative to A17 at higher Al levels. The differences in Al sensitivity among *M. truncatula* genotypes were highest at 50 µM Al, and decreased as the concentration of Al increased.

Hematoxylin staining appears to be a suitable procedure to determine Al tolerance. Staining Al-treated roots allowed easy estimation of Al accumulation in roots, and the rating of intensity was correlated to RRG. The color difference between an Al-sensitive and an Al-tolerant accession has been reported (Chandran et al., 2008b). However, in this study, a range of color intensity between the Al-sensitive and tolerant ecotypes indicated significant variation of Al-sensitivity in germplasm. Variation in Al-accumulation in *M. truncatula* had been reported using lumogallion stain, but the
correlation between the intensity and soil study was low (Narasimhamoorthy et al., 2007).

Soil-based screening is considered the most realistic method of evaluating plants for Al tolerance. We found a positive correlation between RRG in agarose and soil-based screening, although only one ecotype (W6 6037) showed no significant reduction in root growth in the soil experiment, because the Al toxicity level in acid soil was stringent (Foy et al., 1993).

Several germplasm accessions that we studied had been reported previously for their Al-response. Using different screening methods, we found both similar and different results. For example, we found ecotype W6 6037 as consistently Al-tolerant in each method; however, it was reported as Al tolerant in a soil study and a lumogallion staining study, but as Al sensitive in a hydroponic study (Narasimhamoorthy et al., 2007). Several ecotypes, such as PI 535614 and PI 535622, were considered Al sensitive in our studies; however they had high RRG in earlier hydroponic experiments and were considered to be Al tolerant (Sledge et al., 2005). Based on our studies, we selected ecotypes that represented putative Al tolerant, intermediate and Al sensitive ones for further genetic studies.
**Figure 3.7** Root length of a) sensitive lines PI 566889 and PI 535622, and b) tolerant lines PI 577633 and W6 6037, grown in different Al-containing media (0, 50, 100, and 200 µM).

**Acknowledgement**

This project was funded in part by the USDA - CSREES T-STAR program.
References


CHAPTER 4

Genetic study of aluminum tolerance in *Medicago truncatula*

**Introduction**

Crop improvement via traditional breeding can be enhanced through identification of genetic linkages using molecular markers (Ishitani et al., 2004). Molecular markers have been used to monitor DNA sequence variation in and among species to aid in introgression of target genes in plant breeding programs (Landjeva et al., 2007).

Identified genes coding for aluminum (Al) resistance in major crops could help to speed breeding for resistance of other crops to acid soils. Nonetheless, to date the mechanisms of Al resistance have been explored only in a limited number of crop species, mainly cereals with a high economic value (Poschenrieder et al. 2008).

**Aluminum tolerance in Legumes**

The Leguminosae is the third largest family of higher plants, with approximately 20,000 species. Legumes are second to cereal crops in agricultural importance based on area harvested and total production. Grain legumes provide about one third of all dietary protein nitrogen and one-third of processed vegetable oil for human consumption (Gepts et al., 2005). Forage legumes are also important sources of animal feed in both temperate and tropical regions. Often, grasslands for livestock are located on low-fertility acid soils; however, legumes are among the plant species that are least adapted to soil acidity in general. Selection or breeding of forage legumes for better adaptation to low-fertility, acid soils are practical approaches to increase pasture productivity (Rao et al., 2001). In addition, this is a low-input method with reduced cost of liming.

The enhanced exudation of citrate in response to Al stress has been reported in snapbean (*Phaseolus vulgaris* L.) (Miyasaka et al., 1991) and soybean (*Glycine max*) (Yang et al., 2001; Shen et al., 2005). Genotypic differences and the mechanism involved of Al resistance in soybean have been widely studied (Rangel et al., 2010). Quantitative trait loci (QTL) associated with Al-tolerance in soybean has been reported (Bianchi-Hall et al., 2000).

Root characteristics of common bean (*Phaseolus vulgaris* L.) under Al treatment were found to be under polygenic control. Some QTLs were identified at the same
location as QTLs for tolerance to low phosphorus stress, indicating possible cross-links in genetic control of adaptation of common bean to abiotic stresses of acid soils (Lopaz-Marin et al., 2009).

**Aluminum tolerance in *Medicago* species**

Alfalfa (*Medicago sativa*) is the most important and widely grown forage legume in the world. Its global production is seriously affected by Al toxicity (Rechcigl et al., 1998). Several screening procedures have been used to select for Al-resistant cultivars; however, neither alfalfa cultivars nor wild introductions varied greatly in Al resistance when screened in acid soil (Bouton, 1996). This finding indicated that selection for Al tolerance in alfalfa is complex due to tetraploid inheritance, obligate outcrossing and inbreeding depression, which can mask Al tolerance (Bouton, 1996; Dall’Agnol et al., 1996). Recently, two QTLs for Al tolerance were identified on linkage groups 1 and 8 in diploid alfalfa (*Medicago sativa* subsp. coerulea) using RFLP analysis of F₂ and backcross populations (Sledge et al., 2002). Narasimhamoorthy et al. (2007b) studied additional QTLs for Al tolerance by using simple sequence repeats (SSR), and the Al tolerance genes from sugarcane (*Saccharum spp.*), as candidate genes to map the alfalfa genome. Three putative QTLs were mapped on linkage groups 1, 2 and 3, explaining 38, 16 and 27% of the phenotypic variation, respectively.

*Medicago truncatula* Gaetn. (Barrel medic) is a legume species closely related to alfalfa and comparable to it in forage quality. Barrel medic is also an important forage in the integral farming system in Australia. Both *M. sativa* and *M. truncatula* are sensitive to soil acidity and Al toxicity (Rechcigl et al., 1998). Due to reasons stated above, alfalfa is a difficult subject for genomic studies. In recent years, *M. truncatula* has been chosen as a model legume for genomic studies, because of its small diploid genome, fast generation time (from seed-to-seed), self-pollination, and high transformation efficiency (Cook, 1999). An efficient mutagenesis protocol using ethyl-methyl sulfonate (EMS) and a polymorphic ecotype with properties appropriate for use as a mapping parent are established (Penmetsa and Cook, 2000)

Aluminum tolerance in the *M. truncatula* germplasm has been reported based on a hydroponic screening method (Sledge et al., 2005), Al-toxic soil assay, and a lumogallion root staining method (Narasimhamoorthy et al., 2007a). Results from the hydroponic
screening indicated that sufficient variation of Al-tolerance exists within this collection of *M. truncatula* accessions to select Al sensitive and tolerant accessions, which could be used to identify QTLs for Al tolerance, the first step in a map-based cloning approach to discover Al tolerance genes (Sledge et al., 2005). However, the results from these three screening methods differed from each other, with altered rankings for genotypes depending on the method (Narasimhamoorthy et al., 2007a). The physiological mechanisms that are responsible for these reported differences are unknown.

Choi et al. (2004) developed a sequence-based genetic map using 288 sequence-based markers, covering 513 cM on the genetic map of *M. truncatula*. Using a mapping population of genotype A-17 crossed with genotype A-20, Mun et al. (2006) analyzed a total of 1,236 microsatellite markers for polymorphism in gene-rich Bacteria Artificial Chromosome (BAC) sequences, 27 Mbp of non-redundant transcript sequences, 20 Mbp of random whole genome shotgun sequences, and 49 Mbp of BAC-end sequences.

Chandran et al. (2008a, b) studied gene expression in *M. truncatula*, and reported that genes involved in cell-wall modification, abiotic and biotic stress responses were up-regulated in response to Al. In contrast, genes involved in secondary metabolism, protein synthesis, and cell cycle processing were down-regulated in response to Al. Transcriptional profiling using DNA microarray revealed three genes that encode for an ABC transporter, a putative multidrug and toxin efflux (MATE) protein, and a putative metal-binding isoprenylated protein), which have potential roles in binding and/or sequestration of Al in *M. truncatula*.

To date, numerous databases of molecular markers, expression profiles, functional and structural genomics are available ([http://www.medicago.org](http://www.medicago.org)). Thus, *M. truncatula* is an ideal species for studying genes that are involved in Al-tolerance.

Our objective is to generate a population segregating for Al response in order to investigate genetic markers associated with Al tolerance in *M. truncatula*. In this study, we reported on several loci that are possibly associated with the Al tolerance from a segregating $F_2$ population developed from an Al-tolerant ecotype and an Al-sensitive EMS mutant.
Material and methods

Plant materials and crossing procedure

Aluminum tolerant and sensitive lines from the previous experiments (Chapter 3) were grown as parents. Female and male flowers were chosen according to *Medicago truncatula* Handbook (Chabaud et al., 2006). Female flowers (2 days before opening) were cut longitudinally using the tip of a surgical blade. Unopened anthers were emasculated using modified vacuum suction (Cook Lab, UC Davis, California). The stigmas were hand-pollinated with the tip of a freshly harvested sexual column in order to saturate the sticky, receptive surface of the stigma. Flowers were covered with cheesecloth and tagged with an appropriate label. Twelve pairs of crosses were made from 3 Al-tolerant ecotypes (PI 577633, PRT 179, and W6 6037), and 4 Al-sensitive ecotypes or mutant lines (PI 535614, PI 535622, PI 566889, and S69). The male sterile A17 tap (mtapetala mutant) (Penmetsa and Cook, 2000) was also used as a receptor in order to test the compatibility between crosses.

Validation of F1 population

Seedlings obtained from crosses and parental lines were validated using SSR (Simple sequence repeats) and length markers (Direct amplification of length polymorphism) (Appendix A) (Choi et al., 2004; Mun et al., 2006). Leaf tissue of each plant was collected and extracted for DNA using DNeasy plant mini kit (Qiagen, USA). Polymerase Chain Reaction (PCR) products of seedlings and parents were compared on 3% agarose gel. Seedlings from validated crosses were planted, allowed to self-pollinate, and seeds were collected for further genetic analysis.

Evaluation of Al sensitivity in F2 population

The F2 seedlings were screened for Al-sensitivity, using agarose screening methods previously described (Chapters 2, 3) to determine the phenotypic trait of Al-response. After agarose screening, seedlings were grown in a growth chamber, under 16 hours light/ 8 hours dark, 23 °C. Extraction of DNA from leaf tissue of parents and the segregating population were conducted using DNeasy plant mini kit (Qiagen, USA).

Genetic analysis of F2 population

Extracted DNA of 23 Al tolerant and sensitive individuals of the segregating F2 population as well as one parent was analyzed using Restriction site-associated DNA
(RAD) analysis. Analysis of DNA using RAD was conducted by the Cook Laboratory, University of California, Davis (UC Davis). Briefly, genomic DNA from individual samples (1-10 ng) was digested with Bam HI or Hind III 0.5 µl (U) at 37 °C for 1 hour in a 20 µL reaction. Each individual sample was ligated with barcode adapters (Appendix A) and common adapters, and incubated at 22 °C for 1 hour. Then, samples were purified with PEG 8000 (Polyethylene glycol 8000) and digested with Nla III 0.7 µl (U). The products were used as templates for PCR reactions with biotinylated primers. PCR products were purified with Qiagen PCR clean up kit (Qaigen, USA), then passed through streptavidin-agarose bead binding column (Pierce #20349, USA). DNA was eluted with Qiagen RNeasy mini kit RLC buffer (Qaigen, USA), and validated with a high sensitivity DNA assay Bioanalyzer (Agilent Technologies, Inc., USA). The DNA library was sequenced with Solexa sequencer (Illumina Inc., USA) at the University of Southern California, Los Angeles. The sequence data were analyzed using Bowtie program and PLINK software (http://pngu.mgh.harvard.edu/purcell/plink/, Purcell et al., 2007) at the Cook laboratory (UC Davis).

Results and discussion

Plant materials and crossing procedure

Ecotype W6 6037 which appeared the most Al tolerant in acid soil (Chapter 3) was used as the male donor (D), and Al-sensitive lines PI 535614, PI 535622, PI 566889, and S69 were the female receptors (R). However, these crosses did not produce seeds, or the seedlings obtained showed poor vigor, and chlorosis (Figure 4.1). The reciprocal crosses were not successful either. Also, crosses between W6 6037 and A17 tap did not produce seed.

We obtained pods from crosses between other Al-tolerant ecotypes, PI 577633 and PRT 179 with the Al-sensitive lines or ecotypes (Table 4.1). Seeds were collected and subjected to genetic validation to confirm cross-pollination.

Ellwood et al. (2007) used six simple sequence repeat (SSR) loci to analyse the genetic diversity and relationships between randomly selected individuals from 192 accessions in the core collection at the South Australian Research and Development Institute (SARDI). They reported that M. truncatula is composed of three subspecies.
(ssp.): ssp. *truncatula*, ssp. *longeaculeata*, and ssp. *tricycla*. Contingency Chi-squared tests were significant between ssp. *tricycla* and ssp. *truncatula* at four loci, suggesting a barrier to gene flow between these subspecies.

Based on pod characteristics, W6 6037 might be characterized as ssp. *truncatula* (5–8 coils, pods longer than wide, spines more or less appressed to the pod surface) while the others might be ssp. *tricycla* (2.5–4 coils, length of pod less than its diameter, spines usually not appressed to the pod surface) (Ellwood et al., 2007). Our results confirmed that the unsuccessful crosses reported here might be due to the incompatability between subgroups. Genetic and phenotypic correlation were similar within each population, but they were quite different between populations. Very low levels of outcrossing in *M. truncatula*, might be a consequence of a high level of spatial structure of the population (Bonnin et al., 2001).

**Table 4.1.** Results of cross pollination between Al-tolerant (e.g., W6 6037, PI 577633, and PRT 179) and Al sensitive ecotypes or mutant line (PI 535614, PI 535622, PI 566889, and S69)

<table>
<thead>
<tr>
<th>Parent lines</th>
<th>PI 535614</th>
<th>PI 535622</th>
<th>PI 566889</th>
<th>S69</th>
</tr>
</thead>
<tbody>
<tr>
<td>W6 6037</td>
<td>NA ¹</td>
<td>NA</td>
<td>Very poor</td>
<td>NA</td>
</tr>
<tr>
<td>PI 577633</td>
<td>Poor</td>
<td>Fair</td>
<td>NA</td>
<td>Good</td>
</tr>
<tr>
<td>PRT 179</td>
<td>Good</td>
<td>Fair</td>
<td>NA</td>
<td>Fair</td>
</tr>
</tbody>
</table>

¹ Rating system as follows, Good: F₁ seedlings were vigorous, and mature plants produced many seeds; Fair: Seedling were vigorous, mature plant produced many pods, but only a few seeds or none were found inside; Poor: seedling were not vigorous, but survived through the flowering stage, and mature plants produced a few seeds; Very poor: seedlings grew poorly and did not survived to the flowering stage; NA: no seeds were obtained.
**Figure 4.1.** Left, F₁ seedlings from Al-sensitive PI 566889 (Female recipient, R) and Al-tolerant W6 6037 (Male donor, D) grew poorly; Right, parent PI 566889

Validation of F₁ population

Leaf tissues were collected from vigorous, putative F₁ seedlings and extracted for genomic DNA. Polymorphisms among Al tolerant and sensitive lines were observed from genetic markers DK296L, MU10, PPDK and DK049R (Figure 4.2). Using co-dominant makers, true F₁ seedlings from crosses showed the polymorphisms of both parents. We were able to validate F₁ seedlings from the following crosses: PI 577633 x PI 535614; PI 577633 x PI 535622; PI 577633 x S69; PRT 179 x PI 535614; PRT 179 x PI 535622; and PRT 179 x S69.
Figure 4.2. Polymorphisms of markers DK296L, MU10, PPDK and DK049R (Choi et al., 2004) among ecotypes selected as parents.

Evaluation of Al sensitivity in F2 population

Seedlings of parents PI 577633 and S69, as well as the most vigorous progeny from the F2 population were grown in Al containing agarose media. Under control conditions (0 Al), there was no significant differences (P> 0.05) among root lengths of these 3 groups (Figure 4.3a). In contrast, root lengths of PI 577633 and S69 were significantly different when grown in 50 μM Al-containing media (Figure 4.3b). Root lengths of the F2 population varied greatly, ranging from to 0.4 to 3.3 cm (Figure 4.3b).
Figure 4.3. Root length individual seedlings of PI 577633, S69, and F2 populations were significantly different when grown in a) 0 µM, b) 50 µM Al containing media.

We obtained a wide range of segregation in the F2 population, indicating that Al tolerance in *M. truncatula* is not due to a simple dominant-recessive trait. In contrast, barley (*Hordeum vulgare* ssp. vulgare) appeared to have a single dominant gene involved in Al tolerance; the F2 progeny from a cross between an Al-tolerant barley FM-404 and sensitive Harrington cultivars segregated in 3:1 ratio (Echart et al., 2002). Similar results indicating a single recessive gene were reported in rice (*Oryza sativa* L.) (Ma et al, 2005); progeny of a cross between Al-sensitive mutant als1 and wild-type plants showed segregated at a 3:1 dominant ratio. Similarly, F2 hybrids of Al tolerant and sensitive chickpea (*Cicer arietinum*) parents showed segregation for Al tolerance with the 3:1 ratio, indicating a single dominant gene (Singh and Raja, 2011).
**Genetic analysis of F2 population**

Restriction site-associated DNA analysis of the F2 population (PI 577633 x S69) obtained from Bam HI and Hind III were sequenced with Solexa sequencing. The library generated from the Hind III restriction enzyme yielded more loci mapped to the reference (A17 genome); therefore, it was used for further sequence analysis (Table 4.2).

**Table 4.2** Number of sequence reads and loci mapped of RAD library generated from Bam HI and Hind III

<table>
<thead>
<tr>
<th>Read</th>
<th>BamHI</th>
<th>HindIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total # reads</td>
<td>38,323,143</td>
<td>21,193,678</td>
</tr>
<tr>
<td>Loci mapped</td>
<td>73,921</td>
<td>165,745</td>
</tr>
<tr>
<td>Loci with &gt;20 &amp; &lt;1000 reads</td>
<td>33,058</td>
<td>83,868</td>
</tr>
<tr>
<td>Loci with &gt;= 15 individuals</td>
<td>14,718</td>
<td>35,773</td>
</tr>
</tbody>
</table>

Using the Bowtie (0.12.7) program (Langmead et al., 2009) with a restriction of 1 SNP (Single nucleotide polymorphism) per read allowed, a total of 9,458,158 reads were mapped onto 158,272 loci of the Mt3.5 genome. The mapped loci were then filtered with following criteria; loci should be present in 12 or more individuals with at least 4 reads per individual and mapped with 200 total numbers of reads or less. There were 9,128 loci passed and they were used for further analysis. A total of 1,729 alleles showed valid SNPs in at least four different individuals. These alleles were further analyzed for SNP association using PLINK software (Purcell et al., 2007) from the Broad Institute. According to the software manual, the input files were prepared including allele map, SNP calls for each individual, and their phenotype data. Manhattan plot (Figure 4.4) of 1,729 alleles at their locations on 8 chromosomes showed with the association probability value of each allele to the tolerant phenotype. The spots with high probability (above 3 of –\(\log_{10}(P)\)) are taken as candidate alleles which might be associated the phenotype. Based on genotypes and phenotypes, there were 11 alleles that strongly correlated with the Al sensitivity in F2 progeny (Table 4.3).
Our results showed new loci that were associated with Al-tolerance. Hypothetical proteins close to alleles were identified on chromosomes 1, 3, 5, and 7. Glycosyl transferase and glycerophosphoryl diester phosphodiesterase were also located near alleles on chromosome 3. These loci (Figure 4.5) differed from a previous study that reported differential expression of genes in an Al-resistant and an Al-sensitive line as follows: two genes associated with cell death, senescence, cell wall degradation (TC96658, Xyloglucan endotransglycosylase; TC111698 COBRA-like gene), and a multidrug and toxin efflux (MATE) gene (TC105342) (Chandran et al., 2008b). The Al-inducible MATE gene was hypothesized to be involved in organic acid exudation and internal detoxification; however, no organic acid secretion was observed in seedlings exposed to Al (Chandran et al., 2008b). In addition, these genes were different from the set of transcriptional genes MATE efflux family protein, Periaxin-like gene, and unknown gene (TC98950, TC103000, and TC102756, respectively) that up-regulated during the Al treatment of the A17 genotype (Chandran et al., 2008a). However, *M. truncatula* exhibits unusually wide dispersal of genotypes throughout its native Mediterranean region, possibly due to animal and trade-related movements (Ellwood et al., 2006). Using a different set of genetic materials might have resulted in this novel finding.
### Table 4.3 Possible alleles associated with Al tolerance in *M. truncatula* F₂ population

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Allele</th>
<th>Possible gene from Mt. 3</th>
<th>Region on chromosome (estimate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chr1</td>
<td>24672934(G&gt;T)</td>
<td>Medtr1g116690 hypothetical protein</td>
<td>24720000</td>
</tr>
<tr>
<td>Chr3</td>
<td>13339531(A&gt;G)</td>
<td>Medtr3g05926 hypothetical protein</td>
<td>13314000</td>
</tr>
<tr>
<td>Chr3</td>
<td>13946885(G&gt;C)</td>
<td>Medtr3g061660 Glycosyl transferase, family</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medtr3g076710 Glycerophosphoryl diester phosphodiesterase</td>
<td></td>
</tr>
<tr>
<td>Chr3</td>
<td>17790213(T&gt;G)</td>
<td>Medtr3g076720 hypothetical protein</td>
<td>17784000</td>
</tr>
<tr>
<td>Chr5</td>
<td>13776296(T&gt;C)</td>
<td>Medtr5g033810 hypothetical protein</td>
<td>13775000</td>
</tr>
<tr>
<td>Chr5</td>
<td>15229439(A&gt;G)</td>
<td>Medtr5g036520 hypothetical protein</td>
<td>15229000</td>
</tr>
<tr>
<td>Chr5</td>
<td>18172596(C&gt;T)</td>
<td>Medtr5g043890 functional candidate resistance protein KR1, related</td>
<td>18171000</td>
</tr>
<tr>
<td>Chr6</td>
<td>9821564(T&gt;C)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chr6</td>
<td>10578145(G&gt;T)</td>
<td>Medtr6g059140 Methyl-CpG binding</td>
<td>10557000</td>
</tr>
<tr>
<td>Chr7</td>
<td>14146264(A&gt;G)</td>
<td>Medtr7g068540 hypothetical protein</td>
<td>14130000</td>
</tr>
<tr>
<td>Chr8</td>
<td>6474743(G&gt;A)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Six alleles identified were clustered on chromosome 3 and 5 (Figure 4.5), indicating the possibility of genes involved in Al tolerance within these region. Further experimentation is needed to distinguish between linkages, whether they are consistent with Al tolerance in this population. Experiments to confirm whether the alleles were dominant in Al-tolerant PI 535633 need to be conducted. The combination of Al tolerance from other tolerant lines may be observed using the same approach.
Figure 4.5 Genes associated with Al tolerance that had been reported previously and candidate alleles of Al tolerance from PI 56677633 x S69 population

- **TC98950** MATE efflux family protein
- **TC103000** Periaxin-like protein
- **TC102756** Unknown
- **TC111698** COBRA-like gene
- **TC96658** Xyloglucan endotransglycosylase

Candidate alleles for Al tolerance in PI 577633 Xs69 population
References


Dall’Agnol M, Bouton JH and Parrott WA (1996) Screening methods to develop alfalfa germplasms tolerant of acid, aluminum toxic soils. Crop Science 36:64–70


Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MAR, Bender D, Maller J, Sklar P, de Bakker PIW, Daly MJ & Sham PC (2007) PLINK: a toolset for whole-genome association and population-based linkage analysis. American Journal of Human Genetics, 81.


CHAPTER 5
Conclusion and future research

Results from our studies suggested that the *M. truncatula* germplasm expressed a wide range of Al sensitivity. Mutants generated from EMS also varied in Al sensitivity. The screening methods could lead to different results as each technique has distinct advantages and disadvantages; however, we found ecotypes and one mutant line that exhibited consistent responses in each screening assay. Relative root growth, and visual scoring were effective for direct estimation of Al toxicity. A soil-based assay might be the most accurate method as it is the closest to field conditions. Agarose-based assay was fast, but the results also vary dependent on pH, Al concentration, and the particular lines. Correlations were observed between the three methods used. The Al-tolerant and Al-sensitive lines were identified according to the results from all methods. Although there were incompatibilities among the Al-tolerant and sensitive lines that we selected for breeding, we have successfully obtained a segregating F2 population. Genetic analysis of F2 populations between sensitive mutant and tolerant ecotype showed that the Al-tolerant seedlings and Al-sensitive seedlings were not segregated at a 3:1 ratio, indicating that the sensitivity to Al were not controlled by a single recessive gene.

From this population, candidate alleles that might be associated with Al tolerance were identified. Linkages between the alleles needed to be investigated further to confirm the exact location on chromosome. In the future, these results could be used to find novel genes involved in the response to Al toxicity in *M. truncatula*. Genetic analysis should be repeated in an F3 and backcrossed population to confirm the results. With this information, Al-tolerant genes linked with molecular markers can be studied further. Crop improvement of legumes for Al tolerance may be possible based on identification of a genotype or a combination of a genotype and a phenotype.
## Appendix A

### Table 5.1. Microsatellite markers and BAC associations used for progeny validation (Mun et al., 2006)

<table>
<thead>
<tr>
<th>Marker Name</th>
<th>Accession</th>
<th>MtGSP ID</th>
<th>BAC Name</th>
<th>Marker Type</th>
<th>Size (bp)</th>
<th>BAC Name</th>
<th>Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtB007</td>
<td>AC119411</td>
<td>MtGSP_001A08</td>
<td>MtH2 23C14 (TA)14</td>
<td>202</td>
<td>MtH2 23C14</td>
<td>LG7</td>
<td></td>
</tr>
<tr>
<td>MtB030</td>
<td>AC138448</td>
<td>MtGSP_001D05</td>
<td>MtH2 11N13 (TA)23</td>
<td>268</td>
<td>MtH2 11N13</td>
<td>LG2</td>
<td></td>
</tr>
<tr>
<td>MtB035</td>
<td>AC126778</td>
<td>MtGSP_001D10</td>
<td>MtH2 32J21 (AT)35</td>
<td>495</td>
<td>MtH2 32J21</td>
<td>LG2</td>
<td></td>
</tr>
<tr>
<td>MtB044</td>
<td>AC122726</td>
<td>MtGSP_001E08</td>
<td>MtH2 23O24 (AT)14</td>
<td>300</td>
<td>MtH2 23O24</td>
<td>LG8</td>
<td></td>
</tr>
<tr>
<td>MtB051</td>
<td>AC125473</td>
<td>MtGSP_001F04</td>
<td>MtH2 8J13 (AT)30</td>
<td>247</td>
<td>MtH2 8J13</td>
<td>LG1</td>
<td></td>
</tr>
<tr>
<td>MtB056</td>
<td>AC122165</td>
<td>MtGSP_001F10</td>
<td>MtH2 32M22 (AT)24</td>
<td>183</td>
<td>MtH2 32M22</td>
<td>LG1</td>
<td></td>
</tr>
<tr>
<td>MtB058</td>
<td>AC121241</td>
<td>MtGSP_001G03</td>
<td>MtH2 4N3  (AT)21</td>
<td>240</td>
<td>MtH2 4N3</td>
<td>LG8</td>
<td></td>
</tr>
<tr>
<td>MtB059</td>
<td>AC121242</td>
<td>MtGSP_001G04</td>
<td>MtH2 9J3  (TA)15</td>
<td>266</td>
<td>MtH2 9J3</td>
<td>LG7</td>
<td></td>
</tr>
<tr>
<td>MtB082</td>
<td>AC124959</td>
<td>MtGSP_002B08</td>
<td>MtH2 23L11 (TA)24</td>
<td>251</td>
<td>MtH2 23L11</td>
<td>LG4</td>
<td></td>
</tr>
<tr>
<td>MtB084</td>
<td>AC133862</td>
<td>MtGSP_002B10</td>
<td>MtH2 30D7 (AT)22</td>
<td>202</td>
<td>MtH2 30D7</td>
<td>LG2</td>
<td></td>
</tr>
<tr>
<td>MtB110</td>
<td>AC127169</td>
<td>MtGSP_002E11</td>
<td>MtH2 36N3 (AT)16</td>
<td>224</td>
<td>MtH2 36N3</td>
<td>LG8</td>
<td></td>
</tr>
<tr>
<td>MtB127</td>
<td>AC124972</td>
<td>MtGSP_002G08</td>
<td>MtH2 24J4 (AT)23</td>
<td>230</td>
<td>MtH2 24J4</td>
<td>LG5</td>
<td></td>
</tr>
<tr>
<td>MtB135</td>
<td>AC126794</td>
<td>MtGSP_002H08</td>
<td>MtH2 24J7 (AT)26</td>
<td>282</td>
<td>MtH2 24J7</td>
<td>LG8</td>
<td></td>
</tr>
<tr>
<td>MtB211</td>
<td>AC135798</td>
<td>MtGSP_004C03</td>
<td>MtH2 31C16 (AT)31</td>
<td>292</td>
<td>MtH2 31C16</td>
<td>LG2</td>
<td></td>
</tr>
<tr>
<td>MtB254</td>
<td>AC137827</td>
<td>MtGSP_004H06</td>
<td>MtH2 20G23 (AT)26</td>
<td>275</td>
<td>MtH2 20G23</td>
<td>LG3</td>
<td></td>
</tr>
<tr>
<td>MtB304</td>
<td>AC139356</td>
<td>MtGSP_005G06</td>
<td>MtH2 12C11 (AT)17</td>
<td>264</td>
<td>MtH2 12C11</td>
<td>LG2</td>
<td></td>
</tr>
<tr>
<td>MtB309</td>
<td>AC140068</td>
<td>MtGSP_005H01</td>
<td>MtH2 15L24 (AT)22</td>
<td>300</td>
<td>MtH2 15L24</td>
<td>LG5</td>
<td></td>
</tr>
<tr>
<td>MtB311</td>
<td>AC140032</td>
<td>MtGSP_005H05</td>
<td>MtH2 10I23 (AT)18</td>
<td>202</td>
<td>MtH2 10I23</td>
<td>LG8</td>
<td></td>
</tr>
<tr>
<td>MtB320</td>
<td>AC141108</td>
<td>MtGSP_006A03</td>
<td>MtH2 22G6 (AT)19</td>
<td>230</td>
<td>MtH2 22G6</td>
<td>LG2</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5.2. Direct amplification of length polymorphism (length) markers used for progeny validation (Choi et al., 2004)

<table>
<thead>
<tr>
<th>Marker name</th>
<th>Template sequence accession no.</th>
<th>Sequenced BAC accession no.</th>
<th>Type</th>
<th>Different (bp, A17/A20)</th>
<th>Method</th>
<th>Linkage group</th>
</tr>
</thead>
<tbody>
<tr>
<td>DK003R</td>
<td>AQ841082</td>
<td>NA</td>
<td>BEST</td>
<td>550</td>
<td>Length</td>
<td>5</td>
</tr>
<tr>
<td>DK296L</td>
<td>AQ917136</td>
<td>NA</td>
<td>BEST</td>
<td>290</td>
<td>Length</td>
<td>7</td>
</tr>
<tr>
<td>CrS</td>
<td>AI737624</td>
<td>AC122724.13</td>
<td>ESTi</td>
<td>200</td>
<td>Length</td>
<td>6</td>
</tr>
<tr>
<td>78B21L</td>
<td>AZ758038</td>
<td>AC134824.15</td>
<td>BEST</td>
<td>80</td>
<td>Length</td>
<td>6</td>
</tr>
<tr>
<td>MTU10</td>
<td>AI974637</td>
<td>NA</td>
<td>ESTe</td>
<td>50</td>
<td>Length</td>
<td>8</td>
</tr>
<tr>
<td>ENOD40</td>
<td>X80262</td>
<td>NA</td>
<td>GS</td>
<td>41</td>
<td>Length</td>
<td>5</td>
</tr>
<tr>
<td>DK511L</td>
<td>AQ917538</td>
<td>AC126779.10</td>
<td>BEST</td>
<td>40</td>
<td>Length</td>
<td>5</td>
</tr>
<tr>
<td>DK006R</td>
<td>AQ841074</td>
<td>NA</td>
<td>BEST</td>
<td>30</td>
<td>Length</td>
<td>5</td>
</tr>
<tr>
<td>DK049R</td>
<td>AQ841103</td>
<td>NA</td>
<td>BEST</td>
<td>30</td>
<td>Length</td>
<td>5</td>
</tr>
<tr>
<td>DENP</td>
<td>AI974308</td>
<td>NA</td>
<td>ESTe</td>
<td>27</td>
<td>Length</td>
<td>4</td>
</tr>
<tr>
<td>DK313L</td>
<td>AQ917231</td>
<td>AC122728.16</td>
<td>BEST</td>
<td>25</td>
<td>Length</td>
<td>3</td>
</tr>
<tr>
<td>PPDK</td>
<td>AI737496</td>
<td>NA</td>
<td>ESTe</td>
<td>20</td>
<td>Length</td>
<td>8</td>
</tr>
<tr>
<td>DK202R</td>
<td>AQ917161</td>
<td>NA</td>
<td>BEST</td>
<td>15</td>
<td>Length</td>
<td>3</td>
</tr>
<tr>
<td>DK015R</td>
<td>AQ841060</td>
<td>NA</td>
<td>BEST</td>
<td>10</td>
<td>Length</td>
<td>5</td>
</tr>
<tr>
<td>DK018R</td>
<td>AQ841066</td>
<td>NA</td>
<td>BEST</td>
<td>10</td>
<td>Length</td>
<td>5</td>
</tr>
<tr>
<td>DK020R</td>
<td>AQ841084</td>
<td>NA</td>
<td>BEST</td>
<td>10</td>
<td>Length</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 5.3. Barcode adaptors used for RAD library construction (Hind III)

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Barcode adapter</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-A4</td>
<td>ctccR</td>
<td>AGCTggagAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>S-A5</td>
<td>tgeaR</td>
<td>AGCTtgeaAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>S-A8</td>
<td>actaR</td>
<td>AGCTagtaAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>S-B7</td>
<td>gteR</td>
<td>AGCTagacAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>S-B20</td>
<td>gaatR</td>
<td>AGCTattcAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>S-F14</td>
<td>gegrR</td>
<td>AGCTacgeAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>S-F15</td>
<td>tggeR</td>
<td>AGCTgecaAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>S-G27</td>
<td>cgatR</td>
<td>AGCTatcgAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>S-G30</td>
<td>cttgaF</td>
<td>AGCTtcaagAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>S-H4</td>
<td>tcaecR</td>
<td>AGCTgttgaAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-B2</td>
<td>ctageR</td>
<td>AGCTgttagAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-B4</td>
<td>acaaaaR</td>
<td>AGCTttttagAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-B8</td>
<td>cttcaaaR</td>
<td>AGCTttggacgAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-B10</td>
<td>cttccaaR</td>
<td>AGCTtgaagAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-F1</td>
<td>gagaR</td>
<td>AGCTttactcAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-F4</td>
<td>atgcEssR</td>
<td>AGCTtagtgcatAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-F7</td>
<td>ctttcttR</td>
<td>AGCTaagcagAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-F8</td>
<td>atggatcR</td>
<td>AGCTgttcatAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-F16</td>
<td>aaaaagtR</td>
<td>AGCTaacttttagAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-G32</td>
<td>gaacttcR</td>
<td>AGCTgaagttcAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>T-H25</td>
<td>gacactcR</td>
<td>AGCTtaggttccAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
<tr>
<td>P-S69-2</td>
<td>gtegattR</td>
<td>AGCTaatcgagaAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT</td>
</tr>
</tbody>
</table>
### Table 5.4. Barcode adaptors used for RAD library construction (Bam HI)

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Barcode adapter</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-A4</td>
<td>ctccR</td>
<td>GATCggagAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>S-A5</td>
<td>tgcaR</td>
<td>GATCtgcaAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>S-A8</td>
<td>actaR</td>
<td>GATCtagtAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>S-B7</td>
<td>gtctR</td>
<td>GATCagacAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>S-B20</td>
<td>gaatR</td>
<td>GATCattcAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>S-F14</td>
<td>gegtR</td>
<td>GATCagecAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>S-F15</td>
<td>tggcR</td>
<td>GATCgccaAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>S-G27</td>
<td>cgatR</td>
<td>GATCategAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>S-G30</td>
<td>cttgaF</td>
<td>GATCcaagAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>S-H4</td>
<td>tcaacR</td>
<td>GATCggtgaAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-B2</td>
<td>ctageR</td>
<td>GATCgtegAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-B4</td>
<td>acaaaR</td>
<td>GATCtttgtAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-B8</td>
<td>cgtaaaR</td>
<td>GATCttgacgAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-B10</td>
<td>cttcaR</td>
<td>GATCtggagAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-F1</td>
<td>gagataR</td>
<td>GATCtacttcAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-F4</td>
<td>atgcttR</td>
<td>GATCagggatAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-F5</td>
<td>tatatttR</td>
<td>GATCaaaaataAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-F7</td>
<td>cttgcttR</td>
<td>GATCaaageagAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-F8</td>
<td>atgaaacR</td>
<td>GATCgttcatAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-F16</td>
<td>aaagtttR</td>
<td>GATCaaagtttAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-F17</td>
<td>gaattcR</td>
<td>GATCtgaattcAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-G32</td>
<td>gaacctcR</td>
<td>GATCgaagtttcAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>T-H25</td>
<td>ggaccttaR</td>
<td>GATCtaggctcAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
<tr>
<td>P-S69-2</td>
<td>tccR</td>
<td>GATCggagAGATCGGAAGAGCGTCTCGTGTAAGGAAAAGAGTGT</td>
</tr>
</tbody>
</table>
Figure 5.1. Correlation between relative root growth (RRG) and seed weight (g/100 seeds) of the evaluated germplasm (n=91, P< 0.05)

Figure 5.2. Correlation between relative root growth (RRG) grown in different Al concentration (n=91, P< 0.05)
REFERENCES


