MOLECULAR AND GENETIC INVESTIGATIONS OF ALUMINUM TOLERANCE IN WHEAT (TRITICUM AESTIVUM) AND MAIZE (ZEA MAYS)

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MOLECULAR AND GENETIC INVESTIGATIONS OF ALUMINUM TOLERANCE IN WHEAT (TRITICUM AESTIVUM) AND MAIZE (ZEA MAYS)

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Aluminum (Al) toxicity is the primary limiting factor of crop production on acid soils, which affect much of the earth's arable lands. Traditional plant breeding has been successful in improving crop tolerance to Al toxicity. However, further improvements in crop aluminum tolerance will require a deeper understanding of the underlying molecular and physiological mechanisms. Hence, the research described in this thesis focused on the genetic and molecular basis for aluminum tolerance in wheat and maize.

In wheat, two approaches were taken to attempt to isolate Al tolerance genes/proteins. A proteomics-based approach using wheat deletion lines around the major Al tolerance locus was unsuccessful in identifying Al tolerance proteins associated with tolerance. An alternate approach focused on the isolation of putative malate transporters of the CLC (chloride channel) channel family, as wheat Al tolerance is based on Al-activated root malate exudation. Eight representatives of this family were cloned from the Al tolerant wheat cultivar Atlas 66; however, genetic analysis revealed that none of these genes were linked to the wheat Al tolerance locus. The CLC genes exhibit widely different tissue and Al responsive gene expression patterns, suggesting different functions for some of these CLCs. Phylogenetic analysis revealed that the cloned TaCLCs represent only a portion of the entire CLC family present in wheat.

In maize, a quantitative statistical analysis of aluminum tolerance was conducted on recombinant inbred lines of the intermated B73 x Mo17 (IBM) population. Five quantitative trait loci (QTL) were identified using composite interval mapping as having a significant impact on Al tolerance. These five regions were not orthologous to genomic regions associated with Al tolerance in wheat, rice, sorghum, rye, or barley. In three QTL, Mo17, which has an extremely high Al-activated root citrate release, contributes the superior allele, and these QTL are likely to contribute to that mechanism. In the two QTL in which B73, which has virtually no citrate release, contributes the superior allele tolerance is likely conferred through an alternative mechanism.

BIOGRAPHICAL SKETCH

Paul was born in Davis, California July 12, 1974 to Don and Donna Mason. His parents weren't in the academic profession, but had an strong affinity for University towns. Along with Paul's sister Marita, they lived in Davis, Palo Alto, and Urbana, Illinois. Paul steadily developed early interests in conservation, nature, and global citizenship. His family hosted exchange students from Brazil, Japan, Korea, and France. His parents traveled extensively, particularly in repeated trips to the Soviet Union and Malawi. Paul's travels in his youth were mostly limited to the automobile, in which he visited 47 states, 9 Canadian provinces, and a taste of Mexico. More overseas travel would follow.

When it was time to set out on his own, Paul immediately returned west. Paul attended Lewis and Clark College, a school that, in the spirit of its namesake, encourages exploration, of both the natural and civilized worlds. His adventures there included sea kayaking the fourth clearest lake in the world, plenty of ultimate and disc golf, and a semester in Australia. Paul majored in biology, and became inspired by plants particularly by a volunteer trip to Santa Cruz Island with the Nature Conservancy and a course in plant physiology taught by Dr. Ed Florance. His passion for research was also piqued by an NSF Research Experience for Undergraduates program at the University of North Carolina, where he worked with Dr. James Raleigh. After receiving his B.S. Paul took a year-long tour of duty with the Portland institution that is McMenamin's, while preparing for his next adventure out east at Cornell.

Paul's time at Cornell was not just lab work. It also included several years with the Smashed Tomatoes soccer team, continued interest in games of all kinds, a trip to Ecuador, and the celebration of marriage to Alice. Surely there is more to come.

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CHAPTER ONE

Introduction and Literature Review

INTRODUCTION

Aluminum (Al) toxicity is the primary limiting factor of crop production on acid soils. Aluminum has pleiotropic toxic effects, but Al-induced inhibition of root growth is the most limiting to overall plant health and yield. Most crop plants tolerate Al by excluding the toxic Al ionic species from the rhizosphere. Breeding for Al tolerant lines has been quite successful in several crops, however molecular knowledge of resistance mechanisms is still quite limited. Genetics studies show simple inheritance of tolerance in wheat, but complex genetics in maize. Physiologically, Al-activated root organic acid exudation has been shown to be a tolerance mechanism based on root tip Al exclusion in a broad range of species. However, there are likely to be other mechanisms in maize and perhaps other species. The research presented here uses genetics, physiology, molecular biology, and several unique genetic stocks to better understand the genetics as well as physiological mechanisms of Al tolerance in wheat and maize.

Acid Soils and Aluminum Toxicity

Aluminum (Al) is the most abundant metal element in the earth's crust, comprising 8.3% by weight (Greenwood and Earnshaw, 1997). At low pH, several different Al speciesare solubilized into the soil solution, including the trivalent cation Al^{3+}, which is toxic to plant roots (Kochian, 1995). Al^{3+} rapidly inhibits root growth, probably initially by interfering with both cell growth and division. But aluminum also has many other demonstrated toxic effects, including alterations in the cytoskeleton (Blancafor et al. et al., 1998), a decrease in cytosolic Ca^{2+} concentrations

(Jones et al., 1998), and interference with signaling proteins (Jones and Kochian, 1995). The net result of limited root growth is an inhibited ability to take up adequate water and nutrients for good growth and yield.

Acid soils are a prevalent worldwide agronomic problem. Of the estimated 41,274,890 km^2 of potentially arable land, 19,863,000 km^2, or slightly less than half, are affected by Al toxicity (World Soil Resources Report 90, 2000). Manganese toxicity and phosphorus deficiency are secondary stresses often associated with acid soils (Kochian et al., 2004), but Al toxicity is the most significant limiting factor. Acid soils are especially prevalent in subtropical regions such as South America, West Africa, and Southeast Asia. In these regions poverty often magnifies the importance of growing staple crops cheaply. In addition, ameliorative procedures such as liming are less cost effective in such circumstances.

The mechanism of aluminum toxicity has been studied intensively in recent years due to its worldwide agronomic significance. Despite the abundance of aluminum in the earth's crust, plants are often spared the toxic effects because aluminum is largely insoluble in solutions above pH 5.0. In acidic soils, toxic forms of aluminum become soluble, and can severely inhibit root growth, leading to whole plant complications. Many soils in the tropics and sub-tropics are naturally acidic. In addition, some agricultural practices, such as excessive fertilization, can cause soil acidification. The phenomenon of acid rain also abets the continuation of acid soil issues and aluminum toxicity.

Understanding the mechanism of aluminum toxicity requires knowledge about the nature of aluminum in acid soil conditions as well as information about how aluminum ions interact with plant roots. Aluminum has a complex chemistry in that it can interact with numerous compounds to form complexes of various solubilities and toxicities. The nature and abundance of aluminum complexes is very sensitive to pH

and to the activities of other ions. Thus, determining which species of aluminum exist is difficult, even in something as straightforward as a solution of known composition. Furthermore, the pH of the cytoplasm is inevitably higher than the surrounding solution in acid soils, while the apoplasm falls in between and may harbor a pH gradient. An aluminum ion translocating between these various regions may change forms or complexes in response to the changing pH. These sensitivities have made it quite difficult to determine which aluminum species are present in soil or nutrient solution, which are toxic to plants, and which may be transported across the plasma membrane. Nevertheless, experimental evidence suggests many aluminum complexes are not toxic to plants (e.g. aluminate, aluminum hydroxide, aluminum sulphate, aluminum phosphate, and aluminum complexed with various organic acids). Meanwhile, a form of aluminum known as Al_{13} ($AlO_4Al_{12}(OH)_{24}(H_2O)_{12}^{7+}$) is extremely toxic. Computer programs such as GEOCHEM-PC are often used to predict specific activities in solution based on binding constants (Parker et al., 1995). Attempts to determine intracellular aluminum content and speciation have met with some difficulty.

Aluminum Uptake

Al^{3+} has many potential cellular and extracellular targets. Due to interest in determining the primary toxicity targets, the study of aluminum uptake into roots has been a focal point of many studies on aluminum toxicity and aluminum tolerance. A common question or assay is to determine how much aluminum has been taken up into the root apoplast or root symplast of sensitive and tolerant lines at different levels of applied aluminum. One technique for measuring total aluminum content in a tissue is graphite-furnace atomic absorption spectroscopy (GFAAS; Zhang et al., 1989; Delhaize et al., 1993a). Another method uses the ^{26}Al radioisotope (Nagata et al., 1991; 1992). Using these methods, total aluminum can be approximated. However,

most aluminum in the roots is tightly bound to negative charges in the Donnan Free Space of the apoplasm. Many studies have used one of several desorption methods to attempt to remove apoplastically bound aluminum and thus to assay intracellular aluminum in the remaining fraction. Recent studies with the single cell macro-alga *Chara corallina*, where the cell wall can be physically removed from the cytoplasm, have shed some light on these desorption techniques and the accuracy of claimed intracellular aluminum measurements (Rengel and Reid, 1997). Using the Al desorption agent citrate, and a 2 hour desorption period, over 5% of the cell wall aluminum still remained. Also, very sensitive readings of cytosolic aluminum content revealed that intracellular uptake occurs at low rates (<0.3 nmol g^{-1} FW h^{-1}), and that a vast majority of the aluminum is bound in the cell wall relative to the cytoplasm (>10,000X before desorption; >20X after desorption). While *Chara* is a quite different organism from the grasses where most aluminum toxicity work is focused, its growth response to aluminum and cell wall electrical properties are similar to wheat (Rengel and Reid, 1997). Another study on aluminum uptake in *Chara* (Taylor et al., 2000) also indicated that uptake into the cytoplasm occurred at rates 1000-fold less than apoplastic accumulation. This cytoplasmic uptake is followed by vacuolar sequestration. Thus, the overall picture of aluminum uptake in non-accumulator species is of a high percentage of aluminum accumulating in the cell wall apoplasm, with small amounts transported to the cytoplasm and vacuole. Initial putative aluminum toxicity targets in the cytoplasm may be sensitive to these low levels of aluminum. However, the large concentrations of aluminum in the apoplasm definitely impact root growth and physiology.

Wherever the site of aluminum toxicity is, initial effects of aluminum toxicity are detectable on the order of minutes. In protoplasts, effects are observable in a few minutes. In isolated membrane patches, electrophysiological effects are instantaneous.

Even at the whole plant level, responses to aluminum such as root growth and malate release in wheat are detectable after only 15 minutes (Delhaize *et al.*, 1993b; Jones and Kochian, 1997). Using microscopic techniques to monitor root growth with a high degree of temporal resolution, Jones and Kochian (1997) showed that Al began inhibiting root growth in an Al sensitive wheat cultivar within 15 min after exposure to Al in hydroponic media. Other responses to aluminum toxicity detectable in the first half hour include reduced Ca^{2+} uptake, blockage of plasma membrane embedded Ca^{2+} channels, decreased Mg^{2+} and NO_3^- uptake, reduced K^+ efflux, and accumulation of callose. It is possible that all of these effects could be initiated by the presence of extracellular aluminum.

Effects on Intracellular Signaling

One intriguing potential aspect of aluminum toxicity is the effect on signaling pathways. Ca^{2+} is involved in multiple signaling pathways as a second messenger (Sanders et al., 2002). Aluminum has been shown to affect Ca^{2+} homeostasis in *Arabidopsis thaliana* root hairs (Jones *et al.*, 1998). In these experiments the dye Indo-1 was used to visualize intracellular Ca^{2+}. Cells remained living, as evidenced by cytoplasmic streaming. In wild type and sensitive lines, Al-induced increases in the cytosolic concentrations of Ca^{2+} were observed. Another common signaling pathway involves the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2). In response to various stimuli, phospholipase C (PLC) cleaves PIP_2, releasing inositol 1,4,5-triphosphate (IP_3) into the cytosol while the other product, diacylglycerol (DAG) remains in the membrane. Jones and Kochian (1995) observed the specific effects of aluminum on the IP_3 signal transduction pathway. This study used the sensitive wheat cultivar Scout 66. Viability was confirmed by observed respiration throughout the experiment. H_2O_2 was used as a stimulator of a transient IP_3 response. Aluminum application inhibited this response both in whole root application and in

microsomal membranes derived from wheat roots. The effect of this response at the whole root level indicates that it may be initiated by apoplastic aluminum. In addition, $AlCl_3$ and Al-Citrate seemed to have a specific inhibitory action on phospholipase C. Other work on Al-membrane interactions has suggested that aluminum has the strongest affinity for the membrane phospholipid PIP_2 of several lipids studied (Jones and Kochian, 1997). Thus, although aluminum has many long-term effects on root growth and physiology, there may be certain specific initial effects. Knowing that aluminum may have effects on signaling pathways, we may next ask: What cellular processes are altered by these disturbed signaling pathways?

Cytoskeletal Effects

Work on aluminum toxicity has focused on many possible sites of cellular injury. Two recent works on the effects of aluminum on the cytoskeleton are compared and analyzed in this chapter. A common measure of aluminum toxicity (or tolerance) is root growth. The cytoskeleton, along with the cell wall, is one of the major players controlling root growth at the cellular level. These reports reveal the possible key role of cytoskeletal alterations in the response to toxic levels of aluminum.

As we have learned from work on *Chara* cells and other experiments discussed above, it is likely that of the total aluminum to which a root is exposed, very little actually enters the symplasm. While aluminum has been shown to initiate microtubule polymerization *in vitro* (MacDonald *et al.*, 1987), we can not be sure whether specific aluminum species have the opportunity to influence microtubules or microfilaments in such a direct manner *in vivo*.

Blancaflor *et al.* (1998) examined the effects of aluminum (50 μM in 200 μM $CaCl_2$) on root morphology and cytoskeletal structure. They used indirect immunofluorescence to visualize microtubules (rat anti-yeast tubulin, with a

secondary antibody labelled with fluorescein isothiocyanate) and actin microfilaments (mouse monoclonal antibody against phalloidin-stabilized pea actin, with a secondary antibody labelled with fluorescein isothiocyanate). Sections were taken from aluminum-treated maize (cultivar Merit) primary roots in the elongation zone. Light micrographs revealed swelling and tissue disruption as gross morphological effects of the aluminum in simple salt solutions. Aluminum treatment resulted in alterations in the microtubule patterns in the cortex and stele. Al exposure caused transverse microtubule patterns to be altered to a random microtubule orientation in the root stele and inner cortex, but not in the outer cortex. However, evidence in this paper was only presented for microtubule pattern disruption after 3 hours of continuous treatment, much longer than is required even for the gross physiological change of root growth inhibition. The authors then proceeded to test two cytoskeleton treatments on the roots to see if aluminum altered the microtubule response. Auxin reorients MTs from a lateral to a longitudinal alignment and the authors found that aluminum treatment prevented this reorientation. Cold treatment normally results in microtubule depolymerization, with no organized repolymerization. Aluminum pretreatment also appeared to inhibit cold-induced depolymerization as the microtubules were still aligned in a lateral fashion. In parallel experiments, actin microfilament patterns were examined in control and aluminum treated roots. No substantial qualitative differences were observed until 6 hours of Al exposure.

Sivaguru *et al.* (1999) revisited the issue while focusing on a more specific region of the root. Sivaguru et al. used the maize cultivar Lixis (an aluminum sensitive cultivar), and 90 μM activity of Al^{3+} in a full nutrient solution. The monoclonal mouse anti-chick brain alpha-tubulin and a secondary antibody labeled with fluorescein isothiocyanate were used to label microtubules. They used monoclonal mouse anti-chicken gizzard actin and a secondary antibody with

fluorescein isothiocyanate to visualize actin microfilaments. This study focused on the distal part of the root transition zone (DTZ). The transition zone refers to the region just behind the root tip where a transition from primarily cell division to primarily cell elongation as the primary contributor to root growth occurs. After 1 hour of aluminum treatment, the authors observed disintegration of microtubules in the outermost cortical file. It was noted that file-specific microtubule disintegration is observed with ethylene treatment. For comparison with the Blancaflor *et al*. (1998) paper, Sivaguru *et al.* (1999) also looked at microtubules in the root elongation zone, which were found to remain stable in a normal transverse orientation. Microtubule disintegration in the transition zone continued and spread with longer aluminum treatments (up to 12 hours).

A comparison of the results obtained by Sivaguru *et al.* (1999) with Blancaflor *et al.* (1998) reveals several apparent contradictions. The primary effect of aluminum on microtubules observed by Blancaflor and colleagues was microtubule stabilization. However, Sivaguru and coworkers observed a disintegration of microtubule organization upon aluminum exposure, which increased with time. How could such diametrically opposed results be observed? We must first remember that identical experimental conditions were not used. First, different genetic materials were used. The Lixis line used by Sivaguru *et al*. was previously shown to be Al sensitive (Horst et al. 1997). No categorization of the relative Al tolerance of the maize cultivar Merit, used by Blancaflor *et al.* was given, but the observed 50% growth inhibition with Al exposures of between 15 and 20 μM Al in a simple salt solution categorizes it as an Al sensitive maize line. Different cultivars varying in aluminum tolerance and other traits may react differently to the various treatments employed. Blancaflor *et al.* (1998) studied effects of aluminum in the elongation zone, because elongation is suspected to be the first growth step inhibited in Al toxicity. On the other hand, Sivaguru and

coworkers focused on a very specific region of the root transition zone that they had previously determined to be the site of maximal aluminum sensitivity (Sivaguru and Horst, 1998). Furthermore, Blancaflor *et al.* used a simple salt solution, while Sivaguru *et al.* used a full nutrient solution. Since the length of the experiments was short (3-4 days) this should not affect plant nutrient status, although it does affect the speciation of aluminum. Both papers calculated the theoretical specific activity of Al^{3+}. However, the specific activity needed for a comparable response will vary with the aluminum tolerance of the line.

With so many discrepancies between the experiments, we are wise to make conclusions with some caution. However, the universal nature of many plant processes, and especially those within a species permits us to draw some conclusions. The most significant differences in the experiments concerned the cultivars used, and the aluminum levels applied, as they relate to the toxicity level induced in that cultivar. That is, does a specific Al^{3+} activity of 90 μM applied to cv. Lixis invoke the same physiological response as does 27.8 μM Al^{3+} applied to Merit? Direct comparable measurements would be the best way to determine this, but Lixis had >60% growth inhibition in 25 μM Al in full nutrient solution (with only some fraction of that present as Al^{3+}). It follows that 90 μM free Al^{3+} activity would be very toxic to Lixis. Meanwhile, growth inhibition of Merit increased over the time observed, reaching about 75% inhibition by 12 hours, and with growth completely inhibited after 24 hours. While direct comparison of these treatments is impossible, both cultivars were exposed to extreme Al toxicity.

The other major consideration is the root zone under study. As the cells develop from the meristem throughout the transition zone to the elongation zone, they undergo numerous physiological, biochemical, and morphological changes. Given the very specific localization of the aluminum toxicity response to the terminal 3-5 mm of

root tips in maize, these cells will almost certainly be undergoing a change in their response to toxic levels of aluminum. It is possible that aluminum induces microtubule breakdown in transition zone cells while simultaneously inducing microtubule stability in elongation zone cells.

Several of these emerging facts can now be aligned. We know from *Chara* cells that nearly 100% of aluminum is bound in the cell wall. However, a small fraction of the total aluminum does quickly enter the symplasm. On the other hand, there are several possible effects of aluminum on signaling pathways. We know that aluminum affects Ca^{2+} homeostasis. We know that aluminum binds tightly to the plasma membrane and interferes with IP_3 signaling. Also, new work has shown the significance of a continual cytoskeletal-PM-cell wall network for structure and communication (Baluska et al., 2003). Putting all these clues together, we can form a sensible speculative hypothesis about the action of aluminum toxicity. Because little aluminum actually enters the symplasm, the direct effect of aluminum on microtubule formation and stability may be a moot point. However, if observed microtubule disintegration is not artifactual then it may be a direct argument against aluminum entry into the cell, in light of aluminum's *in vitro* effects on microtubule formation. Since both Ca^{2+} and IP_3 signaling are known to affect cytoskeletal regulation, and aluminum is known to affect both Ca^{2+} and IP_3 signaling, it follows that aluminum could conceivably affect cytoskeletal regulation. Since both signaling pathways have been altered by aluminum *in vivo*, it is also conceivable that these interactions can be effected across the plasma membrane. Thus, one can suggest that during the toxicity response, aluminum might be interacting at the cell wall and/or plasma membrane to alter signaling pathways, which can result in varying cytoskeletal responses, depending on the cell state.

Relevance of Al Toxicity to Enhancing Tolerance

Aluminum toxicity has rather dramatic and wide-ranging physiological effects. At the whole root level, root morphology and root growth are altered. Root growth is significantly inhibited. Roots become thickened, cells expand, and root surfaces become damaged (Blancaflor et al., 1998). In addition to the toxicity symptoms discussed previously, Al exposure causes the creation of reactive oxygen species, which cause further damage to cellular components (Richards et al., 1998; Boscolo et al., 2003). However, the toxicity of aluminum is not indiscriminate, and does not kill all of the cells of the root, as respiration is able to continue.

Ultimately, though, efforts at developing more aluminum tolerant crops may not depend on a precise knowledge of the mechanism of aluminum toxicity. Most aluminum tolerant cereal crops seem to use a mechanism of exclusion from the root apex, thus potentially preventing aluminum from reaching its sites of toxicity. This exclusion is not absolute, however, and it is likely that lower levels of aluminum still reach sites of toxicity. The aluminum may then simply be in lower concentrations than is necessary for toxicity or accessory tolerance mechanisms (such as binding and sequestration) may be able to process this aluminum. If whole root zone exclusion turns out to be the primary tolerance mechanism, it will simplify dealing with the agricultural goals, bypassing the complexities of aluminum toxicity.

The Genetics and Molecular Genetics of Aluminum Tolerance

In order to understand the molecular mechanism of Al tolerance, it is first necessary to understand the genetics of Al tolerance. Genetic studies can reveal the number of genes important for Al tolerance, the range of allelic phenotypes, the genomic locations of those genes, and the types of genic interactions which occur. This knowledge can guide both basic and applied research goals. This review focuses

on maize and wheat, the two crops involved in this study. However, information gained from other cereal crops and additional plant species is included and can be useful for assessing the conservation of Al tolerance mechanisms.

Studies on the genetics of Al tolerance are dependent on the assay for Al tolerance. Good assays are fast, accurate, reproducible, and provide data relevant to field studies. Efforts have been made to develop lab assays for Al tolerance which are easy to perform yet accurately predict field tolerance. An obvious starting point is soil-based assays in lab conditions. However, characteristics which are easily observable in these studies, such as shoot growth, have not been shown to always correlate well to Al tolerance. Root growth has been the most common measure of Al tolerance. Solution-based studies make repeated measurements of root length much easier. Lafever and Campbell (1978) assayed the response to Al in terms of root length, leaf length, and the number of roots per plant. They determined that root length was the characteristic most related to Al tolerance. An alternate assay for Al tolerance is the hematoxylin staining method. Hematoxylin is an Al-binding dye and when it reacts with root associated Al, a blue color is produced, revealing the pattern of Al uptake. Al tolerant plants where tolerance is based on Al exclusion will stain a lighter color because they have less Al in their root tips, which leads to less severe toxicity symptoms. This method has the advantage of speed and simplicity, especially for large samples. However, the utility of the dye for differentiating tolerance levels has a limited range, and appropriate Al levels must be empirically determined. Ideally, two or more methods should be used simultaneously to confirm the tolerance phenotype, since there may be differences depending on the assay used (Riede and Anderson, 1996).

Further complicating genetic analysis of Al tolerance is the complex chemistry of Al. Al is capable of forming numerous complexes with other ions and with water.

While some studies have used soil-based growth conditions (Johnson et al., 1997), it is difficult to control Al^{3+} activity in soil. Therefore, many researchers have chosen to use solution cultures to assay Al tolerance. Even in solution culture the complex chemistry of Al often makes it difficult to determine the exact activity of Al^{3+}. This complicates comparisons between studies using different solution formulae. The chemical prediction program GEOCHEM-PC has been used to develop theoretical predictions for the activities of ions in solutions of known compositions (Parker et al., 1995). However, variances in nutrient contents may affect the response to Al in ways other than direct alterations of Al^{3+} activity.

Much of the interpretation of genetic data depends on the characterization of the Al tolerance phenotype as a qualitative or quantitative trait. Depending on the experiment, the trait can be considered either way. Al tolerance can be thought of as a quantitative trait. At a given Al exposure (Al activity x time) the inhibition of root growth (as measured by relative root growth [RRG]) can have a continuous spectrum of variation. And indeed, a wide range of tolerances are seen in wheat and maize using this measure. Alternatively, the tolerance of a plant can be determined by the Al activity necessary for 50% root growth inhibition. Here, too, a continuous variation is possible and many values are observed. Even when tolerance is scored by hematoxylin staining a wide range of tolerance levels can be seen (Polle et al., 1978). However, many genetic studies choose a level of Al exposure which maximizes the phenotypic differential between the parents. The parental phenotypes under these conditions are used to define discrete tolerant and sensitive phenotypic classes. Under these conditions, F_2 segregation data are often interpreted in terms of these distinct classes, and are thus qualitative.

Wheat Genetics

Aneuploid wheat lines

Before delving into the genetics of Al tolerance in wheat, it is important to understand the aneuploid genetic stocks, which are an important tool in wheat genetics research. The hexaploid genomic structure of wheat creates some difficulty for most traditional genetic analyses. However, it simultaneously permits the viability of aneuploid lines that would be lethal or infertile in diploid species. Aneuploid lines are missing one or more entire chromosomes or some fraction of one or more chromosomes. Aneuploid lines may also have extra copies of certain chromosomes, but those are less useful for genetic studies. Because of the hexaploid duplication of genes, many of the deletion stocks remain viable and fertile. The deletion stocks provide unique tools for quickly mapping the gross physical location of genes, gene products, and physiological traits associated with those genes.

All of these lines were created in the wheat cultivar Chinese Spring. Generally, they are formed by making an unnatural genetic cross which disrupts normal chromosome pairing, and then sorting out the karyotypes of the progeny using cytogenetics. Initially, these lines were created by crossing haploid wheat (n=21) with normal diploid wheat (2n=42; Sears, 1939). Progeny from this cross included monosomics (one copy of one chromosome; 2n-1=41) and trisomics (three copies of one chromosome; 2n+1=43). Progeny from trisomic lines sometimes resulted in tetrasomic (four copies of one chromosome; 2n+2=44) aneuploids. Progeny from monosomic lines were occasionally nullisomic (one chromosome completely absent; 2n-2=40). Additionally, monosomic lines occasionally gave rise to telosomic (chromosome containing only one arm) and isochromosomic (chromosome contains two duplicate copies of one arm arranged in mirror-image orientation around the centromere) lines (Sears, 1944).

A plant in which the telosomic condition has become homozygous is referred to as ditelosomic. There are 42 chromosome arms in the haploid component of hexaploid bread wheat. Ditelosomic stocks have been developed representing 35 of those chromosomes. The remaining lines are sterile or lethal in the homozygous state. The ditelosomic lines provide a powerful mapping tool for the difficult hexaploid genomic structure of wheat. Southern analysis using ditelosomic lines as the template with the appropriate cDNA probes can quickly identify the chromosome arm where that gene is located. Of course, due to gene duplication and the similarity of homoeologous genes, the result may be ambiguous. The ditelosomic lines have also been used to determine which chromosome arms harbor genes important for the primary wheat Al tolerance mechanism, Al-activated root malate exudation (Papernick et al., 2001).

More recently Endo (1988) developed another method for creating aneuploid stocks. This method, involving the monosomic addition of a gametocidal chromosome from a related species (*Aegilops cylindrica* L. or *A. speltoides* Tausch), results in deletion stocks with only partial deletions from one or a few chromosome arms. The presence of the gametocidal chromosome is usually lethal to gametophytes normally lacking the chromosome. However, in Chinese Spring, the effect is milder, and these unique aneuploids sometimes result and once homozygous are stably transmitted. These partial deletion lines can be used in similar ways to the ditelosomic lines, but their smaller deletions increase their utility considerably. When investigating the physiological effects of a deletion, the confounding effects of large deletions are minimized. For molecular analyses, the number of genes in the area of interest is decreased. Additionally, using a combination of two deletion stocks with close breakage points can further narrow the genomic region under consideration.

Recent molecular studies have elucidated what is happening with these deletions at the molecular level and have added to the initial cytological characterizations. Sequencing of newly formed deletions reveals that chromosome breakage is followed by partial duplication and inversion of genic DNA along with insertion of new DNA (Tsujimoto et al., 1997). The partial deletion chromosome is then capped with new telomeric repeats by a telomerase. Lili et al. (2002) mapped 101 deletion lines using 526 EST clones. They discovered that many deletion lines harbored secondary deletions that were undetectable cytogenetically. These secondary deletions are relatively minor, but important to consider when interpreting results.

Aluminum Tolerance Genetics in Wheat

Most genetic studies of Al tolerance in wheat have pointed to a single gene controlling the majority of the tolerance. Kerridge and Kronstad (1968) analyzed F1 and F2 individuals from a cross between the Druchamp (tolerant) and Brevor (sensitive) varieties of wheat for Al tolerance and their results indicated that tolerance was due to a single dominant gene. Subsequently, Riede and Anderson (1996) analyzed recombinant inbred (RI) lines from a cross between BH1146 (tolerant) and Anahuac (sensitive) and again found that a single gene controlled Al tolerance. Riede and Anderson also conducted genetic mapping of the Al tolerance locus, which they named Alt_{BH}, using restriction fragment length polymorphism (RFLP) markers, and identified two markers linked to Alt_{BH}. The marker Xbcd1230 mapped 1.1 cM from Alt_{BH} while Xcdo1395 mapped 10.2 cM from Xbcd1230. Treating the root growth data quantitatively, Xbcd1230 explained 85% of the variability in the phenotype. These markers were found to map to chromosome arm 4DL, as determined by probing DNA from nullisomic-tetrasomic and ditelosomic stocks of Chinese Spring (Riede and Anderson, 1996). Further evidence that chromosome 4DL holds the only major Al tolerance gene comes from a study focusing on the molecular mapping of the Al

tolerance locus, Alt2, in Chinese Spring (Luo and Dvorak, 1996). Disomic substitution lines with D genome chromosomes from Chinese Spring substituted for either the A or B genome homoeologous chromosome from the sensitive cultivar Langdon revealed that only chromosome 4D contained loci beneficial to Al tolerance. RFLP mapping using 4D/4B recombinant lines mapped Alt2 to several tightly linked markers on 4DL, including Xpsr914, Xpsr1051, and Xmwg2180 (Luo and Dvorak, 1996). Genetic analysis of a cross between a tolerant 4D/4B recombinant line and the sensitive Langdon revealed that this is a single dominant gene.

Some genetic studies using very Al tolerant genotypes of wheat have revealed the existence of possibly two Al tolerance genes in these tolerant lines. (Camargo, 1981; Berzonsky, 1992). Even in these cases, the effect of the single locus on chromosome 4DL dominates the variation in tolerance.

Wheat Molecular Genetics

Because wheat genetic studies have indicated that Al tolerance can be conferred by one major Al tolerance gene, cloning of this gene became a major research priority. One of the first ways researchers tried to isolate the Al tolerance gene in plants was by looking for Al-induced genes. This method has been tried both at the levels of gene transcription (comparing mRNA levels) (see, for example, Snowden et al., 1993; Richards et al., 1994, 1998; Hamel et al., 1998) and protein expression (Delhaize et al., 1991; Rincon and Gonzales, 1991; Picton et al., 1991). None of these experiments successfully resulted in the identification of an Al tolerance gene. It appears that the major weakness in this approach, at least for wheat where much of the work has been done, is that Al tolerance genes are probably not Al inducible, or they are obscured by other genes which are upregulated to a greater extent. A host of studies have identified genes induced by exposure to Al in wheat (Snowden et al. 1995; Hamel et al. 1998; Hamilton et al. 2001; Sasaki et al. 2002) and

other species (Ezaki et al., 1995; Richards et al., 1998; Drummond et al., 2001; Watt, 2003). Those genes that have been studied in both sensitive and tolerant lines are induced in both, reducing their possible role in differential tolerance (Hamel et al. 1998; Sasaki et al. 2002). Of those that have been studied under other stresses, most or all are not specifically induced by Al (Snowden et al. 1995; Sasaki et al. 2002). Some aluminum induced wheat genes have been overexpressed in *Arabidopsis thaliana* or inducibly expressed in yeast. In neither of these cases did the genes confer significant increases in Al tolerance (Ezaki et al. 1999; Ezaki et al. 2000). Taken together, these results cast doubt on the idea that the major Al tolerance mechanism in wheat is activated through inducible gene expression.

An alternative approach, based on the idea that the Al tolerance gene may have a constitutively higher expression in tolerant lines than in sensitive lines, may have led to the successful isolation of the first Al tolerance gene in plants. An aluminum-activated malate transporter was isolated very recently from wheat (*ALMT1*; Sasaki et al., 2004). It is very tightly linked to aluminum tolerance in segregating populations, suggesting that it may be the gene underlying the *Alt1* locus. *ALMT1* functions as a malate transporter when expressed in *Xenopus* oocytes, and when expressed in rice plants and tobacco-suspension cells, an Al-activated malate release is conferred. *ALMT1* is a novel transporter, not related to any known membrane transporter families. Therefore, *ALMT1* appears to be both the major aluminum tolerance gene in wheat and the plasma membrane malate transporter.

Maize Genetics

In contrast to wheat, where a majority of data supports the major role of a single dominant gene conferring Al tolerance, results of genetics studies in maize have been less clear. Fewer studies have been performed in maize than wheat, finding

evidence for dominant genes, recessive genes, additivity, and heterosis with regards to maize Al tolerance. This collection of evidence suggests that multiple genes contribute to Al tolerance in different maize genotypes.

Rhue et al. (1978) performed one of the early studies on Al tolerance in maize. They surveyed a selection of maize inbreds, hybrids, and composites, and found a wide range of tolerance. Their Al tolerance screen measured the amount of Ca or Mg needed to ameliorate Al toxicity in the nutrient solution as detected by root growth. The authors found distinct classes of tolerance in the F_2 and BC populations, which adhered to 3:1 and 1:1 tolerant:sensitive ratios, respectively. Thus, they inferred the presence of a single gene with a multiple allelic series. This is plausible as the major contribution to Al tolerance. However, no raw data were presented, and it is unclear, therefore, if other genes may have had minor effects in this study. The study may also be criticized because using a strict qualitative screen on a quantitative trait may result in lost information.

Magnavaca et al. (1987) studied the genetics of Al tolerance in several generations derived from American maize inbred lines. They used the root growth metric, relative seminal root length (RSRL) for their Al tolerance assay. RSRL can be used when plants are transferred from a control solution to an Al-containing treatment solution. RSRL is the final seminal root length divided by the initial seminal root length at the time of transfer to the Al treatment solution. This method has been shown to correlate reasonably well with other assays such as the hematoxylin staining method (Cançado et al., 1999). However, theoretically, it may not be the best assay of Al tolerance, as it depends heavily on the growth of roots in nutrient solution, which may be impacted by unrelated genes. Magnavaca et al. (1987) created F_1 hybrid populations from six parental pairs. Four of these were from parents with a distinct difference in tolerance levels, while two were from crosses of two sensitive parents.

Only three of the six F_1 populations were found to be tolerant. Only one of the four F_1 populations deriving from a tolerant x sensitive cross was found to be tolerant. The lack of complete dominance implicates incomplete penetrance, epistasis, or other effects. This implies that complete dominance was not observed in these maize crosses. Statistical analysis of the variance in these crosses by Magnavaca and colleagues confirmed the significant contribution of additivity as well as dominance, with additive gene effects accounting for most of the genetic variation. This supports the concenpt of multigenic inheritence of Al tolerance in maize.

Especially interesting is the effect of inbred line A554 on its hybrid crosses. The phenotypic index used in this study was relative seminal root length (RSRL; the length of the seminal root after a period of treatment divided by its initial length). Higher numbers indicate greater growth under aluminum treatment, after standardizing for the untreated root growth of that individual. A554 was classified as sensitive with an RSRL of 1.24, yet it increased the Al tolerance of many lines it was crossed with, including more tolerant lines. For example, when crossed with the inbred line W117, which has an RSRL of 1.35, the resulting F_1 had a population mean of 1.48, higher and more tolerant than either parental inbred. A554 also contributed to increases in tolerance in crosses with the tolerant lines A635, C103, C16, and H84. While some of this benefit may be due to heterosis, this suggests that A554 contains genes which are beneficial in terms of Al tolerance when expressed with other tolerance genes.

The F_2 frequency distributions published in Magnavaca et al. (1987) did not show discrete classes. Instead, normal and skewed distributions were observed. This also implies the presence of multiple genes conditioning Al tolerance. Further, the F_2 RSRL means from tolerant x sensitive crosses never matched the tolerant parental line.

Therefore, specific combinations of genes present in the tolerant parent may need to be present to express the maximal level of tolerance.

Together, these data suggest that multiple genes may be involved in Al tolerance in maize. There are certainly multiple alleles of tolerance genes conferring a range of tolerances. Also, simple dominance may not explain fully the nature of the genic interactions involved in tolerance. The conflicting evidence regarding the complexity of the trait invites the further pursuit of experiments aimed at clarifying the complexity of Al tolerance in maize and chromosomal locations of genes which confer Al tolerance.

Quantitative Trait Analysis

In maize, where the more genes regulate aluminum tolerance and there is less consensus about regions important for aluminum tolerance, no putative Al tolerance genes have been cloned. For these reasons, quantitative trait locus (QTL) mapping is an excellent tool to shed light on the quantitative genetics of Al tolerance in maize and to begin the molecular search for the genes underlying Al tolerance in maize. Given that Al tolerance is usually assayed on a quantitative scale, it makes sense to analyze Al tolerance in a quantitative manner. QTL mapping is a method used to do genetic mapping of loci contributing to the expression of a quantitative phenotype. QTL analysis was developed for the purpose of studying quantitative traits in which phenotype is influenced by many genes. In this situation, traditional genetic techniques have often been unable to detect contributing genes, especially those with minor influences. QTL mapping uses statistical techniques such as analysis of variance (ANOVA) to detect regions of the genome that contribute to a quantitative trait. Whereas traditional genetics looks for cosegregation between a locus and a trait, QTL analysis looks for the net influence of a locus on a quantitatively variable trait. The r value representing that correlation can be used to determine the proportion of a

phenotypic effect which is accounted for by the presence of that locus. This property also makes QTL analysis useful when approaching a quantitative trait which has been treated as a discrete/qualitative trait. In this case, information about the proportion of phenotypic variation contributed can be ascertained, even for a major gene.

Al tolerance studies which support the presence of a single major dominant gene in their phenotypic ratios still show variance within the phenotypic classes. This variance implies that other genes have some, although relatively small, effect on conditioning the tolerance phenotype. So QTL studies have utility in cases of both polygenic and monogenic inheritance.

Fewer studies have addressed the genetics of Al tolerance and the mapping of Al tolerance genes in maize than in wheat. Thus, more information on the genetic complexity and mapping locations of genes important to Al tolerance in maize are needed to move forward with molecular cloning strategies as well as to understand the mechanistic complexity.

Recombinant Inbred Lines (RILs) are permanent mapping populations which can be used to isolate genotypic variability from environmental or experimental variability (Burr and Burr, 1991). RILs are derived from a mapping population such as an F_2 population by using single seed descent to produce line homogeneity. RILs can be tested in replicate experiments and under varying experimental conditions. In this manner, natural variation can be isolated from genetic variation. In addition, the genetically identical RILs permit multiple assays, including destructive assays, and genotypic data to be gathered on the same lines. This allows replicates to be performed on genetically identical plants. Variation within a specific line/treatment combination is assumed not to be due to genetic differences.

A new type of RIL population was developed recently which further expanded the genetic power of this approach. The Intermated B73 x Mo17 (IBM) mapping

population from B73 and Mo17 was created by intermating members of the F_2, F_3, and F_4 generations. This repeated intermating allows much more recombination to take place than is possible in an F_2 generation. The RILs developed from this population are more densely populated with recombinant sites and are much more powerful for genetic mapping. Because the RILs are selfed to homogeneity, they are still "permanent" and molecular mapping of the IBM members can be used in the same manner as those of a traditional RIL set.

Genetics of Aluminum Tolerance in Cereal Crops and Comparative Mapping

Al tolerance has been studied genetically in most major cereal crops, including wheat, maize, rice, rye, barley, and sorghum. Comparisons of the inheritance of Al tolerance and of the genetic loci which confer tolerance help determine whether similar or varied genetic mechanisms exist in these related plants, and can help guide map-based cloning efforts, especially in the less-studied species. The most fundamental genetic question is: How many genes control aluminum tolerance? Al tolerance is relatively simple genetically in wheat, rye, barley, and sorghum. In contrast, Al tolerance is genetically complex in maize and rice. This influences the kind of research avenues which will be successful with each species. It also provides clues to the complexity of the physiological mechanisms in place.

The species with simple genetics for Al tolerance probably have tolerance genes orthologous to those in species with complex genetics. The major Al tolerance gene in wheat, barley, and rye are all on orthologous loci corresponding to a region of rice chromosome 3, which also harbors an Al tolerance QTL (Kochian et al., 2004). There is also a cluster of Al tolerance QTL identified in several studies on rice chromosome 1, which is orthologous to one of the Al tolerance QTL from maize and the single major Al tolerance locus in sorghum (Kochian et al., 2004).

When examining diverse germplasm in species with quantitative genetics, commonality of tolerance genes may be rare. Five studies have been published examining the quantitative genetics of Al tolerance in rice (Wu et al., 2000; Nguyen et al., 2001, 2002; Ma et al., 2002; Nguyen et al. 2003). The aforementioned region of rice chromosome one was the only region determined to be important for Al tolerance in all five studies. In contrast, when all the QTL from the five studies were summarized, there were 27 total regions identified as important for Al tolerance in at least one of the studies. The five rice studies encompassed a wide variety of genetic backgrounds, including the subspecies *indica* and *japonica*, and a wild relative *Oryza rufipogon*.

In maize, where the quantitative inheritance has been repeatedly supported, only one study has examined the quantitative genetics of Al tolerance. Ninamango et al. (2003) mapped QTLs for Al tolerance in maize using two Brazilian maize inbreds, L53 (Al sensitive) and L1327 (Al tolerant). They used two methods for analyzing their data. Using multiple regression analysis, they identified nine QTLs, while composite interval mapping revealed five Al tolerance QTL.

Multiple genes also appear to contribute to aluminum tolerance in *Arabidopsis*. Two studies have examined the quantitative genetics of aluminum tolerance in *Arabidopsis* (Kobayashi et al., 2002; Hoekenga et al., 2003). Kobayashi et al. detected two significant QTL via composite interval mapping (CIM) on chromosomes one and five. Hoekenga and colleagues also detected two QTL via CIM. One was a shared locus on chromosome one. The other was on chromosome five. Each study also detected other significant loci using less stringent statistical tests. Surprisingly, none of these other loci overlap between the two studies. These two experiments used the same RIL sets from the same parental cross. The different results are likely due to the considerable differences in experimental method. For example, in Hoekenga et al.

(2003) the plants were grown on solid gel media with a complex nutrient solution plus sucrose at pH 4.2. Kobayashi et al. grew their plants in a very dilute nutrient solution at pH 5 with very low Al activities. These results highlight the sensitivity of quantitative analysis to experimental design. Variations in QTL detection may be due to a real diversity of Al tolerance genes present in differing genetic lines, or they may be due to experimental differences which favor one QTL over another.

Al Tolerance Physiology

Plants can tolerate toxic levels of Al either through internal detoxification or through exclusion from the root. At least three plant species that accumulate aluminum have been shown to use organic acid chelation to detoxify internal aluminum. Both *Melastoma* and buckwheat have been shown to chelate internal Al with oxalic acid (Watanabe et al., 1998 and Ma et al., 1998, respectively), while *Hydrangea* uses citrate to chelate internal Al (Ma et al., 1997a). However, for the vast majority of examined plant species, the primary mechanism of Al tolerance that has been seen is conferred through active exclusion of aluminum from the root (as reviewed in Kochian, 1995).

Two primary mechanisms have been proposed for aluminum exclusion: root-mediated increases in rhizosphere pH and the exudation of organic acids or other Al chelators. Because Al becomes less soluble as the solution pH rises, a zone of increased pH in the rhizosphere would cause the Al^{3+} activity in this region to decrease, thus reducing Al accumulation in the root. The only known published account of of this tolerance mechanism based on Al-induced alterations in rhizosphere pH comes from the work of Degenhardt et al. (1998), who reported on an Al tolerant *Arabidopsis* mutant which employs this mechanism to confer tolerance. To date, no natural instances of this Al tolerance mechanism have been observed. The exclusion

mechanism with the most experimental support, however, is Al-activated root organic acid exudation. Organic acids such as malate, citrate, and oxalate form strong chelates with the Al^{3+} ion; this complex is unable to enter the root and is not toxic. Phenolic compounds are another class of organic compounds commonly exuded from roots that have been proposed to aid aluminum tolerance through Al-chelation and exclusion, and may even work in harmony with organic acid exudation (reviewed in Barcelo and Poschenreider, 2002). Thus far, organic acid exudation remains the most experimentally supported mechanism, as an abundance of evidence links organic acid exudation to aluminum tolerance in a wide range of species, as recently reviewed by Kochian et al. (2004).

The first publication demonatrating Al-activatedorganic acid exudation as an Al tolerance mechanism was in snapbean (Misasaka et al., 1991), where a strong Al-activated exudation of citrate was observed exclusively in the Al-tolerant genotype. This work was followed by many supporting studies in other species, summarized in Table 1.1. An early demonstration of the powerful potential of this Al tolerance mechanism came in wheat, where three key supporting pieces of evidence were demonstrated by Delhaize et al. (1993b). They showed that Al tolerant wheat genotypes exhibit a much higher aluminum-activated malate exudation than do the corresponding sensitive genotypes. Furthermore, they showed that root malate exudation cosegregated with Al tolerance in F2 populations. Finally, it was demonstrated that supplying exogenous malate to the growth media ameliorated aluminum toxicity for Al sensitive wheat lines. This study also revealed that the Al-activated malate release is very rapid, detectable within minutes, and therefore the activation must occur at the protein, not gene, level at least in wheat. Also, the release is localized to the first few millimeters of the root tip, which corresponds to the region of Al-exclusion in tolerant lines and the zone of Al toxiticity in sensitive lines.

Table 1.1. Plant species exhibiting Al-activated root carboxylate exudation that is correlated with Al resistance.

Organic Acid Released	**Plant Species (common name)**	**Reference**
Citrate	*Cassia tora* (sickle senna)	Ishikawa et al., 2000 Ma et al., 1997b
Citrate	*Galium saxatile* (heath bedstraw)	Schöttelndreier et al., 2001
Citrate	*Glycine max* (soybean)	Silva et al., 2001 Yang et al,. 2000
Citrate	*Miscanthus sinensis* and *Miscanthus sacchariflorus*	Kayama 2001
Citrate	*Nicotiana tabacum* (tobacco)	Delhaize et al., 2001
Citrate	*Oryza sativa* (rice)	Ishikawa et al,. 2000 Ma et al., 2002
Citrate	*Sorghum bicolor* (sorghum)	Magalhaes 2002
Citrate	*Zea mays* (maize)	Kollmeier et al., 2001 Piñeros et al., 2002 Ishikawa et al., 2000 Jorge and Arruda, 1997 Pellet et al., 1995
Citrate, & Malate	*Avena sativa* (oat)	Zheng et al., 1998a
Citrate & Malate	*Brassica napus* (rape)	Zheng et al., 1998a
Citrate & Malate	*Helianthus annuus* (sunflower)	Saber et al., 1999
Citrate & Malate	*Raphanus sativus* (radish)	Zheng et al., 1998a
Citrate & Malate	*Secale cereale* (rye)	Li et al., 2000
Citrate & Malate	*Triticale ssp* (triticale)	Ma et al., 2000
Citrate, & Oxalate	*Zea mays* (maize)	Kidd et al., 2001
Malate	*Arabidopsis thaliana*	Hoekenga et al., 2003
Malate	*Triticum aestivum* (wheat)	Huang et al., 1996 Pellet et al., 1996 Papernik et al., 2001 Ishikawa et al., 2000 Ryan et al., 1995 Delhaize et al., 1993a Delhaize et al., 1993b
Oxalate	*Colocasia esculenta* (taro)	Ma and Miyasaka, 1998
Oxalate	*Fagopyrum esculentum* (buckwheat)	Ma et al., 1997c Zheng et al., 1998a Zheng et al., 1998b

Al-activated organic acid exudation from the root is also thought to be an important Al tolerance mechanism in maize. However, recent evidence suggests that other physiological mechanisms must also be operating in maize to explain the full range of observed tolerance (Piñeros et al., 2005). Several studies have characterized the organic acid release Al tolerance mechanism in single contrasting tolerant and sensitive maize lines (Pellet et al. 1995; Jorge and Arruda, 1997; Piñeros et al. 2002). In these studies it was shown that citrate release is induced by exposure to Al to a much greater degree in the tolerant lines. The citrate exudation rate tends to increase in response to increasing Al^{3+} activity. In Piñeros et al. (2002), a spatial analysis of root citrate exudation showed that unlike wheat, Al-activated organic acid exudation was not localized to the root apex; instead, it was more broadly distributed, occurring as far back as 5 cm from the root tip. The physiological characterization of this putative tolerance mechanism has been followed up by electrophysiological (patch clamp) analysis of root plasma membrane anion channels capable of transporting citrate out of root tip cells (Piñeros and Kochian, 2001; Kollmeier et al. 2001).

Recently, a study examining Al tolerance in a larger number of maize genotypes (six maize hybrids and inbreds) has cast doubt on the role of citrate exudation as the sole physiological Al tolerance mechanism in maize. Piñeros et al. (2005) examined a range of physiological characteristics of six maize lines and their response to Al treatment. They found little correlation between citrate exudation rates and Al tolerance among the six lines. Of particular interest was the North American inbred Mo17, which grouped with three other Al sensitive lines in the study, yet exhibited the highest Al-activated root citrate exudation rates of any lines included in the study. Another North American inbred, B73, had a similarly low level of Al tolerance, but had almost no detectable Al-activated root citrate release . In this study the authors hypothesized that Al-activated root citrate exudation may be a basal

mechanism of maize Al tolerance, observed in all tolerant and some sensitive maize lines. However in extremely Al tolerant genotypes such as the Brazilian standard for maize tolerance, Cateto-Colombia, other mechanisms may be operating in addition to root citrate release. These paradoxes regarding citrate release emphasize the importance of looking for additional mechanisms of aluminum tolerance in maize.

In *Arabidopsis* Al^{3+}-induced malate release has been shown to be the major physiological mechansim of aluminum tolerance. Hoekenga et al. (2003) reported that malate release in *Arabidopsis* explained 95% of the variation in Al tolerance among the RIL population studied.

Anion Channels and Aluminum Tolerance

Given the large body of evidence supporting root exudation of organic acids as an Al tolerance mechanism, there is considerable interest in the transport mechanism(s) underlying this exudation Possible organic acid release pathways include exocytosis and transport protein-mediated exudation. Because organic acids are almost fully deprotonated at the neutral pH of the cytoplasm, they exist in the cytoplasm as anions. Furthermore, as plant cells have a large inside-negative trans-membrane electrical potential, the transport of organic acids across the plasma membrane is a thermodynamically passive process. Thus, active, energy-consuming transport methods such as exocytosis, pumps, and antiporters would be inefficient. This leaves anion channels as the most efficient method for organic acid release. Experimental evidence also supports the role of anion channels in the transport mechanism. Anion channel inhibitors have been shown to inhibit the aluminum-activated malate release in wheat (Ryan et al., 2003). Also, recent patch clamp studies have revealed the presence of aluminum-activated anion channels in root tip protoplasts from both wheat (Ryan et al., 1997; Zhang et al., 2001) and maize (Piñeros

and Kochian, 2001; Piñeros et al., 2002). The wheat anion channel activity is only detectable in protoplasts from the root tip. The maize studies demonstrated that Al-inducible anion channel activity was present in excised patches of plasma membrane, and therefore did not require cytosolic factors. Thus, it is likely that the Al-activated organic acid exudation tolerance mechanism is a result of aluminum activation of a plasma membrane anion channel.

Several large families of anion channels exist in the cereal crops. Two families that include plasma membrane anion transporters are the CLC (chloride channel) anion channels and a subset of the ABC (ATP-Binding Cassette) transporter superfamily. The CLC anion channel family is evolutionarily ancient. Members of the family are found in plants and animals, as well as bacteria and yeast. The nine mammalian CLCs that have been identified have a wide variety of expression patterns and functionality, as reviewed by Maduke et al. (2000). Several mammalian CLCs have functions which suggest a plasma membrane localization, such as skeletal muscle excitability, renal ion transport, and cell volume regulation. Other CLCs have been identified as intracellular transporters, such as ScCLC from yeast, which helps to regulate pH in post-Golgi vesicles (Gaxiola et al., 1998). The first CLC homolog cloned from plants was reported by Lurin et al. (1996). In that study, which described the cloning of CLC-Nt1 from *Nicotiana tabacum*, the CLC-Nt1 reportedly was functionally expressed in *Xenopus* oocytes and electrophysiological examination of this channel expressed heterologously indicated it exhibited a slowly activating inward current, which would be consistent with an anion efflux. Unfortunately, this transport activity has yet to be reproduced by any other group studying plant CLCs. However, a host of plant CLCs have subsequently been cloned based on this initial discovery. CLC-6 and CLC-7 are the two mammalian CLCs most closely related to the plant CLCs (Maduke et al., 2000). Neither of these have been functionally characterized,

however, mouse CLC-6 is able to complement the phenotype of the yeast ScCLC knockout (Kida et al., 2001) while CLC-7 does not. Thus, it seems likely that CLC-6 is an intracellular membrane protein. To date, the membrane localization of most plant CLCs is unknown, thus we do not know if any of these could be involved in plasma membrane transport processes.

A subset of the ABC protein superfamily act as anion transporters. The Cystic Fibrosis Transmembrane Regulator (CFTR) is a Cl^- channel in mammalian cell membranes (Anderson et al., 1991). Pdr12 is an ABC transporter, which mediates organic acid efflux in yeast (Piper et al., 1998). A slow anion channel detected in the guard cell plasma membrane may be an ABC protein; it mediates the sustained release of anions and has transport properties similar to the ABC anion transporters CFTR and Pdr12 (Leonhardt et al., 1999). Interestingly, the ABC transporter antogonist diphenylamine-2-carboxylic acid inhibits the guard cell slow anion channel, Pdr12, and the Al-activated efflux from from intact wheat roots. So it is likely that an ABC protein or another transporter with similar physical properties mediates this efflux.

The recently discovered ALMT1 gene (Sasaki et al., 2004) may be the malate transporter in wheat. The predicted amino acid sequence does not correspond to any known channels in the public database. However, when expressed in *Xenopus* oocytes ALMT1 conferred an Al-activated electrogenic transport activity, which is likely to be carried by malate transport.

SUMMARY

The Al-induced root exudation of organic acids is the single Al tolerance mechanism with the most experimental support. In wheat, where Al-tolerance is conferred primarily by one gene, malate release is likely the primary tolerance mechanism. ALMT1 appears to be a transporter capable of mediating this malate

release. In maize, where many genes act together to determine total tolerance, citrate release is probably one of several tolerance mechanisms. Other proposed tolerance mechanisms await additional experimental support. The isolation of genes underlying Al tolerance in maize should permit clarification of alternative tolerance mechanisms.

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CHAPTER TWO

Searching for Aluminum Tolerance Proteins Using Wheat Chromosomal Deletion Lines that Harbor or Lack the Major Wheat Al Tolerance Gene

ABSTRACT

In experiments based on high-resolution two dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis of wheat root tip proteins from unique genetic stocks of wheat, an attempt was made to search for putative Al tolerance proteins. In wheat, there is one primary Al tolerance gene, that has been located on the long arm of chromosome 4D. A set of partial deletion lines of the wheat cultivar Chinese Spring are missing sequentially longer segments of chromosome arm 4DL. By analyzing these deletion lines for Al tolerance, we identified the physical location of the tolerance gene between the breakpoints for deletion lines 4DL-2 and 4DL-14. That is, 4DL-2, and all lines with more severe deletions were Al sensitive, while 4DL-14 retains the tolerance of the euploid Chinese Spring.

The experimental plan was to compare protein samples from root tips of the two lines, and screen for proteins present in 4DL-14 and absent in 4DL-2. The research plan was to then use mass spectrometry techniques to identify the genes underlying those proteins. We would then confirm the mapping location of these genes, and then do follow up work to determine if any of these were the Al tolerance gene.

For these experiments, a protocol was developed for extracting protein from wheat root tips. Proteins from root tips of both 4DL-2 and 4DL-14 were separated using one-dimensional SDS-PAGE gels. No differences were observed in the protein profiles between the two deletion lines, so a second set of root tip proteins were analyzed using two dimensional IEF/PAGE gels. Using this technique it was possible

to resolve approximately 300 protein spots/sample. No repeatable differences were found between the lines at this level of resolution. Several attempts were made to enrich the protein extracts for membrane-bound proteins to increase resolution on proteins of interest, but because the technologies for resolving and identifying proteins via 2D-PAGE were still being developed and advanced at the time of this early study, the project was terminated to wait for the development of more reproducible and sensitive proteomics techniques.

INTRODUCTION

Most genetic studies of Al tolerance in wheat have pointed to a single gene controlling the majority of the tolerance. Kerridge and Kronstad (1968) analyzed F1 and F2 individuals from a cross between the Druchamp (tolerant) and Brevor (sensitive) varieties of wheat for Al tolerance and their results indicated that tolerance was due to a single dominant gene. Subsequently, Riede and Anderson (1996) analyzed recombinant inbred (RI) lines from a cross between BH1146 (tolerant) and Anahuac (sensitive) and again found that a single gene controlled Al tolerance. Riede and Anderson also conducted genetic mapping of the Al tolerance locus, which they named Alt_{BH}, using RFLP markers, and identified two markers linked to Alt_{BH}. The marker Xbcd1230 mapped 1.1 cM from Alt_{BH} while Xcdo1395 mapped 10.2 cM from Xbcd1230. Treating the root growth data quantitatively, Xbcd1230 explained 85% of the variability in the phenotype. These markers were found to map to chromosome arm 4DL, as determined by probing DNA from nullisomic-tetrasomic and ditelosomic stocks of Chinese Spring (Riede and Anderson, 1996). Further evidence that chromosome 4DL holds the only major Al tolerance gene comes from a study focusing on the molecular mapping of the Al tolerance locus, Alt2, in Chinese Spring (Luo and Dvorak, 1996). Disomic substitution lines with D genome chromosomes

from Chinese Spring substituted for either the A or B genome homoeologous chromosome from the sensitive cultivar Langdon revealed that only chromosome 4D contained loci beneficial to Al tolerance. RFLP mapping using 4D/4B recombinant lines mapped Alt2 to several tightly linked markers on 4DL, including Xpsr914, Xpsr1051, and Xmwg2180 (Luo and Dvorak, 1996). Genetic analysis of a cross between a tolerant 4D/4B recombinant line and the sensitive Langdon revealed that this is a single dominant gene.

Some genetic studies using very Al tolerant genotypes of wheat have revealed the existence of possibly two Al tolerance genes in these tolerant lines. (Camargo, 1981; Berzonsky, 1992). Even in these cases, the effect of the single locus on chromosome 4DL dominates the variation in tolerance. Because of the importance of this locus to Al tolerance in most wheat lines studied, a strategy to clone this locus on chromosome 4DL was devised.

MATERIALS AND METHODS

Wheat Seed Germination

Seeds were germinated on moistened filter paper in Petri dishes. The seeds were stratified by placing the Petri dishes containing the seeds at 4°C in the dark for 5 days. Subsequently, the Petri dishes were transferred to a dark location at room temperature overnight. The wheat seedlings were then transferred into mesh cups that fitted into holes cut into the lids of plastic tubs containing 8 l of simple salt solution (200 μM $CaCl_2$), such that all three primary roots were submerged in the solution. The seedlings were grown in a 16 hour light - 8 hr dark cycle at 22^{o} C. After 24 hours of growth in this control solution, the simple salt solutions were changed, with the treatment plants getting an Al treatment solution.

Al Tolerance Assay

The wheat cultivar Chinese Spring and the deletion lines of Chinese Spring were assayed for aluminum tolerance in a simple salt solution consisting of 200 μM $CaCl_2$, pH 4.5. Al was added as $AlCl_3$ to a final concentration of 2.5 μM Al. Root length was measured at the time of initiation of treatment (+/- Al) and again 24 hours after growth on Al (Day 1). The two longest roots were measured at each time point. The difference in length between the longest roots at each time point was defined to be the root growth over that 24 hour period. The average root growth in the Al treatment group divided by the average root growth in the control group was calculated to yield the relative root growth (RRG), which is a standard measure of Al tolerance used by researchers (see, for example, Pellet et al., 1996; Papernik et al., 2001).

Hematoxylin Staining

After aluminum treatment, roots were rinsed twice in 8 l of H_2O (30 minutes/rinse) with continued stirring, then immersed in a solution of 0.2% (w/v) hematoxylin and 0.02% (w/v) KIO_3 for 20 minutes, and finally destained by rinsing twice in 8 l of H_2O (30 minutes/rinse), also with stirring. Hematoxylin staining is used to detect Al accumulation in roots, via the formation of a dark blue colored complex (Polle et al., 1978).

Protein Isolation From Root Tips

Wheat seedlings were germinated as described above. After 2 days of growth in the simple salt solution, the terminal 1 cm was excised from each root. Tissue harvest and the initial extraction step were performed at 4°C to minimize protein degradation. Using a plastic pestle in a 1.5 mL tube, the root tips were ground in protein extraction buffer consisting of 62.5 mM Tris.HCl, 100 mM NaCl, 10 mM DTT, 2% v/v Triton X-100, and 1 mM EDTA, pH 6.8. The homogenate was spun at 10,000 X g for 5 minutes to remove cellular debris. The supernatant was added to a

solution consisting of 10 mM DTT and 10% TCA in acetone and incubated at -20°C overnight to precipitate the protein. This solution was then centrifuged at 10,000 X g for 30 minutes to pellet the protein sample. The protein pellet was washed twice with 10 mM DTT in acetone. The pellet was then resuspended in the appropriate loading buffer. Protein levels were quantified using the Bradford assay (Bradford, 1976; Bio-Rad Laboratories, Inc, Hercules, CA).

SDS-PAGE Gel Procedure

For SDS-PAGE analysis, the proteins were resuspended in SDS loading buffer (0.0625 M Tris.HCl, pH 6.8, 2.3% SDS, 5.0% 2-ME, 10% glycerol, bromphenol blue, in H_2O) and loaded on 12% SDS-PAGE gels. Gels were silver stained using the Silver Stain Plus kit (Bio-Rad Laboratories, Inc, Hercules, CA).

2D Gel Procedure

Proteins were resuspended in 200 µL of lysis buffer (9M urea, 0.1 M DTT, 2% w/v CHAPS, 1.7% ampholines [pH ranges of 3-5, 4-6, 6-8]) and loaded onto IEF tube gels with a diameter of 1 mm. The IEF gels contained ampholytes in the pH ranges of 3-5, 4-6, and 6-8. For IEF, the anode electrode solution was 0.01 M H_3PO_4 and the cathode electrode solution was 0.02 M NaOH. Both solutions were degassed under vacuum for 30 minutes immediately before use. IEF gels were run for 10 minutes at 500 V, followed by 4 hours at 750 V and then the gels were removed immediately from their glass tubes and laid horizontally across the top of the second dimension SDS-PAGE gel. The SDS-PAGE gel was then run at 200 V for 40 minutes.

The SDS gel consisted of a 7cm x 5cm 12% acrylamide resolving gel and a 7cm x .5cm 4 % acrylamide stacking gel. Gels were silver stained using the Silver Stain Plus kit (Bio-Rad Laboratories, Inc, Hercules, CA).

RESULTS

The Al tolerance locus of Chinese Spring lies between the breakpoints of the partial deletion lines 4DL-2 and 4DL-14

Eight deletion lines were assayed for Al tolerance using relative root growth in the presence and absence of Al as the standard measure of tolerance. We chose this set of deletion lines based on the even distribution of their breakpoints over the length of chromosome arm 4DL as shown in Figure 2.1 with respect to the chromosomal banding pattern. The quantitative measure of breakpoint location, the fraction length (FL, the ratio of the deletion line arm length to the arm length of the full chromosome), is listed for each deletion line in Table 2.1. Additionally, relative Al content of the root tip was also assayed via hematoxylin staining to confirm that tolerance was associated with root tip Al exclusion as has been shown before for wheat (Delhaize et al., 1993; Papernik et al., 2001). Using both methods, the Al tolerance locus was localized to chromosome 4DL between the breakpoints of 4DL-2 (FL = 0.70) and 4DL-14 (FL = 0.86). As described above, Al tolerance was measured using the relative root growth (RRG) parameter because it compensates for inherited variability in control root growth rates between lines. Even when this type of normalization is used, anomalous results can occur when there are large differences in control root growth rates between lines. In this case it is especially important to determine if these large segmental deletions were causing changes and thus variability in control root growth. Figure 2.2 shows that the average root length on planting day was similar amongst the deletions lines. Therefore, these deletions did not have a large impact on the combined germination and root growth rates. Growth in the control solution over the following two days was also similar among all deletion lines (Figure 2.3). However, there were significant differences between the lines in terms of growth in Al-containing solution (Figure 2.3). These results imply that Al tolerance

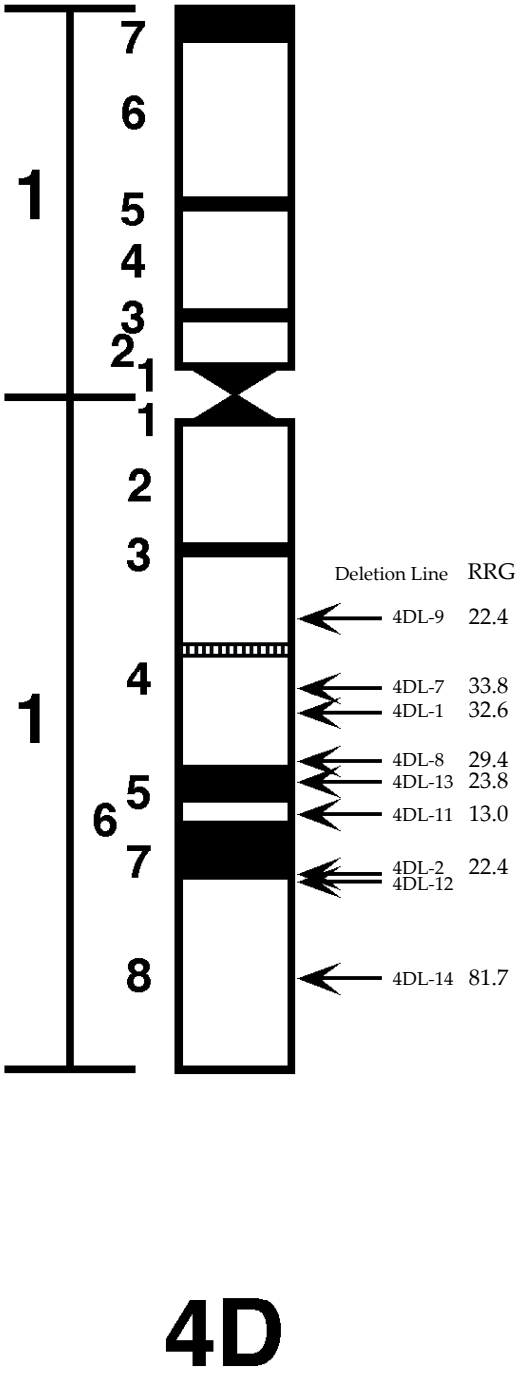

Figure 2.1. Map of chromosome 4D with deletion line breakpoints and RRG marked.

Table 2.1. Deletion line root growth over the two day experimental period. FL (fraction length) is the length of chromosome arm 4DL in the deletion line divided by the length of the euploid 4DL arm. Control growth is the average growth in mm over the two day period in the control solution, ± SE. Aluminum growth is the average growth in mm over the two day period in the Al treatment solution, ± SE. RRG (relative root growth) is the average growth in the Aluminum treatment divided by the average growth in the Control treatment.

Line	FL	Control Root Growth (mm)	Aluminum Treated Root Growth (mm)	RRG (% of control root growth)
4DL-9	0.31	28.3 ± 1.9	6.3 ± 1.1	22.4
4DL-7	0.41	26.7 ± 1.6	6.3 ± 1.4	23.8
4DL-1	0.46	22.5 ± 1.6	7.3 ± 1.4	32.6
4DL-8	0.53	23.8 ± 1.9	7.0 ± 1.1	29.4
4DL-13	0.56	23.8 ± 0.5	5.7 ± 0.6	23.8
4DL-11	0.61	24.3 ± 1.7	3.2 ± 0.8	13.0
4DL-2	0.70	25.3 ± 1.7	5.7 ± 1.0	22.4
4DL-14	0.86	21.0 ± 1.9	17.2 ± 1.0	81.7

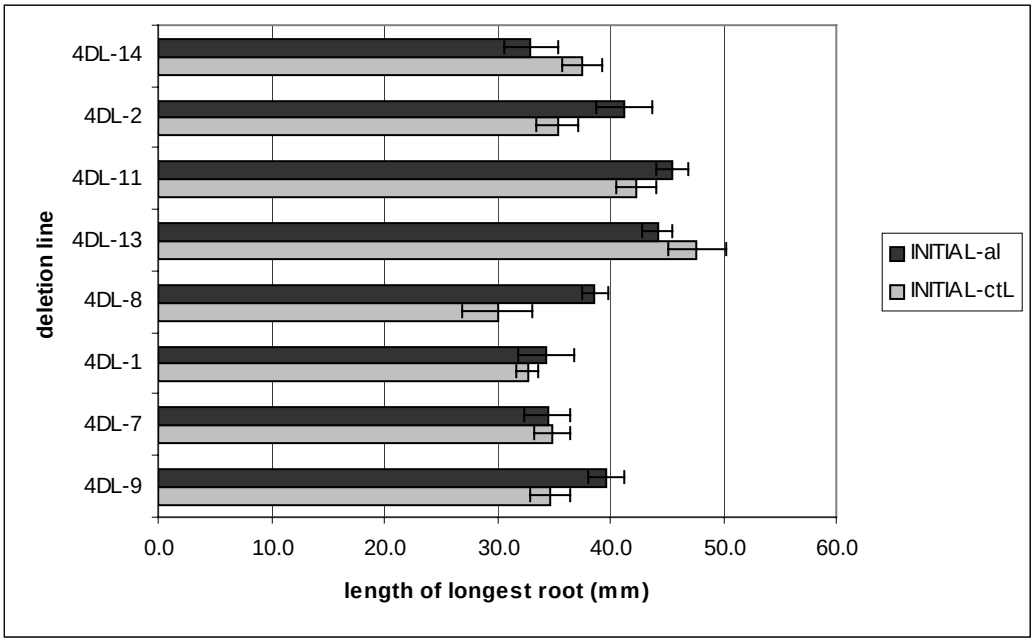

Figure 2.2. Length of longest root at day 0 for treatment and control groups. Black bars show the average length in mm of the treatment group for each deletion line, with SE marked. Grey bars show the average length of the control group. However, this data was collected just before exposure to Al, so neither group has been treated yet. The similarity within lines shows that comparable seeds were used in each treatment. The similarity between lines shows that these deletions do not have a major impact on germination rate or root growth in a simple salt solution.

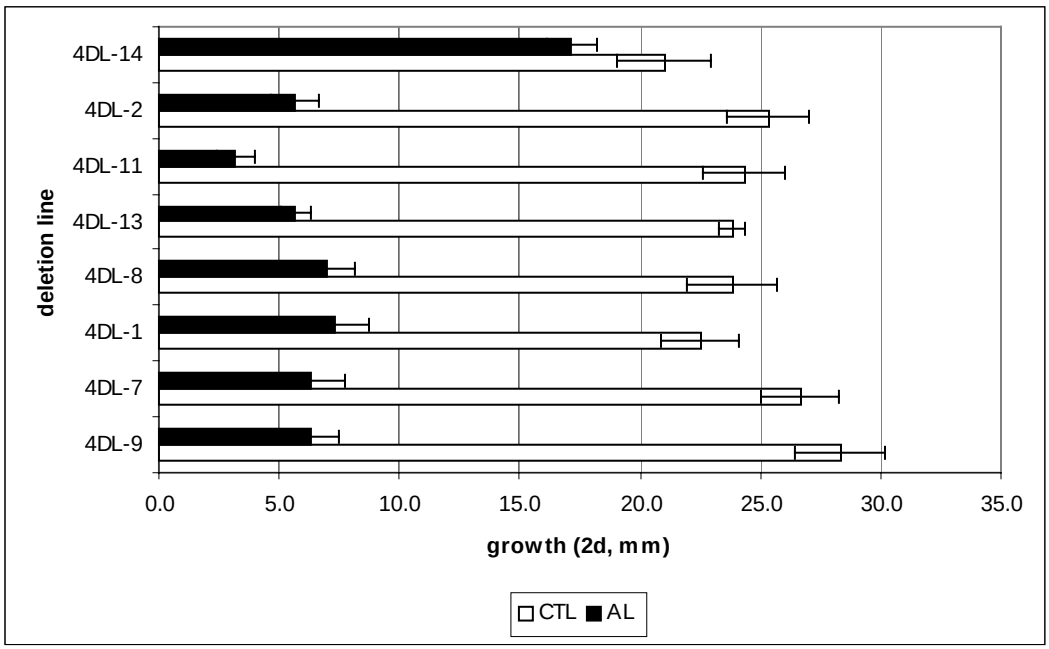

Figure 2.3. Influence of Al exposure on root growth of deletion lines over a two day period. Total growth over the period is shown in mm, with standard error bars. Control-treated groups are white; Al-treated groups are black. These data indicate that the control growth rate is unaffected by the deletions, while the growth rate under Al treatment is significantly reduced for 4DL-2 and all lines with more severe deletions.

is specifically controlled by a gene or genes in this region of chromosome 4DL. Root growth was much less inhibited by aluminum treatment in line 4DL-14 than any of the other deletion lines, and was very similar to the effect of the same Al exposure on root growth in the euploid Chinese Spring (data not shown). As seen in Figures 2.3 and Table 2.1, Al only inhibited root growth in 4DL-14 by 19% while in 4DL-2 and the subsequent deletion lines lacking larger portions of 4DL, Al inhibited root growth by 67-87%. Because 4DL-14 is so much more Al tolerant than the other more severe deletions, the Al tolerance locus must lie between the deletion breakpoints for 4DL-14 and 4DL-2, the closest deletion line. This region contains 16% of the chromosome arm by cytological determination of physical length, and is the same region harboring the Al tolerance locus identified by Rodriquez Milla and Gufstafson (2001). Hematoxylin staining of root tips confirmed Al exclusion as the tolerance mechanism.

The primary mechanism of Al tolerance that has been studied in plants involves Al exclusion from the root tip via Al-activated exudation of Al-chelating organic acids into the rhizosphere (see Kochian et al., 2004 and references therein). This has been well documented as a major Al tolerance in wheat (see, for example, Delhaize et al., 1993; Pellet et al., 1996; Papernik et al., 2001). Therefore, the ability of this set of deletion lines to exclude Al from the root tip was also analyzed. Hematoxylin is a stain that binds to Al in plant tissues and is often used to give an indication of the amount and location of Al accumulation. It has therefore been used as a proxy measure of Al tolerance (Polle et al., 1978). As seen in Figure 2.4, the root tips of deletion line 4DL-14 exhibited very little to no hematoxylin staining in the root tips while the other deletion lines had significant root tip staining. Therefore, line 4DL-14 maintains the ability to exclude Al from the root tip tissue that is also seen in the euploid parent (data not shown) while 4DL-2 and all more severe deletion lines have lost this ability. This is a useful confirmation that exclusion is the mechanism of

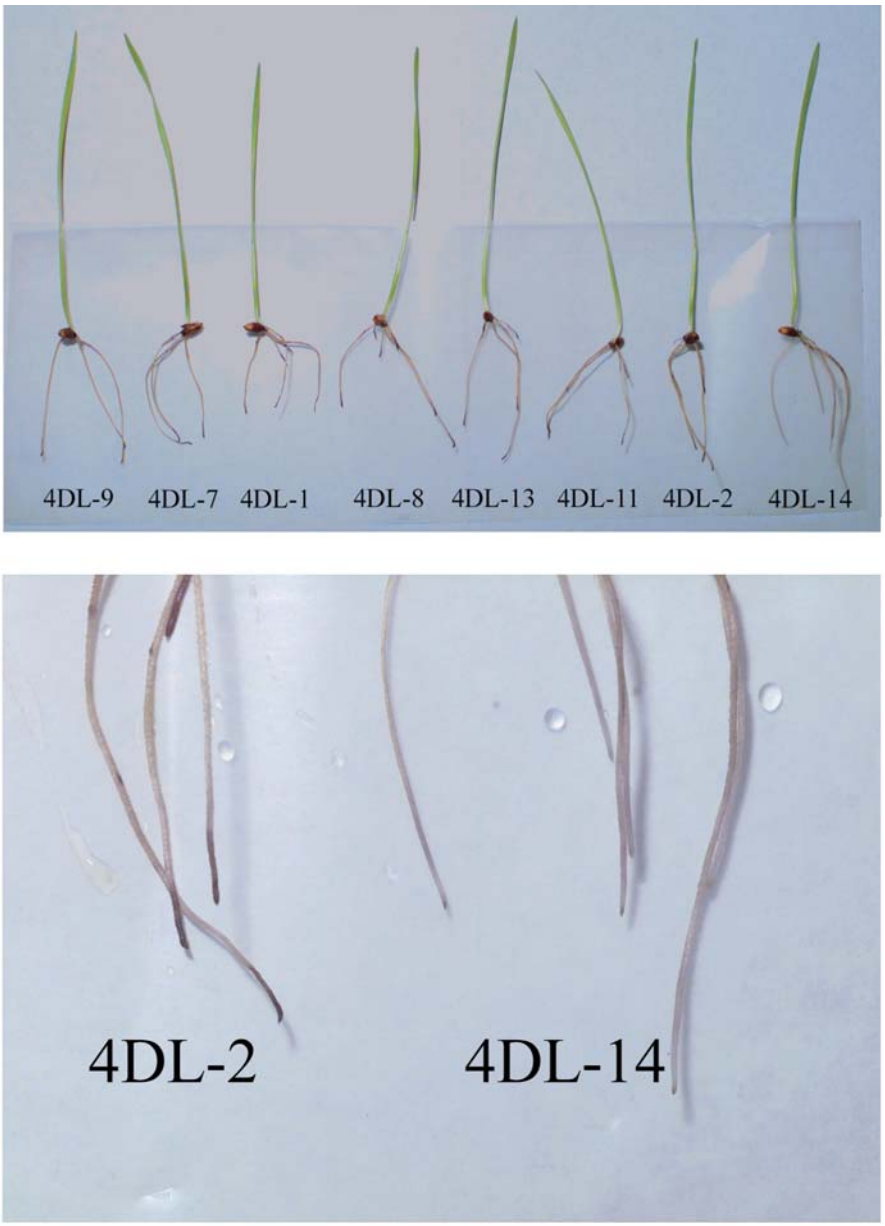

Figure 2.4. Hematoxylin staining of deletion line roots. Hematoxylin binds to Al present in the roots. Dark staining indicates Al uptake and Al sensitivity. Light staining indicates successful Al exclusion and Al tolerance. (A) All 8 deletion lines initially surveyed. (B) Magnified view of 4DL-2 and 4DL-14. All lines except 4DL-14 have darkly stained tips and are Al sensitive.

tolerance in Chinese Spring, and the mechanism is conferred by an Al tolerance locus located between the chromosomal break points for lines 4DL-2 and 4DL-14. As depicted qualitatively in Figure 2.1, the breakpoints of these two lines define a location between 70% and 86% of the distance from the centromere to the telomere of the long arm of chromosome 4D. After establishing that the Al tolerance locus was in this region, our next goal was to use differential approaches with this pair of deletion lines to attempt to identify proteins and genes between these two breakpoints. To do so, protein expression was examined in the root tips of these two deletion lines. Gel-separated protein samples were scanned for binary (presence/absence) differential expression between lines. Candidate proteins would then be analyzed and identified using mass spectrometry techniques, in order to determine their underlying genes, which would theoretically be located in the region of the tolerance locus. Genes so identified would either be the Al tolerance gene or a closely linked gene, which could be useful as a marker, and thus a stepping stone towards identifying the tolerance gene.

SDS-PAGE separation of wheat tip protein samples resulted in about 50 distinct protein bands, with no repeatable differences between deletion lines 4DL-2 and 4DL-14

Initially, to determine sample complexity and similarity, protein samples were separated by molecular weight (MW) only. In order to detect differential protein expression between Chinese Spring, 4DL-2, and 4DL-14, protein was extracted from the root tips of these three lines. These protein samples were separated on a one dimensional SDS-PAGE gel for comparison purposes (Figure 2.5). For these samples, approximately 50 distinct protein bands were resolvable, and no differences in protein expression were observed between lines. These results were not surprising, as these 50 bands presumably represented the most highly expressed proteins in the root tip,

and were unlikely by chance to be encoded by genes residing in the deleted region of interest. In addition, multiple proteins of similar size may not have been resolvable using this technique. As 2D-PAGE separates proteins of similar size based on the second characteristic of isoelectric point (pI), the pH value at which that protein is neutrally charged, the root tip proteins were subsequently analyzed via this technique. 2D-PAGE separation of wheat tip protein samples resulted in about 250 distinct protein spots, with no repeatable differences between deletion lines 4DL-2 and 4DL-14.

When root tip protein samples were separated in two dimensions (by pI and by MW) about 250 distinct protein spots were identifiable (Figures 2.6 through 2.8). Comparing protein samples of 4DL-2 and 4DL-14 run in parallel shows a very similar pattern for most spots (see Figures 2.6 and 2.7). For any given experimental pair of gels, there were sometimes one or more spots that appeared to be present in only one of the two samples. For example, Figure 2.8 shows one pair of gels where a specific protein is missing in 4DL-2, while a second protein is apparently missing in 4DL-14. However, repeated experiments always revealed that these differences were artifacts. The missing spots showed up in subsequent repeated experiments, suggesting that they were lowly expressed proteins subject to variation in extraction or separation.

DISCUSSION

This project was carried out because we had access to a unique set of genetic materials in wheat, and we also had the physiological tools to screen these materials for differences in Al tolerance. This set the stage for a differential approach that may have allowed us to identify an important Al tolerance protein. As discussed previously, a single gene (known as *Alt2* in Chinese Spring) controls most or all genetic variability for Al tolerance in wheat. Isolating this gene would be highly

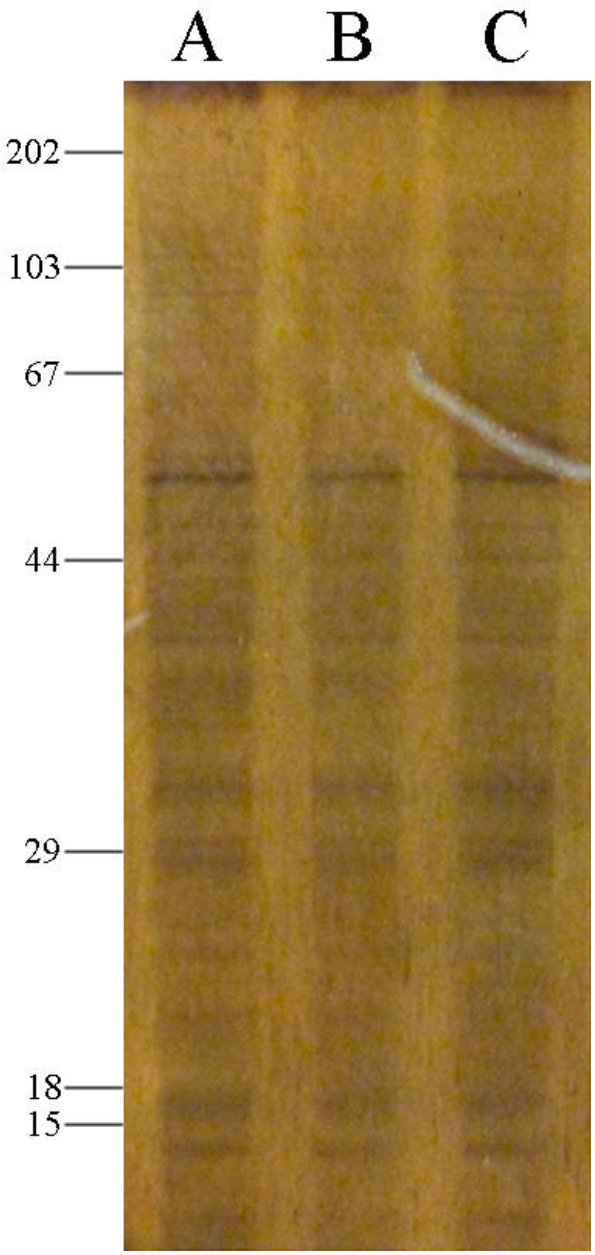

Figure 2.5. Root tip protein samples separated by apparent molecular weight. Lanes include (A) Chinese Spring, (B) 4DL-2, and (C) 4DL-14. Approximately 50 bands are distinguishable. No differences in protein expression between samples are apparent.

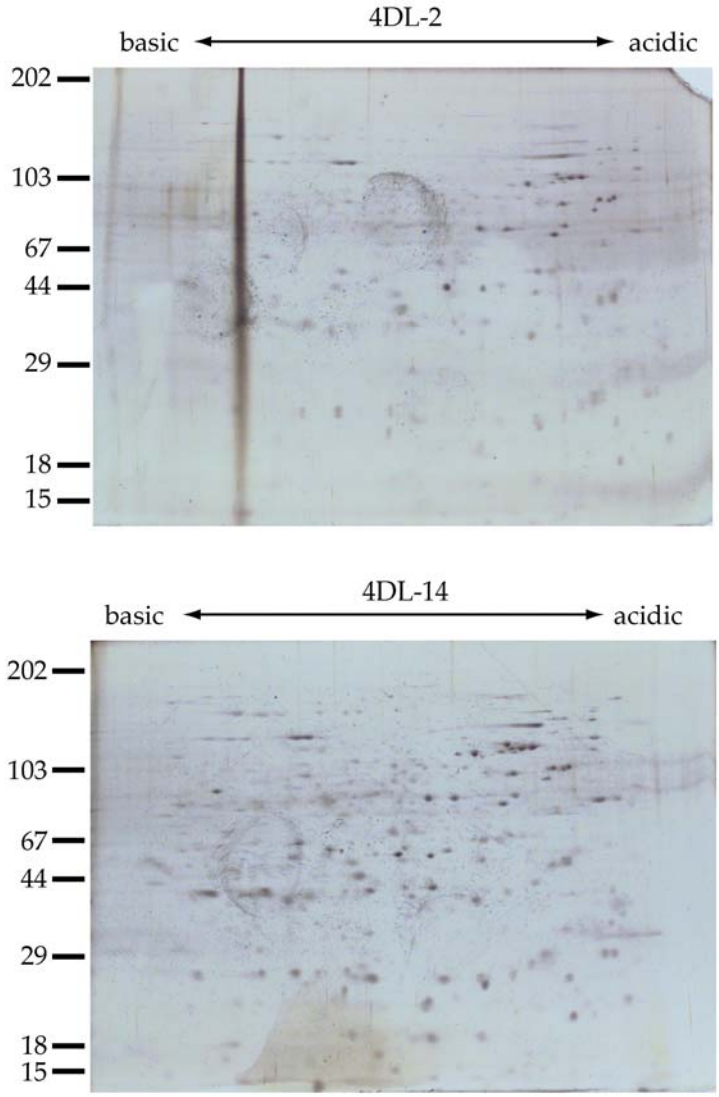

Figure 2.6. 2D-PAGE comparison of 4DL-2 and 4DL-14 root tip proteins. Apparent MW in kDa marked on the left. pH range for IEF marked above. IEF gel was cast with ampholytes in the ranges of 5-7 (1.6%) and 3-10 (.4%). (A) 4DL-2 and (B) 4DL-14 have very similar patterns.

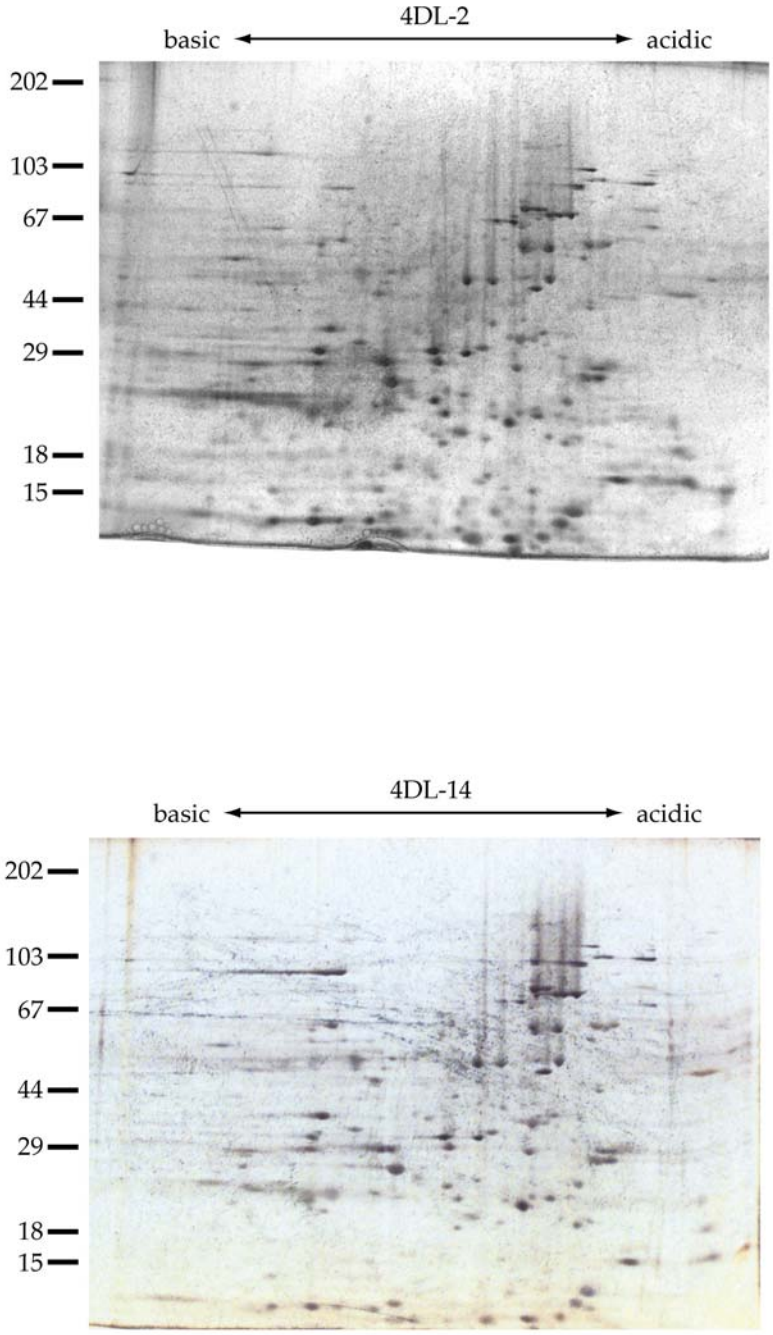

Figure 2.7. 2D-PAGE comparison of 4DL-2 and 4DL-14 root tip proteins. Same conditions as Figure 2.6. These gels are from a replicate experiment with different plants.

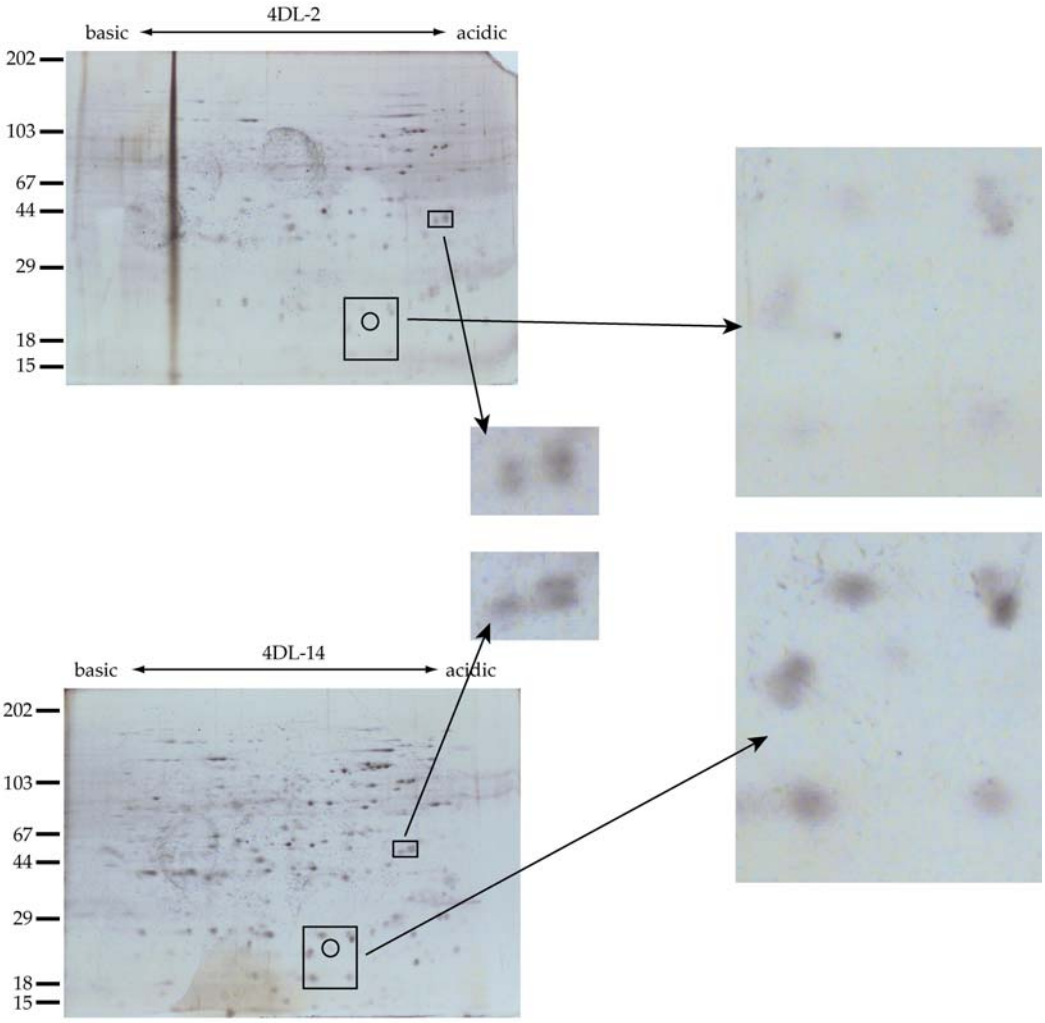

Figure 2.8. Close-up of putative differential protein spots. Same gel pair shown in Figure 2.6. Close-ups are shown at 5x magnification relative to whole gel images. An example of a spot apparently exclusively expressed in 4DL-14 is shown in the circle within a square. An example of a spot apparently exclusively expressed in 4DL-2 is shown in the small rectangle.

desirable for marker-assisted breeding, for basic understanding of the Al tolerance mechanism, and possibly for genetic engineering of Al tolerant varieties of wheat and other crops.

One of the first ways researchers tried to isolate the Al tolerance gene in plants was by looking for Al-induced genes. This method has been tried both at the levels of gene transcription (comparing mRNA levels) (see, for example, Snowden et al., 1993; Richards et al., 1994, 1998; Hamel et al., 1998) and protein expression (Delhaize et al., 1991; Rincon and Gonzales, 1991; Picton et al., 1991). None of these experiments successfully resulted in the identification of an Al tolerance gene. It appears that the major weakness in this approach, at least for wheat where much of the work has been done, is that Al tolerance genes are probably not Al inducible. A host of studies have identified genes induced by exposure to Al in wheat (Snowden et al. 1995; Hamel et al. 1998; Hamilton et al. 2001; Sasaki et al. 2002) and other species (Ezaki et al., 1995; Richards et al., 1998; Drummond et al., 2001; Watt, 2003). Those genes that have been studied in both sensitive and tolerant lines are induced in both, reducing their possible role in differential tolerance (Hamel et al. 1998; Sasaki et al. 2002). Of those that have been studied under other stresses, most or all are not specifically induced by Al (Snowden et al. 1995; Sasaki et al. 2002). Some aluminum induced wheat genes have been overexpressed in *Arabidopsis thaliana* or inducibly expressed in yeast. In neither of these cases did the genes confer significant increases in Al tolerance (Ezaki et al. 1999; Ezaki et al. 2000). Taken together, these results cast doubt on the idea that the major Al tolerance mechanism in wheat is activated through inducible gene expression. In wheat, an Al-inducible malate release from the root apex is highly correlated with Al tolerance. This response is very rapid, occurring within minutes, suggesting that Al tolerance is activated at the protein and not gene level (Delhaize et al., 1993; Kochian et al., 1995, 2004). Therefore, one model for Al

tolerance in wheat is the activation of a previously synthesized protein system rather than an induction of protein synthesis. This could occur, for example, through the opening of a plasma membrane anion channel in response to Al exposure, allowing the organic acid anion to flow out of the root cell into the rhizosphere. In fact, just such Al-triggered anion channels have been observed in both wheat and maize (Zhang et al., 2001; Piñeros and Kochian, 2001).

If the Al tolerance gene is constitutively expressed in wheat, how can it be identified? To address this challenge, we used wheat genetic stocks with physical deletions of chromosome segments. Using a line with a physical deletion of the region containing the tolerance gene creates a presence/absence dichotomy versus the euploid line or a less severe deletion line retaining that chromosome segment. Once this pair of lines is identified, genes on the deleted segment of interest could in theory be identified by comparing DNA, mRNA, or protein differences between the lines. Given the large amount of repeated, non-genic DNA in wheat, DNA comparisons are not ideal. Both mRNA and protein comparisons have their advantages and disadvantages. Comparisons based on mRNA using PCR-based methods have the ability to distinguish among paralogous genes with as little as one nucleotide variation. Protein comparisons have the ability to detect differences in post-translational modification, as well as sequence differences affecting protein structure or pI. A challenge for identifying deleted genes in wheat is the hexaploid genomic structure. It is likely that one or both of the other genomes contains a homoeologue of the genes in this region. This feature is what makes deletions lines so frequently viable in wheat. However, any homoeologues of the tolerance gene are presumably not functional, at least with regards to the Al tolerance phenotype. Therefore, there must be some change in structure, function, or expression of those genes. If the

changes affect protein size, pI, or expression, then protein sample separation by 2D-PAGE should be able to detect that difference.

Unfortunately, in the actual experiments no differential protein expression was detected. There are several factors which likely contributed to this result. There are undoubtably proteins whose expression is lost with the deletion of the chromosome segment bearing the genes encoding those proteins. Our ability to detect these proteins depends on characteristics of the protein and the abilities of the experimental system. The relatively small gel system was limited with regards to protein loading capacity. Thus, lowly expressed proteins were less likely to be loaded at levels detectable by silver staining. Some classes of proteins may not have been efficiently extracted or separated on the gels. Membrane proteins in particular are challenging to extract and separate with traditional protocols. Other classes of proteins may have difficulty entering the first dimension gel, or being transferred to the second dimension gel, or their pI value may fall outside the isoelectric focusing range of the first dimension (Shaw and Riederer, 2003). Protein degradation could also limit the sensitivity of this approach.

While technical limitations can be overcome with more advanced equipment and techniques, a possible biological limitation would be harder to overcome. That is, proteins from homoeologous genes (from chromosome arms 4AL or 4BL) could have the identical MW, pI, and also be expressed in the root tips. We know that these homoeologues are not functional for Al tolerance, so its seems unlikely that this would be the case. However, it is possible that amino acid substitutions or a small insertion or deletion could negatively impact the protein structure while not changing the protein profile detectable by this method.

Finally, the most imposing obstacle was probably the size of this portion of chromosome 4DL which constituted 16% of the long arm of chromosome 4D. Given

the very large size of the wheat genome (16,000 Mb), we still are dealing with a large piece of genomic DNA that could contain a large number of wheat genes. Also, because each gene can result in multiple protein isoforms due to different types of post-translational modification, we could be looking for a low abundance protein, possibly a membrane protein, in a mixture of many thousands of proteins.

Proteomics is one of the fastest progressing fields in biology today. If we were to go forward with this project today, the equipment and techniques would be much more advanced. However, the general methods and research strategy would remain. There are three areas where improvements could be made. Protein isolation could be improved. Membrane fractionation and other cellular fractionation procedures can decrease the complexity of the sample and increase the sensitivity to low-abundance proteins which may be enriched in that fraction. Membrane proteins are also a likely location for Al tolerance related proteins.

Increased gel resolution will allow for better separation of complex samples, and the identification of more protein spots. There are several ways to do this. Immobilized pH gradient strips can spread out a specified pH range in a 2D-PAGE gel. Larger gel rigs also improve resolution. Advanced techniques such as isotope coded affinity tag (ICAT, Gygi, et al, 1999 and recently reviewed in Patton et al., 2002) allow for protein quantification and identification in complex samples without using 2D-PAGE.

Protein identification techniques have also been improved, with increasingly sensitive mass specrometry techniques benefiting from the simultaneous development and expansion of protein databases. Techniques such as LC-MS/MS are capable of giving very specific protein profiles for database matching as well as peptide sequence information to aid the isolation of novel genes (reviewed in Mann et al., 2001).

If this work were repeated today, the specific proteomic techniques employed would be quite different, but the inherent challenges in the approach would remain. Today's proteomics approaches are capable of reproducibly resolving a much larger number of plant proteins. However, problems relating to homoeologous chromosomes encoding similar proteins remains. Also, the number of wheat proteins remains encoded by genes in this region of chromosome 4DL would still be high, and low-abundance and membrane proteins could still remain difficult to identify. Even with today's improved proteomic techniques, the identification of candidate Al tolerance proteins using this experimental strategy would still be a difficult task.

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CHAPTER THREE

Cloning and Molecular Characterization of the CLC Family of Anion Channels From Wheat (*Triticum aestivum*)

ABSTRACT

Aluminum (Al) toxicity is a global problem limiting agricultural yields on acid soils. Tolerant varieties of wheat are able to prevent aluminum from entering the roots by releasing malate into the rhizosphere, which is thought to bind aluminum, and prevent its entry into the root. In this investigation, the *CLC* family (chloride channel) of anion channel genes was chosen as possible candidates for the organic acid transporter involved in wheat Al tolerance. Eight members of the *CLC* gene family were cloned from wheat (*Triticum aestivum* cv Atlas66). These genes were mapped for Al tolerance using deletion lines of wheat, which are missing partial or entire chromosome arms, and through Southern analysis this allows for a rapid determination of the presence or absence of a probe in that deleted region. Deletion line mapping indicated that none of the *TaCLC* genes co-localize with *Alt2*, the major aluminum tolerance locus in wheat. Northern analysis of *CLC* gene expression indicated that there was no correlation between expression of any of the *CLC*s and aluminum tolerance, but the findings did suggest that some of the *CLC* genes may be involved in general stress responses. Differences in expression patterns, especially between shoots and roots, suggest that different members of this family may be involved in diverse functions in the plant.

Phylogenetic analysis of the sequence data for these genes indicated that more members of this family are probably present in wheat. Although functional data are not yet available for these genes, the conservation of amino acid residues essential for anion selectivity suggests they are functional anion channels.

INTRODUCTION

Aluminum (Al) is the most abundant metal element in the earth's crust, comprising 8.3% by weight (Greenwood and Earnshaw, 1997). At low pH, aluminum is solubilized into the soil solution, including the trivalent cation Al^{3+}, which is toxic to plants (Kochian, 1995). Al^{3+} rapidly inhibits root growth, probably initially by interfering with both cell growth and division. But aluminum also has many other demonstrated toxic effects, including alterations in the cytoskeleton (Blancaflor et al., 1998), a decrease in cytosolic Ca^{2+} concentrations (Jones et al., 1998), and interference with signaling proteins (Jones and Kochian, 1995). The net result of limited root growth is an inhibited ability to take up adequate water and nutrients for good growth and yield.

Genetically, aluminum tolerance in wheat is fairly simple, controlled by one or a few genes. A single locus confers the majority of the aluminum tolerance in all genetic crosses studied thus far. This locus is called Alt_{BH} in tolerant cultivar BH1146, *Alt1* in tolerant cultivar Carazinho, and *Alt2* in the moderately tolerant cultivar Chinese Spring. However, all these loci are located in the same region of the long arm of chromosome 4D and are most likely alleles at the same locus (Magalhaes et al., 2004).

Plants can tolerate toxic levels of Al either through internal detoxification or through exclusion from the root. At least three plant species that accumulate aluminum have been shown to use organic acid chelation to detoxify internal aluminum. Both *Melastoma* and *Fagopyrum* (buckwheat) have been shown to chelate internal Al with oxalic acid (Watanabe et al., 1998 and Ma et al., 1998, respectively), while *Hydrangea* uses citrate to chelate internal Al (Ma et al., 1997). However, for the vast majority of examined plant species, tolerance is conferred through active

exclusion of aluminum from the root (as reviewed in Kochian, 1995). Tolerant varieties of wheat, in particular, actively exclude aluminum from the terminal 3 mm of the root tip (Delhaize et al., 1993a).

Two primary mechanisms have been proposed for aluminum exclusion: root-mediated increases in rhizosphere pH and the exudation of organic acids or other Al chelators. Because Al becomes less soluble as the solution pH rises, a zone of increased pH in the rhizosphere would cause the Al^{3+} activity in this region to decrease, thus reducing Al accumulation in the root . The only known instance of this tolerance mechanism based on a rhizosphere pH increase comes from the work of Degenhardt et al. (1998), who reported on an *Arabidopsis* mutant that utilizes this mechanism to improve aluminum tolerance. The exclusion mechanism with the most experimental support, however, is organic acid exudation. Organic acids such as malate, citrate, and oxalate form strong chelates with the Al^{3+} ion, and this complex is not toxic and unable to enter the root. Phenolic compounds are another class of organic compounds commonly exuded from roots that have been proposed to aid aluminum tolerance through exclusion, and may even work in harmony with organic acid exudation (reviewed in Barcelo and Poschenreider, 2002). Thus far, organic acid exudation remains the most experimentally supported mechanism, as an abundance of evidence links organic acid exudation to aluminum tolerance in a wide range of species, as recently reviewed by Kochian et al. (2004). Hoekenga et al. (2003) reported that quantitative trait locus (QTL) mapping of Al tolerance and malate release in *Arabidopsis* showed that Al-activated root malate exudation explained 95% of the variation in Al tolerance observed among the lines studied. There are three supporting pieces of evidence for this tolerance mechanism in wheat, as demonstrated by Delhaize et al. (1993b). They showed that Al tolerant wheat genotypes exhibit a much higher aluminum-activated malate exudation than do the corresponding sensitive

genotypes. Furthermore, they showed that root malate exudation cosegregated with Al tolerance in F2 populations. Finally, it was demonstrated that supplying exogenous malate to the growth media ameliorated aluminum toxicity for Al sensitive wheat lines.

The questions then arise, how is the malate released into the rhizosphere, and how do tolerant lines mediate this increased release? Possible malate release pathways include exocytosis and transport protein-mediated exudation. Because malate is almost fully deprotonated at the neutral pH of the cytoplasm, it exists in the cytoplasm as an anion. Furthermore, as plant cells have a large inside-negative trans-membrane electrical potential, the transport of malate across the plasma membrane is a thermodynamically passive process. Thus, the energy requirement of methods such as exocytosis, pumps, and antiporters should be unnecessary. This leaves anion channels as the most efficient method for organic acid release. Experimental evidence also supports the role of anion channels in the transport mechanism. Anion channel inhibitors have been shown to inhibit the aluminum-activated malate release in wheat (Ryan et al., 2003). Also, recent patch clamp studies have revealed the presence of aluminum-activated anion channels in root tip protoplasts from both wheat (Ryan et al., 1997; Zhang et al., 2001) and maize (Piñeros and Kochian, 2001; Piñeros et al., 2002). The wheat anion channel activity is only detectable in protoplasts from the root tip. Thus, it is likely that the Al-activated malate exudation tolerance mechanism is a result of aluminum activation of a plasma membrane anion channel. Cloning anion channels involved in malate release would be very helpful in dissecting the details of this tolerance mechanism.

For this study, the feasibility of cloning wheat anion channels involved in malate exudation by hybridization screening was assessed. There are three anion channel families with cloned representatives from plants. The VDACs (voltage-

dependent anion channels) are typically located in the mitochondria, and therefore unsuitable for malate release from root cells. The large ATP-binding cassette (ABC) superfamily of solute transporters includes some anion channels. However, most ABC transporters mediate active transport, which is unnecessary for the electrochemical conditions associated with malate release. Some ABC transporters have been shown to transport organic anions (Piper et al., 1998). However, the sheer size of the family increases the difficulty of studying ABC proteins by this method. It is the largest protein family in *Arabidopsis*, and many members are not even transporters. Thus, while a hybridization screen would likely pull out ABC family members, the challenging task of identifying plasma membrane anion channels from that pool would remain.

The third cloned anion channel family from plants is the CLC (chloride channel) family. CLCs are an evolutionarily ancient family. Members of the family are found in plants and animals, as well as bacteria and yeast. The first CLC gene was cloned from the spotted electric ray (*Torpedo marmorata*; Jentsch et al., 1990), where it carries a current in the electric organ discharge used to capture prey. The nine mammalian CLCs have a wide variety of expression patterns and functionality, as reviewed by Maduke et al. (2000). Several mammalian CLCs have functions that suggest a plasma membrane localization, such as skeletal muscle excitability, renal ion transport, and cell volume regulation. Other CLCs have been identified as intracellular transporters, such as ScCLC from yeast, which helps to regulate pH in post-Golgi vesicles (Gaxiola et al., 1998).

The first CLC cloned from plants was reported by Lurin et al. (1996). In that study, which described the cloning of CLC-Nt1 from *Nicotiana tabacum*, the CLC-Nt1 reportedly was functionally expressed in *Xenopus* oocytes and electrophysiological examination of this channel expressed heterologously indicated it

exhibited a slowly activating inward current, which would be consistent with an anion efflux. Unfortunately, this transport activity has yet to be reproduced by any other group studying plant CLCs. However, a host of plant CLCs have subsequently been cloned based on this initial discovery. CLC-6 and CLC-7 are the two mammalian CLCs most closely related to the plant CLCs (Maduke et al., 2000). Neither of these have been functionally characterized; however, mouse CLC-6 is able to complement the phenotype of the yeast ScCLC knockout (Kida et al., 2001) while CLC-7 does not. Thus, it seems likely that CLC-6 is an intracellular membrane protein. To date, the membrane localization of most plant CLCs is unknown, thus we do not know if any of these could be involved in plasma membrane transport processes. . Because a member of the CLC family may be a reasonable candidate for a role in root organic acid anion transport, this study focused on the cloning of wheat root tip CLCs and their possible role in wheat Al tolerance was studied.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Two winter wheat cultivars were used in this study, one possessing high tolerance to Al (*Triticum aestivum* cultivar Atlas-66) and one sensitive cultivar (*Triticum aestivum* cultivar Scout-66). Additionally, deletion lines of the wheat cultivar Chinese Spring (which is moderately tolerant) were used for physical mapping of candidate anion channel genes to specific chromosome arms. Seeds were germinated on moistened filter paper in Petri dishes. The seeds were stratified by placing the Petri dishes containing the seeds at 4°C in the dark for five days. Subsequently, the Petri dishes were transferred to a dark cabinet at room temperature overnight. The wheat seedlings were then transferred into mesh cups that fitted into holes cut into the lids of plastic tubs containing eight l of simple salt solution (200 μM

$CaCl_2$, pH 4.5), such that all three primary roots were submerged in the solution. The seedlings were grown in a 16 hour light - 8 hr dark cycle at 22° C. After 24 hours of growth in simple salt solution (200 μM $CaCl_2$, pH 4.5), Al treatment group Atlas seedlings were transferred to the treatment solution (200 μM $CaCl_2$ and 15 μM $AlCl_3$, pH 4.5) for 24 hours.

Isolation of Wheat Root Tip CLC Clones

Root tip samples (1.0 cm) were harvested and frozen in liquid N_2. Subsequently, mRNA was extracted from the root tips using the Invitrogen FastTrack 2.0 mRNA extraction kit (Invitrogen Corporation, Carlsbad, CA 92008). The root tip mRNA was used to construct a root tip cDNA library in a phage host system with the Stratagene cDNA Synthesis Kit (Stratagene, La Jolla, CA 92037). cDNA was directionally cloned into the Uni-ZAP XR (LambdaZAP II-based) vector using *Eco*RI and *Xho*I adapters. *Arabidopsis* ESTs were obtained for *AtCLC-a* (H2G10T7), *AtCLC-b* (H10G11T7), *AtCLC-c* (40C9T7), and *AtCLC-d* (124F5T7) from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, Columbus, Ohio). The other two *Arabidopsis* genes (*AtCLC-e* and *AtCLC-f*) were not identified at the time of iniation of this study. The cDNA library was screened using a mixture of the four *AtCLC* ESTs. The probes were labeled with [α-^{32}P]dCTP by random hexamer primers and hybridized to the membranes overnight. Library aliquots were plated on NZYM plates (1.5% agar (w/v) in Bacto NZYM Broth {Difco Laboratories, Detroit, Michigan}) and incubated at 30°C overnight. For the initial screen, 20 plates were plated at a density of 50,000 pfu (plaque forming units)/plate, for a total of approximately 1,000,000 plaques screened. Hybond N+ nylon membranes were applied to plates for five minutes to lift plaques. Lifts were treated for 15 minutes in denaturation solution (1.5 M NaCl, 0.5 M NaOH), twice for 15 minutes in neutralization solution (1. 5 M NaCl, 0.5 M Tris-HCl pH 7.5) and finally rinsed in 0.2

M Tris-HCl (pH 7.5), 2x SSC buffer. Plaques isolated based on positive hybridization results were diluted and plated for a second round of plaque lifts. Incubation, plaque lifts, membrane hybridization and plaque picks were repeated. In the third round of screening, the dilution level was sufficient to allow for the clean picking of individual phage plaques. Finally, clones were excised into the bacterial vector and sub-cloned into E. coli strain DH5α for further analyses.

TaCLC cDNA Sequencing, Alignment, and Phylogenetic Analysis

cDNA clones were end sequenced using T7 and T3 primers. The complete cDNA sequence was determined using the primer walk method. Predicted amino acid sequences were aligned using the ClustalW algorithm (Thompson et al., 1994) as implemented in the MegAlign software package (Clewley and Arnold. 1997; MEGALIGN, DNASTAR, Inc, Madison, WI), with the following parameters: gap penalty 10.00; gap length penalty 0.20; delay divergent sequences 30%; the Gonnet series was used for the protein weight matrix. Phylogenetic analyses were performed with PAUP* (Swofford, 1998). For parsimony analysis, 10 random addition sequences of heuristic parsimony searching using TBR branch-swapping were performed with maxtrees set to increase infinitely. A strict consensus tree was constructed from equally parsimonious trees. For bootstrap analysis, 100 replicates of simple addition sequence heuristic parsimony searching using TBR branch swapping were performed, with maxtrees set to 1000.

Northern Blot Analysis

Northern analysis was conducted both with the Al tolerant and sensitive wheat cultivars Atlas 66 and Scout 66, and the chromosome deletion lines 4DL-2 and 4DL-14 of the wheat cultivar Chinese Spring. The wheat seedlings were grown in simple salt solution (200 μM $CaCl_2$, pH4.5) and treated with 0 or 15 μM $AlCl_3$ as described above. For the deletion lines 4DL-2 and 4DL-14, 1 cm root tips were harvested and

stored in liquid N_2. For tissue specific expression analysis in Atlas 66 (tolerant) and Scout 66 (sensitive), samples were taken from the following plant parts and stored in liquid N_2: 1.0 cm root tips from control treatment, 1.0 cm root tips from Al treatment, the remainder of the roots from control treatment, and whole shoots from control treatments. Subsequently, mRNA was isolated using the FastTrack 2.0 mRNA extraction kit (Invitrogen Corporation, Carlsbad, California 92008). Messenger RNA was separated by size on a formaldehyde gel, and transferred to a Hybond-N^+ membrane (Amersham Life Science, England). *TaCLC* probes (complete cDNAs) were labeled with [α-^{32}P]dCTP by random hexamer primers and hybridized to the membrane overnight. Approximately one µg mRNA was loaded per lane and equal loading insured by ethidium bromide staining of ribosomal subunits and by actin probe hybridization. Following hybridization, the membranes were washed three times for 20 minutes each; twice at low stringency (2x SSC, 0.1% SDS), followed by a high stringency wash (0.5x SSC, 0.1% SDS)..

Southern Blot Analysis

A hybridization feasibility study was conducted and involved Southern analysis with *AtCLC* clones and barley DNA. Genomic DNA (~40 µg) was isolated from barley seedlings (*Hordeum vulgare* cultivar Arapoles) and digested with one of six restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Hae*III, *Hind*III, or *Xba*I). Subsequently, Southern analysis was conducted with the *TaCLC* clones and wheat genomic DNA. Genomic DNA (~40 µg) was isolated from Atlas 66 (*Triticum aestivum*) seedlings and digested with one of four restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, or *Hind*III).

Additionally, Southern analysis for the *TaCLC* clones was conducted with genomic DNA isolated from 35 ditelosomic lines of the wheat cultivar Chinese Spring (*Triticum aestivum*). These lines each have lost a specific chromosome arm.

Collectively, their deletions span the majority of the wheat genome. Hybridization of a wheat clone to a Southern blot of these lines can quickly determine the chromosomal arm location of the probe. The wheat ditelosomic lines were obtained from the Wheat Genetics Resource Center at Kansas State University (http://www.ksu.edu/wgrc/). Genomic DNA (~40 μg) was digested with *Eco*RI or *Hind*III.

For all Southerns, digested DNA was fractionated on a 0.8% agarose gel, and blotted onto a Hybond-N^+ membrane (Amersham Life Science, England) according to the manufacturer's instructions. *TaCLC* probes (complete cDNAs) were labeled with [α-^{32}P]dCTP by random hexamer primers and hybridized to the membrane overnight. The membrane was hybridized at 65°C overnight, washed at 65°C with 2×, 1×, or 0.5× SSC (for low, medium, and high stringency, respectively) and 0.1% SDS and visualized by autoradiography.

RESULTS

CLC homologs exist in the grass family (*Poaceae*)

To determine if CLC homologs were present in the Poaceae, Southern blots were prepared using barley (*Hordeum vulgare* cv. Arapoles) genomic DNA. Given that barley is diploid, which makes interpretation of Southern blots easier, and that, being a grass, barley is phylogenetically much closer to wheat than to *Arabidopsis*, barley was used for this test. DNA was digested with six restriction enzymes (*Bam*HI, *Dra*I, *Eco*RI, *Hae*III, *Hind*III, or *Xba*I). Blots were probed with cDNA probes representing four *Arabidopsis* CLCs (AtCLC-a, AtCLC-b, AtCLC-c, and AtCLC-d; two less-related CLCs from *Arabidopsis* had not been identitified at the time this experiment was initiated). All four cDNA probes yielded positive hybridizations (Figure 3.1). AtCLC-c and AtCLC-d had simple banding patterns, whereas the banding patterns for AtCLC-a and AtCLC-b were more complex. This experiment

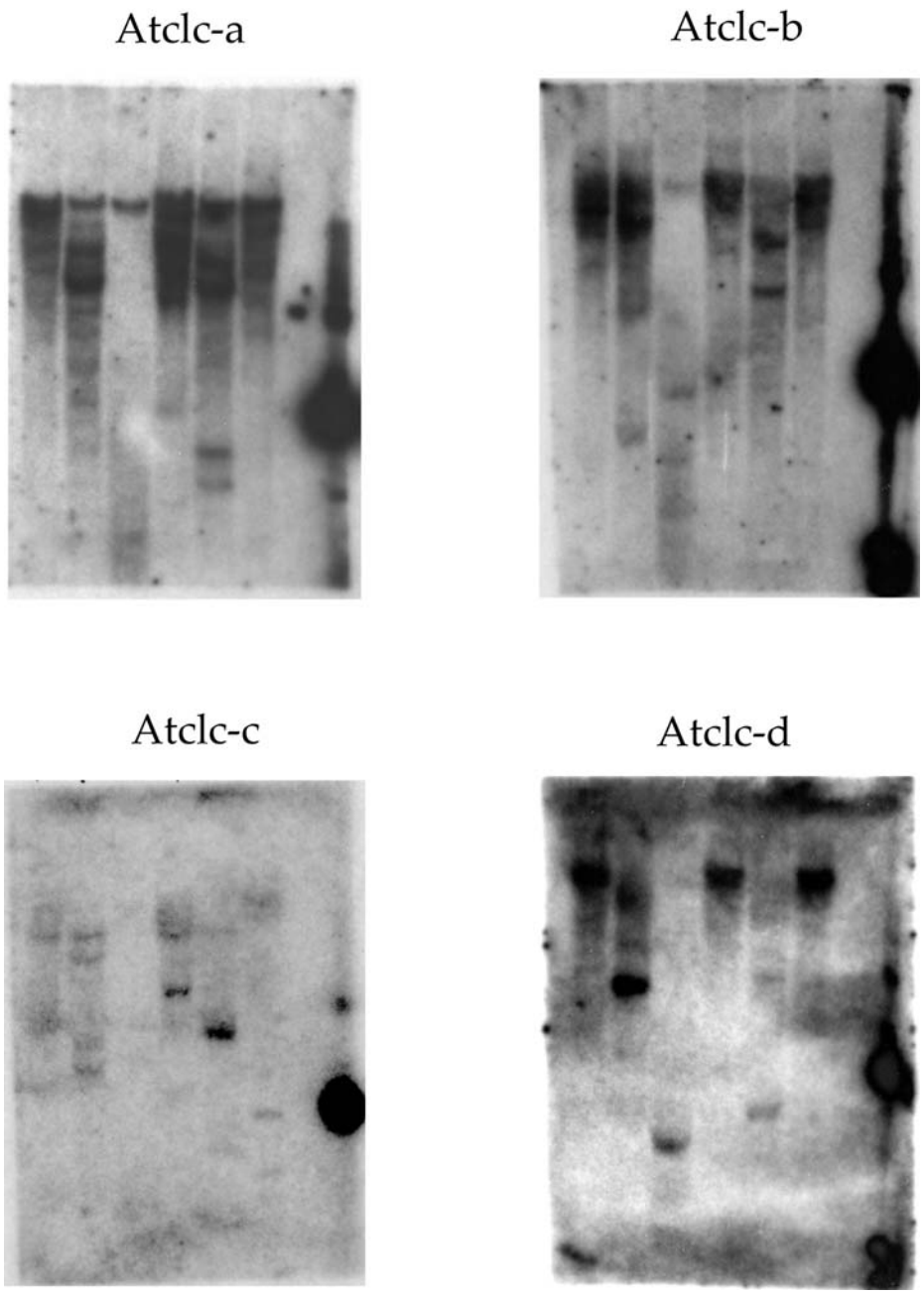


Figure 3.1. Barley Southern blots probed with EST clones of four members of the *Arabidopsis* CLC anion channel family. Each lane was loaded with barley genomic DNA digested with one of six restriction enzymes (from left to right: *Bam*HI, *Dra*I, *Eco*RI, *Hae*III, *Hind*III, or *Xba*I). Each blot was probed with the designated EST. Barley was chosen because it is so closely related to wheat that hybridization results are equivalent. Additionally, the diploid nature of the barley genome makes the results easier to interpret. Hybridization was observed with all four ESTs. Thus, CLC family members exist in the Poaceae and a hybridization-based cloning method is likely to be effective.

established that hybridization between *Arabidopsis* and grass genes was feasible, so a hybridization screen was devised. The complex banding patterns suggested that the CLCs are a multigene family in barley and most likely in wheat (an allohexaploid) as well. Also, because the different *Arabidopsis* ESTs yielded different banding patterns, it was important to use all four available *Arabidopsis* genes as probes, to insure the identification of as wide a selection of *TaCLC*s as possible.

Eight unique TaCLC genes were isolated from an Atlas 66 root tip cDNA library

After confirming that CLCs were present in the Poaceae, a method was devised to isolate homologs from wheat. Plants of the tolerant wheat variety Atlas 66 were grown in simple salt solution and exposed to 15 μM $AlCl_3$, a sub-toxic level for Atlas 66, but a level that induces the malate exudation response that is correlated with tolerance. A cDNA library was constructed from mRNA extracted from the terminal 1.0 cm of root tip tissue. Root apices were used for the purpose of enriching the cDNA library for genes involved in the root tip tolerance mechanism. This library was then screened using EST clones of four *Arabidopsis CLC*s (a,b,c, and d). 25 initially selected clones were screened for duplicates using restriction digest analysis (data not shown). Partial clone sequence was analyzed for homology to other members of the *CLC* family using the BLAST algorithm. Eight unique *CLC* family members from wheat (named *TaCLC-1* through *TaCLC-8*) were identified and characterized.

None of the eight TaCLCs are linked to the *Alt2* Al tolerance locus

Ditelosomic lines of Chinese Spring wheat are special aneuploid stocks of wheat missing one complete chromosome arm. Because of the redundancy conferred by wheat's hexaploid genomic structure, these large deletions are usually not lethal. Southern hybridizations of gene- based probes to these lines can rapidly identify the chromosomal arm location of the probe. Southern blots were prepared using digested

genomic DNA from the 35 ditelosomic lines of Chinese Spring (Table 3.1). The ditelosomic lines are named for the remaining partial chromosome; for example, ditelosomic line Dt1AS is missing the long arm of chromosome 1A. The seven unavailable ditelosomic lines presumably have lethal or infertile phenotypes, despite the hexaploid redundancy that enables the viability of the remaining lines. The blots were probed with labeled *TaCLC* probes. A missing band in a given lane suggests that the gene is located on the chromosome arm deleted from that line. Representative blots for two of the *TaCLC* genes are shown in Figure 3.2. A summary of the chromosomal arm mapping locations is shown in Table 3.2. Due to the hexaploid nature of wheat, as many as three locations could be expected, one in each homoeologous chromosome set. The results shown instead indicate single arm locations for four *TaCLC*s, double arm locations for two *TaCLC*s, and no conclusive physical mapping evidence for two. Although it remains possible that copies of these genes reside on the seven unmappable chromosomal arms, it is likely that homoeologous copies of these genes have diverged sufficiently to prevent cross-hybridization at the stringency used for these Southerns or that the homoeologous copies have been lost as has been observed in the formation of polyploid lines of wheat (Feldman et al., 1997; Ozkan et al., 2001). None of the eight *TaCLC*s mapped to 4DL, the location of the major wheat Al tolerance locus.

Additionally, Southern blot analysis using genomic DNA from the partial deletion lines, 4DL-2 and 4DL-14, were probed with the eight TaCLCs. These partial deletion lines are similar to the ditelosomic lines, but in this case only a fraction of a single chromosome arm is missing. Southern blotting with these lines enables localization to a smaller physical region of the chromosome. As described in Chapter 2, the deletion lines 4DL-2 and 4DL-14 differ in a region of chromosome 4DL that harbors the major wheat Al tolerance locus. Representative blots are shown in Figure

Table 3.1. The ditelosomic lines used in the chromosomal arm mapping experiments. Each row represents one of the 7 chromosomes present in each genome. Each pair of columns represents a genome; the left column including lines with deletions of the long arms of their chromosome while the right column includes lines with deletions of the short arms (for example, Dt1AS lacks the long arm of chromosome 1A). There are 42 chromosome arms in wheat (3 genomes x 7 chromosomes per genome). There are 35 ditelosomic lines each representing a deletion of one of those arms. The missing lines are due to the lethality or infertility resulting from that deletion.

	A genome		B genome		D genome	
	Short arm	Long arm	Short arm	Long arm	Short arm	Long arm
Chromosome 1	Dt1AS	Dt1AL	Dt1BS	Dt1BL	Dt1DS	Dt1DL
Chromosome 2	Dt2AS			Dt2BL	Dt2DS	Dt2DL
Chromosome 3	Dt3AS	Dt3AL	Dt3BS	Dt3BL	Dt3DS	Dt3DL
Chromosome 4	Dt4AS	Dt4AL	Dt4BS		Dt4DS	Dt4DL
Chromosome 5		Dt5AL		Dt5BL		Dt5DL
Chromosome 6	Dt6AS	Dt6AL	Dt6BS	Dt6BL	Dt6DS	Dt6DL
Chromosome 7	Dt7AS	Dt7AL	Dt7BS	Dt7BL	Dt7DS	

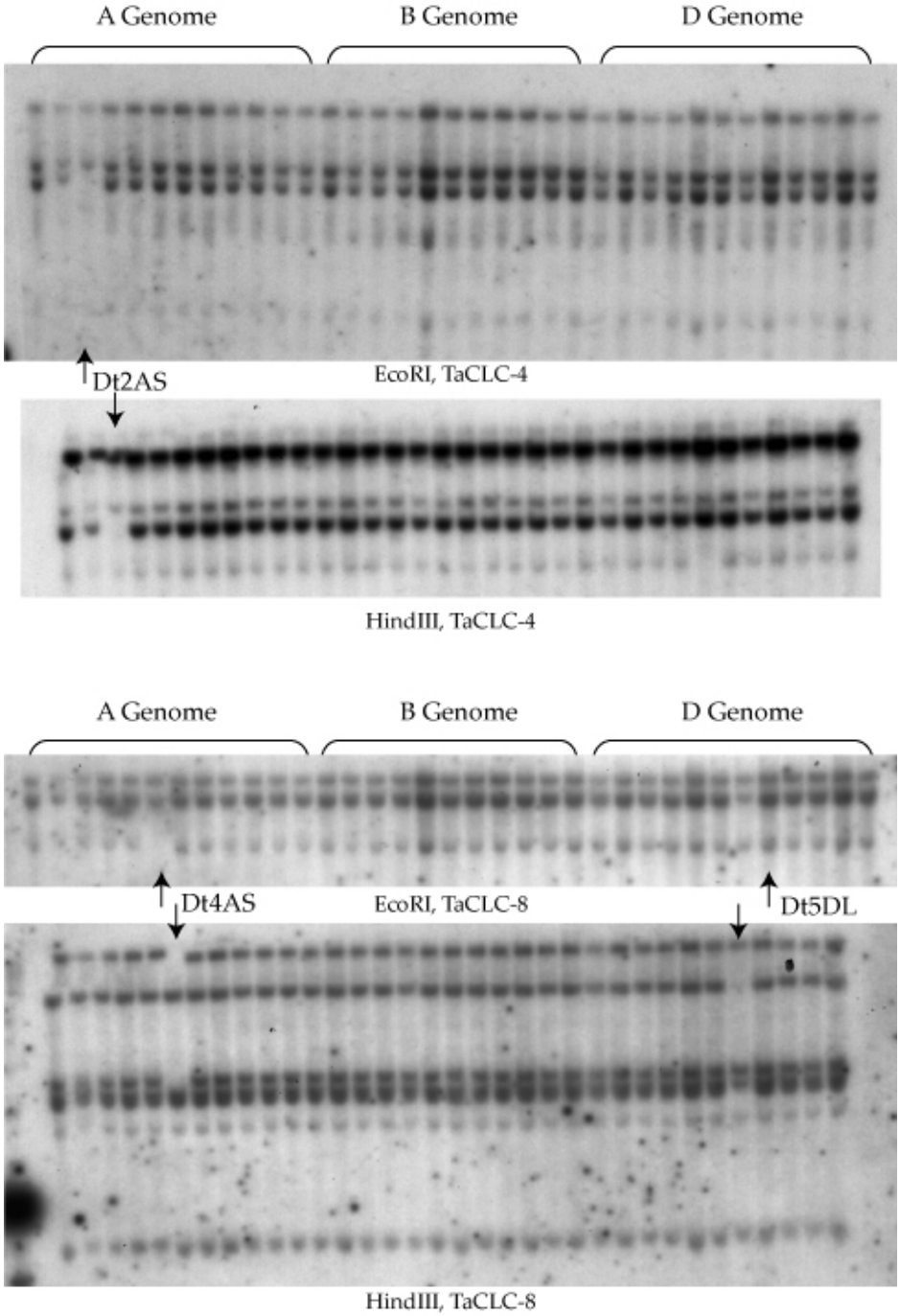


Figure 3.2. Representative Southern blots from the ditelosomic mapping experiment. Digested genomic DNA from ditelosomic lines of wheat was probed with the *TaCLC* cDNA clones. Each lane contains digested DNA from one of the 35 ditelosomic lines listed in Table 3.1, ordered from the left by genome, chromosome, and arm (short, then long). In the figure, arrows point to lanes containing missing bands, which contain genomic DNA from the ditelosomic line indicated. Probing the blots with *TaCLC* probes enabled the one-step localization of a gene to a chromosome arm in some cases. The mapping results are summarized in Table 3.2.

Table 3.2 Putative chromosomal arm localization of *TaCLC* genes based on ditelosomic Southern analysis mapping experiment. Each clone had a missing band on a Southern blot corresponding to the ditelosomic line missing that chromosome arm. Unmapped clones may be localized on chromosome arms not represented among the ditelosomic line deletions. These arms may also harbor homoeologous copies of other clones.

Arm	**Line**
2AL	TaCLC-4
3AS	TaCLC-5
3DS	TaCLC-6
4AL/5DS	TaCLC-1, TaCLC-8
6DL	TaCLC-3
Unmapped	TaCLC-2, TaCLC-7

3.3. None of the eight *TaCLC*s mapped to this region of chromosome 4DL. The results from these two Southern blot experiments clearly indicate that none of these genes are in fact this aluminum tolerance locus. However, this does not preclude a possible role in Al-activated malate release. One way to gain insight into that possible function is to assay the interaction between *Alt2* and the *TaCLC*s as described in the next section.

TaCLC expression is unaffected by the loss of the *Alt2* Al tolerance locus

To determine if the presence or absence of *Alt2* affects expression of the *TaCLC*s, RNA blots of mRNA extracted from root tips of the partial deletion lines 4DL-2 and 4DL-14 were probed with the eight *TaCLC* cDNAs. As seen in Figure 3.4, there were no differences in expression levels for any of the *TaCLC*s between the two deletion lines. This confirms the Southern blot mapping experiments in the sense that a deleted gene would have a missing band or a band of less intensity (if multiple *TaCLC* mRNAs of the same size are simultaneously expressed and hybridize to the same probe). Additionally, it indicates that the deletion of *Alt2* does not directly affect the expression of the *TaCLC*s, which might happen if *Alt2* were necessary for the expression of a *TaCLC*, for example.

Northern analysis shows at least three different expression patterns among *TaCLC* genes

Another question regarding *TaCLC* involvement in the response to aluminum is whether *TaCLC* expression is altered in response to exposure to aluminum. Because aluminum toxicity and tolerance function primarily at the root apex, root tip expression was analyzed in this region both with and without aluminum treatment. For comparison, expression in the remainder of the root (not including the apex) and in the shoot was also analyzed. Northern blots were prepared using mRNA extracted from four different tissue/treatment combinations in both Atlas66 (tolerant) and

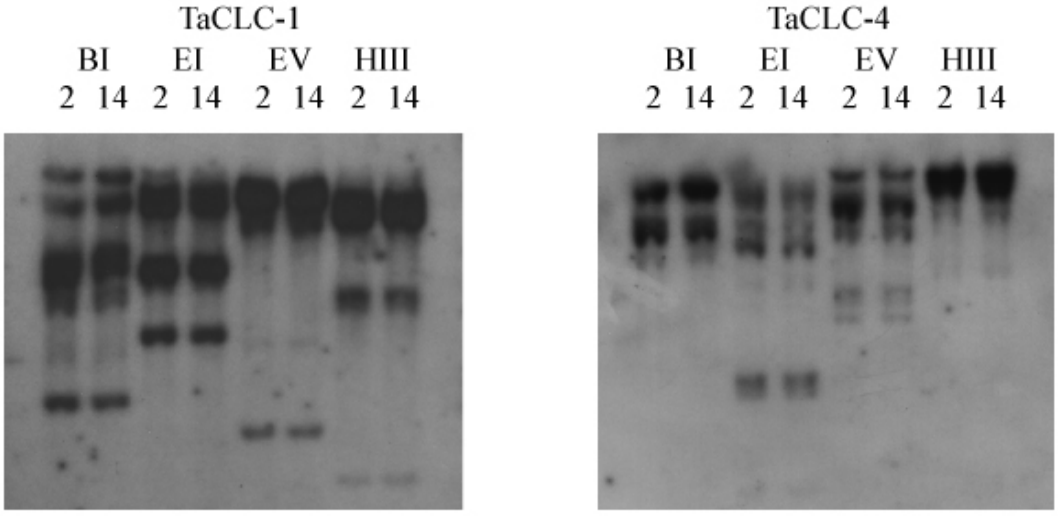


Figure 3.3 Representative Southern blot depicting mapping results from partial deletion line experiment. Blots contain digested DNA from partial deletion lines 4DL-2 and 4DL-14 of wheat cultivar Chinese Spring. DNA was digested with BamHI (BI), EcoRI (EI), EcoRV (EV), or HindIII (HIII), as labeled. Blot DNA is in alternating lanes of 4DL-2 (2) and 4DL-14 (14). None of the 8 *TaCLC*s were indicated to reside in this deletion interval.

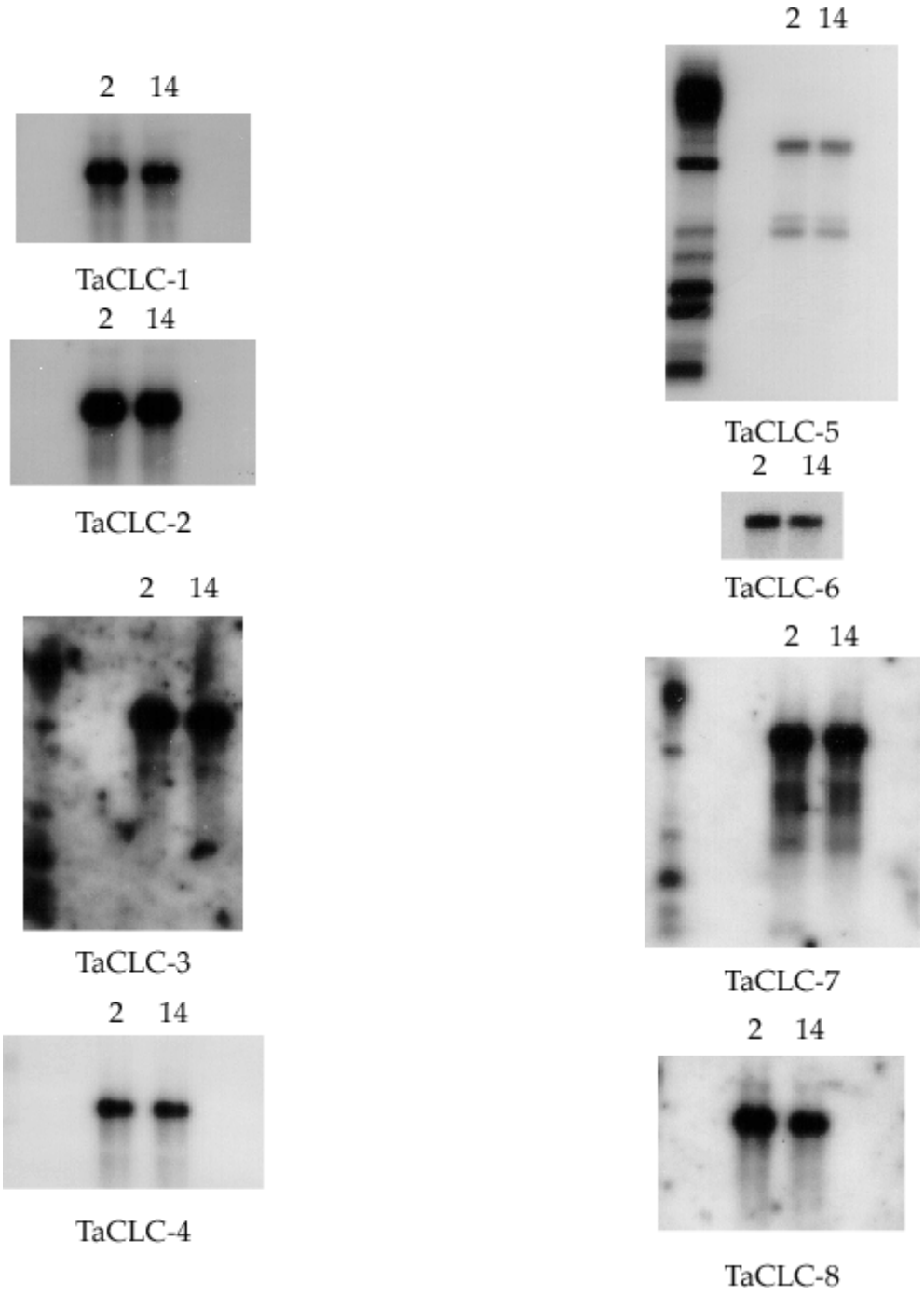


Figure 3.4. Northern blot analysis for expression of *TaCLC*s in the partial deletion lines 4DL-2 (2) and 4DL-14 (14). These Northerns confirm the results from the two Southern mapping experiments. They also show that deletion of the *Alt2* aluminum tolerance locus does not alter expression of any of the *TaCLC*s.

Scout66 (sensitive). As seen in Figure 3.5, there were three broad expression patterns, two of which were observable for multiple *TaCLC*s. *TaCLC-2* had lower expression in the shoots than in the roots and its expression was increased by Al exposure in Atlas. *TaCLC-1* and *TaCLC-4* both had consistent expression across all tissue/treatment combinations. *TaCLC-3*, *TaCLC-5*, and *TaCLC-6* were more highly expressed in shoots than in roots. Among these, *TaCLC-5* also exhibited a small induction upon Al treatment. In fact, *TaCLC-5* may be induced by Al to a greater extent in Scout (the sensitive variety). This pattern would be consistent with the pattern seen with genes involved in general stress response (Hamel et al., 1998). The widely varying expression patterns indicate that the *TaCLC*s probably perform a variety of functions in different locations in the plant, as has been found with animal *CLC*s. The specific role of each *TaCLC* remains to be elucidated.

None of the *TaCLC*s exhibited marked differential expression between the tolerant and sensitive lines of wheat. One possible exception is the root tip expression of *TaCLC-2*, which appears to be higher in Scout (sensitive) both with and without Al. *TaCLC-5*, also induced by Al, seems to be more highly induced in Al sensitive Scout. A number of general stress response genes show greater Al induction in Al sensitive lines than in tolerant lines (Snowden et al., 1995). The expression patterns of *TaCLC-2* and *TaCLC-5* are consistent with that response rather than a tolerance-related response.

CLC Phylogenetic Analysis

BLAST searching (Altschul et al., 1990) revealed 12 *Oryza sativa* CLCs in Genbank. Since rice is a close relative of wheat and is diploid, three homoeologous wheat genes may be expected for each rice gene, although rice duplications or wheat deletions would reduce the actual number. Therefore, there could be between eight and 36 *TaCLC*s in total in wheat. Predicted protein sequences based on ESTs from a

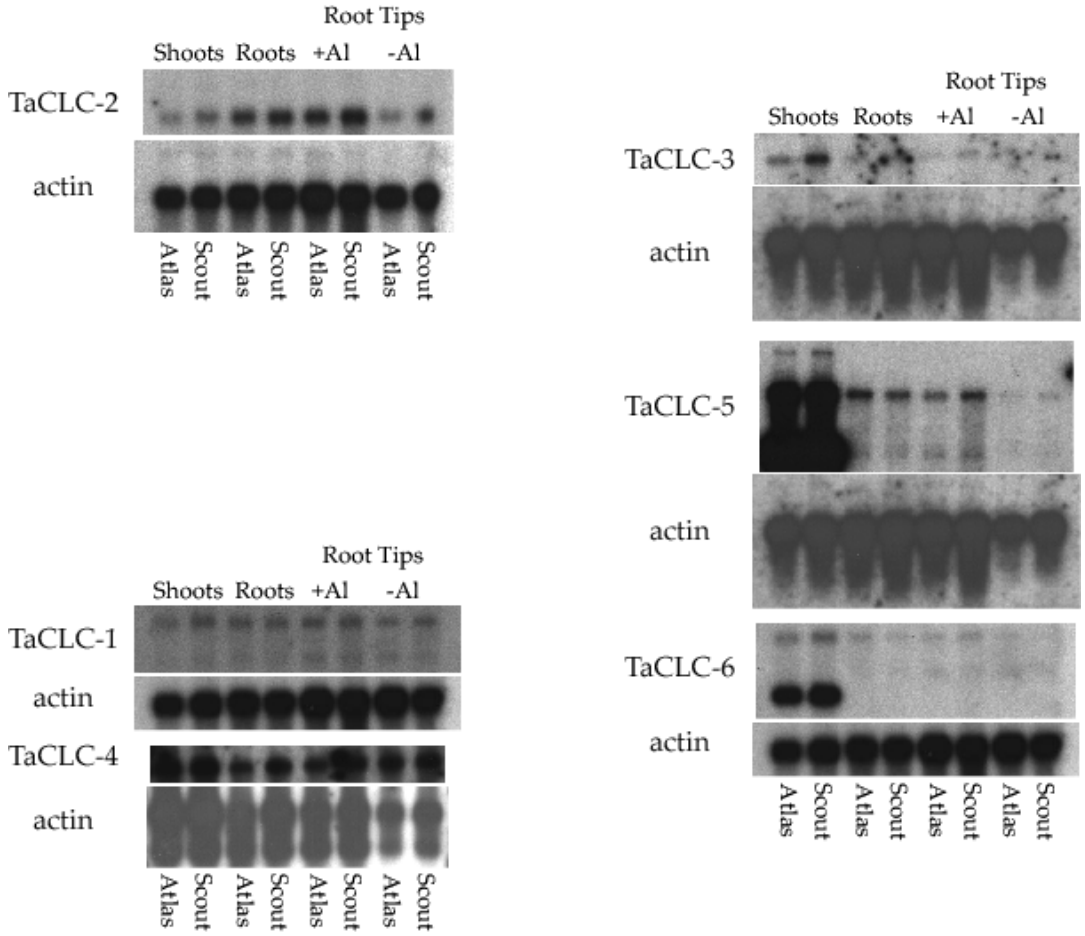


Figure 3.5. Northern blot expression analysis of *TaCLC*s. Each pair of lanes contains mRNA from Atlas66 (tolerant) and Scout66 (sensitive). Messenger RNA was extracted from shoots, roots, root tips of plants treated with aluminum, and root tips from the control (-Al) treatment. The blots and *TaCLC*s are grouped according similarity in broad patterns of expression. *TaCLC*-2 and *TaCLC*-5 both seemed to have slight induction upon treatment with aluminum.

number of species were aligned and parsimony analysis was performed. The alignment of the TaCLCs is shown in Figure 3.6. The phylogenetic tree based on parsimony analysis is shown in Figure 3.7. Interestingly, three rice *CLC*s group with *AtCLCe* and *AtCLCf*. This whole clade is grouped with a *CLC* from *Rhodospirillum rubrum* (a member of the alpha purple bacteria, the likely progenitors of mitochondria). A likely explanation is that the progenitor of this group of genes was transferred from the mitochondria to the nucleus. *AtCLCe* and *AtCLCf* were not available when this screen was undertaken, and it is possible that this difference underlies the lack of *TaCLC*s cloned from this sub-family.

DISCUSSION

Given the likely dependence of the wheat aluminum tolerance mechanism on anion transporters for malate release, the first question after cloning wheat *CLC*s was: do any of these genes represent the major aluminum tolerance gene in wheat? The straightforward mapping results (Figures 3.2 and 3.3) indicate that the eight *TaCLC*s cloned in this study are not located near the Al tolerance locus and therefore do not represent the tolerance gene.

Given that these anion channels are not the tolerance gene in and of themselves, might they play a role in the release of malate even though they themselves are not the regulator of differential tolerance? The clearest answer to this question awaits functional data (through heterologous expression, for example) and the correlation of such data to previously observed Al-induced anion currents *in planta*. In the meantime, the RNA expression data presented here gives some clues. *TaCLC-2* and *TaCLC-5* were both induced in root tips in response to Al. Following a pattern seen in many stress response genes, the induction was higher in the sensitive line as compared to the tolerant line, correlating better with toxicity than with

Figure 3.6. Amino acid alignment of the *TaCLC*s. The light gray shaded boxes indicate CBS domains (as determined by SMART; Letunic et al., 2004), a characteristic cytoplasmic domain of CLC channels, but also found in the eponymous cystathionine ß-synthase and many other proteins.. Medium gray shaded residues represent transmembrane helices (as determined by PHDhtm Combet et al., 2000). The dark gray shaded boxes indicate the conserved regions of the selectivity filter, as determined by alignment to the crystal structures of two prokaryotic CLCs (Dutzler et al., 2002).

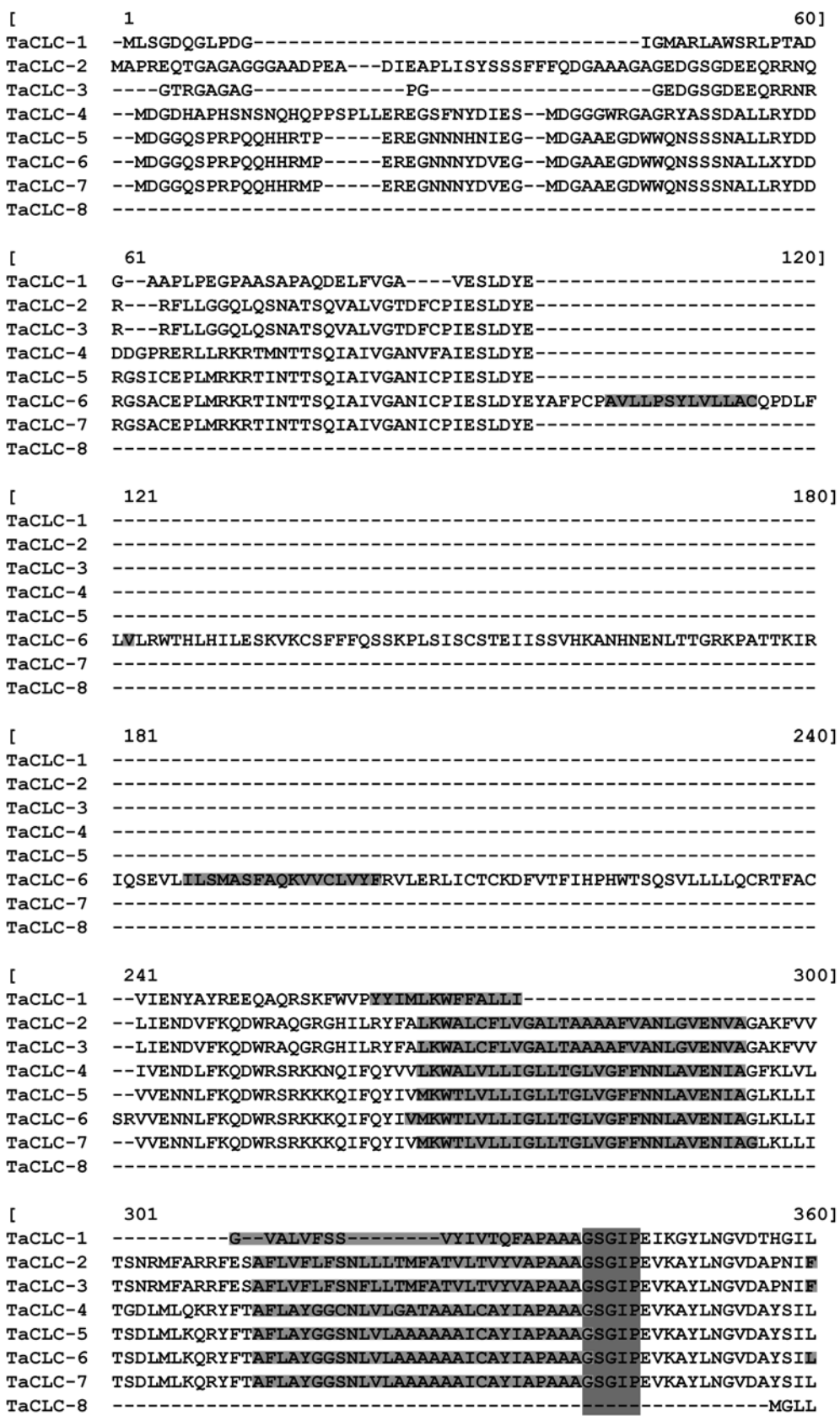

[1 60]
TaCLC-1 -MLSGDQGLPDG----------IGMARLAWSRLPTAD
TaCLC-2 MAPREQTGAGAGGGAADPEA---DIEAPLISYSSSFFFQDGAAAGAGEDGSGDEEQRRNQ
TaCLC-3 ----GTRGAGAG----------PG----------GEDGSGDEEQRRNR
TaCLC-4 --MDGDHAPHSNSNQHQPPSPLLEREGSFNYDIES--MDGGGWRGAGRYASSDALLRYDD
TaCLC-5 --MDGGQSPRPQQHHRTP-----EREGNNNHNIEG--MDGAAEGDWWQNSSSNALLRYDD
TaCLC-6 --MDGGQSPRPQQHHRMP-----EREGNNNYDVEG--MDGAAEGDWWQNSSSNALLXYDD
TaCLC-7 --MDGGQSPRPQQHHRMP-----EREGNNNYDVEG--MDGAAEGDWWQNSSSNALLRYDD
TaCLC-8 ----------
[61 120]
TaCLC-1 G--AAPLPEGPAASAPAQDELFVGA----VESLDYE----------
TaCLC-2 R---RFLLGGQLQSNATSQVALVGTDFCPIESLDYE----------
TaCLC-3 R---RFLLGGQLQSNATSQVALVGTDFCPIESLDYE----------
TaCLC-4 DDGPRERLLRKRTMNTTSQIAIVGANVFAIESLDYE----------
TaCLC-5 RGSICEPLMRKRTINTTSQIAIVGANICPIESLDYE----------
TaCLC-6 RGSACEPLMRKRTINTTSQIAIVGANICPIESLDYEYAFPCPAVLLPSYLVLLACQPDLF
TaCLC-7 RGSACEPLMRKRTINTTSQIAIVGANICPIESLDYE----------
TaCLC-8 ----------
[121 180]
TaCLC-1 ----------
TaCLC-2 ----------
TaCLC-3 ----------
TaCLC-4 ----------
TaCLC-5 ----------
TaCLC-6 LVLRWTHLHILESKVKCSFFFQSSKPLSISCSTEIISSVHKANHNENLTTGRKPATTKIR
TaCLC-7 ----------
TaCLC-8 ----------
[181 240]
TaCLC-1 ----------
TaCLC-2 ----------
TaCLC-3 ----------
TaCLC-4 ----------
TaCLC-5 ----------
TaCLC-6 IQSEVLILSMASFAQKVVCLVYFRVLERLICTCKDFVTFIHPHWTSQSVLLLLQCRTFAC
TaCLC-7 ----------
TaCLC-8 ----------
[241 300]
TaCLC-1 --VIENYAYREEQAQRSKFWVPYYIMLKWFFALLI----------
TaCLC-2 --LIENDVFKQDWRAQGRGHILRYFALKWALCFLVGALTAAAAFVANLGVENVAGAKFVV
TaCLC-3 --LIENDVFKQDWRAQGRGHILRYFALKWALCFLVGALTAAAAFVANLGVENVAGAKFVV
TaCLC-4 --IVENDLFKQDWRSRKKNQIFQYVVLKWALVLLIGLLTGLVGFFNNLAVENIAGFKLVL
TaCLC-5 --VVENNLFKQDWRSRKKKQIFQYIVMKWTLVLLIGLLTGLVGFFNNLAVENIAGLKLLI
TaCLC-6 SRVVENNLFKQDWRSRKKKQIFQYIVMKWTLVLLIGLLTGLVGFFNNLAVENIAGLKLLI
TaCLC-7 --VVENNLFKQDWRSRKKKQIFQYIVMKWTLVLLIGLLTGLVGFFNNLAVENIAGLKLLI
TaCLC-8 ----------
[301 360]
TaCLC-1 ----------G--VALVFSS--------VYIVTQFAPAAAGSGIPEIKGYLNGVDTHGIL
TaCLC-2 TSNRMFARRFESAFLVFLFSNLLLTMFATVLTVYVAPAAAGSGIPEVKAYLNGVDAPNIF
TaCLC-3 TSNRMFARRFESAFLVFLFSNFLLTMFATVLTVYVAPAAAGSGIPEVKAYLNGVDAPNIF
TaCLC-4 TGDLMLQKRYFTAFLAYGGCNLVLGATAAALCAYIAPAAAGSGIPEVKAYLNGVDAYSIL
TaCLC-5 TSDLMLKQRYFTAFLAYGGSNLVLAAAAAAICAYIAPAAAGSGIPEVKAYLNGVDAYSIL
TaCLC-6 TSDLMLKQRYFTAFLAYGGSNLVLAAAAAAICAYIAPAAAGSGIPEVKAYLNGVDAYSIL
TaCLC-7 TSDLMLKQRYFTAFLAYGGSNLVLAAAAAAICAYIAPAAAGSGIPEVKAYLNGVDAYSIL
TaCLC-8 ----------MGLL

Figure 3.6 (continued)

Figure 3.6. (continued).

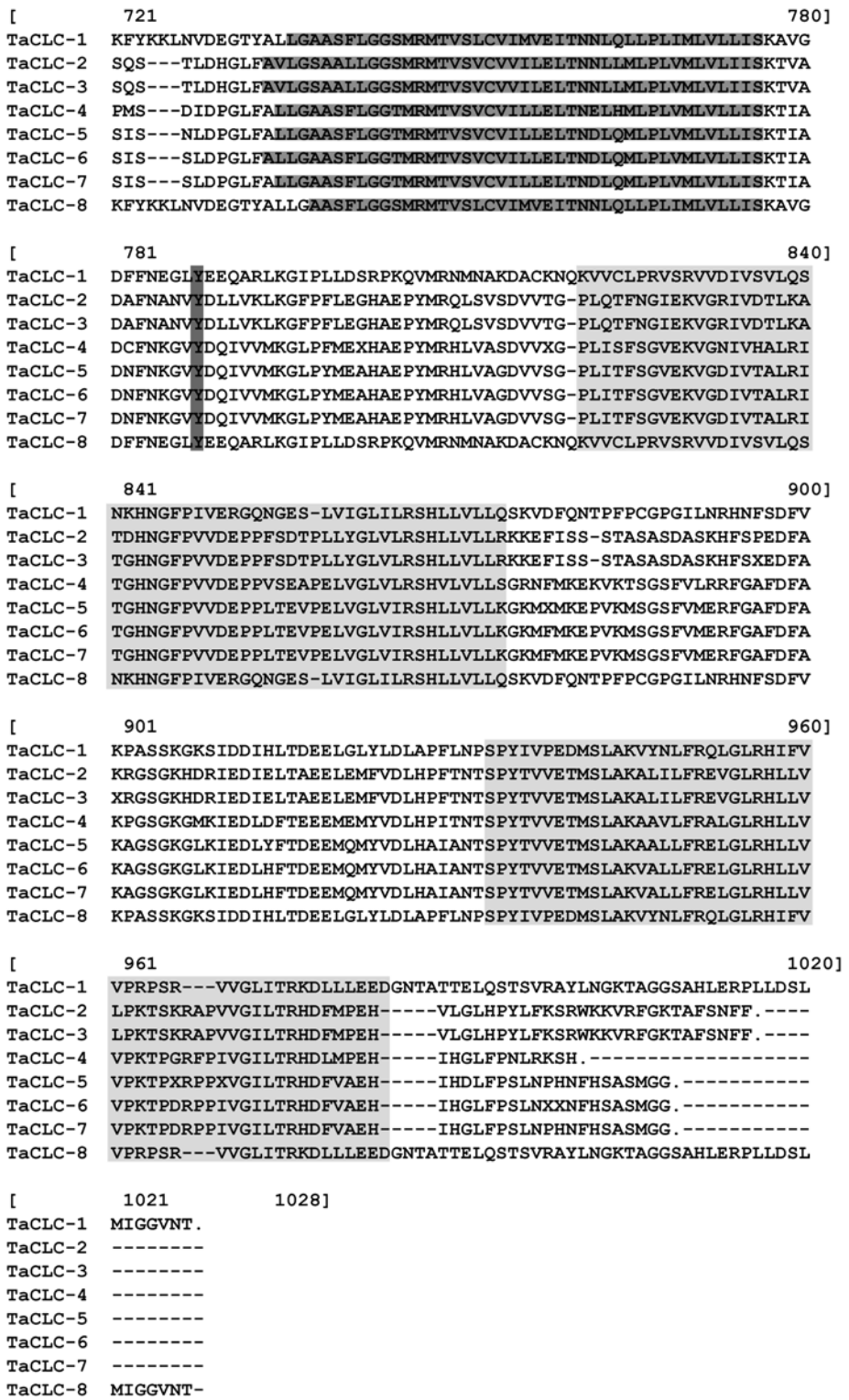
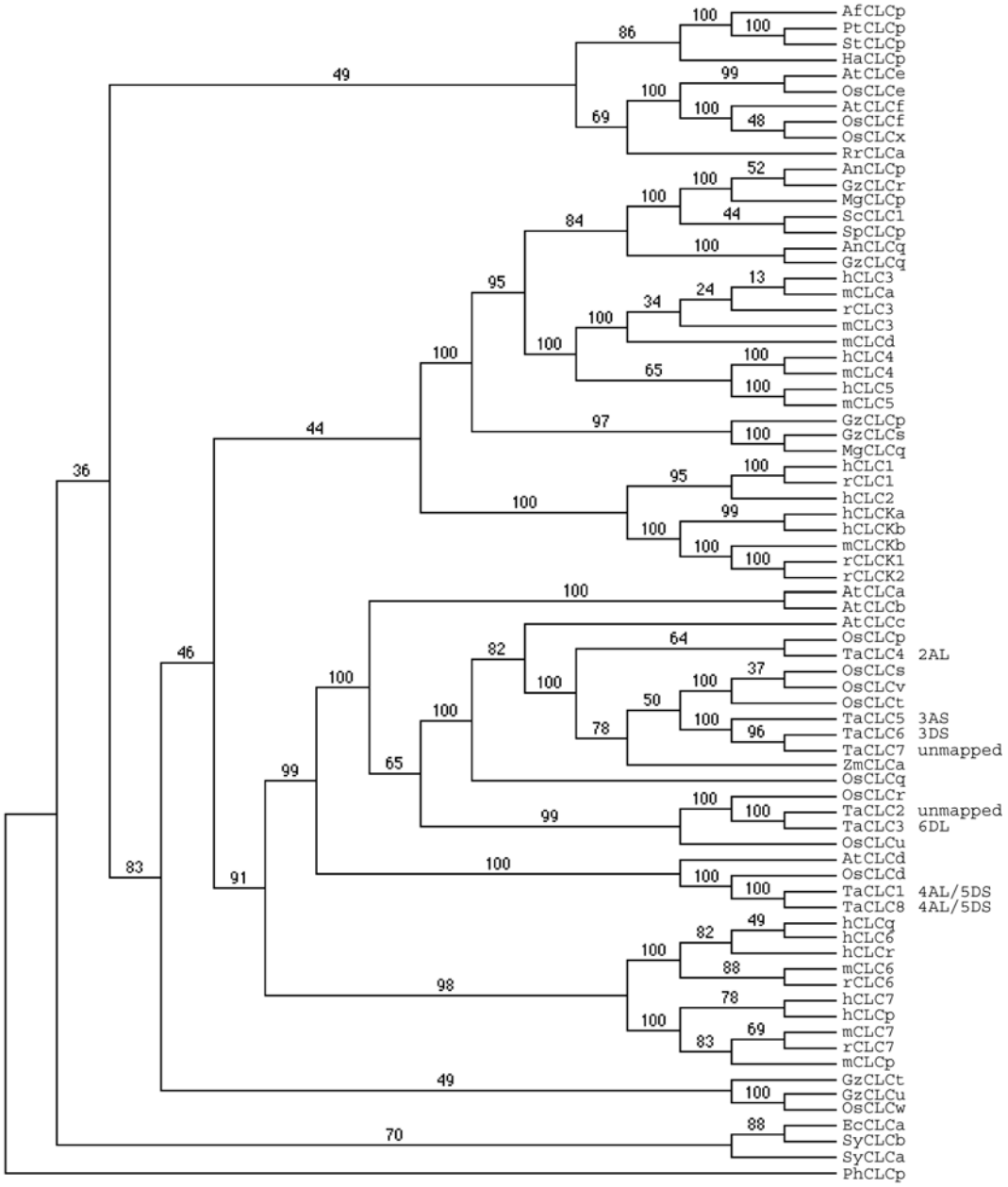


Figure 3.7. CLC phylogenetic tree. Amino acid sequences were aligned using the ClustalW algorithm. Parsimony and bootstrap analysis was performed with PAUP* (Swofford, 1998), and the tree was rooted with an EST from the archea *Pyrococcus horikoshii*. Protein sequences from plants, humans, yeast, bacteria, and archebacteria were included. The following table indicates the sequences used, their NCBI GI numbers, and their taxonomic groups. Some of the alpha suffixes are arbitrary designators for unnamed genes from the EST database. Chromosome arm mapping locations as determined by ditelosomic line Southern blots are included for the TaCLCs.

Name	GI	Species	Domain	Subgroup
StCLCp	15623533	*Sulfolobus tokodaii*	Archea	Crenarchaeota
AfCLCp	11499010	*Archaeoglobus fulgidus*	Archea	Euryarchaeota
HaCLCp	10581031	*Halobacterium sp.*	Archea	Euryarchaeota
PhCLCp	3256417	*Pyrococcus horikoshii*	Archea	Euryarchaeota
PtCLCp	48430876	*Picrophilus torridus*	Archea	Euryarchaeota
SyCLCa	16331172	*Synechocystis sp.*	Eubacteria	Cyanobacteria
SyCLCb	16330386	*Synechocystis sp.*	Eubacteria	Cyanobacteria
RrCLCa	22967595	*Rhodospirillum rubrum*	Eubacteria	Proteobacteria (alpha)
EcCLCa	30749968	*Escherichia coli*	Eubacteria	Proteobacteria (gamma)
EcCLCb	26246101	*Escherichia coli*	Eubacteria	Proteobacteria (gamma)
LmCLCp	2995581	*Leishmania major*	Eukaryota	Euglenozoa
AnCLCp	40745263	*Aspergillus nidulans*	Eukaryota	Fungi
AnCLCq	40739505	*Aspergillus nidulans*	Eukaryota	Fungi
GzCLCp	46117512	*Gibberella zeae*	Eukaryota	Fungi
GzCLCq	42552425	*Gibberella zeae*	Eukaryota	Fungi
GzCLCr	42552696	*Gibberella zeae*	Eukaryota	Fungi
GzCLCs	46122355	*Gibberella zeae*	Eukaryota	Fungi
GzCLCt	46110298	*Gibberella zeae*	Eukaryota	Fungi
GzCLCu	42552083	*Gibberella zeae*	Eukaryota	Fungi
MgCLCp	38106704	*Magnaporthe grisea*	Eukaryota	Fungi
MgCLCq	38104469	*Magnaporthe grisea*	Eukaryota	Fungi
ScCLC1	1077068	*Saccharomyces cerevisiae*	Eukaryota	Fungi
SpCLCp	19112959	*Schizosaccharomyces pombe*	Eukaryota	Fungi
hCLC1	544024	*Homo sapiens*	Eukaryota	Metazoa
hCLC2	18204325	*Homo sapiens*	Eukaryota	Metazoa
hCLC3	854103	*Homo sapiens*	Eukaryota	Metazoa
hCLC4	4502871	*Homo sapiens*	Eukaryota	Metazoa
hCLC5	4557473	*Homo sapiens*	Eukaryota	Metazoa
hCLC6	1705910	*Homo sapiens*	Eukaryota	Metazoa
hCLC7	14149607	*Homo sapiens*	Eukaryota	Metazoa
hCLCKa	31753083	*Homo sapiens*	Eukaryota	Metazoa
hCLCKb	4557475	*Homo sapiens*	Eukaryota	Metazoa
hCLCp	13436311	*Homo sapiens*	Eukaryota	Metazoa
hCLCq	40789076	*Homo sapiens*	Eukaryota	Metazoa

Figure 3.7 legend (continued)

hCLCr	1770376	*Homo sapiens*	Eukaryota	Metazoa
mCLC3	34785552	*Mus musculus*	Eukaryota	Metazoa
mCLC4	3182962	*Mus musculus*	Eukaryota	Metazoa
mCLC5	23271431	*Mus musculus*	Eukaryota	Metazoa
mCLC6	6753434	*Mus musculus*	Eukaryota	Metazoa
mCLC7	6753436	*Mus musculus*	Eukaryota	Metazoa
mCLCa	22023503	*Mus musculus*	Eukaryota	Metazoa
mCLCd	22023505	*Mus musculus*	Eukaryota	Metazoa
mCLCKb	18043439	*Mus musculus*	Eukaryota	Metazoa
mCLCp	26328487	*Mus musculus*	Eukaryota	Metazoa
rCLC1	6978663	*Rattus norvegicus*	Eukaryota	Metazoa
rCLC3	1705905	*Rattus norvegicus*	Eukaryota	Metazoa
rCLC6	34872458	*Rattus norvegicus*	Eukaryota	Metazoa
rCLC7	1177613	*Rattus norvegicus*	Eukaryota	Metazoa
rCLCK1	27465537	*Rattus norvegicus*	Eukaryota	Metazoa
rCLCK2	1705861	*Rattus norvegicus*	Eukaryota	Metazoa
AtCLCa	1742953	*Arabidopsis thaliana*	Eukaryota	Viridiplantae
AtCLCb	1742955	*Arabidopsis thaliana*	Eukaryota	Viridiplantae
AtCLCc	1742957	*Arabidopsis thaliana*	Eukaryota	Viridiplantae
AtCLCd	1742959	*Arabidopsis thaliana*	Eukaryota	Viridiplantae
AtCLCe	14039799	*Arabidopsis thaliana*	Eukaryota	Viridiplantae
AtCLCf	14039802	*Arabidopsis thaliana*	Eukaryota	Viridiplantae
OsCLCd	27552547	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCf	42407340	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCp	21321022	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCq	34907322	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCr	38344896	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCs	46390910	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCt	21321024	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCu	34015377	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCv	21321026	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCw	46391116	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCx	45735842	*Oryza sativa*	Eukaryota	Viridiplantae
OsCLCy	34910358	*Oryza sativa*	Eukaryota	Viridiplantae
TaCLC1	n/a	*Triticum aestivum*	Eukaryota	Viridiplantae
TaCLC2	n/a	*Triticum aestivum*	Eukaryota	Viridiplantae
TaCLC3	n/a	*Triticum aestivum*	Eukaryota	Viridiplantae
TaCLC4	n/a	*Triticum aestivum*	Eukaryota	Viridiplantae
TaCLC5	n/a	*Triticum aestivum*	Eukaryota	Viridiplantae
TaCLC6	n/a	*Triticum aestivum*	Eukaryota	Viridiplantae
TaCLC7	n/a	*Triticum aestivum*	Eukaryota	Viridiplantae
TaCLC8	n/a	*Triticum aestivum*	Eukaryota	Viridiplantae
ZmCLCa	48374433	*Zea mays*	Eukaryota	Viridiplantae


AfCLCp
PtCLCp
StCLCp
HaCLCp
AtCLCe
OsCLCe
AtCLCf
OsCLCf
OsCLCx
RrCLCa
AnCLCp
GzCLCr
MgCLCp
ScCLC1
SpCLCp
AnCLCq
GzCLCq
hCLC3
mCLCa
rCLC3
mCLC3
mCLCd
hCLC4
mCLC4
hCLC5
mCLC5
GzCLCp
GzCLCs
MgCLCq
hCLC1
rCLC1
hCLC2
hCLCKa
hCLCKb
mCLCKb
rCLCK1
rCLCK2
AtCLCa
AtCLCb
AtCLCc
OsCLCp
TaCLC4 2AL
OsCLCs
OsCLCv
OsCLCt
TaCLC5 3AS
TaCLC6 3DS
TaCLC7 unmapped
ZmCLCa
OsCLCq
OsCLCr
TaCLC2 unmapped
TaCLC3 6DL
OsCLCu
AtCLCd
OsCLCd
TaCLC1 4AL/5DS
TaCLC8 4AL/5DS
hCLCg
hCLC6
hCLCr
mCLC6
rCLC6
hCLC7
hCLCp
mCLC7
rCLC7
mCLCp
GzCLCt
GzCLCu
OsCLCw
EcCLCa
SyCLCb
SyCLCa
PhCLCp

tolerance. So it is possible that these two *TaCLC*s play some role in the cellular response to aluminum. Whether they are directly involved in malate exudation remains to be determined.

The very recent discovery of an aluminum-activated malate transporter in wheat (*ALMT1*; Sasaki et al., 2004) sheds doubt on the likelihood that the *TaCLC*s are directly involved in the aluminum tolerance response. *ALMT1* was cloned based on its higher expression in a tolerant variety of wheat relative to a sensitive variety. It is very tightly linked to aluminum tolerance in segregating populations, suggesting that it may be the gene underlying the *Alt1* locus. *ALMT1* functions as a malate transporter when expressed in *Xenopus* oocytes, and when it is expressed in rice plants and tobacco suspension cells, an Al-activated malate release is conferred. *ALMT1* is a novel transporter, not related to any known channel families. Even though *ALMT1* appears to be both the major aluminum tolerance gene and the plasma membrane malate transporter, there still may be a role for the *TaCLC*s in Al tolerance. Intracellular malate distribution may be a critical component of aluminum tolerance, and vacuolar or vesicular anion channels presumably play a role in these processes.

Meanwhile, clues to other functions for the *TaCLC*s arise from differences in the gene expression patterns. *TaCLC-3*, *TaCLC-5*, and *TaCLC-6* show higher expression in the shoots than in the roots. *TaCLC-1* and *TaCLC-4* exhibit equal expression in shoots and roots, whereas *TaCLC-2* exhibits lower expression in the shoots. The significance of these expression differences is uncertain, but they suggest differences in specialization among the *TaCLC*s.

From examination of the *TaCLC* sequences and their relationship to other members of the CLC family, it is almost certain that there are more than eight wheat representatives of this family. For example, no wheat genes from the putative mitochondrial clade are represented. There may also be missing orthologues of

*OsCLC*q, *OsCLC*u, and *OsCLC*w, among others. So, it remains a possibility that an uncloned homolog is linked to or represents the major aluminum tolerance gene. However, the ditelosomic line mapping experiments covered considerably more ground than the six clones specifically mapped, as paralogues with similar sequences could have revealed themselves with missing bands.

There are many unanswered questions about the role and function of the *TaCLC*s. One pertinent question in ascertaining the possible involvement of these genes in malate exudation is to determine their subcellular localization. Recent work in the Kochian lab indicates that *TaCLC-1*, when tagged with GFP, and expressed in mammalian cells, is expressed in the plasma membrane (Piñeros et al., 2003). This would be consistent with a role in malate exudation. However, membrane localization may differ between wheat and human cells. Transforming wheat roots with GFP-tagged *TaCLC*s could clarify which membranes the *TaCLC*s are targeted to. *In situ* hybridization could clarify cellular and tissue distribution of expression.

Functional data have been elusive for plant CLCs despite the many studies from non-plant CLCs. Heterologous expression options include *Xenopus* oocytes, yeast, and mammalian cells. Tobacco *NtCLC-1* was initially reported to be functional when expressed in oocytes (Lurin et al., 1996). However, *Xenopus* oocytes have subsequently been shown to exhibit native anion fluxes similar to those reported for *NtCLC-1*. Mammalian cells or artificial lipid bilayers may offer a better "substrate" for these studies.

Recent structural information has elucidated the general mechanism of anion selectivity in CLCs and has offered some explanation for the unusual electrophysiological properties of the channels. For years there was debate on the gating properties of the CLC channels. The multi-state bursting behaviour observed in single-channel patches (e.g. Lin et al., 1999) was alternately interpreted as a result of

two conformations of a single pore or as a novel channel configuration containing two pores. The first definitive evidence for the homodimer/two pore structure came from the projection structure of the E. coli CLC at 6.5Å (Mindell et al., 2001). Cryo-electron microscopic analysis of two-dimensional protein crystals shows the two-dimensional distribution of protein density, which revealed two separate water-filled pores. This study was shortly followed by the three-dimensional X-ray crystal structure at 3.0Å (Dutzler et al., 2002), which confirmed the homodimer double pore architecture. The crystal structure reveals that Cl^- anion stabilization in the core of CLCs is conferred by interaction with α-helix dipole partial positive charges and by chemical coordination with nitrogen atoms and hydroxyl groups. The four peptide segments identified as important for the selectivity filter by the crystal structure are highlighted in dark grey in Figure 3.6. Excluding *TaCLC-8*, which is a truncated cDNA clone, the first region (GSGIP) and the last (Y) are 100% conserved in the *TaCLC*s. The second region (GKEGP in five of the seven TaCLCs), is altered by a single amino acid (to GKAGP) in *TaCLC-2* and *TaCLC-3*. This substitution of a negatively charged glutamate with a smaller and neutral alanine could have a significant impact on anion permeability or selectivity. It may impact the size of solutes transported through the pore. Also, the neutral alanine would increase the affinity of the selectivity filter to negatively charged solutes. The third region (G[Q/K]F[V/I]P), is conserved throughout the *TaCLC*s with the similar substitutions glutamine/lysine and valine/isoleucine. While the functional characteristics of the *TaCLC*s have not yet been determined, the conservation of these amino acids important for anion channel activity indicates that they are likely to be functional anion channels in wheat.

Another method for obtaining functional data is through the complementation of knockout lines. Yeast has only one *CLC* in its genome, *ScCLC*, formerly known as

GEF1. *AtCLC-d* functionally complements the yeast knockout of this gene, as does mouse *CLC-6*. The known function of *ScCLC* is not in the plasma membrane, but in post-Golgi vesicles, preceding fusion with the plasma membrane. Knockout lines of *Arabidopsis* also exist for some of the *AtCLC*s. Geelen et al. (2000) identified an *Arabidopsis* line harboring a T-DNA insertion in *AtCLC-a*. Plants with this mutation showed diminished ability to accumulate nitrate under conditions of nitrate excess. The authors conclude that *AtCLC-a* may play a general role in controlling intracellular nitrate status. Functional complementation of these lines would be informative.

The future remains wide open for elucidation of the function of the *TaCLC*s. While the CLC functions in wheat are unlikely to resemble the powerful electrical organ discharge of their relatives in the electric ray, there are a bevy of other possible roles for anion channels. Could the *TaCLC*s play roles in mineral nutrition such as in nitrate homeostasis? Could the *TaCLC*s play a role in intracellular compartmental pH regulation? And will the pleiotropic effects observed in yeast also be found in plants or will each the TaCLCs have diverged into more specialized roles? The CLCs were named after their chloride fluxes observed in animal systems. But the anion composition of plant cells is very different, dominated by nitrate, sulfate, phosphate, and organic anions. What are the permeabilities and selectivities of these plant CLCs? Functional data and subcellular localization will be essential pieces in answering these questions.

To answer these questions, follow up work is being planned. To obtain functional data, stable transformed lines of mammalian cells expressing the *TaCLC*s are being developed. These lines will be studied using the patch clamp method to search for novel anion channel currents. To determine membrane localization of the TaCLC proteins, in-frame TaCLC-GFP chimeras will be transiently expressed in wheat roots. Confocal laser scanning microscopy will be used to assay membrane

localization. Finally, to determine cell-type expression patterns, fluorescently-labeled gene-specific oligonucleotides will be hybridized to fixed wheat root tissue using a novel quantitative *in situ* hybridization technique (Küpper et al. 2005) recently developed in Dr. Kochian's lab. These results may give hints as to the functions of TaCLCs in wheat.

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CHAPTER FOUR

Identification of Quantitative Trait Loci Important for Maize Aluminum Tolerance Using the Intermated B73 x Mo17 Population

ABSTRACT

Aluminum (Al) toxicity is a global problem limiting agricultural yields on acid soils. In maize, Al tolerance is a genetically complex trait. Furthermore, Al-activated root tip citrate exudation is a well-characterized Al tolerance mechanism in maize, but does not completely explain the observed range of tolerance when a number of maize genotypes are compared. In this study, a quantitative genetic analysis of Al tolerance in the Intermated B73 x Mo17 (IBM) population revealed five genomic regions important for Al tolerance. Three of these QTL combine to explain 42% of the variation in Al tolerance as determined by the net seminal root length (NSRL) phenotypic index. Mo17 contributes the superior allele at three of the five genomic locations, while B73 contributes the superior allele at the other two. Because Mo17 has much higher rates of Al-induced citrate release, the B73 QTL may represent genes conferring Al tolerance through novel mechanisms not associated with Al-activated organic acid exudation.

INTRODUCTION

Aluminum (Al) is the most abundant metal element in the earth's crust, comprising 8.3% of the lithosphere by weight (Greenwood and Earnshaw, 1997). At low pH values, aluminum is solubilized into the soil solution, including the trivalent cation Al^{3+}, which is toxic to plants (Kochian, 1995). Al^{3+} rapidly inhibits root growth, probably initially by interfering with both cell growth and division. But aluminum also has many other demonstrated toxic effects, including causing

alterations in the cytoskeleton (Blancafor et al., 1998), changes in cytosolic Ca^{2+} activities (Jones et al., 1998), and interferring with signaling proteins (Jones and Kochian, 1995). The net result of this Al-induced limitation of root growth is an inhibited ability to take up adequate water and nutrients for good growth and yield. Acid soils are a prevalent worldwide agronomic problem. Nearly half of the world's potentially arable land is affected by Al toxicity, which is the most yield-limiting stress associated with acid soils (Kochian et al., 2004). Maize is a widely grown crop in many of the regions where acid soils are especially prevalent, such as South America and West Africa. As such, maize is a crop of particular interest for Al tolerance improvement as well as for a better understanding of the underlying tolerance mechanisms.

Genetically, maize aluminum tolerance in is a complex trait. While some early studies posited simple inheritance, these studies tended to have population structures or experimental designs that were biased towards detecting single genes. For example, Rhue et al. (1978), who used Ca or Mg amelioration of Al toxicity as their phenotype, categorized their populations into discrete classes, necessarily eliminating any smaller quantitative effects that may have been present in their populations. In a second example of a study claiming qualitative inheritance, Sibov et al. (1999) used a somoclonal mutant of the Al tolerant maize inbred Cat-100-6. While the two genes thusly identified may be important or essential for Al tolerance, they may not represent genes that explain variations in tolerance between maize lines. Also, they may not represent the complete array of tolerance genes present in Cat-100-6 or other maize lines.

In contrast, most inheritance studies have concluded that Al tolerance in maize is a complex, quantitative trait (Magnavaca et al. 1987; Giaveno et al. 2001; Ninamengo et al. 2003). Magnavaca et al. (1987) crossed several tolerant and

sensitive varieties and concluded that additive gene effects had the most significant contribution to Al tolerance. Giaveno et al. (2001) performed one generation of selection on an open-pollinated tropical maize population. Statistical analysis of the progeny indicated that Al tolerance is a highly heritable quantitative trait. In the only published QTL analysis of maize Al tolerance, Ninamengo et al. (2003), working with recombinant inbred lines derived from a cross of tolerant and sensitive South American maize genotypes, identified five distinct genomic regions important for Al tolerance.

As in many other plant species, Al-activated organic acid exudation from the root is thought to be an important Al tolerance mechanism in maize. However, recent evidence suggests that other physiological mechanisms must also be operating in maize to explain the full range of observed tolerance. Several studies have characterized the organic acid release Al tolerance mechanism in single contrasting tolerant and sensitive maize lines (Pellet et al. 1995; Jorge and Arruda, 1997; Piñeros et al. 2002). In these studies it was shown that citrate release is induced by exposure to Al to a much greater degree in the tolerant lines. The citrate exudation rate tends to increase in response to increasing Al^{3+} activity. In Piñeros et al. (2002), a spatial analysis of root citrate exudation showed that unlike wheat, Al-activated organic acid exudation was not localized to the root apex; instead, it was more broadly distributed, occurring as far back as 5 cm from the root tip. The physiological characterization of this putative tolerance mechanism has been followed up by electrophysiological (patch clamp) analysis of root plasma membrane anion channels capable of transporting citrate out of root tip cells (Piñeros and Kochian, 2001; Kollmeier et al. 2001).

Recently, a study examining Al tolerance in a larger number of maize genotypes (six maize hybrids and inbreds) has cast doubt on the role of citrate exudation as the sole physiological Al tolerance mechanism in maize. Piñeros et al.

(2005) examined a range of physiological characteristics of six maize lines and their response to Al treatment. They found little correlation between citrate exudation rates and Al tolerance among the six lines. Of particular interest was the North American inbred Mo17, which grouped with three other lines in the study with low Al tolerance, yet exhibited the highest Al-activated root citrate exudation rates of any lines included in the study. Another North American inbred, B73, had a similarly low level of Al tolerance, but had almost no detectable Al-activated root citrate release. In this study the authors hypothesized that Al-activated root citrate exudation may be a basal mechanism of maize Al tolerance, observed in all tolerant and some sensitive maize lines. However in extremely Al tolerant genotypes such as the Brazilian standard for maize tolerance, Cateto-Columbia, other mechanisms may be operating in addition to root citrate release. These paradoxes regarding citrate release emphasize the importance of looking for additional mechanisms of aluminum tolerance in maize. How does Cateto-Columbia gain such a superior tolerance to Mo17 without a higher rate of root citrate release? How does B73 achieve a similar tolerance to Mo17 with vanishingly low citrate release rates?

The present study uses a recombinant inbred line population ideally structured to answer questions about the genetics and physiology of Al tolerance in maize. The intermated B73 x Mo17 (IBM) population is a set of recombinant inbred lines (RILs) developed by single seed descent after five generations of random intermating (syn5), as opposed to the typical development of RIL from an F2 population (Lee et al. 2002). This approach increased the number of recombination events 2.7-fold in this RIL population, and the size of the genetic map was increased nearly five-fold. The result of this increased recombination is a much finer genetic map, with more allelic combinations amongst the population in most chromosomal regions. This permits more precise localization of genetic factors when mapping quantitative trait loci

(QTL). Because the parents of the IBM population, B73 and Mo17, represent the extremes in Al-activated root citrate release, yet achieve similar levels of Al tolerance, this population may provide insights into the importance of citrate release for Al tolerance in these lines, as well as provide a platform for investigating other Al tolerance mechanisms.

MATERIALS AND METHODS

Genetic Stocks

Maize inbred lines B73 and Mo17 were used in this study along with 88 recombinant inbred lines of the intermated B73 x Mo17 (IBM) population. IBM lines were a generous gift from Dr. Michael Lee (Iowa State; Lee et al., 2002).

Plant Growth Conditions

Seeds were surface-sterilized in 1.25% NaOCl and 0.01% Triton X-100 for 20 minutes with shaking. Seeds were rinsed 7 times briefly in 18 MΩ H_2O, with a final 20 minute rinse with shaking. To prevent fungal growth, seeds were suspended in germination solution containing 0.1% (w/v) Captan , a fungicide. Seeds were placed between layers of sterile filter paper moistened with germination solution on sterile petri dishes, and incubated at 25°C in the dark for three or four days for hybrid or inbred lines, respectively (as the maize hybrids grew more rapidly). The nutrient solution composition as well as the methods used to evaluate aluminum tolerance were as described in Magnavaca et al. (1987), with modifications. The nutrient solution contained the following macronutrients (in mmol L^{-1}): Ca - 3.53, K - 2.35, Mg - 0.85, N (as NO_3) - 10.86, N (as NH_4) - 1.3, P - 0.045, S - 0.59. The solution also contained the following micronutrients (in μmol L^{-1}): B - 25, Mn - 9.1, Cu - 0.63, Mo - 0.83, Zn - 2.29, FeHEDTA - 77, and Cl - 600. For the appropriate treatments, aluminum was added as $AlK(SO_4)_2.12H_2O$ to the desired final concentration. Al^{3+} activities of 0,

20,30, 35, and 40 μM were achieved by maintaining a total Al concentration in the nutrient solution of 0, 110, 148, 190, and 222 μM, respectively, and Al^{3+} activities were estimated using GEOCHEM-PC (Parker et al., 1995). The pH was adjusted to 4.0 by titration with 6 N HCl and the nutrient solution was constantly aerated throughout the experimental period. Experiments were carried out in a walk-in growth chamber (Environmental Growth Chambers; Chagrin Falls, Ohio 44022) with 26°C day and 23°C night temperatures, a light intensity of 550 μmol photons $m^{-2}s^{-1}$ at the shoot level and a 16 hour photoperiod.

Analysis of Aluminum Tolerance

Al tolerance was measured using three phenotypic traits. The length of the primary root was recorded in control and Al-treatment groups just before treatment (day 0) and 24, 72, and 96 hours after treatment (days 1,3, and 5). From these primary parameters, three derived parameters were calculated. Net seminal root length (NSRL) is the growth in mm over the two day period from 24 to 72 hours after treatment with Al. Relative root growth (RRG) is the ratio of the NSRL under Al treatment to the NSRL in control solution. Relative seminal root length (RSRL) is the ratio of the root length at 72 hours after Al treatment to the root length at 24 hours after treatment. Each of these derived parameters focuses on the time period from 24 to 72 hours after treatment, which is the period which has the most vigorous growth of the primary roots in control conditions, and the most consistent response to Al in the Al-treatment conditions.

QTL Analysis

Single marker and composite interval mapping analysis was performed using QTL Cartographer (Version 2.0, Windows XP; Wang et al., 2001-2004). Significance thresholds for composite interval mapping of α=0.01, 0.05 and 0.2 were determined

using a 1000 repetition permutation test. Likelihood ratios for each trait at each significance threshold are shown here in tabular format.

α	NSRL	RSRL	RRG
0.01	21.85	20.78	22.49
0.05	18.80	17.97	18.63
0.20	15.10	14.75	15.04

RESULTS

Determining the optimal Al^{3+} activity to maximize differential Al tolerance

To determine an appropriate Al toxicity level for QTL analysis, a random sample of eight RILs were assayed for root growth under +/- Al conditions at three Al^{3+} activities. Figures 4.1-4.3 show the results for those eight lines, along with the parental lines B73 and Mo17 and the maize hybrid, Pioneer 3335 (a reference line commonly used in our lab), for each of the three traits (NSRL, RRG, and RSRL, respectively). QTL analysis requires variability in phenotype among individuals in the population. Variability in Al tolerance among RILs was observed at all three Al levels for all three traits (Figures 4.1-4.3). The Al^{3+} activity must be high enough to expose differential Al tolerance between the RILs, but must not be so high that even the roots of tolerant RIL be strongly inhibited. Thus determining the appropriate Al^{3+} activity is important for detection of QTL providing significant increases in Al tolerance. RRG is a good intuitive measure for determining the severity of the Al toxicity response. As seen in Figure 4.2, six of the eight RILs used in the preliminary study were inhibited less than 20% at 30 μM Al^{3+} activity relative to the controls, while all eight lines were inhibited by at least 40% at 40 μM Al^{3+} activity, with half inhibited over 60%. Thus, 35 μM was chosen as a single appropriate Al^{3+} activity level in terms of root growth inhibition and variation in tolerance among RILs.

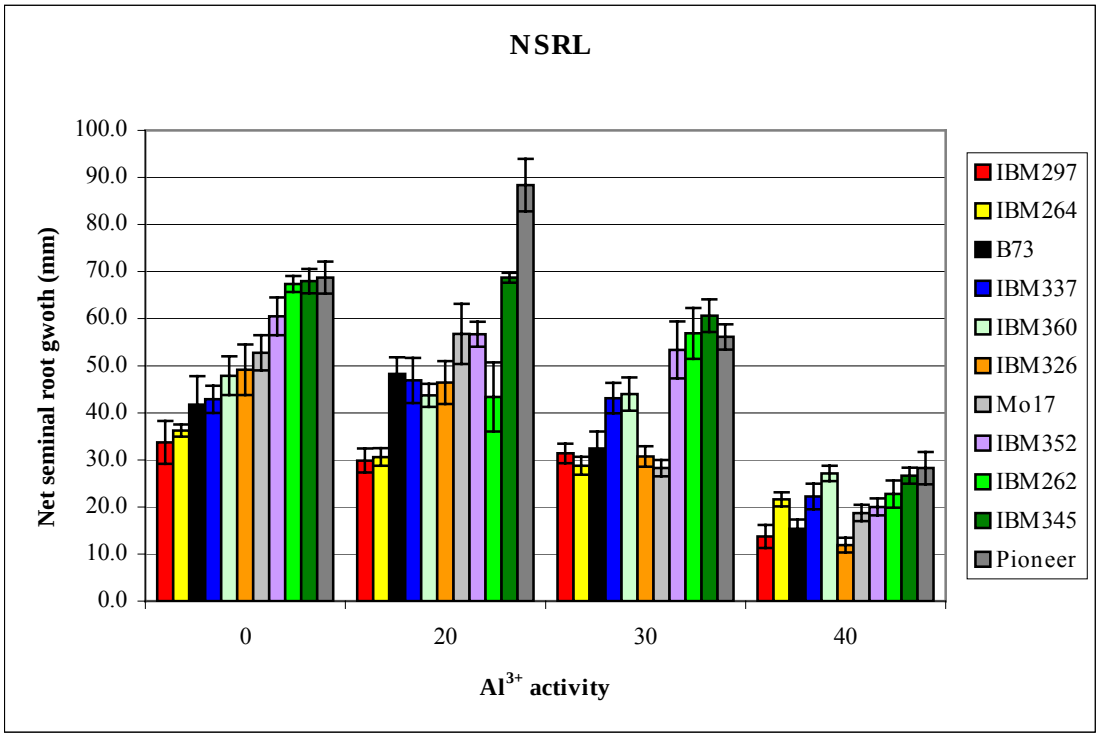

Figure 4.1. Al tolerance determined as Net Seminal Root Length over a range of Al^{3+} activities of eight RILs from the IBM population, along with the parents B73 and Mo17, and a Pioneer hybrid line for reference. NSRL is root growth between days 1 and 3. For Figures 4.1-4.3, the RILs are ordered from left to right by increasing NSRL at zero Al^{3+} activity. The bars representing root growth for the parental lines (B73 and Mo17) are black (B73) and light grey (Mo17, the reference line (Pioneer hybrid) is dark grey, and the IBM RILs are in color.

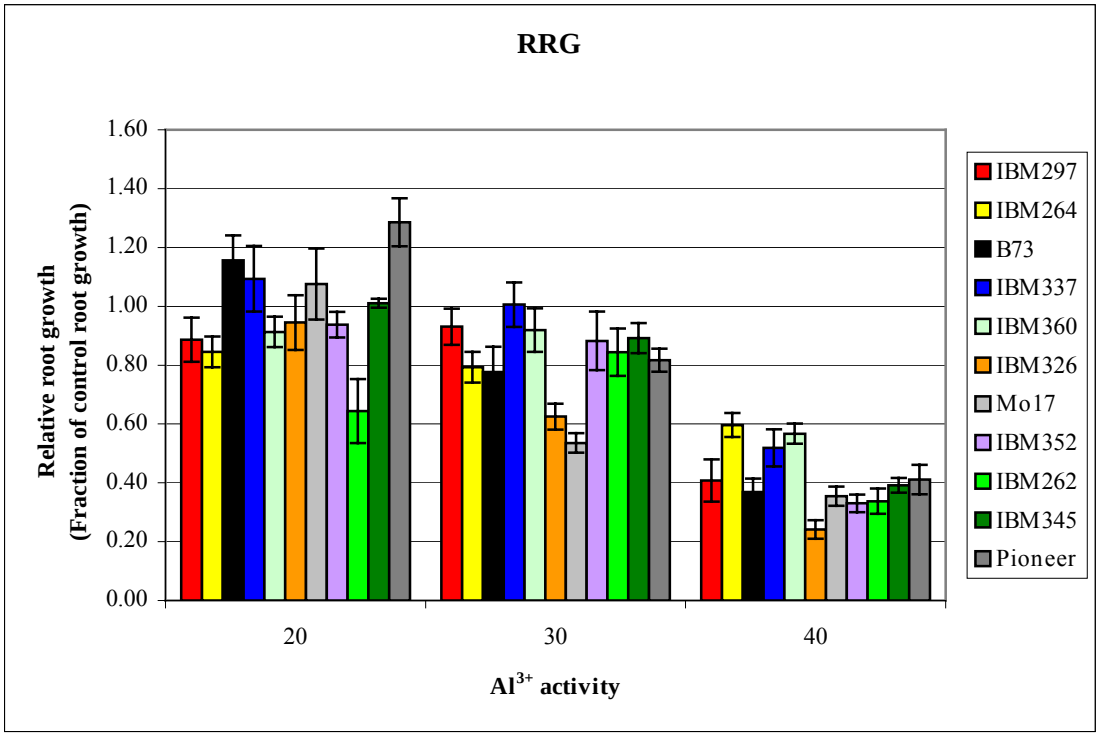

Figure 4.2. Al tolerance determined as Relative Root Growth (RRG) over a range of Al^{3+} activities for the same eight IBM RILs depicted in Figure 4.1, along with the parents B73 and Mo17, and a Pioneer hybrid line for reference. Relative Root Growth is the ratio of root growth in Al divided by root growth for the same genotype without Al. The bar color scheme and layout is the same as in Figure 4.1.

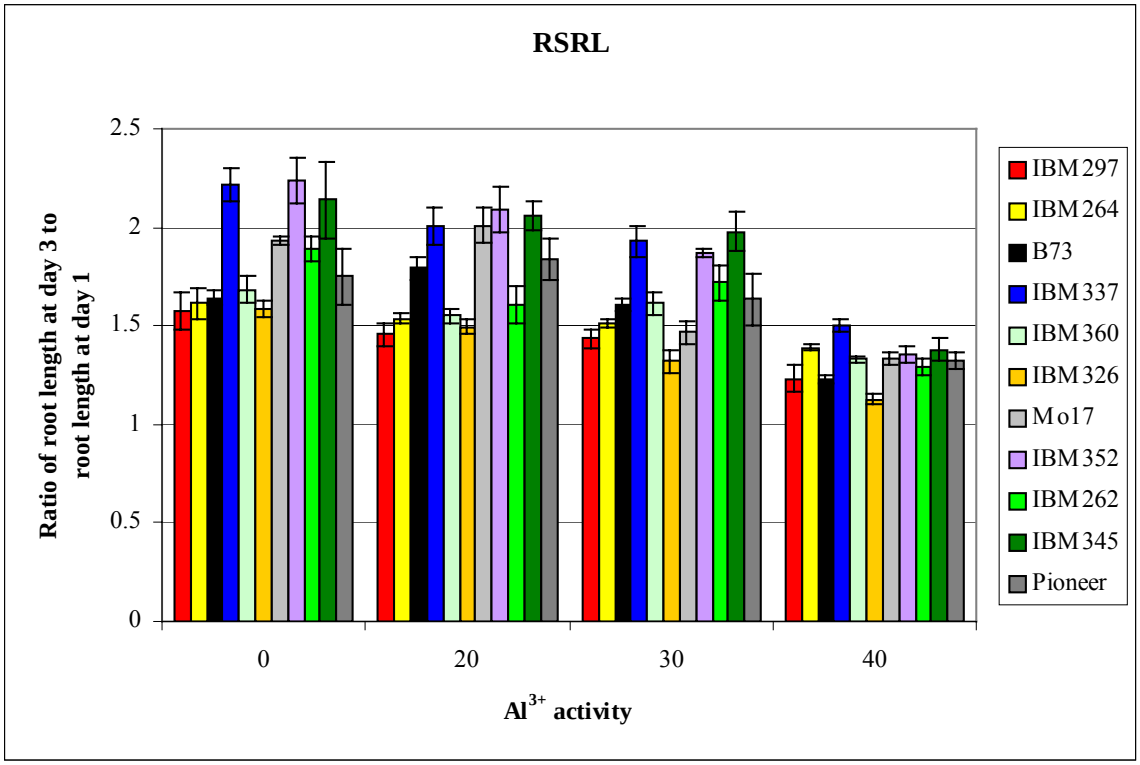

Figure 4.3. Al tolerance as determined by Relative Seminal Root Length (RSRL) over a range of Al^{3+} activities for the same eight IBM RILs depicted in Figure 1, along with the parents B73 and Mo17, and a Pioneer hybrid line for reference. Relative Seminal Root Length is the ratio of the root length after three days of Al treatment divided by the root length of the same individual plant after one day of treatment. The bar color scheme and layout is the same as in Figure 4.1.

The distribution of Al tolerance among IBM RILs suggests quantitative genetics underly Al tolerance in the IBM population

A subset of the IBM mapping population consisting of 88 recombinant inbred lines (RIL) was assayed for Al tolerance at 35 μM Al^{3+} activity using the three phenotypic indices. Parental performance and distribution of the RILs is shown in Figures 4.4-4.6. The parental lines B73 and Mo17 exhibited similar Al tolerance using all three phenotypic indices. However, a high degree of transgressive segregation was observed for all three traits in both tolerant and sensitive directions. For example, a number of RIL demonstrated significantly higher Al tolerance than either parent, and the most tolerant RIL were as tolerant as the Brazilian standard for maize Al tolerance, Cateto-Columbia, which does not exhibit root growth inhibition at these levels of Al^{3+} exposure. Phenotypic distributions were approximately normal for all three traits, suggesting quantitative inheritance.

Statistical analysis reveals several strong QTL

Composite interval mapping of all three root growth parameters revealed four putative QTL at the $p = 0.05$ experiment-wise error rate for the three traits. Two additional NSRL QTL passed the less stringent $p = 0.20$ significance threshold. The likelihood ratio (LR) curves from composite interval mapping are shown in Figures 4.7 through 4.12. Figures 4.7 through 4.9 show curves for NSRL. Figure 4.10 shows the curve for RRG. Figures 4.11 and 4.12 show curves for RSRL. Table 4.1 lists the six putative QTL along with information about their position and effect. Each QTL was named based on the phenotypic index it represents and given a suffix based on its chromosomal location (e.g. NSRL-4 or RRG-5). NSRL-4 is the strongest NSRL QTL and explains 21% of the genetic variance for Al tolerance in the population. This QTL, along with the two weaker NSRL QTL (NSRL-10 and NSRL-8), in total explain

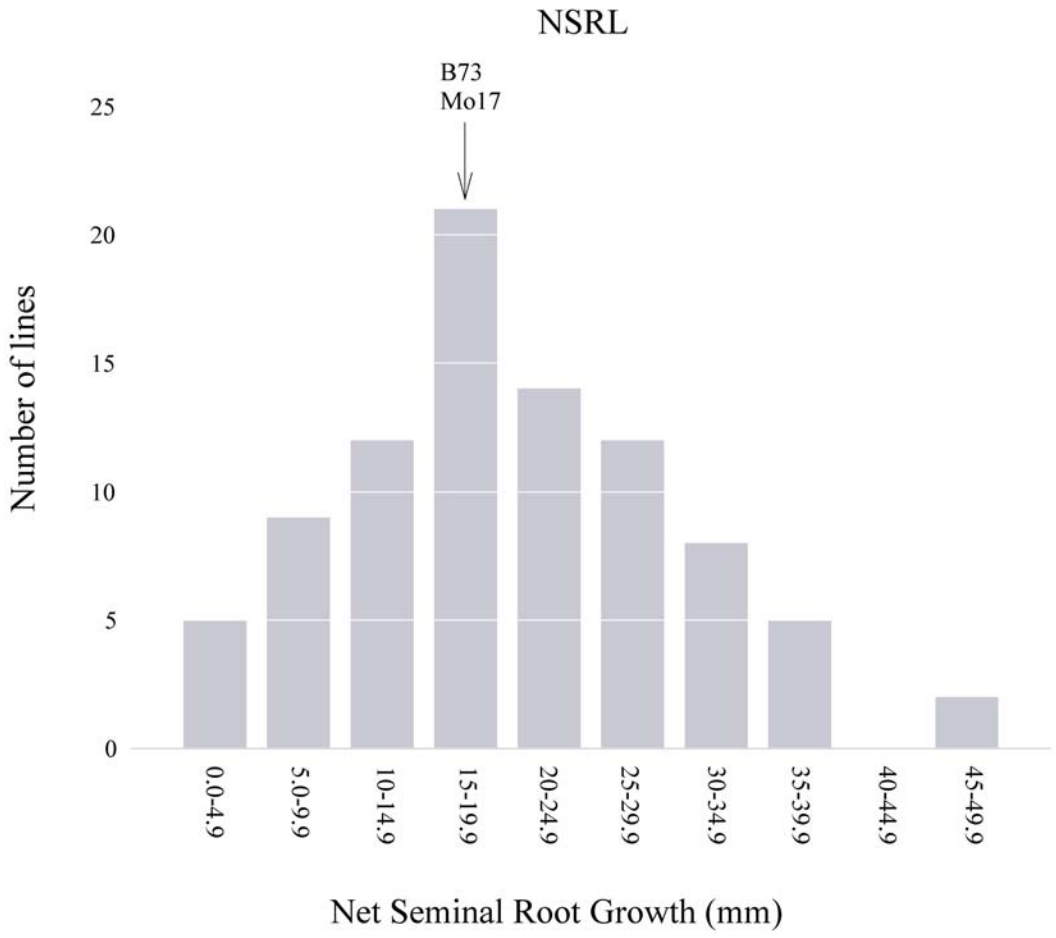

Figure 4.4 Distribution of Al tolerance among the 88 IBM RILs as measured by Net Seminal Root Length (NSRL) at 35 μM Al^{3+} activity. NSRL mean values and standard errors were 19.1±2.1 for Mo17 (N=47) and 19.0±1.4 for B73 (n=48). NSRL values for the individual IBM RILs are the means of up to 16 individuals. The normal distribution of this phenotype indicates that it is a quantitative trait likely influenced significantly by multiple loci and thus appropriate for QTL analysis.

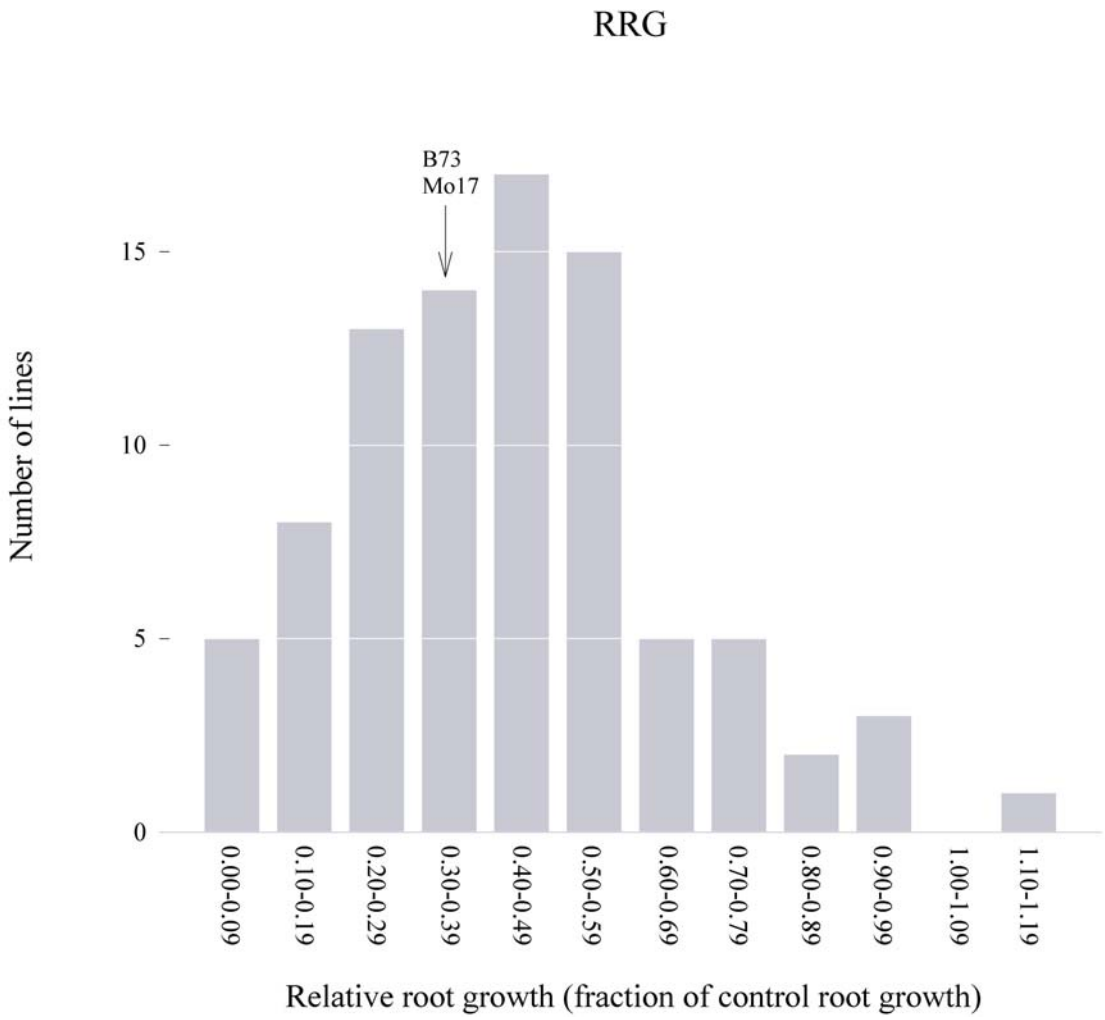

Figure 4.5 Distribution of Al tolerance among the 88 IBM RILs as measured by Relative Root Growth (RRG) at 35 μM Al^{3+} activity. RRG mean values and standard errors were .39±0.04 for Mo17 (N=47) and .35±0.03 for B73 (n=48). RRG values for the individual IBM RILs are the means of up to 16 individuals. The normal distribution of this phenotype indicates that it is a quantitative trait likely influenced significantly by multiple loci and thus appropriate for QTL analysis.

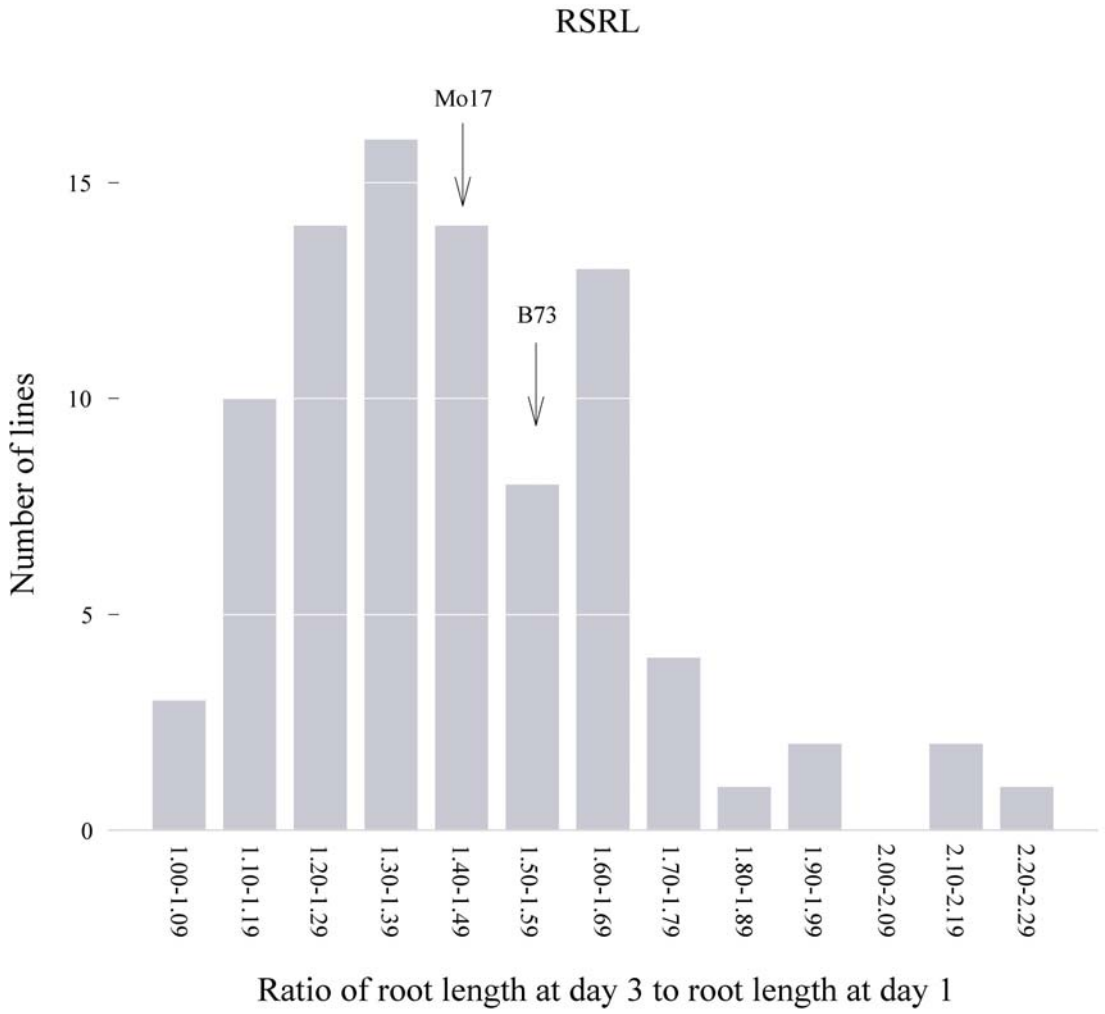

Figure 4.6. Distribution of Al tolerance among the 88 IBM RILs as measured by Relative Seminal Root Length (RSRL) at 35 μM Al^{3+} activity. RRG mean values and standard errors were 1.45±0.05 for Mo17 (N=47) and 1.50±0.04 for B73 (n=48). RRG values for the individual IBM RILs are the means of up to 16 individuals. The normal distribution of this phenotype indicates that it is a quantitative trait likely influenced significantly by multiple loci and thus appropriate for QTL analysis.

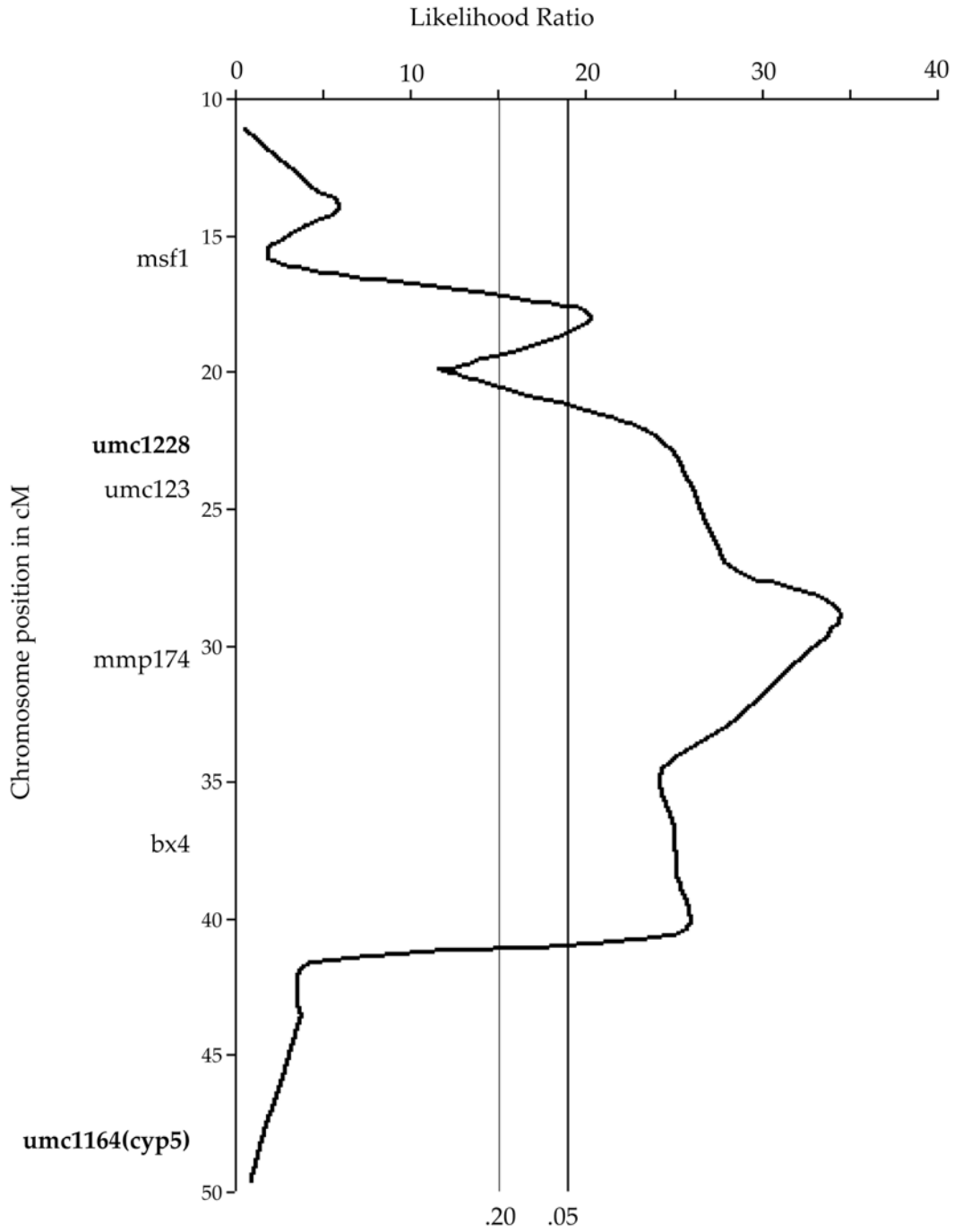

Figure 4.7. Composite interval mapping (CIM) analysis of Net Seminal Root Length (NSRL) on chromosome 4. Likelihood ratio is plotted against genetic distance from the distal end of chromosome 4S to show the location of an NSRL QTL using CIM. Confidence thresholds are shown as the vertical gray lines (dark grey, alpha = 0.05; light grey, alpha = 0.20) based on empirically determined values. **Bold** markers denote SSRs, plain text markers are RFLPs, and names followed by * are probes from plant species other than maize.

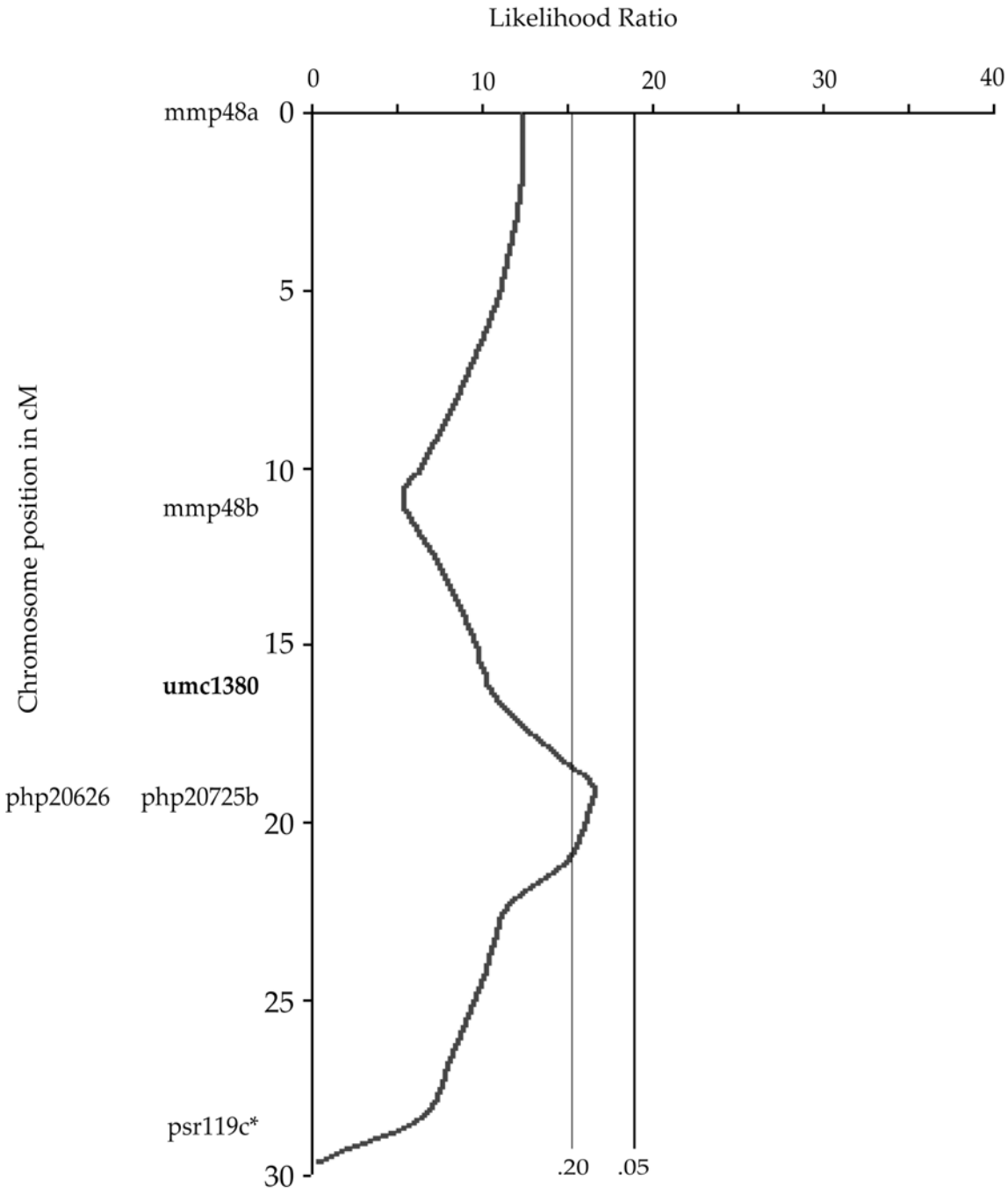

Figure 4.8. Composite interval mapping (CIM) analysis of Net Seminal Root Length (NSRL) on chromosome 10. Likelihood ratio is plotted against genetic distance from the distal end of chromosome 10S to show the location of an NSRL QTL using CIM. Confidence thresholds and marker conventions are as in Figure 4.7.

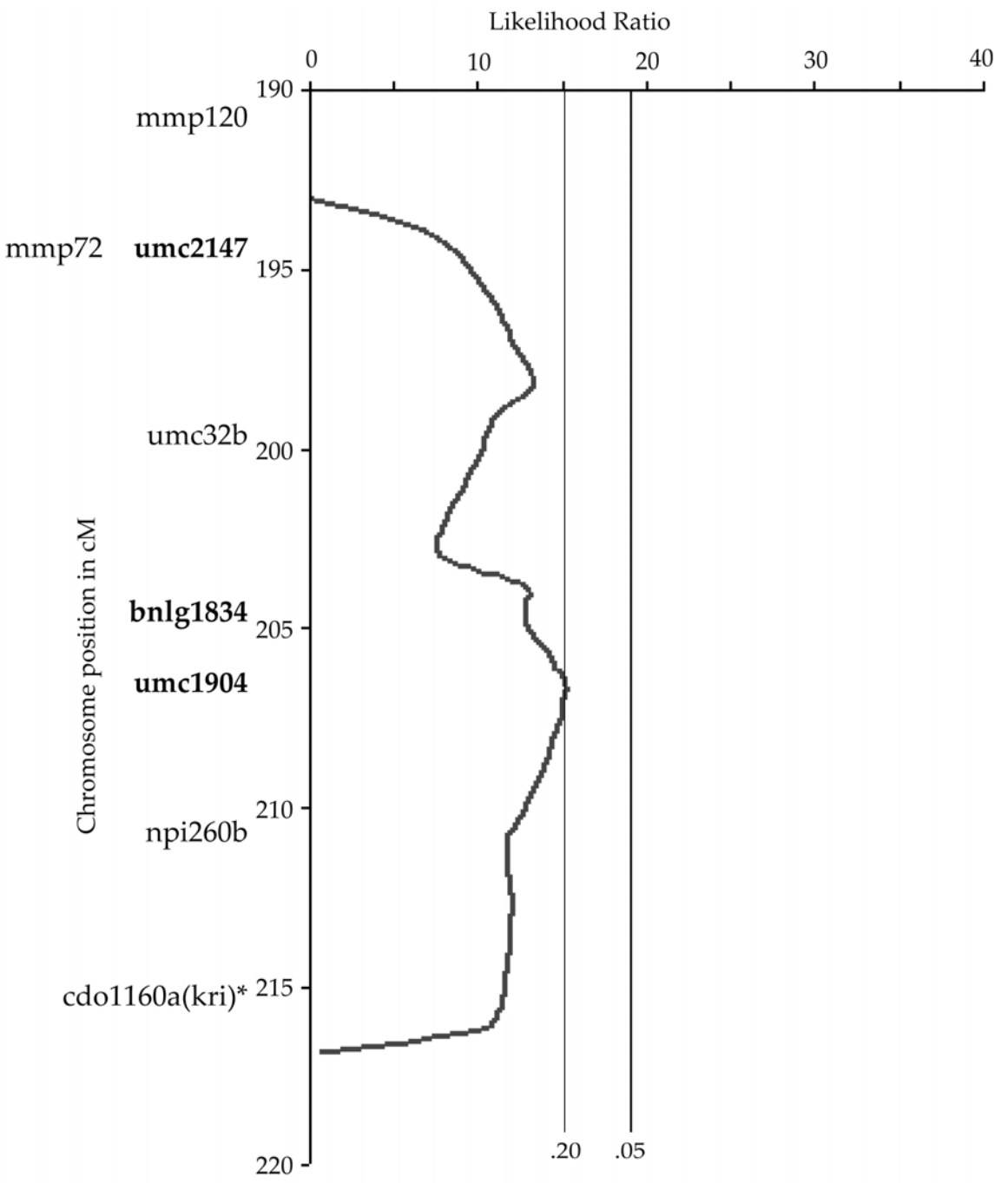

Figure 4.9. Composite interval mapping (CIM) analysis of Net Seminal Root Length (NSRL) on chromosome 8. Likelihood ratio is plotted against genetic distance from the distal end of chromosome 8S to show the location of an NSRL QTL using CIM. Confidence thresholds and marker conventions are as in Figure 4.7.

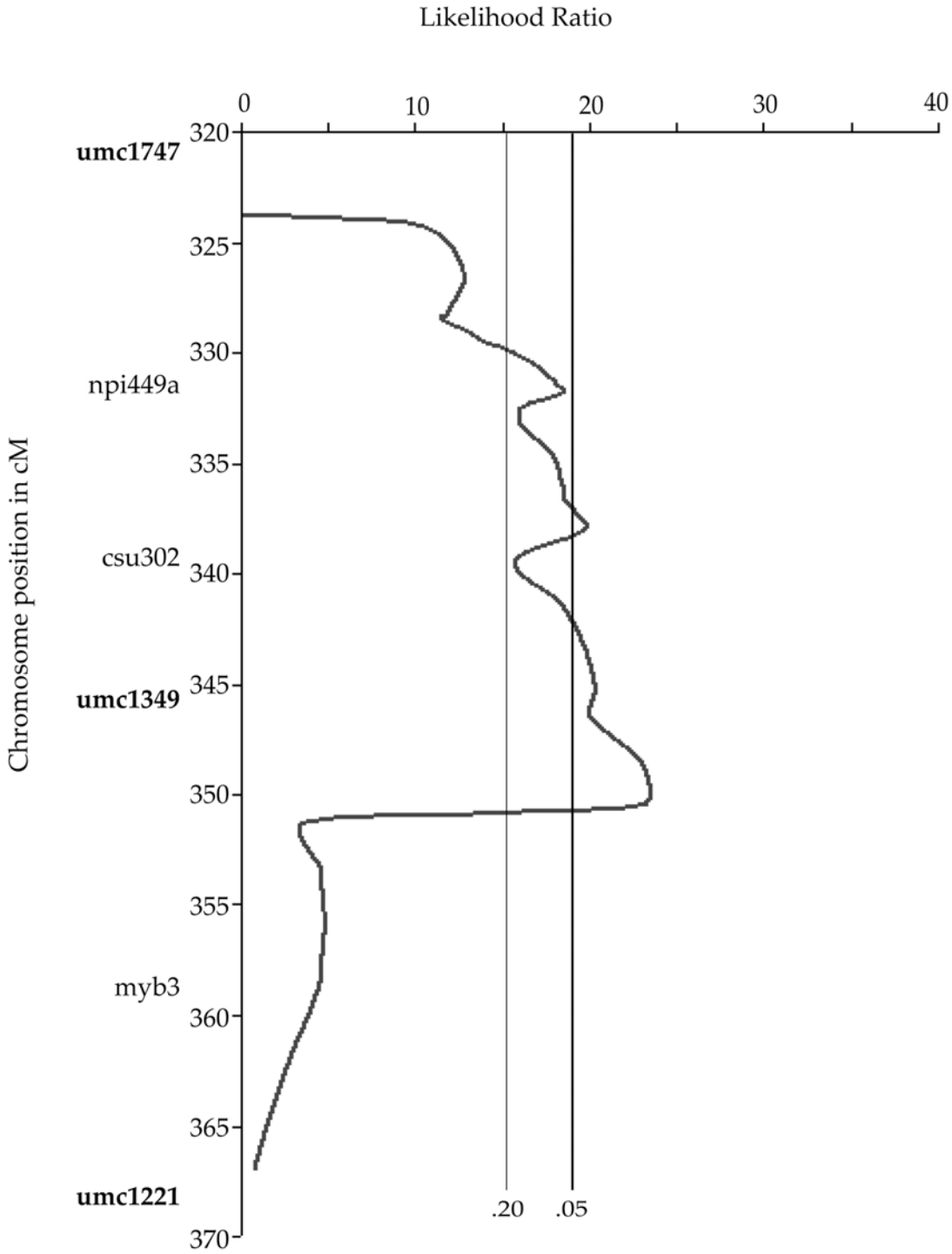

Figure 4.10. Composite interval mapping (CIM) analysis of Relative Root Growth (RRG) on chromosome 5. Likelihood ratio is plotted against genetic distance from the distal end of chromosome 5S to show the location of an RRG QTL using CIM. Confidence thresholds and marker conventions are as in Figure 4.7.

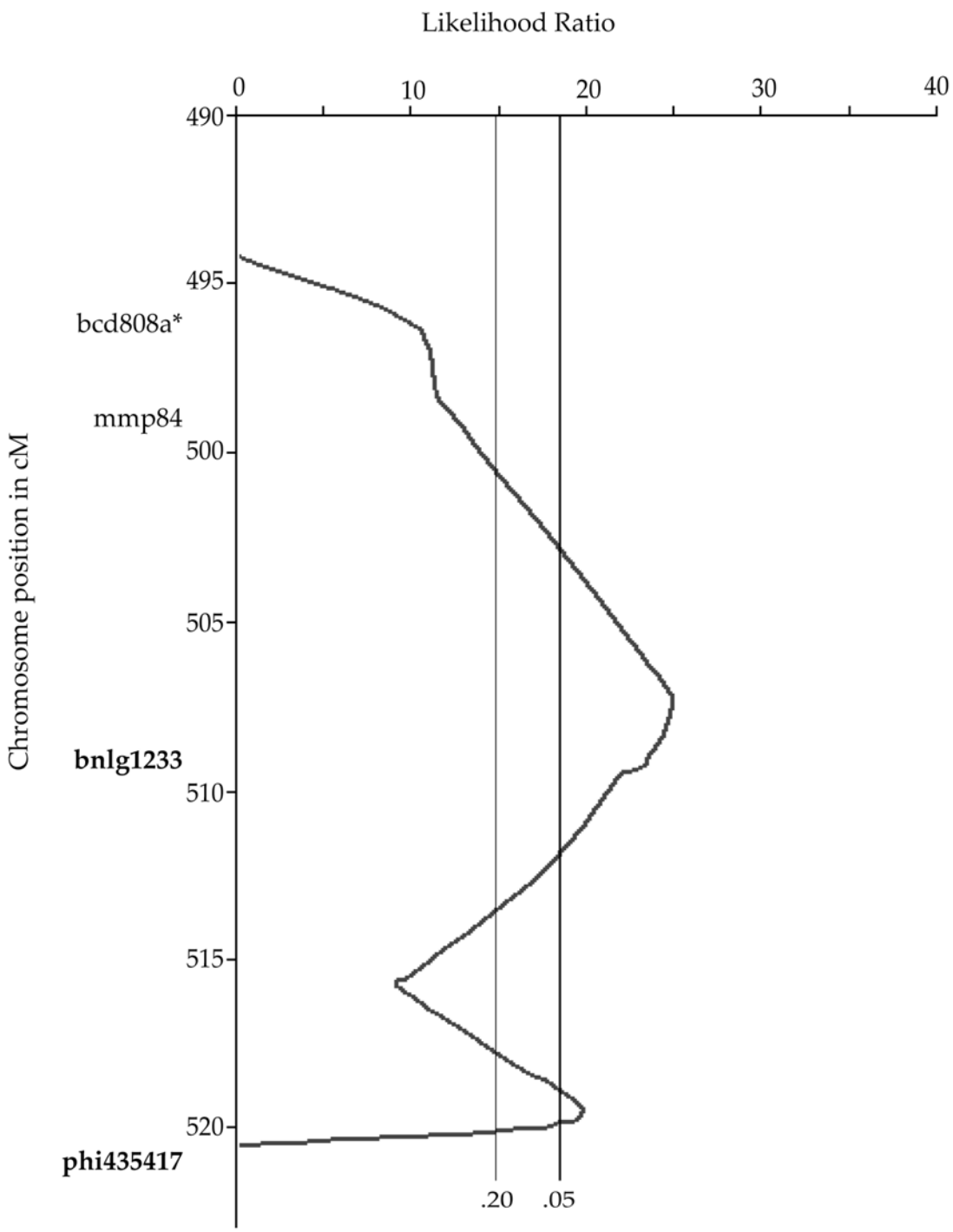

Figure 4.11. Composite interval mapping (CIM) analysis of Relative Seminal Root Length (RSRL) on chromosome 2. Likelihood ratio is plotted against genetic distance from the distal end of chromosome 2S to show the location of an RSRL QTL using CIM. Confidence thresholds and marker conventions are as in Figure 4.7.

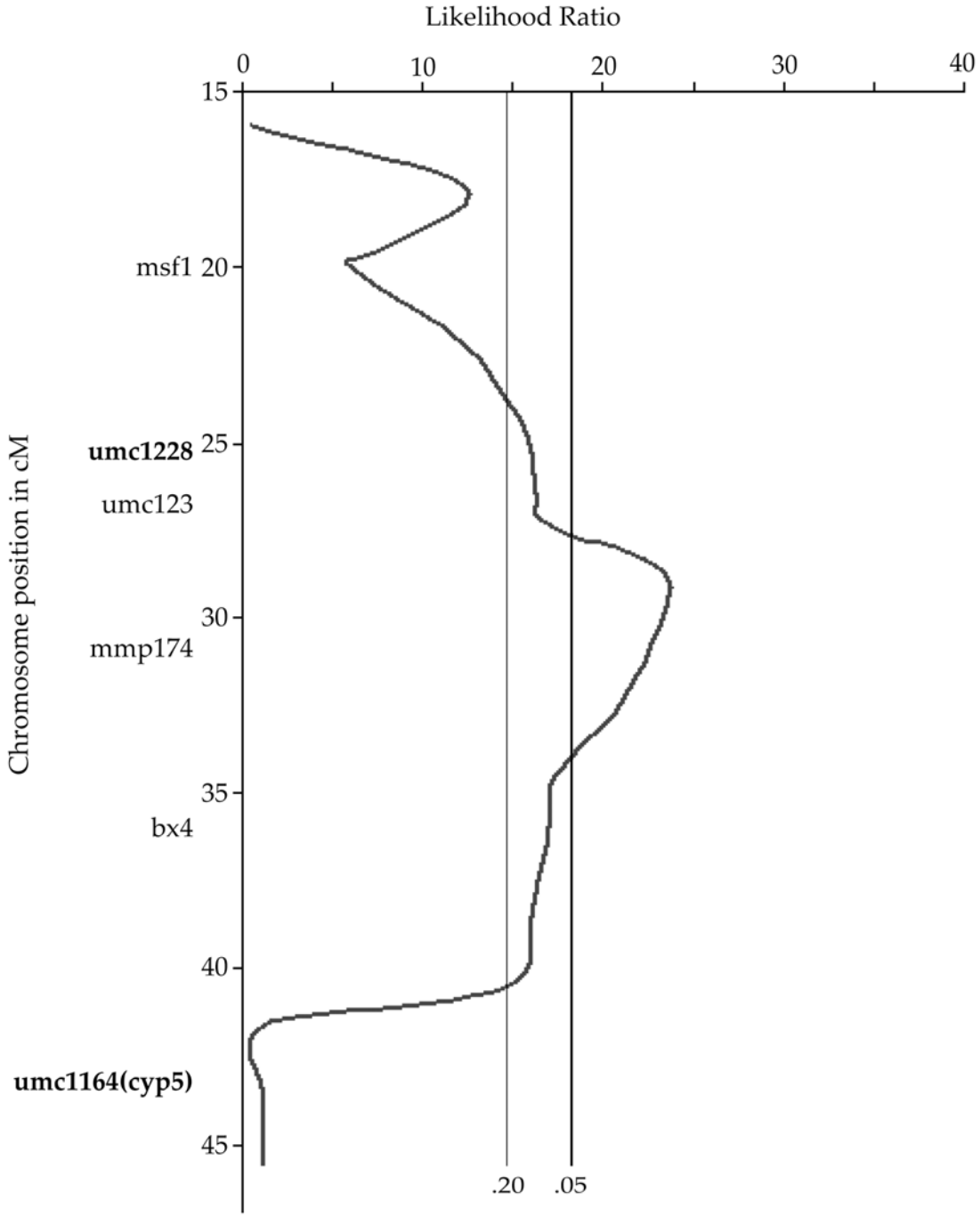

Figure 4.12. Composite interval mapping (CIM) analysis of Relative Seminal Root Length (RSRL) on chromosome 4. Likelihood ratio is plotted against genetic distance from the distal end of chromosome 4S to show the location of an RSRL QTL using CIM. Confidence thresholds and marker conventions are as in Figure 4.7.

Table 4.1. Characteristics of all Al tolerance QTLs with p values of less than 0.20. Listed are: the trait for which the QTL was detected; the location of the QTL, including the chromosome, nearest marker, and cM position; the likelihood ratio; the p value (as determined by a 1000-fold permutation test); the r^2 value (the percentage of variance explained by that QTL); the additive effect of that QTL on the trait; the parental line contributing the superior allele; and the dominance effect of that allele. The r^2 values in bold are the combined variance explained by all QTL for that trait.

Trait	Chrom	marker	cM	LR	p value	R^2	Additive	positive allele	Dominance
NSRL	4	mmp174	28.7	34.4	0.00	0.21	-4.5	Mo17	0.7
NSRL	10	php20626	19.1	16.5	0.12	0.09	-3.0	Mo17	0.7
NSRL	8	umc1904	206.6	15.3	0.18	0.08	2.9	B73	0.8
						0.42			
RSRL	2	AY109583	507.4	25.0	0.00	0.16	0.11	B73	11.2
RSRL	4	mmp174	28.7	23.3	0.01	0.15	-0.10	Mo17	10.5
						0.29			
RRG	5	umc1349	348.5	22.9	0.01	0.18	-0.10	Mo17	5.8

42% of the genetic variance for this trait. For NSRL-4 and NSRL-10, Mo17 contributes the superior allele, while for the NSRL-8, B73 contributes the superior allele.

For RSRL, two putative QTL, RSRL-2 and RSRL-4, explain 16% and 15%, of the variation in that trait. A model based on those two QTL together explains 29% of the genetic variation. RSRL-4 is coincident with NSRL-4. The superior allele for RSRL-4 is again contributed by Mo17, while the superior allele for the RSRL-2 is contributed by B73.

Composite interval mapping for RRG revealed only one putative QTL, RRG-5, which explains 18% of the variance in that trait.

With no single QTL explaining more than 21% of the variance in the population by any of the three phenotypic indices, this study supports the idea that Al tolerance is a complex trait in maize, conferred by several or many genes working together. At the same time, individual genes clearly have a major impact. For example, the three NSRL QTL explain 42% of the variance in the NSRL trait, with NSRL-4 explaining 21%. Similarly, the two RSRL QTL contribute approximately equally, explaining 29% of the variance together.

DISCUSSION

Determining optimal experimental conditions to maximize detection of aluminum tolerance QTL

During the first days of maize seedling growth, both the primary root growth rate and the response of root growth to Al treatment vary over time. The growth of the primary root accelerates after germination before reaching an approximately linear phase, then later deccelerates while approaching its final length. The sensitivity to aluminum varies over these physiological stages. Therefore, the time period chosen to

assess the tolerance phenotype is a highly important aspect of the experimental plan. The natural root growth habit of maize in nutrient solution is dominated in the early days by a single primary root and several secondary roots emerging from the embryo. As time goes on, primary root growth slows, while lateral root growth (branches off the primary and secondary roots) increases. Eventually, lateral roots dominate the root structure. The timing of the reduced growth rate of the primary root is of critical concern, as this could be mistaken for Al sensitivity under treatment conditions. Preliminary experiments on the RILs of the IBM population revealed that the majority of primary roots had a growth rate that was steady through day three, but began to slow by day four or five (data not shown). These same experiments showed that some lines that had clear Al inhibition by day three were not strongly inhibited by day one. For these reasons, the time frame of day one to day three in Al was chosen for making measurements of root growth.

Several root growth indices have been used to assess Al tolerance in various plant species. Some are based solely on root growth in a medium containing Al (such as NSRL). Others compare growth under Al exposure to root growth in a control medium lacking Al (such as RRG and RSRL). As a further distinction, RRG is based on two parallel treatment populations, while RSRL is based on a single population under two phases of treatment. There is a wide range among maize lines with respect to root growth rates when grown in nutrient solutions. Because of this variability, it is often desirable to include a comparison to control growth rates in the tolerance analysis. This variability is evident both between lines (as measured by population means) and within lines (as measured by population standard deviations). Relative Root Growth (RRG) in particular, which takes the ratio of root growth in Al treatment to root growth in the control solution, attempts to normalize for the variability between lines. Relative Seminal Root Length (RSRL), which takes the ratio of root length after

a specified period of Al treatment to an initial root length in the absence of Al, also attempts to normalize for the variability within lines. Net Seminal Root Length (NSRL), which is simply the growth of the primary root over the period of Al treatment in question, does not attempt to normalize for untreated root growth, but simply looks at the ability of the root to grow under Al stress. As Al^{3+} activity increases, the correlation of treated root growth with untreated root growth weakens (Figure 4.1). As seen in Figure 4.2, dividing the root growth of Al-treated plants by the root growth of untreated plants (to get RRG) alters the inferred relative tolerances of the various lines by apparently increasing the tolerance of those lines with slower root growth in solution lacking aluminum and decreasing the assayed tolerance of those lines with higher rates of root growth under control conditions. The RSRL statistic (Figure 4.3) gives an intermediate result due to its more subtle adjustment for the pre-treatment growth of each individual plant. While each derived phenotypic index has its own advantages and disadvantages, none can clearly be stated to be the best for all situations. The different tolerance rankings present in the initial experiment summarized in Figures 4.1 – 4.3 presage differences in tolerance values among the entire IBM population, which manifest themselves in different QTL loci detected using different phenotypic indices.

Al Tolerance Mechanisms

There is uncertainty at the present time as to the relative contribution of organic acid exudation to aluminum tolerance in maize. The parental lines of the IBM population provide a perfect example as to why this particular hypothesized mechanism for Al tolerance is being re-evaluated. B73 and Mo17 have similar levels of Al tolerance as measured by any of the three phenotypic indices (Figures 4.4-4.6), yet Mo17 maintains a much larger Al-activated root citrate exudation compared with B73 over a range of Al^{3+} activities and time periods (Piñeros et al., 2005). When

exposed to 40 μm Al^{3+}, Mo17 releases about 800 pmol citrate $root^{-1}$ $hour^{-1}$, more than any other maize line tested in the study. Yet Mo17 is relatively Al sensitive, and is roughly equal in tolerance to B73, which exhibits the lowest rate of Al activated citrate release. One possible explanation for these observations is that B73 employs an Al tolerance mechanism that does not depend on root organic acid exudation to achieve its equivalent tolerance level. Some alternative mechanisms that have been proposed in the literature include Al-induced modifications of rhizosphere pH, Al detoxification in the root symplasm possibly via chelation or sequestration, and the exudation of other Al-chelating compounds, such as phenolics. If this is true, it could explain the transgressive segregation observed between the RILs as seen in Figures 4.4 - 4.6, which could be due to the additive effect of two (or more) distinct tolerance mechanisms that results in the increased tolerance observed in some RILs. In future work planned for this project, QTL mapping of citrate exudation across the same RIL population should help determine which of the existing tolerance QTLs are associated with the citrate release mechanism. Any Al tolerance QTLs not associated with citrate release will become putative QTL for alternate mechanisms. But of particular immediate interest are the tolerance QTL NSRL-8 and RSRL-2, which gain their positive effect from alleles contributed by B73. Because B73 has extremely low Al-activated root citrate release, it presumably has an alternate tolerance mechanism, and these QTL presumably confer Al tolerance through a non-citrate exudation mechanism. Near isogenic lines (NIL) are currently being constructed from all of the Al tolerance QTL identified in this study. Some of these NIL will contain the B73 QTL allele in an otherwise Mo17 genomic background, and should be excellent research materials for identifying novel Al tolerance mechanisms. For example, does the NIL generated for the RSRL-2 QTL have a higher citrate release than Mo17? If so, then perhaps it is part of the citrate exudation mechanism which is not operational

in a fully B73 genome. If not, then perhaps it does contribute an alternative tolerance mechanism. In that case, physiological studies will be undertaken to elucidate possible mechanisms. The reciprocal NIL are also being constructed. In contrast to the previous example, in this case the genomic makeup would be fully B73 but with the Mo17 allele at RSRL-2. This should decrease Al tolerance relative to B73. How would it affect the various physiological parameters?

The newly developed NIL will represent several genetic loci which control Al tolerance. These few lines will permit rapid and efficient tests for an array of proposed physiological Al tolerance mechanisms; tests which might be prohibitively expensive and time-consuming on a larger genetic population. These tests may indicate which physiological tolerance mechanisms are associated with a given genetic locus. If novel tolerance mechanisms appear from this analysis, it may be informative to assay the complete IBM population for those physiological traits. This would permit complete genetic analyses of the control of those traits. Comparison of the genetic loci contributing to the various tolerance mechanisms in the IBM population to each other and to tolerance loci in other grasses will help identify the number of mechanisms used by the various grass species. This will also spotlight targets for transgenic improvements of crop Al tolerance. Finally, advanced NIL will also be useful tools for further comparative molecular studies, as well as for the map-based cloning of the genes underlying these tolerance QTLs.

Comparative Mapping

The inheritance and genetics of Al tolerance has been studied in most agronomically important grass crops, including maize, rice, sorghum, rye, wheat, and barley. Al tolerance is relatively simple genetically in wheat, rye, barley, and sorghum. In contrast, Al tolerance is genetically complex in maize and rice. The major Al tolerance gene in wheat, barley, and rye are all on orthologous loci

corresponding to a region of rice chromosome 3, which also harbors an Al tolerance QTL (Kochian et al., 2004). There is also a cluster of Al tolerance QTL identified in several studies on rice chromosome 1, which occur in a region orthologous to one of the Al tolerance QTL identified in maize and the single major Al tolerance locus in sorghum (Kochian et al., 2004). When the Gramene database (Ware et al, 2002) was used to compare the orthologous regions in each of the above mentioned grass species to the different maize Al tolerance QTL identified in this study , no common loci were identified. To help understand how this could occur, it is informative to examine the exisiting literature on the quantitative genetics of Al tolerance.

Five studies have been published examining the quantitative genetics of Al tolerance in rice (Wu et al., 2000; Nguyen et al., 2001; Nguyen et al., 2002; Ma et al., 2002; Nguyen et al. 2003). The aforementioned region of rice chromosome 1 was the only region determined to be important for Al tolerance in all five studies. In contrast, when all the QTL from the five studies were summarized, there were 27 total regions identified as important for Al tolerance in at least one of the studies. In maize, only one study previous to the work described here has examined the quantitative genetics of Al tolerance. Ninamango et al. (2003) mapped QTLs for Al tolerance in maize using two Brazilian maize inbreds, L53 (Al sensitive) and L1327 (Al tolerant). They used two methods for analyzing their data. Using multiple regression analysis, they identified nine QTLs, while composite interval mapping revealed five Al tolerance QTL. None of these QTL co-localizes with the five QTL regions identified in the present study. The closest is a QTL identified by multiple regression analysis on chromosome 8 (marker umc103a) which is about 60 cM from NSRL-8.

Why would two separate experiments in maize identify completely different sets of QTL for Al tolerance, and five different experiments in rice identify only one concensus QTL? One possible reason for this might be due to a real diversity of Al

tolerance genes present in differing genetic lines. The two maize studies used very dissimilar genetic stocks, with the current study based on RIL generated from two North American maize inbreds, while the Brazilian analysis was based on two South American inbred lines. It is possible that the Al tolerance genes operating in L1327, which has been bred for generations on the acid soils of Brazil could be entirely different than the genes important for Al tolerance extant in the less extreme North American plains. The five rice studies also encompassed a wide variety of genetic backgrounds, including the subspecies *indica* and *japonica*, and a wild relative *Oryza rufipogon*. All of the maize and rice studies confirm the genetic complexity of Al tolerance in these species. A second possible cause of the variation in detected QTL may be based on the details of the experimental design. With many genes having a small contribution to Al tolerance, experimental details may significantly impact the resultant QTLs detected. Growing conditions (such as light, humidity, temperature, and nutrient solution composition) and experimental design (such as the time period observed and the phenotypic indices employed) may impact which QTL are detected as significant.

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CHAPTER FIVE

General Conclusions and Future Research

GENERAL CONCLUSIONS

Wheat

Two approaches were taken to attempt to clone genes relevant to aluminum (Al) tolerance in wheat (*Triticum aestivum*). A proteomics-based approach using deletion lines of the wheat cultivar Chinese Spring was used, and this approach was unsuccessful in identifying proteins encoded by genes in the region of chromosome 4DL where the major Al tolerance locus resides, due to the lack of appropriate proteomics technologies at the time this research was conducted. An alternate approach used our knowledge of the physiological mechanisms of Al tolerance in wheat to target genes that might encode the Al-activated root malate transporter from a potentially relevant family, the CLC anion channels. Eight representatives of this family were cloned from the Al tolerant wheat cultivar Atlas 66 using a hybridization screen. Genetic tests revealed that none of these eight genes were linked to the major Al tolerance locus in wheat, although another role in the malate exudation process was not ruled out. These different genes exhibited widely different expression patterns, with some showing high root expression, others high expression in the shoot, and some also Al inducible. Phylogenetic analysis revealed that the cloned *TaCLC*s only represent a portion of the entire *CLC* family that is likely present in wheat. In particular, a class of plant *CLC*s with a likely mitochondrial origin are not represented.

Maize

Previous work revealed Al tolerance to be a genetically complex trait in maize. Al tolerance was measured in 88 recombinant inbred lines (RIL) of the intermated B73 x Mo17 (IBM) population and a quantitative statistical analysis was performed to

identify genetic factors contributing to Al tolerance. Five quantitative trait loci (QTL) were identified using composite interval mapping (CIM) as having a significant impact on at least one of the three phenotypic traits analyzed. Three QTL combined to explain 42% of the variation in net seminal root length (NSRL) while two QTL combined to explain 29% of the variation in relative seminal root length (RSRL). A single QTL identified for relative root growth (RRG) explained 18% of the variation in that trait. These five regions are not orthologous to genomic regions associated with Al tolerance in wheat, rice, sorghum, rye, or barley. Also, they do not coincide with the results of another maize QTL mapping study (Ninamengo et al., 2003), which used a different mapping population. In three of the five QTL, Mo17 contributes the superior allele. Since Mo17 has a much higher Al-activated citrate release than any other maize genotype studied to date (Piñeros et al., 2005), these QTL are likely to contribute to that mechanism. Of course, it is also possible that they contribute through some other mechanism. In two of the five QTL, B73 contributes the superior allele. Since B73 has virtually no citrate release, these QTL are likely to contribute to Al tolerance through an alternative mechanism not based on Al-activated root organic acid exudation.

FUTURE RESEARCH

Wheat

The primary future research goals in wheat, where malate release is clearly the dominant physiological mechanism for Al tolerance, is the molecular and cellular characterization of that tolerance mechanism. The recent cloning and initial characerization of *ALMT1* (Sasaki et al., 2004), which has subsequently been shown to be tightly linked to the Al tolerance locus (Raman et al, 2005) and a putative malate transporter, will likely guide this research in the coming years. While the genetic

evidence presented here excludes the *TaCLC*s as candidates for the Al tolerance locus in wheat, the roles that the *TaCLC*s may play in root organic acid release or other cellular processes related to plant mineral nutrition remain to be elucidated.

To determine the roles of the *TaCLC*s in plant processes, functional data and information about the cellular localization of gene and protein expression will be required. To date, no convincing functional evidence for any of the CLC family members from plants has been presented indicating that they are in fact bona fide plant anion channels. Several approaches are possible to gain such evidence, including heterologous expression (*Xenopus* oocytes, insect cell lines, yeast, etc.), electrophysiological investigation of protoplasts isolated from plants transformed with specific *CLC*s, and expression of CLC proteins in artificial lipid membranes. The chance for success with any of these approaches can vary signiifcantly, so we may have to try several techniques. If channel activity is detected, alterations in solute and electrical conditions will be used to determine the electrophysiological properties of the channel. Spatial expression data will be used to complement functional data. From Northern analysis, we already know that some *TaCLC*s are more highly expressed in the shoots, some in the roots, and some have consistent expression across tissue types. More precise localization data would be helpful in the interpretation of functional data. Are those *TaCLC*s with high shoot expression preferentially expressed in guard cells, epidermal cells, mesophyll cells, or some other cell type? Are those more highly expressed in the roots preferentially expressed in epidermal, stellar, or cortical cells? A recently developed quantitative in situ hybridization technique (Küpper et al., 2005) is one method to ascertain such information while attempting to distinguish the various members of the family. A second aspect of spatial expression especially critical for transporters is membrane localization. Are the expressed proteins targeted to the plasma membrane, vacuolar membrane, Golgi ,

or some other cellular compartment? We may be able to answer this question by fusing a green fluorescent protein (GFP) reporter to the TaCLCs. Such fusion proteins, transgenically expressed in wheat or *Arabidopsis*, may permit the visualization of protein localization *in vivo*.

Maize

There appear to be multiple genetic factors and multiple physiological tolerance mechanisms in maize. The twin priorities of maize Al tolerance research are identifying the genes responsible for Al tolerance and the mechanisms conferred by those genes. The results presented here, along with previous maize experiments (e.g. Ninamengo et al., 2003) form a good basis for identifying those genes. The contrasting genomic regions identified may be resolved by examining more diverse genotypes simultaneously under identical experimental conditions. The near isogenic lines (NIL) being developed based on the QTL reported here will be useful for both research priorities. To match phenotypes to QTL, we plan to test each NIL for Al-induced organic acid exudation and root tip Al accumulation as well as for a battery of other putative tolerance mechanisms. These may include the exudation of phenolic compounds (which are Al chelators), changes in rhizosphere pH, and changes in cellular organic acid concentrations. It will be informative to discover which mechanisms are conferred by which QTL. Especially interesting will be the results for the two loci at which B73 holds the superior allele, since these likely act through an alternative mechanism to Al-activated organic acid exudation. QTL mapping of phenotypes related to various proposed tolerance mechanisms across the entire IBM population will reveal the complete genomic contributions to these mechanisms. High resolution mapping of specific QTL using the advanced NIL will also facilitate the isolation of genes underlying the Al tolerance QTL.

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