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Selection of asparagine substrate analog and sodium chloride resistant mutants in *Arabidopsis thaliana*

Chen, Futai, M.S.

The University of Arizona, 1988

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SELECTION OF ASPARAGINE SUBSTRATE ANALOG AND SODIUM CHLORIDE RESISTANT MUTANTS IN ARABIDOPSIS THALIANA

by

Futai Chen

A Thesis Submitted to the Faculty of the

DEPARTMENT OF PLANT SCIENCES

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCES
WITH A MAJOR IN AGRONOMY AND PLANT GENETICS

In the Graduate College

THE UNIVERSITY OF ARIZONA

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STATEMENT BY AUTHOR

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This thesis has been approved on the date shown below:

Fredric R. Lehle — November 15, 1988

Fredric R. Lehle — Date

Assistant Professor of Plant Sciences

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ABBREVIATIONS

AAH--beta-aspartyl hydroxamate

AMP--adenosine 5'-monophosphate (adenylic acid)

ANOVA--analysis of variance

AS--asparagine synthetase

Asn--asparagine

AsnRS--asparaginyl-transfer ribonucleic acid synthetase

ATP--adenosine triphosphate

C--centigade

cDNA--complementary deoxyribonucleic acid

CHO--Chinese hamster ovary

d--day

DNA--deoxyribonucleic acid

EDTA--ethylene dinitrilo tetraacetic acid

EMS--ethyl methane sulfonate

Fe--iron

Kd--kilodalton

kM--Michaelis constant

L--liter

LSD $_{0.05}$--least significant difference at the 0.05 level of probability

m--meter

mg--milligram

ml--milliliter

mm--millimeter

mM--millimolar

Abberviations- (Continued)

mol--mole

mRNA--messenger ribonucleic acid

N--nitrogen

PPi--inorganic pyrophosphate

s--second(s)

tRNA--transfer (soluble) ribonucleic acid

tRNAAsn--transfer ribonucleic acid specific for asparagine

uE--microeinstein

ug--microgram

v--volume

w--weight

LIST OF ILLUSTRATIONS

ABSTRACT

The inhibitory effects of NaCl, L- and D-asparagine, and asparagine substrate analogs, beta-aspartyl hydroxamate (AAH) and albizziin, alone or in combination on Columbia Arabidopsis seed germination and seedling survival were characterized under aseptic conditions. Germination on an agar medium supplemented with inorganic nutrients was prevented by 200 mM NaCl, 20 mM L-asparagine, 60 mM D-asparagine, 1.4 mM AAH, or 8 mM albizziin. Established seedlings were generally more tolerant to these chemicals than germinating seeds. Exogenous L- and D-asparagine partly reversed the inhibitory effects of NaCl on seed germination. L-asparagine also partly reversed AAH inhibition of germination. A M_2 seed bank was created from the self-pollinated progeny of ethyl methane sulfonate treated seeds. Arabidopsis mutants having increased tolerance to NaCl and AAH, but not albizziin, were successfully selected from this seed bank.

INTRODUCTION

The accumulation of salts in agricultural soils is a long standing problem (Epstein et al., 1980). Salinity of the arable land is an increasing problem in many arid and semiarid regions of the world that are irrigated. It is a significant factor in reducing crop productivity from salt-affected lands. Agriculture in those large areas of the world which depend on irrigation faces a serious challenge; crop productivity must be improved upon or at least maintained on ever more saline soils and waters.

In arid and semiarid regions, insufficient precipitation results in extensive reliance on irrigation. Continual irrigation can result in a concentration of salts (mainly NaCl) in soils and drainage water that is high enough to impair the growth of plants. At low concentrations, salt suppresses plant growth and at higher concentrations, it can cause plant death. The problem exists even in some of the world's subhumid regions, but is most widespread, severe, and threatening in arid and semiarid lands (Epstein et al., 1980). For example, severe salinization has occurred in over 50% of the Eurphrates valley and over 80% of the irrigated land in Pakistan is affected to some degree (Toenniessen, 1984).

There are various ways to solve the soil salinity problem in agriculture. One approach is to improve the land itself to minimize the extent and spread of saline soils (Epstein et al., 1979). This is accomplished by upgrading the land through improvements in subsoil drainage and the avoidance of low quality water for irrigation. The use of irrigation is dependent on the availabilities of high quality water

and systems to deliver it. The initial high cost and power requirements of the latter limits its use in impoverished areas. If irrigation is available, it can contribute to the soil salinity problem. If not properly managed, this occurs through an accumulation of salts following evaporation of the irrigation water.

Where land reclamation and irrigation are slow to be implemented or correctly managed, improvements in the salt tolerance of crop plants themselves represents the only practical near term solution to soil salinity. Breeding crops for increased salt tolerance is a promising, energy-efficient approach that would not require the quality of water typically associated with irrigation. Scientists could potentially reach the goal either by conventional breeding (Epstein et al., 1980; Kelly et al., 1979) or by genetically engineering NaCl resistance into crops (Marx, 1979; Mielenz et al., 1979; Csonka, 1980).

Conventional breeding appears to be an appropriate approach for improving crop salt tolerance since genetic diversity in salt tolerance has been reported in several crops (Greenway and Munns 1980). Despite this, there are few breeding programs which focus on salt tolerance. If genetic diversity of salt resistance is limited or lacking in superior backgrounds, then breeders could attempt to transfer the genes from wild salt-resistance relatives through sexual matings (Norlyn, 1980; Rush and Epstein, 1981). The disadvantage of this approach is that many unwanted traits would be introduced which may be difficult to remove by breeding. This would be especially true when these unwanted traits were closely linked to the salt-resistance gene or genes. Another disadvantage of this approach is that the time required to increase the homozygosity of

a trait isolated from a wild species and breed into superior backgrounds is lengthy. Usually, a trait controlled by two single alleles takes 8 to 10 generations to be expressed in a homogeneous population. If a trait is controlled by more than one gene, it takes much longer than 10 generations. These disadvantages may explain the reason why few commercial crop varieties adapted to saline growth conditions have been released.

Since plant tissue culture may produce somatic variations in established lines, it was thought tissue culture might overcome the slow pace of a conventional breeding approach. Unfortunately, this approach has also not yielded any major commercial crop varieties having increased salt tolerance. A number of unexpected problems have been encountered in selection for increased salt tolerance in cell or callus cultures. NaCl was found to interfere with the regeneration process and induce gross changes in the morphology and genome of regenerated plants (Bressan et al., 1985; McCoy, 1987a). In addition, salt-tolerance expressed at the callus stage was not always expressed in whole plants (McCoy, 1987b). For plant tissue culture technologies to succeed in improving salt tolerance in whole plants, more studies are needed to explore how the regeneration process itself interferes with the transmission of the salt tolerance trait.

The improvement of salt tolerance in crop plants has also been approached by studying gene expression during salt stress (King et al., 1986). These studies typically consist of comparisons of mRNA transcription and protein translation both in the presence and absence of salt (Gulick and Dvorak, 1987; Ragamopal, 1987; Singh et al., 1987).

For example, a 26-Kd protein termed osmotin has been isolated from the roots of tomato plants (King et al., 1986, 1988) and in tobacco suspension cultures (LaRosa et al., 1985, 1987; Singh et al., 1985) adapted to NaCl. While a study of gene expression during salt stress is promising, the functions of salt stress-induced gene products are largely unknown.

The accumulation of intracellular low molecular weight osmoprotectants in response to stress has been observed in a wide variety of higher plants (Stewart and Lee, 1974; Stewart and Hanson, 1980; Petolino and Leone, 1980). While proline is the primary amino acid which accumulates during water and salt stress in plants, other amino acids such as asparagine also accumulate (Bar-Nun and Poljakoff-Mayber, 1979; Barnett and Naylor, 1966; Ebercon, 1976; Fukutoku and Yamada, 1981; Helal and Mengel, 1979; Imamul Huq and Larher, 1983; Labanuskas et al., 1978).

The study of the adaptive significance of asparagine accumulation seems to be a promising approach to improving salt-tolerance in crop plants. There has not, however, been any evidence that asparagine accumulation in higher plants is an adaptive response to salt stress. Such a hypothesis might be tested in several ways. First, it could be demonstrated that exogenous application of asparagine protects whole plants from salt injury. Alternately, mutants having alterations in asparagine biosynthesis could be isolated. Particularly relevant would be the selection of mutants which overproduce asparagine. Strategies for the selection of such mutants have already been worked out for animal cells (Andrulis and Siminovitch, 1982b), but have not previously

been applied to whole plants. Thus, the unavailability of suitable mutants in higher plants delays progress in determining the adaptive significance of asparagine accumulation during stress.

The overall objective in this project was to determine if the intracellular accumulation of asparagine during salt stress is an adaptive response in higher plants. To reach this goal, my first objective was to determine the suitability of Arabidopsis to study salt stress and the adaptive significance of asparagine accumulation during salt stress in higher plants. This objective included a) characterization of the toxicities of NaCl on Columbia Arabidopsis, seed germination and seedling survival, b) demonstration that the natural level of salt tolerance in Arabidopsis can be altered by mutation, c) characterization of the tolerance of Arabidopsis to exogenous asparagine, d) demonstration that exogenous asparagine can reverse the inhibitory effects of NaCl at the whole plant level, and e) demonstration that D-asparagine, like L-asparagine, can reverse the inhibitory effects of salt stress. My second objective was to demonstrate the feasibility of isolating mutants in Arabidopsis which have altered regulation of the biosynthesis of asparagine. This objective included a) characterization of the inhibitory effects of the asparagine substrate analogs, beta-aspartyl hydroxamate (AAH) and albizziin on Arabidopsis, b) demonstration that the inhibitory effect of these asparagine substrate analogs can be reversed by asparagine in Arabidopsis, and c) demonstration that mutants resistant to both these compounds can be isolated in Arabidopsis.

In this thesis, I demonstrate the suitability of Arabidopsis as a

model system to study salt stress adaptation, evidence that asparagine may serve an adaptive function during NaCl stress and, the feasibility of selecting AAH, NaCl, or albizziin-resistant mutants in Arabidopsis.

LITERATURE REVIEW

Arabidopsis as a Model Plant System. Arabidopsis thaliana (a member in the family Cruciferae) has been a convenient subject for studies in classical genetics (Laibach, 1943; Redei, 1975), molecular genetics (Leutwiler et al., 1984; Meyerowitz, 1987), biochemistry (Last and Fink, 1988; Chang and Meyerowitz, 1986), and plant physiology (Moffatt and Somerville, 1988). The versatility of Arabidopsis derives from its minimum cultural requirements and genetic simplicity.

Arabidopsis thaliana has a simple life-cycle and growth habit which make it convenient for the study of plant biology (Koornneef et al., 1980). Normally, the plant flowers self-pollinate under natural conditions, but they can be easily cross-pollinated in the laboratory. The plant produces a large amount of seeds compared with most higher plants; generally each silique contains 30-60 seeds at maturity. It is possible to collect over 10,000 seeds from an individual plant owing to the fact that the plants continue to produce flowers for months. The seed size is very small, each weighing, on average, less than 20 ug. Once the seeds have matured, they may be stored in dry, cold (0-4C) conditions for years without loss of germinability. The plant has the advantage of a short life cycle. The commonly used strains Columbia and Landsberg have a generation time of only six weeks.

The simplicity of the Arabidopsis genome makes it useful for both traditional and modern studies of plant genetics (Meyerowitz, 1987). Cytogenetically speaking, this plant has a small number of chromosomes. The haploid chromosome number is only 5 chromosomes, which is one of the lowest number of chromosomes in the higher plant kingdom. Its genome

size is also one of the smallest identified so far among higher plants. Its haploid nuclear genome consists of only about 70,000 kilobase pairs which is only fifteen times the size of the Escherichia coli chromosome. Owing to the small size of its genome, it is easy to make a genomic library that is rapid and inexpensive to screen. This advantage, combined with the small genomic size, make it easier and faster to induce, select, and characterize mutations in this species than in most other angiosperms. These same features makes chromosome-walking experiments possible in Arabidopsis (Meyerowitz, 1987). There is also a method for the molecular cloning of genes from Arabidopsis and transferring them to other plant cells. This has been done by the methods that became standard for dicotyledonous plants (Caplan et al., 1983), involving recombinant constructions based on the Ti-plasmid of Agrobacterium tumefaciens. Transformation takes advantage of the ability of the bacterium to cause the insertion of the T-DNA region of the plasmid into chromosomes of infected plant cell.

Little is known about the salt tolerance of Arabidopsis and whether variability exists either naturally among ecotypes or can be induced by different mutagens. Thus, despite its many advantages, its suitability for studying salt tolerance in higher plants has not been addressed.

Accumulation of Asparagine in Stressed Plants. Proline accumulation under stress has been the focus of much research over the last two decades (Singh et al., 1973; Ebercon, 1976; Mali and Mehta, 1977; Hanson et al., 1977; Hanson and Nelsen, 1980; Stewart and Hanson, 1980). There are many reports, however, where other amino acids such as asparagine also accumulate in some higher plants under stress. For

example, Braun and Fluckiger (1984) found that low concentrations of NaCl sprayed on hawthorn Crataegus spp. caused large increases in the leaf content of asparagine, glutamine, and aspartic acid. Imamul Huq and Larher (1983) used 150 mM NaCl to stress Phaseolus aureus. and found free amides (expressed as asparagine equivalent) increased in the root with increasing salinity. Proline, asparagine, glutamine, and serine accumulate in Agrositis stolonifera under saline conditions and at high salinities, asparagine is accumulated to levels greater than proline (Fukutoku and Yamada, 1981). Salt-grown plants of Lupinies vulgare accumulate large quantities of glutamine, while those of Triglochin maritima accumulate both asparagine and glutamine.

Asparagine accumulation and that of other amino acids are found not only in NaCl-stressed plants (Braun and Fluckiger, 1984; Petoline and Leone, 1980), but also in water, drought, and freezing stressed plants. Hanower and Brzozowska (1975) studied the effect of water stress on the free amino acids in cotton leaves and found that the major differences between treated and untreated leaves were in the levels of gamma-aminobutyric acid, asparagine, proline, and glutamic acid. Fukutoku and Yamada (1981) investigated water stress in soybean and found that water stress induced a remarkable accumulation of free amino acids including histidine, phenylalanine, isoleucine, leucine, tyrosine, valine, and especially proline and asparagine. The maximum contents of proline and asparagine were 31% and 24% of the total free amino acid nitrogen, respectively. Fukutoku and Yamada (1981) studied severely water-stressed plant tissue and found proline and asparagine accumulated extensively, especially in the younger green leaves. Fifty-four percent

of the loss of leaf protein ^{15}N was in the free amino acids; 41% was present in proline and asparagine. Munns et al. (1979) used an enclosed elongating leaf and an unenclosed expanded leaf of wheat plants to study the effect of water stress on amino acid accumulation. They found that among the amino acids, the largest increases were in asparagine and proline. Thankur and Rai (1982) investigated amino acid contents in two maize cultivars with different sensitivities to drought and observed that a substantial accumulation of asparagine, serine, glycine, glutamic acid, and aspartic acid occurred in both cultivars. Also water-stress induced accumulation of asparagine was recorded in both cultivars with increasing drought.

Tanabe et al. (1982) studied the effect of water stress in tobacco leaves and asparagine was reported to be the primary amino acid accumulated in a few water-stressed plants. They also found asparagine accumulation was greater in younger leaves at the upper positions on the stalks. More interesting, they found asparagine, but not proline, accumulated in leaves under turgid conditions. In the case of immature leaves, water stress strongly induced proline accumulation, but more dramatically affected an accumulation of amides (mostly asparagine). In contrast to wilting leaves, turgid leaves did not accumulate proline during the stress period, although an appreciable amount of amino acids and amides were accumulated. Amides, especially asparagine, were produced in excised tobacco turgid leaves as well as in wilting leaves. Similarly, asparagine accumulated greatly in detached young tobacco leaves both under wilting and turgid conditions (Thompson et al., 1966b).

Despite the fact that asparagine accumulation has been associated with abiotic stresses, the adaptive significance of this response is uncertain. Three roles of beneficial amino acid accumulation during stress have been suggested. First, when electrolytes are lower in the cytoplasm than in the vacuole, the accumulated amino acids can adjust the osmotic balance (Stewart and Lee, 1974). Second, the accumulated amino acids can have a protective effect on enzymes in the presence of high electrolytes in the cytoplasm (Ahmad et al., 1982; Paleg et al., 1981). Third, plants may accumulate amino acids as a storage form of nitrogen during stress (Thompson et al., 1966a).

Asparagine accumulation could result from several possible mechanisms, but the exact means is not yet known. Some reports stated that amino acids accumulation under stress may be the result of de novo synthesis (Barnett and Naylor, 1966; Boggess et al., 1976). Others claim that amino acid accumulation under stress may be the result of protein hydrolysis (Kudrew, 1967; Fukutoku and Yamada, 1981, Goring and Thien, 1978) or may be the result of inhibition of certain amino acid oxidation (Stewart and Boggess, 1978). Although much speculation regarding amino acids accumulation has been proposed, the physiological significance of changes in nitrogenous compound levels during stress conditions remains uncertain. Imamul Huq and Larher (1983), demonstrated that NH_4^+ accumulated in Phaseolus aureus with externally increased salinity. Excessive amounts of NH_4^+ in the plant resulted in an increased accumulation of ureides and asparagine. Asparagine and some other inorganic ions, K^+ and NO_3^-, played a key osmoregulatory role in Phaseolus aureus. In general, the role of amino acid accumulation

during stresses is uncertain.

Progress in understanding the function of asparagine metabolism has been hindered by the absence of *in vitro* assays for the anabolic and catabolic plant enzymes involved, namely asparagine synthetase and asparaginase, respectively. Enzyme stability is one of the major problems in developing a reliable assay for these enzymes from plants (Reitzer and Magascanik, 1982).

Although some research work have been done on AAH or albizziin resistance in Chinese hamster ovary (CHO) cells (Andrulis, 1985), no similar work has been reported in higher plants. Progress has also been especially hampered by a lack of proper biochemical mutants in higher plants. Isolation of the proper mutants is a prerequisite for understanding the regulation of asparagine metabolism in higher plants. Such mutants would be useful for several reasons. First, mutants which had an altered regulation of asparagine biosynthesis could be employed to determine the adaptive significance of asparagine accumulation during salt stress. This could accomplished by assessing the effect of the mutation of salt-tolerance of the mutants relative to the wild type. Second, such mutants would have a major impact in the elucidation of regulatory mechanisms of basic molecular processes in higher plants. The proper mutants are also needed for elucidating the relationship between control of biological processes at molecular and whole organism levels.

Asparagine Synthetase (E C 6.3.5.4.). Asparagine synthetase (AS) catalyzes the amidation of aspartate by glutamine to form asparagine and glutamate as illustrated below:

$$\text{Aspartic Acid} + \text{Glutamine} + \text{ATP} + H_2O \xrightarrow{AS} \text{Asparagine} + \text{Glutamic Acid} + \text{AMP} + PP_i$$

In this reaction, asparagine synthetase catalyzes the stoichiometric generation of asparagine, AMP, and inorganic pyrophosphate from aspartate, ATP, and glutamine (Reitzer and Magasanik, 1982).

Asparagine is generally thought to be acylated to $tRNA^{Asn}$ prior to being incorporated into protein by asparaginyl-tRNA synthetase (AsnRS) as diagramed below (reviewed by Andrulis, 1985).

$$\text{Asparagine} + tRNA^{Asn} + \text{ATP} \xrightarrow{AsnRS} \text{Asparaginyl-}tRNA^{Asn} + \text{AMP} + PP_i$$

$$\text{Asparaginyl-}tRNA^{Asn} \longrightarrow \text{protein}$$

Alternatively, asparagine can be degraded to aspartate and ammonia, which is toxic, by the enzyme asparaginase.

$$\text{Asparagine} + H_2O \xrightarrow{\text{Asparaginase}} \text{aspartate} + NH_4^+$$

Asparagine synthetase is divided into glutamine-dependent and ammonia-dependent (Reitzer, 1983). The reactions catalyzed by each are illustrated as follows:

$$\text{Aspartate} + \text{glutamine} + \text{ATP} \longrightarrow \text{Asparagine} + \text{Glutamate} + \text{AMP} + PP_i$$

$$\text{Aspartate} + \text{Ammonia} + \text{ATP} \longrightarrow \text{Asparagine} + H_2O + \text{AMP} + PP_i$$

The first route occurs in all organisms. The second route, however, is present only in bacteria (Reitzer, 1983).

The glutamine-dependent asparagine synthetase is controlled by asparagine through repression and inhibition. For example, crude beef

pancreatic asparagine synthetase is inhibited by asparagine. In addition, asparagine synthetase from pancreas appear to be ATP specific. Asparagine synthetase in plants seems only slightly more resistant to asparagine than the enzymes in bacteria (Reitzer, 1983). The regulation of the ammonia-dependent asparagine synthetase is more complex (Reitzer, 1983). Asparagine may inhibit both as glutamine analog and as a product inhibitor (Reitzer, 1983). Asparagine also inhibits the glutaminase and the synthetase activity of the glutamine dependent enzymes (Horowitz and Meister, 1972; Reitzer and Magascanik, 1982). The concentration of Cl^- is also important for regulating the activity of the enzyme from plants (Rognes, 1980) To my knowledge, regulation of asparagine synthetase under stress conditions has not been previously studied.

Depending upon its source, the two forms of asparagine synthetase differ in their kinetic parameters, (Horowitz and Meister, 1972; Holcenberg, 1969), ion dependence, (Horowitz and Meister, 1972), and molecular weight. Markin and Schuster (1979) reported that rat liver asparagine synthetase exists in three different forms (57 Kd, 110 Kd 113 Kd) depending on diet. Hepatic asparagine synthetase was found to exist in either a 110 Kd or a 57 Kd form. If the rat diet was deficient in asparagine, both of these enzyme forms were present. If, on the other hand, the diet contained asparagine, only the lower molecular weight form was observed. The glutamine-dependent asparagine synthetase isolated from Klebsiella aerogenes has subunits of 57 Kd and a total molecular weight of about 230 Kd indicating that the enzyme is a tetramer. The plant enzyme has a molecular weight of 320 Kd in the

presence of ATP and 160 Kd in the absence of ATP (Rognes, 1975). Chloride ions are essential for optimum activity of some plant forms of asparagine synthetase (Oaks and Ross, 1984; Rognes, 1980). It is clearly understood that Escherichia coli requires two genes to code for asparagine synthetase and that asparagine auxotrophy demands that both genes be defective (Felton et al., 1980; Humbert and Simon, 1980).

Asparagine synthetase has been isolated from the cotyledons of a number of germinating seeds (Rognes, 1970; Streeter, 1973), maize roots, (Stulen and Oaks, 1977, Stulen et al., 1979), and root nodules (Scott et al., 1976). There were some important differences in the properties of asparagine synthetase extracted from various tissues of corn and soybean cotyledons (Oaks and Ross, 1984). The enzyme activity was very sensitive to chemical and physical factors such as iron concentration, pH, and temperature. The presence of inhibitors complicates the in vitro measurement of asparagine synthetase activity (Reitzer, 1983).

Analogs of Aspartic Acid and Glutamine—AAH and Albizziin. The substrates of asparagine synthetase, aspartic acid and glutamine, share structural similarities with the toxic analogs AAH and albizziin, respectively (Fig. 1) The molecular structures of aspartic acid, beta-aspartyl hydroxamate, glutamine, albizziin, and asparagine are shown below (next page).

Due to these chemical structural similarities, both AAH and albizziin have been studied as potential selection agents for mutants having altered regulation of asparagine biosynthesis. Their biological activities are consistent with such a role. Norton and Chen (1969)

working with Lactobacillus arabinosus, presented evidence which suggested that AAH represses asparagine synthetase formation in vivo and is a false feedback inhibitor in vitro. The effect of AAH on asparagine metabolism has been well investigated in the CHO cell system by Andrulis (1985). Brynes et al. (1978) tested the ability of AAH to inhibit asparagine-resistant tumor formation. Andrulis et al. (1979; Andrulis and Siminovitch, 1981) has exploited these properties of AAH and albizziin to select asparagine over-producing mutants in CHO cells.

Andrulis and her colleagues have succeeded on isolating and characterizing AAH or albizziin resistant mutants in CHO cells (Andrulis et al., 1979, 1980; Andrulis and Siminovitch, 1981, 1982; Andrulis et al, 1985). Andrulis et al. (1983) used the chemical mutagen ethyl methane sulfonate to induce mutations in CHO cells and isolated three albizziin-resistant mutants. The albizziin-resistant mutants were approximately 10-fold more resistant to albizziin than the parental cell lines and expressed 8- to 17-fold elevations in asparagine synthetase activity. The mechanism of albizziin resistance of CHO cells was shown to be due to gene amplification of asparagine synthetase (Andrulis, 1985).

Andrulis and Siminovitch (1982) also reported that mutants of CHO cells with alterations in asparagine synthetase activity could be obtained after selection for resistance to AAH. All isolated mutants expressed elevated levels of asparagine synthetase activity (up to 20-fold). Some of the mutants exhibited structural changes in the enzyme (i.e. increased thermostability or increased kinetics for AAH). Gantt (1980) found that CHO cells resistant to AAH also had elevated levels of

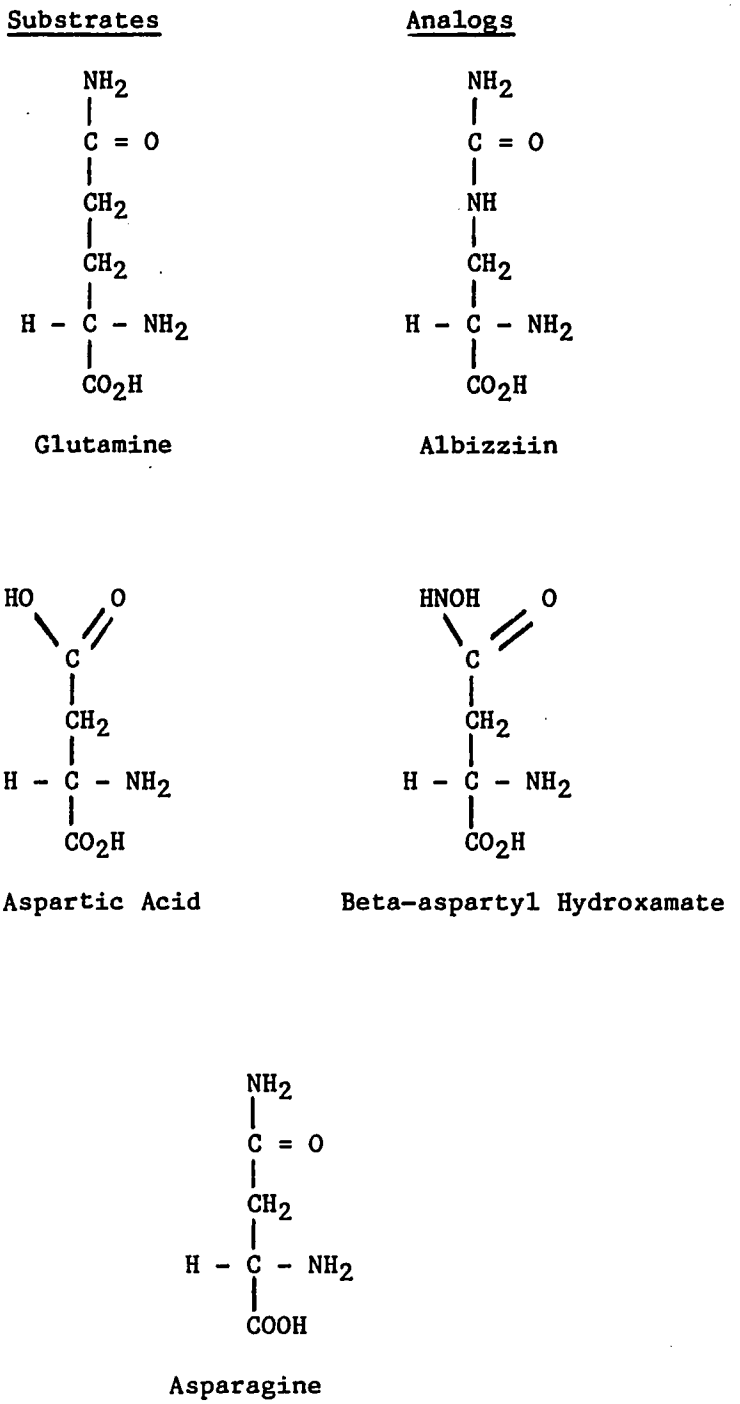

Figure 1. Molecular structures of asparagine, aspartic acid, beta-aspartyl hydroxamate, glutamine, and albizziin.

an apparently normal asparagine synthetase. When AAH resistance was transferred to AAH sensitive recipient cells by DNA transfection, some of the transferants expressed highly increased levels (60 to 70 fold) of the enzyme (Andrulis and Siminovitch, 1981). They concluded that AAH inhibited the activity of both asparagine synthetase and asparagine-tRNA synthetase in vitro. The molecular mechanism of AAH resistance in CHO cells is believed to be caused by an alteration in the structural gene for asparagine synthetase (Andrulis, 1985). I was unable to locate any publicated work concerning AAH or albizziin resistance in higher plants.

The analogs differ in the extent in which they interfere with normal metabolism. Albizziin is a glutamine analog and as such interferes with many glutamine-utilizing enzymes. In contrast, AAH affects fewer enzymes, reflecting the lesser but nevertheless essential role of aspartic acid in cellular metabolism (Pinkus, 1977).

The metabolic pathways of glutamine are listed below:

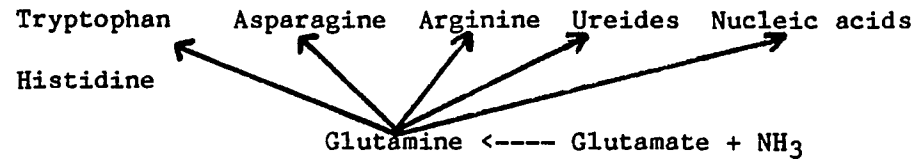

Figure 2. The metabolic pathways involving glutamine.

MATERIALS AND METHODS

Plant Material. The Columbia strain of Arabidopsis thaliana Heynh. homozygous for the recessive trait glabra-1 (Koornneef et al., 1980) was used for all experiments. Original seed samples were obtained from Dr. C. R. Somerville of the MSU-DOE Plant Research Laboratory, East Lansing, MI. Seeds were multiplied in a evaporated-cooled greenhouse at the Agricultural Experiment Station at University of Arizona in 1986 by two cycles of self-pollination.

Seed Mutagenesis. About 70,000 Columbia seeds were mutagenized in 0.25% ethyl methane sulfonate (EMS) for 12.5 hr according to the procedure of Somerville and Ogren (1982). Due to the volatility of EMS, all operations were done in a flume hood. The EMS mutagenic solution at room temperature was stirred with a paper clip driven by a water-powered magnetic stirrer. After the mutagenic treatment, the seeds (M_1 generation) were rinsed with water 15 times in order to remove the residual EMS from the seed surface. Waste EMS and water rinses were added to a covered glass jar containing 100 grams NaOH initially dissolved in a minimum amount of water. Following rinsing, the seeds were suspended in 3075 ml of sterile 0.15% (w/v) agar autoclaved the night before. The seeds were then distributed evenly on a potting mixture of vermiculite, perlite, and peat moss (1:1:1) among 60 plastic flats (27.9 by 539 by 603 cm) in an evaporative-cooled greenhouse. The potting mixture was first soaked with water and then fertilized with nutrient solution prepared according to Somerville and Ogren (1982). Each flat received 50 ml of the agar solution containing about 1,000 M_1 seeds. Dispersion was with a pasteur pipet. Flats were finally covered

with plastic film under 63% shade cloth. Plants were sub-irrigated daily and fertilized about once a week and allowed to self-pollinate as they matured. The seeds were sowed on June 8, 1987 and harvested on August 18, 1987. The entire growth cycle was 71 days. The M_2 seeds were bulked by flats into parental groups designated C1 to C60. Each parental group contained the self-pollinated progeny from about 1000 M_1 parents. After the seeds had been cleaned with a screen (3.55 mm mesh opening) and a seed blower, they were allowed to dry and after-ripen at room temperature for 3 weeks in paper envelopes. Following after-ripening, seeds were transferred to glass vials and stored at 5C.

Seed Sterilization. The seeds were surface-sterilized by the following immersion schedule: 1 hr in sterile water; 5 min in 95% ethanol; and 10 min in 1.6% (w/v) NaOCl. The seeds were then rinsed with sterilized water 5 times and suspended in a seed transfer solution of sterile 0.15% (w/v) agar which had stood overnight after autoclaving. Seeds were always sterilized just before use.

Medium Preparation and Petri Dish Preparation. Inorganic components were added to a nutrient medium prepared according to Somerville and Ogren (1982). Each of the inorganic components was prepared as a separate stock solution (Griffin et al., 1986). To prevent precipitation, stock solutions of $MgSO_4$ and $Ca(NO_3)_2$ were mixed together with 1.37% (w/v) agar and stock solutions of KNO_3, KH_2PO_4, micronutrient mixture, and Fe-EDTA were mixed together following the procedure developed by Griffin et al. (1986). These two mixtures were autoclaved separately at 121C for 20 minutes and then mixed together prior to pouring the medium. To prevent contamination, 2 ml of an

aqueous ampicillin solution (5 mg/ml) were added to each 100 ml of medium. Where appropriate, different concentrations of NaCl, L- and D-asparagine, AAH, and albizziin were added to the inorganic medium. NaCl, AAH, albizziin, L-asparagine, and D-asparagine were filter sterilized as solutions using disposable 0.2 micron filters (ACRODISC, Gelman Sciences). To make the medium nutritionally identical, 4 ml of 1.37% (w/v) agar nutrient solution plus different volumes (0 to 1 ml) of NaCl, AAH, albizziin, L-asparagine, or D-asparagine dissolved in nutrient solution without agar and different volumes (0 to 1 ml) of nutrient solution were added to make the final volume 5 ml per petri dish (60 by 15 mm). The final agar concentration of the medium was 1.1% (w/v). A 50 mm diameter of autoclaved nylon netting (1-mm mesh opening) was placed on the agar surface after the medium solidified. An appropriate number of seeds, suspended in 0.5 ml seed transfer solution was pipetted onto the agar surface and spread evenly with a sterile glass rod. The nylon mesh prevented seed clumping. The pouring of media and sowing of seeds were done in a laminar flow hood using sterile technique.

Inhibitory Effects of L-Asparagine, Its Substrate Analogs, D-Asparagine, and NaCl to Arabidopsis Seed Germination. Solutions of NaCl, L-asparagine, D-asparagine, AAH, and albizziin dissolved in nutrient solution were filter-sterilized for all experiments. NaCl, L-asparagine, D-asparagine, AAH, or albizziin solutions was added directly to the petri dishes prior to pouring the medium. Planting density was about 200 seeds per dish. All petri dishes were covered and sealed with Parafilm after sowing. All treatments were replicated at least twice.

After 7 d incubation at 23 $\pm$ 1C in continuous fluorescent light (100 to 150 uE m^{-2} s^{-1}), the percent germination was recorded. The criteria for germination was seedlings with expanded cotyledons which were 2 mm or more from tip to tip when viewed from the top. An additional requirement was that the cotyledons still retained over 50% of their green color. This criteria was used for all experiment.

Comparison of the Inhibitory Effects of L- and D-Asparagine on Arabidopsis Seed Germination. The procedures used were the same as described above except that the dishes contained either 0 to 25 mM L-asparagine or 0 to 60 mM D-asparagine. L-asparagine and D-asparagine were dissolved in nutrient solution and filter-sterilized separately. The sterilized solutions were added directly to the different treatment dishes prior to pouring the medium.

Inhibitory Effects of L-Asparagine, Its Substrate Analogs and NaCl to Arabidopsis Seedling Survival. For seedling experiments, the initial sowing medium consisted of 4 ml 1.37% (w/v) nutrient agar. Seeds were sown in 0.5 ml of 0.15% agar (w/v). Germination and seedling establishment were allowed to occur for 7 d prior to the aseptic addition of a 1 ml solution containing asparagine, AAH, albizziin, or NaCl to the agar surface. It was necessary to heat solutions containing asparagine at concentrations above 30 mM to effect solubilization. Dishes were left unsealed and incubated for an additional 7 d. Seedling survival was recorded 14 d after imbibition onset using the same criteria as germination.

Reversal of the AAH Inhibition of Arabidopsis Seed Germination by L-Asparagine. Procedures were the same as above except the medium

contained 0 to 20 mM L-asparagine and 1.3 mM AAH. Different concentrations of AAH and L-asparagine were added prior to the medium pouring. Germination was counted 7 d after seeding.

Reversal of Albizziin Inhibition of Arabidopsis Seed Germination by L-Asparagine. Procedures were the same as that in reversal of AAH inhibition by L-asparagine except that the dishes contained 0 to 8 mM of L-asparagine and 8 mM of albizziin. L-Asparagine and albizziin were added to the dishes prior to medium pouring.

Reversal of NaCl Inhibition of Arabidopsis Seed Germination by L-Asparagine. Procedures used were the same as that in reversal of AAH inhibition by L-asparagine experiment, except that the dishes contained 0 to 8 mM L-asparagine and 120 mM NaCl. L-Asparagine and NaCl, dissolved in nutrient solution, were added prior to medium pouring.

Reversal of the Inhibitory Effect of 120 mM NaCl by L- and D-Asparagine. Procedures used were the same as those in the reversal of NaCl inhibition by L-asparagine experiment, except that the dishes contained 0 to 20 mM L- and D-asparagine and 120 mM NaCl.

Selection of Arabidopsis Resistant Mutants to AAH, Albizziin, and NaCl. The final concentration of agar in the selection experiment was 0.8% (w/v). Selections were conducted on medium concentrations either 1.4 to 1.6 mM AAH, 14 mM albizziin, or 180 to 250 mM NaCl. The seed density was about 2000 seeds per dish which represented about 333 M_1 parents per petri dish (90 by 20 mm). The seeds were germinated aseptically in Parafilm sealed dishes at 23C in continuous fluorescent light for 10 d.

Variant Rescue. Rescue medium for AAH resistant and albizziin

resistant variants consisted of 0.6% (w/v) agar medium containing 2 mM asparagine. Rescue medium for NaCl resistant variants consisted of 0.6% (w/v) agar. Variants were rescued from 10 to 15 d after the onset of inhibition.

Statistics: All percent germination and seedling survival data was transformed by the arcsin transformation prior to ANOVA (Steel and Torrie, 1960). ANOVA for all experiments assumed a completely randomized design with two or more replications (see APPENDIX I).

RESULTS

Inhibition of Arabidopsis Seed Germination and Seedling Survival by L-Asparagine. L-Asparagine was inhibitory to Arabidopsis seed germination (Fig. 3). Significant ($P<0.0001$) inhibition occurred at L-asparagine concentrations greater than 4 mM. Germination was prevented entirely by L-asparagine concentrations above 20 mM in the medium. Symptoms of L-asparagine inhibition included reduction in seedling size and primary root formation. Some severely inhibited seedlings lacked radicles. Increased leaf chlorosis was also evident at L-asparagine concentrations inhibitory to growth. Leaf chlorosis of emerged seedlings was first noticeable on dishes containing 8 mM L-asparagine. In contrast to its effects on seed germination, exogenous L-asparagine was not very toxic to established seedlings. In one experiment, 7-day-old established seedlings continued to grow and showed only slight leaf yellowing after the medium concentration of L-asparagine was increased to 55 mM. In another experiment, only about 20% of established seedlings were killed by a saturated L-asparagine solution, i.e. about 150 mM final concentration.

Inhibition of Arabidopsis Seed Germination by D-Asparagine. Like L-asparagine, D-asparagine was inhibitory to Arabidopsis seed germination (Fig. 4). Significant ($P<0.0001$) inhibition of germination was first shown at medium concentrations of about 20 mM D-asparagine. Germination percentage declined increasingly as the concentration of D-asparagine in the medium was increased. Germination was completely prevented at 60 mM D-asparagine. The first significant reductions in seedling size occurred at medium concentrations of 25 mM D-asparagine,

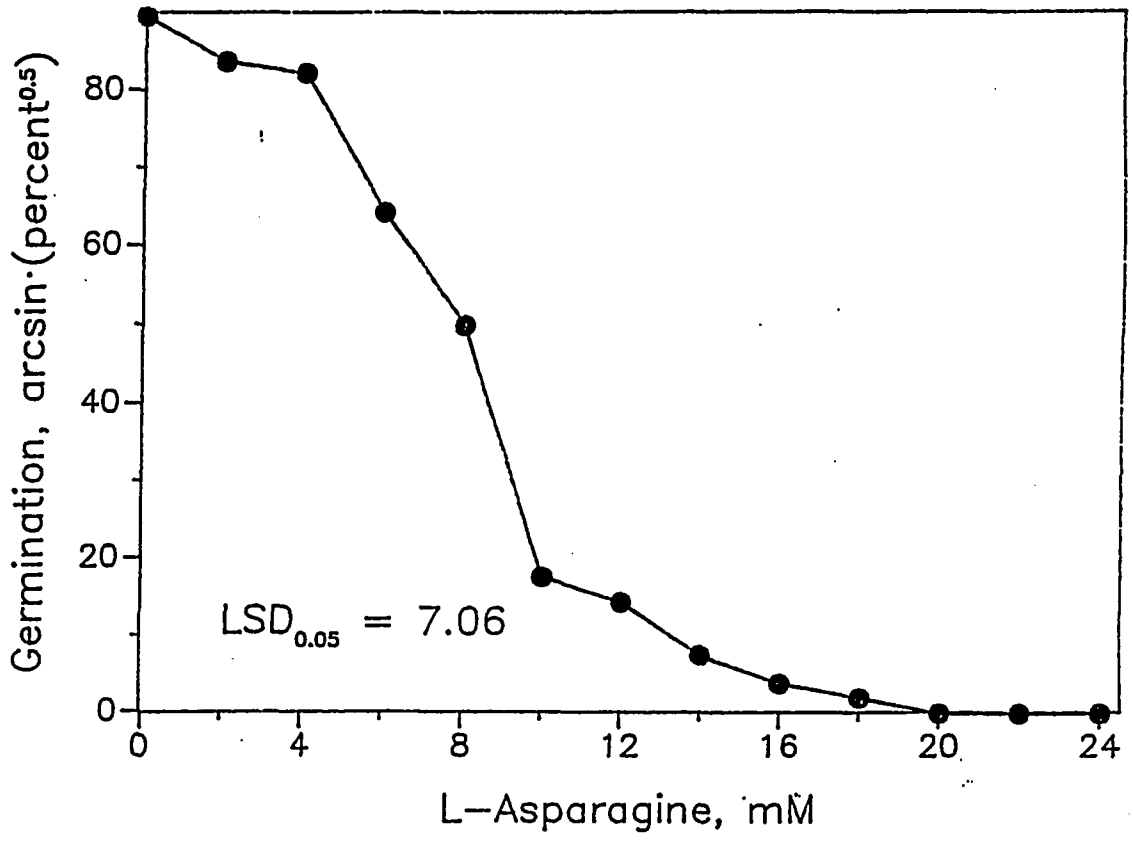

Figure 3. The effect of L-asparagine on Arabidopsis seed germination. Experiment was conducted aseptically in petri dishes containing 1% agar (w/v) supplemented with inorganic nutrients and the indicated range of L-asparagine concentrations. About 200 seeds were sown in each dish before incubation at 23 ± 1C in continuous fluorescent light. The number of germinated and ungerminated seeds were recorded 7 days after inhibition onset. Each point represents the mean of two replications.

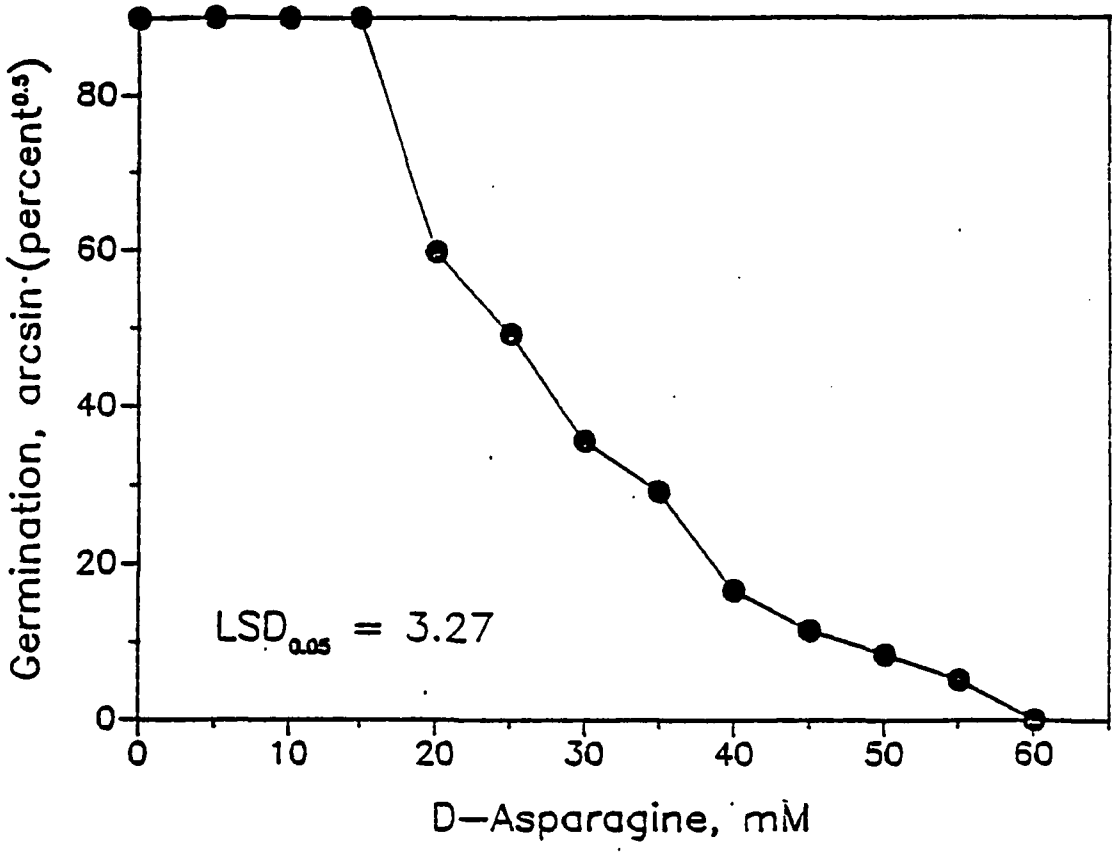

Figure 4. The effect of D-asparagine on Arabidopsis seed germination. Methods were the same as in Figure 3.

which was 5 mM higher than the lethal concentration for L-asparagine. Chlorosis on the leaves of emerged seedlings was not observed even at medium concentrations of 55 mM D-asparagine.

The Relative Toxicities of L- and D-Asparagine to Arabidopsis Seed Germination. Although both L-asparagine and D-asparagine were inhibitory to Arabidopsis seed germination, the results indicated that D-asparagine was much less inhibitory than L-asparagine (Fig. 5). For D-asparagine, no inhibition of seed germination was evident at concentrations up to 15 mM. In contrast, at this same concentration of L-asparagine, there was a significant ($P<0.0001$) inhibitory effect on Arabidopsis seed germination. D-asparagine was less inhibitory than L-asparagine at all concentrations tested (Fig. 5). For example, the prevention of germination required 60 mM D-asparagine, which was 3 times higher than the level required of L-asparagine to achieve similar inhibition. Overall, results showed that D-asparagine was much less inhibitory to Arabidopsis seed germination than was L-asparagine.

Inhibition of Arabidopsis Seed Germination and Seedling Survival by NaCl. The results indicated that NaCl was inhibitory to both Arabidopsis seed germination and seedling survival. The inhibitory effect of NaCl on seed germination is shown in Figure 6. Germination was prevented entirely at concentrations of 200 mM or was increasingly inhibited by NaCl concentrations at or above 20 mM; seed germination was prevented entirely at concentrations of 200 mM or above. Germination rate and established seedling size were reduced by increases in the NaCl concentration.

Leaf chlorosis increased as the concentration of NaCl in the medium

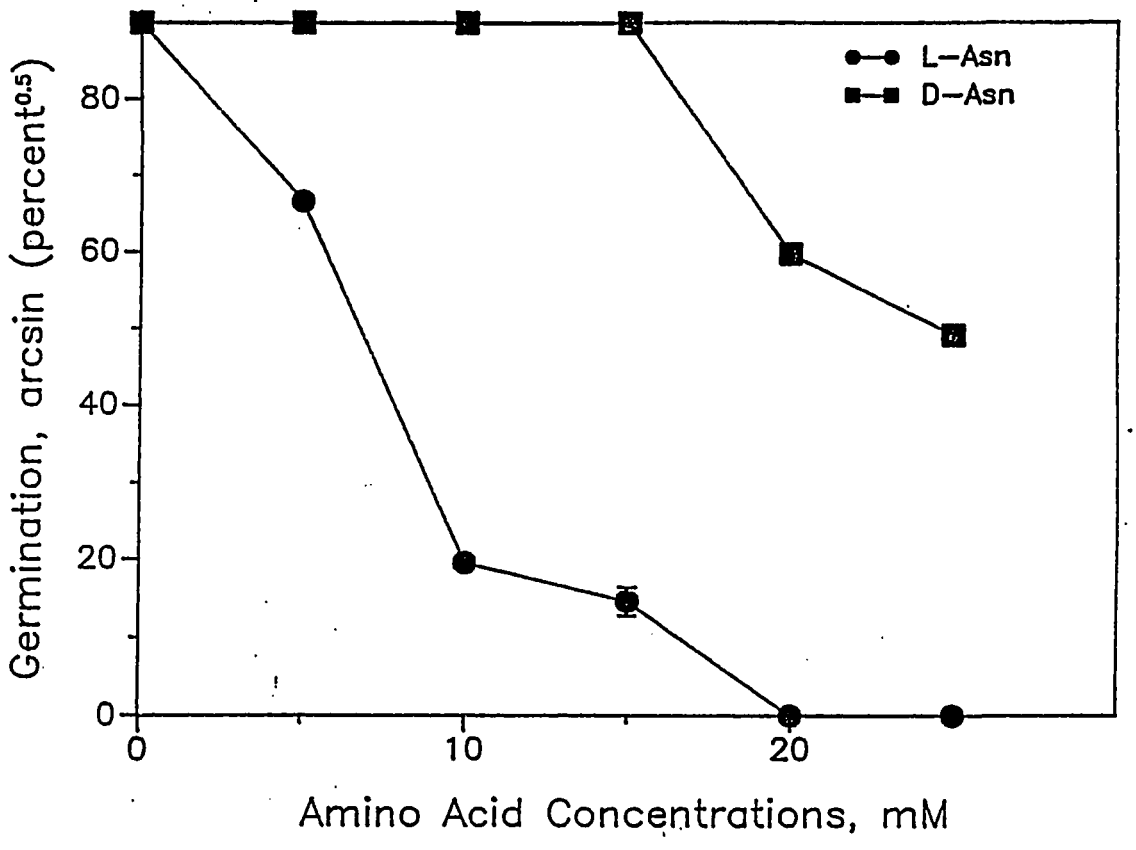

Figure 5. Comparison of inhibition of Arabidopsis seed germination by L-and D-asparagine. Seeds were germinated on aseptic nutrient agar medium containing the indicated ranges of either L- or D-asparagine concentrations. The number of germinated and ungerminated seeds were recorded after 7 days of inhibition at 23 ± 1C in continuous fluorescent light. Each point represents the mean of two replications.

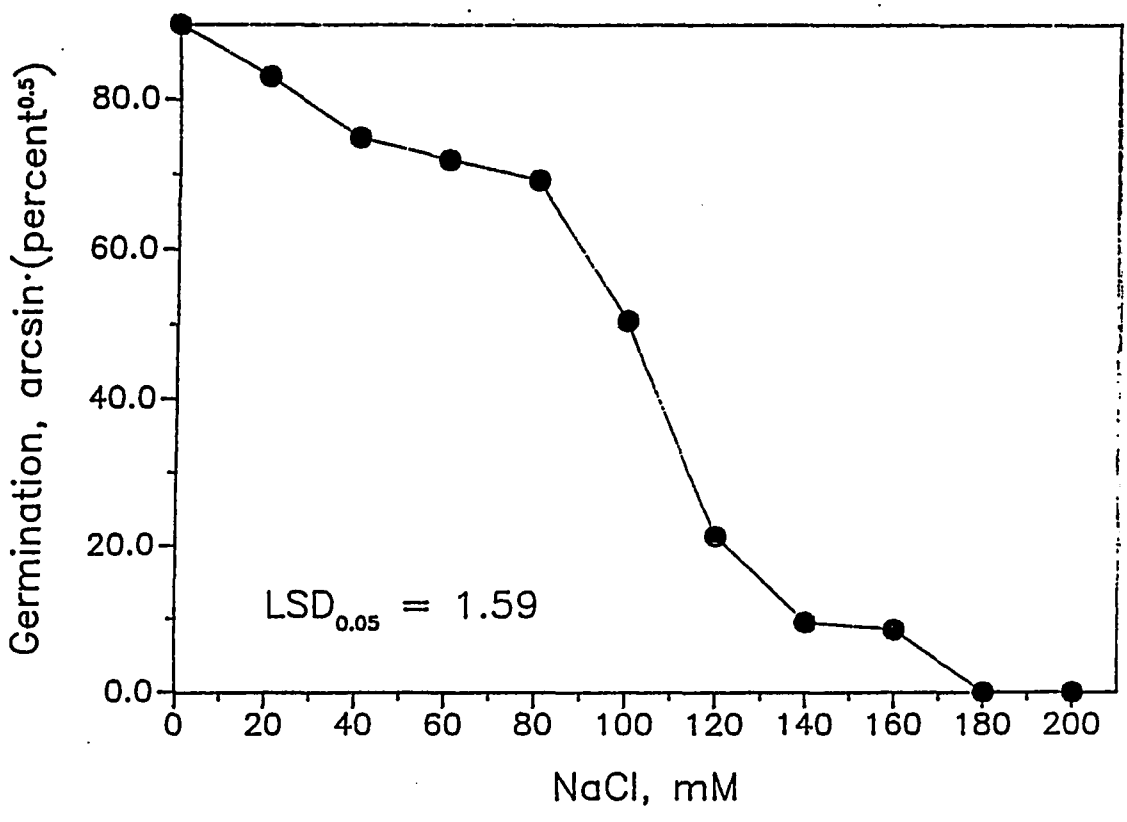

Figure 6. The relationship between Arabidopsis seed germination and the inhibitory effects of NaCl. The experiment was conducted in petri dishes under aseptic conditions. There were about 200 seeds in each dish. The germinated and ungerminated seeds were recorded after 7 days of inhibition at 23 $\pm$ 1C in continuous fluorescent light. Each point represents the mean of two replications.

increased. There was no visual leaf chlorosis at or below 60 mM NaCl. At NaCl concentration of 80 mM or above, the emerged seedlings became increasingly chlorotic. Leaves of seedlings killed by high concentrations of NaCl were frequently without any color.

The inhibitory effect of NaCl to established Arabidopsis seedlings is shown in Figure 7. NaCl additions to the medium were stimulatory to the growth of established seedlings at lower levels (20-40 mM), but were inhibitory and eventually lethal to established seedlings at higher levels (60 mM and above). NaCl had no effect on the growth of established seedlings at concentrations of 20 mM NaCl or below. Seedling growth increased after the medium concentration was increased from 20 to 40 mM NaCl. The size and the color of the leaves of seedlings grown on medium containing 20 or 40 mM NaCl appeared darker than those grown on media without NaCl. Seedling survival declined as the medium concentration of NaCl was suddenly increased to 60 mM or higher NaCl. Established seedlings were killed on medium where the NaCl concentration was increased suddenly from 0 to 220 mM NaCl or above.

Reversal of the Inhibitory Effects of 120 mM NaCl on Arabidopsis Seed Germination by L-Asparagine. L-Asparagine reversed the inhibition of 120 mM NaCl on Arabidopsis seed germination (Fig. 8). Maximum reversal of the inhibition caused by 120 mM NaCl occurred at about 3 mM L-asparagine. Asparagine concentrations higher than 4.5 mM did not show any significant reversal effect. Germination was prevented on medium containing 120 mM NaCl and 8 mM L-asparagine.

Reversal of the Inhibitory Effect of 120 mM NaCl to Arabidopsis Seed Germination by L- and D-Asparagine. Although L-asparagine was more

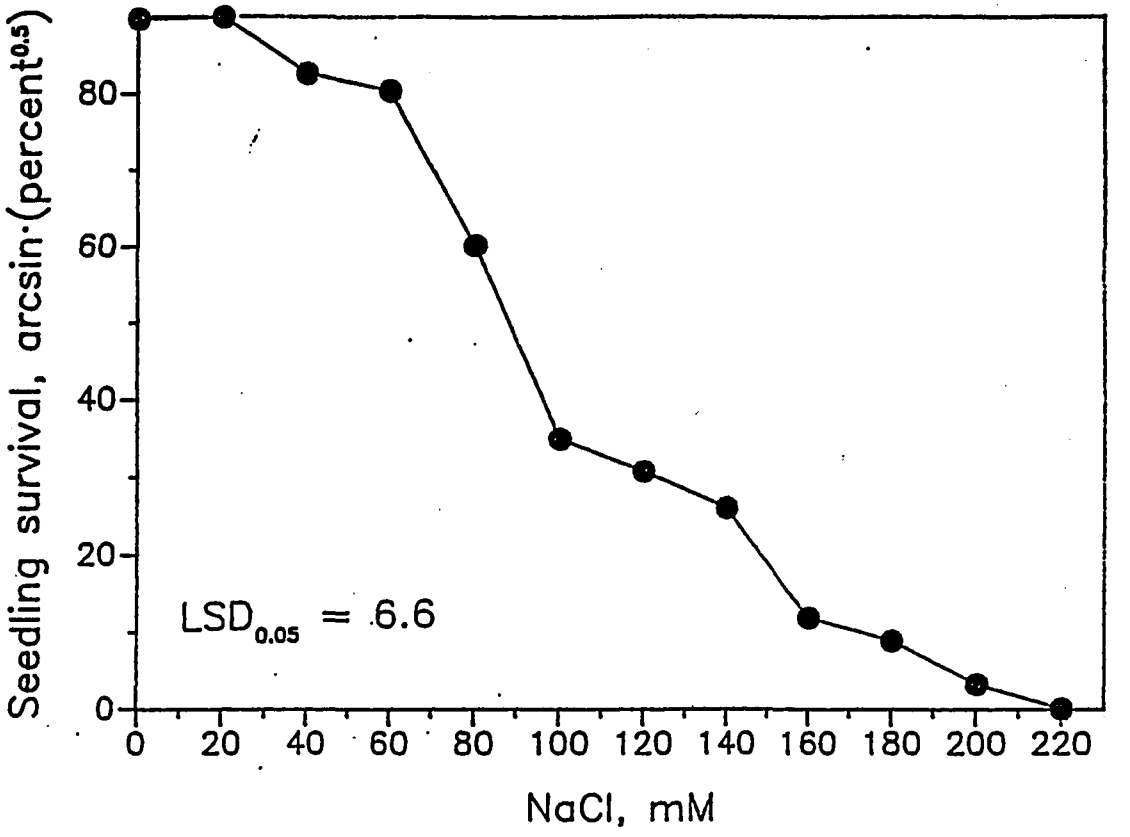

Figure 7. The inhibitory effect of NaCl to Arabidopsis seedling survival. Seedlings were established for 7 d at 23 ± 1C in continuous fluorescent light from seeds in petri dishes containing aseptic nutrient agar media. There were about 100 seeds in each dish. The concentration of NaCl was increased suddenly to the indicated levels 7 d after inhibition onset by adding a NaCl concentrate to the agar surface. The NaCl concentrations indicated are those expected at equilibrium following diffusion. Seedling survival was recorded 7 later. Each point represents the mean of two replications.

inhibitory to Arabidopsis seed germination than D-asparagine, the inhibitory effects on seed germination by 120 mM NaCl were reversed a similar degree by both compounds at similar concentrations (Fig. 9). Optimal reversal occurred from 2 mM to 4 mM for either L- or D-asparagine. Reversal of NaCl inhibition by either form of asparagine did not occur at concentrations of 6 mM or above. The effect of each form of asparagine, however, differed at concentrations above 4 mM. The addition of 6 mM or more of L-asparagine to 120 mM NaCl inhibited seed germination more than 120 mM NaCl alone. Seed germination was prevented at a concentration of 10 mM L-asparagine in the presence of 120 mM NaCl. In contrast, the level of inhibition of 120 mM NaCl was not increased by the addition of D-asparagine at concentrations up to 20 mM.

Inhibition of Arabidopsis Seed Germination and Seedling Survival by AAH. The inhibitory effect of AAH to Arabidopsis seed germination is shown in Figure 10. Only about half of the seeds germinated on media containing 0.9 mM AAH. Germination was inhibited further at greater concentrations of AAH in the medium. Germination was prevented at a concentration of 1.4 mM AAH. The symptoms of inhibition included a reduction in seedling size and a reduction in primary root formation. The inhibition of root growth was the most pronounced response. Some of the emerged seedlings lacked any visible radicle growth on media containing 1.2 mM AAH or above. Leaf chlorosis was another characteristic symptom of AAH inhibition of seedling development.

Similar to its effect on seed germination, AAH was potentially lethal to established seedlings (Fig. 11). About 50% of established 7-day-old seedlings were killed if the medium concentration was increased

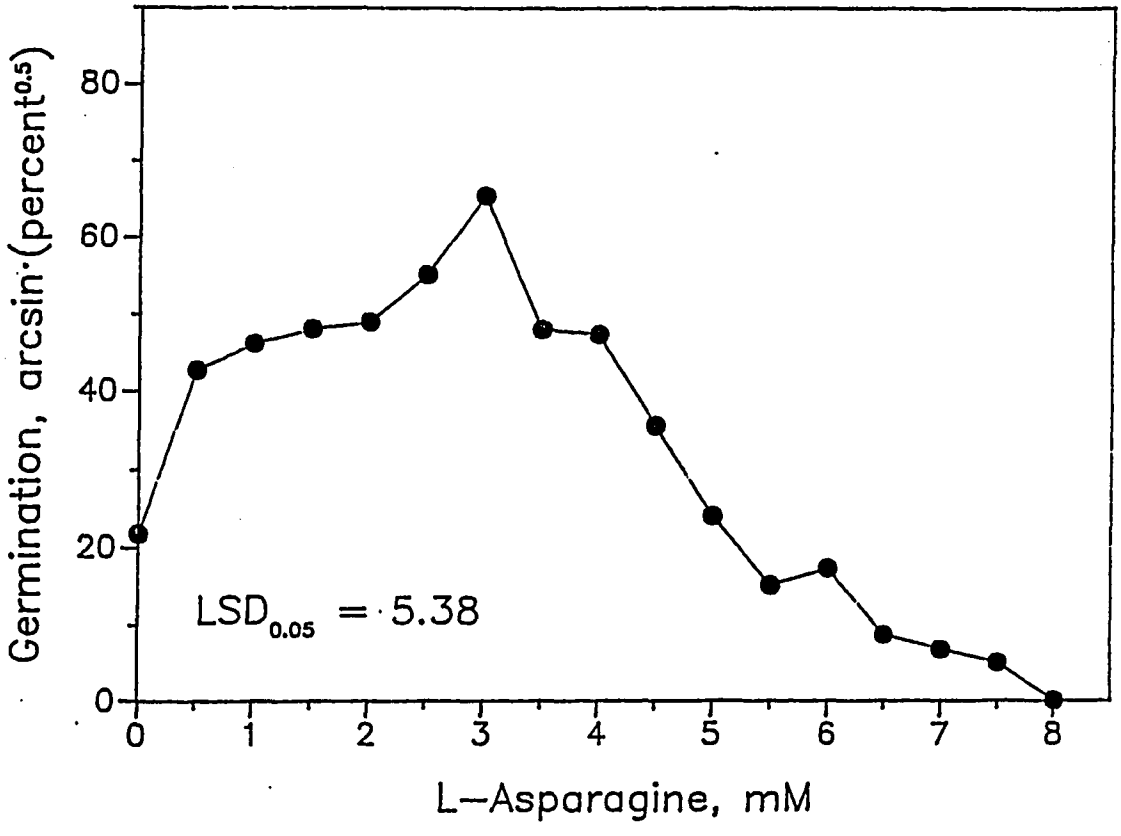

Figure 8. Reversal of the inhibitory effect of 120 mM NaCl to Arabidopsis seed germination by exogenous L-asparagine. About 200 seeds were sown on aseptic nutrient media containing 120 mM NaCl and the indicated range of L-asparagine concentrations. The number of germinated and ungerminated seeds were recorded after incubating dishes for 7 days of at 23 ± 1C in continuous fluorescent light. Each point represents the mean of two replications.

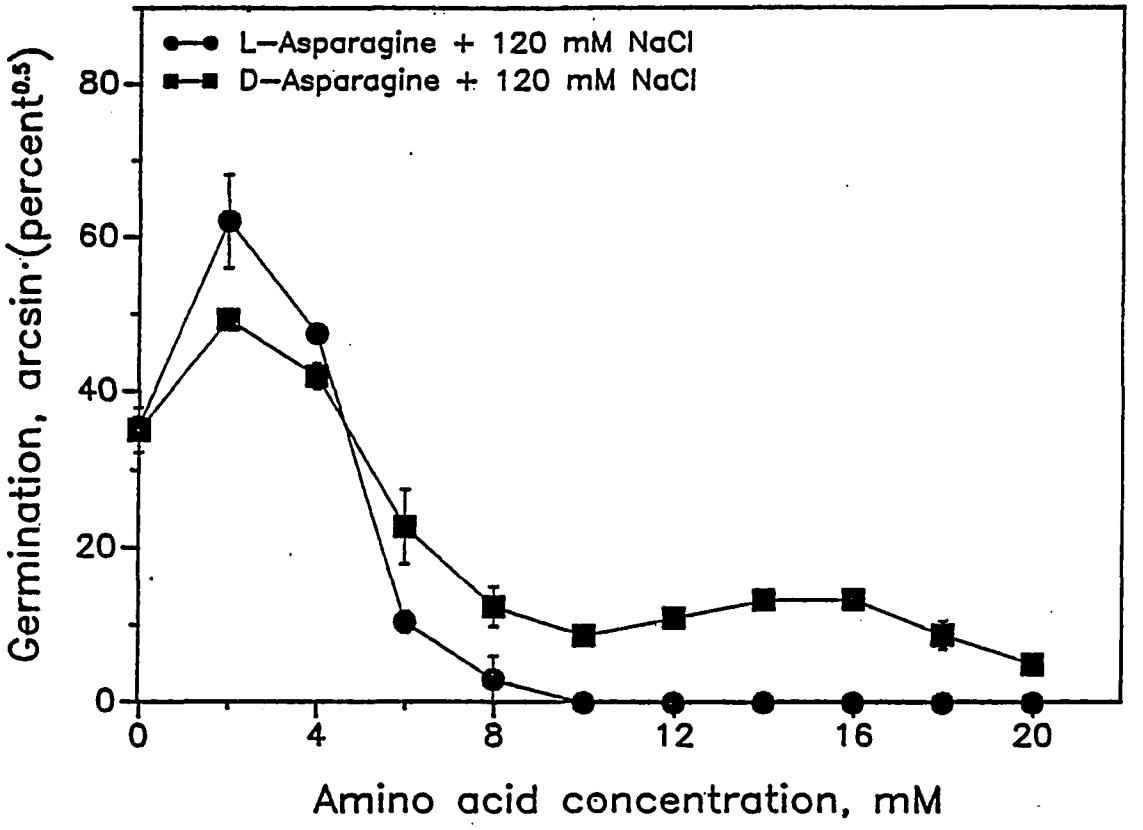

Figure 9. Reversal of the inhibitory effect of 120 mM NaCl to Arabidopsis seed germination by L- and D-asparagine. About 200 seeds were sown on aseptic nutrient agar medium containing 120 mM NaCl and the indicated range of L- or D-asparagine concentrations. The number of germinated and ungerminated seeds were recorded after incubating dishes 7 days at 23 ± 1C in continuous fluorescent light. Each point represents the mean of two replications.

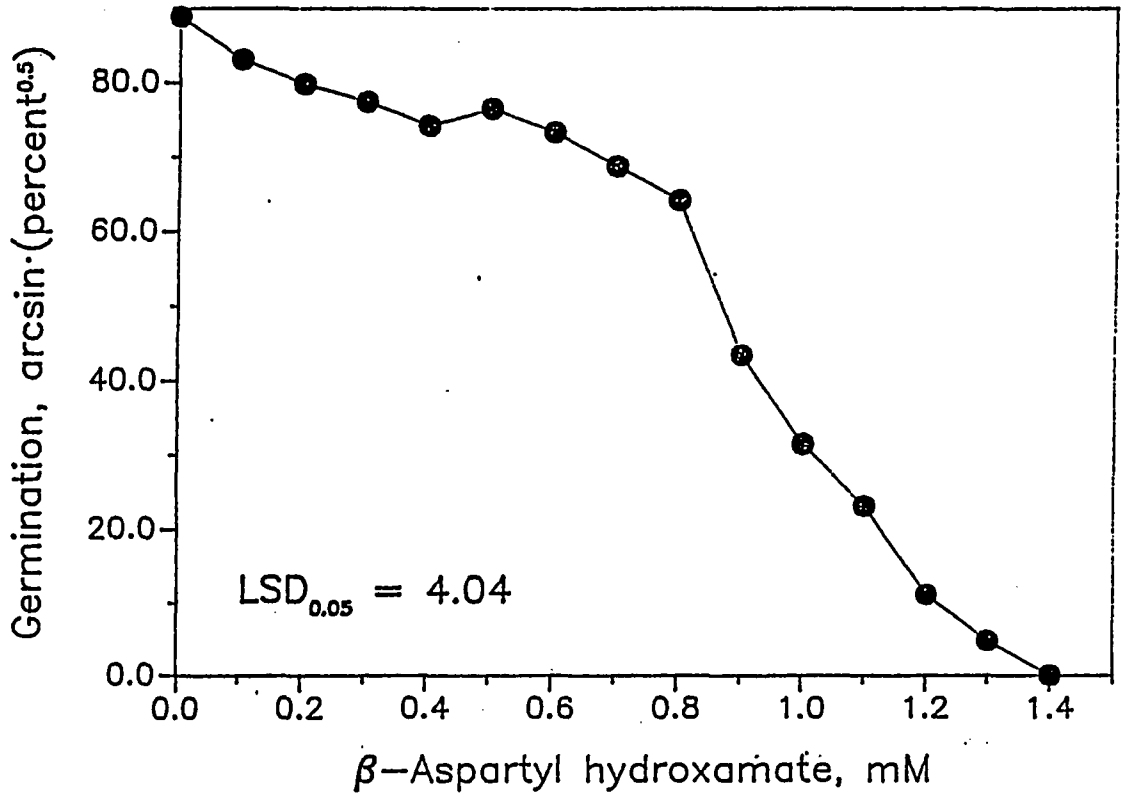

Figure 10. The inhibition of Arabidopsis seed germination by beta-aspartyl hydroxamate (AAH). The experimental methods were the same as in Figure 3.

suddenly from 0 to 2 mM AAH. All established seedlings were killed if the medium concentration was suddenly increased from 0 to 14 mM AAH. The results showed that Arabidopsis was much more tolerant of AAH at the seedling stage than at the germination stage of growth.

Leaf chlorosis increased in established seedlings receiving exogenous AAH. The extent of chlorosis appeared directly correlated to the level of added AAH. In contrast to its effects on inhibiting seedling growth during germination, no obvious reductions in seedling size were noted in established seedlings receiving AAH.

Reversal of the Inhibitory Effect of AAH to Arabidopsis Seed Germination by L-Asparagine. Exogenous L-asparagine reversed the inhibitory effects of 1.3 mM AAH on Arabidopsis seed germination (Fig. 12). The maximal reversal of inhibition by 1.3 mM AAH occurred at an L-asparagine concentration of 8 mM (Fig. 12). Higher concentrations of L-asparagine up to 20 mM were increasingly less effective in reversing the inhibition to seed germination caused by 1.3 mM AAH.

AAH appeared to reverse some of the toxicity of high concentrations of L-asparagine. Germination was typically prevented at a medium concentration of 20 mM L-asparagine (Fig. 3). Some germination occurred at this concentration of L-asparagine, however, if 1.3 mM AAH was also present in the medium (Fig. 12).

Inhibition of Arabidopsis Seed Germination and Seedling Survival by Albizziin. Albizziin was also inhibitory to Arabidopsis seed germination (Fig. 13). Germination percent showed a negative correlation with the medium concentration of albizziin. About half of the seeds germinated on media containing 1.0 mM albizziin. Germination

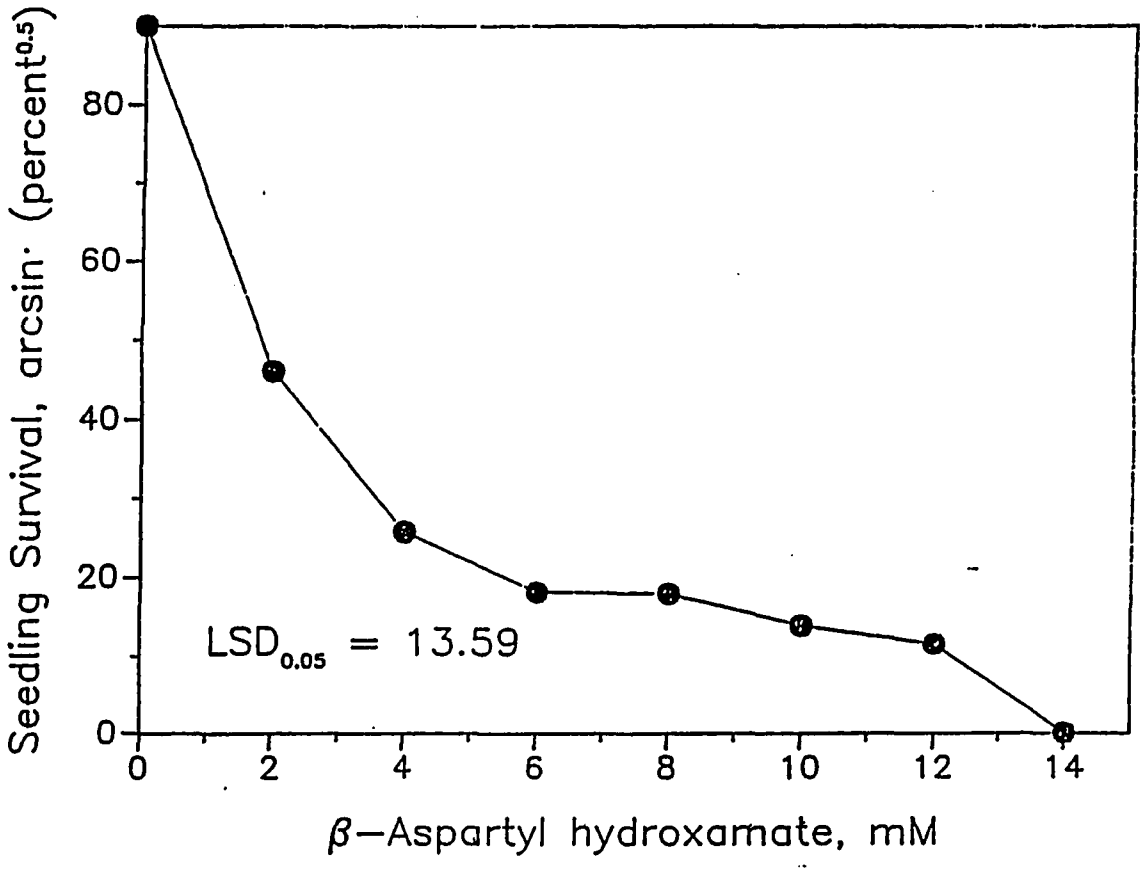

Figure 11. Inhibition of Arabidopsis seedling survival by beta-asparty1 hydroxamate. Methods were the same as in Figure 7.

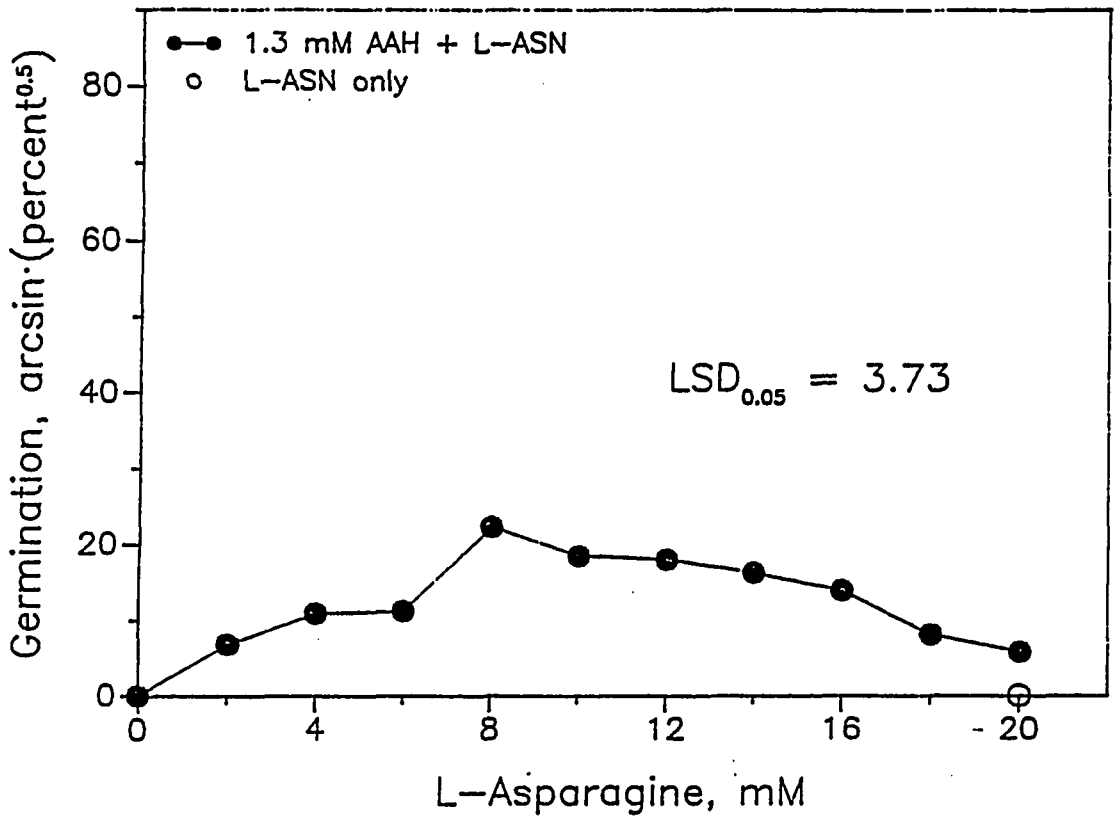

Figure 12. Reversal of the inhibitory effect of beta-aspartyl hydroxamate on Arabidopsis seed germination by L-asparagine. About 200 seeds were sown on aseptic nutrient agar media containing 1.3 mM AAH and the indicated range of L-asparagine concentrations. The numbers of germinated and ungerminated seeds were recorded after 7 d of incubating dishes at 23 ± 1C in continuous fluorescent light. Each point represents the mean of two replications.

was prevented at medium concentrations of 8.0 mM albizziin or above (Fig. 13). Leaf chlorosis was a characteristic symptom of albizziin inhitition of seedling growth. Chlorosis increased at albizziin concentrations of 0.2 mM or above in the medium. Other symptoms of albizziin inhibition included reductions in emerged seedling size and in primary root formation. While cotyledons expanded during germination, many inhibited seedlings were lacking significant radicle growth. The intensity of injury symptoms caused by albizziin was more pronounced than that expressed with AAH inhibition.

Albizziin was also toxic to established Arabidopsis seedlings (Fig. 14). About 50% of established 7-day-old seedlings were killed by a sudden increase in the albizziin concentration of medium from 0 to 4 mM. Seedling survival was reduced further as more albizziin was added to the medium. A sudden increase in the medium concentration of albizziin from 0 to 24 mM was lethal to all established seedlings (Fig. 14). A characteristic symptom of albizziin toxicity to established seedlings was leaf chlorosis. The size of established seedlings was not obviously inhibited by albizziin.

Reversal of Albizziin Inhibition of Arabidopsis Seed Germination by L-Asparagine. The addition of L-asparagine to the medium did not reverse the inhibition of Arabidopsis seed germination by 8 mM albizziin (Appendix I). Seeds responded, however, differently to the presence of different concentrations L-asparagine in the presence of 8 mM albizziin. Seeds started to germinate on media containing from 0.1 to 0.3 mM L-asparagine in addition to 8 mM albizziin. No evidence of seed germination occurred on media containing a combination of 8 mM

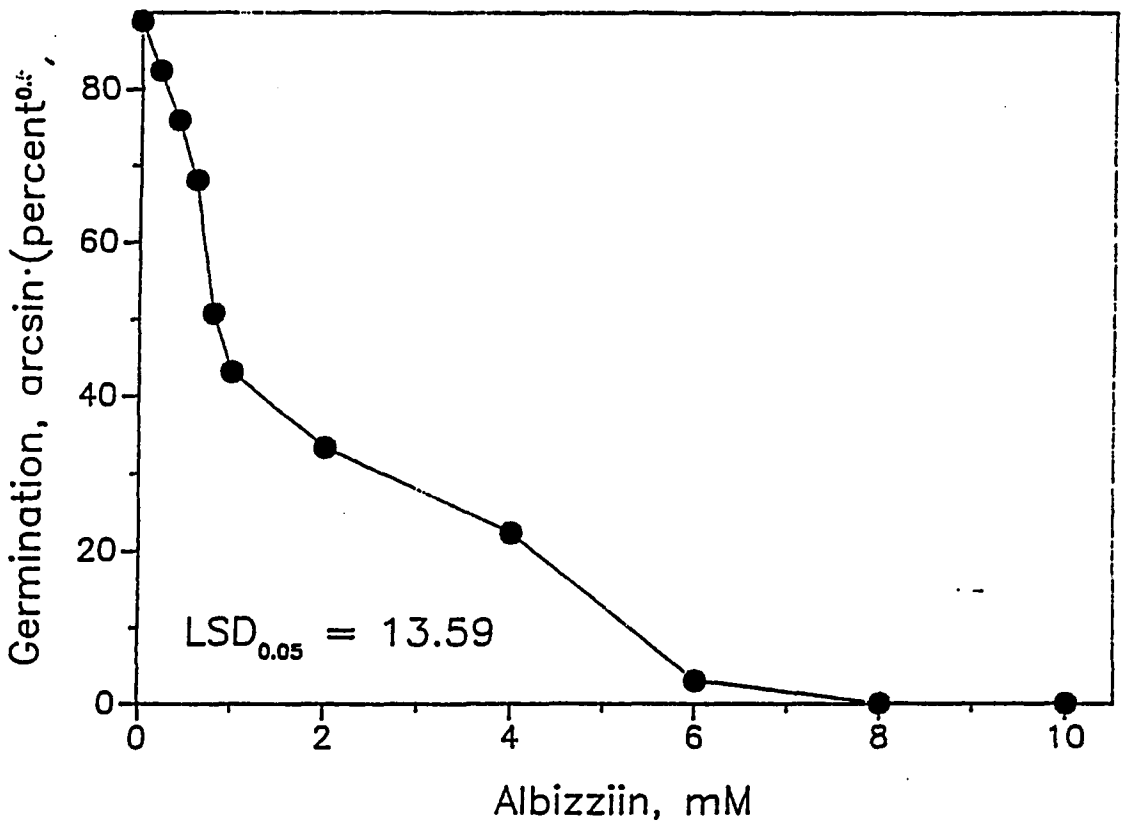

Figure 13. The inhibition of Arabidopsis seed germination by albizziin. Methods were the same as in Figure 3.

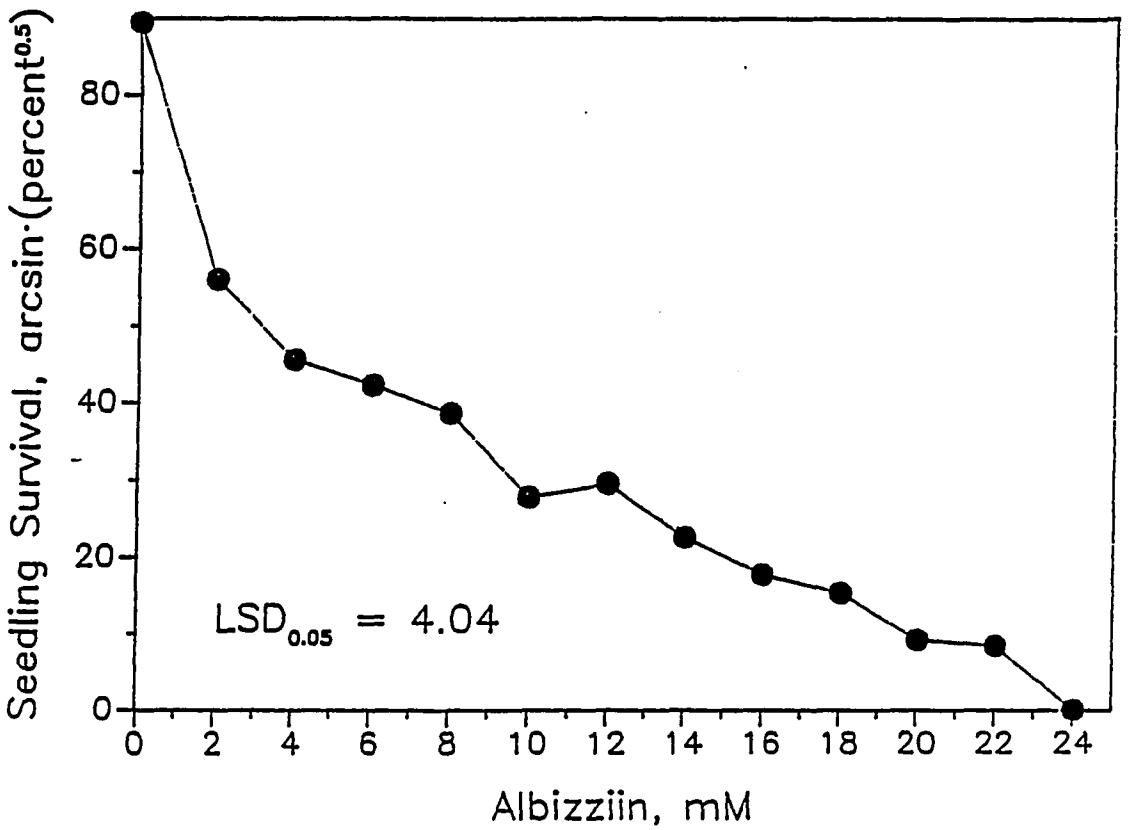

Figure 14. The toxicity of albizziin to Arabidopsis seedling survival. Methods were the same as Figure 7 except each dish was sown with only 100 seeds.

albizziin and concentrations of L-asparagine of 0.4 mM or above.

Selection of AAH Resistant Mutants. A total of 360,000 M_2 seeds representing 60,000 M_1 parents were selected on media containing 1.4 to 1.6 mM AAH. A total of 217 variants resistant to 1.4 to 1.6 mM AAH were rescued and survived to produce self-pollinated seeds (APPENDIX III). Nine of the 217 M_3 families (FC2, FC260, FC264, FC271, FC273, FC379, FC387, FC470, and FC552) retained resistance to 1.5 mM AAH upon reselection.

Selection for Albizziin Resistance Variants. A total of 152,000 M_2 seeds representing 25,000 M_1 parents were selected on a media containing 14 mM albizziin. Twenty-one resistant variants were rescued but none of the rescued variants survived to produce seeds.

Selection of NaCl Resistant Mutants. From a total of 360,000 M_2 seeds representing 61,000 M_1 parents, 380 variants were selected based on resistance to 180 to 250 mM NaCl (APPENDIX II). On control plates, seeds usually germinated within 72 hours after sowing, whereas, seeds sown on media containing 250 mM NaCl usually germinated after about 120 hours. All 97 M_3 families selected on 250 mM NaCl at the M_2 generation were reselected on 250 mM NaCl at the M_3 generation. Only one family (FC445) was still resistance to 250 mM NaCl.

DISCUSSION

Results of this thesis show that the primary effect of NaCl on Arabidopsis seed germination is one of inhibition. Although NaCl inhibition of later growth stages shows characteristic symptoms such as leaf chlorosis, I did not observe any symptoms characteristic of NaCl inhibition of seed germination other than a reduction in growth. Some variation in NaCl tolerance was observed in what was considered an essentially isogeneic strain of Arabidopsis. The basis of this variability in NaCl tolerance is not known. While Mozafar and Goodin (1986) found 0.02 and 0.04 mol L^{-1} of NaCl stimulated the germination of two wheat cultivars, I did not observe any stimulation of Arabidopsis seed germination by NaCl.

The effect of NaCl on established Arabidopsis seedlings was more complicated than its effect on seed germination. The size of seedlings growing on media containing from 20 to 40 mM NaCl was physically larger than control seedlings grown without NaCl. Thus, low levels of exogenous NaCl appear to stimulate the growth of established seedlings. At NaCl concentrations above 40 mM, seedling survival was reduced with further increases in the NaCl content of the medium.

The physiological basis of NaCl toxicity in Arabidopsis is unknown. Despite numerous studies concerning NaCl injury in plants, it is not known whether injury is caused by high Cl^- or high Na^+. Since both ions appear to be taken up in similar amounts by plants (Greenway et al., 1965), it is probable that both ions contribute to the toxicity of NaCl. In order to answer this question, measurements of ion uptake before and after injury would have to be made. Therios and Misopolinos (1988)

studied the response to NaCl of several genotypes of olive (Olea europaea) and found that, apart from various toxic symptoms, salinity significantly reduced water absorption. Although I did not measure the water absorption of Arabidopsis seedlings, I did observe in treatments receiving higher levels of NaCl, more water remained on the agar surface.

Even though the mechanism of NaCl injury in plants is unknown, progress will likely come from studies which focus on the inhibitory effects of Na^+ and Cl^- ions on metabolism and photosynthesis. There are many biological reactions occurring in vivo. Na^+ stimulates some of these reactions and inhibits others. Each reaction has its own optimum Na^+ concentration. Thus, excessive amounts of Na^+ and Cl^- can potentially interfere with many biological reactions.

Excessive salinity is inhibitory to many crop plants as a result of its adverse effects on photosynthesis. For example, some evidence shows that high levels of NaCl affect ribulose biosphosphate carboxylase activity (Cheeseman, 1988). Downton (1977) provided the evidence that 75 mM NaCl caused a 20 to 40% drop in the concentration of carbohydrate in grapevine leaves. This suggests that one aspect of NaCl inhibition in Arabidopsis is a reduction in photosynthesis. Such an inhibition of photosynthesis would mean that less fixed carbon would be available for growth and ultimately this would result in a reduction in seedling size.

It is not known with certainty how Na^+ and Cl^- ions exert their adverse effects in higher plants. Limited information exists concerning how these ions effect membrane permeability and the activities of different enzymes in salt stressed plant tissues (Greenway and Munns,

1980).

Salt tolerance may be defined generally as sustained growth of plants in an environment containing NaCl or combinations of mixed salts (Epstein et al., 1979). Salt tolerance is not to be confused with salt-avoidance mechanisms in which the plant life cycle is altered to limit exposure to salt. Salt-avoidance strategies include delayed germination or maturity until favorable conditions prevail. In practice, the terms salt tolerance or salt resistance have been used interchangeably to define true cytoplasmic resistance to salinity or in conjunction with salt avoidance, to describe all mechanisms that may give the plant a selective advantage during saline stress. Salt tolerance can be measured by a number of criteria. Survival at high salt concentrations has recently been the fundamental selection criterion for barley, wheat and tomato (Epstein et al., 1979). I used the same criterion to select salt resistant mutants in Arabidopsis.

The results suggest that salt tolerance in Arabidopsis can be increased by mutation. A number of variants were selected from the M_2 population that had substantially increased NaCl tolerance. Only in a few cases does the increased tolerance appear to be heritable. I have reselected 97 M_3 families and one family still showed resistance to 250 mM NaCl. This mutant should prove useful in understanding the mechanisms involved in regulating the natural levels of NaCl tolerance and altered asparagine synthetase in Arabidopsis.

In this thesis, determination of the toxicity of exogenous asparagine to Arabidopsis at various developmental stages was necessary before its effects on reversing NaCl, AAH, and albizziin could be

determined. The results show that exogenous L-asparagine was potentially inhibitory to seed germination. In contrast, the results indicated that sudden exogenous applications of L-asparagine were less toxic to established seedlings, even when the medium was saturated with L-asparagine. It cannot be determined on the basis of these experiments alone, why exogenous asparagine is inhibitory to seed germination but less inhibitory to established seedlings. Several explanations are possible.

One possible explanation of the inhibitory effect of asparagine to Arabidopsis seed germination is that the uptake of exogenous L-asparagine by seeds interferes with their normal nitrogen metabolism. Since exogenously supplied L-asparagine can inhibit Arabidopsis seed germination, this suggests Arabidopsis seeds directly uptake asparagine from the medium. As Arabidopsis seeds germinate, they rely on the mobilization of storage protein as their principle source of nitrogen. The excessive uptake of exogenous asparagine during germination would presumably accumulate in the cell. Since aspartate and ammonia are the products of L-asparagine, excessive accumulation of toxic ammonia may accompany excessive L-asparagine uptake. In this regard my results are consistent with previous findings in plant (Magalhaes and Wilcox, 1984), fungus (Kostintsyn and Popov, 1982), and animal cells (Guerrini, 1988). Considerable progress in elucidating the mechanism of toxicity of L-asparagine to seed germination would most likely result from a study of L-asparagine uptake in Arabidopsis seeds and seedlings.

Other explanations are also possible to explain why established seedlings have a high degree of tolerance to exogenous L-asparagine.

First, there is a physical difference in size between the developmental stages. Seedlings are considerably larger than seeds and their larger body mass may account for their higher level of tolerance. An average 7-day-old seedling is about 5- to 10-fold more massive than that of a seed. Such a difference in size is sufficient in itself to account for the difference in tolerance between the two stages of growth. An alternative explanation would be that established seedlings have a greater ability than seed tissue to assimilate and dispose of excessive levels of L-asparagine than do seeds. The larger vegetative mass of seedlings suggests that more L-asparagine could be utilized for growth and development. Saarelainen and Mikola (1987) separately injected eight ^{14}C-labelled amino acids (asparagine was one of them) into the endosperm of germinating barley. They found that L-asparagine was rapidly transported in an intact form through the scutellum to the embryo axis. Uptake experiments are needed in Arabidopsis to determine the rate of L-asparagine assimilation in seedling versus seed tissue.

Results clearly indicate that D-asparagine is less inhibitory to Arabidopsis seed germination than is L-asparagine. The difference in toxicity between the two forms of asparagine can be explained in several ways. First, there may be differences in the uptake of both forms. Some preferential uptake of L-asparagine would be expected since it is the natural form. D-asparagine is not typically present in plants. The fact that, at sufficiently high concentrations, both forms were toxic to seed germination, suggests that there was uptake of both forms by the seeds. So the question remains-Do Arabidopsis seeds uptake L- and D-asparagine differently? Radiotracer studies using labeled L- and D-

asparagine should be able to answer this question.

A second explanation for the difference in the toxicity between the L- and D-form of asparagine is that the toxicity of L-asparagine is related to its metabolism following uptake. This explanation would be independent of differences in the uptake of both forms which could be the same. Rather, if toxicity required recognition or metabolism of the natural form, it is easy to see that D-asparagine, not normally present, would be less toxic either due to a lack of recognition or metabolism specific for the L-form. D-Asparagine is not involved in protein synthesis, so how seeds would utilize or store D-asparagine is not clear.

Results clearly show that exogenous L-asparagine can reverse the inhibition caused by NaCl. It was shown that the reversal was concentration dependent and was maximal at 3 mM L-asparagine. These results imply that exogenous L-asparagine can reduce the inhibitory effects of NaCl in vivo. The mechanism of reversal of NaCl inhibition by L-asparagine is unknown. Several mechanisms are possible. First, external asparagine may reduce the uptake of NaCl from the medium either by seeds or by seedlings. Secondly, it is possible that L-asparagine, accumulated intracellularly, forms a intercellular complex with Na^+ or Cl^- thereby decreasing the inhibitory effect of NaCl. Such a explanation is supported by the observation that Cu^+ binds L-asparagine and histidine in soybean (White et al., 1981). A similar binding of Na^+ to L-asparagine may occur in Arabidopsis. How this would reduce the toxicity of NaCl is unclear.

The results showed that D-asparagine was as effective as L-

asparagine in reversing NaCl inhibition of seed germination. Both forms of asparagine had essentially identical effectiveness at the same concentration. While compositionally similar, the D-form is an optical isomer of the L-form. All protein amino acids having one or more chiral centers are of the L-configuration. Amino acids of the L-configuration are the predominant forms found in living organisms, but amino acids of the D-configuration are found occasionally in higher plants. Most enzymes catalyzing amino acid transformations, however, exhibit a high degree of specificity for the L-form (Larsen, 1980). Thus, the fact that D-asparagine was as equally effective as L-asparagine in reversing NaCl inhibition suggests that the reversal response does not require recognition or metabolism of the natural L-configuration. Obviously, more study is needed to elucidate the mechanism of L- and D-asparagine reversal of NaCl inhibition in Arabidopsis.

The experiments showed that NaCl was inhibitory to both Arabidopsis seed germination and seedling survival. The results also showed that although L- and D-asparagine were inhibitory to Arabidopsis seed germination, both were capable of reversing the inhibitory effect of NaCl to Arabidopsis seed germination. Results also suggest that salt tolerance in Arabidopsis can be increased by mutation. These facts suggest that Arabidopsis is the suitable organism to study salt tolerance and possibly the adaptive significance of asparagine accumulation during salt stress in higher plants.

AAH is an analog of aspartic acid, which inhibits the activity of both asparagine synthetase and asparagine-tRNA synthetase in vitro (Andrulis, 1979). Analogs such as AAH, which physically resemble a

natural compound with which they complete, are frequently toxic to biological systems. Cartier et al. (1987) found that asparagine synthetase from both animal and bacterial sources was inhibited by AAH. AAH has been shown to inhibit asparagine synthetase *in vitro* and *in vivo* (Andrulis, 1982). The results of this thesis are consistent with these previous findings.

My experiments showed that AAH was inhibitory to both *Arabidopsis* seeds and seedlings which suggests that AAH was both taken up from the medium by both seeds and seedlings and competed with aspartic acid for the catalytic site of asparagine synthetase. *Arabidopsis* seeds were relatively tolerant of 0.3 mM AAH for 7 days which implys that this level of AAH does not inhibit all asparagine synthetase activities *in vivo*. In sufficient concentrations of AAH, it is quite possible that, *in vivo* asparagine synthetase molecules are prevented from binding with their normal substrate, aspartic acid. The level of AAH required to accomplish this is unknown. Regardless, the consequence of such inhibition would be to prevent asparagine biosynthesis by asparagine synthetase. Seeds and seedlings starved of asparagine would rapidly lose their ability to maintain their normal metabolic activities. Prolonged inhibition of asparagine biosynthesis would lead to eventual tissue death.

Since AAH inhibits asparagine synthetase and *Arabidopsis* seeds and seedlings appear to uptake both AAH and asparagine from the medium, I hypothesized that exogenously supplied L-asparagine would reverse the inhibitory effects of AAH in *Arabidopsis*. Results showed that L-asparagine was capable of reversing the inhibitory effects of AAH. The

extent of reversal was not dramatic, however, suggesting that AAH may be inhibiting more than asparagine biosynthesis. It is interesting that the reverseal of AAH inhibition was maximal over a range of L-asparagine concentrations, that alone, are very inhibitory to germination in Arabidopsis. One interpretation of this result is that AAH, in an unknown manner, reverses some of the toxic effects of L-asparagine.

L-Asparagine has been shown to reverse AAH inhibition of CHO cells. Gantt et al. (1980) found that the inhibition of asparagine-tRNA formation by AAH was competitive with L-asparagine. The same phenomena was observed in Arabidopsis seed germination. The ability of L-asparagine to relieve AAH inhibition of seed germination implys that exogenous AAH inhibits asparagine synthetase in Arabidopsis. These results suggest that AAH could used to isolate mutants in Arabidopsis which had altered regulation of asparagine biosynthesis. Since the analog is a competitive inhibitor of asparagine synthetase and asparaginyl-tRNA synthetase, a mutation resulting in an increase in the intracellular concentration of asparagine would presumably increase AAH tolerance in Arabidopsis seeds. If correct, then AAH could be used in a positive selection scheme to obtain asparagine overproducing mutants in Arabidopsis.

Results showed that albizziin is inhibitory to Arabidopsis seed germination and at sufficient concentrations can be lethal to established seedlings. As an analog of glutamine, albizziin can potentially inhibit the biosynthesis of asparagine by competing for the glutamine binding site of asparagine synthetase (Pinkus, 1977; Schnoeder et al., 1969). Thus, one cause of its toxicity is similar to that of

AAH, i.e. interference with asparagine biosynthesis.

Besides serving as substrate for asparagine biosynthesis, glutamine is involved in at least five other major metabolic pathways. As an analog of glutamine, the toxicity of albizziin can not be assumed to be limited to inhibition of asparagine biosynthesis. Kubik (1987) found that 25 mM albizziin reduced the content of glutamine in the incubation mixture of Pisum arvense roots. This suggested that albizziin inhibited glutamine biosynthesis as well as metabolic pathways requiring glutamine. Andrulis (1985) showed that albizziin inhibits both glutamine synthetase and glutamate synthetase. Albizziin almost completely blocked the incooperation of ^{15}N-labeled amino acids into protein which caused an excessive NH_4^+ accumulation in the ectomyrrhizal roots of Zea mays (Oaks and Ross, 1984). Presumably albizziin has similar metabolic activity in Arabidopsis.

Since albizziin is a analog of glutamine and glutamine is a substrate of asparagine synthetase, asparagine could potentially reverse the inhibitory effects of albizziin in vivo. To test this hypothesis in Arabidopsis, the effectiveness of L-asparagine in reversing the inhibition of seed germination by 8 mM albizziin was assessed. Results showed that L-asparagine was unable to significantly reverse the inhibitory effects of albizziin on seed germination in Arabidopsis. However, relative to the control, some seed germination was evident on media containing from 0.1 to 0.3 mM L-asparagine. One interpretation of this result is that 8 mM albizziin was much too inhibitory a concentration to be reversed by L-asparagine alone. This is because at this concentration, albizziin would presumably be

inhibitory to several metabolic pathways in addition to asparagine biosynthesis.

Andrulis (Andrulis et al., 1979; Andrulis, 1985) used 0.1 mM of AAH to select AAH resistant mutants and 2 mM of albizziin to select albizziin resistant mutants in CHO cells. In these studies, the concentration difference between AAH and albizziin was about 20-fold. I used 1.4 to 1.6 mM of AAH to select AAH resistant mutants and 14 mM of albizziin to select albizziin resistant variants. The concentration difference between AAH and albizziin in my selections was about 9- to 10-fold. This is consistent with evidence that the effects of these analogs in Arabidopsis is similar to the more thoroughly characterized CHO. My results suggest that the two analogs have different effects on cellular metabolism even though both of them are competitive inhibitors of the substrates of asparagine synthetase. The explanation of this difference appears to lie in the greater range of metabolic pathways that glutamine has involvement in and thus its analog albizziin would be toxic to.

As neither AAH nor albizziin had been used previously to isolate mutants in Arabidopsis, the appropriate selection conditions were determined by germinating seeds in various concentrations of the analogs. About 98% of the seeds did not germinate on media containing either 1.4 mM AAH or 14 mM albizziin. Selections for variants from an M_2 population were conducted at these concentrations of AAH and albizziin.

From a total of 360,000 M_2 seeds selected, 217 variants resistant to AAH were rescued and survived to produce self-pollinated progeny. A

major problem during selection was that AAH strongly inhibited root formation making rescue difficult. Thus, many potentially valuable variants died before they matured to produce seeds. Of the reselected M_3 families, nine families still showed AAH resistance. The inheritance of AAH resistance remains to be determined for the isolated mutants. Complementation analysis also needs to be done to determine the number of genes and alleles involved in AAH resistance in the isolated mutants. It would also be of interest to determine if any of the AAH resistant mutants accumulate intercellular asparagine. Such characterization was, however, beyond the scope of this thesis.

From a total of 152,000 M_2 seeds selected, 21 variants resistant to 14 mM albizziin were rescued. Similar to selection on AAH, root formation in albizziin-inhibited plants was severely inhibited. Some plants had no primary root at all. As a result, none of my rescued variants survived to produce seeds. As glutamine is involved in five metabolic pathways besides asparagine synthesis (Fig. 2), more variants would likely be recovered if the medium was supplemented with the primary products of these other inhibited pathways. Potential supplements would include arginine, ureides, tryptophan, histidine and nucleotides. Of these, nucleotides appear to be the most important and were used by Andrulis (1985) in her selections in CHO.

The inhibitory effect of AAH to wild Arabidopsis seed germination showed that germination was prevented at a medium concentration of 1.4 mM AAH. My selection experiments showed that the mutants germinated on medium containing from 1.4 to 1.6 mM AAH. Also, wild type seeds were prevented from germinating by 8 mM albizziin, yet some variants

temporarily tolerated up to 14 mM albizziin. Obviously, these mutants and variants were more resistant to these two analogs than the Columbia wild type. The extent of the increase in tolerance remains to be determined. Since AAH and albizziin inhibit asparagine biosynthesis, one possible mechanism of resistance to these inhibitors would be mutants which overproduce asparagine. Resistance could also be explained by reduced uptake of the analogs. The exact basis of the AAH resistance will require further study. Combined biochemical and genetic studies and a better understanding of the mechanisms of NaCl, AAH, and albizziin resistance are essential for further progress.

SUMMARY

My experiments show that NaCl is inhibitory to both Arabidopsis seed germination and seedling survival. Seed germination was completely prevented at a medium concentration of 200 mM NaCl or above. Established seedlings were killed if the medium concentration of NaCl was suddenly increased from 0 to 220 mM NaCl. The effects of NaCl on estabilished Arabidopsis seedlings were more complex than its effects on seed germination. Lower levels (20 to 40 mM) of NaCl stimulate the growth of established seedlings. Higher levels (60 to 220 mM) of NaCl inhibit the growth of established seedlings.

From a total of 360,000 M_2 seeds, 380 variants were selected based on resistance to 180 to 250 mM NaCl. Of 97 M_3 families reselected, one family still showed resistance to 250 mM NaCl. This suggests that salt tolerance in Arabidopsis can be increased by mutation. Increased tolerance was infrequently heritable; only one of 97 M_3 families retained increased NaCl tolerance.

The results show that exogenous L- and D-asparagine were inhibitory to Arabidopsis seed germination. Seed germination was completely prevented at 20 mM L-asparagine. In contrast, seed germination was completely prevented at 60 mM D-asparagine. Dispite differing toxicities, L- and D-asparagine reverse the inhibitory effect of 120 mM NaCl to Arabidopsis seed germination at the same concentrations. The maximal reversal effect occurred at 3 mM L- and D-asparagine. The fact that D-asparagine was an equally effective as L-asparagine in reversing 120 mM NaCl inhibition suggests that the reversal response does not require recognition or metabolism of the natural L-configuration.

Estabiilished seedlings were more tolerant to L-asparagine than were seeds. Only about 20% of established seedlings were killed by saturating the medium with L-asparagine (i.e. 150 mM). These results suggest that Arabidopsis is the suitable organism to study salt tolerance and the adaptive significance of asparagine accumulation during salt stress in higher plants.

Asparagine substrate analogs, AAH and albizziin, are inhibitory to both Arabidopsis seed germination and seedling survival. Arabidopsis seed germination was completely prevented at 1.4 mm AAH. Additional inhibitory effects of AAH to Arabidopsis include inhibition of radicle formation and reduction of seedling size. The inhibition of root formation was the most pronounced response. Established seedlings were killed at a medium concentration of 14 mM AAH. An additional inhibitory effect of AAH to established seedlings included leaf chlorosis. No obvious reduction in seedling size was noted in established seedlings. L-asparagine was capable of reversing the inhibitory effects of AAH. The maximal reversal effect occurred at 6 mM L-asparagine. From a total of 360,000 M_2 seeds selected on media containing 1.4 to 1.6 mM AAH, a total of 217 variants were rescued and survived to produce self-pollinated seeds. Nine of the 217 M_3 families retained resistance to 1.5 mM AAH upon reselection.

Albizziin was inhibitory to Arabidopsis seed germination and seedling survival. Seed germination was completely prevented at a medium concentration of 8 mM albizziin. Additional inhibitory effects of albizziin to Arabidopsis included severe inhibition of root formation, leaf chlorosis, and reduction of seedling size. The

intensity of injury symptoms caused by albizziin was more pronounced than that expressed with AAH inhibition. Exogenous L-asparagine did not significantly reverse the inhibitory effect of 8 mM albizziin. From a total of 152,000 M_2 seeds selected on a medium containing 14 mM albizziin, 21 resistant variants were rescued but none survived to produce seeds. Established seedlings were killed by a sudden increase in the medium concentration of albizziin from 0 to 24 mM. An additional inhibitory effect of albizziin to established seedlings was leaf chlorosis.

By establishing that AAH is toxic to Arabidopsis and by isolating mutants that are resistant to AAH, I have demonstrated the feasibility of using this asparagine substrate analog to attempt the isolation of mutants which have altered regulation of asparagine biosynthesis. The molecular basis and inheritance of all mutations, however, were not determined. Such mutants may be helpful in understanding both NaCl tolerance and the regulation of stress-induced asparagine accumulation in higher plants.

APPENDIX I. Analysis of Variance for Different Experiments.

Table 1. ANOVA for the inhibition of Arabidopsis seed germination by L-asparagine.

	SS	df	MS	F	P
Main Effect					
Asparagine	31515.15052	12	2626.262543	245.89744321	.0000 ***
Error	138.8441158	13	10.6803166		
Total	31653.99464	25			

Student-Newman-Keuls Test
Error mean square = 10.6803166
Degrees of freedom = 13
Significance level = .05
LSD .05 = 7.060247589

Rank	Treatment #	Mean	n	Non-Significant ranges
1	11	0	2	a
2	12	0	2	a
3	13	0	2	a
4	10	2.058721375	2	a
5	9	3.934500899	2	a
6	8	7.652076384	2	ab
7	7	14.476674790	2	bc
8	6	17.771419680	2	c
9	5	50.034245070	2	d
10	4	64.340731970	2	e
11	3	82.278085970	2	f
12	2	83.757872090	2	f
13	1	89.629525350	2	f

Table 2. ANOVA for the inhibition of Arabidopsis seed germination by D-asparagine.

Source	SS	df	MS	F	P
Main Effect					
D-Asparagine	31057.10129	12	2588.091774	1111.503956	.0000 ***
Error	30.26997149	13	2.328459345		
Total	31087.37126	25			

Student-Newman-Keuls Test
Error mean square = 2.328459345
Degrees of freedom = 13
Significance level = .05
LSD .05 = 3.296569207

Rank	Treatment #	Mean	n	Non-Significant ranges
1	13	0	2	a
2	12	5.160050831	2	b
3	11	8.426883720	2	bc
4	10	11.492307870	2	c
5	9	16.561572670	2	d
6	8	29.146782230	2	e
7	7	35.534334990	2	f
8	6	49.081880370	2	g
9	5	59.687973810	2	h
10	4	89.949329510	2	i
11	3	89.949329510	2	i
12	2	89.949329510	2	i
13	1	89.949329510	2	i

Table 3. ANOVA for factorial experiment of D-asparagine vs L-asparagine effects on Arabidopsis seed germination.

Source	SS	df	MS	F	P
Main Effects					
Concentration	13312.6861	5	2662.537221	2978.180298	.0000 ***
Asparagine	12894.71374	1	12894.71374	14423.37861	.0000 ***
Interaction					
Conc X Asn	4283.32882	5	856.6657639	958.2232617	.0000 ***
Error	10.72817743	12	0.8940147857		
Total	30501.45684	23			

Student-Newman-Keuls Test
Factor: concentration
Error mean square = 0.8940147857
Degrees of freedom = 12
Significance level = .05
LSD .05 = 1.456724005

Rank	Treatment #	Mean	n	Non-Significant ranges
1	6	24.54094018	4	a
2	5	29.84398690	4	b
3	4	52.23603785	4	c
4	3	54.71636928	4	d
5	2	78.20473556	4	e
6	1	89.94932951	4	f

Student-Newman-Keuls Test
Factor: asparagine
Error mean square =0.8940147857
Degrees of freedom = 12
Significance level = .05
LSD .05 = 0.8410399962

Rank	Treatment #	Mean	n	Non-Significant ranges
1	1	31.73593773	12	a
2	2	78.09452870	12	b

Table 4. ANOVA for the inhibition for Arabidopsis seed germination by NaCl.

Source	SS	df	MS	F	P
Main Effect					
NaCl	25753.67268	10	2575.367268	4945.439703	.0000 ***
Error	5.728315711	11			
Total	25759.40100	21			

Student-Newman-Keuls Test
Error mean square = 0.5207559737
Degrees of freedom = 11
Significance level = .05
LSD .05 = 1.588306249

Rank	Treatment #	Mean	n	Non-Significant ranges
1	10	0.0	2	a
2	11	0.0	2	a
3	9	8.494451649	2	b
4	8	9.469090704	2	b
5	7	21.227301780	2	c
6	6	50.422111850	2	d
7	5	69.132456220	2	e
8	4	71.852564950	2	f
9	3	74.817810070	2	g
10	2	83.021885220	2	h
11	1	89.988509600	2	i

Table 5. ANOVA for the inhibition of *Arabidopsis* seedling survival by NaCl.

Source	SS	df	MS	F	P
Main Effect					
NaCl	27374.28847	11	2488.571679	268.8993768	.0000 ***
Error	111.0558920	12	9.254657666		
Total	27485.34436	23			

Student-Newman-Keuls Test
Error mean square = 9.254657666
Degrees of freedom = 12
Significance level = .05
LSD .05 = 6.628268661

Rank	Treatment #	Mean	n	Non-Significant ranges
1	12	0.0	2	a
2	11	3.209878462	2	ab
3	10	8.843570652	2	bc
4	9	11.834783000	2	c
5	8	26.033707070	2	d
6	7	30.770364820	2	de
7	6	35.014600020	2	e
8	5	60.123723420	2	f
9	4	80.325484420	2	g
10	3	82.532365650	2	gh
11	1	89.629525350	2	h
12	2	89.825430720	2	h

Table 6. ANOVA for reversal 120 mM NaCl inhibition of Arabidopsis seed germination by L-asparagine.

Source	SS	df	MS	F	P
Main Effect					
Asparagine	13022.66625	16	813.9166409	125.0582386	.0000 ***
Error	110.6411145	17	6.508300851		
Total	13133.30737	33			

Student-Newman_Keuls Test
Error mean square = 6.508300851
Degrees of freedom = 17
Significance level = .05
LSD .05 = 5.382428939

Rank	Treatment #	Mean	n	Non-significant ranges
1	17	0.00	2	a
2	16	4.987568318	2	ab
3	15	6.762568258	2	b
4	14	8.676553086	2	b
5	12	15.233410530	2	c
6	13	17.362592480	2	c
7	11	24.138212520	2	d
8	10	35.657473700	2	e
9	1	21.721444450	2	e
10	2	42.659715060	2	f
11	3	46.087056940	2	f
12	9	47.330346070	2	f
13	8	47.980579780	2	fg
14	4	48.032683530	2	fg
15	5	48.943277570	2	fg
16	6	55.105484010	2	g
17	7	65.377294050	2	h

Table 7. ANOVA for reversal of 120 mM NaCl *Arabidopsis* seed germination by L- and D-asparagine.

Source	SS	df	MS	F	P
Main Effects					
Asparagine	364.9349153	1	364.9349153	40.43061947	.0000 ***
Concentration	14306.34221	10	1430.634221	158.4979276	.0000 ***
Interaction					
Asn X Conc	726.9984041	10	72.69984041	8.054311768	.0000 ***
Error	198.5764315	22	9.026201431		
Total	15596.85196	43			

Student-Newman-Keuls Test
Factor: asparagine
Error mean square = 9.026201431
Degrees of freedom = 22
Significance level = .05
LSD .05 = 1.878617397

Rank	Treatment #	Mean	n	Non-Significant ranges
1	1	14.44891378	22	a
2	2	20.20876642	22	b

Student-Newman-Keuls Test
Factor: concentration
Error mean square = 9.026201431
Degrees of freedom = 22
Significance level = .05
LSD .05 = 4.405748322

Rank	Treatment #	Mean	n	Non-Significant ranges
1	11	2.503611879	4	a
2	6	4.348200349	4	a
3	10	4.399260942	4	a
4	7	5.510000115	4	a
5	8	6.676534511	4	a
6	9	6.704885198	4	a
7	5	7.767933283	4	a
8	4	16.66881257	4	b
9	1	35.39090013	4	b
10	3	44.85932199	4	c
11	2	55.78778058	4	d

Table 8. ANOVA for the inhibition of *Arabidopsis* seed germination by beta-aspartyl hydroxamate.

Source	SS	df	MS	F	P
Main Effect					
Beta-AAH	27226.15706	14	1944.725504	542.2172899	.0000 ***
Error	53.79924822	15	3.586616548		
Total	27279.95631	29			

Student-Newman-Keuls Test
Error mean square = 3.586616548
Degrees of freedom = 15
Significance level = .05
LSD .05 = 4.036616868

Rank	Treatment #	Mean	n	Non-Significant ranges
1	15	0.0	2	a
2	14	4.694561042	2	b
3	13	11.033319800	2	c
4	12	23.138929510	2	d
5	11	31.477421530	2	e
6	10	43.431098610	2	f
7	9	64.098982100	2	g
8	8	68.711344430	2	h
9	7	73.239166870	2	i
10	5	74.116162940	2	i
11	6	76.377572590	2	ij
12	4	77.322474580	2	ij
13	3	79.754209050	2	jk
14	2	83.034513920	2	k
15	1	88.813981130	2	l

Table 9. ANOVA for the inhibition of Arabidopsis seedling survival by beta-aspartyl hydroxamate.

Source	SS	df	MS	F	P
Main Effect					
Beta-AAH	11231.92253	7	1604.560362	46.19827875	.0000 ***
Error	277.8563021	8	34.73203776		
Total	11509.77883	15			

Student-Newman-Keuls Test
Error mean square = 34.73203776
Degrees of freedom = 8
Significance level = .05
LSD .05 = 13.59018016

Rank	Treatment #	Mean	n	Non-Significant ranges
1	8	0.0	2	a
2	7	11.57897845	2	ab
3	6	13.92296721	2	ab
4	4	17.92706111	2	ab
5	5	18.12830216	2	ab
6	3	25.75375838	2	b
7	2	45.95953743	2	c
8	1	89.94932951	2	d

Table 10. ANOVA for asparagine reversal of inhibition of beta-aspartyl hydroxamate to *Arabidopsis* seed germination.

Source	SS	df	MS	F	P
Main Effect					
Asparagine	1124.090951	11	102.1900865	34.87824061	.0000
Error	35.15891330	12	2.929909442		
Total	1159.249865	23			

Student-Newman-Keuls Test
Error mean square = 2.929909442
Degrees of freedom = 12
Significance level = .05
LSD .05 = 3.72946918

Rank	Treatment #	Mean	n	Non-Significant ranges
1	1	0.0	2	a
2	12	0.0	2	a
3	11	5.754264241	2	b
4	2	6.771961930	2	b
5	10	8.060746523	2	b
6	3	10.851432950	2	b
7	4	11.172834070	2	bc
8	9	13.910330000	2	cd
9	8	16.230016780	2	d
10	7	17.936378510	2	de
11	6	18.429032860	2	de
12	5	22.263449120	2	e

Table 11. ANOVA for the inhibition of Arabidopsis seed germination by albizziin.

Source	SS	df	MS	F	P
Main Effect					
Albizziin	22474.32728	10	2247.432728	787.6544558	.0000 ***
Error	31.38655514	11	2.853323194		
Total	22505.71383	21			

Student-Newman-Keuls Test
Error mean square = 2.853323194
Degrees of freedom = 11
Significance level = .05
LSD .05 = 3.717856282

Rank	Treatment #	Mean	n	Non-Significant ranges
1	10	0.0	2	a
2	11	0.0	2	a
3	9	3.008864053	2	a
4	8	22.326948250	2	b
5	7	33.276573540	2	c
6	6	43.116491870	2	d
7	5	50.689118780	2	e
8	4	68.090580220	2	f
9	3	75.895375100	2	g
10	2	82.381520230	2	h
11	1	88.813981130	2	i

Table 12. ANOVA for the inhibition of Arabidopsis seedling survival by albizziin.

Source	SS	df	MS	F	P
Main Effect					
Albizziin	13791.68508	12	1149.30709	329.636193	.0000 ***
Error	45.32570297	13	3.486592536		
Total	13837.01078	27			

Student-Newman-Keuls Test
Error mean square = 3.486592536
Degrees of freedom = 13
Significance level = .05
LSD .05 = 4.033931004

Rank	Treatment #	Mean	n	Non-Significant ranges
1	13	0.0	2	a
2	12	8.277340879	2	b
3	11	9.057230744	2	b
4	10	15.273176610	2	c
5	9	17.690275230	2	c
6	8	22.551873340	2	d
7	6	27.728914570	2	e
8	7	29.514200980	2	e
9	5	38.551872050	2	f
10	4	42.190412200	2	fg
11	3	45.497378220	2	g
12	2	55.929716680	2	h
13	1	89.433619980	2	i

Table 13. ANOVA for the inhibition of *Arabidopsis* seedling survival by asparagine.

Source	SS	df	MS	F	P
Main Effect					
Asparagine	1521.9629	5	304.3925799	927.1921598	.0000 ***
Error	1.969770193	6	0.328295033		
Total	1523.93267	11			

Student-Newman-Keuls Test
Error mean square = 0.3282950332
Degrees of freedom = 6
Significance level = .05
LSD .05 = 1.402007962

Rank	Treatment #	Mean	n	Non-Significant ranges
1	6	61.56557933	2	a
2	5	69.16493128	2	b
3	4	75.70939778	2	c
4	1	89.69147474	2	d
5	2	89.82543072	2	d
6	3	89.88738011	2	d

Table 14. ANOVA for the reversal effects to the inhibition of 8.0 mM albizziin to Arabidopsis seed germination by asparagine.

Source	SS	df	MS	F	P
Main Effect					
Asparagine	14.40909362	13	1.108391817	0.9980593673	.4987
Error	15.54765773	14	1.110546981		
Total	29.95675135	27			

Student-Newman-Keuls Test
Error mean square = 1.110546981
Degrees of freedom = 14
Significance level = .05
LSD .05 = 2.260229679

Rank	Treatment #	Mean	n	Non-Significant ranges
1	1	87.0462335	2	a
2	2	89.43361998	2	a
3	14	89.62952535	2	a
4	4	89.82543072	2	a
5	5	89.82543072	2	a
6	6	89.82543072	2	a
7	8	89.82543072	2	a
8	10	89.82543072	2	a
9	11	89.82543072	2	a
10	12	89.82543072	2	a
11	13	89.82543072	2	a
12	7	89.88738011	2	a
13	9	89.88738011	2	a
14	3	89.88738011	2	a

APPENDIX II. List of All Recovered Variants Resistant to 180 mM to 250 mM NaCl.

All variants listed survived to produce self-pollinated seeds. Refer to Seed Mutagenesis Selection of Materials and Methods for an explanation of parental group. M_4 progeny of M_3 families which retained resistance to 250 mM NaCl are marked with an asterisk (*).

Parental group M_3 family

(Following variants were rescued from selection of M_2 generation on 180 mM NaCl.)

C4	FC34
	FC35
	FC37
	FC39
	FC41
C5	FC46
	FC47
	FC48
	FC49
	FC50
	FC51
	FC52
	FC53
	FC54
	FC55
	FC56
	FC57
	FC58
C6	FC59
	FC60
	FC61
	FC62
	FC63
C7	FC64
	FC65
	FC66
	FC67
	FC68
	FC69
	FC70
	FC71
	FC72
	FC73
	FC74
	FC75
	FC76
	FC77
	FC78

C9	FC79
	FC80
	FC81
	FC82
	FC83
	FC84
	FC85
C9	FC86
	FC87
	FC88
	FC89
	FC90
	FC91
	FC92
	FC93
	FC94
C10	FC95
	FC97
	FC98
	FC99
	FC100
C12	FC103
	FC104
	FC105
	FC106
	FC107
C13	FC108
	FC109
	FC110
C15	FC112
	FC113
	FC114
	FC115
	FC116
	FC117

(Following variants were rescued from selection of M_2 generation on 200 mM NaCl.)

C15	FC118
	FC119
	FC120
	FC121
	FC122
C16	FC124
	FC125
	FC126
	FC128
	FC129
	FC130
	FC131
	FC132

	FC133
	FC134
	FC135
	FC136
	FC137
C17	FC138
	FC140
	FC141
	FC142
	FC143
	FC144
	FC145
C18	FC146
	FC147
	FC148
	FC149
C19	FC150
	FC151
	FC152
	FC153
	FC154
	FC155
	FC156
	FC157
	FC158
C20	FC159
	FC160
	FC161
	FC162
	FC163
	FC164
	FC165
	FC166
	FC167
	FC168
	FC169
	FC170
	FC171
C21	FC172
	FC173
	FC174
C22	FC175
	FC176
	FC177
	FC178
	FC179
	FC180
	FC181
	FC182
	FC183
	FC184
	FC185

	FC186
	FC187
C23	FC188
	FC189
	FC190
C42	FC191
	FC192
C45	FC193
	FC194
C45	FC195
	FC196
	FC197
	FC198
	FC199

(Following variants rescued from selection of M_2 Generation on 220 mM NaCl.)

C35	FC200
	FC201
	FC202
	FC203
	FC204
	FC206
	FC208
	FC209
	FC210
	FC211
	FC212
	FC213
	FC214
	FC215
C36	FC216
	FC217
	FC218
	FC219
	FC220
	FC221
	FC223
	FC224
	FC225
	FC226
	FC227
	FC228
C37	FC233
C39	FC229
	FC232
C41	FC234
	FC235
	FC236
	FC237
C38	FC238

	FC240
	FC241
	FC242
	FC243
	FC244
C40	FC245
	FC246
	FC247
	FC248
	FC249
	FC250
	FC251
	FC252
	FC253
C44	FC254
	FC255
	FC256
	FC257
	FC258

(Following variants rescued from selection of M_2 generation on 240 mM NaCl.)

C25	FC287
	FC289
	FC290
	FC292
	FC293
C29	FC295
	FC296
	FC297
	FC298
	FC299
	FC300
C39	FC307
	FC308
	FC309
	FC310
	FC311
	FC313
	FC314
	FC316
	FC317
	FC318
C28	FC319
	FC320
	FC321
	FC322
	FC323
	FC324
	FC326
	FC327

	FC328
C27	FC330
C26	FC337
	FC338
	FC339
	FC340
	FC341
	FC342
	FC343
	FC344
	FC345
	FC346
C40	FC348
	FC349
	FC350
	FC351
C44	FC367
	FC368
	FC369
	FC370
	FC371
C46	FC376
	FC377
C48	FC410
C49	FC411
	FC412

(Following variants rescued from selection of M_2 generation of 250 mM NaCl.)

C40	FC413
	FC414
	FC415
C50	FC416
	FC417
	FC418
	FC419
	FC420
	FC421
	FC422
C49	FC423
	FC424
C25	FC427
	FC428
	FC429
	FC430
	FC431
	FC432
	FC433
C51	FC434
	FC435
	FC436

	FC437	
	FC439	
C52	FC441	
	FC442	
	FC443	
	FC444	
	FC445	
	FC446*	236FC, 237FC, 238FC, 239FC, 240FC, 241FC, 242FC, 243FC, 244FC, 245FC, 246FC, and 247FC
	FC447	
	FC448	
C26	FC450	
	FC451	
C54	FC453	
	FC454	
	FC455	
	FC456	
	FC457	
	FC459	
	FC460	
	FC461	
	FC462	
C29	FC463	
	FC464	
	FC465	
	FC466	
	FC467	
C51	FC468	
C50	FC469	
C25	FC470	
C52	FC471	
	FC472	
	FC473	
	FC474	
	FC475	
	FC476	
	FC477	
	FC478	
	FC479	
	FC480	
	FC481	
	FC482	
	FC483	
	FC484	
	FC485	
	FC486	
	FC487	
	FC488	
	FC489	
	FC490	
	FC492	
	FC493	

	FC493
	FC494
	FC495
	FC496
	FC497
	FC498
C26	FC499
	FC500
	FC503
	FC504
C49	FC505
	FC506
C54	FC508
	FC509
	FC510
	FC511
	FC512
C52	FC513
	FC514
	FC515
	FC516
	FC517
C25	FC518
	FC519
	FC520
	FC521
	FC522
C51	FC523
	FC525
	FC526
	FC527
	FC528
	FC529
	FC530
	FC531
C47	FC555
B1	N26
	N27
	N28
	N29
	N30
	N31
	N32
	N33
	N34
	N35
	N36
	N37

APPENDIX III. List of All Recovered AAH Resistant Mutants. All variants listed survived to produce self-pollinated seeds. Refer to Seed Mutagenesis Section of Materials and Methods for an explanation of parental groups. All listed variants were selected at M_3 generation on 1.5 mM AAH. Self-pollinated progeny of M_3 families are marked with an asterisk.

Parental group	M_3 family	
(Following variants rescued from selection of M_2 generation on 1.4 mM AAH.)		
C4	FC4	
	FC6	
	FC7	
	FC8	
	FC9	
	FC16	
	FC17	
	FC19	
	FC20	
	FC22	
	FC23	
	FC24	
	FC25	
	FC28	
	FC29	
	FC30	
	FC31	
	FC32	
	FC263	
	FC264*	99FC,100FC
	FC265	
C1	FC266	
	FC269	
	FC271*	249FC, 250FC
	FC273*	104FC, 105FC, 106FC, 109FC,110FC,112FC, 114FC, 115FC, 115FC, 116FC, 117FC, 119FC, 120FC, 121FC, and 122FC
	FC274	
	FC278	
	FC279*	135FC, 136FC
C9	FC357	
	FC358	
	FC359	
	FC360	
	FC364	
	FC366	
C5	FC379*	137FC
C6	FC384	
	FC385	
C7	FC386	

	FC387
	FC390
C8	FC391
	FC393
	FC394

(Following variants rescued from selection of M_2 generation on 1.6 mM AAH. All listed variants were selected at M_3 generation on 1.5 mM AAH.)

C16	FC440
C25	FC470
C26	FC541
	FC554
C31	FC543
	FC545
	FC546
	FC547
C40	FC552
	FC553

(Following variants rescued from selection of M_2 generation on 1.6 mM AAH. All listed variants have not been selected at M_3 generation yet.)

C32	N10
	N11
	N12
C33	N13
C9	N44
	N45
	N46
	N47
	N48
	N49
	N50
	N51
	N52
	N53
	N54
	N55
	N56
	N57
	N58
	N59
	N60
	N61
	N62
	N63
	N64
	N65
	N66
	N67

N68
N69
N70
N71
N72
N73
N74

All the variants and mutants listed above are maintained in the laboratory of Dr. Fredric R. Lehle, Department of Plant Sciences, University of Arizona, Tucson, AZ 85721.

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