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THE INHERITANCE OF TWO GLYPHOSATE TOLERANT MUTANTS IN THE FERN *CERATOPTERIS*

A Thesis

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ABSTRACT

The herbicide glyphosate ([N-phosphonomethyl] glycine) is a highly effective and widely used broad spectrum herbicide which also possesses properties that reduce its potential harmful effect on the environment. Because of this, there is considerable interest in developing glyphosate tolerant crop plants. However, genes that confer tolerance to glyphosate must first be identified. The fern *Ceratopteris richardii* can be used to select for and characterize mutations conferring herbicide tolerance. In this study two different confirmed glyphosate tolerant mutant strains, HαG492 and HαG343, of the fern *Ceratopteris richardii* were characterized in both the haploid gametophyte and diploid sporophyte generation.

The effects of glyphosate were assayed by the comparison of spore germination in gametophytes and growth both in the gametophyte and sporophyte generation. Germination of the wild type and one mutant strain, HαG343, was affected by increasing concentrations of glyphosate while the HαG492 mutant strain was unaffected. Both mutants showed increased growth in comparison to the wild type in the presence of glyphosate. One strain, HαG492, showed high tolerance to glyphosate while the HαG343 strain showed moderate tolerance.

The mutant strains were analyzed to determine the inheritance of the tolerance traits. Segregation in the gametophyte generation showed that for both strains, tolerance to glyphosate is due to a single nuclear gene. Segregation analysis of the double mutant hybrid indicated that the mutations involve separate loci but are linked. Genetic analysis in the

sporophyte generation showed that the mutation carried by the HαG343 strain is recessive while that in the HαG492 strain is expressed as a semidominant trait. The expression of this semidominant mutation is also evident in sporophytes containing both mutations in the trans configuration. The mutations that are responsible for conferring tolerance to the HαG343 and HαG492 strains have been designated *g343* and *G492*, respectively.

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I. INTRODUCTION

Genetically engineering herbicide tolerance into crop plants is of great agronomic interest because this would greatly reduce expenditure on labor and fuel costs for weed control before and during crop growth. The herbicide Roundup^{Tm} possesses several properties that make it an important target for such genetic engineering research. Glyphosate (N-[phosphono-methyl]glycine) (Figure 1), the active ingredient in Roundup, is a widely used broad-spectrum post-emergence herbicide which is also effective against some types of bacteria and algae (Gresshoff, 1979).

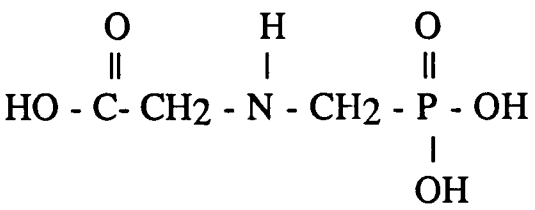

Figure 1. The Structure of Glyphosate.

Glyphosate's wide use stems from the fact that it effectively controls 76 of the worlds 78 worst weed species (della-Cioppa et al., 1987). In addition, glyphosate possesses several properties which reduce it's potential harmful effect on the environment. Glyphosate is readily translocated in plants (Gottrup et al., 1976) and when it comes into contact with soil is rapidly inactivated through (1) microbial degradation by those microbial organisms which can utilize phenoxyacetic acid as a carbon source to

produce the plant nutrients phosphoric acid, ammonia, and carbon dioxide, (2) by reversible adsorption to clay and organic matter and (3) possibly by chemical degradation (Sprankle et al., 1975; Torstensson and Aamisepp, 1977). Glyphosate also exhibits very low toxicity in fish and aquatic invertebrates (Folmar et al., 1979). Obtaining glyphosate tolerant crop plants would, therefore, increase the range of application of the herbicide without significantly increasing the potential damage to the environment associated with other herbicides.

One way to obtain glyphosate tolerant crop plants is through genetic engineering. In order to do this, genes that confer tolerance to the herbicide must first be identified. Once these genes have been identified, a mechanism for their efficient transfer into agriculturally useful crops must be developed. The use of microorganisms presents a very efficient and effective mechanism for rapidly screening for a wide variety of mutants. Glyphosate tolerance has been reported for several microorganisms (Schulz et al., 1984; Amrhein et al., 1983; Comai et al., 1983; Rogers et al., 1983), and a recent study documents the successful transfer of glyphosate tolerance from a microorganism to a higher plant (Fillatti et al., 1987). Through this and other studies significant progress is being made in overcoming barriers to gene exchange between species. Glyphosate tolerance has also been reported for cell cultures of higher plants (Amrhein et al., 1983; Nafziger et al., 1984; Smith et al., 1986; Steinrucken et al., 1986).

Although the microbial and tissue culture approaches above have considerable promise, several potential difficulties are evident. For instance, in microbial selection systems the level of expression ultimately achieved in diploid vascular sporophytes cannot be assessed until the successful transfer of these traits into first cell cultures and followed by the regeneration plants. In tissue culture, glyphosate tolerance has been associated with a similar inability to directly assess the level of expression in diploid vascular sporophytes (Hauptmann et al., 1988). Problems also exist with fitness in the absence of the herbicide (Nafziger et al., 1984; Singer and MacDaniel, 1985), a reduced ability to regenerate glyphosate tolerant plants (Smith et al., 1986), variability in the level of expressed tolerance in individually regenerated plants (Dyer et al., 1988) and the limited applicability of tissue culture selection systems to a wide variety of plants. Clearly, additional studies involving the selection and characterization of mutations that confer glyphosate tolerance would expand our knowledge in this area.

A selection system for herbicide tolerance utilizing the fern *Ceratopteris richardii* offers several advantages over the two systems previously outlined. These include, the ability to rapidly and efficiently screen for both recessive and dominant mutations which confer tolerance to glyphosate, and the ability to use this system for rapid and direct assessment of the effects of the resistance traits in morphologically differentiated gametophytes and in homozygous vascular sporophytes (Hickok and Schwarz, 1986a). This system can also be readily utilized for genetic studies. Each haploid gametophyte is derived from a single meiotic

product and can be self fertilized to produce completely homozygous sporophytes. Cross fertilizations can easily be carried out to obtain desired hybrid combinations. These homozygous and hybrid sporophytes can then be vegetatively cloned to produce large quantities of genetically defined diploid material and haploid gametophytes for use in genetic and/or physiological studies (Hickok et al., 1987).

This research project was undertaken to study selected glyphosate tolerant mutants of *Ceratopteris richardii* through detailed characterization of responses to specific concentrations of glyphosate and by the determination of the inheritance of the mutations. Two glyphosate tolerant mutant lines, HαG492 and HαG343, were chosen from available confirmed mutants (Hickok et al., 1987) by carrying out preliminary dose response tests with gametophytes over a range of glyphosate concentrations (0-1.0 mM). These mutant lines were chosen because they exhibited different levels of tolerance in comparison to the wild type over specific concentrations of glyphosate and were therefore likely to represent different mutations.

II. REVIEW OF LITERATURE

Mode of Action of Glyphosate

The aromatic amino acid pathway (Figure 2) was first proposed as the site of action for glyphosate by Jaworski (1972) because growth inhibition by glyphosate in *Rhizobium japonicum* and *Lemna gibba* could be reversed by the addition of phenylalanine and tyrosine or phenylalanine, respectively. Because tryptophan was not essential for reversal of inhibition, this suggested that glyphosate blocked a reaction after the formation of chorismate. Chorismate mutase and prephrenate dehydratase were suggested as possible sites of inhibition for glyphosate. This hypothesis was later refuted by Roisch and Lingens (1974). Their studies indicated that three amino acids, tryptophan, plenylalanine and tyrosine, were required to reverse inhibition in *E. coli* and that prephrenate dehydratase and chorismate mutase were insensitive to inhibition by glyphosate. Two enzymes, 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase (DAHP synthase) and 3-dehydroquinic acid synthase (DHQ synthase), which catalyse early steps in the aromatic amino acid pathway, were found to be sensitive to inhibition by glyphosate. This inhibition was, however, too slight to account for the adverse effects on growth by glyphosate.

Further studies (Gresshoff, 1979) showed that phenylalanine and tryrosine were required for the reversal of growth inhibition by glyphosate in a variety of organisms. It was also suggested that glyphosate may act by affecting processes or compounds that are derived from phenylalanine or

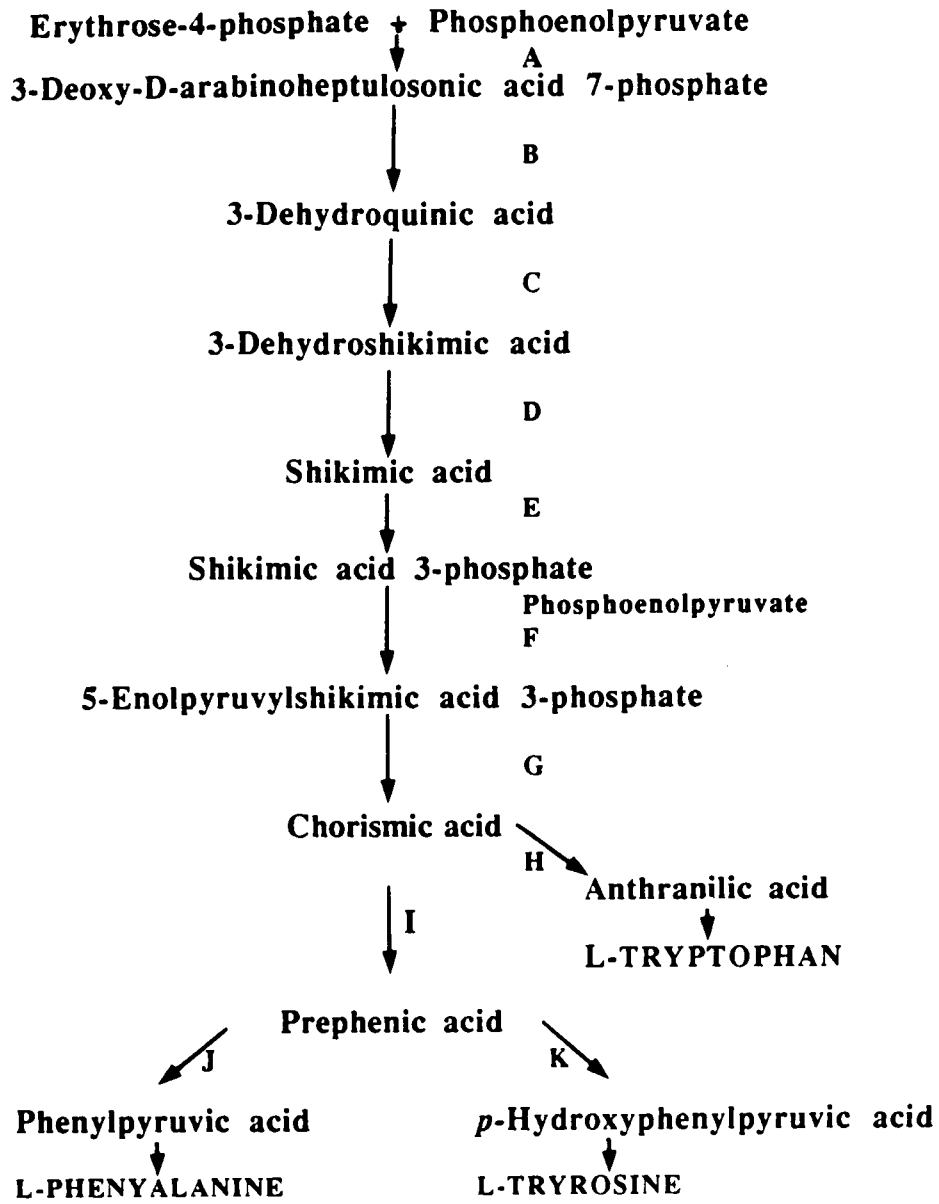

Figure 2. General Outline of The Shikimic Acid Pathway. A= 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; B= 3-dehydroquinate synthase;C= 3-dehydroquinate dehydratase;D= shikimate dehydrogenase;E=shikimate kinase;F= 5-Enoylpyruvyl shikimic acid-3-phosphate synthase;G= chorismate synthase;H= anthranilate synthase;I= Chorismate mutase;J=Prephenate dehydratase;K= Prephenate dehydrogenase. Note: 5-dehydroquinic acid, 5-dehydroshikimic acid, shikimic acid 5-phosphate, 3-enoylpyruvylshikimic acid 5-phosphate= alternative names for some intermediates. (modified from Goodwin and Mercer, 1983; Lehninger, 1971).

tyrosine. Duke et al., (1979) detected an increase in the extractable amount of L-phenylalanine ammonia-lyase (PAL) in soybean treated with glyphosate and therefore hypothesized that glyphosate may function through the induction of PAL activity. The induction of PAL could lead to a depletion of phenylalanine with adverse effects on protein synthesis, a build up of toxic levels of ammonia and the accumulation of growth limiting phenolics which are derived from trans-cinnamic acid, the product of PAL. Induction of PAL activity, however, does not explain the growth inhibitory action of glyphosate in organisms such as bacteria which do not have PAL (Hollander and Amrhein, 1980). In addition, a potent inhibitor of PAL activity, l-α-aminooxy-β-phenylpropionic acid, failed to relieve glyphosate toxicity in soybean. This suggested that induction of PAL activity may only partially contribute to amino acid depletion and may not be the primary target for glyphosate (Duke et al., 1980).

From other studies, a biosynthetic step between shikimate and chorismate was implicated as the possible enzyme target for glyphosate because glyphosate inhibited the incorporation of [^{14}C]shikimate into the three aromatic amino acids, L-tryptophan, L-phenylalanine and L-tyrosine, and caused the accumulation of radioactively labeled shikimate in buckwheat hypocotyls (Hollander and Amrhein, 1980). Glyphosate was also shown to inhibit the formation of anthraquinoid compounds, which are directly derived from chorismate, in *Galium mollugo* and to inhibit the formation of chorismate from shikimate in a multiply blocked auxotroph of *Aerobacter aerogenes* (Amrhein et al., 1980). This provided conclusive

evidence that glyphosate inhibits the formation of chorismate from shikimate.

The biosynthetic pathway between shikimate and chorismate is catalyzed by three enzymes. Of these three enzymes, the specific enzymatic site for glyphosate was identified to be 5-enoylpyruvyl-shikimate-3-phosphate synthase (EPSP synthase) in *Aerobacter aerogenes* (Steinrucken and Amrhein, 1980). EPSP synthase is also the sensitive enzyme target in *Neurospora crassa* (Boocock and Coggins, 1983), *Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa* (Fischer et al., 1986) and in tissue cultures of *Nicotiana sylvestris* (Rubin et al., 1984), *Corydalis sempervirens* (Amrhein et al., 1983), and *Pisum sativum* (Mousedale and Coggins, 1984). These reports include prokaryotes, lower eukaryotes and higher plants, indicating that EPSP synthase may be the general enzyme target in nature.

Inhibition by glyphosate is associated with the excretion of large amounts shikimic acid and/or shikimate-3-phosphate (S-3-P) in bacteria (Schulz et al., 1984; Fischer et al., 1986). In higher plants and tissue culture, inhibition by glyphosate is associated with the accumulation of free shikimate while shikimate-3-phosphate is less often detected (Rubin et al., 1984). Shikimate-3-phosphate is probably the main product accumulated, since it directly precedes the block in the aromatic amino acid pathway by glyphosate, but may then be converted to shikimate by phosphatase activity in the vacuole, as shown for buckwheat (Hollander-Czytko and Amrhein, 1983).

The accumulation of shikimate rather than shikimate-3-phosphate may also be associated with the end product inhibition of shikimate kinase by shikimate-3-phosphate (Rubin et al., 1984).

Kinetic Effects of Glyphosate

EPSP synthase catalyses the addition of the phosphoenoylpyruvyl moiety of phosphoenoylpyruvate to shikimate-3-phosphate for the formation of 5-enolpyruvyl-shikimate-3-phosphate. EPSP synthase leads to the formation of chorismate and therefore the biosynthesis of aromatic compounds. Glyphosate is a potent competitive inhibitor of EPSP with respect to PEP in various bacteria (Steinrucken and Armhein, 1984b; Duncan et al., 1984; Anton et al., 1983) in fungi (Boocock and Coggins, 1983) and in higher plants (Mousedale and Coggins, 1984; Rubin et al., 1984; Steinrucken et al., 1986). This indicates that PEP and glyphosate compete for the same binding site on the enzyme and that inhibition can be reversed by high concentrations of the substrate. The interaction of glyphosate with S-3-P in the forward reaction is, however, varied. Glyphosate is uncompetitive with respect to S-3-P in *N . crassa* (Boocock and Coggins, 1983) and *Nicotiana silvestris* (Rubin et al., 1984) but is non competitive with respect to S-3-P in *Corydalis sempervirens* (Amrhein et al., 1983) and *Klebsiella pneumoniae* (Steinrucken and Amrhein, 1984b).

Mechanistic studies of bacterial EPSP synthase, (Grimshaw et al., 1982) indicate that the condensation reaction catalyzed by EPSP synthase occurs through an addition elimination mechanism in which the enoylpyruvyl moiety of of PEP is transferred to S-3-P. Data from kinetic

studies of EPSP synthase in *N. crassa* (Boocock and Coggins, 1983), *E. coli* (Lewendon and Coggins, 1983) and *N. silvestris* (Rubin et al., 1984) indicate that the enzyme proceeds by an ordered sequential mechanism in which EPSP synthase binds first to S-3-P and then to PEP. It has been proposed that glyphosate may act as a transition state analogue of PEP (Steinrucken and Amrhein, 1984a) and the inhibitory effect of glyphosate on EPSP synthase is therefore thought to result from reversible interaction with the enzyme and S-3-P complex, thereby forming a dead end complex. The inhibition of EPSP synthase by glyphosate appears to be highly specific because compounds that are structurally related to glyphosate do not inhibit the enzyme. In addition, other PEP utilizing enzymes from various sources are not inhibited by glyphosate (Steinrucken and Amrhein, 1984b).

Glyphosate Inhibition of The Shikimic Acid Pathway

In the shikimic acid pathway, L-phenylalanine, L-tyrosine and L-tryptophan are the aromatic end products in bacteria, fungi and plants. In higher plants, the aromatic amino acids are precursors for alkaloids, flavanoids, lignin precursors, coumarins, indole derivatives and other phenolic compounds (Rubin and Jensen, 1985). The shikimic acid pathway is therefore essential for protein synthesis and for the synthesis of a variety of secondary metabolites. These secondary compounds serve as intermediates in the metabolism of other compounds and are essential for a wide range of plant processes including embryo development, flower color and in plant defense mechanisms. The highly effective herbicidal action of

glyphosate, because of it's inhibition of EPSP synthase, can be attributed to several factors: (1) the interruption of aromatic amino acid synthesis resulting in an imbalance in protein synthesis, (2) the accumulation of toxic phosphorolated pathway intermediates such as 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (DAHP) and shikimate-3-phosphate, (3) a decrease in the synthesis of secondary metabolites of the shikimic acid pathway derived from chorismate, and (4) an energy drain effect resulting from the use of 1 PEP and ATP molecule for every molecule of shikimate-3-phosphate accumulated, resulting in a severe depletion of respiratory substrates (Fischer et al., 1986). A deficit of the respiratory intermediates such as α–ketoglutarate would limit the substrate available for ammonia assimilation resulting in elevated and possibly toxic levels of ammonia.

A dual herbicidal action has also been proposed for glyphosate in which inhibition of EPSP synthase by glyphosate leads to end product starvation, thereby relieving early pathway allosteric enzymes from feedback inhibition (Rubin et al., 1984). This could result in the unchecked, wasteful flow of carbons into the shikimic acid pathway. This theory stems from the fact that in *N. silvestris*, DAHP synthase, which catalyses the condensation of PEP and erythrose-4-phosphate (E4P) to DAHP, exists as two isozymes which are differentially regulated. One isozyme which requires magnesium for activity (DS-Mn) is located in the chloroplast and is insensitive to glyphosate while the other which requires cobalt magnesium or manganese for activity (DS-Co) is located in the cytosol and is sensitive to inhibition by glyphosate. Recent *in vitro* studies indicate that the sensitivity of this isozyme to glyphosate is dependent on the presence of

the cobalt cation as the activating moiety. The presence of glyphosate results in the formation of a cobalt glyphosate complex which binds tightly to the enzyme, resulting in inactivity (Ganson and Jensen, 1988). It has been suggested that in the presence of glyphosate the massive accumulation of shikimate results from the action of the insensitive form of the enzyme DS-Mn. DS-Mn is subject to feedback inhibition by L-arogenate which is thought to be the precursor of phenylalanine and tyrosine in higher plants (Jensen, 1986). Glyphosate inhibition of EPSP synthase is thought to result in the depletion of L-arogenate leading to a loss of feedback control of DS-Mn promoting a wasteful flow of carbons into the aromatic amino acid pathway. In addition, this would result in a depletion of PEP, thus, enhancing the inhibition of EPSP synthase by glyphosate (Rubin et al., 1984). Inhibition of the glyphosate sensitive form of the enzyme in the cytosol may be significant enough to prevent the flow of carbon into the shikimic acid pathway, thereby enhancing the growth inhibition caused by glyphosate. Differentially regulated isozymes of DAHP synthase have also been found in *Vigra radiata* (Rubin and Jensen, 1985), *Solanum tuberosum* (Morris et al., 1989), and a variety of other higher plants (Ganson et al., 1986). Thus, this may be the general enzyme makeup in higher plants.

The Effect of Glyphosate on Chlorophyll

Chlorosis and bleaching are usually observed in plants that are treated with glyphosate. N,N-bis(phosphonomethyl) glycine, an analogue of glyphosate, caused chlorosis in emerging leaves of maize but not in mature leaves (Croft et al., 1974). Glyphosate has also been reported to reduce chlorophyll in maize seedlings (Ali and Fletcher, 1978) and to affect both the synthesis and degradation of chlorophyll in tobacco and soybean cells (Lee, 1981). In ultrastructural studies, glyphosate has been reported to disrupt chloroplasts, to cause swelling of the endoplasmic reticulum and to reduce starch grains in quackgrass (Campbell et al., 1976). These studies indicate that a second mode of action of glyphosate may involve the synthesis and degradation of chlorophyll, secondarily resulting in the disappearance of starch and protein.

It has been proposed that chlorophyll content may be affected by glyphosate through either photobleaching and/or peroxidation or by the inhibition of chlorophyll synthesis (Villanueva et al., 1985). Evidence to support the first hypothesis comes from the finding that caroteniod levels are more greatly affected than chlorophyll in purple nutsedge (Abu-Irmaileh and Jordan, 1978). This result is significant because caroteniods protect chlorophyll from light and from attack by molecular oxygen. Therefore, a reduction in the level of caroteniods would be expected to subsequently result in a reduction of chlorophyll (Villanueva et al., 1985). In support of the second hypothesis, studies by Kitchen et al., showed that chlorophyll accumulation was inhibited by glyphosate (Kitchen et al., 1981a) and that there was a reduction in the incorporation of ^{14}C-

glutamate ^{14}C-α-ketoglutarate and ^{14}C-glycine into δ–aminolevulinic acid (ALA) in barley shoots treated with glyphosate. The incorporation of ^{14}C-ALA into chlorophyll was not affected. This finding indicates that the site of action of glyphosate may involve either the conversion of glutamate to ALA, the condensation of glycine with succinyl CoA to form ALA, or a site common to both of these pathways (Kitchen et al., 1981b).

The inhibition of chlorophyll synthesis by glyphosate at the level of ALA can affect many physiological processes in the plant, since the formation of ALA is the rate limiting step in the synthesis of chlorophyll and a variety of other porphyrin containing compounds. Two molecules of ALA combine to form porphobilinogen. Four molecules of porphobilinogen combine to form protoporphyrin IX. Protoporphyrin IX is the precursor of Fe protoporphyrin which is utilized to form cytochromes, peroxidases, catalases and Mg protoporphyrin, which is used in the synthesis of chlorophyll (Kitchen et al., 1981b). Processes such as the electron transport system involving cytochromes, photosynthesis and the conversion of toxic peroxides to water and oxygen by peroxidases would therefore be severely affected. If the synthesis of chlorophyll is affected by glyphosate before the formation of ALA, it is expected that glyphosate would cause a reduction in the biosynthesis of other porphyrin containing compounds. In fact, catalase activity has been found to be reduced in purple nutsedge after treatment with glyphosate (Abu-Irmaileh and Jordan, 1978), which further lends support to this hypothesis.

Glyphosate is readily translocated to the roots in plants (Gottrup et al., 1976) and root elongation has been found to be severely affected in

soybean (Hoagland, 1980) and maize (Ali and Fletcher, 1978). It has been proposed that the primary site of action for glyphosate may be in the roots, due to the inhibition of respiration resulting in death of the plant (Ali and Fletcher, 1978).

Multiple Enzyme Sites

There is evidence to show that chloroplasts can catalyse all of the reactions necessary to form aromatic amino acids and it is possible that enzymes for the shikimate pathway are located in both the chloroplast and the cytosol (Rubin et al., 1984). Isozymes located in the chloroplast and the cytosol have been shown to exist for DAHP synthase in *N. silvestris* (Rubin et al., 1984), *Vigna radiata* (Rubin and Jensen, 1985), *Solanum tuberosum* (Morris et al., 1989) and a variety of other higher plants (Ganson et al., 1986). Isozymes have also been shown to exist for chorismate mutase in *N. silvestris* (d' Amato et al., 1984), *Sorghum bicolor* (Singh et al., 1985) and in mung bean (Morris et al., 1989). Both these enzymes are located at important branch points in the aromatic amino acid pathway and there is also evidence to suggest that there are isozymes of the enzymes that catalyse intervening steps in the pathway. Isozymes have been found for DHQ dehydratase (Jensen, 1986; Mousedale and Coggins, 1985), shikimate oxidoreductase (SORase) (Rothe et al., 1983; Mousedale and Coggins, 1985) and EPSP synthase (Mousedale and Coggins, 1985; Ream et al., 1988). Isozymes located in the chloroplast and cytosol have also been found for anthranilate synthase which catalyses the

conversion of chorismic acid to anthranilic acid (Brotherton et al., 1986).

The isozymes of DAHP synthase (Rubin et al., 1984; Rubin and Jensen, 1985; Ganson et al., 1986; Morris et al., 1989), chorismate mutase (Goers and Jensen, 1984a,b; Singh et al., 1985; Morris et al., 1989) and anthranilate synthase (Brotherton et al., 1986) are all differentially regulated with the unregulated forms of the enzymes existing in the cytosol and the regulated forms exiting in the plastid. A dual pathway for aromatic biosynthesis has been proposed consisting of two separate shikimic acid pathways. One which is subject to tight allosteric control and is thought to be located in the chloroplast, is primarily responsible for the synthesis of amino acids and the other, which is located in the cytosol, is unregulated and is primarily responsible for the synthesis of secondary metabolites (Jensen, 1985). However, there are still questions regarding the uniformity of this enzyme makeup and regulation in higher plants.

The presence of a dual aromatic amino acid pathway in the chloroplast and cytosol may present multiple enzyme targets for glyphosate: EPSP synthase in the cytosol and plastid and DAHP synthase in the cytosol (Jensen, 1985). This may make genetically engineering glyphosate resistance into crop plants more complex than previously thought.

Mechanisms of Tolerance to Glyphosate

EPSP synthase-related tolerance to glyphosate has been reported for both bacteria and higher plants. EPSP synthase tolerance is associated with the over-production of the wild type sensitive enzyme or with an alteration of the EPSP synthase enzyme, resulting in a reduced sensitivity to glyphosate. These reports provide further evidence that EPSP is a primary enzyme target for glyphosate.

There are only two cases where tolerance to glyphosate is associated with a mutant enzyme. EPSP synthase with reduced sensitivity to glyphosate has been reported for a glyphosate resistant strain of *Aerobacter aerogenes*. EPSP synthase activity in crude extracts form this strain was found to be insensitive to a 50 mM concentration of glyphosate (Schulz et al., 1984). Further characterization of the glyphosate insensitive enzyme showed a reduced ability to bind to glyphosate and an increased Km for PEP and S-3-P, resulting in a reduction in the general performance of the enzyme (Sost et al., 1984). In *Salmonella typhimurium*, glyphosate insensitive EPSP synthase activity has been correlated with a mutation in the Aro A locus which codes for EPSP synthase enzyme (Comai et al., 1983). The point mutation results in a pro-ser change at residue 101 of the enzyme. The mutant enzyme was found to have a decreased affinity for glyphosate but an increased affinity for PEP and S-3-P, suggesting that the mutation does not result in a kinetically deficient enzyme (Stalker et al., 1985).

Tolerance due to over expression of the wild type EPSP synthase enzyme in *E. coli* has been achieved through gene amplification via a multicopy plasmid carrying the EPSP synthase gene (Rogers et al., 1983). In *Aerobacter aerogenes* (Amrhein et al., 1983) and in cell cultures of *Corydalis sempervirens* (Amrhein et al., 1983; Smart et al., 1985) there was an increase in EPSP synthase activity in cultures adapted to glyphosate. Similarly, in cell cultures of *Daucus carota* (Nafziger et al., 1984), tomato cells (Smith et al., 1986), and a *Petunia hybrida* cell line (Steinrucken et al., 1986), tolerance to glyphosate has been associated with the overproduction of the EPSP synthase enzyme. A glyphosate tolerant cell line has also been obtained in *N. silvestris* (Dyer et al., 1988). In this instance, tolerance in cell culture and in regenerated plants is also thought to result from increased levels of EPSP synthase and DAHP synthase. While increased levels of EPSP synthase are thought to be mainly responsible for the observed tolerance to glyphosate, DAHP synthase may also contribute by increasing the flow of carbon through the shikimic acid pathway.

There are reports of genetically engineered glyphosate tolerance in crop plants. Glyphosate tolerant tobacco plants have been obtained through the introduction of a mutant allele of an aroA gene from *Salmonella typhimurium* which encodes a glyphosate tolerant EPSP synthase. This was accomplished by co-cultivation using an agrobacterium. This bacterial gene, however, only gave rise to the cytoplasmic form of the enzyme because it lacks a transit polypeptide sequence (Comai et al., 1985). Since most EPSP synthase reportedly occurs in the chloroplast (Mousedale and

Coggins, 1985), it was suggested that higher levels of tolerance may be achieved by targeting EPSP synthase tolerant enzymes to the chloroplast (Comai et al., 1985). The successful transfer of glyphosate tolerance targeted for the chloroplast has been achieved for *Petunia hybrida* (Shah et al., 1986). This was accomplished by fusing a cauliflower mosaic virus 35s promoter to a complementary DNA clone encoding EPSP synthase which was obtained from a *P. hybrida* cell line that already overproduced the enzyme. This chimeric gene was then introduced into a *P. hybrida* cell line by co-cultivation with an agrobacterium and resulted in a high level of expression of the EPSP synthase enzyme. The overproduced enzyme was thought to have been transported into the chloroplast of the petunia cells by a transit polypeptide sequence. della-Cioppa (1987) has also reported the successful targeting of a glyphosate resistant EPSP enzyme from *E. coli* to the intact chloroplast of *Latuca sativa* in suspension cell culture. In *P. hybrida,* EPSP synthase is localized in the chloroplast but is first synthesized as a cytoplasmic precursor with a transit peptide sequence that directs it to the chloroplast (della-Cioppa et al., 1986). A stable chimeric glyphosate resistant EPSP enzyme was produced by fusing a gene from *E. coli* encoding a mutant EPSP synthase enzyme behind a portion of cDNA that encodes a transit polypeptide sequence of *P. hybrida* EPSP synthase. The chimeric enzyme was imported into the intact chloroplast of *Latuca sativa* and processed to produce a stable glyphosate resistant enzyme. Tobacco plants were also transformed using an *Agrobacterium tumefaciens* vector containing the same chimeric gene. This demonstrates the *in vivo* as

well as *in vitro* targeting of a glyphosate resistant bacterial enzyme to the chloroplasts of higher plants.

Glyphosate tolerant plants have also been generated in *Arabidopsis thaliana* due to the overproduction of the EPSP synthase enzyme. This was accomplished by cloning the *Arabidopsis* EPSP synthase gene by homology to a petunia gene probe, fusing it to a cauliflower mosaic virus 35S promoter in a plant transformation vector and reintroducing the chimeric gene into *Arabidopsis* by co-culture with an agrobacterium. The 35S promoter resulted in an approximated twenty-fold over-expression of the EPSP transcript in comparison with the endogenous promoter. Transformed callus tissue and regenerated plants showed tolerance to normally lethal levels of glyphosate. Seeds from regenerated plants that were heterozygous for the transformed DNA showed segregation of the resistant phenotype when cultured on glyphosate containing medium (Klee et al., 1987).

III. MATERIALS AND METHODS

Spore Source

Spores from three completely homozygous lines of *C . richardii* Brongn. were used in this study: the diploid wild type, Hn-n (Hickok et al., 1987), and two glyphosate tolerant lines. Spores of the Hn-n line, which had been collected on 2/15/85, were used throughout the study. The two glyphosate tolerant lines were chosen from available confirmed mutants (Hickok et al., 1987) by carring out a preliminary dose response test over a range of glyphosate concentrations (0-1.0 mM) and assaying gametophyte growth responses. From this preliminary dose response test, two mutants, HαG492 and HαG343, which could clearly be distinguished from each other and the wild type, were chosen for further studies. Spores of the HαG343 line were available from an M1 sporophyte generated by selfing the original M1 tolerant gametophyte. These were used for all preliminary studies and hybridizations. However, because spore quantity was limited, an M2 gametophyte generated from the HαG343 M1 sporophyte was self fertilized to produce an M2 generation sporophyte, HαG343-1. Spores from this sporophyte were used throughout the rest of the study. Since the generation of the M2 sporophyte involved self fertilization of a haploid gametophyte, the M2 sporophyte was considered to be genetically identical to the M1 sporophyte. For the HαG492 line, the M2 sporophyte, HαG492-10, was available at the start of the study. Spores from the two glyphosate tolerant lines were collected continuously throughout the study.

Medium Preparation

The gametophyte cultures used for these experiments were grown in 60 x 20 mm petri plates containing 10-15 ml of agar-solidified (1%, w:v) Parker/Thompson inorganic nutrient medium (Table 1). The pH was adjusted to 5.4 before autoclaving. Sporophyte cultures were grown in styrofoam cups or 250 ml flasks in 125-150 ml of Parker/Thompson inorganic nutrient medium. The pH was adjusted to 6.0 prior to autoclaving.

Glyphosate supplemented media was prepared by the addition of specific quantities of a stock solution of glyphosate to the media after adjusting to pH 5.4 or 6.0 and autoclaving. The stock solution of glyphosate (m.w. 169g) was prepared from 96.4% or 98% pure Technical glyphosate, which was a gift from Monsanto, by the addition of 0.169g of 96.4% pure glyphosate or 0.166g of 98% pure glyphosate to 100 ml of Parker/Thompson inorganic nutrient medium. The pH of the medium was adjusted to 5.4 (solidified medium) or to a pH of 6.0 (liquid medium) before the addition of glyphosate. The stock solution was then cold filter sterilized using a 0.2 micron Acrodisc^{R} filter. Although the original intention was to use a 10^{-2} M stock solution, failure to initially account for the percent purity of the glyphosate resulted in a stock solution of 0.0964 x 10^{-2} M. Because this difference is quite minimal with respect to the responses observed over the range of concentrations used (0-1.0 mM), data are reported as whole unit concentrations, eg. 0.01 mM, 0.05 mM, etc.

Table 1. Composition of Stock Nutrient Solutions[a] (Modified from Klekowski 1969)

Code	Stock Solutions	g/l	molar
	Thompsons Macronutrients		
A	NH_4NO_3	25.0	0.312
B	KH_2PO_4	20.0	0.147
C	$MgSO_4$ (anhydrous)	4.8	0.040
D	$CaCl_2.H_2O$	13.0	0.117
E	Parker's Micronutrients		
	$MnSO_4$ (anhydrous)	0.022	0.00015
	$CuSO_4.H_2O$	0.037	0.00021
	$ZnSO_4.7H_2O$	0.052	0.00018
	H_3BO_3	0.186	0.00301
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0037	2.99×10^{-6}
F	Iron Solution		
	$FeSO_4.7H_2O$	2.78	0.010
	$Na_2EDTA.2H_2O$	3.73	0.010

[a]Basal medium is prepared by adding 5 ml of stock A, 25 ml of stock B, 12 ml of stock C, 2 ml of stock D, 10 ml of stock E, and 10 ml of stock F to 900 ml of deionized, distilled, twice polished water and then bringing to 1 liter volume. This solution is then added to a two liter Erlenmyer flask containning ten grams of tissue culture grade agar, and then the pH is adjusted to 5.4 before autoclaving. Liquid medium was prepared as above without agar and adjusted to a pH of 6.0 before autoclaving.

Spore Sterilization and Sowing

Spores were sterilized by soaking them in sterile distilled water in a conical 15 ml centrifuge tube for 12-24 hrs. The spores were then treated with a 1:5 solution of commercial bleach (5.25% Na hypochlorite by weight) and water for 3 minutes. After 3 minutes, the chlorox solution was removed using a sterile Pasteur pipette and the spores washed 2 times with ca. 4 ml of sterile distilled water. The water and sterilizing solution were removed from the the conical centrifuge tube by placing the sterile pipette on the bottom of the tube and withdrawing the liquid by aspiration, leaving the spores in the tube (Warne et al., 1986). The spores were resuspended in a measured amount of sterile distilled water, sown onto the medium and spread with a sterile wire loop. A constant spore density was maintained for all experiments by sowing 5 drops of a standard spore suspension containing ca. 0.036 mg spores/0.1459 ml water (ca. 0.1459 ml/5 drop), giving a spore density in cultures of ca. 20 spores/cm^2. For some experiments, gametophytes were transferred to and cultured in 60 x 20 mm petri plates. Twenty gametophytes were cultured in each 60 x 20 mm plate. All plates were placed in clean 24" x 48" plastic trays which were covered with clear plastic domes.

All experiments were timed from the initial soaking of the spores (time 0) to a specified number of days following soaking (DFS) or from the initial transfer of gametophytes (time 0) to a specified number of days from transfer (DFT). For gametophyte experiments, cultures were maintained under standard conditions of light (ca. 90 µmoles m^{-2} s^{-1}) and temperature 27 $\pm$ 2° C for the duration of the experiment.

Germination Counts

The effect of glyphosate on spore germination was determined by sowing spores obtained from glyphosate tolerant mutant lines and the wild type on media supplemented with glyphosate (0-1.0 mM). The percentage of germinated spores was determined by viewing these culture plates with a dissecting microscope at a magnification of 25x. Spores in successive fields of view were counted until 100 spores were scored for germination. Germination was defined as the visible emergence of the primary rhiziod. Germination counts were taken at 4, 6, 8, 10, and 12 DFS.

Measurement of Gametophyte Growth

Gametophyte growth was measured at specific days from transfer (DFT). Multispore cultures of gametophytes contained two morphological types: meristematic bisexual cordate forms which are essentially two dimensional, and small ameristic male gametophytes (Hickok et al., 1987). Male gametophytes were not scored since their growth was determinate. Cordate gametophytes sampled from each experiment were mounted on slides and stained using a 4:1 (v:v) mixture of Hoyers and 0.5% acetocarmine and covered with a cover slip. Slides were viewed at a magnification of 25x and gametophyte areas determined using a Bioquant IV^{Tm} image analysis system with a hipad digitizer connected to an IBM personal computer.

Generation and Maintenance of Homozygous Sporophytes

M2 generation sporophytes of the selected mutant lines were obtained by culturing spores of M1 generation sporophytes on nutrient medium under standard conditions. Individual mature bisexual gametophytes were isolated at days 10-14 and watered with sterile distilled water to stimulate the release of spermatozoids in order for self fertilization to occur. Gametophytes were watered repeatedly at 7 day intervals until young sporophytes were observed.

Hybrid combinations of the selected mutants were generated by isolating young cordate gametophytes of the intended female parent, possessing 2-4 archegonia and few or no antheridia, and inundating them with a spermatozoid solution obtained from the gametophyte culture of the intended male parent. The spermatoziod solution was obtained by flooding the male stock culture plate with sterile distilled water. The plate was monitored for the release of spermatozoids and, after five to ten minutes, the sperm suspension was collected using a sterile pastuer pipette and bulb and transferred to the isolates. After three days, the attempted crosses were examined under a dissecting microscope for the presence of embryos. Unsuccessful crosses which did not produce an embryo were discarded. Homozygous and putative hybrid sporophytes were cultured under standard conditions until they were large enough to be transplanted to the greenhouse in styrofoam cups containing a mixture of peat, vermiculite and perlite (2:1:1). The cups were continuously kept moist by making holes in the base of the cups and placing them in trays with water. The

water in the trays was periodically replaced by an influx of fresh water. All sporophytes were fertilized weekly.

Hybrid Confirmation

Putative hybrid combinations of the selected mutant strains and the wild type were confirmed by the segregation of mutant (tolerant) to wild type (sensitive) type gametophytes derived from the F1 sporophyte generation on nutrient media supplemented with 0.1 mM glyphosate. All sporophytes which showed mutant and wild type segregation at this concentration of glyphosate were considered true hybrids. On this basis, two reciprocal hybrid sporophytes between HαG492-10 and the wild type, F1(HαG492 x Hn-n)2 and F1(Hn-n x HαG492)10 were selected for further studies. For the HαG343 mutant, two hybrid sporophytes, F1(HαG343 x Hn-n)12 and F1(Hn-n x HαG343)3, were selected. Several reciprocal crosses between the mutants, F1(HαG492 x HαG343)n and F1(HαG343 x HαG492)n were also selected using this technique. In each combination the egg parent is listed first and underlined.

Gametophyte Dose Response Test

Gametophyte dose response tests were carried out using spores from the selected glyphosate tolerant mutant lines and the wild type over glyphosate concentrations of 0, 0.01, 0.05, 0.1, 0.5 and 1.0 mM. The gametophytes were first cultured on basal medium. At the visible emergence of a primary rhiziod, the gametophytes were randomly sampled and transferred to glyphosate supplemented medium. Twenty isolated

gametophytes were cultured in a 60 x 20 mm petri plate and a total of 60 gametophytes were sampled at each concentration of glyphosate for each strain. Gametophytes were cultured for 14 DFT and then stained and mounted on slides. For all gametophyte dose response tests, the areas of the gametophytes were measured and quantified as previously described.

Segregation Analysis of Hybrid Combinations

Segregation analysis was carried out on reciprocal hybrid combinations of the selected mutants and the wild type. Spores obtained from the hybrid combinations and the parental strains Hn-n, HαG492-10 and HαG343-1 were sown and cultured under standard conditions on basal medium. At the visible emergence of a primary rhiziod, individual gametophytes were transferred to 60 x 20 mm petri plates containing media supplemented with 0.1 mM or 0.4 mM glyphosate. Transfers of developmentally uniform gametophytes were carried out over a 24 hour period at approximately 20, 40, and 60% germination. Twenty gametophytes were transferred to each 60 x 20 mm petri plate and a total of 50 or 200 gametophytes were transferred for each hybrid combination. Cultures were maintained under standard conditions for 14 DFT. At 14 DFT gametophytes were visually (qualitatively) scored for sensitivity or tolerance to 0.1 mM or 0.4 mM glyphosate: Sensitive gametophytes were dead after ca. four days culture while the tolerant gametophytes were alive after two weeks. The gametophytes were then stained and mounted on slides. Chi square analysis using Yates correction was carried out on mutant and wild type hybrid combinations of both mutants to test a single

gene mode of inheritance for the two mutations. For quantitative assessment, areas of the gametophytes were measured as previously described.

Sporophyte Dose Response Test

Sporophytes of the selected mutants, HαG492-10, HαG343-1, and the wild type were generated by sowing and culturing for twelve days gametophytes of the individual lines under standard conditions on basal medium adjusted to a pH 5.4. The plates were then flooded with sterile distilled water to stimulate the release of swimming spermatozoids. After 2-3 weeks, small sporophytes were transferred to liquid culture on full strength Parker/Thompson inorganic nutrient medium (pH 6.0) and acclimated under continuous illumination (ca. 87 μMoles m^{-2} s^{-1}) and at ca. 31° C for 3 days. The sporophyte dose response test was carried out over glyphosate concentrations of 0, 0.5, and 1.0 mM glyphosate. Nine small sporophytes of each strain were collectively weighed under sterile conditions in moist, preweighed small petri plates and then subjected to a 4 hr pulse treatment in 125 ml of liquid medium adjusted to a pH of 6.0 and supplemented with glyphosate and 0.01% Tween. Flasks were shaken every 1/2 hr to facilitate complete wetting of the sporophytes. After four hours, the sporophytes were rinsed 3 times with sterile distilled water using vacuum filtration. The sporophytes from each stock at each concentration of glyphosate were separated into groups of 3 sporophytes and placed into 250 ml flasks containing 150 ml of media. The sporophytes were then cultured under continuous illumination (ca. 87 μMoles m^{-2} s^{-1}) and at 31°

C for 8 days. Fresh weights of the sporophytes were obtained by weighing them in small, preweighed aluminum weighing boats. No initial dry weights were taken. Eight days after pulse treatment with glyphosate, the dry weights of the individual sporophytes at each concentration of glyphosate were obtained after they were dried over night at 80^0 C. Mean Relative growth rate (fresh weight basis) was calculated for each sporophyte using the equation:

$$R = \frac{Ln(W_2) - Ln(W_1)}{t_2 - t_1}$$

where W_2 = final mass, W_1 = initial mass (calculated as the average of the nine preweighed individuals) and t_2 and t_1 are final and initial times respectively (Singer, 1985).

Sporophyte Dose Response of Hybrids

Two approaches were used to test the response of hybrid sporophytes to glyphosate. In one approach, sporophyte dose response tests were carried out on sporophytes of the confirmed hybrid F1(HαG343 x HαG492)8 and the wild type, Hn-n. Sporophytic tissue for this sporophyte dose response test was obtained by vegetatively cloning the F1 hybrid and wild type sporophytes. Very small buds were collected so that a triangle of sporophyte tissue was left around the bud. The buds were sterilized in a 1:4 solution of commercial bleach (5.25% Na hypochlorite by weight) and water for one minute. The buds and chlorox solution were

then poured into a Buchner funnel and washed with 200 ml of sterile distilled water. The buds were cultured in 150 ml of full strength Parker/Thompson inorganic medium (pH 6.0) in styrofoam cups for 2-3 weeks. The sporophytes were transferred to new media every 5-7 days. Cultures were maintained under continuous illumination (ca. 87 μmoles m^{-2} s^{-1}) and at 31° C. After 2-3 weeks the sporophytes were weighed, pulsed in media supplemented with 0 and 1.0 mM glyphosate and 0.01% Tween, as previously described, and cultured under standard conditions of light and temperature for 8 days. The fresh and dry weights of the sporophytes were obtained and the mean relative growth rates calculated and analyzed as previously outlined. While this technique yielded good results, there were many difficulties in obtaining and culturing developmentally uniform sporophytes. In preliminary experiments (data not shown) this resulted in high variability in the response of the sporophytes to glyphosate within treatments and strains. Because of the difficulties inherent in this technique a second method was developed to obtain more developmentally uniform sporophytes.

With the second technique, sporophyte dose response tests were carried out on the parental sporophyte strains Hn-n, HαG492-10, HαG343-1 and 57-45. The 57-45 strain is a synthesized marker stock containing a recessive gene for paraquat tolerance (pq45) (Hickok and Schwarz, 1986) and a mutation which results in 100% cordate gametophytes (HaC57) (Warne et al., 1988). Included in this dose response test were confirmed hybrid sporophytes F1(57-45 x HαG492) and F1(57-45 x HαG343) which were synthesized from 57-45 and the mutant strains

HαG492-10 and HαG343-1. These sporophytes were generated by sowing and culturing for twelve days gametophytes of the selected strains under standard conditions on basal medium adjusted to a pH of 5.4. At day twelve, sperm was obtained from HαG492-10 and HαG343-1 and used to fertilize cordate gametophytes of 57-45 in order to obtain putative F1(57-45 x HαG492) and F1(57-45 x HαG343) hybrids. The remaining plates of Hn-n, HαG492-10 and HαG343-1 and 57-45 were watered with glass distilled sterile water to obtain completely homozygous sporophytes. After two weeks, small sporophytes were transferred to liquid culture on full strength Parker/Thompson inorganic nutrient medium (pH 6.0) and acclimated under continuous illumination (ca. 87 μmoles m^{-2} s^{-1}) at 31° C for one week.

Putative hybrids of F1(57-45 x HαG492) and F1(57-45 x HαG343) were confirmed by testing their sensitivity (bleaching) to 0.5 μM paraquat; self fertilized 57-45 sporophytes would be homozygous recessive for the paraquat marker and would therefore exhibit tolerance (no bleaching) to 0.5 μM paraquat while true hybrid sporophytes, heterozygous for the recessive paraquat marker, would show sensitivity to paraquat. Putative hybrids were confirmed by removing a young leaf from the sporophytes and placing each leaf separately in 2 ml of 0.5 μM paraquat in 24 cell well plates. Sporophyte leaves were randomly sampled from wild type and mutant strains, floated in plain distilled water and used as a control. In order to maintain uniformity among all treatments and strains, leaves were snipped from the sporophytes of the remaining strains. Sporophytes that were being tested for their sensitivity to paraquat were cultured separately

in 20 ml scintillation vials over night. Putative hybrid sporophytes that showed sensitivity to 0.5 μM paraquat were considered to be confirmed hybrids. The confirmed hybrid sporophytes and the parental stocks were then cultured for an additional week under standard conditions of light and temperature.

The sporophyte dose response test was carried out at glyphosate concentrations of 0 and 1.0 mM. Nine small sporophytes from each strain were collectively weighed under sterile conditions in moist preweighed petri plates and then subjected to a four hour pulse treatment with glyphosate. Pulse treatment with glyphosate was carried out as previously described. Fresh and dry weights were obtained and the mean relative growth rates calculated as previously described. Statistical comparisons were made with Tukey's Studentized Range (HSD) test using PC-SAS^{R}.

IV. RESULTS

Characterization of Mutants

Gametophyte Characterization

The selected mutant lines HαG492-10 and HαG343-1 were sown and cultured on media supplemented with glyphosate (0-1.0 mM) and then quantitatively characterized. In order to determine the effect of glyphosate on spore germination, germination counts were taken at days 4, 6, 8, 10, and 12. The data presented in Figure 3 show the comparison of germination at 0 mM glyphosate for the two mutant lines and the wild type over days 4-12 . The HαG343-1 mutant line showed slower initial germination than the HαG492-10 mutant and the wild type. However, all three strains exhibited similar maximum germination at 12 DFS in the absence of glyphosate. The percentage of maximum germination at day 12 over glyphosate concentrations of 0-1.0 mM is shown in Figure 4. Germination of the wild type and HαG343-1 was affected by increasing concentrations of glyphosate. In comparison, germination of the HαG492-10 mutant line was relatively unaffected.

The growth of the gametophytes at 14 DFT expressed as size versus treatment concentration is shown in Figure 5. In the absence of glyphosate, both mutants closely paralleled the wild type in gametophyte size with the HαG492-10 mutant showing slightly enhanced growth over the wild type. Both mutants showed greater tolerance to increasing concentrations of glyphosate than the wild type with the HαG343-1 mutant showing slightly less tolerance than HαG492-10. To eliminate absolute

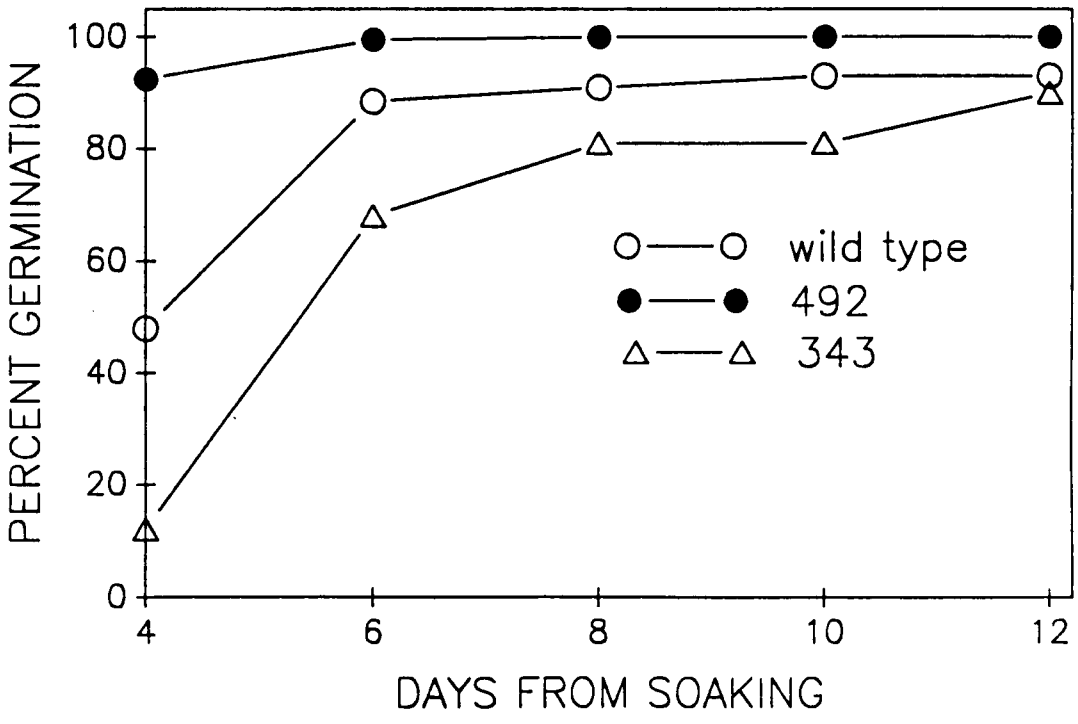

Figure 3. Germination in multispore sowings of the wild type and two mutant strains at 0 mM glyphosate over days 4-12 (Symbols represent means of two samples).

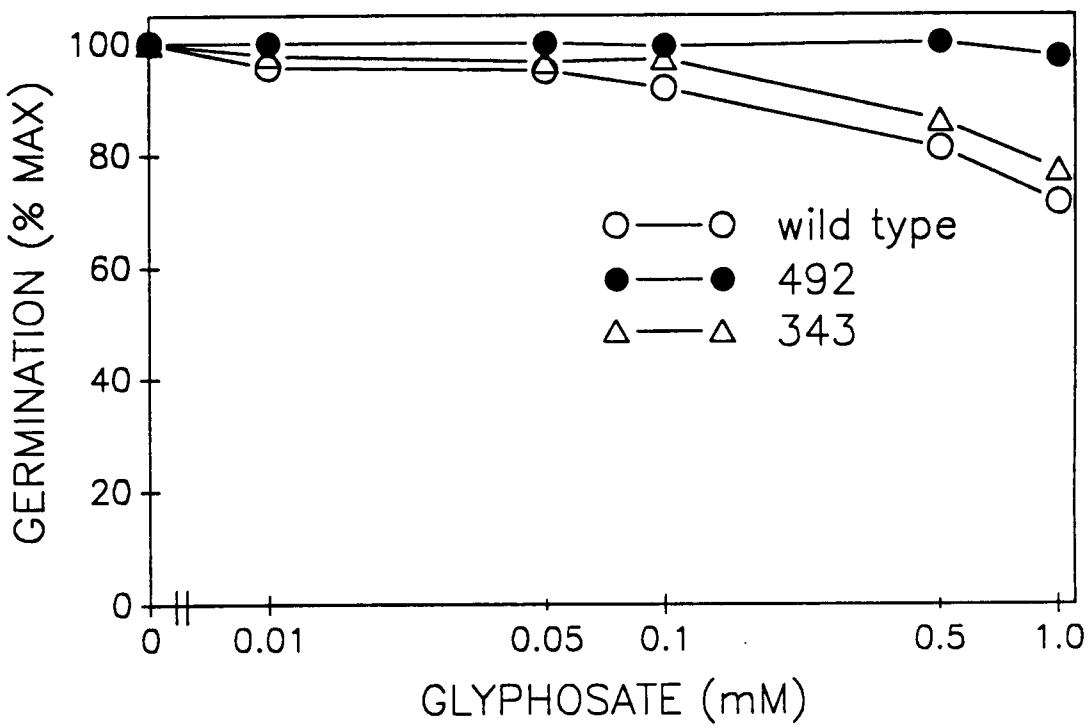

Figure 4. Germination response to glyphosate, in multispore sowings, of the wild type and two mutant strains expressed as a percent of the maximum germination at 12 DFS (Symbols represent means of two samples).

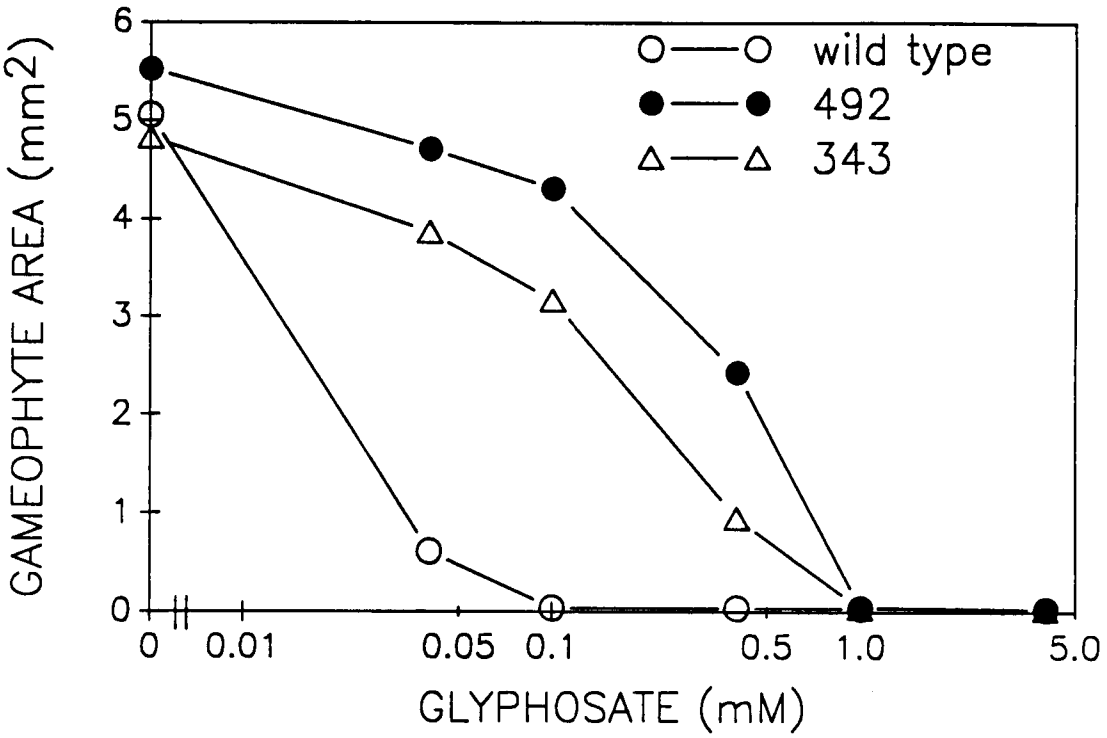

Figure 5. Gametophyte area at 14 DFT of surface growing cordates of the wild type and two mutants over a range of glyphosate concentrations (Symbols represent means ± SE, note that the error bars are smaller than the symbols); n ranged from 37-60.

growth differences, the data in Figure 6 are presented as a percent of the 0 mM control. Both mutants clearly showed increased tolerance to glyphosate over the wild type with the HαG343 mutant showing slightly less tolerance than the HαG492 mutant.

Sporophyte Characterization

Homozygous sporophytes of the selected mutants and the wild type were quantitatively characterized by weighing, pulse treating sporophytes with media supplemented with glyphosate (0, 0.5, 1.0 mM) and then culturing in liquid medium for 8 days. After 8 days the sporophytes were again weighed and the mean relative growth rates calculated. Comparison of the mean relative growth rates for the selected mutants and the wild type is shown in Figure 7. All strains showed similar mean relative growth rates in the absence of glyphosate. Both mutants exhibited increased tolerance relative to the wild type over glyphosate concentrations of 0-1.0 mM with the HαG343-1 mutant showing less tolerance than the HαG492-10 mutant. The effect of glyphosate on sporophyte growth was also visually apparent. The wild type showed severe chlorosis of the meristem and new shoot growth, a reduction in the growth of new roots and severe necrosis in older roots when compared to the control. Sporophytes of the HαG343-1 mutant were less affected by treatment with glyphosate than the wild type, showing only slight chlorosis in older leaves while the meristem and new growth remained green. New shoot and root growth in these sporophytes was only slightly reduced in comparison to the control.

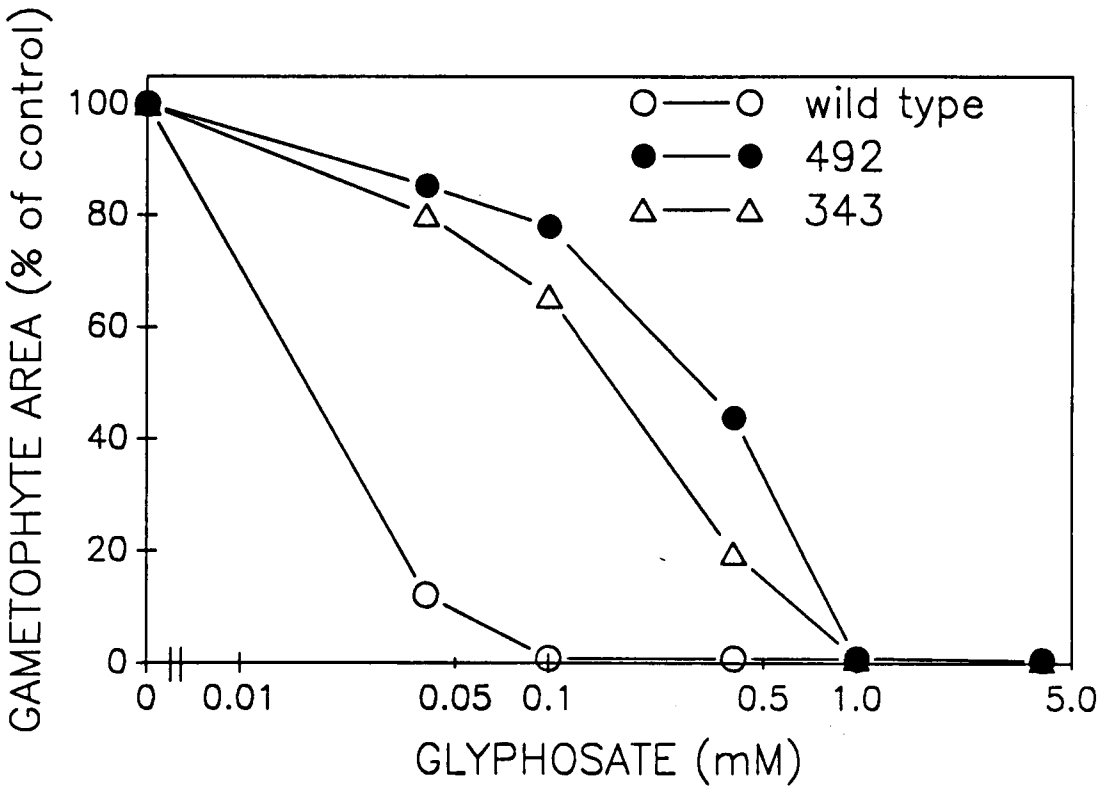

Figure 6. Gametophyte area at 14 DFT of surface growing cordates of the wild type and two mutants over a range of glyphosate concentrations, presented as a percent of the 0 mM control (Symbols represent means ± SE, note that the error bars are smaller than the symbols); n ranged from 37-60.

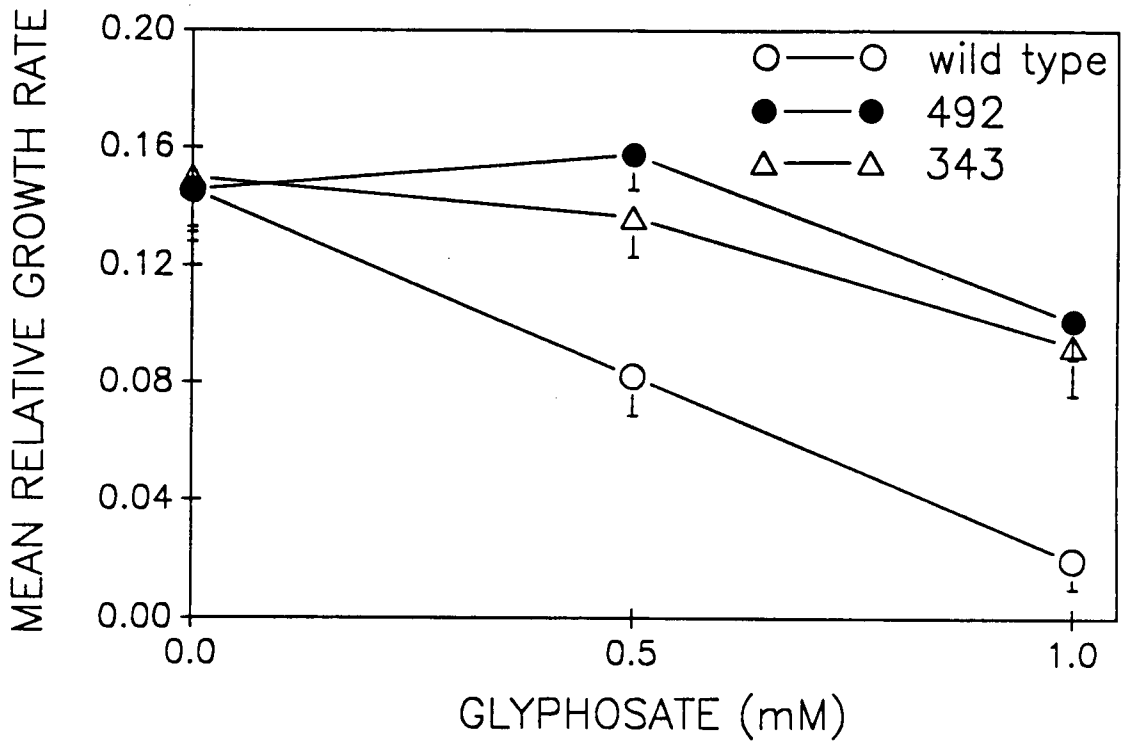

Figure 7. Mean relative growth rates determined from the fresh weights of sporophytes from homozygous parental strains eight days after pulse treatment with 0, 0.5 and 1.0 mM glyphosate (Symbols represent means $\pm$ SE); n=9 at each concentration of glyphosate for each strain.

The HαG492-10 mutant appeared to be relatively unaffected by treatment with glyphosate, showing no chlorosis and negligible reduction in shoot and root growth.

Genetic Analysis

Gametophytic Genetic Analysis

Segregation tests were carried out in order to determine the inheritance of both mutations. Gametophytes from F1 hybrid sporophytes were monitored for their response to glyphosate in comparison to the parental types.

Analysis of Single Mutant Hybrids. Because of the effect of glyphosate on spore germination (Figure 4), gametophytes were first sown on basal medium and then, at the visible emergence of a primary rhiziod, transferred to media supplemented with glyphosate. Transfers were carried out over a 24 hr. period at approximately 20, 40 , and 60% germination. Slides were made at 14 DFT. Gametophytes were assessed both visually (qualitatively) and quantitatively by measuring as previously described.

In initial gametophyte dose response tests, qualitative assessment of the reciprocal crosses for the HαG492-10 mutant, F1(Hn-n x HαG492)10 and F1(HαG492 x Hn-n)5 and for the HαG343-1 mutant, F1(Hn-n x HαG343)3 and F1(HαG343 x Hn-n)12, showed an approximate 1:1 segregation ratio of tolerant and sensitive gametophytes (Table 2). The behavior

Table 2. Segregation of Glyphosate Tolerant and Sensitive Gametophyte Types from F1 Hybrid Sporophytes[a].

F1 Hybrid Combination	Segregation[b]		
	Sensitive	Tolerant	X^{2c}
(Hn-n x HαG492)10	24	26	1:1 (P > 0.5)
(HαG492 x Hn-n) 2	25	23	1:1 (P > 0.5)
(Hn-n x HαG343) 3	20	25	1:1 (P > 0.5)
(HαG343 x Hn-n)12	26	22	1:1 (P > 0.5)

[a]Spores were sown on basal medium and at the visible emergence of a primary rhiziod transferred to media supplemented with 0.1mM Glyphosate.

[b]Hybrids between the mutants and the wildtype segregated for two gametophyte types: sensitive (dead after ca. four days culture) and tolerant (alive after two weeks).

[c]X^2 tests, using Yeats correction for continuity, were based on a hypothesis of segregation at a single locus and a resulting 1:1 ratio.

of the reciprocal crosses in each combination was similar, suggesting a nuclear basis for both mutations. Because of this, only the F1(Hn-n x HαG492)10 and F1(Hn-n x HαG343)3 hybrids were included in subsequent gametophyte tests to further document the inheritance of the two mutations.

Quantitative comparisons of parental responses and segregation from F1 hybrids were made by measuring segregating gametophyte types from the selected strains at a specific concentration of glyphosate. Figure 8 shows the responses to 0.1 mM glyphosate for the HαG492-10 mutant and the wild type. The size ranges of the two parental types were clearly distinguished. The wild type had a mean of 0.048 ± 0.013 mm^2. The mutant showed a wider range of response than the wild type with a mean of 6.649 ± 1.090 mm^2. The response of the F1(Hn-n x HαG492)10 mutant is shown in Figure 9. Two distinct size classes were evident, one with a mean of 0.046 ± 0.012 mm^2 and the other with a mean of 5.590 ± 0.987 mm^2. Although the size range for the tolerant segregants is somewhat smaller than that observed for the HαG492 mutant (Figure 8) visual observations (prior to slide preparation) clearly indicated that they were HαG492-10 types. The size difference may be associated with slight environmental differences between the two experiments or with some undefined epigenetic factor(s). Of 181 gametophytes, 94 showed a wild type response suggesting a 1:1 ratio and a single gene basis of inheritance. Chi-square analysis indicated that this deviation from expected is within the range expected by chance alone ($X^2 = 0.199$, $p > 0.5$ and $df=1$). Figure 10 shows the parental response for the HαG343-1 mutant and the wild type. The wild

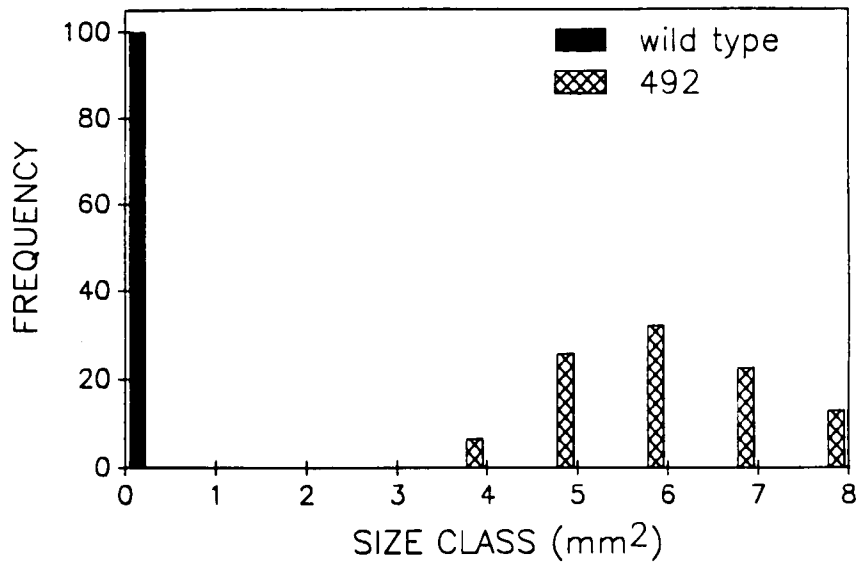

Figure 8. Growth of gametophytes at 14 DFT from homozygous parental strains on medium supplemented with 0.1 mM glyphosate; n=38 for the wild type and n=32 for HαG492-10.

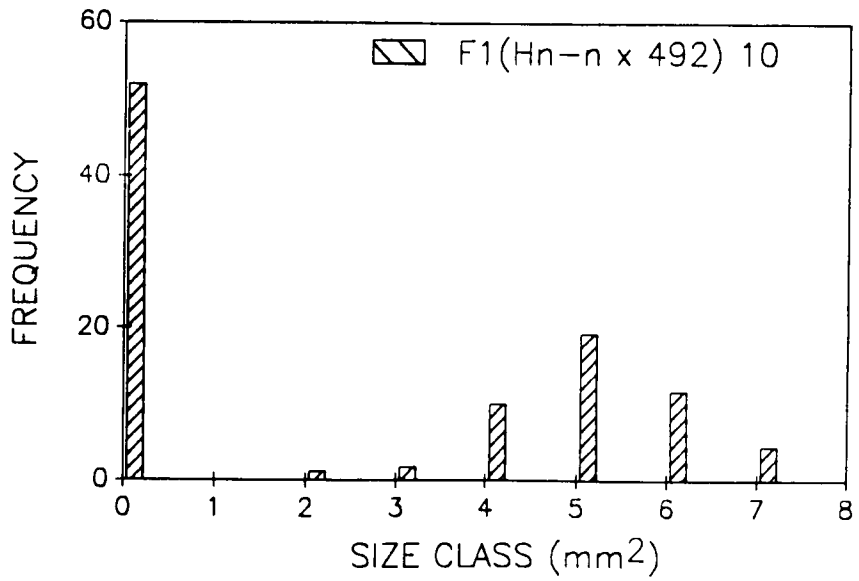

Figure 9. Growth of gametophytes at 14 DFT from the F1(Hn-n x HαG492)10 hybrid on medium supplemented with 0.1 mM glyphosate; n = 181.

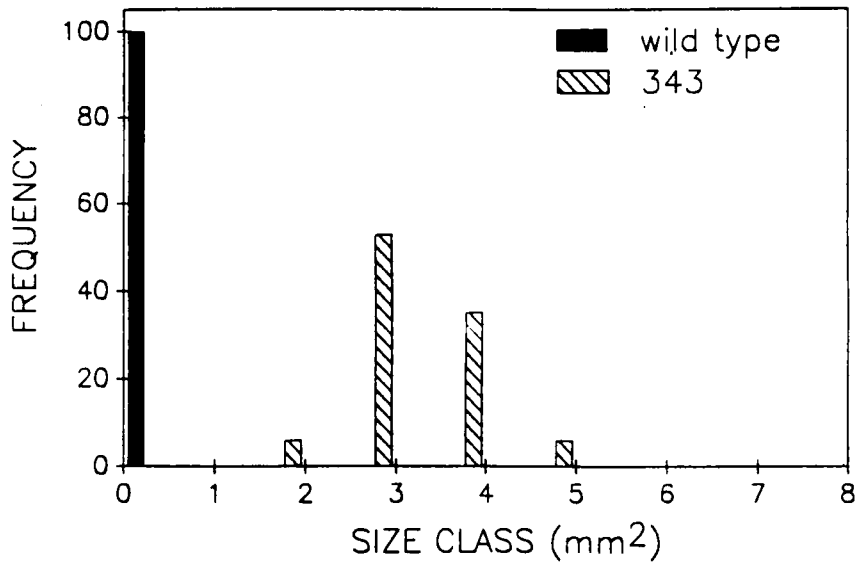

Figure 10. Growth of gametophytes at 14 DFT from homozygous parental strains on medium supplemented with 0.1 mM glyphosate; n=38 for the wild type and n=17 for HαG343-1.

type had a mean of 0.048 ± 0.013 mm^2 and the mutant had a mean of 3.981 ± 0.679 mm^2. A wider range of response types was evident for the mutant. The response of the F1(Hn-n x HαG343)3 hybrid is shown in Figure 11. The segregation pattern from this hybrid was more continuous than that observed for the parental types (Figure 10). Several gametophytes segregated into a 1 mm^2 size class which does not overlap segregation patterns from either HαG343-1 or the wild type. With qualitative analysis (prior to slide preparation), clear distinction could be made between the wild type, which were dead 14 DFT at this concentration of glyphosate, and tolerant gametophytes which were still green. The gametophytes which segregated into the 1 mm^2 size class were clearly tolerant and were therefore grouped into the HαG343-1 class for purposes of analysis. Thus, two size classes were evident, one with a mean of

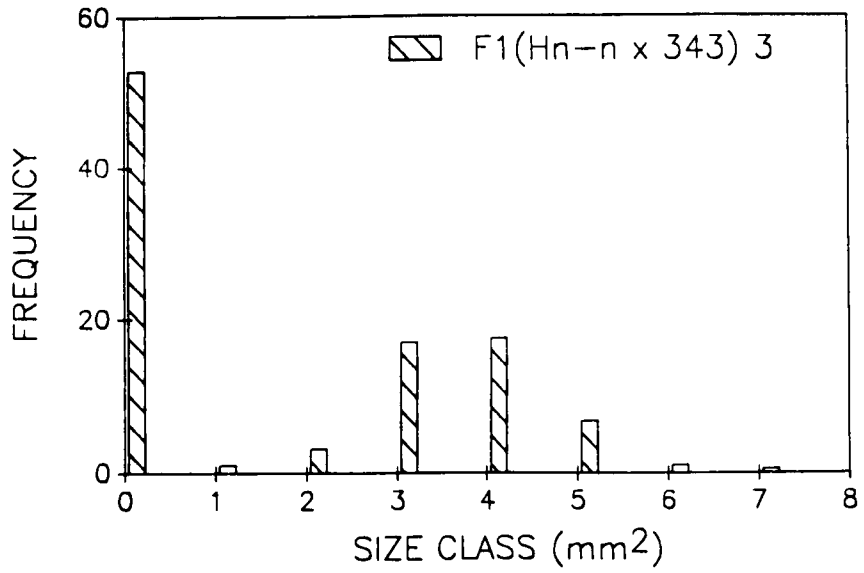

Figure 11. Growth of gametophytes at 14 DFT from the F1(Hn-n x HαG343)3 hybrid on medium supplemented with 0.1 mM glyphosate; n=193.

0.053 ± 0.059 mm^2 and the other with a mean of 4.169 ± 0.976 mm^2. Of 193 gametophytes, 102 showed a wild type response suggesting a 1:1 segregation ratio (X^2=0.518, p> 0.1 and df = 1) and a single gene basis of inheritance.

Analysis of Double Mutant Hybrids. Segregation analyses of the double mutant hybrid were carried out by sowing gametophytes on basal medium and then, at the visible emergence of a primary rhizoid, transferring them to media supplemented with glyphosate. Transfers were carried out over a 24 hr period at approximately 20, 40, and 60% germination.

Qualitative analysis were carried out prior to the preparation of slides which were made at 14 DFT. Gametophytes were also assessed quantitatively by measuring the gametophytes as previously described.

Initial segregation tests were carried out at 0.05 and 0.1 mM glyphosate. Sensitive wild type and tolerant mutant gametophytes types could clearly be visually (qualitatively) distinguished at these concentrations of glyphosate. However, no distinction could be made between the mutants. The combined data from several experiments, which included several different hybrid sporophytes of the same combination, showed that the reciprocal crosses for the HαG492-10 and HαG343-1 mutants at these concentrations of glyphosate had a similar segregation pattern of sensitive and tolerant gametophyte types. Of 339 gametophytes, 252 were tolerant and 87 sensitive for the F1 (HαG492 x HαG343)n combination and for the F1(HαG343 x HαG492)n combination, 317 were tolerant and 63 sensitive out of a total of 380 gametophytes. Because of the similarity from reciprocal crosses only a single hybrid, (HαG343 x HαG492)8, was used in additional tests for segregation.

In order to more clearly separate segregating tolerant gametophyte types additional tests for segregation were attempted at a higher concentration of glyphosate (0.4 mM) and the data were evaluated both quantitatively and qualitatively. Figure 12 shows the parental responses to 0.4 mM glyphosate. The wild type and tolerant gametophyte types could be clearly distinguished but there was still an area of overlap between the HαG343-1 and HαG492-10 parental types.

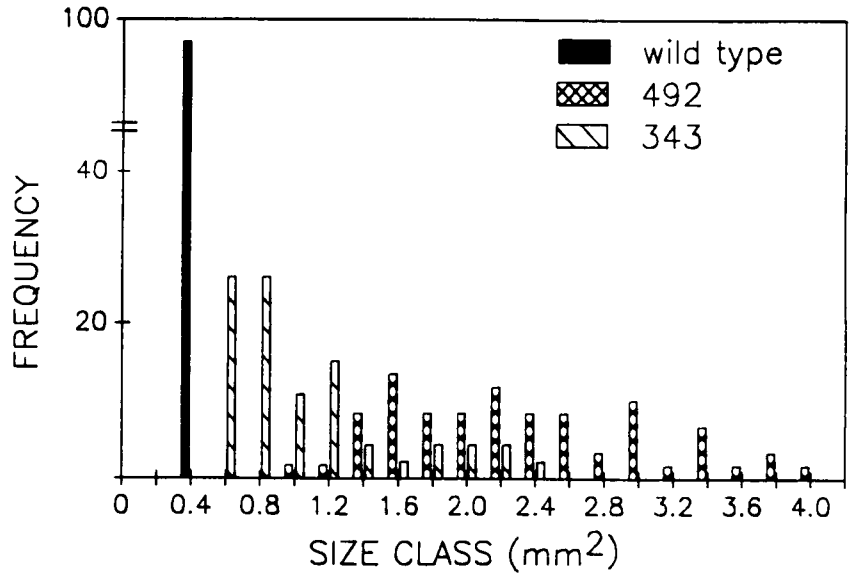

Figure 12. Growth of gametophytes at 14 DFT from homozygous parental strains on medium supplemented with 0.4 mM glyphosate; n=59 for the wild type, n=59 for HαG492-10 and n=46 for HαG343-1.

Figure 13 shows the segregation pattern for the F1(HαG343 x HαG492)8 hybrid on 0.4 mM glyphosate. Out of 148 gametophytes 25 showed the wild type phenotype and had a mean of 0.049 mm^2 (Figure 12). The remaining tolerant gametophytes could not be separated into distinct classes, although the size ranges of both parental types were evident. Because there was a significant area of overlap in the size ranges, accurate counts of these size classes could not be made. It was evident that the distribution was highly skewed, with a large group of gametophytes segregating into the size range representing the HαG343-1 parental type (Figure 12).

With visual analysis a more accurate count of the separate segregating tolerant gametophyte types versus sensitive gametophytes was obtained. The wild type could clearly be distinguished from the mutant

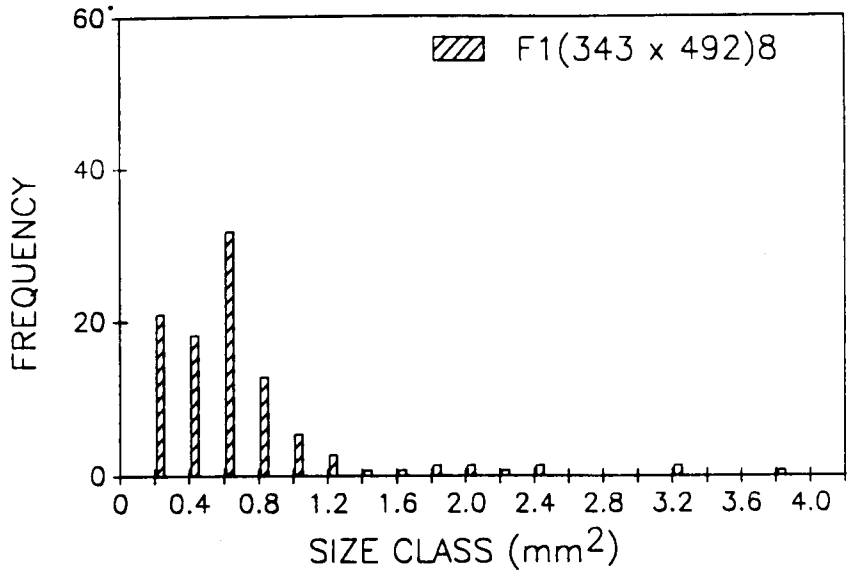

Figure 13. Growth of gametophytes at 14 DFT from the F1(HαG343 x HαG492)8 hybrid on medium supplemented with 0.4 mM glyphosate; n=148.

gametophytes and was dead 14 DFT on 0.4 mM glyphosate. The HαG492-10 type was alive 14 DFT and still retained a green color and normal morphology. Gametophytes of the HαG343-1 mutant were bleached and exhibited abnormal morphology 14 DFT. Qualitative data from three similar experiments carried out at 0.4 mM glyphosate have been combined. Of 447 gametophytes, 300 (67.1%) showed the HαG343-1 phenotype, 54 (12.1%) exhibited the HαG492 -10 phenotype and 93 (20.8%) showed the wild type phenotype. Since a fourth class of gametophytes could not be resolved, gametophytes were grouped into sensitive and tolerant gametophyte types for chi square analysis. If the mutants involve different and unlinked loci, then an F1 hybrid of the genotype (343/+, +/492) would segregate for ++, G343 +, + G492 and G343 G492 types in a 1:1:1:1 genotypic ratio or a 1:3 phenotypic ratio of sensitive to tolerant gametophytes. Chi square analysis does not support the hypothesis of a 1:3

phenotypic ratio (X^2 = 3.974, $p<0.05$, df=1). Therefore, the mutations may be linked.

Further analysis of the data from several segregation tests of the double mutant hybrid lends support to this interpretation. Although these segregation tests were carried out at different concentrations of glyphosate (0.05, 0.1, 0.4, 0.5 mM), in each case clear visual distinction could be made between tolerant and sensitive gametophyte types. On this basis, data from seven separate experiments have been combined. Of 1491 gametophytes, 1192 were tolerant and 299 sensitive. The sensitive gametophytes represent 20.1% of the total sample size. Chi square analysis of this data does not support the 1:3 phenotypic ratio (X^2=19.193, $P<0.001$, df=1). The mutations appear to be linked and approximately 40 map units apart.

The segregation of approximately 20% wild type (++) gametophytes indicates that there should be a corresponding percentage of gametophytes carrying both mutations (G343 G492). Sixty percent of the total sample should segregate as parental type gametophytes, 30% being (G343 +) types and 30% (+ G492) type gametophytes. However, in all segregation tests of the double mutant hybrid, based on both quantitative and qualitative assessments, there was a consistent segregation of a much greater number of HαG343 type gametophytes than HαG492. At the present time, a clear explanation for this segregation behavior is not apparent.

In a recent experiment, gametophytes exhibiting abnormal morphology were detected at a low frequency in cultures of the HαG343 mutant and in hybrid combinations derived from it. This occurrence was

not taken into account in any of the afore mentioned experiments and it is not clear at this point whether this finding is responsible for the unusual segregation pattern observed in the double mutant hybrid.

Sporophytic Genetic Analysis

Sporophyte dose response tests were carried out using hybrid sporophytes of the mutants and the wild type and the homozygous parental sporophyte. These tests were carried out to determine expression of the mutations in the homozygous and heterozygous diploid sporophytes. Sporophyte responses were assessed both quantitatively and qualitatively after pulse treatment with glyphosate. Quantitative assessment was carried out by comparing the mean relative growth rates (RGR) of cloned or hybrid sporophytes to the response of the parental types. Statistical comparisons were carried out using Tukey's Studentized Range (HSD) test with PC-SAS.

Comparison of Parental Types, Single and Double Mutant Heterozygotes. Sporophyte Dose response tests were carried out on newly generated homozygous sporophytes of the parental strains, Hn-n, 57-45, HαG492-10 and HαG343-1 and on hybrid sporophytes newly synthesized using 57-45 as the egg parent and either HαG343-1 or HαG492-10 as the sperm parent. These sporophytes were tested for hybridity (heterozygosity) for the pq45 marker as previously described. In addition, in order to compare the response of the double mutant hybrid [F1(HαG343 x HαG492)8] buds from a single confirmed mature hybrid were cloned and

their response to glyphosate compared to clones of the wild type sporophyte. These sporophytes are designated by an asterisk in Tables 3 and 4.

The RGR of the parental and hybrid sporophytes was calculated eight days after pulse treatment with 1.0 mM glyphosate and compared to the 0 mM controls (Table 3). The RGR of the single mutant hybrids and the parental strains was similar in the absence of glyphosate, ranging from 0.129 to 0.156. The cloned double mutant hybrid and the wild type (*), also showed similar mean relative growth rates in the absence of glyphosate, although they were somewhat lower than the newly generated sporophytes. On 1.0 mM glyphosate, a wide range of response types was evident with the mean relative growth rate ranging from 0.012 to 0.134 in the newly synthesized sporophytes and from 0.021 to 0.038 in the cloned material (*). For the synthesized sporophytes, both mutants showed greater tolerance to 1.0 mM glyphosate than the wild type and 57-45. Strain 57-45 exhibited greater sensitivity than the wild type to glyphosate. The HαG492-10 mutant showed the highest level of tolerance while the HαG343 mutant showed somewhat less. The F1(57-45 x HαG492) hybrid exhibited greater tolerance than the wild type and 57-45, but less tolerance than HαG492-10 mutant. In contrast, the F1(57-45 x HαG343) hybrid showed similar RGR in comparison to the wild type. The double mutant hybrid, F1(HαG343 x HαG492)8, showed tolerance to 1.0 mM glyphosate greater than the cloned wild type and similar to the (57-45 x HαG492) hybrid. Although statistical comparisons indicated that the percentage of control values for the strains, with the exception of HαG492,

Table 3. Relative Growth Rates of Sporophytes (fresh weight basis).

	Mean Relative Growth Rate ± SE[1]		
Strain	0.0 mM Glyphosate	1.0 mM Glyphosate	% of Control
Hn-n	0.129 ± 0.015	0.038 ± 0.006	[c]29.422 ± 4.823
57-45	0.149 ± 0.011	0.012 ± 0.009	[c]7.826 ± 6.048
HαG492-10	0.155 ± 0.007	0.134 ± 0.015	[a]86.206 ± 9.488
HαG343-1	0.140 ± 0.010	0.094 ± 0.040	[a,b]67.307 ± 10.029
F1(57-45 x 492)n	0.144 ± 0.010	0.063 ± 0.009	[b,c]43.786 ± 6.345
F1(57-45 x 343)n	0.156 ± 0.004	0.037 ± 0.009	[c]23.704 ± 5.638
*Hn-n	0.088 ± 0.009	0.021 ± 0.011	[c]23.678 ± 12.577
*F1(343 x 492)8	0.090 ± 0.006	0.038 ± 0.008	[b,c]42.561 ± 9.261

[1] n= 9 for each strain at each concentration of glyphosate.

[abc]Tukey's Studentized Range (HSD) test. Letters indicate means that are significantly different when tested at a 0.5% level of significance.

*Data represent results from a separate hybrid sporophyte dose response test in which sporophytic tissue was obtained by vegetatively cloning the selected F1 hybrid and wild type sporophytes. All other data were derived from newly synthesized sporophytes (see Materials and Methods).

were not significantly different, the strains were grouped in an order consistent with the interpretation stated above.

A comparison of the final dry weights for the various strains after pulse treatment with 0 and 1.0 mM glyphosate is shown in Table 4. Although comparison of the final dry weights expressed as a percentage of the zero control for the parental types and the hybrid combination show HαG343 and HαG492 to have slightly higher final dry weights than the wild type, no clear pattern of tolerance is apparent, in either the cloned or synthesized material. This finding is also supported by statistical comparisons of the % of control values.

Growth responses were also assessed qualitatively. Differences in the response of the sensitive and tolerant sporophyte types could be clearly distinguished. The wild type and 57-45 were visibly affected by pulse treatment with 1.0 mM glyphosate in comparison to the control. Sporophytes of the wild type and 57-45 showed slight chlorosis and some necrotic spots in older leaves and severe chlorosis in new leaves and the meristem. Treated sporophytes showed a severe reduction in the growth of new leaves in comparison to the control. Root growth in these sporophytes was also affected in that older roots were brown and necrotic and new root growth was inhibited. There was no visible difference between the responses of the wild type and 57-45. In contrast, the HαG492-10 mutant appeared to be relatively unaffected by pulse treatment with glyphosate, showing no chlorosis or necrosis and only a slight reduction of root and shoot growth growth in comparison to the control. Sporophytes of the HαG343-1 mutant showed slight chlorosis of older leaves but the

Table 4. Dry Weights of Sporophytes Following Glyphosate Treatments.

	Mean Dry Weight ± SE[1]		
Strain	0.0mM Glyphosate	0.1mM Glyphosate	% of Control
Hn-n	0.016 ± 0.002	0.011 ± 0.001	**b**72.456 ± 4.236
57-45	0.015 ± 0.001	0.010 ± 0.001	**b**65.568 ± 5.266
HαG492-10	0.017 ± 0.001	0.017 ± 0.002	**a**104.960 ± 12.630
HαG343-1	0.016 ± 0.002	0.014 ± 0.001	**a,b**88.051 ± 7.036
F1(57-45 x 492)n	0.016 ± 0.001	0.012 ± 0.001	**a,b**77.355 ± 6.806
F1(57-45 x 343)n	0.015 ± 0.001	0.011 ± 0.001	**a,b**75.344 ± 4.412
*Hn-n	0.023 ± 0.002	0.015 ± 0.002	**b**65.923 ± 7.430
*F1(343 x 492)8	0.020 ± 0.001	0.017 ± 0.001	**a,b**77.753 ± 5.444

[1] n = 9 for each strain at each concentration of glyphosate.

abcTukey's Studentized Range (HSD) test. Letters indicate means that are significantly different when tested at a 0.5% level of significance.

*Data represent results from a separate hybrid sporophyte dose response test in which sporophytic tissue was obtained by vegetatively cloning the selected hybrid sporophytes, pulse treating them with glyphosate and obtaining the dry weights after eight days.

meristem and new shoot growth remained green. Root growth was slightly affected, although some necrotic areas were present, new root growth remained green and was only slightly reduced in comparison to the control. Hybrid combinations F1(57-45 x HαG492) and F1(57-45 x HαG343) showed slight chlorosis in older leaves, new growth and meristem. Roots were also visibly affected, showing areas of necrosis. Both shoot and root growth was reduced in comparison to the control. Although treated sporophytes of the F1(57-45 x HαG492) hybrid appeared to have less reduction in shoot growth in comparison to the F1(57-45 x HαG343) hybrid, no clear visible distinction could be made between them. Sporophytes of the cloned wild type and F1(HαG343 x HαG492)8 hybrid were both visibly affected by pulse treatment with glyphosate. However, the meristem and the new shoot growth of the F1(HαG343 x HαG492)8 sporophytes remained green and these sporophytes showed less reduction in root growth in comparison to the control.

V. DISCUSSION

Characterizations of the two mutant lines (HαG492, HαG343) in both the haploid gametophyte and diploid sporophyte generation show enhanced tolerance relative to the wild type. The mutant strains showed different degrees of tolerance, both quantitatively and quantitatively, with the HαG492 mutant showing high tolerance and the HαG343 mutant showing moderate tolerance to glyphosate. With the exception of some slight developmental irregularities in gametophytes of HαG343, both mutants were indistinguishable from the wild type in the absence of glyphosate and tolerance is therefore not thought to be related to morphological differences. For both mutants, tolerance to glyphosate did not result in reduced fitness in the absence of the herbicide, with the HαG492 mutant consistently showing enhanced growth over the wild type. Germination of the wild type and one mutant strain, HαG343, was affected by increasing concentrations of glyphosate while the HαG492 mutant was unaffected.

The growth response of gametophytes from F1 hybrids for both mutants was analyzed relative to the response of the parental types to determine the mode of inheritance for tolerance. Segregation analysis of F1 (Hn-n x HαG492) and F1 (Hn-n x HαG343) reciprocal crosses clearly showed approximate 1:1 segregation of two gametophyte types in each case. This suggests that tolerance for each mutant strain is controlled by a single gene, with one response representing the wild type and the other representing the mutant allele response. Reciprocal crosses of both mutant

strains exhibited similar segregation patterns, indicating a nuclear gene basis for tolerance.

Segregation analysis of the double mutant hybrid F1(343 x 492)8 indicated the segregation of 3 gametophyte types: sensitive, low and moderate tolerant types. Attempts to resolve a fourth potentially highly tolerant class of gametophytes, presumably containing both mutations, were unsuccessful. The evidence suggests that the two mutations are in separate loci that are linked and are approximately forty map units apart.

From sporophyte dose response tests, both mutants showed enhanced tolerance to glyphosate over the wild type and 57-45, with the HαG343-1 mutant showing moderate tolerance. The 57-45 strain was more sensitive than the wild type to glyphosate. Although the reason for this is unclear, a similar pattern has been observed in 57-45 gametophytes under salt stress (Warne and Hickok, 1987). The F1 (57-45x HαG492) mutant showed greater tolerance to glyphosate but less tolerance than the HαG492-10 parental type. This indicates that the mutation is expressed as a semidominant trait. The F1(57-45 x HαG343) mutant and the wild type showed a similar sensitivity to glyphosate, indicating that the mutation is recessive. In tests of the double mutant hybrid, F1 (HαG343 x HαG492)8 showed greater tolerance to glyphosate than the wild type, apparently showing the expression of the semidominant mutation carried by the HαG492 mutant line. The mutations carried by the HαG343 and HaG492 strains have been assigned the designations, *g343* and *G492*, respectivity.

In the haploid gametophyte generation, both mutants are tolerant to 0.5 mM glyphosate whereas the wild type is dead at 0.01 mM glyphosate.

This is a fifty fold increase in tolerance over the wild type. In the diploid sporophyte generation both mutants were still alive up to 2 mM glyphosate (data not shown) whereas growth in the wild type was considerably affected by 0.5 mM glyphosate. Greenhouse tests to determine phenotypic tolerance levels in the sporophytes of the two mutant strains were not carried out. It is therefore difficult to determine if tolerance to glyphosate is at a commercial level. In other studies, the level of tolerance obtained in cell culture is varied, ranging from 0.5 mM in petunia cells (Shah et al., 1986) to 25 mM levels in a carrot cell line (Nafziger et al., 1984). In microbial systems, levels of tolerance of up to 50 mM glyphosate have been obtained (Sost et al., 1984).

This study reports the complete Mendelian genetic characterization of two mutations conferring tolerance to glyphosate at the whole plant level. Mendelian analysis has also been reported in a study of tomato plants that were transformed through the introduction of a bacterial gene which codes for a mutant EPSP synthase enzyme. Transformed regenerated plants were self pollinated and the progeny sprayed with the equivalent of 0.84 Kg/ ha of glyphosate. The ratio of tolerant to sensitive plants fit the expected 3:1 segregation ratio indicating that the mutant Aro A gene was inherited as a single locus (Fillatti et al., 1987). In another study, a carrot cell line which was doubly resistant to DL-5-methyl tryphtophan (5MT) and azedtidine-2-carboxylate (A2C) was fused with an amplified EPSP resistant cell line. The fused lines expressed resistance to 5MT in a semidominant fashion and expressed the A2C and glyphosate mutations as a dominant or semidominant trait in a line specific manner. This genetic analysis could

only be carried out at the somatic level because of the inability to regenerate plants from cell culture (Hauptmann et al., 1988).

A major problem with genetically engineered herbicide tolerance is that it often results in reduced fitness in comparison to the wild type in the absence of the herbicide. A selection system which screens for mutations conferring tolerance to glyphosate that can be directly assessed and genetically characterized in the haploid gametophyte and diploid vascular sporophyte plants has obvious advantages. In this study, one mutant, HαG492, consistently showed enhanced growth over the wild type in the absence of glyphosate and will therefore be of significant interest in further studies.

The genetic characterization of these mutants, provides a foundation for further studies to examine the molecular and biochemical basis for the mutations. It must first be determined whether tolerance in these mutants results from a change in the ability to uptake glyphosate. Tolerance to glyphosate has been associated with either an increase in the levels of EPSP or with a mutation in that enzyme which reduces it's affinity for glyphosate. Therefore, once it has been demonstrated that tolerance does not result form the inability to uptake glyphosate then further studies can be carried out to determine if there are increases in the levels of EPSP synthase or if changes have occurred in the enzyme which results in a reduced affinity for glyphosate.

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VITA

Paulette Ann Tai Chun was born February 14, 1964 in Balaclava, Saint Elizabeth, Jamaica. In 1980 she graduated from Mount Saint Joseph High School, in Mandeville, Jamaica. In 1985 she received a Bachelor of Science Degree in biology from Denison University, Granville, Ohio. In the Fall of 1986 she began her post-graduate studies at The University of Tennessee, Knoxville in the Botany Department.