1991

The Molecular Basis of Imidazolinone Herbicide Resistance in Arabidopsis Thaliana Var. Columbia.

Kanagasabapathi Sathasivan
Louisiana State University and Agricultural & Mechanical College

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

U·M·I

University Microfilms International
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
313/761-4700 800/521-0600

Order Number 9200089

The molecular basis of imidazolinone herbicide resistance in *Arabidopsis thaliana* var. Columbia

Sathasivan, Kanagasabapathi, Ph.D.

The Louisiana State University and Agricultural and Mechanical Col., 1991

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

THE MOLECULAR BASIS OF IMIDAZOLINONE HERBICIDE RESISTANCE IN *ARABIDOPSIS THALIANA* VAR. COLUMBIA

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment for the degree of
Doctor of Philosophy
in
The Department of Plant Pathology and Crop Physiology

By

KANAGASABAPATHI SATHASIVAN

B.Sc.(Ag)., Tamil Nadu Agricultural University, Coimbatore, India, 1978.
M.Sc.(Ag)., Tamil Nadu Agricultural University, Coimbatore, India, 1980.

May 1991

To my father,

with love, respect, and memory

of his hard work, perseverance, and honesty

ACKNOWLEDGEMENTS

I am very grateful to my major advisor Dr. N. Murai, Department of Plant Pathology and Crop Physiology for the excellent opportunity and training he provided me to learn plant molecular biology and for his valuable guidance and support. I thank Dr. L. M. Kitchen, my former major advisor, for initiating me on a graduate program at Louisiana State University (LSU) and providing me a departmental research assistantship. I am grateful to Dr. J. B. Baker, Professor and Head, Department of Plant Pathology and Crop Physiology for serving as an advisory committee member, his fatherly advise, sincere care and the research assistantship support he provided. I thank my minor advisor Dr. S. G. Bartlett, Department of Biochemistry for introducing me to biochemistry as an interesting subject and for providing technical guidance on my research. I am indebted to Dr. M. A. Cohn, Department of Plant Pathology and Crop Physiology, who served as a member in my advisory committee, for his constant appreciation, valuable suggestions and timely advice. Also, I thank Dr. J. V. Moroney, Department of Botany for his suggestions on enzyme assays and constructive criticism on my research project as an advisory committee member.

I am grateful to Dr. R. W. Schumacher of Monsanto Company, St.Louis, who served as a mentor in my career development in Monsanto. I thank Dr. C.R. Somerville, Michigan State University, East Lansing and Dr. G. W. Haughn, Department of Biology, University of Saskatchewan, Saskatoon, Canada for their generous cooperation to provide the imidazolinone-resistant *Arabidopsis thaliana* (GH90) and pGH1 ALS gene used in this study. Thanks are also due to Dr. S. Chang, Department of Biochemistry, LSU, for his

support and guidance in cloning the ALS gene. I thank Dr. M. C. Mehdy, Department of Botany, The University of Texas at Austin for her support and encouragement towards the completion of this project.

I thank the Department of Plant Pathology and Crop Physiology and the College of Agriculture, LSU for the research assistantship support. I thank the American Cyanamid Chemical Company for providing the herbicides imazapyr and imazaquin used in this research. I acknowledge the support of this research in part by the Louisiana State Educational Quality Support fund.

I immensely thank my friends and colleagues, Drs. K. V. Subbarao, W. S. Lee, A. Hayashimoto, P. Sen, C. Chlan and M. Burow and messrs S. Footit, Z. Li, Y. Kawagoe, P. Mohan and A. Sankaralingam for all their help and encouragement during my graduate program.

I am thankful for the blessings, basic education and integrity my late father, Mr. T. Kanagasabapathi bestowed on me. I am very grateful to my mother, Mrs. K. Sunthara-thammal for the love, sacrifice, confidence, and her best wishes for my success. I express profound thanks to my father-in-law, Mr. T.M. Nellaiyappan for taking care of all my family affairs in India and my mother-in-law, Mrs. N. Rathinaparvathi for helping us in the United States during our son's birth. I thank my children, Kanaka and Sarathi, for their love and sacrifice to let me concentrate on my research and dissertation work whenever needed. Most of all, I have no words to appreciate and thank the love, sacrifice, confidence, and constant encouragement of my wife Kanthimathi in the completion of this major ambition.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (Continued)

ABSTRACT

Acetolactate synthase (ALS) is a key regulatory enzyme in the biosynthetic pathway of leucine, isoleucine, and valine. ALS is inhibited by four classes of structurally unrelated herbicides viz. sulfonylureas, imidazolinones, triazolopyrimidines, and pyrimidyl-oxy-benzoate. To understand the molecular basis of imidazolinone resistance, the ALS gene was isolated from an imazapyr-resistant mutant GH90 of *Arabidopsis thaliana* (Haughn and Somerville, 1990. Plant Physiol. 92:1081-1085). DNA sequence analysis of the mutant gene demonstrated a single point mutation from G to A at the 1958th nucleotide of the ALS coding sequence (Sathasivan et al.,1990. Nucl. Acids Res. 18:2188). This would result in serine to asparagine substitution at the 653rd amino acid, near the carboxyl terminal of matured ALS. The mutant ALS gene was introduced into tobacco using *Agrobacterium*-mediated transformation. The presence of the introduced ALS gene was confirmed by a southern hybridization analysis of transgenic plant DNA. Imidazolinone resistance of transformed calli and leaves of transgenic plants was 100 fold greater than that of wild type. The level of resistance of the ALS activity *in vitro* correlated with the amount of resistance in the leaves of transgenic plants. These results demonstrated that the single amino acid substitution from serine to asparagine at the 653rd amino acid near the carboxyl terminus of ALS is the molecular basis of imidazolinone herbicide resistance.

CHAPTER I

INTRODUCTION

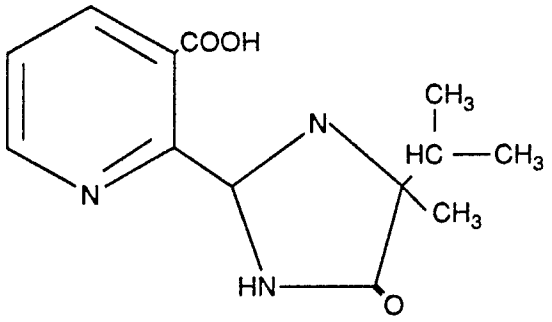
Chemical weed control has become an essential part of modern agriculture in all the developed and many developing nations of the world. Herbicides help farmers minimize an estimated $ 7.47 billion annual losses due to weeds in United States alone (Brosten and Simmons, 1989). Biochemical processes unique to plants such as photosynthesis and essential amino acid biosynthesis have been used as good targets for herbicides because this approach reduces the chances for mammalian toxicity. These herbicides include triazines and ureas which inhibit the quinone binding protein Qb in photosystem II (Pfister et al., 1981), glyphosate which inhibits the 5-enolpyruvil-shikimic acid-3-phosphate synthase in aromatic amino acid biosynthetic pathway (Steinrucken and Amrhein, 1980), as well as sulfonylureas (Ray, 1984), imidazolinones (Shaner et al., 1984) and triazolopyrimidines (Gerwick et al., 1987) which inhibit the acetolactate synthase (ALS) in branched chain amino acid biosynthetic pathway.

Herbicides can be broadly classified into selective and nonselective, based on their toxicity to many crops and weeds. Selectivity of a herbicide may be due to lack of uptake or rapid metabolism of the herbicide into non-toxic compounds (Hathway, 1989). For example, tolerance of soybean to imazaquin is attributed to active metabolism of the herbicide into nontoxic compounds (Shaner and Robson, 1985). Resistance to a herbicide may be developed by gene amplification (Shaw et al., 1986) or an alteration in the target site (Yadav et al., 1986). Such resistance may be developed naturally among the weeds by altered sensitivity of the target site after repeated application of the herbicide (Hall and Devine, 1990; Saari et al., 1990). Resistant plants may also be identified by selecting

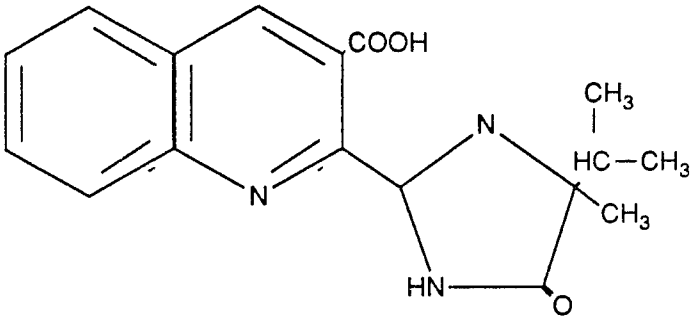
spontaneously or chemically mutated seeds or cells in tissue culture by screening with increasing amounts of a herbicide (Chaleff and Mauvais, 1984; Haughn and Somerville, 1986; 1990). Herbicide resistance may be obtained by increased transcription from the target site gene by using Cauliflower Mosaic virus 35S promoter (Shaw et al., 1986; Odell et al., 1990). Alternately, recombinant herbicide detoxification genes from bacteria may be introduced into plants to develop herbicide resistance (De Block et al., 1987; Stalker et al., 1988; Streber and Willmitzer, 1989). The excellent reviews by Kishore and Shaw (1988), Mazur and Falco (1989), and Shultz et al. (1990) discusses the extensive research done at the biochemical and molecular level on herbicide-resistance and the development of herbicide resistant crops.

Imidazolinones

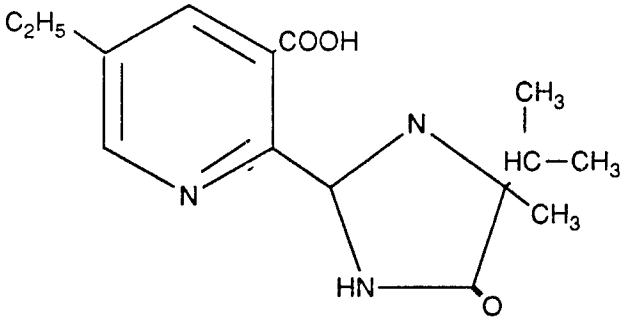
Imidazolinones are a group of potent herbicides with long term broad spectrum activity and low mammalian toxicity, discovered by American Cyanamid Chemical Company (Peoples, 1984). This class of herbicides are characterized by the presence of imidazole ring on an aromatic nucleus *ortho*-substituted by a carboxyl group as shown in Figure 1.1. (Wepplo, 1987). The imidazolinone class of herbicides includes imazaquin (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2yl]-3-quinolinecarboxylic acid), imazapyr (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2yl-}-3-pyridinecarboxylic acid), and imazethapyr (2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1H-imidazol-2-yl]-5-ethyl-3-pyridine-carboxylic acid). Important characteristics of selected imidazolinone herbicides used in this research are summarized in Table 1.



1. Imazapyr



2. Imazaquin



3. Imazethapyr

Figure 1.1. Chemical structures of imidazolinone herbicides which inhibit acetolactate synthase as the primary target site.

Table 1.1. Characteristics of selected imidazolinone herbicides imazapyr and imazaquin (Herbicide Handbook, 1989).

Character	Imazapyr	I m a z a q u i n
1. Trade name	Arsenal	Scepter
2. Code numbers	AC 252925 and AC 243997	AC 252214
3. Molecular weight	320.4	311.3
4. Solubility of the technical	1 - 1.5 % in water	15.9 % in DMSO 6.8 % in DMF 0.5 % in MeOH 0.3 % in EtOH
5. Selectivity	Non-selective	Selective to soybean
6. Uses	For weed control in industrial and right of way areas	For grass and broad leaf control in soybean.
7. Usage rates	0.63 - 2.0 kg/ha	0.14 kg/ha
8. Application time	Postemergence	Preplant incorporated, preemergence & postemergence
9. Absorption	Foliar and root	Foliar and root
10. Translocation	Via xylem and phloem	Via xylem and phloem
11. Soil residues	3 months to 2 years	4 to 11 months
12. Soil degradation	Photodecomposition	Microbial
13. Leaching in soil	Strongly adsorbed to soil	Limited movement
14. Toxicity		
Acute		
oral LD_{50} rats	> 5000 mg/kg	> 5000 mg/kg
dermal LD_{50} rabbit	> 2000 mg/kg	> 2000 mg/kg
Chronic rats	In progress	No effect level 5000 ppm

Imidazolinones are applied either as preemergence or postemergence on both broadleaf and grass weeds (Peoples, 1984). Imazaquin can be applied at 140 g/ha to obtain 90 % control of the target weeds without any damage to soybeans (Congleton et al., 1987). Imazapyr is recommended at 500 to 750 g/ha to control most of annual and perennial target weeds (Peoples, 1984). Once applied, they are absorbed by both roots and foliage, translocated in xylem and phloem and accumulate in growing meristematic regions (Shaner and Robson, 1985).

Physiological and biochemical responses observed after imidazolinone treatments were: inhibition of DNA synthesis, decrease in soluble protein levels and reduced levels of branched chain amino acids in maize tissue culture (Shaner and Reider, 1986); reduced biomass synthesis and growth inhibition in wheat, radish, corn and bean leaves (Devlin and Koszanski, 1986); and inhibition of mitotic activity in sunflower root tips (Birk and Duke, 1986).

The primary target site of imidazolinones is the enzyme ALS (EC 4.1.3.18), that catalyses the first common step in the biosynthetic pathway of branched chain amino acids (Shaner et al., 1984; Anderson and Hibberd, 1985). Imazapyr was observed to bind ALS slowly and tightly, and the binding could be reversed *in vitro* by diluting the enzyme-imazapyr complex with 100 fold assay solution (Muhitch et al., 1987).

Acetolactate synthase

Branched chain amino acids leucine, isoleucine and valine are synthesized by a common pathway that involves a series of enzymes. Acetolactate synthase or acetohydroxy acid synthase (AHAS) is the key regulatory and first common enzyme in

this pathway (Magee and DeRobichon-Szulmajster, 1968). As shown in Figure 1.2, ALS catalyses the condensation of two pyruvates to form acetolactate and also of pyruvate and alpha-ketobutyrate to form acetohydroxy acid (Miflin, 1971). ALS requires Mg++, flavoadenine dinucleotide (FAD) and thiamine pyrophosphate (TPP) as cofactors in microorganisms and higher plants. It is regulated by feedback inhibition by the end products of the biosynthetic pathway valine, leucine, and isoleucine. ALS from maize has been partially purified and characterized (Muhitch et al., 1987). Since ALS is a labile enzyme it is very difficult to purify the active protein to homogeneity for further characterization and preparation of antibodies.

Genetic analysis indicated that ALS genes are located at a single locus in the nuclear genomes of yeast and *Arabidopsis* (Falco and Dumas, 1985; Haughn and Somerville, 1986). The precursor of the mature enzyme contains a transit peptide at the N terminus and the mature enzyme is presumably located in plastids (Jones et al., 1985; Miflin,1974). The first eukaryotic ALS gene was isolated from a herbicide-resistant yeast by screening plasmid DNA libraries for sulfonylurea-resistance and then by sequencing the shortest region in that plasmid conferring such resistance (Falco and Dumas, 1985). ALS genes from tobacco and *Arabidopsis thaliana* were isolated by screening the genomic DNA libraries with the heterologous yeast gene (Mazur et al., 1987). The ALS genes from *Arabidopsis* and tobacco have coding sequences of 2,013 and 2,004 nucleotides which correspond to proteins of apparent molecular weight 72,593 and 72,877, respectively. These plant ALS genes lack introns and share 74% nucleic acid homology among themselves. The ALS gene from *Brassica napus* was identified by

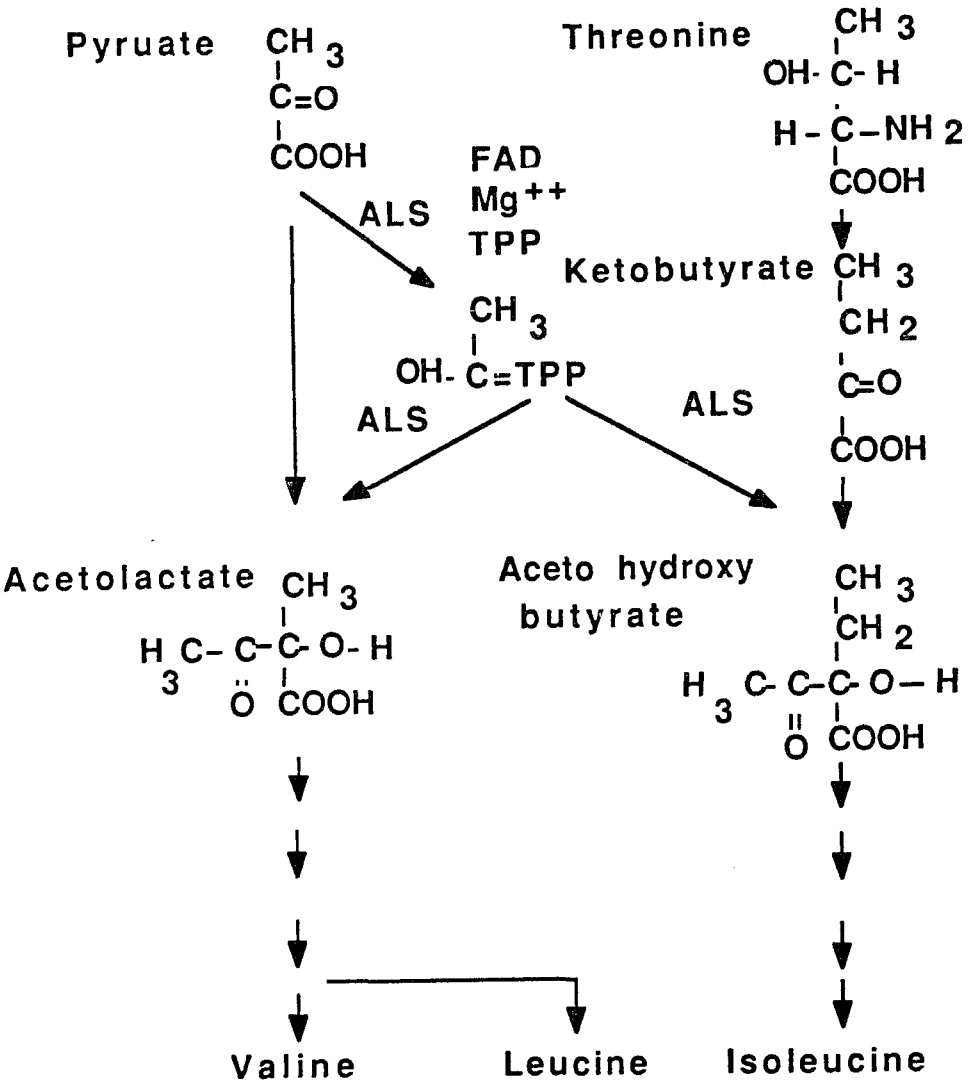
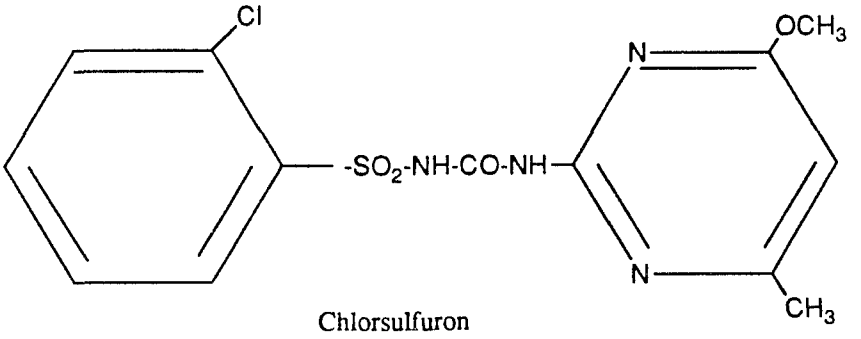


Figure 1.2. Reactions catalyzed by acetolactate synthase in the biosynthetic pathway of branched chain amino acids.

screening a genomic DNA library with the *Arabidopsis* ALS gene (Wiersma et al., 1989). The *Brassica* ALS gene has an open reading frame of 1911 bp with no intron, and it homology among themselves. The ALS gene from *Brassica napus* was identified by screening a genomic DNA library with the *Arabidopsis* ALS gene (Wiersma et al., 1989). The *Brassica* ALS gene has an open reading frame of 1911 bp with no intron, and it 1codes for an mRNA of approximately 2.2 kb size based on northern hybridization analysis. The deduced amino acid sequence of *Brassica* ALS shares 85% and 77% similarity to *Arabidopsis* and tobacco ALS, respectively. Odell et al. (1990) reported that when a tobacco ALS gene was placed under the CaMV35S promoter, the increased levels of ALS transcripts did not result in increased ALS activity. This suggested that plant enzyme levels are probably regulated at the posttranscriptional level or the enzyme activity is feedback regulated.

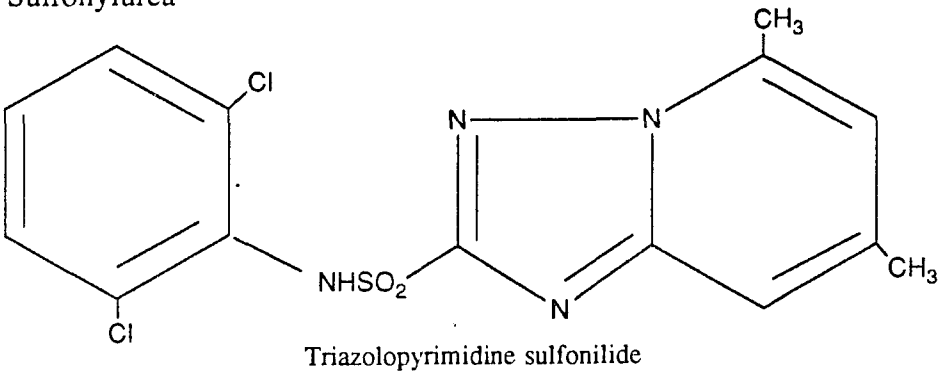
Inhibition of ALS by structurally different herbicides:

The discovery that sulfonylureas and imidazolinones inhibit ALS as the primary target site took enzymologists and biochemists working on their mechanism of action by surprise. This led to the discovery of other chemicals inhibiting this enzyme, and an increased research focus on the ALS and its genes in many plants including *Arabidopsis*. Since ALS is the key regulatory enzyme in the biosynthetic pathway of branched chain amino acids that is absent in animals it has become an excellent target of many herbicides like imidazolinones (Shaner et al., 1984), sulfonylureas (Ray, 1986), triazolopyrimidines , and pyrimidyl-oxy-benzoate (Subramanian et al., 1990). Chemical structures of these ALS inhibitors are shown in Figure 1.1. and 1.3. Several lines of evidence suggested that



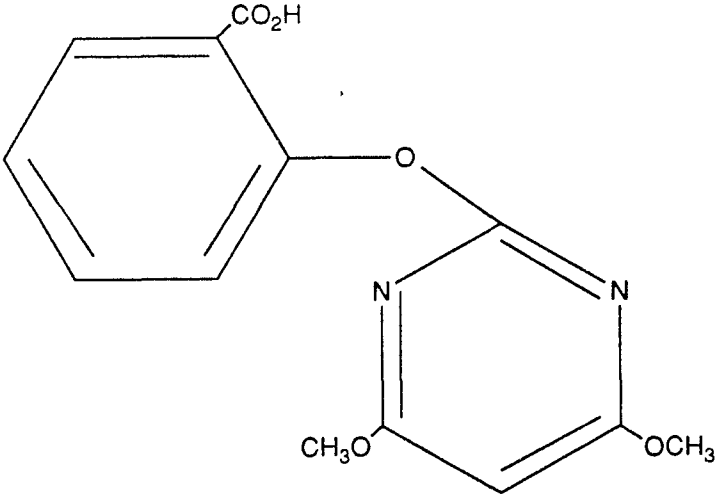
Chlorsulfuron

A. Sulfonylurea



Triazolopyrimidine sulfonilide

B. Triazolopyrimidine



C. Pyrimidyl-oxy-benzoate

Figure 1.3. Chemical structures of examples of other classes of herbicides inhibiting acetolactate synthase as the primary target site.

sulfonylureas and imidazolinones are potent inhibitors of ALS. Growth inhibition of pea root culture by sulfonylureas was reversed by the addition of valine, isoleucine and leucine (Ray, 1984). Imazapyr inhibited the growth of corn suspension culture and decreased the levels of branched chain amino acids (Shaner et al., 1984; Anderson and Hibberd, 1985). Growth inhibition by imidazolinone was reversed by the exogenous supply of the branched chain amino acids valine, leucine, and isoleucine. Also, the ALS activity *in vitro* was inhibited by sulfonylureas and imidazolinones. In addition, the mutant plants resistant to sulfonylureas (Chaleff and Mauvais, 1984) and imidazolinones (Shaner and Anderson, 1985) had an altered form of ALS insensitive to the herbicide inhibition.

The concentration of herbicides required to inhibit 50% of ALS activity (I_{50}) in plants varies among these different classes of ALS inhibitors. Chlorsulfuron and sulfometuron methyl (sulfonylureas) have I_{50} values ranging from 6 to 46 nM (Ray, 1986); whereas imazapyr and imazaquin (imidazolinones) have I_{50} values of 3 to 5 μM (Shaner and Anderson, 1985). Imidazolinones inhibit ALS uncompetitively (Shaner et al., 1984) whereas sulfonylureas inhibit ALS competitively (LaRossa and Schloss, 1984). The mutant forms of ALS are not necessarily cross resistant to both imidazolinones and sulfonylureas, except in a few cases, (Haughn and Somerville, 1986; Saxena and King, 1988; 1990; Winder and Spalding, 1988; Subramanian, 1990), indicating their binding sites on ALS may be different. Schloss et al. (1988) proposed that sulfonyl- ureas, imidazolinones, and triazolopyrimidines share a common binding site on a bacterial ALS based on herbicide-enzyme binding studies.

Sulfonylurea-resistant mutants of *Escherichia coli* (Yadav et al., 1986), Saccharomyces cerevisiae (Falco and Dumas, 1985), *Nicotiana tabacum* (Chaleff and Mauvais, 1984) and *Arabidopsis thaliana* (Haughn and Somerville, 1986) have been identified. Single amino acid substitutions in the ALS gene have been shown to confer resistance to sulfonylureas in *E. coli*, yeast (Yadav et al., 1986), *Arabidopsis* (Haughn et al., 1988), and tobacco (Lee et al., 1988).

Imidazolinone resistance

Imidazolinone-resistant mutant cell lines and plants have been identified in maize (Shaner and Anderson, 1985), *Datura innoxia* (Saxena and King, 1988), *Chlamydomonas reinhardtii* (Winder and Spalding, 1988), and *Arabidopsis thaliana* (Haughn and Somerville, 1990). Imidazolinone-resistant weeds had been reported among the fields treated with sulfonylureas (Hall and Devine, 1990; Saari et al., 1990). These mutants have been characterized genetically and physiologically.

The imidazolinone-resistant maize cell line XA17 was selected by Shaner and Anderson (1985) by exposing the suspension culture to 0.03 mg/l (0.1 μM) imazaquin initially and increasing to 0.1 mg/l (0.33 μM) in the fifth subculture. The selected line XA17 was approximately 30-fold resistant to imazaquin over the unselected tissue. The ALS activity in XA17 was 1000-fold less sensitive to imazapyr and imazaquin. Saxena and King (1988) isolated sulfonylurea-resistant cell lines of *Datura innoxia* after chemical mutagenesis with ethyl methane sulfonate (EMS). Some of these cell lines SMR1, CSR1, and CSR6 showed 100- to 1000-fold resistance to imazapyr-inhibition of the cell growth and ALS activity. Imazaquin-resistant mutants of *Chlamydomonas reinhardtii* were

nitrosoguanidine (NG). The growth of the mutant IMR-13 was approximately 100-fold resistant to imazaquin over the control cells. A homozygous mutant GH90 of *Arabidopsis thaliana* resistant to imazapyr was identified and genetically characterized by Haughn and Somerville (1990). The resistance in GH90 was due to an altered form of ALS, and the mutation was closely linked to the *csr1* locus of Arabidopsis. GH90 and its ALS were resistant to imidazolinone and not to sulfonylurea herbicide. The ALS gene from the GH90 *Arabidopsis* mutant was isolated, sequenced, and characterized in transgenic tobacco in this dissertation research.

Arabidopsis thaliana

There are several plant model systems such as tobacco, maize, petunia, and *Arabidopsis thaliana* to study the cellular and molecular aspects of herbicide resistance. *Arabidopsis thaliana* (L.) Heynh., a member of the Brassicaceae family, was selected as a model system in this research for the following reasons. Its small genome size (7 X 10^7 base pairs per haploid genome), low percentage of repetitive DNA, and minimal levels of DNA methylation make the isolation of single copy genes much easier than in any other plants (Leutwiler et al., 1984). It is sufficient to screen 16,000 recombinant lambda clones with an average insert size of 20 kb to achieve 99% probability of isolating a single copy gene from a genomic DNA library (Leutwiler et al., 1984; Estelle and Somerville, 1986). In addition, it has a short regeneration time of only five weeks; producing more than 10,000 seeds per plant, and dozens of plants could be grown in a small pot (Meyerowitz and Pruitt, 1985). It is typically self-fertilized, and cross-pollinations can also be made for genetic analysis. Mutants could be created by soaking

selected by Winder and Spalding (1988) after mutagenizing with N-methyl-N'-nitro-N- the seeds in ethyl methane sulfonate or irradiating the seeds. Such mutated seeds could be screened for a phenotype of interest, such as herbicide resistance. Most of all, a well characterized imazapyr-resistant *Arabidopsis* mutant GH90 (Haughn and Somerville, 1990) was available at the beginning of this research.

Significance:

Identification of the mutation site(s) in the ALS gene conferring imidazolinone-resistance would be of considerable scientific and practical significance. First, it will help to elucidate the molecular basis of imidazolinone herbicide resistance and enhance our understanding of the cross resistance of ALS mutants to the structurally different imidazolinones and sulfonylureas. Also, it might help explain how weeds develop resistance to imidazolinone herbicides (Silva, 1989; Holt and LeBaron, 1990; Saari et al., 1990; Hall and Devine 1990). Understanding such problems at the molecular level might provide a sound basis of weed management.

Second, isolation and characterization of genes conferring herbicide resistance will provide the opportunity to introduce such traits into crop varieties that are otherwise injured. Application of a particular herbicide to a tolerant crop may be harmful to a sensitive crop planted next season due to its residual activity. For example, imazaquin is safe to soybean but is toxic to corn, tobacco, and sugarbeet planted in following season (American Cyanamid, 1990). If a herbicide-resistance trait could be introduced into crop varieties the market area of a herbicide could be considerably increased (Netzer, 1984). This approach will also help the farmers if the herbicide is cost effective, and it will

broaden his choices for other similar herbicides.

Finally, the isolated ALS gene could serve as a potential selection marker in plant transformation experiments. The ALS gene could also be expressed in prokaryotic systems such as *E. coli* and *Salmonella* and used for *in vitro* mutagenesis experiments. Such mutants could be screened for resistance to other groups of herbicides, and mutations in feedback regulation or catalysis.

Objectives:

The specific objectives of this dissertation research were:

1. To isolate the ALS gene from the GH90 mutant of *Arabidopsis thaliana* var. Columbia.
2. To identify the mutation site(s) by sequencing both the coding and noncoding strands of the ALS gene.
3. To introduce the mutant *Arabidopsis* ALS gene into tobacco and to test its ability to confer imidazolinone resistance.

LITERATURE CITED

American Cyanamid Co. (1990) Sceptor herbicide for use in soybeans. Product label. Princeton, New Jersy.

Anderson, P. C., and Hibberd, K. A. (1985). Evidence for the interaction of imidazolinone herbicide with leucine, valine and isoleucine metabolism. Weed Science 33: 479-483.

Brosten, D., and Simmons, B. (1989). Crops gone wild. Agrichem. Age. 33:6-7, 26, 28.

Birk, J. H., and Duke, W. B. (1986). Imazaquin induced mitotic arrest in sunflower root tips. 40th Proc. of Northeastern Weed Sci. Soc. p186.

Chaleff, R. S., and Mauvais, C. J. (1984). Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. Science 224:1443-1445.

Congleton, W. F., Vantacatfort, A. M., and Lignowski, E. M. (1987).Imazaquin (Sceptor): A new soybean herbicide. Weed Technology 1:186-188.

DeBlock, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gossele, V., Rao Movva, N. Thompson, C., Van Montagu, M., and Leemans, J. (1987). Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. 6:2513-2518.

Devlin, R. M., and Koszanski, Z. K. (1986). Some physiological effects of AC 252-214 on several plant growth systems. Proc. Norteastern Weed Sci. Soc. 40:99-103.

Estelle, M. A., and Somerville, C. R. (1986). The mutants of *Arabidopsis thaliana.* TIBS 11:89-93.

Falco, S. C., and Dumas, K. S. (1985). Genetic analysis of mutants of *Saccharomyces cerevisiae* resistant to the herbicide sulfometuron methyl. Genetics 109:21-35.

Gerwick, B. C., Loney, V., and Subramanian, M. (1987). Mechanism of action of 1,2,4-triazolo [1,5A] pyrimidines. American Chem. Soc. 194th National Meeting. New Orleans, LA. Abstract No. 163.

Hall, L. M., and Devine, M. D. (1990). Cross resistance of a chlorsulfuron-resistant biotype of *Stellaria media* to a triazolopyrimidine herbicide. Plant Physiol. 93:962-966.

Hathway, D. E. (1989). Molecular Mechanisms of Herbicide Selectivity. Oxford Univ. Press. New York. pp 224.

Haughn, G. W., Smith, J., Mazur, B., and Somerville, C. R. (1988). Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol. Gen. Gen. 211:266-271.

Haughn, G. W., and Somerville, C. R. (1986). Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol. Gen. Gen. 204:430-434.

Haughn, G. W., and Somerville, C. R. (1990). A mutation causing imidazolinone resistance maps to *csr1* locus in *Arabidopsis thaliana* var. Columbia. Plant Physiol. 92:1081-1085.

Herbicide Handbook, (1989). published by Weed Sci. Soc. America. Urbana Champaign, Illinois.

Holt, J. S. and LeBaron, H. M. (1990). Significance and distribution of herbicide resistance. Weed Technol. 4:141-149.

Jones, A. V., Young, R. M., and Leto, K. J. (1985). Subcellular localization and properties of acetolactate synthase, target site of the sulfonylurea herbicides. Plant Physiol. Suppl. 77:Abstract 55.

Kishore, G. M., and Shaw, D. M. (1988). Amino acid biosynthesis inhibitors as herbicides. Ann. Rev. Biochem. 57:627-623.

LaRossa, R. A., and Schloss, J. V. (1984). Potent and selective inhibitor of acetolactate synthase in *Salmonella typhimurium*. J. Biol. Chem. 259:8753-8757.

Lee, K. Y, Townsend, J., Tepperman, J., Black, M., Chui, C. F., Mazur, B., Dunsmuir, P., and Bedbrook, J. (1988). The molecular basis of sulfonylurea resistance in tobacco. EMBO J. 7:1241-1248.

Leutwiler, L. S., Haugh Evans, B. R., and Meyerowitz, E. M. (1984). The DNA of *Arabidopsis thaliana*. Mol. Gen. Gen.194:15-23.

Magee, P. T. and DeRobichon-Szulmajster, H. (1968). The regulation of isoleucine-valine biosynthesis in *Saccharomyces cerevisiae* 3. Properties and regulation of the activity of acetohydroxyacid synthetase. European J. Biochem. 3:507-511.

Mazur, B., Chui, C., and Smith, J. K. (1987). Isolation and characterization of plant genes coding for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiol. 85:1110-1117.

Mazur, B. J. and Falco, S. C. (1989). The development of herbicide resistant crops. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:441-470.

Meyerowitz, E. M. and Pruitt, R. E. (1985). *Arabidopsis thaliana* and plant molecular genetics. Science 229:1214-1218.

Miflin, B. J. (1971). Cooperative feedback control of barley acetohydroxy acid synthase by leucine, isoleucine, and valine. Arch. Biochem. Biophys. 146:542-550.

Miflin, B. J. (1974). The location of nitrite reductase and other enzymes related to amino acid biosynthesis in the plastids of roots and leaves. Plant Physiol. 54:550-555.

Muhitch, M. J., Shaner, D. L., and Stidham, M. A. (1987). Imidazolinones and acetohydroxy acid synthase from higher plants. Properties of the enzyme from maize suspension culture cells and evidence for the binding of imazapyr to acetohydroxy acid synthase *in vivo*. Plant Physiol. 83:451-446.

Netzer, W. J. (1984). Engineering herbicide tolerance: When is it worthwhile? Bio/Technology 2:939-944.

Odell, J. T., Caimi, P. G., Yadav, N. S., and Mauvais, C. J. (1990). Comparison of increased expression of wild-type and herbicide-resistant acetolactate synthase genes in transgenic plants, and indication of posttranscriptional limitation on enzyme activity. Plant Physiol. 94:1647-1654.

Peoples, T. R. (1984). Arsenal herbicide (AC 252925) : A developmental overview. Proc. South. Weed Sci. Soc. 37:378.

Pfister, K., Steinback, K. E., Gardner, G., and Arntzen, C. J. (1981). Photoaffinity labelling of an herbicide receptor protein in chloroplast membranes. Proc. Natl. Acad. Sci. U.S.A., 78:981-985.

Ray, T. B. (1984). Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. Plant Physiol. 75:827-831.

Ray, T. B. (1986). Sulfonylurea herbicides as inhibitors of amino acid biosynthesis in plants. TIBS. 11:180-183.

Saari, L. L., Cotterman, J. C., and Primiani, M. M. (1990). Mechanism of sulfonylurea herbicide resistance in the broadleaf weed *Kochia scoparia*. Plant Physiol. 93:55-61.

Saxena, P. K. and King, J. (1988). Herbicide resistance in *Datura innoxia*. Cross-resistance of sulfonyl urea-resistance lines to imidazolinones. Plant Physiol. 86: 863-867.

Saxena, P. K., and King, J. (1990). Lack of cross resistance of imidazolinone-resistant cell lines of *Datura innoxia* P.Mill. to chlorsulfuron. Evidence for separable sites

of action on the target enzyme. Plant Physiol. 94:1111-1115.

Schloss, J. V., Ciskkanik, L. M., and VanDyk, D. E. (1988). Origin of the herbicide binding site of acetolactate synthase. Nature 331:360-362.

Schultz, A., Wengenmayer, F. and Goodman, H. (1990). Genetic engineering of herbicide resistance in higher plants. Critical Rev. Plant Sci. 9:1-15

Shaner, D. L., Anderson, P. C., and Stidham, M. A. (1984). Imidazolinones. Potent inhibitors of acetohydoxy acid synthase. Plant Physiol. 76:545-546.

Shaner, D. L., and Anderson, P. C. (1985). Mechanism of action of the imidazolinones and cell culture selection of tolerant maize. In Biotechnology in Plant Science. Eds. M. Zaitlin, P. Day and A. Hollanender. Academic Press. NY. pp 287-299.

Shaner, D. L. and Reider, M. L. (1986). Physiological responses of corn (*Zea mays)* to AC 243,997 in combination with valine, leucine, and isoleucine. Pest. Biochem. and Physiol. 25:245-257.

Shaner, D. L. and Robson, P. A. (1985). Absorption, translocation and metabolism of AC252-214 in soybean *(Glycine max),* common cocklebur *(Xanthium strumarium)* and velvet leaf *(Abutilon theophrastii).* Weed Sci. 33:469-471.

Shaw, D. M., Horsch, R. B., Klee, H. J., Kishore, G. M., Winter, J. A., Tumer, N. E., Hironaka, C. M., Sanders, P. R., Gasser, C. S., Aykent, S., Siegal, N. D., Rogers, S. G., and Fraley, R. T. (1986). Engineering herbicide tolerance in transgenic plants. Science 233:478-481.

Silva, D. (1989). The war of the weeds. Agrichem. Age. 33:12-13.

Stalker, D. M., McBride, K. E., and Malyj, L. D. (1988). Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science 242:419-423.

Steinrucken, H. C. and Amrhein, N. (1980). The herbicide glyphosate is a potent inhibitor of 5-enolpyruvil-shikimic acid 3-phosphate synthase. Biochem. and Biophys. Res. Comm. 94:1207-1212.

Streber, W. R., and Willmitzer, L. (1989). Transgenic tobacco plants expressing a bacterial detoxifying enzyme are resistant to 2,4-D. Bio/Technology 7:811-816.

Subramanian, M., Hung, H. U., Dias, J. M., Miner, V. M., J. H. Butler and Jachettia, J. J. (1990). Properties of mutant acetolactae synthases resistant to triazolopyrimidine sulfonanilide. Plant Physiol. 94:239-244.

Yadav, N., McDevitt, R. E., Bernard, S., and Falco, S. C. (1986). Single amino acid

substitutions in the enzyme acetolactate synthase confer resistance to the herbicide sulfometuron methyl. Proc. Natl. Acad. Sci. USA. 83: 4418-4422.

Wepplo, P. J. (1987). Imidazolinone herbicides: synthesis and novel chemistry. American Chem. Soc. Symp. New Orleans. Abs. No: 139.

Wiersma, P. A., Schmiemann, M. G., Condie, J. A., Crosby, W. L., and Moloney, M. M. (1989). Isolation, expression and phylogenetic inheritance of an acetolactate synthase gene from *Brassica napus*. Mol. Gen. Gen. 219:413-420.

Winder, T., and Spalding, M. (1988). Imazaquin and chlorsulfuron resistance and cross resistance in mutants of *Chlamydomonas reinhardtii*. Mol. Gen. Gen. 213:394-399.

CHAPTER II

CLONING AND SEQUENCING OF A MUTANT ACETOLACTATE SYNTHASE GENE FROM AN IMAZAPYR-RESISTANT *ARABIDOPSIS THALIANA* VAR. COLUMBIA.

ABSTRACT

Acetolactate synthase (ALS), the key enzyme in the biosynthesis of leucine, isoleucine, and valine is a primary target site of imidazolinones. To understand the molecular basis of imidazolinone herbicide-resistance, an *Arabidopsis thaliana* mutant GH-90 (Haughn and Somerville, 1990. Plant Physiol. 92:1081-1085) was analyzed. Southern hybridization of genomic DNA from the mutant and the wild-type *Arabidopsis* indicated that gene amplification or major structural change was not responsible for conferring imidazolinone resistance. A genomic DNA library was constructed from the GH90 in the phage vector EMBL3 and screened with a homologous ALS probe. Three positively hybridizing clones were identified and one of them (designated as Imr3ALS3) was purified further. The selected clone contained a full length ALS gene in a 11.5 kb insert, as shown by southern hybridization with 5' and 3' ALS probes. The ALS gene was subcloned as a 5.8 kb XbaI fragment into pUC18 and designated as pKS1. DNA sequences of both the coding and noncoding strands were determined, and a single point mutation from G to A was identified in the ALS gene in pKS1 at the 1958th nucleotide which predicted an amino acid substitution of asparagine to serine at the 653rd residue (Sathasivan et al., 1990. Nucl. Acids Res. 18:2188).

INTRODUCTION

Imidazolinone herbicides inhibit ALS as the primary target site (Shaner et al., 1984). A homozygous mutant line (GH90) of *Arabidopsis thaliana* resistant to imidazolinone was isolated after chemical mutagenesis and characterized by Haughn and Somerville (1990). GH90 is approximately 100-fold more resistant to the imidazolinone herbicide imazapyr than the wild-type plant. The mutant enzyme showed at least 100 times less inhibition by imazapyr *in vitro* than the wild type ALS. Genetic analysis indicated that the imidazolinone-resistance is due to a single dominant nuclear mutation closely linked to the ALS locus. The mutant GH90 however, is not resistant to sulfonylureas and triazolo pyrimidines (Sathasivan and Murai, unpublished). A southern hybridization analysis of the mutant and wild type *Arabidopsis* DNA indicated that gene amplification was not responsible for imidazolinone-resis-tance. To determine the molecular basis of imidazolinone- resistance, the ALS gene from GH90 was isolated from a genomic DNA library and sequenced. A single point mutation was observed near the 3' region of the ALS coding region resulting in a single amino acid substitution .

MATERIALS AND METHODS

Plants, bacterial strains and plasmids.

The mutant GH90 *Arabidopsis* was received from G. W. Haughn and C. R. Somerville at Michigan State University. The wild- type *Arabidopsis thaliana* var. Columbia was obtained from T. S. Moore, Department of Botany, Louisiana State University (LSU). Seeds of both the wild-type and the mutant GH90 *Arabidopsis* were germinated and grown on an autoclaved perlite/ vermiculite/ spaghnum mixture (1:1:1)

as described (Haughn and Somerville, 1986). Plants were grown at 21-23 C and 16 hrs light/8hrs dark cycle and harvested just before flowering for DNA isolation. *Escherichia coli* strains K803, GM2163, NM522, NM538, and NM539 were obtained from E. Achberger, Department of Microbiology at LSU. The plasmid pGH1 containing the ALS gene from the sulfonylurea-resistant *Arabidopsis* was also provided by G. W. Haughn and C.R. Somerville, Michigan State University, East Lansing. *E. coli* strain XL1 blue and pBluescript phagemid are from Stratagene.

Plant DNA isolation

Total DNA from wild-type and GH90 *Arabidopsis* was isolated according to the method of Leutwiler et al. (1984), with some modifications. Approximately 10 g of *Arabidopsis* plants were rinsed with water, frozen with liquid nitrogen, and ground in a mortar and pestle. The powder was mixed with 100 ml of extraction buffer containing 150 mM Tris HCl (pH 8.5), 100 mM EDTA, 2% (w/v) N-lauryl sarcosine and 10 mg proteinase K (freshly prepared). After incubation at 37 C for 30 min, the mixture was centrifuged at 10,000 g for 10 min at 4 C. The supernatant was filtered through autoclaved cheese cloth and centrifuged again to remove the residues. The supernatant was mixed with two volumes of EtOH and 0.1 volume of 3.0 M sodium acetate to precipitate nucleic acids. The pellet was resuspended in 16 ml of 10 mM Tris(pH 8.0), 1 mM EDTA and the DNA band was isolated and dialysed after CsCl density centrifugation. Standard nucleic acid techniques used in the following experiments were as described in Maniatis et al (1982).

Southern Hybridization Analysis of Genomic DNA.

The purified DNA was completely digested with appropriate restriction enzymes as described in the figure legends and fractionated on a 0.8% (w/v) agarose gel overnight along with copy number controls. Fractionated DNA was then transferred to nitrocellulose filter (Schleicher and Schull) and hybridized to ^{32}P-labelled 2.1 kb and 2.4 kb EcoRI probes containing the 5' and 3' regions of ALS gene from pGH1, respectively, according to the manufacturers instructions.

Construction and screening of genomic DNA library.

DNA library construction and screening were basically as described by Kaiser and Murray (1985). Thirty μg of total DNA from GH90 mutant was subjected to partial digestion by Sau3A, size fractionated by sucrose density gradient centrifugation and the fractions of 15-20 kb were pooled. The lambda vector EMBL3 DNA was self-ligated and digested with BamHI, and the central fragment was removed by sucrose density gradient centrifugation. The EMBL3 arms and the 15-20 kb fragments from Imr3 were ligated and packaged using an *in vitro* packaging kit from Promega. The library contained approximately 3.2 x 10^4 Plaque Forming Units/ml that was enough to screen twice for a single copy gene with 99% probability, twice. The library was plated on *E. coli* strain K 803 and probed with 2.1 and 2.4 kb EcoRI fragments containing the ALS gene from pGHI. Three positively hybridizing clones were identified, and one of them designated as Imr3ALS3 was purified further by screening two more times with the same probes.

Subcloning and sequencing

A 5.8 kb XbaI fragment from Imr3 ALS 3 containing the ALS gene, and its

flanking sequences was subcloned into pUC18 and designated as pKS1. The same fragment was also subcloned into pBluescript SK + with the ALS in both orientations. Nested deletions of the pBluescript clones were created using Exonuclease III and mung bean nuclease (Henikoff, 1984) as described in Stratagene's Exo/Mung-DNA sequencing instruction manual. The nested deletions were sequenced by double-stranded DNA sequencing protocol using the Sanger's dideoxy sequencing method (Sanger et al., 1977) with ^{35}S-dATP and buffer gradient gels (Biggins et al., 1983) or standard polyacrylamide gels. Some of the missing regions were individually subcloned into pBluescript and sequenced using the single strand DNA isolated with M13Ko7 helper phage (Promega). The DNA sequence was compiled and analyzed by DSAS program written by J. Niegel, University of Southwestern Louisiana, Lafayette, LA. The DNA sequnce was compared with the wild-type ALS sequence (Mazur et al., 1987).

RESULTS

Cloning of the ALS gene from GH90

A genomic DNA library from GH90 was constructed for cloning and characterizing the ALS gene. Approximately 32,000 plaque forming units from the GH90 genomic DNA library in EMBL3 were screened with the EcoRI 2.1 and 2.4 kb fragments from pGH1 containing the 5' and 3' of the *csr1-1* gene (Haughn et al., 1988). Three positively hybridizing clones were identified and one of them designated as Imr3ALS3 was selected and purified by two more screenings. DNA from the selected phage Imr3ALS3 was hybridized to 5' and 3' ALS probes separately as shown in Figures 2.1A. and 2.1B, respectively. A single 5.8 kb XbaI band and the 2.1 kb EcoRI band in the 5'

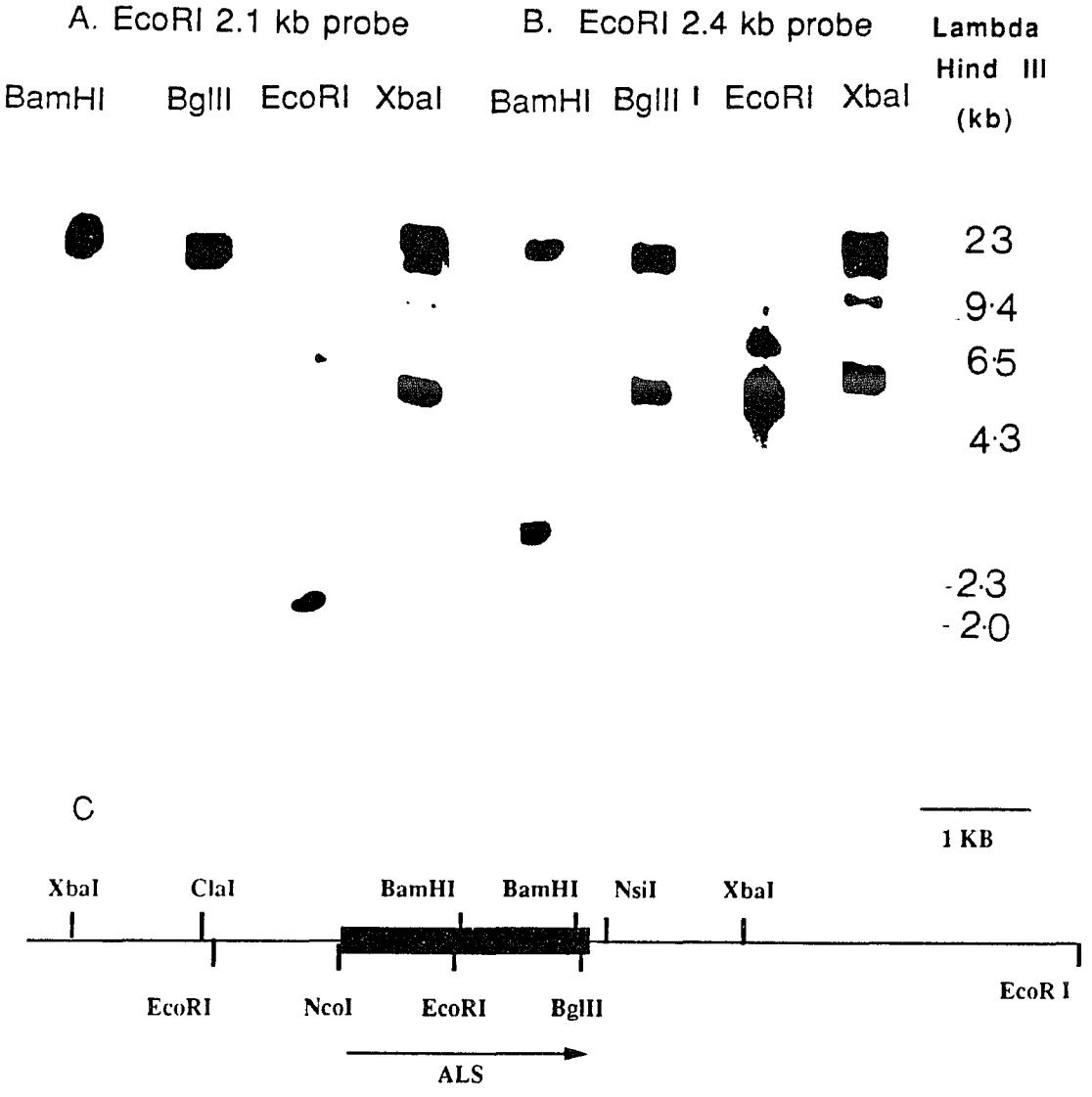


Figure 2.1. Southern hybridization analysis and restriction map of the lambda clone Imr3ALS3.

Approximately 1 μg of the phage Imr3ALS3 DNA was digested with BamHI, BglII, EcoRI, and XbaI and fractionated on an 0.8 % (w/v) agarose gel, transferred to nitrocellulose filter. The filter was then separately probed with an EcoRI 2.1 kb 5' probe (A) or an EcoRI 2.4 kb 3' probe (B) of ALS gene from pGH1. The restriction map of the Imr3ALS3 (C) was obtained by digestion of the DNA with respective enzymes and size fractionating on an 0.8 % agarose gel and mapping the sites.

and 5.5 kb EcoRI band in the 3' region indicated that a complete ALS gene has been cloned. Further restriction analysis of Imr3ALS3 suggested that it contains the ALS gene in a 11.5 kb insert. The restriction map of part of the lambda clone is shown in Figure 2.1C. The 5.8 kb XbaI fragment was subcloned into pUC18 creating the plasmid pKS1. The 5.8 kb XbaI fragment from pKS1 was further subcloned into pBluescript for creating nested deletions and sequencing. The restriction map of the subclone pKS1 is shown in Figure 2.2. The restriction sites in the coding and surrounding regions of the ALS gene in pKS1 were essentially identical to the wild-type *csr1* gene (Mazur et al., 1987) and its allele *csr1-1* in pGH1 (Haughn and Somerville, 1990). However, there was an additional ClaI site approximately 1.2 kb 5' upstream of the ALS coding sequence, the importance of which is not known.

Sequence analysis

To identify if there are any mutation(s) in the ALS gene from GH90, the DNA sequence of the genomic clone in pKS1 was determined. Both the coding and noncoding strands of the ALS gene were sequenced from 300 bases upstream to 30 bases downstream of the coding region. The entire DNA sequence of this mutant ALS gene was published in 1990 (Sathasivan et al., 1990). A single point mutation from guanosine to adenosine was identified at the 1958th nucleotide position as shown in Figure 2.3. This would result in serine to asparagine substitution at the 653rd residue near the carboxyl terminal of the mature protein. This mutation in the ALS gene from the imidazolinone-resistant *Arabidopsis thaliana* is different from the reported mutations in ALS gene conferring for sulfonylurea-resistance (Mazur and Falco, 1989). This mutation is

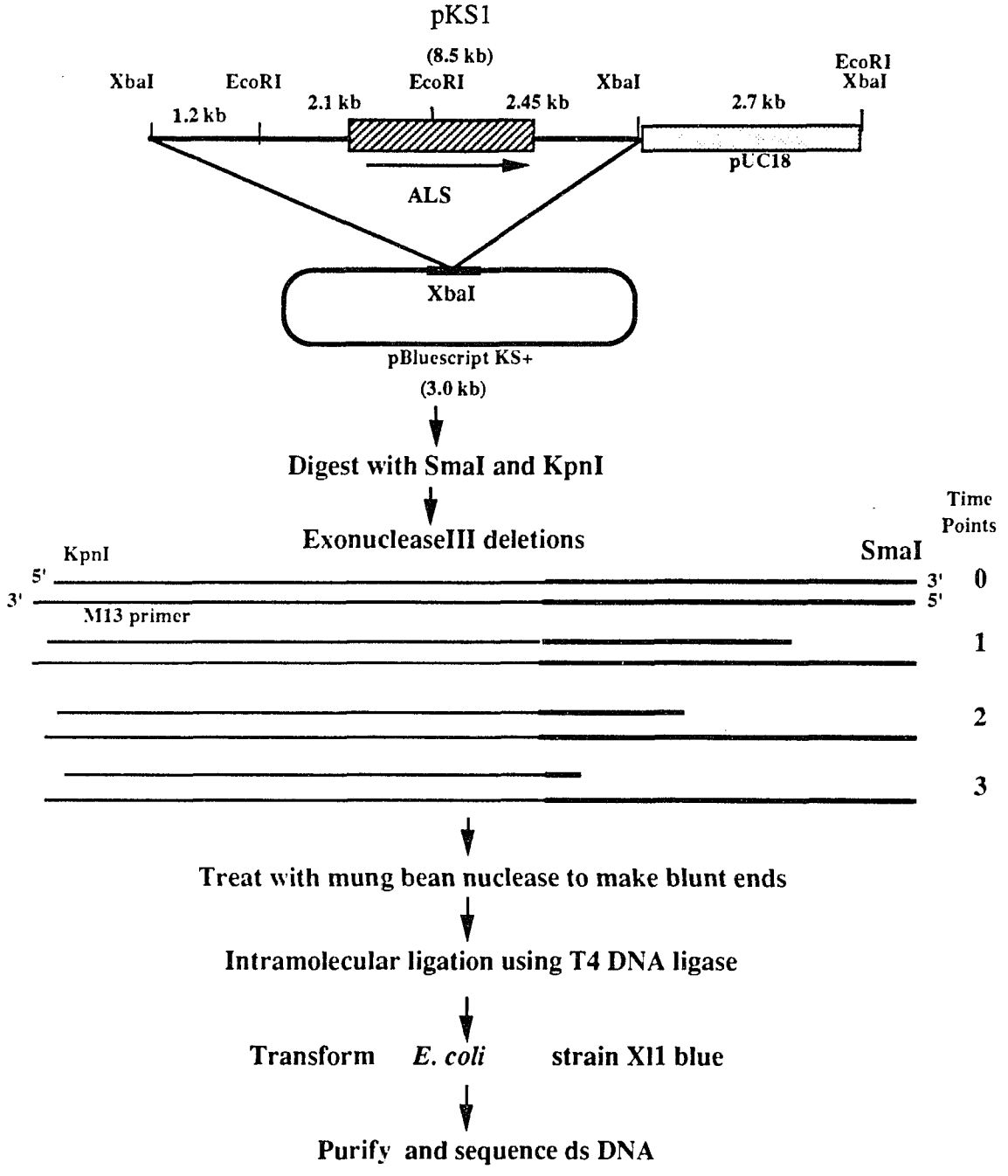


Figure 2.2. Subcloning and DNA sequencing strategy for the mutant ALS gene.

```
CTTGTATCCATTCTCTTAACCAATAAAAAAAGAAAGAAAGATCAATTTGATAAATTT   -248
 TCAGCCACAAATTCTACATTTAGGTTTTAGCATATCGAAGGCTCAATCACAAATACA   -192
 TAGATAGACTAGAGATTCCAGCGTCACGTGAGTTTTATCTATAAATAAAGGACCAAA   -135
 ATCAAATCCCGAGGGCATTTTCGTAATCCAACATAAAACCCTTAAACTTCAAGTCTC   -77
 TTTTTAAACAAATCATGTTCACAAGTCTCTTCTTCTTCTCTGTTTCTCTATCTCTT    -21

                              +1
 GCTCATCTTTCTCCTGAACCATGGCGGCGGCAACAACAACAACAACAACATCTTCTT   37
                               M  A  A  A  T  T  T  T  T  T  S  S  S

 CGATCTCCTTCTCCACCAAACCATCTCCTTCCTCCTCCAAATCACCATTACCAATCT   94
   I  S  F  S  T  K  P  S  P  S  S  S  K  S  P  L  P  I  S

 CCAGATTCTCCCTCCCATTCTCCCTAAACCCCAACAAATCATCCTCCTCCTCCCGCC   151
   R  F  S  L  P  F  S  L  N  P  N  K  S  S  S  S  S  R  R

 GCCGCGGTATCAAATCCAGCTCTCCCTCCTCCATCTCCGCCGTGCTCAACACAACCA   208
   R  G  I  K  S  S  S  P  S  S  I  S  A  V  L  N  T  T  T

 CCAATGTCACAACCACTCCCTCTCCAACCAAACCTACCAAACCCGAAACATTCATC    264
   N  V  T  T  T  P  S  P  T  K  P  T  K  P  E  T  F  I

 TCCCGATTCGCTCCAGATCAACCCCGCAAAGGCGCTGATATCCTCGTCGAAGCTTTA   321
 S  R  F  A  P  D  Q  P  R  K  G  A  D  I  L  V  E  A  L

 GAACGTCAAGGCGTAGAAACCGTATTCGCTTACCCTGGAGGTGCATCAATGGAGATT   378
 E  R  Q  G  V  E  T  V  F  A  Y  P  G  G  A  S  M  E  I

 CACCAAGCCTTAACCCGCTCTTCCTCAATCCGTAACGTCCTTCCTCGTCACGAACAA   435
 H  Q  A  L  T  R  S  S  S  I  R  N  V  L  P  R  H  E  Q

 GGAGGTGTATTCGCAGCAGAAGGATACGCTCGATCCTCAGGTAAACCAGGTATCTGT   492
 G  G  V  F  A  A  E  G  Y  A  R  S  S  G  K  P  G  I  C

 ATAGCCACTTCAGGTCCCGGAGCTACAAATCTCGTTAGCGGATTAGCCGATGCGTTG   549
 I  A  T  S  G  P  G  A  T  N  L  V  S  G  L  A  D  A  L

 TTAGATAGTGTTCCTCTTGTAGCAATCACAGGACAAGTCCCTCGTCGTATGATTGGTA   607
 L  D  S  V  P  L  V  A  I  T  G  Q  V  P  R  R  M  I  G  T

 CAGATGCGTTTCAAGAGACTCCGATTGTTGAGGTAACGCGTTCGATTACGAAGCATA   664
   D  A  F  Q  E  T  P  I  V  E  V  T  R  S  I  T  K  H  N
```

Figure 2.3. The nucleotide and deduced amino acid sequence of the acetolactate synthase gene from GH90 (Continued).

The ALS gene in pKS1 was further subcloned into pBluescript and sequenced as described in methods. A single point mutation from G to A was identified at the 1958th nucleotide position (underlined) as compared to the wild-type *Arabidopsis* gene (Mazur et al., 1987).

```
ACTATCTTGTGATGGATGTTGAAGATATCCCTAGGATTATTGAGGAAGCTTTCTTTTT  722
  Y  L  V  M  D  V  E  D  I  P  R  I  I  E  E  A  F  F  L

AGCTACTTCTGGTAGACCTGGACCTGTTTTGGTTGATGTTCCTAAAGATATTCAACAA  780
  A  T  S  G  R  P  G  P  V  L  V  D  V  P  K  D  I  Q  Q

CAGCTTGCGATTCCTAATTGGGAACAGGCTATGAGATTACCTGGTTATATGTCTAGGA  838
Q  L  A  I  P  N  W  E  Q  A  M  R  L  P  G  Y  M  S  R  M

TGCCTAAACCTCCGGAAGATTCTCATTTGGAGCAGATTGTTAGGTTGATTTCTGAGTC  896
  P  K  P  P  E  D  S  H  L  E  Q  I  V  R  L  I  S  E  S

TAAGAAGCCTGTGTTGTATGTTGGTGGTGGTTGTTTGAATTCTAGCGATGAATTGGGT  954
 K  K  P  V  L  Y  V  G  G  G  C  L  N  S  S  D  E  L  G

AGGTTTGTTGAGCTTACGGGGATCCCTGTTGCGAGTACGTTGATGGGGCTGGGATCTT  1012
R  F  V  E  L  T  G  I  P  V  A  S  T  L  M  G  L  G  S  Y

ATCCTTGTGATGATGAGTTGTCGTTACATATGCTTGGAATGCATGGGACTGTGTATGC  1070
  P  C  D  D  E  L  S  L  H  M  L  G  M  H  G  T  V  Y  A

AAATTACGCTGTGGAGCATAGTGATTTGTTGTTGGCGTTTGGGGTAAGGTTTGATGAT  1128
 N  Y  A  V  E  H  S  D  L  L  L  A  F  G  V  R  F  D  D

CGTGTCACGGGTAAGCTTGAGGCTTTTGCTAGTAGGGCTAAGATTGTTCATATTGATA  1186
R  V  T  G  K  L  E  A  F  A  S  R  A  K  I  V  H  I  D  I

TTGACTCGGCTGAGATTGGGAAGAATAAGACTCCTCATGTGTCTGTGTGTGGTGATGT  1244
  D  S  A  E  I  G  K  N  K  T  P  H  V  S  V  C  G  D  V

TAAGCTGGCTTTGCAAGGGATGAATAAGGTTCTTGAGAACCGAGCGGAGGAGCTTAA  1301
 K  L  A  L  Q  G  M  N  K  V  L  E  N  R  A  E  E  L  K

GCTTGATTTTGGAGTTTGGAGGAATGAGTTGAACGTACAGAAACAGAAGTTTCCGTTG  1359
  L  D  F  G  V  W  R  N  E  L  N  V  Q  K  Q  K  F  P  L

AGCTTTAAGACGTTTGGGGAAGCTATTCCTCCACAGTATGCGATTAAGGTCCTTGATG  1417
S  F  K  T  F  G  E  A  I  P  P  Q  Y  A  I  K  V  L  D  E

AGTTGACTGATGGAAAAGCCATAATAAGTACTGGTGTCGGGCAACATCAAATGTGGG  1474
  L  T  D  G  K  A  I  I  S  T  G  V  G  Q  H  Q  M  W  A
```

Figure 2.3. Continued.

```
CGGCGCAGTTCTACAATTACAAGAAACCAAGGCAGTGGCTATCATCAGGAGGCCTTG  1531
  A  Q  F  Y  N  Y  K  K  P  R  Q  W  L  S  S  G  G  L  G

GAGCTATGGGATTTGGACTTCCTGCTGCGATTGGAGCGTCTGTTGCTAACCCTGATGC  1589
  A  M  G  F  G  L  P  A  A  I  G  A  S  V  A  N  P  D  A

GATAGTTGTGGATATTGACGGAGATGGAAGCTTTATAATGAATGTGCAAGAGCTAGC  1646
  I  V  V  D  I  D  G  D  G  S  F  I  M  N  V  Q  E  L  A

CACTATTCGTGTAGAGAATCTTCCAGTGAAGGTACTTTTATTAAACAACCAGCATCTT  1704
  T  I  R  V  E  N  L  P  V  K  V  L  L  L  N  N  Q  H  L

GGCATGGTTATGCAATGGGAAGATCGGTTCTACAAAGCTAACCGAGCTCACACATTT  1761
G  M  V  M  Q  W  E  D  R  F  Y  K  A  N  R  A  H  T  F

CTCGGGGATCCGGCTCAGGAGGACGAGATATTCCCGAACATGTTGCTGTTTGCAGCA  1818
L  G  D  P  A  Q  E  D  E  I  F  P  N  M  L  L  F  A  A

GCTTGCGGGATTCCAGCGGCGAGGGTGACAAAGAAAGCAGATCTCCGAGAAGCTAT  1874
A  C  G  I  P  A  A  R  V  T  K  K  A  D  L  R  E  A  I

TCAGACAATGCTGGATACACCAGGACCTTACCTGTTGGATGTGATTTGTCCGCACCA  1931
  Q  T  M  L  D  T  P  G  P  Y  L  L  D  V  I  C  P  H  Q

AGAACATGTGTTGCCGATGATCCCGAATGGTGGCACTTTCAACGATGTCATAACGGA  1988
  E  H  V  L  P  M  I  P  N  G  G  T  F  N  D  V  I  T  E

AGGAGATGGCCGGATTAAATACTGAGAGATGAAACCGGTGATTATCAGAACCTTTTA  2045
  G  D  G  R  I  K  Y  Stop

TGGTCTTTGTAT                                               2057
```

Figure 2.3. Continued.

observed in a region conserved among the plant ALS sequences (Mazur et al., 1987; Wiersma et al., 1989) and is much closer to the carboxyl terminal of ALS than the mutations conferring sulfonylurea-resistance.

DISCUSSION

Herbicide-resistance may be due to lack of uptake, detoxification, an alteration in the target site or amplification of the target site genes (Hathway, 1989). Amplification of EPSP synthase has been shown to confer glyphosate resistance (Shaw et al., 1986; Steinrucken et al., 1986). A transient duplication of the ALS gene in *Datura innoxia* has been shown to confer sulfonylurea resistance (Xiao et al., 1987). To distinguish among these possibilities, the ALS enzyme in GH90 was assayed and found to be insensitive to imazapyr-inhibition (Haughn and Somerville, 1990). Furthermore, southern analysis of wild-type and GH90 genomic DNA indicated no gene amplification was involved. Hence, cloning and sequencing the ALS gene from GH90 was undertaken.

The ALS gene from an imazapyr-resistant *Arabidopsis thaliana* GH90 was isolated from a genomic DNA library, and both strands were sequenced. A single base substitution from G to A was observed at the 1958th nucleotide. This would result in the substitution of serine to asparagine at the 653rd residue. This mutation is observed in a conserved region of ALS, and it is considered to be the probable cause of imidazolinone resistance. To demonstrate that the ALS gene in pKS1 encodes an imazapyr-resistant enzyme and confers imidazolinone-resistance, the mutant ALS gene was introduced into tobacco. The tobacco transformation and the analysis of imidazolinone-resistance of the transgenic tobacco are described in the following chapter.

LITERATURE CITED

Biggins, M. D., Gibson, T. J., and Hong, G. F. (1983). Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA. 80:3963-3965.

Hathway, D. E. (1989). Molecular mechanisms of herbicide selectivity. Oxford Univ. Press. New York. pp 224.

Haughn, G. W., Smith, J., Mazur, B., and Somerville, C. R. (1988). Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol. Gen. Gen. 211:266-271.

Haughn, G. W., and Somerville, C. R. (1986). Sulfonylurea-resistant mutants of *Arabidopsis thaliana.* Mol. Gen. Gen. 204:430-434.

Haughn, G. W., and Somerville, C. R. (1990). A mutation causing imidazolinone resistance maps to *csr1* locus in *Arabidopsis thaliana* var. Columbia. Plant Physiol. 92:1081-1085.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351-359.

Kaiser, K. and Murray, N. E. (1985). The use of phage lambda replacement vectors in the construction of representative genomic DNA libraries. *In* DNA Cloning. Vol I. Eds. D. M. Glover, IRL press Ltd. Oxford, England, p 1-41.

Leutwiler, L. S., Haugh Evans, B. R., and Meyerowitz, E. M. (1984). The DNA of *Arabidopsis thaliana.* Mol. Gen. Gen. 194:15-23.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular cloning : A Laboratory Manual. Cold Spring Harbor Laboratory, p 545.

Mazur, B. J., Chui, C., and Smith, J. K. (1987). Isolation and characterization of plant genes coding for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiol. 85:1110-1117.

Mazur, B. J. and Falco, S. C. (1989). The development of herbicide resistant crops. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:441-470.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.

Sathasivan, K., Haughn, G. W., and Murai, N. (1990). Nucleotide sequence of a mutant acetolactate synthase gene from imidazolinone resistant *Arabidopsis thaliana* var. columbia. Nucleic Acids Res. 18:2188.

Shaner, D. L., Anderson, P. C., and Stidham, M. A. (1984). Imidazolinones. Potent inhibitors of acetohydroxy acid synthase. Plant Physiol. 76:545-546.

Shaw, D. M., Horsch, R. B., Klee, H. J., Kishore, G. M., Winter, J. A., Tumer, N. E., Hironaka, C. M., Sanders, P. R., Gasser, C. S., Aykent, S., Siegal, N. D., Rogers, S. G., and Fraley, R. T. (1986) Engineering herbicide tolerance in transgenic plants. Science 233:478-481.

Steinrucken, H. C., Schulz, A., Amrhein, N., Porter, C. A., and Fraley. R. T. (1986). Overproduction of 5-enolpyruvilshikimic acid-3-phosphate synthase in a glyphosate tolerant *Petunia hybrida* cell line. Arch. Biochem. Biophys. 244:169-178.

Wiersma, P. A., Schmiemann, M. G., Condie, J. A., Crosby, W. L., and Moloney, M. M. (1989). Isolation, expression and phylogenetic inheritance of an acetolactate synthase gene from *Brassica napus*. Mol. Gen. Gen. 219:413-420.

Xiao, W., Saxena, P. K., King, J., and Rank, G. H. (1987). A transient duplication of acetolactate synthase gene in a cell culture of *Datura innoxia*. Theor. Appl. Genet. 47:417-422.

CHAPTER III

THE MOLECULAR BASIS OF IMIDAZOLINONE HERBICIDE RESISTANCE IN *ARABIDOPSIS THALIANA* VAR. COLUMBIA.

ABSTRACT

Acetolactate synthase (ALS), the first enzyme in the biosynthetic pathway of leucine, isoleucine, and valine, is inhibited by imidazolinone herbicides. To understand the molecular basis of imidazolinone-resistance, we isolated the ALS gene from an imazapyr-resistant mutant GH90 of *Arabidopsis thaliana* (Haughn and Somerville, 1990. Plant Physiol. 92:1081-1085. DNA sequence analysis of the mutant ALS gene demonstrated a single point mutation from G to A at the 1958th nucleotide of the ALS coding sequence (Sathasivan et al., 1990. Nucl. Acids Res. 18:2188). This would result in serine to asparagine substitution at the 653rd residue, near the carboxyl terminal of the matured ALS. The mutant ALS gene was introduced into tobacco using *Agrobacterium*-mediated transformation. Imidazolinone-resistance of transformed calli and leaves of transgenic plants was 100-fold greater than that of wild type. The relative levels of herbicide resistance of ALS activity correlated with the amount of herbicide resistance in the leaves of transgenic plants. Southern hybridization analysis confirmed the presence of the introduced ALS gene in a transformant showing high imazapyr-resistance. The present results demonstrate that the mutant ALS gene confers resistance to imidazolinone herbicides. To our knowledge, this is the first report on the molecular basis of imidazolinone herbicide-resistance in plants.

INTRODUCTION

Imidazolinones are a group of potent herbicides with long term broad spectrum activity and low mammalian toxicity (Peoples, 1984), including the non-selective herbicide imazapyr and the selective herbicides, imazaquin and imazethapyr. Several lines of evidence suggest that the primary target site of imidazolinones is the enzyme acetolactate synthase (ALS; EC 4.1.3.18), which catalyses the first common step in the biosynthetic pathway of leucine, isoleucine and valine. Imidazolinone treatment of a maize suspension culture resulted in reduced biosynthesis of the branched-chain amino acids and the imidazolinone-toxicity could be reversed by exogenous supply of the branched-chain amino acids (Shaner and Anderson, 1985). Imidazolinones inhibit ALS activity *in vitro* (Shaner et al., 1984). Finally, many mutant plants resistant to imidazolinone herbicides also have an imidazolinone-resistant ALS activity [*Zea mays*, Shaner and Anderson, 1985; *Datura innoxia,* Saxena and King, 1988; *Chlamydomonas reinhardtii*, Winder and Spalding, 1988; and *Arabidopsis thaliana*, Haughn and Somerville, 1990].

In addition to imidazolinones, ALS is also inhibited by other structurally diverse group of herbicides *viz.* sulfonylureas (Ray, 1984; Chaleff and Mauvais, 1984), triazolo-pyrimidines (Gerwick et al., 1987), and pyrimidyl-oxy-benzoate (Hawkes, 1989 and Subramanian et al., 1990). Schloss et al., (1988) proposed that sulfonylureas, imidazolinones, and triazolopyrimidines compete for a common binding site on a bacterial ALS based on herbicide-enzyme binding studies. However, several spontaneous and chemical mutant plants and their enzymes showed varied levels of cross-resistance to

these ALS inhibitors (Haughn and Somerville, 1986; Winder and Spalding, 1988; Saxena and King, 1988; and 1990; Saari et al., 1990; Hall and Devine, 1990 and Subramanian et al., 1990) suggesting that these herbicides may interact with non-overlapping binding sites on ALS.

We were interested in determining the molecular basis of imidazolinone-resistance for four reasons. First, such information will enhance our understanding of the interaction between imidazolinones and ALS. Second, it might elucidate the causes for lack of cross resistance in many mutant plants to both imidazolinones and sulfonylureas, indicating their sites of interaction on ALS may be different. Third, it will provide an explanation as to how weeds develop resistance to imidazolinones (Saari et al., 1990; Hall and Devine, 1990) and suggest a basis for developing sound weed control programs. Finally, isolation of a plant ALS gene conferring resistance to imidazolinones will permit the introduction of imidazolinone-resistance trait to crop varieties and provide a useful selectable marker in plant transformation experiments.

A homozygous mutant line (GH90) of *Arabidopsis* has been isolated and characterized by Haughn and Somerville (1990). GH90 is approximately 100-fold more resistant to imazapyr than the wild-type plants due to an imazapyr resistant ALS activity. Genetic analysis indicated that the imidazolinone- resistance is due to a single dominant nuclear mutation, *imr*, closely linked to the ALS locus. The mutant GH90 however, is not resistant to sulfonylureas (Haughn and Somerville, 1990) and triazolopyrimidines (Sathasivan and Murai, unpublished results). We isolated the ALS gene from the mutant GH90 by screening a genomic DNA library with an ALS probe from pGH1 (Sathasivan

et al., 1990). The ALS gene in a 5.8 kb XbaI fragment was subcloned into pUC18 creating plasmid pKS1. We sequenced both strands of this ALS gene and identified a single point mutation from G to A at the 1958th nucleotide position of the coding sequence. This mutation predicted the substitution from Ser to Asn at the 653rd residue of ALS. In this report, we provide evidence that the isolated mutant ALS gene from GH90 confers imazapyr-resistance to transgenic tobacco plants. Our results demonstrated that the single amino acid substitution from Ser to Asn near the carboxyl terminus of ALS is the molecular basis for imazapyr-resistance in the GH90 mutant.

MATERIALS AND METHODS

Plants, Bacterial Strains and Plasmids.

Seeds of both wild-type and mutant GH90 (csr1-2/csr1-2, Haughn and Somerville, 1990) *Arabidopsis thaliana* var. Columbia were germinated and grown on an autoclaved perlite/ vermiculite/ spaghnum mixture (1:1:1) as previously described (Haughn and Somerville, 1986). *E. coli* strain XL1 blue and the plasmid pBluescript were obtained from Stratagene and used for all vector construction and subcloning purposes. *Agrobacterium tumefaciens* strain LBA4404 containing the disarmed Ti plasmid (Hoekema et al., 1983) was used for tobacco transformation. Plasmid pGH1 contains an ALS gene encoding for sulfonylurea-resistance as described by Haughn et al. (1988). Plasmid pKS1 was created by subcloning the 5.8 kb XbaI fragment from the lambda clone Imr3ALS3 containing the ALS gene from GH90 into pUC18 (Sathasivan et al., 1990). The plant transformation vector pTRA409 (11.5 kb size) was constructed by Murai and coworkers (unpublished) containing the right and left border of Ti plasmid

flanking the NPTII gene with tml promoter and terminator in a broad host range vector pTJS75. This vector has a prokaryotic tetracyclin selection marker and an unique XbaI site.

Chemicals

Imazapyr and imazaquin were obtained from American Cyanamid Chemical Company, Princeton, New Jersey. Triazolopyrimidine was a gift from Dr. M. Subramanian, Dow-Elanco Chemical Company, Walnut Creek, California.

Nucleic acid techniques:

To compare the DNA sequence of the mutant with the wild-type ALS gene, the 3' region of wild-type and mutant ALS gene were subcloned into pBluescript at the BglII-NsiI restriction site. The plasmid DNA was sequenced with sequenase according to the manufacturers instructions by the chain termination method (Sanger et al., 1977). The DNA from tobacco plants was isolated as described by Nagao et al. (1983). The *Arabidopsis* DNA was isolated as described by Leutwiler et al. (1984). DNA from the non-transformed and transgenic plant 435-3A was restricted with EcoRI, fractionated on a 0.8% (w/v) agarose gel, transferred to nitrocellulose membrane (Schleicher and Schull) and hybridized to 2.1 kb EcoRI probe containing the 5' region of ALS gene from pKS1 according to the manufacturers recommendations. Standard techniques were followed according to Maniatis et al. (1982).

Construction of plant transformation vector pTRA435

A 4.6 kb ClaI-XbaI fragment from pKS1 containing 2.03 kb ALS coding, 1.25 kb upstream and 1.35 kb downstream regions was subcloned into pBluescript to create

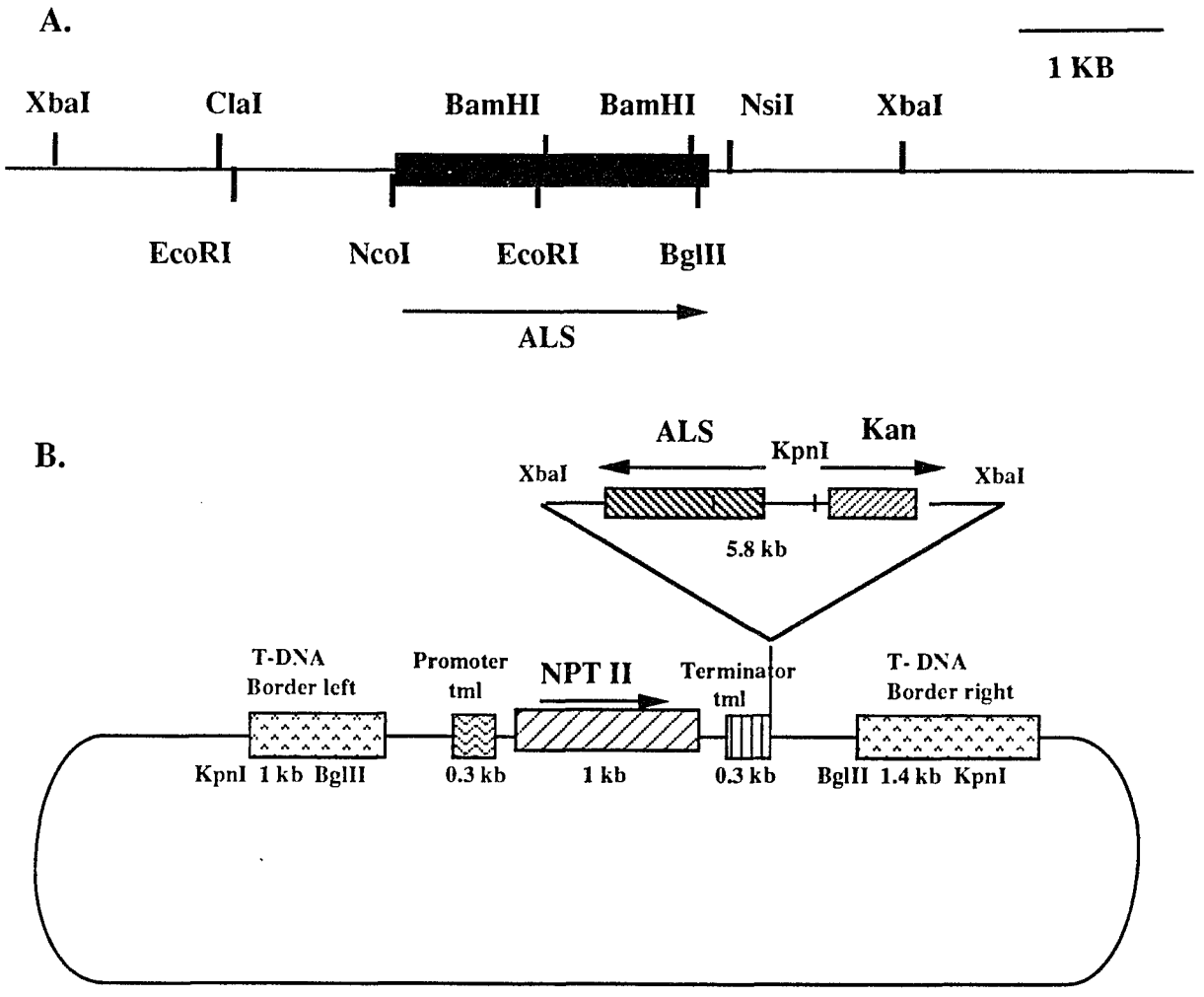


Figure 3.1. Restriction endonuclease maps of the lambda clone and the plant expression vector containing the mutant ALS gene.

A 1.2 pBKS1. This generated a KpnI site close to the ClaI site upstream of ALS gene. kb EcoRI fragment containing a Tn903-derived neomycin phosphotransferase gene from kSAC (Pharmacia) was subcloned into pBluescript to generate pBKan with KpnI-XbaI sites on both sides of the kanamycin marker. A 4.6 kb KpnI-XbaI fragment from pBKS1 containing the ALS gene and its flanking region was ligated with the 1.2 kb KpnI-XbaI from pBKan containing kanamycin marker gene. The resulting 5.8 kb XbaI fragment was cloned into a XbaI site of pTRA409 to generate the plant expression vector pTRA435. The prokaryotic kanamycin marker gene facilitated the selection of subclones containing the ALS gene in the 11.5 kb pTRA409 vector and helped for stable maintanence of the pTRA435 in both *E. coli* and *A. tumefaciens*. The pTRA435 containing the mutant ALS gene was transferred to *A. tumefaciens* by the freeze-thaw method as described by Hofgen and Willmitzer (1988).

Tobacco transformation:

Leaf discs from *Nicotiana tabacum* cv. Xanthi were transformed with the *A. tumefaciens* containing pTRA435 by cocultivation follwed by kanamycin selection, as described by Burow et al. (1990). As controls, tobacco leaf discs were also cocultivated with *A. tumefaciens* which are either non-transformed or transformed with pTRA409 only and regenerated in a similar fashion. Leaf discs transformed with pTRA435 and pTRA409 were transferred to callus media containing 300 mg/l kanamycin and 500 mg/l carbenicillin and maintained for three weeks. Calli from independent leaf discs were transferred to shooting media with 100 mg/l kanamycin and 300 mg/l carbenicillin and numbered. Shoots and their corresponding calli were transferred to rooting and callus

maintenance media, respectively, containing 100 mg/l kanamycin. Non-transformed calli and their shoots were maintained in appropriate media without kanamycin. Rooted plants were transferred to soil and grown to maturity.

Evaluation of herbicide resistance

Approximately ten-day old calli were placed in callus maintenance media containing various concentrations of imazapyr. All plates contained 500 mg/l carbenicillin in addition to the herbicides to suppress the growth of residual *A. tumefaciens*. Imazapyr was dissolved in sterile 10 mM potassium phosphate buffer pH 7.5 to prepare 50 mM stock solution and the stock was diluted to obtain 1 mM, 100 μM, 10 μM, 1μM, 100 nM, and 10 nM final concentrations. Each treatment had four replications and the experiments were conducted at three times. The callus fresh weight was taken after 21 days of incubation at 25 C under continuous fluorescent lighting.

For the leaf cutting assay, healthy young leaves from regenerated plants were surface sterilized with 40 % (v/v) commercial bleach, 0.02 % Triton X-100 (v/v) and rinsed with sterile water. Sterilized leaves were cut into approximately 0.25 cm squares and placed in callus maintenance media containing various concentrations of herbicide. The experiment was conducted twice with five replications per treatment. After 21 days incubation at 25 C under continuous fluorescent lighting, the leaf cuttings were photographed.

ALS assay

Activity of ALS was measured based on the method described by Chaleff and Mauvais (1984) and as modified by Haughn et al. (1988) and Singh et al. (1988). Three

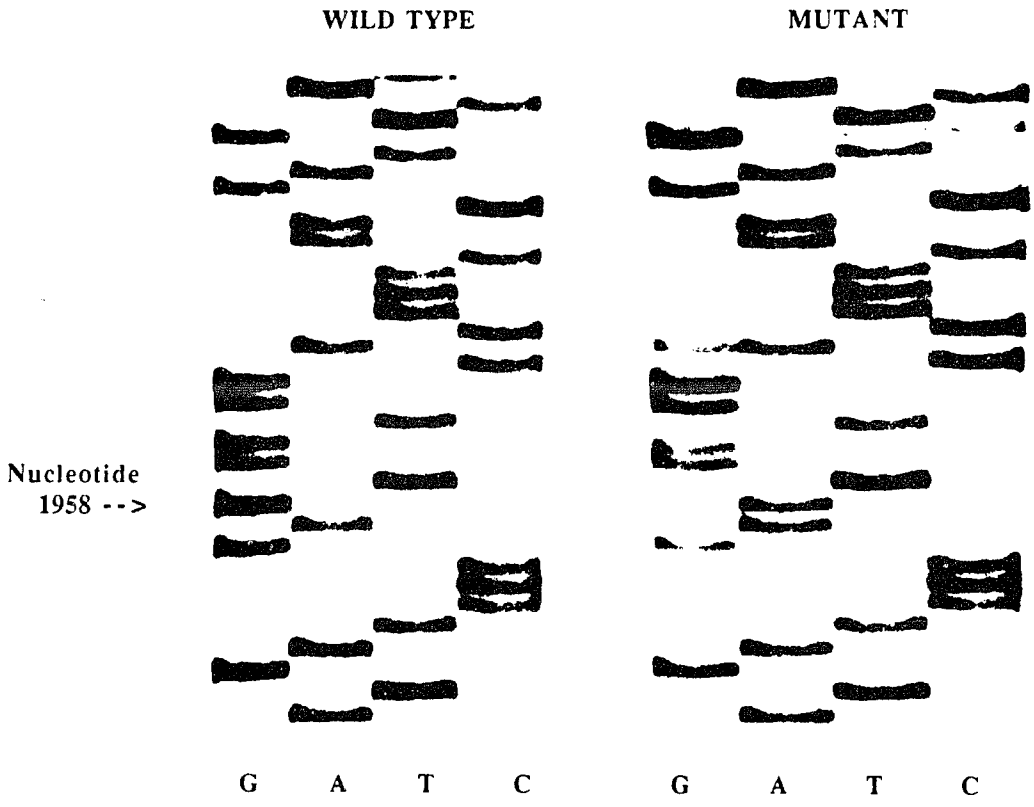
g of fresh leaf samples were ground to a fine powder in liquid N_2. The powder was mixed in 10 ml of cold extraction buffer containing 100 mM potassium phosphate buffer, pH 7.5, 10 mM $MgCl_2$, 40 mM Na pyruate, 500 µM thiamine pyrophosphate, 23 µM FAD, and 1 % polyvinyl pyrolidone. The homogenate was passed through cheese cloth and centrifuged at 27,000 g at 4 C for 10 min and the supernatant was aliquoted into 200 µl volumes in microcentrifuge tubes. Fifty µl of 10X herbicide concentrations, and 250 µl of 2X assay buffer (65 mM potassium phosphate, pH 7.5, 10 mM $MgCl_2$, 100 mM sodium pyruvate, 250 µM thiamine pyrophosphate, and 23 µM FAD) were added to each aliquot. The reaction mixtures were incubated at 37 C for 90 min. The conversion of acetolactate to acetoin and the measurement of acetoin produced were as previously described (Chaleff and Mauvais, 1984).

RESULTS

A single point mutation near the 3' end of ALS gene

The cloning and entire DNA sequence of the mutant ALS gene from GH90 has been reported previously (Sathasivan et al., 1990). A single point mutation from G to A was identified at the 1958th nucleotide position of the ALS coding region. The restriction map of a part of the lambda clone containing the ALS gene is shown in Figure 1A. To confirm the mutation reported earlier, the 3' region of the mutant and wild type ALS gene were subcloned in pBluescript, and the plasmid DNA was sequenced as shown in Figure 2A. A mutation from guanosine and adenosine was observed at the 1958th nucleotide in the mutant ALS gene. This would result in serine to asparagine substitution at the 653rd residue of the matured protein as shown in Figure 3.2B.

A.



B.

Nucleotide		1958
		Ser
Wild type	ATG ATC CCG	AGT GGT GGC ACT TTC
		I
Imr3	ATG ATC CCG	AAT GGT GGC ACT TTC
		Asn
Amino acid		653

Figure 3.2. A single point mutation in the ALS gene from the imidazolinone resistant GH90 mutant.

A. The BglII/NsiI fragment at the 3' region was subcloned into pBluescript and the plasmid DNA was sequenced by dsDNA sequencing technique and fractionated on a 6 % polyacrylamide gel. B. Comparison of the wild-type and mutant sequences.

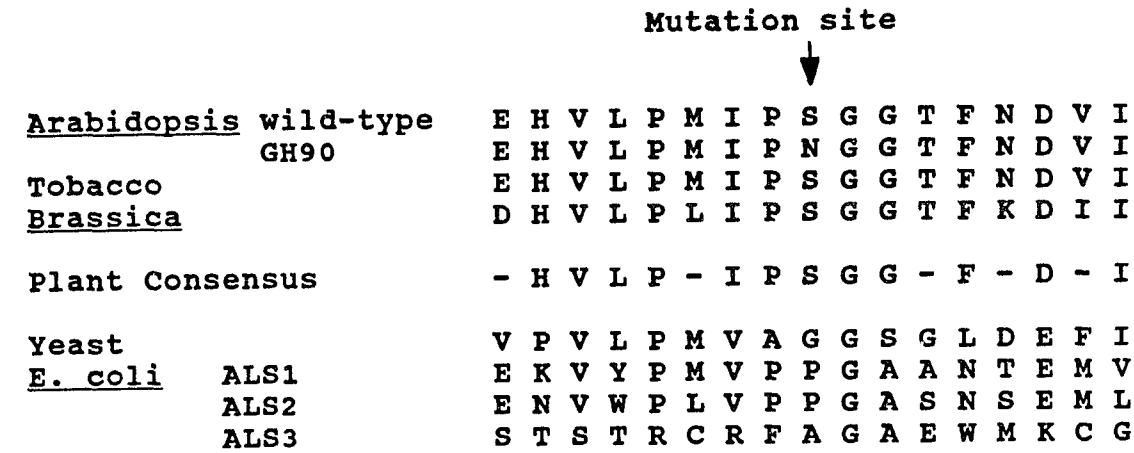


Figure 3.3. Conservation of amino acids in the ALS around the mutation conferring imidazolinone-resistance.

The nucleotide sequences were obtained from Mazur et al. (1987) and Wiersma et al. (1989) and the comparisons were made.

This ALS mutation in the imazapyr-resistant GH90 is unique in that its position is closer to the carboxyl terminus when compared to the other ALS mutations conferring sulfonylurea resistance in higher plants (Lee et al., 1988; Mazur and Falco, 1989) as summarized in Table 3.1. The mutation is observed in the 653rd residue Ser which is conserved among the known plant species but not among yeast and procaryotes (Mazur et al., 1987; Wiersma et al., 1989), implying the importance of this region to plant ALS, as shown in Figure 3.3.

Expression of herbicide resistance in transgenic tobacco

The ALS gene from GH90 was introduced into *Nicotiana tabacum* var. Xanthi to test its ability to confer imidazolinone-resistance phenotype in the transformants. A 4.6 kb ClaI/XbaI fragment containing a 2.03 kb ALS coding, 1.25 kb upstream and 1.35 kb downstream flanking regions was subcloned along with a 1.2 kb prokaryotic kanamycin marker gene into a 11.5 kb plant transformation vector pTRA409 to generate pTRA435 shown in Figure 3.1B. The pTRA435 was introduced into *A. tumefaciens* by the freeze-thaw method (Hofgen and Willmitzer, 1988). Tobacco leaf discs were transformed by cocultivation with recombinant *Agrobacterium* and the transformants were selected by screening with 300 mg/l kanamycin and 500 mg/l carbenicillin (Burow et al., 1990). The transformed calli from independent leaf discs were transferred to shooting media containing 100 mg/l kanamycin for secondary selection. The regenerated shoots and the corresponding calli were transferred to rooting media and callus maintenance media respectively containing 100 mg/l kanamycin. The transformed calli maintained in callus maintenance media were initially tested for herbicide resistance even though they

represented a mixture of transformants. Since, the primary and secondary selection of the transformants was based on the cotransformed NPTII gene conferring kanamycin resistance and not based on imidazolinone resistance, it minimized the chances of selecting spontaneous mutants resistant to the herbicide.

Calli derived from independently transformed leaf discs 435-6A, 435-12A, and 435-13 were plated on callus maintenance media containing different concentrations of imazapyr. As shown in Figure 3.4, the nontransformed control calli grew in concentrations up to 100 nM imazapyr. Transformants 435-13, 435-12A, and 435-6A survived and grew on media containing up to 1 μM, 10μM, and 100 μM imazapyr, respectively, showing 10, 100, and 1000-fold resistance to the non-selective herbicide imazapyr. That imazapyr-resistance of the transformed calli is due to the introduced mutant ALS gene rather than a higher growth rate because growth of nontransformed calli was greater than the transformants in control media lacking herbicide. The difference in the levels of resistance to imazapyr among the transformed calli may be due to the chromosomal position, or copy number of the introduced ALS gene in the tobacco genome.

Imazapyr-resistance in leaves of transgenic plants

Since the primary transformed calli tested above for herbicide-resistance may have represented several independent transformants, the leaves of the regenerated plants were tested to confirm the herbicide resistance of cells with a homogeneous genetic background. Similar tests of using leaf cuttings to assay for herbicide resistance have been described by Stalker et al., (1988) and Olszewski et al. (1988). Leaf cuttings of the

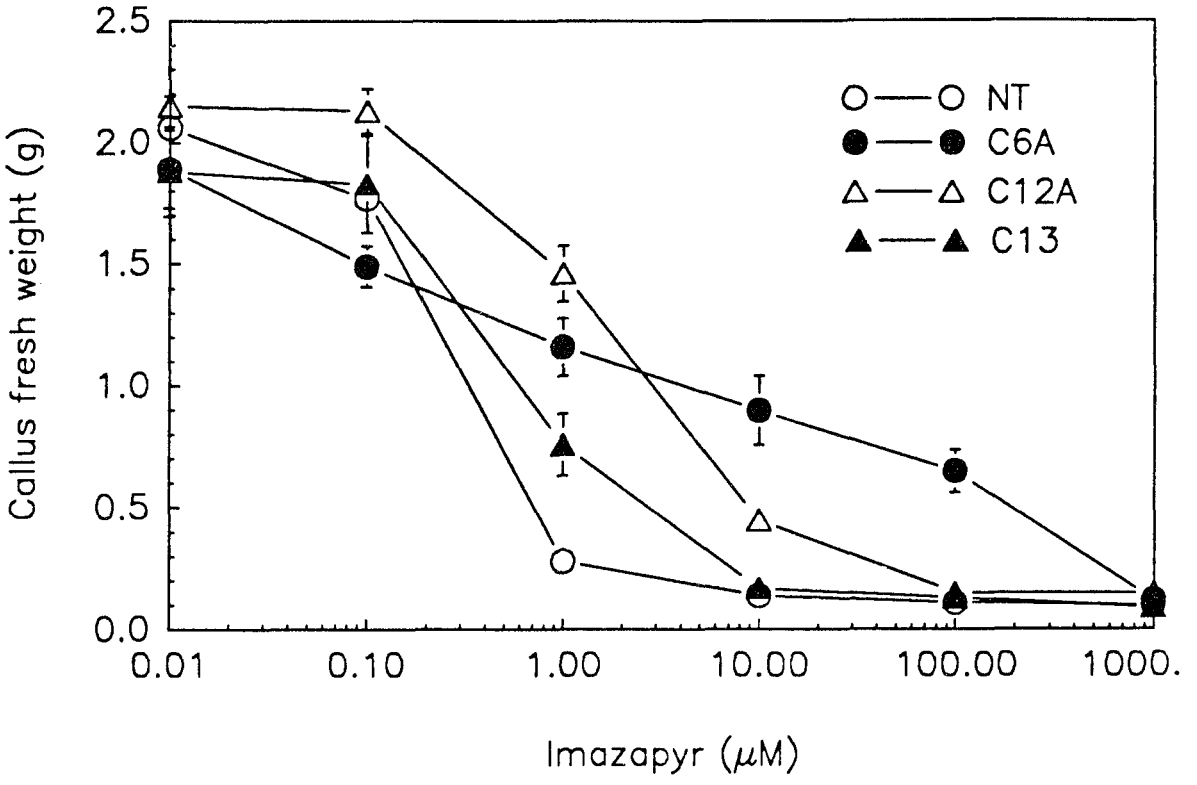


Figure 3.4. Expression of imazapyr-resistance in transformed tobacco calli.

The calli derived from independently transformed leaf discs 435-6A (C6A), 435-12A (C12A), and 435-13 (C13) were grown along with non-transformed control (NT) in various concentrations of imazapyr for 21 days before measuring the fresh weight. The experiment was conducted three times and each data point represents an average of four replications. The average callus fresh weight of NT, C6A, C12A, and C13 in the control media without imazapyr were 2.04, 1.49, 2.37, and 2.16 g, respectively.

transformed and non-transformed plants were surface sterilized and placed on callus maintenance medium containing various concentrations of the herbicide. As shown in Figure 3.5, non-transformed control leaf formed calli up to the imazapyr concentration of 100 nM. Transformants 435-8A and 435-8D showed 10-fold resistance by growing up to 1 μM imazapyr. The transformant 435-3A grew up to 10 μM showing 100-fold resistance. It should also be mentioned that the surface-sterilization treatment of leaf with 40 % bleach and 0.02 % Triton X-100 appeared to increase the susceptibility to the herbicide. The lack of resistance in other transformed plants could be due to the surface-sterilization, absence of active resistant ALS gene due to rearrangement during integration or a leaky selection with kanamycin. Similar variations in the herbicide resistance levels was observed by Stalker et al. (1988) in tobacco plants transformed with the bromoxynil detoxification gene. The level of imazapyr resistance in the transformants 435-3A and 435-8A showing 100- and 10-fold imazapyr-resistance was well correlated with the levels of resistance at the enzyme level as shown below.

Expression of imazapyr-resistance at the enzyme level

ALS activity was assayed in leaves of non-transformed and transformed plants to show that the imazapyr resistance is due to the altered enzyme activity. Crude extracts were prepared from young leaves as described in the methods section and used directly for herbicide inhibition studies. The percent uninhibited activity of ALS in response to various imazapyr concentrations from 100 nM to 1 mM is shown in Figure 3.6. ALS activity in non-transformed plant (NT) and plant transformed with pTRA409 (409-H1) were greatly inhibited at the concentration of 10 μM imazapyr.

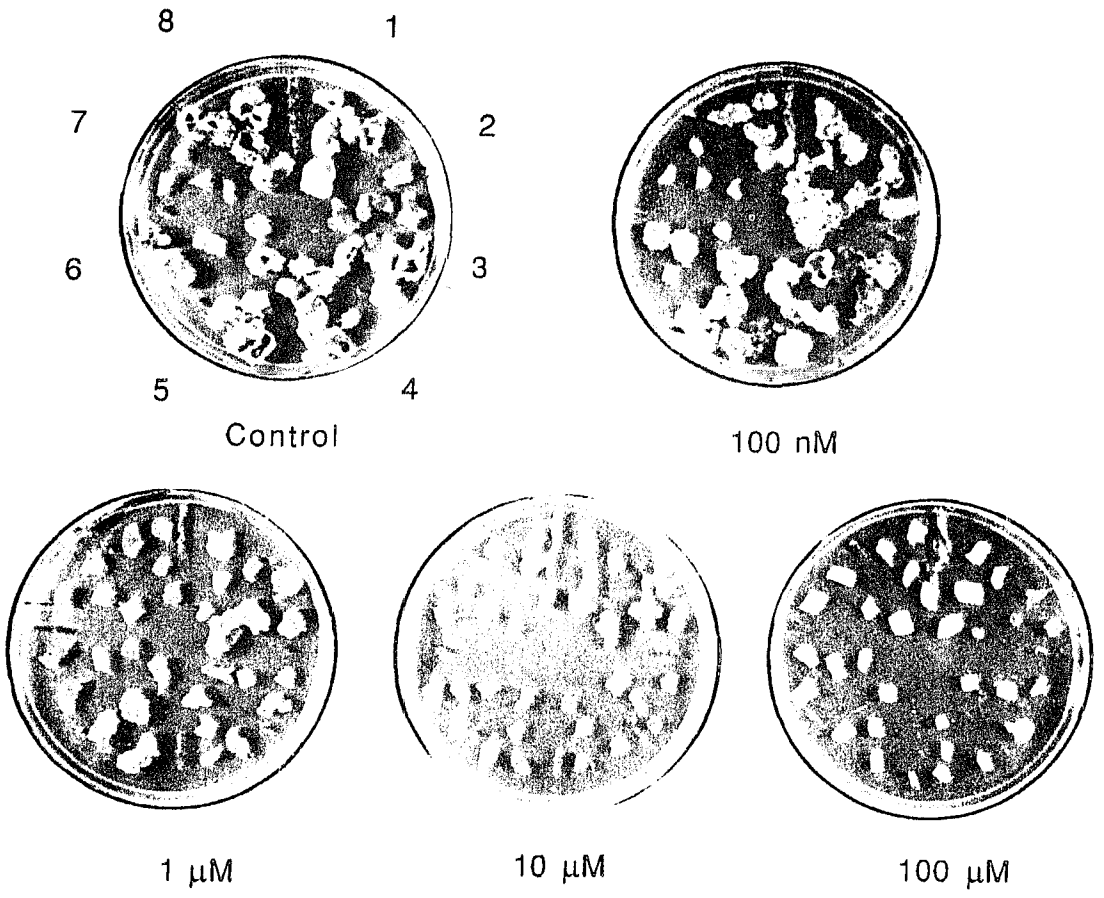


Figure 3.5. Expression of imazapyr-resistance in leaves of regenerated tobacco plants.

The leaf cuttings of non-transformed (1), and transformants 435-3A (2), 435-6B (3), 435-8A (4), 435-8D (5), 435-9B (6), 435-11A (7), and 435-12A (8) were placed on callus maintanence media containing various imzapyr concentrations and photographed. The experiment was conducted twice. Representative plates out of five replications are shown.

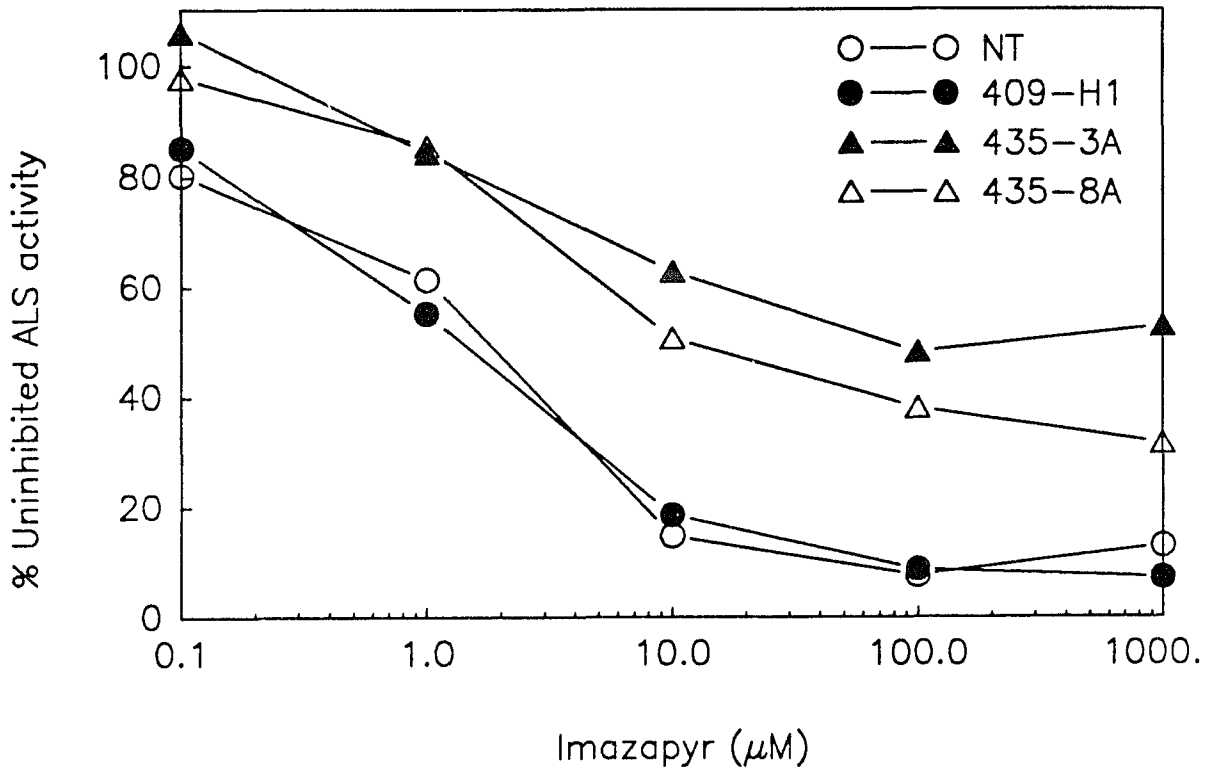


Figure 3.6. Assay of acetolactate synthase activity in tobacco leaf extracts.

The ALS activity was expressed as percentage of control without any herbicide in crude leaf extracts from the non-transformed (NT), transformed with pTRA409 (pTRA409-H1) or pTRA435 (435-3A and 435-8A). The ALS activity in the leaf extracts of all the plants tested above were similar in the control without herbicide and were treated as 100 % for each plant.

The ALS activity in transformants 435-3A and 435-8A was approximately 50 % of the control at 10 μM imazapyr. The 50 % ALS activity in transformants was not inhibited even up to a concentration of 1 mM imazapyr showing a 1000-fold resistance. That 50 % of the ALS activity was inhibited may be accounted for by the fact the transformed plants contains the endogenous tobacco ALS genes coding for imazapyr-sensitive ALS. The observation that transformant 435-3A showed slightly higher ALS activity than transformant 435-8A correlates well with the herbicide-resistance levels in leaf cutting assay. Plants transformed with the vector only (409-H1) showed similar inhibition as the nontransformed plant, indicating that the imazapyr resistance in the transgenic plants was due to the introduced mutant ALS gene coding for an imazapyr resistant ALS.

Southern analysis:

To confirm that the transformants contain the mutant ALS gene from GH90, southern hybridization analysis was performed. The presence of the introduced *Arabidopsis* gene in transgenic tobacco plant 435-3A showing the maximum level of imazapyr-resistance is shown in Figure 3.7. Plant genomic DNA digested with EcoRI was hybridized to a 2.1 kb EcoRI fragment from pKS1, which includes 0.9 kb of coding and 1.2 kb upstream regions of ALS gene. The expected 2.1 kb band was seen only in the *Arabidopsis* and the transformed tobacco 435-3A. Moreover, transformant 435-3A showed additional bands of 3.2 kb and 5.4 kb sizes probably due to multiple integration of the introduced pTRA435 plasmid into the tobacco genome. Non-transformant showed a faint 3.2 kb band which may be due to heterologous hybridization since the ALS genes of *Arabidopsis* and tobacco shares 80 % nucleotide homology (Mazur et al., 1987).

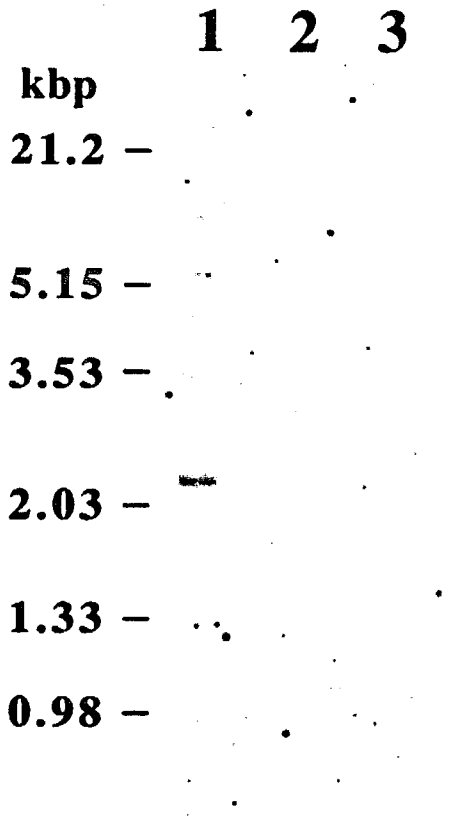


Figure 3.7. Southern hybridization analysis of the DNA from the transformant showing maximum resistance to imazapyr.

Ten μg of total DNA each from the transformant 435-3A (lane 1), non-transformed tobacco (lane 2) and 2 μg of total DNA from wild-type *Arabidopsis (lane 3)* were digested with EcoRI and fractionated on a 0.8 % agarose gel, transferred to nitrocellulose filter and hybridized to EcoRI 2.1 kb ALS probe from pKS1.

DISCUSSION

We have expressed the mutant ALS gene from GH90 in tobacco and showed that it can confer imazapyr-resistance in transformed plants. The ALS gene contained a point mutation from G to A at the 1958th nucleotide which predicted a substitution of Asn to Ser at the 653rd residue of the ALS. This mutant ALS gene transformed imidazolinone-sensitive tobacco into an imidazolinone-resistant phenotype and encodes an imazapyr-resistant ALS activity. We believe that the imazapyr-resistant ALS activity is due to the single amino acid substitution identified at the 653rd residue. This finding has been independently confirmed by P. Stougaard, Denmark (Personal communication) by introducing this mutation in sugar beet ALS gene and expressing it in *E. coli*, showing it confers imazethapyr resistance. The evidence of the Asn-653 mutation in ALS showing imazapyr-resistant ALS activity in the transgenic plants indicates that this unique mutation is the molecular basis of imidazolinone resistance in *A. thaliana*.

Probable effects of point mutations:

The mutations listed in Table 3.1 might cause an alteration in the herbicide binding sites on ALS directly or indirectly through changes in the secondary structure of the protein. A structural preference and hydropathy analysis of these mutations (data not shown) indicated that all the mentioned amino acid substitutions are probably in reverse turns on the external surface of ALS molecule. These data combined with the fact that the mutation does not severely affect ALS function suggests that this mutation does not cause a significant change in the secondary or tertiary structure of ALS. The most probable effect of these mutations is that they cause a minor alteration in electrical charge

Table 3.1. Mutations in acetolactate synthase gene conferring herbicide resistance in plants.

Mutant	Amino acid	Mutation	Resistance to	Reference
Arabidopsis thaliana				
GH 50	197	CCT ------>TCT Pro Ser	Sulfonylurea	Haughn et al.,1988
GH 90	653	AGT ------>AAT Ser Asn	Imidazolinone	This paper
Nicotiana tabacum				
SuRA-C3	196	CCA ------>CAA Pro Gln	Sulfonylurea	Lee et al., 1988
SuRB-S4 HRA	196 and	CCA ------>GCA Pro Ala	Sulfonylurea and Imidazolinones	Lee et al., 1988 and Mazur and Falco, 1989
	573	TGG ------>TTG Trp Leu		

or stearic hinderance at the enzyme-herbicide interaction site, thus interfering with the herbicide binding to ALS.

The above discussion might also explain the probable causes for the lack of cross-resistance to other ALS-inhibitors. Haughn and Somerville (1987, 1990) showed that the *Arabidopsis* mutants resistant to chlorsulfuron are not cross-resistant to imazapyr and vice versa. Saxena and King (1988, 1990) showed similar results of lack of cross-resistance to both sulfonylureas and imidazolinones in *Datura innoxia* cell lines. In addition, Subramanian et al. (1990) reported spontaneous mutants of tobacco and cotton cell lines showing varied levels of cross-resistance to four different types of ALS-inhibiting herbicides and suggested non-overlapping binding domains for these structurally diverse ALS-inhibitors. Here, we show the first evidence at molecular level that mutations which do not result in cross-resistance in *Arabidopsis* lie in distinctly different parts of the gene. However, we can not conclude that the binding domains of imidazolinones and sulfonylureas are different. Since two amino acids located far apart in a coding sequence can still fold together to form a single herbicide binding domain, as suggested by Schloss et al. (1988). The amino acid residues specifically interacting with imidazolinone and sulfonylurea herbicides or for each individual herbicide within each class of the ALS inhibitors may be different in the same binding region. Further conclusions on the binding sites of these herbicides must await more detailed studies on the ALS structure.

Herbicide-resistance in weeds

Hall and Devine (1990) observed cross-resistance of sulfonylurea-resistant biotype of *Stellaria media* to a triazolopyrimidines and not to imidazolinones suggesting that

sulfonylurea and triazolopyrimidine may share a common binding site different from that of imidazolinone. The results presented here and by Mazur and Falco (1989), showing that resistance to sulfonylurea and imidazolinone herbicides are caused by different mutations imply that the frequency of weeds developing resistance to both these herbicides (i.e. the chance of getting a double mutant, for example 10^{-12}) is lower than the chances of developing resistance to only one group (ie. single point mutant, for example 10^{-6}). However, considering the large population of weed seeds present in the soil, the probability of developing resistance to more than one ALS inhibiting herbicides over several cropping seasons is high. Hence, we suggest that herbicides with a target site other than ALS should be rotated or used in combination with these ALS inhibitors.

Other implications

This mutant ALS gene could be introduced into other crop species to develop imidazolinone-resistant crop varieties. Also, similar mutations could be introduced at the corresponding nucleotide positions of other ALS genes to confer imidazolinone resistance as shown by P. Stougarrd (Personal communication). This mutant gene could also be used as a selection marker in plant transformation systems with its native promoter in dicots and with a modified promoter in monocots. Moreover, this gene could be expressed in prokaryotic systems and site-directed mutagenesis could be performed to study how the different groups of herbicides inhibit ALS.

LITERATURE CITED

Burow, M. B., Chlan, C. A., Sen, P., Lisca, A, and Murai, N. (1990). High frequency generation of transgenic tobacco plants after modified leaf disc cocultivation with *Agrobacterium tumefaciens*. Plant Mol. Biol. Reporter 8:124-139.

Chaleff, R. S., and Mauvais, C. J. (1984). Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. Science 224:1443-1445.

Gerwick, B. C., Loney, V., and Subramanian, M. (1987). Mechanism of action of 1,2,4-triazolo [1,5A] pyrimidines. American Chem. Soc. 194th National Meeting. New Orleans, LA. Abstract No. 163.

Hall, L. M., and Devine, M. D. (1990). Cross resistance of a chlorsulfuron-resistant biotype of *Stellaria media* to a triazolopyrimidine herbicide. Plant Physiol. 93:962-966.

Haughn, G. W., Smith, J., Mazur, B., and Somerville, C. R.(1988). Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. Mol. Gen. Gen. 211:266-271.

Haughn, G. W., and Somerville, C. R. (1986). Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol. Gen. Gen. 204:430-434.

Haughn, G. W., and Somerville, C. R. (1990). A mutation causing imidazolinone resistance maps to *csr1* locus in *Arabidopsis thaliana* var. Columbia. Plant Physiol. 92:1081-1085.

Hawkes, T. R. (1989). Studies of herbicides which inhibit branched chain amino acid biosynthesis. *In* L. G. Copping, J. Dalziel, and A. D. Dodge, eds, Prospects for Amino Acid Biosynthesis Inhibitors in Crop Protection and Pharmaceutical Chemistry. BCPC monograph No 42, pp 131-138.

Hoekema, A., Hirsch, P. R., Hooykaas, P. J., and Shilperoort, J. (1983). A binary plant vector strategy based on separation of *vir* and T region of the *Agrobacterium tumefaciens* Ti plasmid. Nature 303:179-180.

Hofgen, R., and Willmitzer, L. (1988). Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids. Res. 16:9877.

Lee, K. Y, Townsend, J., Tepperman, J., Black, M., Chui, C. F., Mazur, B., Dunsmuir, P., and Bedbrook, J. (1988). The molecular basis of sulfonylurea resistance in tobacco. EMBO J. 7:1241-1248.

Leutwiler, L. S., Haugh Evans, B. R., and Meyerowitz, E. M. (1984). The DNA of *Arabidopsis thaliana*. Mol. Gen. Gen. 194: 15-23.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, p545.

Mazur, B., Chui, C., and Smith, J. K. (1987). Isolation and characterization of plant genes coding for acetolactate synthase, the target enzyme for two classes of herbicides. Plant Physiol. 85:1110-1117.

Mazur, B. J. and Falco, S. C. (1989). The development of herbicide resistant crops. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:441-470.

Nagao, R. T., Shaw, D. M., Eckenrode, V. K. and Meagher, R. B. (1983). Multigene family of actin related sequences isolated from soybean genomic library. DNA 1:1-9.

Olszewski, N. E., Martin, F. B., and Asubel, F. M. (1988). Specialized binary vector for plant transformation: Expression of the *Arabidopsis thaliana* AHAS gene in *Nicotiana tabacum*. Nucl. Acids Res. 16:10765-10782.

Peoples, T. R. (1984). Arsenal herbicide (AC 252925) : A developmental overview. Proc. South. Weed Sci. Soc. 37:378

Ray, T. B. (1984). Site of action of chlorsulfuron. Inhibition of valine and isoleucine biosynthesis in plants. Plant Physiol. 75:827-831.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.

Saari, L. L., Cotterman, J. C., and Primiani, M. M. (1990) Mechanism of sulfonylurea herbicide resistance in the broadleaf weed *Kochia scoparia*. Plant Physiol. 93:55-61.

Sathasivan, K., Haughn, G.W., and Murai, N. (1990). Nucleotide sequence of a mutant acetolactate synthase gene from imidazolinone resistant *Arabidopsis thaliana* var. columbia. Nucleic Acids Res. 18-2188.

Saxena, P. K. and King, J. (1988). Herbicide resistance in *Datura innoxia*. Cross-resistance of sulfonyl urea resistance lines to imidazolinones. Plant Physiol. 86: 863-867.

Saxena, P. K., and King, J. (1990). Lack of cross resistance of imidazolinone-resistant cell lines of *Datura innoxia* P.Mill. to chlorsulfuron. Evidence for separable sites

of action on the target enzyme. Plant Physiol. 94:1111-1115.

Schloss, J. V., Ciskkanik, L. M., and VanDyk, D. E. (1988). Origin of the herbicide binding site of acetolactate synthase. Nature 331:360-362.

Shaner, D. L., Anderson, P. C., and Stidham, M. A. (1984). Imidazolinones. Potent inhibitors of acetohydroxy acid synthase. Plant Physiol. 76: 545-546.

Shaner, D. L., and Anderson, P. C. (1985). Mechanism of action of the imidazolinones and cell culture selection of tolerant maize. In Biotechnology in Plant Science. Eds. M. Zaitin, P. Day and A. Hollanender. Academic Press. NY. pp 287-299.

Singh, B. K., Stidham, M. A., and Shaner, D. L. (1988). Assay of acetohydroxy acid synthase. Anal. Biochem. 171:173-179.

Stalker, D. M., McBride, K. E., and Malyj, L. D. (1988). Herbicide resistance in transgenic plants expressing a bacterial detoxification gene. Science. 242:419-423.

Subramanian, M., Hung, H.U., Dias, J.M., Miner, V.M., J.H.Butler and Jachettia,J.J. (1990). Properties of mutant acetolactate synthase resistant to triazolopyrimidine sulfonanilide. Plant Physiol. 94:239-244.

Wiersma, P. A., Schmiemann, M. G., Condie, J. A., Crosby, W. L., and Moloney, M. M. (1989). Isolation, expression and phylogenetic inheritance of an acetolactate synthase gene from *Brassica napus*. Mol. Gen. Gen. 219:413-420.

Winder, T., and Spalding, M. (1988). Imazaquin and chlorsulfuron resistance and cross resistance in mutants of *chlamydomonas reinhardtii*. Mol. Gen. Genet. 213: 394-399.

CHAPTER IV

SUMMARY AND CONCLUSION

An ALS gene was isolated from an imazapyr-resistant mutant (GH90) of *Arabidopsis*, and its nucleotide sequence was determined. There was a single point mutation at the 1958th nucleotide of the ALS coding region. This would result in the substitution of Ser to Asn at the 653rd residue of the ALS. The mutant ALS gene, when expressed in tobacco, conferred resistance to imazapyr up to 1000-fold in calli and up to 100-fold in leaves of the transformed plants. This resistance was due to the altered ALS activity, resistant to imazapyr. The relative level of resistance in the leaves of transgenic plants was correlated with the relative level of imazapyr-resistant enzyme activity. The presence of the introduced mutant ALS gene was confirmed by southern hybridization analysis of the transformant 435-3A, showing 100-fold resistance. This mutation is unique when compared to the mutations conferring sulfonylurea-resistance in its position and nature of conferring-imidazolinone resistance.

The mutation sites of the *Arabidopsis* ALS gene causing sulfonylurea resistance and imidazolinone resistance in *Arabidopsis thaliana* are different. This implies that if a weed develops resistance to sulfonylurea, it may not always have cross-resistance to imidazolinone and vice versa. Hence for such sulfonylurea resistant weeds application of imidazolinone with similar efficacy can be applied as a short term solution. However, with large population of weed seeds in soil, it may not take many generations to develop cross-resistance to imidazolinones since a single base pair change in the appropriate coding region of ALS is sufficient to develop imidazolinone resistance. Hence, it is recommended that herbicides with target sites other than ALS should be rotated or used

in combination with imidazolinones or sulfonylureas.

Based on the results of this research it can not be concluded that sulfonylureas and imidazolinones bind to two separate sites in the ALS. We would need to know the 3 dimensional structure of ALS to correctly predict how close or far are the two mutations at 196th (sulfonylurea resistance) and 653rd residue (imidazolinone resistance) are in the tertiary structure of ALS. However, we can conclude that the effects of these mutations on the binding and/or inhibition of these two herbicides are different. To confirm that the mutations affect the binding of the herbicide, additional experiments need to be performed and details on the structure of ALS needs to be obtained.

This mutant ALS gene in pKS1 can be introduced into crop plants under the control of its native promotor or other promoters such as CaMV35S to confer imidazolinone resistance. If the *Arabidopsis* ALS gene does not express well in transformed plants, an endogenous ALS gene from such plant could be cloned, and similar mutations could be introduced to confer imidazolinone-resistance. Such an approach has been tried successfully in sugar beet by P. Stougarrd in Denmark (personal communication). Development of imidazolinone-resistant crop plants would increase the spectrum of herbicides a farmer could use in different crops and increase the market potential of these herbicides. Herbicides to which the resistance is introduced should be environmentally safe, least toxic to mammals, and not an added environmental concern such as ground water contamination. In the development and use of imidazolinone-resistant crop varieties, caution needs to be exercised when selecting the herbicide, crop varieties, cropping area, cultivation practice, chances for resistance transfer to weeds, and

metabolite or herbicide accumulation in resistant plants. Especially with long- term residual activity in the soil, the crop grown in rotation also has to be considered.

This gene can also be used as selection marker in the transformation experiments where antibiotics such as kanamycin or hygromycin are presently being used. This gene could be used for homologous recombination experiments in which a part of the ALS gene containing the mutation is introduced into *Arabidopsis,* and herbicide resistant transformants are selected and analyzed for homologous recombination event.

In the future, the progenies of the imazapyr-resistant transformants need to be tested for the segregation of the resistance phenotype to understand if the gene has been introduced into one locus or multiple loci. Also, they should be evaluated for cross-resistance to other imidazolinone herbicides, such as imazaquin, imazethapyr, and other ALS inhibitors. Residue studies need to be performed to determine the accumulation of herbicides such as imazapyr, in the meristems of resistant plants when the chemical is applied postemergent or preemergent. Since very little is known about the herbicide detoxification enzymes in plants at molecular level, more studies need to be done to characterize such enzymes and clone their genes to understand their regulation.

In conclusion, a single point mutation at the 1958th nucleotide from G to A in the ALS gene resulting in an amino acid substitution from Ser to Asn at the 653rd residue in the enzyme, forms the molecular basis of imidazolinone resistance in *Arabidopsis thaliana* var Columbia.

VITA

The author was born at Tirunelveli in South India to (late) Mr. T. Kanagasabapathi and Mrs. K. Suntharathammal on April 21, 1957. The author married Ms. Kanthimathi in 1983, and they have a daughter, Kanaka, and a son, Sarathi.

He completed his high school in M. D. T. Hindu College School in 1973 and Pre University Certificate at St. Xavier's College in 1974 at Tirunelveli. He entered Tamil Nadu Agricultural University at Coimbatore, India, where he completed a Bachelor of Science degree in Agriculture in 1978 and a Master of Science degree in Agronomy in 1980.

He worked as Product Development Supervisor for rice herbicides for Monsanto Chemicals of India Ltd. from 1980 to 1983. He was in charge of the rice herbicides development in South India. He was promoted as Product Manager (Acetanilides) and assigned to Monsanto Singapore Ltd, Singapore from 1983 to 1985. At Singapore, he coordinated the acetanilide herbicide development and registration in Southern Asia.

He entered Louisiana State University, Baton Rouge in 1985 for a Ph.D. program in weed science with Dr. L. M. Kitchen. After Dr. Kitchen left Louisiana State University in January 1987, he started working under Dr. N. Murai's guidance on this herbicide resistance research project. He is now a candidate for Ph.D. and currently working at the University of Texas at Austin, Austin, Texas.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: *Kanagasabapathi Sathasivan*

Major Field: *Plant Health*

Title of Dissertation: *The molecular basis of imidazolinone herbicide resistance in Arabidopsis thaliana VAR. Columbia*

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

April 19, 1991