Theor Appl Genet (1991) 83:65–70

THEORETICAL AND APPLIED GENETICS

Mutations in corn (*Zea mays* L.) conferring resistance to imidazolinone herbicides

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Received December 11, 1991; Accepted February 4, 1991
Communicated by J.W. Snape

Summary. Three corn (*Zea mays* L.) lines resistant to imidazolinone herbicides were developed by in vitro selection and plant regeneration. For all three lines, resistance is inherited as a single semidominant allele. The resistance alleles from resistant lines XA17, XI12, and QJ22 have been crossed into the inbred line B73, and in each case homozygotes are tolerant of commercial use rates of imidazolinone herbicides. All resistant selections have herbicide-resistant forms of acetohydroxyacid synthase (AHAS), the known site of action of imidazolinone herbicides. The herbicide-resistant phenotypes displayed at the whole plant level correlate directly with herbicide insensitivity of the AHAS activities of the selections. The AHAS activities from all three selections have normal feedback regulation by valine and leucine, and plants containing the mutations display a normal phenotype.

Key words: Herbicide resistance – *Zea mays* L. – Acetohydroxyacid synthase – Imidazolinones

Introduction

Four agronomic practices that have facilitated dramatic increases in grain production over the past 30 years include high yielding crop varieties, nutrient supplementation with fertilizers, the use of chemical agents for pest control, and improved mechanization for planting and harvesting. Each of these practices is presently an integral and essential component of commercial food production in developed countries.

In recent years producers and consumers alike have begun to question whether feasible alternatives to the external inputs currently used in contemporary agriculture systems are available. Plant biotechnology is poised to deliver products to the agricultural industry that may provide genetic alternatives to some pesticide applications. For example, by introducing into crops a gene that produces the natural insecticidal toxin produced by *Bacillus thuringiensis*, the need for some chemical insecticide applications may be eliminated (Gasser and Fraley 1989).

These same technologies do not appear to provide similar alternatives to the use of herbicides for weed control. Herbicides have been, and for the foreseeable future will continue to be, a major component of weed control practices. Genetic modification can currently be used to allow the use of different herbicides for weed control (Botterman and Leemans 1988; Gasser and Fraley 1989; Mazur and Falco 1989). The introduction of herbicide-resistance traits into crops will enable farmers to use products that are both more effective for weed control and safer than many of the herbicides currently in use.

The imidazolinones are a relatively new class of herbicides with favorable environmental and commercial characteristics. Their high biological potency makes them effective at low application rates. Sensitive plants are killed by the inhibition of acetohydroxyacid synthase (AHAS, E.C.4.1.3.18; also known as acetolactate synthase), which catalyses the first step in the biosynthesis of the branched amino acids valine, leucine, and isoleucine (Shaner et al. 1984). Because this biosynthetic pathway is not present in animals, the imidazolinones are relatively nontoxic to animals. These characteristics could make the imidazolinones useful for weed control in corn (*Zea mays L.*); however, corn is inherently sensitive to currently used imidazolinone herbicides. In the present paper,

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three mutations in corn selected in vitro that confer resistance to imidazolinone herbicides are described.

Materials and methods

Development of imidazolinone-resistant corn inbreds

The selection of the imidazolinone-resistant corn lines used in this study has been described by Anderson and Georgeson (1989). In vitro selection in tissue culture was used to identify resistant cell lines. Resistant plants were regenerated from resistant cell lines. The mutant regenerated plants were crossed with the inbred B73, and have subsequently been backcrossed to B73 for two, four, and six additional generations for the mutant lines QJ22, XI12, and XA17, respectively. Two generations of selfing after backcrossing produced nonsegregating resistant lines. F_2 and testcross progenies from the most recent generation of the backcrossing procedure were used for the segregation data reported in this paper.

Greenhouse tests

All herbicide treatments applied in greenhouse tests used commercial formulations of the herbicides involved and included a 0.25% vol./vol. concentration of the nonionic surfactant Tween 20. All plants were grown using Metromix 350 (W.R. Grace Co) as a growth medium for the plants. Herbicide treatments were applied with a custom designed belt sprayer. Herbicide treatments were applied broadcast in a spray volume of 960 l/ha using a 40015E nozzle tip (Spraying Systems Co, Wheaton, Ill.) for applications made to plants in greenhouse flats. For tests conducted in pots, a 65015E nozzle tip (Spraying Systems Co, Wheaton, Ill) and a spray volume of 400 l/ha was used.

Inheritance of the herbicide resistance trait was evaluated by planting F_2 and testcross progenies in greenhouse flats and treating the resulting seedlings postemergence at the two- to four-leaf stage. A rate of either 150 or 200 g/ha of imazethapyr was used to evaluate the segregation of the resistance trait. At these rates susceptible plants are killed, whereas plants containing one or more copies of a resistance gene are either undamaged or may suffer minor plant damage and deformation, but recover. Since environment and genetic background can affect expression of the trait, such as residual deleterious genes induced via tissue culture, plants surviving the herbicide treatment were merely scored as resistant and plants killed by the herbicide treatment were scored as susceptible.

The levels and spectra of herbicide tolerance for the different lines was determined by treating plants grown individually in 6-inch azalea pots. Herbicides were applied at the three-to four-leaf stage. Three replications were used for each treatment combination.

Assay for acetohydroxyacid synthase activity

The procedures used for the in vitro assays of AHAS activity have been previously published by Singh et al. (1988).

Results

Inheritance studies

Plants from progenies nonsegregating for herbicide resistance, with resistance from the XA17, XI12, and QJ22 lines, were crossed with the susceptible inbred line B73. F_1 hybrid plants derived from these crosses were uniformly herbicide resistant. These hybrids were self pollinated and also crossed back to the susceptible parent. Following treatment with an imidazolinone herbicide, selfed progenies segregated in a 3-resistant: 1-susceptible ratio, and testcross progenies segregated in a 1-resistant: 1-susceptible ratio (Table 1). These results indicate that resistance is conferred by a single dominant nuclear allele for each of the three independent mutations.

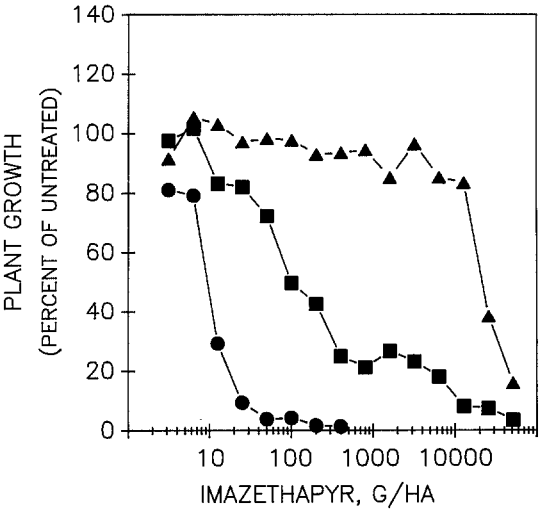

Fig. 1. The effect of treatment with the imidazolinone herbicide imazethapyr on growth of wild-type hybrid corn (without imidazolinone resistance) (●—●), hybrids heterozygous for the XA17 resistance gene (■—■), and hybrids homozygous for the XA17 resistance gene (▲—▲). Rates of active ingredient applied ranged from 3.1 to 51,200 g/ha. Growth measurements for the treatments are expressed as percentage of growth in the 4 weeks following treatment as compared to unsprayed controll

At higher rates of herbicide application, individuals homozygous for the XA17 alleles are more resistant than individuals heterozygous for XA17, indicating that the trait expresses semidominant inheritance at higher rates of herbicide application (Fig. 1). Similar expression patterns occur for the XI12 and QJ22 alleles (data not shown). This pattern of inheritance is consistent with that first observed for sulfonylurea-resistant tobacco by Chaleff and Ray (1984) and subsequently has been reported in other plant species selected for sulfonylurea or imidazolinone resistance (Haughn and Somerville 1986; Swanson et al. 1989; Sebastian et al. 1989; Haughn and Somerville 1990)

Characterization of resistance

The three herbicide-resistant genes were evaluated in the B73 inbred background to allow comparison and assessment of the resistance alleles in a uniform background of an elite inbred. Homozygous resistant plants were tested for their resistance to the imidazolinone herbicides

Table 1. Inheritance patterns observed for imidazolinone-resistance traits, and the fit of observed frequencies to those expected for segregation of a single dominant allele. Seedlings were treated postemergence at the three-leaf stage with 150 g/ha of imazethapyr, and the number of resistant (R) and susceptible (S) individuals were determined at approximately 3 weeks after treatment. Plants that survived the herbicide treatments were scored as resistant, whereas those plants killed by the herbicide treatment were scored as susceptible. F_1 hybrids are the cross between susceptible inbreds and homozygous resistant selections, F_2 progenies are produced by self pollination of individual F_1 plants, and testcross progenies are individual ears produced by making plant to plant crosses between the F_1 hybrids and a susceptible inbred

Genotype	Observed ratio, R:S	Expected ratio, R:S	χ^2 fit of observed to expected	Number of different F_2 or testcross progenies tested
Susceptible inbreds:				
B73	All S	All S		
Mo17	All S	All S		
XA17 selection:				
Homozygous	All R	All R		
F_1	All R	All R		
F_2	634:232	649.5:216.5	1.48, ns*	12
Testcross	800:859	829.5:829.5	2.10, ns	20
XI12 selection:				
Homozygous	All R	All R		
F_1	All R	All R		
F_2	823:285	831:277	0.30, ns	15
Testcross	838:839	838.5:838.5	0.001, ns	15
QJ22 selection/				
Homozygous	All R	All R		
F_1	All R	All R		
F_2	944:341	963.75:321.25	1.62, ns	18
Testcross	622:704	683:683	1.29, ns	18

* The 'ns' indicates that the observed segregation is not significantly different from the expected segregation at the 0.05 probility level when tested by the χ^2 distribution. A χ^2 value of 3.84 or larger would be needed to disprove the 1:1 or 3:1 segregation ratio null hypothesis indicated

imazethapyr and imazaquin, and to the sulfonylurea herbicide sulfometuron methyl. Both imidazolinone and sulfonylurea herbicides have been shown to inhibit AHAS activity (Shaner et al. 1984; Chaleff and Mauvais 1984).

Corn is normally sensitive to all three of these herbicides. The sensitive inbred B73 was killed by the lowest rates of imazaquin and sulfometuron methyl applied and severely stunted by the lowest rate of imazethapyr (Fig. 2). The mutation from line XA17 provides high levels of resistance to all three herbicides (Fig. 2). The imidazolinone herbicides did not inhibit growth of the XA17 selection at any rate used, and slight growth inhibition was observed only at the highest rate of sulfometuron methyl. In contrast, the resistance conferred by the mutation from line XI12 provides high levels of resistance only to imazethapyr. Resistance to imazaquin, although increased relative to the sensitive inbred B73, is less than for the XA17 mutation. Little or no resistance to sulfometuron methyl is conferred with the XI12 allele. The QJ22 resistance allele provides a spectrum of resistance similar to that of XI12; i.e., greater resistance to imazethapyr than to imazaquin, and little resistance to sulfometuron methyl. However, the level of resistance to the imidazolinones conferred by the QJ22 mutation is approximately one-fourth that provided by the XI12 mutation.

Enzyme assays indicate that AHAS activity from the XA17 line is highly resistant to all three herbicides (Fig. 3). AHAS activity from XI12 and QJ22 is resistant to imazethapyr and imazaquin, but is only slightly resistant to sulfometuron methyl. AHAS activity from the inbred line B73 is more sensitive to inhibition by these three herbicides than are AHAS activities from the three mutant lines.

AHAS activity in plants is normally feedback regulated by valine and leucine (Miflin 1971; Miflin and Cave 1972). The feedback regulation by valine and leucine appear normal for the herbicide-resistant alleles (Fig. 4). In vitro assays for the feedback inhibition of AHAS activity from sensitive B73 and the three mutants give coincidental inhibition curves.

Discussion

Nearly all plant mutants resistant to the imidazolinone, sulfonylurea, or triazolopyrimidine herbicides isolated to

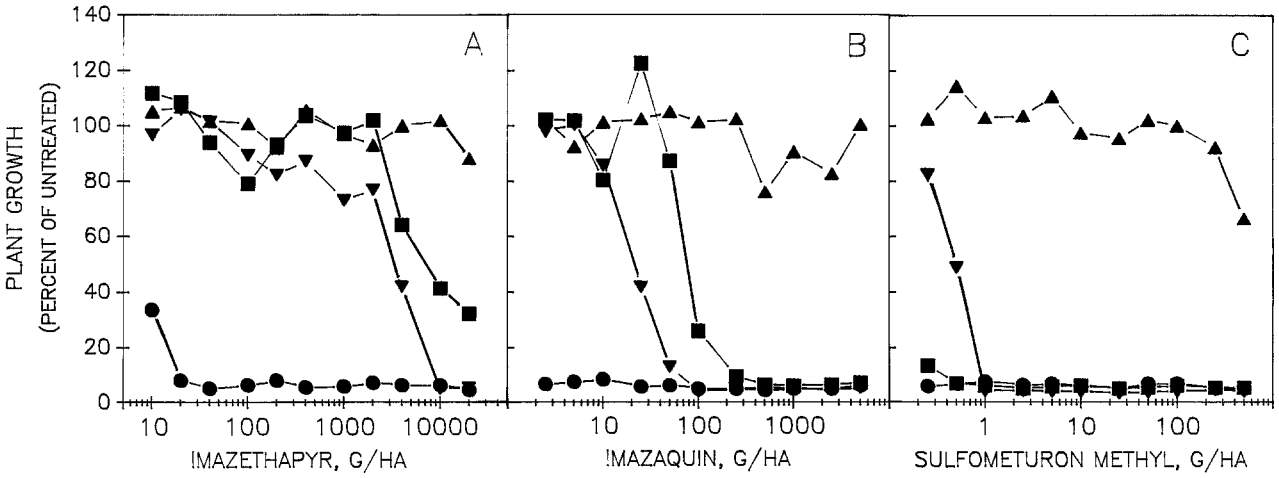

Fig. 2A–C. Growth of wild type corn (B73 ●—●) and three imidazolinone-resistance mutants (XA17 ▲—▲, XI12 ■—■, and QJ22 ▼—▼) after treatment with imazethapyr (**A**), imazaquin (**B**), and sulfometuron methyl (**C**). Rates of active ingredient applied ranged from 10 to 20,000 g/ha for imazethapyr, from 2.5 to 5,000 g/ha for imazaquin, and from 0.25 to 500 g/ha for sulfometuron methyl. Growth measurements for the treatments are expressed as percentage of growth in the 4 weeks following treatment as compared to unsprayed controls

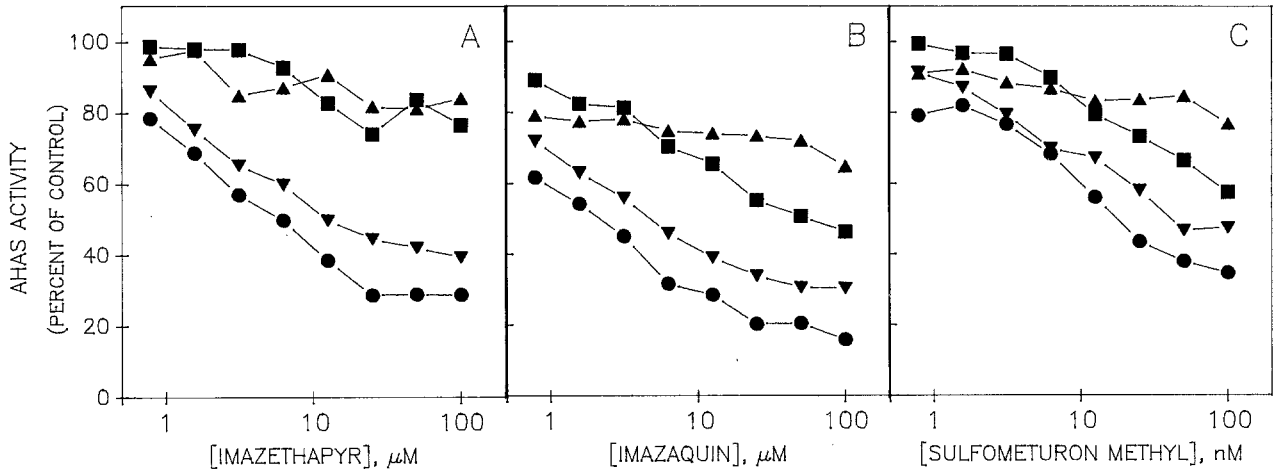

Fig. 3A–C. Inhibition of acetohydroxyacid synthase from the wild-type corn (B73 ●—●), as well as from three corn mutants (XA17 ▲—▲, XI12 ■—■, and QJ22 ▼—▼) by imazethapyr (**A**), imazaquin (**B**), and sulfometuron methyl (**C**). AHAS was extracted from corn seedlings and assayed under standard conditions

date have been shown to have numerous similarities: a single semidominant gene conferring resistance, herbicide-resistant acetohydroxyacid synthase enzyme activity, and a direct correlation between the in vivo, whole plant cross-resistance spectrum and the in vitro AHAS activity cross-resistance spectrum. The mutations described in this paper also follow these general characteristics.

Herbicide-resistant mutants with an altered site of primary action offer an unique opportunity to determine whether secondary sites of action exist for that herbicide. Since no morphological effects have been noted for imidazolinone-treated plants homozygous for the XA17 mutation, it is unlikely that a major secondary site of action for the imidazolinones and sulfonylurea herbicides exists.

The direct correlation between resistance at the whole plant level and the herbicide insensitivity of mutant AHAS activity also supports the hypothesis that AHAS is the only site of action for these herbicides, and that herbicide-resistant AHAS activity is the mechanism of resistance for these resistance mutations. However, factors such as absorption, metabolism and translocation that affect movement of the herbicide to the active site have a major effect on the expression of herbicide tolerance at the whole plant level (Beyer et al. 1988; Shaner and Robson 1985). These factors also play a major role in determining the level of resistance to a particular herbi-

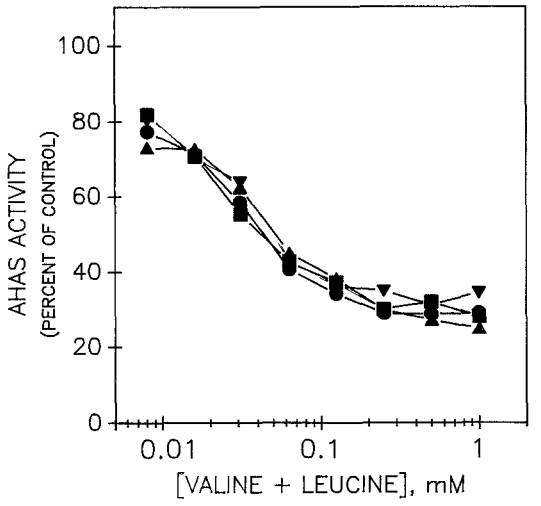

Fig. 4. Inhibition of acetohydroxyacid synthase from wild-type corn (B73 ●—●), as well as from the three corn mutants (XA17 ▲—▲, XI12 ■—■, and QJ22 ▼—▼) by valine and leucine. AHAS activity was assayed under standard conditions in the presence of varying concentrations of the amino acids as indicated. The concentrations presented here represent the concentration of each amino acid

cide within a class of herbicides. For this reason, the resistance of the resistant plants' AHAS activity to herbicides measured by in vitro AHAS assays is an inexact predictor of whole plant responses to those herbicides.

Mutations such as those described herein also provide information about herbicide binding sites on the AHAS enzyme. The mutation in XA17 confers resistance to both imidazolinone and sulfonylurea herbicides; however, the mutations in XI12 and QJ22 distinguish between resistance to imidazolinones and sulfonylureas. These observations may indicate that binding sites for the two herbicide classes are in close proximity, or are similar, but are not identical. Differences in the binding of different imidazolinones to the AHAS enzyme appear to exist as well because the XI12 and QJ22 mutations confer different levels of resistance to imazethapyr than to imazaquin, whereas the XA17 mutation confers strong resistance to both herbicides. Alternatively, these differences in the spectrum of resistance displayed by the three mutations could be the result of substituting different amino acids at a single position in the AHAS enzyme, as has been reported for AHAS mutations in yeast conferring resistance to sulfonylurea herbicides (Mazur and Falco 1989).

Feedback regulation of AHAS activity by leucine and valine is also unaffected in all three corn mutants described. Unaltered feedback regulation has been the usual finding for imidazolinone-resistant or sulfonylurea-resistant mutant lines (Creason and Chaleff 1988; Subramanian et al. 1990). Similarly, valine-resistant tobacco mutants with altered feedback regulation are not resistant to sulfonylurea herbicides (Relton et al. 1986).

Table 2. The degree of safety to comparable use rates of imazethapyr, imazaquin, and sulfometuron methyl provided by the herbicide-resistance alleles from selections XA17, XI12, and QJ22. A safety factor of less than 1X will not permit commercial use of the herbicide

Herbicide	Resistance allele		
	XA17	XI12	QJ22
Imazethapyr	>300X	300X	75X
Imazaquin	>50X	2X	<1X
Sulfometuron methyl	>50X	<1X	<1X

Therefore, the herbicide binding sites for these herbicides appear to be distinct from feedback regulation sites on the AHAS enzyme. However, some exceptions to this general finding have been reported (Rathinasabapathi et al. 1990; Subramanian et al. 1990). In these cases, insensitivity to feedback inhibition might be attributed to a separate mutational event from that conferring herbicide resistance.

The use of plant cell culture to select imidazolinone-resistant mutations has been successful for a number of different plant species (Anderson and Georgeson 1989; Saxena and King 1990; Swanson et al. 1989; Subramanian et al. 1990). However, the mutants described in this paper are unique in that they are the only reported example of imidazolinone resistance in any of the major cereal crops of the world. As such, the mutants provide the means to allow application of imidazolinones to a major cereal grain crop for which selective imidazolinone herbicides are presently unavailable.

The degree of safety that a herbicide-resistant mutation provides in a farmer's field involves a number of factors (Newhouse et al. 1990). One factor is the level of increased tolerance to the herbicide provided by the mutation. A second factor is the rate of the herbicide that must be applied for effective weed control. A third factor is the inherent tolerance of the crop to the herbicide under consideration, in the absence of any resistance gene. As mentioned above, this inherent tolerance (or sensitivity) is dependent on the ability of the plant to absorb, metabolize, and translocate the herbicide. When these factors are taken into consideration for the use of imazethapyr, imazaquin, and sulfometuron methyl on the imidazolinone-resistant corn mutants described in this paper, each of the mutations can be shown to provide a unique spectrum of herbicide safety (Table 2). First, the three mutations are each different in the level and spectrum of resistance that they provide, both in terms of AHAS activity and whole plant responses. Second, comparable commercial use rates for the three herbicides are quite different; the rates used to achieve comparable levels of weed control would probably be on the order of 70 g/ha, 140 g/ha, and 10 g/ha for imazethapyr,

imazaquin, and sulfometuron methyl, respectively. And third, corn has some inherent tolerance to imazethapyr, but little or no inherent tolerance to imazaquin or sulfometuron methyl. The combination of these factors explains the reason that each of the three mutations provides a different opportunity.

Several reviews have been published on the potential of in vitro selection as a tool for plant breeding (Chaleff 1983; Flick 1983). Even though useful traits have been identified in somaclonal variants from tissue cultures (Evans and Sharp 1986), direct selection of traits with agronomic value has had limited success. Selection of herbicide-resistant crops is one area where success has been achieved (Chaleff and Parsons 1978; Miller and Hughes 1980; Chaleff and Ray 1984; Anderson and Georgeson 1989). The mutations discussed in this paper confer levels of resistance sufficient to allow safe application of the recommended use rates for the herbicides tested, and resistant corn hybrids will be marketed internationally. Imidazolinone-resistant corn will be the first cereal crop modified by plant biotechnology to reach the market place. The standard plant breeding technique of backcrossing is being used by Pioneer Hi-Bred International, Inc. (Johnston, Iowa) to introduce the resistance mutations into elite, modern corn hybrids (Fincher 1989).

Acknowledgements. The authors thank Drs. Karen Armstrong, Roy Chaleff, Jerry Ranch, and Wendy Smith for their careful reading of this manuscript and useful suggestions for its improvement.

References

Anderson PA, Georgeson M (1989) Herbicide-tolerant mutants of corn. Genome 31:994–999

Beyer EM Jr, Duffy MJ, Hay JV, Schlueter DD (1988) Sulfonylurea herbicides. In: Kearney PC, Kaufmann DD (eds) Herbicides: chemistry, degradation, and mode of action. Marcel Dekker, New York, pp 117–189

Botterman J, Leemans J (1988) Engineering herbicide resistance in plants. Trends Genet 4:219–222

Chaleff RS (1983) Isolation of agronomically useful mutants from plant cell cultures. Science 219:676–682

Chaleff RS, Mauvais CJ (1984) Acetolactate synthase is the site of action of two sulfonylurea herbicides in higher plants. Science 224:1443–1445

Chaleff RS, Parsons MF (1978) Direct selection in vitro for herbicide-resistant mutants of *Nicotiana tabacum*. Proc Natl Acad Sci USA 75:5104–5107

Chaleff RS, Ray TB (1984) Herbicide-resistant mutants from tobacco cell cultures. Science 223:1148–1151

Creason GL, Chaleff RS (1988) A second mutation enhances resistance of a tobacco mutant to sulfonylurea herbicides. Theor Appl Genet 76:177–182

Evans DA, Sharp WR (1986) Applications of somaclonal variation. Bio/technology 4:528–532

Fincher RR (1989) Transfer of in vitro selected imidazolinone resistance to commercial maize hybrids. In: Copping LG, Rodgers P (eds) Prospects for amino acid biosynthesis inhibitors in crop protection and pharmaceutical chemistry. British Crop Protection Council, Farnham, England, pp 69–73

Flick CE (1983) Isolation of mutants from cell culture. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds) Handbook of plant cell culture. John Wiley & Sons, New York, pp 393–441

Gasser CS, Fraley RT (1989) Genetically engineering plants for crop improvement. Science 244:1293–1299

Haughn GW, Somerville C (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol Gen Genet 204:430–434

Haughn GW, Somerville CR (1990) A mutation causing imidazolinone resistance maps to the *Csr1* locus of *Arabidopsis thaliana*. Plant Physiol 92:1081–1085

Mazur BJ, Falco SC (1989) The development of herbicide resistant crops. Annu Rev Plant Physiol Plant Mol Biol 40:441–470

Miflin BJ (1971) Cooperative feedback control of barley acetohydroxyacid synthase by leucine, isoleucine, and valine. Arch Biochem Biophys 146:542–550

Miflin BJ, Cave PR (1972) The control of leucine, isoleucine, and valine biosynthesis in a range of higher plants. J Exp Bot 23:511–516

Miller OK, Hughes KW (1980) Selection of paraquat-resistant variants of tobacco from cell cultures. In Vitro 16:1085–1091

Newhouse KE, Shaner DL, Wang T, Fincher R (1990) Genetic modification of crop responses to imidazolinone herbicides. In: Green MB, LeBaron HM, Moberg WK (eds) Managing resistance to agrochemicals. American Chemical Society, Washington, pp 474–481

Rathinasabapathi B, Williams D, King J (1990) Altered feedback sensitivity to valine, leucine, and isoleucine of acetolactate synthase from herbicide resistant variants of *Datura innoxia*. Plant Sci 67:1–6

Relton JM, Wallsgrove RM, Bourgin JP, Bright SWJ (1986) Altered feedback sensitivity of acetohydroxyacid synthase from valine-resistant mutants of tobacco (*Nicotiana tabacum*). Planta 169:46–50

Saxena PK, King J (1990) Lack of cross-resistance of imidazolinone-resistant cell lines of *Datura innoxia* P. Mill. to chlorsulfuron. Plant Physiol 94:1111–1115

Sebastian SA, Fader GM, Ulrich JF, Forney DR, Chaleff RS (1989) Semidominant soybean mutation for resistance to sulfonylurea herbicides. Crop Sci 29:1403–1408

Shaner DL, Robson PA (1985) Absorption, translocation, and metabolism of AC 252,214 in soybean (*Glycine max*), common cocklebur (*Xanthium strumarium*), and velvetleaf (*Abutilon theophrasti*). Weed Sci 33:469–471

Shaner DL, Anderson PC, Stidham MA (1984) Imidazolinones: potent inhibitors of acetohydroxyacid synthase. Plant Physiol 76:545–546

Singh BK, Stidham MA, Shaner DL (1988) Assay of acetohydroxyacid synthase. Anal Biochem 171:173–179

Subramanian MV, Hung HY, Dias JM, Miner VW, Butler JH, Jachetta JJ (1990) Properties of mutant acetolactate synthases resistant to triazolopyrimidine sulfonanilide. Plant Physiol 94:239–244

Swanson EB, Herrgesell MJ, Arnoldo M, Sippell DW, Wong RSC (1989) Microspore mutagenesis and selection: Canola plants with field tolerance to the imidazolinones. Theor Appl Genet 78:525–530