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Session 7A

Herbicide-tolerant Crops: A Genetic Approach

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Papers	7A-1 to 7A-4

HERBICIDE TOLERANT CROPS - A PERSPECTIVE

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ABSTRACT

Plant biotechnology incorporating recombinant DNA (rDNA) technology has generated a series of new transgenic crop plants. Because the technology has demanded the use of selection techniques it is not surprising that herbicide tolerance is one of the first agronomic properties to be incorporated into transgenic crops. The impending commercialisation of these crops has caused much discussion, some of which is engendered by the technology itself and some by the property of herbicide tolerance. This brief introduction attempts to set out some of the key points which should be taken into account in seeking to clarify these controversial issues.

INTRODUCTION

Herbicide tolerant crops are amongst the first products of rDNA technology to be approved for commercial use. For example, bromoxynil tolerant tobacco has been cleared for commercial use in Europe although it is not yet on sale. The advances in glyphosate and glufosinate tolerant crops are presented in the following papers. These crops have attracted the attention of many critics of biotechnology and have been subject to debate in the public arena and in the patent courts. For example, Greenpeace challenged the patent awarded to PGS for phosphinotricin (glufosinate) resistance. This attention has lead to many allegations and counter allegations which have often served to confuse rather than clarify the issues by drawing into the argument aspects which are not strictly related to rDNA technology. This brief introduction to this session is an attempt to identify some key points that should be borne in mind when discussing herbicide tolerant crops in general and to identify which of these are related to such crops produced by rDNA technologies.

GENETIC CONSIDERATIONS

Herbicide tolerant crops have existed since the discovery of the first selective herbicide

Selective herbicides depend on genetic differences between the crop and the weeds that enable the crop to escape the toxic effects of the chemical. The fact that such herbicides are discovered by screening the effects of a large number and wide range of chemicals does not contradict the fact that selectivity is a property determined by the genetics of the crop rather than something inherent in the chemical.

The mechanism of 'pre-existing' herbicide tolerance may be determined by one or a small number of genes

For example, variation in inbred lines of maize to sulphonyl ureas is dependent on a single gene. Herbicides which inhibit acetyl co-A carboxylase are selective in graminae against dicot weeds because of the difference in the target enzyme between the two broad groups. These genetic tolerance mechanisms may also pass into related species by normal sexual crossing and hybridisation. This can cause problems with closely related weedy species in crops (e.g. sugar-beet see Timmerman this symposium.) The experience gained with 'conventional' herbicide tolerant crops and their matching herbicide can be used to provide useful information on the potential risks or rDNA produced herbicide tolerance. For example, the billions of hectare/years of application of atrazine on maize have failed to provide any evidence of transfer of the genes for herbicide tolerance from maize into weeds. Different results are found with different crop/herbicide/weed combinations, for example in brassicas.

Despite the publicity given to herbicide tolerance developed by rDNA technology, this is only one of the ways in which herbicide tolerance in a crop can be developed where it does not already exist

Examples exist where tolerance has been selected by exerting selection pressure on plant cells tissue culture, on pollen or on plants in the field. In other cases tolerance may be moved into the crop from a related species. Many of the considerations of the use of herbicide tolerant crops are independent of which method was used to generate them although the regulatory controls differ. Maize specifically selected for tolerance to imidazolinone herbicides has been on the market in the US for several years.

COMMERCIAL CONSIDERATIONS

Where herbicide tolerance has been introduced into a crop, the necessity to maintain it through breeding populations and into released varieties places an extra burden on the plant breeder and slows the rate of gain in other traits

It is unlikely that breeders will embrace the technology in their crops unless there is a real possibility that by doing so they will provide a better solution for the farmer. The commercial pressure and competition on breeders will ensure that any 'frivolous' introduced tolerance traits will soon be dropped. Some of the problems faced by breeders are covered in a subsequent paper by Timmerman.

The major factor that determine whether a farmer will choose a given variety will be its ability to deliver either a probable economic advantage or less risk as compared to other available varieties

It is unlikely that farmer will choose inferior varieties just because they are herbicide tolerant unless the weed problem cannot be solved in any other way. On the other hand, where varieties are nearly equivalent he is likely to choose the herbicide tolerant one if it provides him with extra ways of overcoming potential weed problems.

A farmer will choose a herbicide according to its value in solving the weed problems affecting the crop

Just because a herbicide tolerant crop has been planted it is unlikely that the farmer will use the related herbicide unless circumstances dictate it is necessary. Again the experience already gained in farmer behaviour, for example, in choosing dicurane tolerant wheat cultivars, can provide a guide as to how they will behave when faced with new tolerance/herbicide combinations.

Weeds are a significant agronomic problem and herbicides generate value for the farmer and cheaper prices for the consumer; companies will only continue to develop herbicides (and herbicide-tolerant crops) if they share in that value and make a profit

Many critics talk as if there was some crusade by chemical companies to force this technology on the agricultural industry. While it is true that there has been considerable enthusiasm for the use of rDNA technology to produce such crops this will wane quickly if companies do not recover their investments and make a profit. This may well happen in some of the crops/chemical combinations currently being developed.

REGULATORY ASPECTS

The risks in growing a tolerant variety and using a specific herbicide will depend on the characteristics of the specific products independent of the generic technology used to produce them

It is accepted that one set of regulations govern the production of chemicals according to the principles of health and safety legislation, and another the final use of the product. It is perfectly possible and accepted that a product may be relatively harmless even though some of the intermediate chemicals and processes could be very dangerous; the converse could also be true. However, the decision on the product is taken independently of the process involved. Similarly, I would argue that the nature of herbicide tolerant crop should be considered independently of the process that produced it. Any method of producing genetic change in a crop may cause unsuspected mutations, introduce extra pieces of DNA which may specify known or unknown traits or come from sources with which we are familiar or unfamiliar. Any risk assessment therefore needs to concentrate on the specific product and attempt to use scientifically based methods to determine if a risk exists. Regulations put in place for rDNA technology in a generic sense should be considered separately from specific herbicide tolerant crops.

Some of the postulated 'concerns' raised in relation to herbicide tolerant crops produced by rDNA technology also exist for conventional selective herbicides, if these are genuine then regulations proposed to govern them should be applied consistently to both

For example, it has been suggested that because herbicide tolerant crops might lead to wide spread use of a single herbicide they should be specially regulated. This may be so but it is also true that wide spread herbicide use has been encouraged by the identification of very good herbicides that are selective on existing crops e.g. atrazine. Alternatively one target site might be affected by a wide range of herbicides that work in many crops -

this could also lead to selection pressure for resistance as in fact has happened for herbicides that inhibit acetolactate synthase. The problem is 'should we put in place regulations that limit the area that can be sprayed by a given herbicide?' - if we should (and I have doubts that we should) such a regulation should cover all herbicides irrespective of the reason for the excess use and not limited to those linked to herbicide tolerant crops produced by rDNA technology.

Recombinant DNA technology is fiercely opposed by a small minority, viewed with concern by a larger number and is a technology which the vast majority of European citizens consider should be regulated

This conclusion is based on the Eurobarometer surveys and the concerns should be seriously considered and addressed (Marlier, 1992). Some of the major issues have also been identified in the Consensus Conference on Biotechnology (Durant, 1994). These are not specific to herbicide tolerance but generic to the technology, which has always been subject to regulation since its inception in 1974. Any regulation on the technology should be based on the technology and not just because one of the products might be a herbicide tolerant crop.

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DEVELOPMENT OF GLYPHOSATE TOLERANT CROPS INTO THE MARKET

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ABSTRACT

Two approaches have been used to identify genes which confer tolerance to commercial levels of glyphosate (N-[phosphonomethly]glycine)in several crops. One method is target site modification where a herbicide-insensitive EPSPS (*Agrobacterium* sp. CP4 EPSPS) was identified and introduced by genetic modification techniques. A second method involved metabolic inactivation of the active ingredient. The gene which encodes the enzyme that catalyzes glyphosate degradation to AMPA, glyphosate oxidoreductase (GOX), was cloned and used to confer glyphosate tolerance in plants.

Currently, Monsanto is developing glyphosate tolerant soybeans, canola, cotton, corn, winter oil seed rape and sugar beets. Commercially, the most advanced crops are soybeans and canola. Field studies have shown that there is no yield penalty with glyphosate tolerance, either from the gene insertion per se or from applications of glyphosate at up to 2 times the commercial use rates. Weed control studies have shown that glyphosate, applied 1 to 2 times per season will provide effective season long weed control in soybeans and canola. Food and feed safety studies have confirmed that these crops are nutritionally, compositionally and functionally equivalent to the non-genetically modified crops.

INTRODUCTION

Glyphosate is the most widely used herbicide in the world, and has been used for more than 20 years in all types of production, from perennial tree crops to soybeans to home gardens. The wide use of this herbicide is attributable to its effectiveness in providing broad spectrum weed control as well as its excellent environmental profile. It is readily broken down in the soil, does not leach into ground water and has extremely low toxicity to humans and animals.

Since the early 1980s, Monsanto has been working to build selectivity to glyphosate into major food and fiber crops of the world through genetic modification. Glyphosate tolerant crops would provide the farmer with a new weed control option with a product that is cost effective while taking advantage of this herbicide's excellent environmental and safety profile.

DEVELOPMENT OF GLYPHOSATE RESISTANCE

The mode of action of glyphosate is based on the inhibition of aromatic amino acid biosynthesis (Amrhein *et al.*, 1980, Steinrucken & Amrhein, 1980). Specifically, it is a

nuclear-encoded enzyme in the shikimic acid pathway. The development of EPSPS enzymes which are tolerant to glyphosate has been central to developing glyphosate-tolerant crops.

The primary metabolic degradation route for glyphosate in soil appears to be through the cleavage of the glycyl moiety and formation of aminomethylphosphoate (AMPA) plus glyoxylate (Jacob *et al.*, 1988, Pipke & Amrhein, 1988). Tolerance to glyphosate imparted by metabolic inactivation of the active ingredient has also been a primary target to developing glyphosate tolerant crops.

Target site modification approach

EPSPS catalyzes the reversible reaction of shikimate-3-phosphate and phosphoenolpyruvate to produce EPSP and inorganic phosphate. Numerous studies on the effects of EPSPS have demonstrated that glyphosate is a potent inhibitor of the enzyme in all plant, all fungus and the majority of bacteria examined (Franz *et al.*, 1993). In plants, EPSPS is localized in the chloroplasts or plastids.

An EPSPS was identified from a bacterial screen of glyphosate-degrading bacteria. An *Agrobacterium* sp. strain, CP4 EPSPS was found that exerted extremely high glyphosate tolerance while maintaining high catalytic efficiency (Padgette *et al.*, 1994). The gene for CP4 EPSPS from *Agrobacterium* sp. coding sequence was fused to the chloroplast transit peptide coding sequences to target the protein to the plastids. Commercial tolerance to glyphosate has been demonstrated with the introduction of CP4 EPSPS in several crops.

Metabolic inactivation approach

Glyphosate is known to be readily degraded by certain soil and water bacteria (Rueppel, *et al., 1977).* The glyphosate-to-AMPA pathway appears to be the primary degradation route for glyphosate in soil (Jacob *et al.*, 1988, Pipke & Amrhein, 1988, Torstensson, 1985). In Monsanto, efficient glyphosate degrading bacteria were first isolated from a collection of putative glyphosate-to-AMPA bacteria from a from a glyphosate waste stream facility (Hallas *et al.*, 1988). The *Achromobacter* sp. strain LBAA was chosen from this screen. The enzyme, named glyphosate oxidoreductase (GOX) from *Achromobacter* sp. strain LBAA, catalyzes the cleavage of the C-N bond of glyphosate yielding AMPA and glyoxylate. The gene expressing GOX was cloned and inserted into several plants. The expression of GOX imparts a high level of tolerance in these plants (Barry et al., 1992).

GLYPHOSATE TOLERANT CROPS

Glyphosate tolerance in soybeans, canola, cotton, and corn are currently under development by Monsanto. Additionally winter oil seed rape and sugar beet are also being evaluated.

Soybeans

The gene encoding for CP4 EPSPS was introduced into soybean tissue by the particle acceleration method. The glyphosate tolerant soybean line has been field tested since 1991. Commercial introduction is targeted for 1996 in the United States and Argentina, and 1998 in Brazil.

Yield studies conducted in 1992 and 1993 confirmed that glyphosate tolerant soybeans impart a very high level of tolerance to glyphosate. No significant yield reductions were noted after single or sequential broadcast applications of glyphosate at various crop stages, from early growth to past flowering at rates ranging from 0.84 kg a. e. ha^{-1} to 1.68 kg a. e. ha^{-1}. Tolerance to two times the average use rate with a very wide application window was therefore confirmed. Additionally, isopopulation studies conducted in 1993 and 1994 verified that there is no yield penalty associated with the insertion of the gene. Application rates ranging from 0.63 kg a. e. ha^{-1} to 0.84 kg a. e. ha^{-1} have been shown to provide effective season long, broad spectrum weed control in soybeans.

Food and feed safety studies of glyphosate tolerant soybeans have shown that CP4 EPSPS is chemically, nutritionally, and functionally equivalent to EPSPS *in planta*. The glyphosate tolerant soybean is substantially equivalent to current commercial soybean varieties.

Glyphosate tolerant soybeans will be commercialized in collaboration with leading soybean seed companies. In the United States, all required regulatory approvals (USDA, FDA, and EPA) for glyphosate tolerant soybean production have been received.

Other crops

Two glyphosate tolerant canola lines are under commercial development by Monsanto and seed company partners. These lines express both the CP4 EPSPS gene as well as the GOX glyphosate degradation gene. The canola lines were transformed using *Agrobacterium* and transformants were selected directly on glyphosate. No yield penalty has been noted from broadcast applications of up to 2 times the commercial glyphosate rates. Glyphosate tolerant canola is being developed primarily for the western Canadian market by Monsanto. Monsanto is planning to commercialize glyphosate tolerance in collaboration with leading canola seed companies. Commercial introductions will be in high-yielding elite germplasm. Commercial introduction of glyphosate tolerant canola in Canada is anticipated in 1996.

Lead lines of glyphosate tolerant cotton express only the CP4 EPSPS gene. These lines have shown no yield reduction to glyphosate applied at the 2-5 leaf stage at up to 1.68 kg a. e. ha^{-1}.

In corn, CP4 EPSPS only lines as well as CP4 and GOX lines are being evaluated. These lines are showing high levels of field tolerance to glyphosate.

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THE DEVELOPMENT OF GLUFOSINATE AMMONIUM TOLERANT CROPS INTO THE MARKET

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ABSTRACT

Since several years Glufosinate Ammonium is successfully used in many countries as a non selective herbicide. It conforms well with ever increasing safety standards for human beings, animals and the environment.
Crop tolerance was achieved some years ago by transferring a resistance gene (PAT - gene) into previously susceptible crop plants, allowing a new approach for the development of an almost ideal selective herbicide.
Many different crops have been successfully transformed with the PAT - gene. However, AgrEvo development work has been focused on four major crops: oilseed rape, corn, soybean and sugar beet.
Major seed companies in North America and Europe are developing Glufosinate Ammonium tolerant varieties, which will become commercially available soon.
Field development work for the registration of Glufosinate Ammonium tolerant varieties and the selective weed control with Glufosinate Ammonium has intensified tremendously over the last few years. Field trial data confirmed excellent crop safety and superior post emergent weed control with Glufosinate Ammonium in these tolerant crops.
In Spring 1995 Canadian authorities fully approved the first two Glufosinate Ammonium tolerant spring oilseed rape varities. They also registered the selective weed control with the herbicide Glufosinate Ammonium in this tolerant crop. The unique selective weed control technology with Glufosinate Ammonium was launched the first time this season in Canada.

INTRODUCTION

Without weed control, crop growing is impossible. In today's conventional control methods, chemical weed control has become established worldwide as the method of choice. Other methods, especially mechanical control are almost always inferior in economic and management terms on the farm.
No system of weed control, even mechanical, is without side-effects. The principal drawbacks of using herbicides are possible residues in and on the harvested crop, the adverse impact on flora and fauna, and possible contamination of the soil, water and the air. These drawbacks are associated with any form of chemical weed control; they cannot be completely eliminated but they can be minimized.
Increasing requirements for the safety of agrochemicals for human beings, animals and the environment have resulted in ever increasing registration standards of authorities. The development of new herbicides has become more and more difficult, expensive and risky.

PROFILE FOR NEW HERBICIDES

The primary aim of modern herbicide research is therefore to develop active ingredients which meet the farmer's requirements and have the smallest possible side-effects. An essential criterion for the usefulness of a herbicidal active ingredient is its tolerance by the crop. This is the first requirement for widespread use in suitable tolerant crops.
A modern herbicide ideally should have the following profile:

- broad spectrum weed control
- post emergent activity
- high level of efficacy
- high level of tolerance by crops
- suitable to „crop / weeds management system"
  - application flexibility
  - re-cropping flexibility
  - weed resistance management

GLUFOSINATE AMMONIUM - A MODERN HERBICIDE

The herbicide Glufosinate Ammonium meets this profile with one exception: it is broad acting on all plants and cannot be used for selective weed control in most crops. So far it is sold successfully as a non selective herbicide since many years now in over 50 countries. Glufosinate Ammonium has proven its favourable ecological and safety properties and is regarded as one of the most attractive herbicides.
Since genetic engineering succeeded in transferring a gene coding for resistance to the herbicide Glufosinate Ammonium into plants it was logical to develop it also for selective weed control in major agricultural crops.
Glufosinate Ammonium is the ammonium salt of the amino acid Phosphinothricin which has been derived from the natural compound L-phosphinothricyl-L-alanyl-L-alanine. This tripetide was obtained from *Streptomyces viridochromogenes* by Bayer et al (1972) in Germany and from *Streptomyces hygroscopicus* by Kondo et.al. (1973) in Japan and got the common name Bialaphos. Biological screening by the agrochemical research department of Hoechst AG showed that the amino acid Phosphinothricin was the biologically active moiety of the tripeptide. It proved to have strong herbicidal effect on mono- and dicot plants when applied on leaves and other green parts. Hoechst developed the compound for non selective weed control uses (Rupp et.al., 1977).

MODE OF HERBICIDAL EFFICACY

The phytotoxic activity of Glufosinate Ammonium is caused by the inhibiton of glutamine synthetase (Leason et.al. 1982). For plants glutamine synthetase is crucial for the assimilation of ammonia and for reassimilation of ammonia released by photorespiration. Inhibition of glutamine synthetase leads to increasing concentrations of ammonia in plant cells. This is the reason for the phytotoxic effect of this herbicidal glutamic acid analogue.

MECHANISM OF TOLERANCE TO GLUFOSINATE AMMONIUM

The Streptomyces species producing the tripeptide Bialaphos containing the herbicidal active amino acid Phosphinothricin simultaneously possess an enzyme - and thus a gene - which protects themselves against intoxication by their own metabolite.
De Block et al (1987) demonstrated that the Bialaphos resistance gene (BAR gene) isolated from *Streptomyces hygroscopicus* was expressed in plants as well and protected them against the herbicidal activity. Further glasshouse and field trials confirmed these results (De Greef et.al., 1988; Botterman, 1989; Botterman et.al. 1991).
Independently Strauch et al (1988) isolated and characterized a Phosphinothricin resistance gene from *Streptomyces viridochromogenes*. This gene codes -comparable to the BAR gene - for an enzyme which acetylates Phosphinothricin. Thus it has been named Phosphinothricin-Acetyl-Transferase or PAT enzyme. Correspondingly the gene has been given the name PAT gene. Nucleotide sequences of BAR and PAT show sequence homology. They code for similar enzymes which inactivate Glufosinate Ammonium by specific acetylation of the amino group. N-Acetyl-Glufosinate is formed which has lost any herbicidal activity.

The fact that both the herbicide Glufosinate Ammonium as well as the PAT resistance mechanism have evolved in the same microorganism is an enormous advantage. They complement each other like lock and key. An adverse impact of PAT on plant metabolism is therefore highly unlikely. This view is supported strongly by performance data of tolerant crop species in extensive field tests over several years now.

TRANSFORMATION OF CROP PLANTS

After insertion of the PAT gene into *Agrobacterium tumefaciens* as transformation vector a range of dicot crops could be transformed. (Donn et.al. 1990).
In addition to transformation of dicot species, a maize protoplast transformation system has been developed and fertile transformed maize plants have been regenerated. (Morocz et.al., 1990; Donn et.al., 1990).
These reliable and efficient transformation techniques yielded a large number of transformants. Tolerant calli were selected on Glufosinate Ammonium containing media and regenerated to plantlets. After potting these plants were sprayed with Glufosinate Ammonium. They were completely tolerant to 2kg a.i./ha which is about four to six times the rate used for effective weed control.
Because the transformants contain the PAT enzyme, they can be selected on media containing Glufosinate Ammonium. It was shown, that plant extracts from transformants which were incubated with radioactive Glufosinate Ammonium converted 0,5m M completely into the inactive N-Acetyl-Compound within one hour.

Extensive research on transformation of several different plant species demonstrated that the PAT gene is stably integrated into the genome of receiver plants. As a consequence its inheritance follows mendelian segregation. The PAT gene is furthermore inherited as a dominant gene. Hybrid plants which inherited the PAT gene only from one parent line, and are heterozygous in regard to the PAT gene, are as tolerant as respective homozygous plants (Donn, Eckes, 1992). As Glufosinate Ammonium tolerance can be easily and reliably detected, the PAT and BAR genes are widely used today on as selection markers for transformation of different plant species. They have become tools of biotechnology and modern plant breeding.

DEVELOPMENT OF GLUFOSINATE AMMONIUM TOLERANCE TECHNOLOGY

Since the PAT gene has proven to be stably integrated into plant genomes the door was open, for a new dimension of selective weed control by future uses of Glufosinate Ammonium, the broad acting herbicide. In 1989 a decision was taken to focus respective research and development work on four major crops. Oilseed Rape, Corn, Soybean and Sugar beet have been selected as core crops.

Cooperation and Licence agreements with major breeding institutions and seed companies around the world have been initiated. They are incorporating the PAT gene into their elite germplasm. New Glufosinate Ammonium tolerant parental lines, hybrids and varieties are in development.
Seed companies will register and sell newly developed Glufosinate Ammonium tolerant varieties.

The seed will be regularly tested for Glufosinate Ammonium tolerance. The level and homogenicity of Glufosinate Ammonium tolerance will be regularly tested during all steps of breeding and seed multiplication in order to secure the highest seed quality standard possible. Only seeds which meet the standard will be approved, clearly labelled, and sold.

Seed bags of Glufosinate Ammonium tolerant varieties will show in addition to the variety name an easy recognizable Glufosinate Ammonium tolerance logo. This kind of label will provide farmers with the proper information that they can use Glufosinate Ammonium safely for efficient weed control.

Although the herbicide Glufosinate Ammonium is registered and sold since several years its registration has to be extended to future uses as a selective herbicide in genetically modified tolerant crops. Therefore data on metabolism, toxicity, residues, field performance etc. are generated for evaluation by registration authorities.
Metabolism studies with different Glufosinate Ammonium tolerant plant species confirm, that Glufosinate Ammonium is taken up by green leaves and stems and immediately converted to N-Acetyl-L-Glufosinate, an inactive metabolite. N-Acetyl-L-Glufosinate is shown to be a new metabolite of L-Glufosinate Ammonium which will be further evaluated.
Metabolism studies in soil demonstrated, that N-Acetyl-L-Glufosinate Ammonium is quickly deacetylated by microorganisms to Glufosinate which is known already to be completely degraded to phoshate, carbon dioxide, nitrogen and water (Dorn et.al., 1992). Degradation of the plant metabolite N-Acetyl-L-Glufosinate takes place the same way and as quickly as the originally applied active ingredient Glufosinate Ammonium.

Preliminary results from toxicity and ecotoxicity studies with N-Acetyl-L-Glufosinate show no adverse effects - even at very high dose rates - as one would expect from an inactive metabolite.

FIELD TRIALS

First field tests in 1989 with Glufosinate Ammonium tolerant tobacco plants confirmed excellent crop tolerance also under field conditions. Hence there was proof for the applicability of the new approach for selective weed control with Glufosinate Ammonium.In the following years field trial work was extended tremendously as it can be seen from table 1.

Tab 1: Number of Field tests with Glufosinate Ammonium tolerant crops.

Crop	Region	1991	1992	1993	1994	1995	Sum
Oilseed Rape	North America	14	40	125	65	*105	349
	Europe	1	1	4	10	20	36
	Sum	15	41	129	75	125	385
Maize	North America	1	7	70	113	174	365
	Europe	2	5	8	15	47	77
	Sum	3	12	78	128	221	442
Soybean	North America	3	5	45	69	141	263
Sugar Beet	North America			1	2	4	7
	Europe				1	11	12
	Sum			1	3	15	19
	Total Sum	21	58	253	275	502	1109

* registration and first sales

Generally weed control trials have been conducted as post emergence treatments with single applications of 150 to 600g a.i./ha Glufosinate Ammonium and with sequential applications of 2x150g a.i./ha to 2x600g a.i./ha Glufosinate Ammonium. The timings of treatments were choosen primarily according to the growth stages of weeds.
They are described as follows:

- Early Post, 2 - 4 leaves stage
- Mid Post, 3 - 5 leaves stage
- Late Post, 5 - 8 leaves stage

Sequential or split applications were done at Early Post or Mid Post followed by one further treatment after a new flush of weeds had emerged.

The spectrum of most important weeds in Oilseed Rape, Maize, Soybean and Sugarbeet which are controlled by Glufosinate Ammonium is listed in the following table 2.

Tab 2: Most important weeds controlled by Glufosinate Ammonium

Dicot weeds		Monocot weeds
Abutilon theophrasti	*Mercurialis annua*	*Agropyron repens*
Amaranthus retroflexus	*Papaver rhoeas*	*Alopecurus myosuroides*
Ambrosia spec.	*Polygonum convolvulus*	*Avena fatua*
Capsella bursa-pastoris	*Polygonum spec.*	*Digitaria sanguinalis*
Chenopodium album	*Raphanus raphanistrum*	*Echinochloa crus galli*
Cirsium arvense	*Senecio vulgaris*	*Hordeum vulgare*
Galeopsis tetrahit	*Sinapis arvensis*	*Setaria spec.*
Galium aparine	*Solanum nigrum*	*Sorghum bicolor*
Geranium spec.	*Sonchus spec.*	*Sorghum halepense*
Ipomea spec.	*Stellaria media*	
Kochia scoparia	*Veronica spec.*	
Laminum spec.	*Xanthium strumarium*	
Matricaria spec.		

OILSEED RAPE

A lot of data have been generated with spring oilseed rape (Canola) in Canada since 1990. A single application of 300 to 600 g a.i./ha Glufosinate Ammonium achieved consistantly more than 90% efficacy. In comparison to standard herbicide treatments Glufosinate Ammonium got superior season long weed control.

Higher dose rates or split applications are recommended for heavy infestations of perennial weeds like *Cirsium arvense* or *Agropyron repens*.
Yield assessments with the same Glufosinate Ammonium tolerant Canola variety treated either with Glufosinate Ammonium or with standard herbicides were conducted over the last 4 years. Glufosinate Ammonium treated plots consistantly outyielded standard herbicide treatments. Since Spring 1995 Glufosinate Ammonium is registered for selective use in Canada. Glufosinate Ammonium tolerant Canola was commercially grown on about 40.000 acres. Weed control on farmers fields has been very good this year.

Field trials with Glufosinate Ammonium tolerant winter oilseed rape were conducted the last 3 years in Europe. Dose rates of 300 to 600g a.i./ha Glufosinate Ammonium have been applied as single and sequential applications Early or Mid Post in autumn and in early spring. They showed on average total weed control from 85% to 97%. The level of weed control with Glufosinate Ammonium was in most cases superior to standard herbicides treatments. Crop safety in Glufosinate Ammonium tolerant oilseed rape was excellent. Crop injuries did not occur.
Weed spectrum controlled see table 2.

MAIZE

Extensive field testing of Glufosinate Ammonium tolerant maize hybrids took place the last 3 years particularly in the Midwest of the USA and Western Europe. Glufosinate Ammonium has been applied in single and sequential treatments, in tank mixtures with residual herbicides and as single treatments followed by cultivation. Dose rates tested ranged from 250 to 500 g a.i./ha

Glufosinate Ammonium. Lower rates of 150 to 350g a.i./ha. Glufosinate Ammomnium were tested either in tank mixtures, sequential applications or as treatments followed by cultivation. Early Post or/and Mid Post applications of Glufosinate Ammonium achieved in most cases on average weed control of more than 90%. At locations with heavy weed pressure and long periods of weed germination sequential applications of Glufosinate Ammonium were most efficient and outstanding treatments.
Field trials from 1993 in France and from 1994 in France and Germany with single and sequential applications of Glufosinate Ammonium gave comparable results.
Weed spectrum controlled see table 2.

Yield assessments were conducted since 1993 in the USA and in Europe. First field trials from 1994 in Germany confirm the superior results for selective weed control with Glufosinate Ammonium in tolerant maize hybrids. They revealed top yields for Glufosinate Ammonium treatments. Crop safety for Glufosinate Ammonium was excellent.

SOYBEAN

Glufosinate tolerant soybeans have been tested extensively during the last 3 years in the Midwest of the USA. Trial protocols were similar to those in corn. Weed control achieved with Glufosinate Ammonium treatments was very well in line with results from maize. Early or Mid Post applications with 300 to 400g a.i./ha gave overall weed control of 90 to 95%. Consistently best weed control has been achieved with split applications of 2x250g a.i./ha Glufosinate at Early Post and about 2 weeks later. Excellent crop safety in Glufosinate Ammonium tolerant soybean was achieved. Yield assessments showed also top yields for Glufosinate Ammonium treatments confirming results from oilseed rape and maize.
Weed spectrum controlled see table 2.

SUGAR BEET

First results are available from limited field tests in the USA and Europe. They indicate, that 2 post emergence treatments of 300 to 600g a.i./ha Glufosinate Ammonium achieve excellent broad spectrum weed control. The best timing seems to be a first treatment at Early Post (2-4 leaves of weeds) followed by a second treatment of newly germinated weeds at similar stages. Until now efficient weed control in sugar beets need mixtures of several herbicides which have to be applied 3 to 4 times. Glufosinate Ammonium offers the chance for an easy and superior weed control with 2 applications, which will be a great improvement. Crop safety being a critical issue in sugar beets was excellent with Glufosinate Ammonium.
Weed spectrum controlled see table 2.

ADDITIONAL CROPS OF INTEREST

Cotton, Rice, Lupins, Tomatoes and others have been transformed elsewhere and are of interest in some countries. The selective use of Glufosinate Ammonium will be developed locally. Cereals, except maize, however, are considered to be a risk concerning volunteers, because cereals are widely grown in rotations with maize, oilseed rape, soybeans and sugar beets.

DISCUSSION

WEED RESISTANCE TO GLUFOSINATE AMMONIUM

Due to the mode of action of Glufosinate Ammonium it is very unlikely that weeds become resistant. The reason is that this would require a mutation of the target enzyme glutamine synthetase. However, mutated glutamine synthetase which looses its binding affinity for Glufosinate Ammonium simultaneously looses its binding affinity for glutamate, a structural analogue of Glufosinate Ammonium. A mutated enzyme can not catalyze the amidation of glutamate to glutamine, the essential detoxification of ammonia. As this type of mutation is supposed to be lethal, weeds will not develop resistance to Glufosinate Ammonium.
This hypothesis is well supported:

1. Glufosinate Ammonium has been used on some areas since over 17 years several times a season. No observations of resistant weeds have been made.
2. Extensive in vitro selection programms for maize and alfalfa failed to yield Glufosinate Ammonium tolerant plants. On the other hand it is easy to select for mutants tolerant to other herbicides namely to sulfonylureas.

POLLEN TRANSFER

Concerns that cross pollination between Glufosinate Ammonium tolerant crop plants and their wild relatives may cause problems for weed control have been raised particularly with oilseed rape.
Research at INRA in France resulted in artificial crosspollination of oilseed rape with wild relatives like *Sinapis arvensis*. Most of the interspecific hybrids were however sterile. Therefore the conclusion was that the risk of cross pollination with wild relatives under natural conditions will be very minimal (Chevre et.al. 1992, Kerlan et.al.).
However, even if cross pollination would occur under field conditions this would not cause any environmental problems, because Glufosinate Ammonium tolerance is not a competitive advantage in natural habitats where Glufosinate Ammonium is not sprayed (Crawley et.al. 1993).

TECHNOLOGY ASSESSMENT

Extensive research work on Glufosinate Ammonium tolerance in labs, greenhouses and fields internally as well as externally with institutions and seed companies has shown that this technology is applicable and not more risky than methods traditionally used in agriculture. This has been also confirmed by the conclusion of an extensive and lengthy technology assessment procedure for herbicide tolerant plants in Germany (Van den Daele, 1994) and of course by official permits granted for field tests in North America, West Europe and elsewhere. From the extensive knowledge of Glufosinate Ammonium and experience from many years of field trials, a number of agronomic, economic and ecological benefits can be identified - in all cases in comparison with the currently available weed control systems:

1. The farmer acquires an additional option for controlling weeds after they have emerged. Nonetheless, all previous methods or products continue to be available to him.

2. Optimal olerance by the plant ensures maximum protection of yield. The dependence of

treatment on the growth stage of the crop is reduced, thus making application easier to time. Technical management requirements can be taken into consideration to a greater degree. It is easier to keep weeds below damage thresholds.

3. The new system can also make some treatments completely unnecessary (if weeds are below damage thresholds) or reduce sequential sprayings. Decisions based solely on the level of weed infestation and its development help to reduce the amount of herbicides applied.

4. Crop rotation benefits from reduced herbicide residues in the soil.

5. The opportunities for different growing methods, e.g. erosion control using undersowing or similar techniques, can be improved in combination with the new system.

This new approach will permit the development of herbicides with a more beneficial toxicological and exotoxicological profile enabling weed control to be carried out with as few side-effects as possible.

OUTLOOK

Glufosinate Ammonium and two Glufosinate Ammonium tolerant oilseed rape varieties have been registered and launched in Canada 1995. Glufosinate Ammonium is sold under the brand name Liberty. The responding tolerance label used is Liberty Link together with a logo. Further Glufosinate Ammonium tolerant varieties of oilseed rape, maize, soybean and sugar beet are in development and will be marketed by seed companies soon. In parallel the registration process for the selective weed control with Glufosinate Ammonium in these crops is ongoing. Next launches are projected to follow from 1997 onwards in North America and Europe.

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SUGAR BEETS TOLERANT TO NON-SELECTIVE HERBICIDES - A SEED COMPANY'S PERSPECTIVE.

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ABSTRACT

Genetic modification of sugar beets to tolerate glyphosate or glufosinate, two non-selective herbicides, is expected to provide the grower with economic advantages and increased flexibility. Whilst the use of these herbicides has been shown to be both health-wise and environmentally benign, breeders and agro-chemical companies collaborate to confirm the safety of these products in the field. This paper addresses four issues which are of particular importance to a seed company, namely : gene management, product liability, seed production and agronomic performance.

INTRODUCTION

From a technical point of view, the use of glyphosate or glufosinate for weed control of sugar beets, engineered for tolerance to these herbicides, is competitive with the existing selective herbicides commonly used. I will not address the mode of action of glyphosate nor glufosinate, nor how tolerance to these herbicides can be achieved. A good review on these topics is given by Dekker & Duke, 1995. In addition, my agro-chemical colleague's of Monsanto and Agrevo will undoubtfully address these important issues.

In what follows I would like to share with you four issues which are of major concern to a seed company when developing herbicide tolerant crops via genetic modification :

-> GENE MANAGEMENT
-> PRODUCT LIABILITY
-> SEED PRODUCTION
-> AGRONOMIC PERFORMANCE

Three issues which are equally important, but which strongly depend on how well one manages the above are:

-> ECONOMIC PERFORMANCE
-> ENVIRONMENTAL IMPACT
-> PUBLIC PERCEPTION

GENE MANAGEMENT

Single versus multiple (commercial) transformants

By choosing to develop a single tranformant in all herbicide tolerant hybrids, one can reduce the amount of characterization required, accumulate much more data on this single event and reduce registration costs.

However, we also create linkage drag in all germplasm genetically modified with this trait: the chromosome arm around the introduced gene will to a large extend be inherited when selecting for the herbicide tolerance trait. This reduces genetic variability. The effect of " *negative alleles* " linked to the transgene may only become apparent much later.

If, as some believe, herbicide tolerance becomes a standard trait in sugar beets in the next century, using a single transformant would be an undesirable reduction of the genetic diversity available to the breeders.

Stacking of multiple herbicide tolerance traits

The non-selective herbicides glufosinate and glyphosate have excellent agronomic performance as well as environmental and animal toxicity profiles. (Devine *et al.*, 1993a; Duke, 1988) Consequently their registration may be extended for post-emergence use on sugar beet (genetically modified for tolerance).

One could argue that a sugar beet grower may want the freedom to choose one of both herbicides, depending on the weed spectrum that develops in a given year or the discount which his herbicide supplier offers on one or both products. If the beets carry the tolerance genes for both glyphosate and glufosinate, he could make that choice at the last minute.

Another argument would be that a combination of both herbicides might represent an ideal weed control regime: full weed spectrum coverage and a reduced risk that weeds develop tolerance to both classes of herbicides simultaneously.

However, stacking such herbicide tolerance genes has drawbacks: sugar beet is currently cultivated in rotation with cereals, potatoes, oil seed rape and other species. Some of these crops are also being modified for tolerance to the same non-selective herbicides. With each herbicide tolerance trait that is stacked in sugar beet, control of volunteer sugar beets in the rotation crops would be further complicated.

Gene pool management

When a breeder succeeds to select for a very favorable trait, such as seed monogermity or rhizomania resistance, it is often transferred to a major part of the gene pool and fixed (if possible). As such it becomes an intrinsic quality trait of the gene pool which is used to generate new hybrid parents. However, engineered herbicide tolerance is entirely new, and while we are so far very optimistic, no-one can exclude that undesirable aspects of this weed management approach may appear and lead to the abandoning of herbicide tolerant plants.

This uncertainty may incline breeders, for a certain period of time, not to integrate herbicide tolerance into their main breeding populations but to store it in (semi) elite lines. As a result, special care to backcross such lines to appropriate recurrent parents will be required, thus enhancing both the cost and complexity of breeding programs.

PRODUCT LIABILITY

Environmental damage

Because pollution does not respect national boundaries and envriromental issues have an effect, the European Community decided to integrate the protection of the environment into its own law and regulation, which are binding for its member states. **Articles 130R (paragraphs 2 to 5) and 130T** of the Treaty now fix the principles which are to guide the actions of the European Union:

1) Principle of preventive action.
2) Principle of correction to the original state.
3) Principle of the polluter pays.
4) Principle to integrate environmental concerns in other aspects of communitary policies (such as the CAP).
5) Principle to minimally abide to the communitary norms.
6) Principle to cooperate with international organizations and other countries.

While today, the directives and executive regulations following from these principles have not all be implemented, by the year 2000 this is likely to be the case and seed companies need to consider potential environmental liability of their activities and products very seriously.

A primordial part of Risk Management is PREVENTION. The European Union has enforced *a priori* environmental safety measures through the Directive 90/220/ EEC on the **"Deliberate release into the environment of genetically modified organisms (GMO's)"**. This case-by-case, step-by-step procedure consists of a separate requirement list of safety measures for R & D purposes **(part B)** and a requirements list for placing GMO's on the market **(part C)**. It undoubtfully contributes to the safety of the GMO's to be released.

Defective products

Directive N°85/374/E.E.C. by the Council of 25/07/85 regulates responsibility for *defective* products. As any directive, its provisions are binding for the EU member states who have the obligation to translate them as minimal standards into their national law. Central to this directive is the principle of **Objective Responsibility**. What this means is that the producer of the defective product is responsible for damages whether he is at fault or not. In other words, even if a product has passed all safety and quality controls, has been properly handled or recommended to be handled by the producer, if the product fails, the producer is primarily responsible.

Food and feed safety

Unfortunately, no EU Directive has been approved which properly regulates food and feed safety of entirely novel products. This is due to the strong protests of industry to the proposals thus far. They in fact contained many requirements which are not related to safety or proper consumer information. They focussed on the *way such products have been obtained* and imposed regulation, including indiscriminate labeling, whether or not the products contained novel ingredients or were shown to be as safe as similar existing products. Such regulations could indeed have a negative impact on the competitiveness of European industries.

The UK has been a pioneer in Europe in pragmatic and adequate regulation of biotechnology. In February 1995, the Ministry of Agriculture, Fisheries and Food (MAFF), has applied national legislation to approve 3 Novel Foods for consumption (MAFF News Release, 1995). Additional good news is that the last Novel Food proposal of the EU Council has received good comments by the Committee of Permanent Representatives (COREPER) and may be presented in final form to the European Parliament by end 1995.

Product liability of herbicide tolerant sugar beets, related to environmental and agronomic damages or health hazards are of capital importance to a seed company. It is thus not surprising that a major part of the research coordinated with our agro-chemical collaborators concentrates on these issues.

SEED PRODUCTION

Sugar beet or *Beta vulgaris* subsp. *vulgaris* belongs to the *Chenopodiaceae* family. A taxonomic division of the genus *Beta* is given in table 1. Whilst sugar beets may hybridize and produce fertile hybrids with other subspecies of *B. vulgaris*, weed beets are almost exclusively due to crosses with *B. vulgaris* subsp. *maritima* (Van Geyt *et al.*, 1990a).

Interspecific hybridizations between B. vulgaris (section *Beta)* and members of the section *Corollinae* are very difficult and if successful, result in sterile hybrids or apomictic reproduction (Van Geyt *et al.*, 1990a). It is virtually impossible to hybridize the three species of section *Procumbentes* with *B. vulgaris*. Lethality, high hybrid sterility, irregular meiosis and inadequacy of chromosome pairing were reported (Van Geyt *et al.*, 1990a). Crosses of *B. vulgaris* with *B. nana* (Section *Nanae*) have not been reported.

Beta vulgaris subsp. *maritima* contains two geographical entities in the biosystematic sense, the sea beet and the ruderal beet. Due to its adaptation to the saline, sandy, coastal biotope, the sea beet or its hybrids do not present weed problems in sugar beet fields.

Table 1. Taxonomic division of the genus *Beta L.*

Section	Species	Subspecies
Beta		
	B. vulgaris	*vulgaris*
		maritima
		adanensis
	B. patula	
	B. macrocarpa	
Nanae		
	B. nana	
Procumbentes		
	B. procumbens	
	B. webbiana	
	B. patellaris	
Corollinae		
	B. macrorhiza	
	B. iomatogona	
	B. corolliflora	
	B. trigyna	

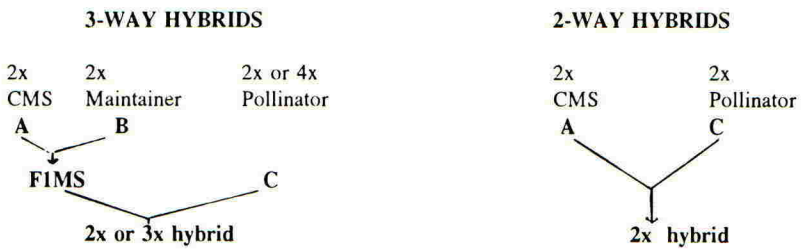
Of agronomical concern are the so-called "weed beets", which are the result of recurrent hybridization between cytoplasmic male sterile sugar beet seed production components and the annual ruderal beet, carrying the dominant gene B for annual growth habit (Ford-Lloyd & Hawkes, 1986; Horsney & Arnold, 1979). The presence of annual beets in a commercial sugar beet field is highly undesirable as they will bolt and flower. As such they compete with (vegetative) beets for light and metabolites and interfere with proper harvesting (Longden, 1989). As weed beets are naturally tolerant to the traditional herbicides used on sugar beets, only strict seed production control and plant-by-plant weeding methods are applicable (Institut Technique de la Betterave, 1989; Boudry *et al.*, 1993).

Farmers, who do not control bolting beets, allow accumulation of seed carrying the annual growth habit (weed beet) in their fields and may, over rotation cycles, accumulate more and more bolters. Glyphosate or glufosinate tolerant sugar beets will give such farmers a unique chance for a "fresh start". However, it is clear that such control is not a complete nor final solution to bolters: (i) bolting-sensitive sugar beet varieties may, in years with cold springs, bolt at a certain frequency and (ii) with time a portion of the annual ruderal beet population surrounding production fields will inherit the herbicide tolerance trait. Male sterile sugar beet lines pollinated by such ruderal beets in production fields will produce herbicide tolerant weed beet seed.

Seed production schemes of herbicide tolerant sugar beet

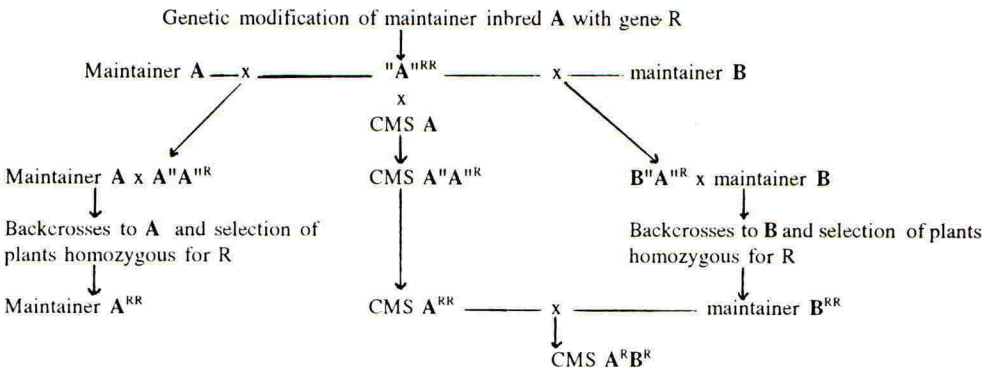
Most commercial sugar beet varieties are three-way hybrids: a maintainer line (O-type) is crossed with a cytoplasmic male sterile (cms) conversion of a *different O-type, producing a F1 male sterile hybrid* (F1MS). The F1MS is subsequently crossed with a pollinator thereby generating a three-way hybrid (figure 1). Sugar beets are recessive for the B gene causing the annual growth habit and require vernalization to induce bolting and flowering. Hence the seed drilled in april will produce a large vegetative storage root by october.

Figure 1. Commercial sugar beet hybrids



In three-way hybrid sugar beet varieties, a novel gene may be introduced via the female or seed parent or via the male or pollen parent. In the first case, the gene must be incorporated into the two O-type lines **A** and **B**, as well as in the cms equivalent of the **A** (or **B**) O-type. All three lines should have the introduced gene in the same position in the sugar beet genome in order to guarantee transmission of the gene to all offspring plants of the F1MS female. Consequently, if the gene was introduced into the maintainer **A** through genetic transformation, it will need to be transferred to maintainer **B** and to the cms-**A**, via sexual crossing and repeated backcrossing (figure 2). In single hybrid varieties, it will suffice to have the transgene in one O-Type (**A** or **B**) and its cms equivalent.

Figure 2. Genetic modification of F1MS (CMS **AB**) for hybrid seed production



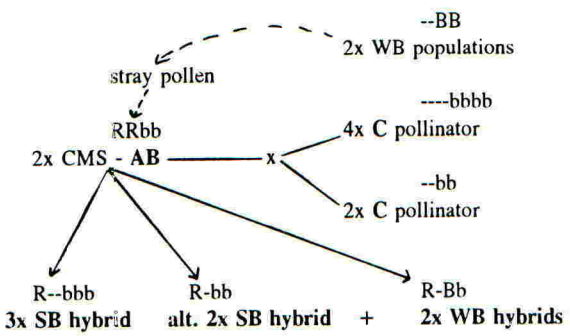
If the novel gene is introduced into the pollinator parent (**C**), the procedures are equivalent to those required to develop the genetically modified O-type $\mathbf{A}^{RR}$ in figure 2, but for **C**. Although cheaper and faster than the first described, it is applicable only in case of diploid pollinators. The reason is the practical difficulty to develop a broad-based, heterozygous tetraploid population which is simultaneously homozygous for the introduced gene.

The sexual component in which the herbicide tolerance trait is integrated may influence the frequency of undesired gene transfer. Below both pro's and con's of each are given.

Introduction into the male sterile female component

If the transgene is introduced into the 2x cms-$\mathbf{AB}^{bb}$ (see figure 3), pollen transfer and direct gene flow from the genetically modified sugar beet plants to the annual ruderal beets in the seed production areas can be prevented. This is possible as basic seed of the cms **AB** component is tightly controlled for male sterility and is produced in regions where no ruderal beets are present. Basic seed is planted in a plantlet or steckling nursery, which can be sprayed with the herbicide to which tolerance was engineered. A crop rotation cycle of our steckling nurseries is 15 years. Such tight controls should safeguard that only herbicide tolerant, male-sterile stecklings are transplanted in seed production fields.

Figure 3. Outcrossing with herbicide tolerance trait (R) in cms female parent



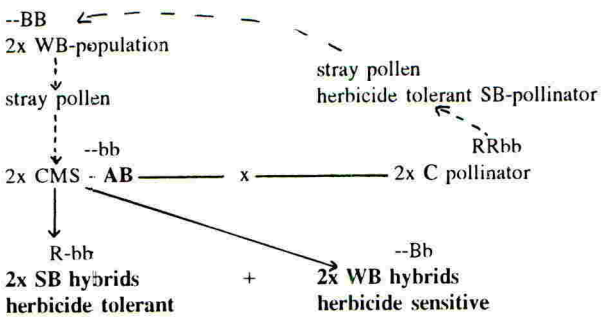
Key: SB : sugar beet ; WB : weed beet ; BB : homozygocity for gene B for annual growth habit.

Whilst the above scenario may appear attractive from a conservative ecological standpoint, it has some drawbacks as well: pollen of annual ruderal beets, growing around production fields, could pollinate the genetically modified cms females. These hybrids seeds will be harvested and sold. As they carry the B gene for the annual growth habit, they will give rise to flowering beets in the farmer's field, thus causing an opportunity for spread of the herbicide tolerance gene to weed beet populations nearby the commercial field.

Introduction into the pollinator component

If the transgene is introduced into the 2x pollinator (figure 4), pollen of annual ruderal beets, which fertilize F1MS lines in the production fields, will give rise to seeds being herbicide sensitive. Plants derived from such seeds will be killed in the farmer's field before they can bolt. This will be a very desirable situation for the farmer. However, control of such weed beets would not remain 100% for long: the genetically modified pollinators in the production fields could also pollinate annual ruderal beets in the surroundings. The frequency of such an event will depend on the degree of self-fertility of wild beets, the synchronization of flowering, the size and nearness of the ruderal beet population to the pollen source and the viability of the hybrid (ruderal) beet seed. And although the herbicide tolerance trait offers no obvious advantage under non-sprayed conditions (BRIDGE report, 1995), some fraction of the ruderal population will probably carry the herbicide tolerance trait. However, it still represents an improvement in comparison with the current situation. For example, if after 10 years, 20 % of wild beets surrounding production fields were herbicide tolerant and thus 20 % of the contamination would not be eliminated by herbicide treatment in the farmers field, then this method of bolting control will still take out 80 % of the outcrosses.

Figure 4. Outcrossing with the herbicide tolerance trait (R) in pollen parent



Key: SB: sugar beet ; WB : weed beet ; BB : homozygocity for gene B for annual growth habit.

While this argument holds for herbicide tolerance, the selective advantage may be entirely different for other traits such as disease resistance. Hence, ecological and agronomic consequences need to be considered case by case.

Seed losses in production fields

In a typical production field, 4 to 6 rows of cms females are planted for every 2 rows of pollinators. An ha produces on average 2.5 ton uncleaned hybrid seed. It is estimated that up to 300 kg is dropped during harvest. As synchronization of flowering between the sexes is crucial, this process is closely monitored. Often flowering is attuned by cutting back inflorescence. Pollination occurs during 2 to 3 weeks, after which (and before seed ripening), the pollinators are cut down completely as their seed set is undesirable. Hence, while we have a very strong control over seed loss of pollinators, control over the loss of hybrid seed, is mainly by tillage to stimulate preliminary germination followed by eradication and by crop rotation. A crop rotation cycle in sugar beet seed production fields is 6 or 7 years. Rotation crops are typically wheat, maize and sunflower, but also peas, soybean and oil seed rape. 6 to 7 cultivations and weed control using herbicides which kill sugar beets effectively, eliminates dropped seed as monitored by the frequency of beets which grow between rows in the following production cycle.

AGRONOMIC PERFORMANCE

Today, sugar beet growers use up to 9 different herbicides in 4 or 5 applications per ha (May, 1994; Institut Technique de la Betterave, 1994). Some herbicides are mixed, others are used in sequence to cover the entire weed spectrum. The reason for this is dual: (i) sugar beet closes canopy rather slowly and has a low (vegetative) stature. Hence, it competes badly with weeds which can cause an important yield penalty (ii) weeds complicate harvesting of sugar beets.

Weed control

Roundup® and Basta®, commercial formulations of respectively glyphosate and glufosinate, control a major part of the weed population at 2 or 3 applications of 2 l per ha. By adding an appropriate traditional herbicide or raising the dose rate of the above mentioned non-selective herbicides, good weed control can be achieved. The total amount of herbicides used will undoubtfully be reduced while at least one less application will necessary. Hence a farmer can look forward to reduced herbicide costs and increased flexibility.

Development of tolerant weeds

A concern associated with the use of a single herbicide on large acreage's is that one will select for tolerant weeds or for a shift in weed spectrum. Glyphosate has been used world-wide for over 20 years and no resistance has been documented. Glufosinate has perhaps been used less frequently and for a smaller period of time, but no cases of weed tolerance were reported (Dekker & Duke, 1995). Ways to avoid the break-down of herbicide efficacy are (i) to add an unrelated herbicide, selective for sugar beet. The additional herbicide may also help control weeds which are naturally less affected by glyphosate or glufosinate; (ii) to rotate the use of glyphosate tolerant beet with glufosinate tolerant beets or with traditional beets.

Crop rotation

Under "Gene Management" it is stated that rotating crops which are tolerant to the same herbicides will complicate weed management. Indeed, this new generation of products will require growers to keep track of what varieties which they grow in a rotation. Recent experiments have shown that volunteer oil seed rape, tolerant to Roundup®, can be controlled in a Roundup® tolerant sugar beet crop by including besides 2 times 2 liters per ha Roundup® other registered herbicides.

Yield

To be agronomically competitive with conventional sugar beets, this novel generation of herbicide tolerant varieties should be equivalent on yield. Hence, the importance to integrate the transgene in elite lines; a difficult task when one realizes that between the original transformation event and commercialization approximately a decade will have elapsed. Such issues may be the actual determinants of the success of these products.

CONCLUSIONS

For a new weed management system to be of value to the sugar beet grower, a number of agronomic and economical criteria need to be fulfilled. Glyphosate or glufosinate tolerant sugar beets may satisfy them. Both herbicides have better environmental and toxicity profiles, will control weeds at lower doses and fewer applications and will to a certain extend eliminate weed beets. The overall regime is likely to be less costly than current practices.

Breeding companies like Hilleshog NK who have invested in the development of herbicide tolerant sugar beets, have a number of important concerns. They relate to germplasm management, seed production constraints, product liability questions and the substantial costs to develop such plants.

Thus far our research and that provided by our collaborators has not demonstrated problems related to human or animal health or environmental hazard. We have however not finished all studies and have not yet applied for regulatory approval.

The uninformed public is cautious to skeptical about GMO's and in particular herbicide tolerant plants (Chamberlain Partnership, 1994). This is because perception is one of increased herbicide use. It is important that in the years that lie before potential commercialization of these products, seed companies make serious efforts to properly inform the general public about the environmental advantages which the use of either glyphosate or glufosinate on sugar beet will present.

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Session 7B

Recent Development in Herbicide Mode of Action and Metabolism

Chairman and Session Organiser	Dr K E Pallett
Papers	7B-1 to 7B-4

HERBICIDE METABOLISM AND SELECTIVITY: ROLE OF CYTOCHROME P450

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ABSTRACT

Cytochrome P450s (P450s) form a large family of heam-thiolate proteins catalysing phase I metabolism of many xenobiotics, including herbicides and other phytopharmaceuticals. P450 catalysed oxygenations usually result in the detoxification of the active molecules and have recently been shown to play an important role in herbicide resistance and selectivity. It is thus not surprising if the occurence of herbicide-resistant weeds is often correlated to modifications in the activity or expression of their P450 oxygenases. P450s are highly inducible enzymes. Their induction, which is largely controlled at the transcriptionnal level, can be obtained by chemical treatments, i.e. with safeners, drugs or pollutants. Moreover, the design of specific tight-binding or mechanism-based inhibitors is made possible by the unique catalytic properties of these heamproteins. Therefore, 1) selective induction or inhibition of plant P450s, 2) interspecies transfer or modification of the genes coding for these enzymes, 3) manipulation of the expression of these genes, offer wide possibilities to manage herbicide resistance in crops and weeds.

INTRODUCTION

P450s constitute the most extensively studied class of enzymes. This is largely due to their prominent role in hormone and drug metabolism, resulting in numerous medical and pharmacological applications. They have been used as drug targets, and their activity was shown to be essential for drug tolerance and clearance, as well as for drugs compatibility. As the knowledge of plant P450s increases similar applications can be foreseen in plant health and management.

GENERAL PROPERTIES AND FUNCTIONS OF PLANT P450S

P450s form a large familly of ubiquitous heam-thiolate proteins. They usually function as monooxygenases, i.e. bind molecular oxygen, catalyse its activation, and incorporate one of its atoms into a substrate, the second oxygen atom being reduced to form water.

$$RH + O_2 + NADPH+H^+ \rightarrow ROH + H_2O + NADP^+$$

The electrons necessary for the activation of oxygen are provided by NADPH via flavoproteins, the NADPH-P450 reductases. In plants, both P450s and their reductases are membrane-bound, microsomal proteins. In their reduced state, P450s can bind carbon monoxide instead of oxygen, forming a complex which maximally absorbs at 450nm. This complex is very stable. CO thus blocks the enzymatic reaction. The inhibition can be reversed by light with a maximum of efficiency at 450 nm.

A part of the catalytic centre is highly conserved in all P450s: the heam prosthetic group and a very few (less than ten) amino acids which surround it. The latter include a cysteine serving as fifth ligand to the heam iron and located about 15% in from the carboxy-terminus of the protein. These conserved elements are responsible for the oxygen binding and activation, and for the transfer of the protons needed to form water. The overall apoprotein sequence is however highly variable among P450s, identity in their amino acid sequences being sometimes less than 20%. This variability in protein sequence accounts for the great versatility of P450

enzymes, and for their capability to metabolise a wide range of substrates and to catalyse very different reactions. The reaction carried out is dependent on the protein and on its substrate. It is very often hydroxylation, but epoxidations, heteroatom dealkylations, deformylations, isomerisations, ring formations, dimerisations, C-C cleavages, dehydrations and reductions can also be obtained.

P450s are encoded by a superfamilly of genes (Nelson *et al.*, 1993). The sequences of more than 400 of them are already recorded in data banks. Those are named and classified according to the identity in amino acid sequences of the deduced protein. Protein with ≤ 40% amino acid identity are usually considered encoded by different gene families, while protein within a same subfamily are > 46-55% identical. There are, however, some exceptions to this rule. The P450 classification was an arbitrary decision, but has turned out to be genetically significant, since genes belonging to a same subfamily usually lie within a same gene cluster. Since the cloning of the first plant cDNA (Bozac *et al*, 1990), more than 60 plant P450 genes have been registered, forming 18 families. Less than ten of them have an identified function.

Little more than 50 physiological substrates of the plant P450s have been characterised (Durst & Benveniste, 1993; Bolwell *et al*, 1994). Most are plant-specific secondary metabolites, phenylpropanoids, isoprenoids, alkaloids and cyanogenic glucosides. A few, membrane sterols and fatty acids, are also natural substrates of fungal and animal P450s. These substrates are precursors of signalling molecules and of compounds essential for the plant structure or defense against light and pathogens. Considering the number of metabolites and reactions in plant secondary metabolism which are still to be characterised, the range of reactions catalysed by plant P450s will very likely be enormous. Depending on their function within metabolism, plant P450s are differentially expressed in response to developmental and environmental cues. At the present stage, our knowledge concerning plant P450s regulation is only rudimentary. Several were however shown to be expressed in an organ- or tissue-specific manner, in shoots, flowers, developing seeds or in lignin synthetising tissues. Many of them are induced by light, osmotic stress, wounding, or following infection by various pathogens. In the absence of specific developmental or environmental induction, the expression of plant P450s might be very low or undetectable.

HERBICIDE METABOLISM AND SELECTIVITY

Data accumulated in the last five years indicate that microsomal P450 monooxygenases constitute the major oxidative pathway involved in herbicide metabolism and detoxification in higher plants. Alkyl and aryl hydroxylations, *N*- and *O*-dealkylations of the active molecules increase their polarity, allow their further conjugation and storage in the cell-wall or in the vacuole, and limit their activity at the sensitive target sites (Sandermann, 1994).

The *N*-demethylation of monuron in cotton seedlings (Frear *et al.*, 1969) was the first P450-dependent reaction characterised *in vitro* using microsomal preparations from higher plants. However, direct evidence for the involvement of P450s in the metabolism of representative members from other major classes of herbicides has only been obtained recently (Table I). This metabolism is usually more efficient and was in most cases detected in crop plants. For example in wheat, P450s have been shown to metabolise at least eleven different wheat selective herbicides with differing modes of action (Frear, 1995). An active P450-dependent herbicide metabolism thus seems to determine herbicide tolerance in major crops.
Most of the studies, performed with wheat, maize, barley, sorghum, mung bean or weeds, provide evidence that qualitative and quantitative P450 contents largely differ from plant to plant. Species and cultivars associated differences in herbicide metabolism, and differential induction or inhibition have been reported (Gonneau *et al.*, 1988; Mougin *et al*, 1991; Moreland *et al.*, 1993, 1995; Barret *et al.*, 1993; Frear, 1995). They indicate that 1) several P450 isoforms contribute to the oxidation of different herbicides in major crops, 2) different P450s metabolise a given herbicide in single or different plant species.

Table I: Herbicides metabolised by plant P450s.

Reaction	Herbicide	Reference
Alkyl hydroxylation	Chlorotoluron	Mougin *et al*, 1990 Fonné-Pfister & Kreuz, 1990
	Flumetsulam	Frear *et al*, 1993
	Prosulfuron	Frear, 1995
Aryl hydroxylation	2,4-D	Makeev *et al*., 1977
	Diclofop	McFadden *et al*.,1989 Zimmerlin & Durst, 1990
	Primisulfuron	Fonné-Pfister *et al*, 1990
	Triasulfuron	Frear *et al*., 1991
	Prosulfuron	Frear, 1995
	Bentazon	McFadden *et al*, 1990
	Flumetsulam	Frear *et al*, 1993
Heteroatom dealkylation	Monuron	Frear *et al*., 1969
	Chlorotoluron	Fonné-Pfister, 1988 Mougin *et al*, 1990
	Prosulfuron	Frear, 1995
	Metolachlor	Moreland *et al*., 1990
	Alachlor	Moreland *et al*., 1995
	CGA-25704	Moreland *et al*., 1995

The metabolism of the herbicides listed here was successfully measured *in vitro* in plant microsomal preparations. Direct involvement of P450 was demonstrated by CO inhibition. Evidence of P450-dependent metabolism of many other herbicides, in microsomes or intact plants, has also been reported.

Evidence has been reported that a single P450 with a broad specificity could metabolise bentazon, imidazolinones, sulfonylureas, chlorotoluron and organophosphate insecticides in maize (Barrett *et al*., 1995). However, most of the data available suggest that the oxygenation of herbicides in plants usually results from their fortuitous binding to the active site of one or several P450s involved in normal plant metabolism. This scheme is supported by a study of the diclofop and ω-1 lauric acid hydroxylases in wheat microsomes (Zimmerlin *et al*., 1990), where both activities were shown to have similar responses to induction, inhibition and autocatalytic inactivation. Diclofop and laurate hydroxylases also displayed similar K_m for their respective substrates, and both substrates acted as competitive inhibitors of each other with nearly identical K_i values. Molecular modeling studies indicated that their low energy structures can be easily superimposed. It seems thus very likely that diclofop and ω-1 lauric acid hydroxylations are catalysed by a single P450 enzyme. This P450 does not seem to contribute to the metabolism of 2,4D, chlortoluron and chlorsulfuron in wheat.

P450 AND WEED CROSS RESISTANCE TO HERBICIDES

In the last years, many cases of weed resistance resulting from increased P450-dependent metabolism have been reported (Powles & Preston, 1995). Such herbicide resistance in plants seems to develop in a manner similar to that observed for insecticide resistance in insects (Brattsten *et al*., 1986). There are, at the moment, fewer cases of resistance related to enhanced metabolism than cases of mutation of the target sites, and the level of herbicide tolerance achieved by increased metabolism is usually lower than the resistance resulting from a target

modification. However, the development of increased metabolism often results in cross-resistance to several herbicides classes and leads to multiple resistant weed populations (i.e. populations wich resist to one or several herbicides by different mechanisms).

Acquired resistance conferred by P450-dependent metabolism following intensive use of herbicides in monocultures has been best documented for grass weeds: Australian biotypes of *Lolium rigidum* or European biotypes of *Alopecurus myosuroides*. One of the most striking features of these biotypes is that they developped resistance to an herbicide as consequence of a selection pressure from an herbicide of an unrelated chemical group (Powles & Preston, 1995). Cases of resistance to ALS-inhibiting herbicides following selection with ACCase-inhibiting diclofop methyl have for example been reported (Christopher *et al.*, 1991; Cotterman & Saari, 1992). The resistant biotypes showed an increased metabolism of both diclofop and chlorsulfuron, the metabolism of chlorsulfuron being increased in greater proportions than the metabolism of diclofop. The nature of the metabolites formed and inhibition experiments strongly suggested involvement of P450 in the oxidation of the sulfonylurea (Christopher *et al.*, 1994). Other *L. rigidum* biotypes, selected with atrazine or diuron, acquired cross resistance to both phenylurea and triazine herbicides (Burnet *et al.*, 1993a, 1993b). In these biotypes, strong evidence of the involvement of P450 in the deethylation of simazine was obtained using inhibitors. Two pathways (ring-hydroxylation and *N*-demethylation) for the detoxification of chlortoluron coexist in the plant, both of which are apparently supported by P450s and increased in resistant biotypes. Coexistence of these two pathways provides an explanation 1) for the broad resistance of the biotypes to many phenylurea analogues which have *N*-alkyl groups to be dealkylated, 2) for their greater resistance to chlorotoluron which can be also ring-hydroxylated.

Despite intensive efforts, involvement of P450 in the metabolism of herbicides by *L. rigidum* could never been confirmed *in vitro* using microsomal preparations. Direct evidence that P450 was responsible for resistance to substituted urea was, however, obtained using microsomal membranes from *A. myosuroides* susceptible or cross-resistant to phenylurea, sulfonylurea, aryloxyphenoxypropanoate, triazine and other classes of herbicides (Kemp &Caseley, 1991).

The mechanism of acquisition of herbicide resistance in weeds has not yet been investigated. Resistance seems to result from the enhancement of a metabolism already detectable in susceptible biotypes. The products of metabolism are usually the same in susceptible and resistant weeds as in crop plants. It suggests that similar mechanisms could be at the basis of the resistance of weeds and crops. A reasonable scenario seems to be a gene amplification or an alteration of gene regulation which would explain the simultaneous increase in expression of the several P450s responsible for the metabolism of a variety of herbicides. This means that any crop-selective herbicide where selectivity is P450-based is a likely candidate for metabolism endowed resistance. It also implies that the already existing metabolism-based resistant weeds are potentally resistant to yet-to-be discovered herbicides.

RATIONAL DESIGN OF HERBICIDE SELECTIVITY

P450 induction and herbicide safeners

As with their animal counterparts, plant P450s are also highly inducible by chemical effectors. Increased activities have been reported following plant treatment with drugs which also induce animal and bacterial P450s (phenobarbital, aminopyrine, clofibrate...), plant secondary metabolites, metals (Mn^{++}, Hg^{++}, Cd^{++}), solvants (ethanol, DMSO), pollutants (benzo(a)pyrene, bis-(2-ethylhexyl)-phthalate...) and agrochemicals, including lindane, biphenyl, procloraz, herbicides and various herbicide safeners (Reichhart *et al.*, 1980; Mougin *et al.*, 1991; Batard *et al.*, 1995; Frear, 1995). Herbicide safeners or antidotes are a group of chemically diverse compounds with the ability to selectively protect crop species from otherwise phytotoxic doses of herbicides. They are exploited 1) to improve tolerance of newly developed herbicides with limited selectivity, 2) to extend the use of available herbicides on

additional crops or on varying environmental conditions. Safeners have been used for a number of years without real knowledge of their mode of action, but there is now increasing evidence that they mostly act by enhancing herbicide metabolism in target crops (Hatzios, 1991; Kreuz, 1993; Farago *et al.*, 1994). This process leads to a reduction of the amount of herbicide reaching its target site in an active form. It was first demonstrated that safeners enhance herbicide conjugation with glutathione, but it is now clear that P450s and glucosyltransferases also play an important role in the safening mechanism of phenylurea, aryloxyphenoxypropanoate, sulfonylurea triazolopyrimidine sulfonanilide, chloroacetanilide and imidazolinone herbicides.

Safeners were initially shown to stimulate oxidative metabolism of herbicides in plants *in vivo*, in particular in large-seeded crops such as maize, grain sorghum, rice and wheat. First direct evidence for an increase in microsomal P450 content and bentazon hydroxylase activity was obtained in maize shoot microsomes treated with naphthalic anhydride (NA) (McFadden *et al.*, 1990). Induction of P450 and P450-dependent herbicide metabolism by NA and other safeners like benoxacor, dichlormid or oxime ethers in several plants or plant cell cultures was then described (Hatzios, 1991; Mougin *et al.*,1991; Moreland & Corbin, 1991; Moreland *et al.* , 1993; Frear, 1995). In many cases, safener pretreatment of crop shoots permitted the *in vitro* characterisation of enzymes involved in herbicide metabolism, their activities being too low to be accurately measured in untreated plant microsomes. Interestingly, safeners allows for differential induction of the P450s which metabolise endogenous molecules and different herbicides (Moreland & Corbin, 1991; Moreland *et.al*, 1993; Frear, 1995). In addition they may act specifically with respect to the plant species, if they are applied directly to crop seeds prior to planting, or when different metabolic routes are present in different plants.

The molecular mechanisms of the safener-promoted induction of plant P450s is still poorly understood, the most recent data, however, indicate that the increases in P450-dependent activities in response to most chemicals, including NA, is correlated to an increase in P450 proteins and corresponding transcripts (Batard, 1995). The induction of P450s by safeners, like the induction of glutathione *S*-transferases, might thus be exerted through enhanced gene expression.

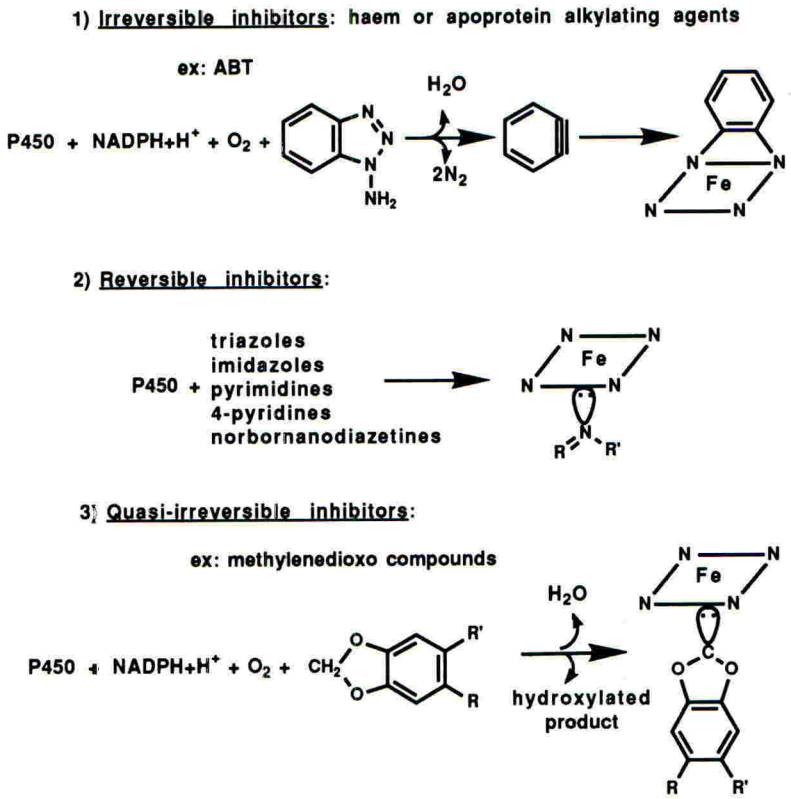
P450 inhibitors and herbicide synergists

Three main categories of inhibitors based on P450 specific properties and catalytic mechanism are currently used to characterise and manipulate the activities of P450 enzymes (Figure 1).

1) Mechanism-based inactivators are molecules which are catalytically activated by P450s to transient species that irreversibly bind and inhibit the enzyme. Such inactivators include a variety of sulfur or halogen containing structures, molecules with terminal olefin or acetylene, dihydropyridines and dihydroquinolines, compounds with N-N functions (Ortiz de Montellano & Reich, 1986). Among the latter figures 1-aminobenzotriazole (ABT), shown to be metabolised by P450s in animals into a benzyne which reacts with the heme porphyrin nitrogen to form N,N-bridged species (Ortiz de Montellano & Mattews, 1981). ABT, wich also promotes inactivation of plant P450s (Reichhart *et al*, 1982), is now one of the most widely used molecules for the characterisation of P450-dependent reactions *in vivo* and *in vitro*. It has been shown to increase the phytotoxicity of phenylurea in wheat and weeds (Gaillardon *et al.*, 1985; Powles & Preston, 1995) and can be used as a synergist of most herbicides metabolised by P450 enzymes (Moreland *et al*, 1993).
ABT inhibits with slightly different efficiencies most plant P450s. Other molecules have recently been shown to inactivate plant P450s with higher selectivity. Some belong to the group of sulfur coumpounds i.e. organophosphosphate herbicides. Many insecticides have long be known to interact synergistically with herbicides to increase their phytotoxicity. Work performed with malathion, terbufos, terbufos sulfoxide and terbufos sulfone strongly suggests that such interaction may result from the autocatalytic inactivation from herbicide-metabolising P450s during desulfuration of the herbicide to the corresponding P -> O analogue (Kreuz & Fonné-Pfister, 1992; Christopher *et al.*, 1994; Diehl *et al*, 1995). The mechanism of this

inactivation was studied in the case of animal P450s, and apparently involves atomic sulfur released during the reaction which covalently binds to the P450 apoprotein. It implies that the herbicide and the insecticide need to compete for the same active site, and that the efficiency of inactivation and synergy will rely on an effective metabolism of the insecticide by a given P450.

Figure 1: The three main categories of P450 inhibitors.



2) Heterocyclic molecules with a lone electron pair on an sp_2-hybridized nitrogen tend to coordinate as sixth ligand with the heme iron, thus shifting heme absorption to 430 nm (so called "type II" ligands). This binding is accompanied by a modification of the spin and of the redox potential of the enzyme that makes its reduction by P450 reductase more difficult (Ortiz de Montellano & Reich, 1986). Some of these molecules are very strong ligands to individual P450s, like lanosterol 14α-demethylase or *ent*-kaurene oxidase, and have been exploited as fungicides or plant growth retardants (Rademacher, 1992, Van den Bossche & Jansen, 1992). A few of them, for example the plant growth retardant tetcyclacis, are commonly used to characterise P450-dependent metabolism. Tetcyclacis was shown to inhibit herbicide breakdown (Cole & Owen, 1987; Moreland & Corbin, 1991; Burton & Maness, 1992; Moreland *et al.*, 1993) and to increase herbicide toxicity *in vivo* (Powles & Preston, 1995).

3) Methylenedioxy compounds have properties of the two other kinds of inhibitors. They need to be oxidized by P450s to form carbenes which tightly coordinate as sixths ligand with heme

iron (Ortiz de Montellano & Reich, 1986). Some of these molecules are commercially employed as insecticide synergists (Casida, 1970). One of them, piperonyl butoxide (PBO) which efficiently reduces herbicide degradation (Mougin *et al.*, 1991; Burton & Manes, 1992; Moreland *et al.*, 1993), was extensively used to demonstrate P450-dependence their metabolism. PBO also enhances herbicide toxicity in crops and weeds (Gaillardon *et al.*, 1985; Burnet *et al.*, 1993).

Genetic engineering

The P450 proteins responsible for the metabolism of herbicides in crops and weeds have not yet been isolated. Data available suggest that they usually represent a very small proportion of the total P450 pool in plants. The trend is, therefore, at a direct isolation of the corresponding genes using differential screening or PCR approaches. The isolation of these genes would open the possibility to transfer herbicide tolerance to susceptible crops or to increase the resistance of already tolerant species.

Several P450 genes have already been isolated from various plants. A very few of them, have been expressed in heterologous systems to study their catalytic properties toward endogenous and exogenous molecules. A cinnamate 4-hydroxylase from *Helianthus tuberosus* (CYP73A1), enzyme whose physiological function is the synthesis of lignin, pigments and phytoalexins, has thus been expressed in yeast and shown to catalyse with low efficiency the ring-methyl hydroxylation of chlorotoluron (Pierrel *et al.*, 1994). It demonstrates that the detoxification of herbicides can be, at least in part, supported by P450s involved in physiological processes. This work also confirmed that several P450s contribute to the metabolism of chlorotoluron in plants, the major pathway for chlorotoluron detoxification in *H. tuberosus* being *N*-demethylation.
The efficiency of the hydroxylation of chlorotoluron by CYP73A1 is too low to confer herbicide resistance to a normal plant. The effect of a strong expression of the enzyme in the whole plant, however, remains to be tested. That the tranformation of P450 genes into plants can confer herbicide resistance was recently demonstrated by the expression in tobacco of both animal and bacterial P450s (O'Keefe *et al.*, 1994; Shiota *et al.*, 1994). Rat CYP1A1 was shown to confer resistance to chlorotoluron, while plant expressing CYP105A1 from *Streptomyces griseolus* activated the sulfonylurea R7402 pro-herbicide and detoxified chlorimuron ethyl 2 times faster than control plants. CYP105A1 was expressed in the whole plant, or from a tapetum-specific promoter. Treatment of immature flower buds with R7402, in the last case resulted in production of non-viable pollen, and thus could be useful as a chemical male-sterilizing agent for hybrid seed selection. Despite use of a strong promoter, the efficiency of the expression of both bacterial and animal P450s seemed relatively low. This is likely due to the need to target the CYP105A1 to the chloroplast and to coexpress CYP1A1 as a fusion protein with yeast P450 reductase to achieve efficient electron tranfer. The expression of plant microsomal P450s should be more straightforward and efficient.

CONCLUSION

It is now well established that the selectivity of several classes of herbicides, as well as the cross-resistance of many weeds biotypes to these herbicides, result from differences in P450-dependent metabolism of the active molecules between weeds and crops, or between susceptible and resistant weeds. Several P450s are clearly involved the detoxification of the different herbicides. The P450 proteins and genes responsible for this metabolism have not yet been isolated. Their characterisation is needed to elucidate the molecular mechanisms of herbicide resistance and selectivity, which may result from plant to plant differences in P450 isoforms or from differences in the regulation of these enzymes. In addition, pure P450s are required to determine their substrate specificity and help the design of specific and potent inhibitors. Availability of isoform specific inhibitors and inducers should significantly help weed management, using both existing and newly developed herbicides.

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GLUTATHIONE TRANSFERASES IN PLANTS INVOLVED IN HERBICIDE DETOXIFICATION.

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ABSTRACT

Glutathione transferases (GSTs) catalyse the detoxification of electrophilic substrates, including herbicides, by catalysing their conjugation with glutathione. Multiple isoenzymes of GST appear to be present in all plants and are all dimers with subunits of relative molecular mass in the range 23- 30 kD. This multiplicity is reflected in a remarkable species-specific portfolio of substrate specificities which allows herbicides to be detoxified by GSTs in some species but not in others. Recent significant advances in the biochemistry and molecular biology of plant GSTs and the contribution this has made to understanding their role in herbicide selectivity in plants will be reviewed, together with prospects for the future.

INTRODUCTION

Glutathione transferases, also termed glutathione *S*-transferases and commonly abbreviated to GSTs, are a ubiquitous group of enzymes catalysing the conjugation of electrophilic substrates with the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) following nucleophilic substitution or more rarely nucleophilic addition. If the substrate is sufficiently electrophilic, GSTs will act on them directly. Alternatively the co-substrate can be bioactivated, most typically by oxidation. The resulting *S*-linked glutathione derivatives are generally polar and non-toxic and in plants are actively transported into the vacuole (Gaillard *et al.*, 1994) and/or further metabolised to a complex range of peptide derivatives (Lamoureux and Rusness 1989). Many studies have unambiguously assigned a role for GSTs in the detoxification of synthetic electrophilic compounds in plants (Lamoureux and Rusness 1989, 1993). However, the activity of GSTs toward endogenous substrates and their functions in cellular metabolism are largely unknown. Herbicide substrates of plant GSTs include several chloro-*s*-triazines and chloroacetanilides, the diphenylethers fluorodifen and formesafen, the sulphoxide of EPTC and chlorimuron (Lamoureux and Rusness 1993). Rather than review all the literature concerning plant GSTs the following sections will consider recent and potential future developments using examples arising from the current work of the author and other researchers in the field.

GLUTATHIONE TRANSFERASES AND HERBICIDE SELECTIVITY

The relative rates of herbicide detoxification in plants is a major determinant of herbicide selectivity and a role for GSTs in the selectivity of atrazine within susceptible and tolerant maize cultivars and between susceptible species, such as peas, and tolerant species, such as maize, has long been established (Lamoureuex and Rusness 1993). In general the more tolerant the plant the higher the GST activity toward the herbicide substrate. Thus, maize contains GSTs which are highly active in detoxifying atrazine while peas are deficient in this activity and metabolise the herbicide by *N*-dealkylation. However, few studies have critically evaluated the role of these enzymes in the selectivity of herbicides between crops and their associated weeds. Instead, their importance has been inferred from whole plant

metabolism studies, with the tolerant crop conjugating more of the herbicide as glutathione conjugates than the susceptible weed. Although such studies are useful they normally employ doses of the herbicides well below the recommended field application rates and other factors such as uptake and bioavailability are also likely to play significant roles. Alternatively, the relative capacities of crops and weeds to conjugate herbicides with glutathione has been determined by assaying for GST activities with unrepresentative substrates such as 1-chloro-2,4-dinitrobenzene (CDNB). As will be discussed below CDNB is not an ideal substrate to base such extrapolations upon. To address this point we have recently completed a study in which relative GST activities toward the herbicides atrazine, alachlor, metolachlor and fluorodifen were determined in maize and associated weed species and correlated with the selectivity of these herbicides (Hatton *et al.*, 1995a). In addition the availability of glutathione was determined in all the species and in the case of atrazine the GST activities compared with the rates of metabolism in excised leaves. With all the herbicides an excellent correlation was obtained between relative GST activities and tolerance in maize and the weeds *Abutilon theophrasti*, *Digitaria sanguinalis*, *Echinochloa crus-galli*, *Panicum miliaceum* , *Setaria faberi* and *Sorghum bicolor*. The metabolism studies with atrazine confirmed that GST activities correlated well with the relative rates of glutathione conjugation *in planta*. In contrast glutathione availability was similar in most of the species and only appeared to influence selectivity with alachlor. Although limited by the correlative nature of the results, this study illustrates the useful information that can be gained by such comparative studies and helps confirm the crucial role of GSTs in determining relative herbicide tolerance in crops and weeds. Clearly, the next step in confirming the importance of GSTs in selectivity will be to genetically engineer a given plant species to express varying activities of GSTs active in herbicide detoxification and determine the relative tolerance to the herbicide in each transformant. Such experiments will also help distinguish between the relative importance of GST activity and glutathione availability in controlling the rates of herbicide detoxification.

REGULATION OF GSTs BY HERBICIDE SAFENERS

Several commercially important herbicide safeners act to protect cereals from subsequent injury by herbicides by increasing the detoxification capacity of the crop. In the case of herbicides metabolised by glutathione conjugation this appears to be brought about by both an increase in the levels of GSTs with activity toward the herbicide and in the size of the reduced glutathione pool (Farago *et al.*, 1994). In addition, the active transporter involved in the sequestration of the glutathione conjugates into the vacuole is also induced by safeners (Gaillard *et al.*, 1994) , suggesting that the whole glutathione detoxification system is under coordinated control. Several studies with GSTs in maize have shown that the safener-mediated increases in the activity of specific isoenzymes results from the accumulation of the corresponding mRNAs and associated increased *de novo* protein synthesis (Timmerman 1989, Jepson *et al.*, 1994, Miller *et al.*, 1994). Significantly, the cell signalling system involved in this response remains largely uncharacterised but seems to be distinct from the signalling system responsible for the induction of GSTs to biotic stress in cereals (Mauch and Dudler 1993).

The induction of GSTs by safeners is normally associated with large seeded cereal crops, such as maize and sorghum (Farago *et al.*, 1994). We have recently confirmed that safeners also induce GSTs with activities toward herbicides in a range of wheat species (see below). It is also clear that safening of GST activities can occur in legume crops though the safeners are different from those with activity in cereals (Hunaiti and Ali 1991). Recent studies in the authors laboratory have confirmed that the safeners dichlormid and fenchlorazole-ethyl had negligible effects

on the specific activity of GSTs in the grass weeds *E.crus-galli*, *D.sanguinalis*, *S.faberi* and *Setaria viridis* (Haile and Hatton, unpublished results). The fact that grass weeds do not respond to safeners in a similar manner to cereals is intriguing and suggests that in non-responsive weeds either safeners are not bioavailable, or that the safener-signalling system is impaired in some way. Interestingly, our studies did suggest that safeners could increase the glutathione content in *Setaria* species and this in turn implies that safeners have partial safening activity in some grass weeds. It will be of particular interest to compare the promoters of GST genes in responsive cereals and related non-responsive weeds to determine whether these differences in inducibility result from differences in the organization of the respective promoters. In addition the promoters from GSTs in unresponsive weeds could be coupled to reporter genes and these constructs introduced into a safener-responsive species such as maize. On treatment with safeners expression of the reporter gene would demonstrate that weeds are deficient in some aspect of their cell signalling system, while a lack of safener induction would suggest that the GST genes from weeds are unresponsive. Similar molecular techniques are now required if we are to make significant progress in determining the mode of action of safeners. As GSTs remain the best characterized safener-responsive genes it is likely that they will play a key role in unravelling the related signalling system. Approaches could include the dissection of safener-inducible GST promoters and the subsequent expression of the truncated promoters coupled to reporter genes in transient assays in safener-treated maize tissue or cell cultures. Alternatively the *cis*-elements which bind transacting factors which are responsive to safeners could be identified by DNAse footprinting or following competition assays in transformed cell cultures using methods which are now well established in the field of plant molecular biology. All of these experiments would be directed at identifying the DNA sequences in the promoter which bind the safener-responsive transacting factor. Once this DNA sequence is identified the transacting proteins may be purified and in turn used to identify the other proteins which interact with it until the signal-transduction chain is elucidated. Additional effort should also be employed in identifying mutants of maize or other cereals which are non-responsive to safeners as analysis of the mutation would provide valuable information on the safener-signalling system.

OCCURRENCE AND SUBSTRATE SPECIFICITIES OF GST ISOENZYMES

In a review in 1993 Lamoureux and Rusness reported that GST enzyme activities had been observed in 33 species of higher plants. Recent studies from the research group of Peter Schröder would suggest that this list could now be extended to include at least 20 species of lower plants including fungi, mosses and algae (Pflugmacher *et al.*, 1995). Similarly the increasing incidence of expressed DNA sequences apparently encoding GSTs in diverse plant species (Droog *et al.*, 1995) suggests that GSTs are likely to be present in all plants. However, eventhough all plants contain GSTs their capacity to detoxify electrophilic herbicides is not universal.

Multiple isoenzymes of GST

The reason for the great diversity in the capacity of plants to conjugate electrophilic xenobiotics is that GSTs exist in multiple isoenzymic forms with varying substrate specificities. In both plants and animals, all of the GSTs identified to date are composed of two protein subunits each with relative molecular masses of between 23kD and 30kD. Each subunit contains a binding domain for reduced glutathione and a binding site for the electrophilic, and often hydrophobic, co-substrate (Wilce and Parker 1994). Native enzymes may be composed of homodimers or

heterodimers. Each subunit is encoded by a distinct gene and in animals, based on their evolutionary similarity, four classes of cytosolic GSTs have been described (Lopez *et al.*, 1994). In plants, until recently there has been an inadequate data base to classify the GSTs. Based on their physical characteristics and substrate specificities some attempts have been made to characterise and classify plant GSTs along similar lines to those used in mammals (Singhal *et al.*, 1991, Lopez *et al.*, 1994). Most recently Droog *et al.*, (1995) have proposed that as determined from their DNA sequences, plant GSTs can be divided into three groupings based on their evolutionary relatedness. Following Droog's classification all of the GSTs with activities toward herbicides which have been sequenced to date would be described as type I GSTs. As increasing numbers of GST enzymes and their respective DNA sequences are isolated from plants their classification should become increasingly refined.

Soluble GST isoenzymes may be readily resolved by anion exchange chromatography, due to their differences in isoelectric points. Such chromatography may even resolve closely related mammalian GSTs which show minor differences in amino acid sequence and/or post-translational modifications such as the relative degrees of glycosylation, methylation and acetylation (Lopez *et al.*, 1994). It is less clear whether the complexity of GST isoenzymes in plants is really due to a multiplicity of gene products or whether some of the isoenzymes arise from post-translational modifications to a more restricted range of GSTs. We have been unable to demonstrate that GSTs in maize or peas are modified by methylation as has been determined in animals. However, it would seem likely that GSTs do undergo alternative post-translational modifications, such as glycosylation, and this is an area which warrants further investigation as it may have implications regarding the cellular targeting and longevity of these enzymes. One of the results of the multiplicity in the forms and substrate specificities of plant GSTs is that it is extremely difficult to characterise all of the GSTs in a plant using a single substrate. CDNB has frequently been used as a general substrate for assaying GSTs in plants, but in the case of herbicides it has been fortuitous that any of the enzymes showing activity toward this colorimetric substrate also show activity toward the substrates of interest. The limitations of using CDNB was shown in the range of GST activities which could be resolved in maize treated with the safener CGA-154281 (Dean *et al.*, 1991). Using CDNB as substrate only two GST isoenzymes could be determined in extracts from the treated maize. In contrast, an additional three isoenzymes could be resolved with atrazine as substrate and two additional isoenzymes with metolachlor as substrate. Furthermore the safener-mediated increase in the GSTs with activity toward metolachlor was much greater than that determined with CDNB while the activities toward atrazine were unaffected demonstrating that CDNB has very limited usefulness in predicting the likely regulation of other GST activities. It is therefore clear that reliable information regarding GSTs with activity toward herbicide substrates can only be obtained using the herbicides of interest, eventhough this is frequently less convenient than using CDNB.

Subcellular localisation

Another aspect of the multiplicity of GST isoenzymes which should be considered is their subcellular distribution. Although the majority of the GSTs present in mammalian cells are soluble, a significant and distinct population of the enzymes are associated with the membrane fraction (Wilce and Parker 1994). In plants the situation is less well defined, with microsomal GSTs with activity toward CDNB being reported in a wide range of lower and higher plants (Pflugmacher *et al.*, 1995). GSTs with activity toward the endogenous substrate cinnamic acid have been partially characterised in the microsomal fraction from a range of legumes (Edwards

and Dixon 1991). In contrast, there have been no definitive accounts showing that GSTs with activity toward herbicides are membrane bound in plants and we have been unable to show any significant activities toward herbicides in washed microsomes prepared from maize or *S.faberi* (Dixon and Hatton, unpublished results). However, our studies using hydrophobic interaction chromatography have shown that the soluble GSTs are among the most hydrophobic of the cytosolic proteins and it is possible that these enzymes could become associated with the membranes of organelles either normally, or artifactually during extraction. Subcellular immunolocalisation studies with antibodies to specific GST isoenzymes will address these questions and it will also be of interest to determine whether all isoenzymes are cytosolic or whether some GSTs are associated with the organelles, particularly the chloroplast, which contains high concentrations of the co-substrate glutathione. The relative compartmentation of GSTs and glutathione is a particularly relevant area of study as it is currently unclear as to whether glutathione availability could be limiting during herbicide detoxification. Determining the subcellular localisation of GSTs would also be helpful in identifying their potential for binding xenobiotics, as most GSTs have binding sites for hydrophobic ligands at domains distinct from the catalytic site. This ligand binding function may regulate the bioavailability of herbicides, such as 2,4-D, which are not GST substrates but are tightly bound by several plant GSTs (Singhal *et al.*, 1991, Droog *et al.*, 1995)

GSTs WITH ACTIVITIES TOWARDS HERBICIDES

The following examples give an up-to-date account of the GSTs with activities toward herbicides which have been characterised in plants. The recent studies of Pflugmacher *et al.*, (1995) and Hatton *et al.*, (1995a) suggest that many additional species will also be shown to have similar activities.

Maize

GSTs have been extensively studied in this species and the purification of four isoenzymes has been described (Timmerman 1989, Irzyk and Fuerst 1993, Holt *et al.*, 1995) and three DNA coding sequences elucidated (Timmerman 1989, Jepson *et al.*, 1995). At present the best estimate for the total number of GST isoenzymes with activity toward herbicides in maize is six. Four of these isoenzymes are constitutively expressed and two are only observed at significant levels in plants treated with safeners such as dichlormid. Maize contains at least two constitutively expressed isoenzymes with activity toward atrazine (Timmerman 1989). Their activity increases in response to safener treatment in some cultivars but not in others (Dean *et al.*, 1991, Dixon *et al.*, 1995). The enzyme activity is also preferentially expressed in light grown rather than etiolated leaves (Dixon *et al.*, 1995). Two isoenzymes with activity toward atrazine have been partially purified (Timmerman 1989) though the final purification and sequencing of either isoenzyme has not been reported. Maize also contains four isoenzymes with activity towards the chloroacetanilide herbicides alachlor and metolachlor which have been termed GSTs I-IV based on their order of discovery. (Timmerman 1989, Irzyk and Fuerst 1993, Holt *et al.*, 1995). GST I is a constitutive isoenzyme consisting of two identical 29 kD subunits (Timmerman 1989). When using CDNB as substrate this is the major isoenzyme determined in maize, with the specific activity of the enzyme (katals/ unit protein) being higher in the roots than in the foliage (Dixon *et al.*, 1995). The enzyme in the roots is identical to that in the shoots and accumulates to a modest degree in response to safeners in both tissues (Dixon *et al.*, 1995). This isoenzyme has low activities toward the chloroacetanilide herbicides alachlor and metolachlor and low activity towards fluorodifen (Holt *et al.*, 1995 , Dixon *et al.*, 1995). Holt *et al.*, (1995) reported that GST I had negligible activities toward atrazine, but we

have determined measurable activity with the pure isoenzyme with this substrate as suggested by earlier studies (Timmerman 1989). GST I has also been reported to be the major isoenzyme in maize catalysing the isomerisation of thiadiazolidin-one herbicides to the more active triaolidin-one-thiones (Iida *et al.*, 1994). GST II is an inducible isoenzyme which is only observed at significant levels in safener-treated roots and shoots (Dixon *et al.*, 1995, Holt *et al.*, 1995). The isoenzyme is a heterodimer of a safener-inducible 27 kD subunit and the constitutive 29 kD subunit also found in GST I (Holt *et al.*, 1995). The enzyme elutes after GST I on anion exchange chromatography columns and has much higher specific activities than GST I toward chloroacetanilides and fluorodifen but negligible activities toward atrazine and lower activities toward CDNB. These results suggest that the herbicide detoxifying capacity of this isoenzyme is attributable to the 27 kD subunit while the 29 kD subunit is largely responsible for the CDNB conjugating activity. This proposition is further confirmed by the discovery of GST IV, a homodimer of the two 27kD subunits, which elutes after GST II by anion exchange chromatography and which is highly active toward metolachlor and alachlor but has negligible activities toward CDNB and atrazine (Fuerst *et al.*, 1993, Holt *et al.*, 1995). GST IV is only determined in healthy plants following the application of safeners. The final isoenzyme, GST III, is perhaps the most intriguing of all the GSTs characterized in maize. This enzyme was first described when the cloning and expression of the respective cDNA was reported, though the enzyme was subsequently purified form maize and shown to be more active toward alachlor than metolachlor (see Timmerman 1989). Recent studies have reported that GST III coelutes with GST II by anion exchange chromatography but our results (Dixon *et al.*, 1995) and the earlier studies of Timmerman and Tu (Timmerman 1989) suggest that GST III can not be readily resolved from GST I. The full substrate specificity of this isoenzyme is currently in progress but it is known to be highly active toward chloroacetanilides (Timmerman 1989). GST III is constitutively expressed in maize, but as is the case with GST I, may also accumulate following treatment with dichlormid (Timmerman 1989).

From the above account it is clear that the molecular and biochemical characterization of GSTs is far from complete, even in this relatively well characterized species. Thus, despite over ten years of research there continue to be several major ambiguities regarding both the numbers and types of GSTs with activities toward herbicide substrates. Many of the discrepancies have probably arisen because different research groups have used differing separation systems to resolve the isoenzymes and tested them with a variety of substrates. It is also likely that the maize hybrid lines used vary considerably in their complement of GSTs (Timmerman 1989). However, it is also obvious that the unexpected genetic and biochemical complexity of the system has been a major stumbling block. Recently, this complexity has been demonstrated by the GST isoenzyme studies in inbred lines of maize which suggest that the multiple GSTs observed show high degrees of polymorphism and are controlled by at least five genes which are under complex developmental regulation (Sari-Gorla *et al.*, 1993). In addition, the GSTs which have been isolated have been inadequately characterised, particularly with respect to their substrate specificities. We are currently addressing these points by characterising the spectrum of GST activities in safener-treated maize using a wide range of herbicide and non-herbicide substrates (Dixon *et al.*, 1995). Similarly, we are also determining structure-activity relationships for individual isoenzymes, both isolated from plants and expressed in recombinant bacteria, with the intention of building up a full picture of the range of the GST activities available to detoxify pesticides in maize.

Wheat and other cereals

Detoxification of herbicides in wheat is more commonly associated with metabolism by P450 monooxygenases than by GSTs. However, several recent developments have led us to reconsider this position. Firstly, wheat has been shown to have multiple and abundant GST isoenzymes encoded by multiple genes, some of which are responsive to exposure to xenobiotics (Mauch and Dudler 1993). Secondly, the herbicide fenoxaprop-ethyl used to control grass weeds in cereals appears to owe its selectivity to rapid detoxification by glutathione conjugation in wheat (Tal *et al.*, 1993).

Following on from these initial observations we have now embarked on a full characterisation of the GSTs in wheat, at both the biochemical and molecular level. Current studies have shown that both roots and shoots of wheat seedlings contained negligible activities toward atrazine and low activities toward chloroacetanilide herbicides. However, both tissues contained appreciable GST activities toward fluorodifen and fenoxaprop, the metabolite of fenoxaprop-ethyl released after ester hydrolysis. Activities of 103 and 55 nmols of herbicide conjugated s^{-1} g^{-1} protein were determined in the roots and shoots respectively with fenoxaprop as substrate and 1.6 and 0.8 nmols s^{-1} g^{-1} respectively with fluorodifen as substrate. Fenoxaprop was readily conjugated with glutathione in the absence of enzyme, though this chemical rate was typically 3 to 5 times lower than the rate observed in the presence of the enzyme extract. In contrast, the intact ester fenoxaprop-ethyl was a poor substrate for both enzymic and non-enzymic conjugation. The wheat-safener fenchlorazole-ethyl increased the extractable GST activities toward fluorodifen and fenchlorazole 3-fold in both tissues but had no effect on the activities toward alachlor, metolachlor or atrazine. Dichlormid was also an active inducer of GSTs in wheat but naphthalic anhydride and butylate were less effective. Similar GST activities were observed in a range of other *Triticum* species of varying degrees of ploidy and genotype. However, the degree of safening of GST activities among these other species was variable with some species such as *Triticum durum* (Durum wheat), showing only slight enhancement of GST activities when treated with fenchlorazole-ethyl. Purification and characterization of the GST responsible for fluorodifen cleavage suggested that the enzyme was identical to that catalysing the detoxification of fenoxaprop.

GSTs also seem to be abundant in other cereals, notably grain sorghum (*Sorghum bicolor*). In common with maize and wheat these activities are induced by a range of safeners (Dean *et al.*, 1990). Untreated plants are reported to contain only one isoenzyme with activity toward metolachlor, while safener treatment could induce the appearance of an additional five isoenzymes which could conjugate the herbicide. The activity of GSTs in sorghum toward other herbicides was not determined. Rice is also reported to contain a GST active in conjugating the chloroacetanilide pretilachlor (Han and Hatzios 1991). In this case the safener fenclorim was unable to elevate this GST activity without the coincident treatment with the substrate pretilachlor.

Legumes and other crops

Peas were one of the first plant species shown to contain GST activities toward the herbicide fluorodifen (see Lamoureux and Rusness 1989) but few studies have exploited these early observations. This is despite the prevailing evidence which would suggest that glutathione conjugation is important in determining the detoxification, and hence selectivity, of major herbicides such as chlorimuron-ethyl, in other legumes such as soybean (Brown and Neighbors 1987). In contrast, our recent studies have shown that the GSTs in maize are unable to act on chlorimuron-ethyl and have much lower activities toward fluorodifen than the enzyme in peas (Edwards, unpublished results). We are currently characterising the GSTs in

legumes and have concentrated our initial studies on the enzymes in peas. To date, a purification scheme for the GSTs has been developed and peas shown to contain at least three different GSTs with activities toward fluorodifen, CDNB and lipid hydroperoxides each composed of subunits with relative molecular masses of 30 kD, 29 kD and 27.5 kD respectively. Interestingly, the fluorodifen cleaving activity was also high in a range of *Phaseolus* and clover species but not in alfalfa. A similar activity has been identified in spruce trees (*Picea abies* L.) and the GST responsible, which was also able to conjugate alachlor, was purified and found to be composed of 23kD subunits (Schröder and Berkau 1993).

Weeds

GSTs with activities toward herbicide substrates have only been identified in a limited number of weeds, though there is increasing interest in examining the role of these enzymes in the evolution of herbicide resistance. In particular, an atrazine-resistant biotype of *A. theophrasti* has been shown to have four-fold higher GST activities toward the herbicide than those determined in susceptible biotypes (Anderson and Gronwald 1991). Both biotypes contained identical concentrations of glutathione confirming that the more rapid detoxification of atrazine in the resistant plants was due to more highly active GSTs rather than a greater availability of the co-substrate. The resistant biotype contained elevated levels of two constitutive GST isoenzymes with activity toward atrazine which appeared similar to the isoenzymes from the susceptible plants. This suggested that resistance had arisen through a mutation involved in regulating the expression of pre-existing GSTs. Recent studies from our research group have shown that a range of weed species contain a range of activities toward herbicide substrates (Hatton *et al.*, 1995a) suggesting the potential for similar mutations arising in other weeds. In addition to *A.theophrasti*, GSTs active in detoxifying atrazine have been implicated in the tolerance of *Panicum miliaceum* and *Setaria faberi* to this herbicide (Ezra and Stephenson 1985, Lamoureux and Rusness 1989). Most of the studies have concentrated on *S.faberi* which contains several isoenzymes capable of conjugating herbicides (Hatton *et al.*, 1995b). However, recent studies have suggested that the role of GSTs in selectivity between the weed and maize are dependant on the relative developmental stage of the two species (Hatton, unpublished results). Studies emphasising the differences in the spectrum of GST activities between crops and weeds may be very useful in developing; i) new herbicides which are selectively conjugated in the crop, but not in the weed, and ii) synergists, such as tridiphane (Lamoureux and Rusness 1993), which specifically inhibit the GSTs in the weed.

FUTURE PROSPECTS

The recent observations regarding the involvement of GSTs in the responses of plants to infection, senescence, hormones and development (Droog *et al.*, 1995) have ensured that these enzymes have become the focus of attention of many research groups around the world. Such studies are concentrating on the respective genes and the supporting biochemistry is now required to identify the functions of GSTs in endogenous metabolism. Identifying the natural substrates of GSTs will in turn provide new insights on the potential activities of these enzymes toward herbicides. With regard to herbicide research, the full spectrum of GST activities in both crops and competing weeds should now be determined and their role in selectivity assessed. GSTs will also provide a valuable molecular tool in unpicking the mode of action of safeners. Finally, the structure activity studies being carried out on GSTs from animals (Wilce and Parker 1994) show the future potential of this area in herbicide development. In particular the definition of active sites following X-Ray crystallography will greatly assist in the design of new selective herbicides

directed to specific GSTs. As an alternative approach, it may be possible to evolve GST activities toward herbicides using the methods of directed mutagenesis and forced molecular evolution. The "designer" GSTs could then be genetically introduced into crops of interest with the intention of introducing a new mechanism for selectivity.

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INHIBITION OF ACETOHYDOXY ACID ISOMEROREDUCTASE BY REACTION INTERMEDIATE ANALOGUES: HERBICIDAL EFFECT, KINETIC ANALYSIS AND 3-D STRUCTURAL STUDIES

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ABSTRACT

N-hydroxy-*N*-isopropyloxamate (IpOHA), is a very potent and selective inhibitor of acetohydroxy acid isomeroreductase (KARI), behaving as a reaction intermediate analogue of the enzyme catalysed reaction. Although IpOHA has been described as a far more potent inhibitor than any of the herbicides that inhibit acetohydroxy acid synthase (ALS), the previous enzyme in the branched-chain amino acid pathway, this compound only presents very poor herbicidal action with application rates that are about a hundred fold higher than those used for ALS inhibitors. Here, we try to identify the reasons for the apparent ineffectiveness of IpOHA. We also evaluate the potential of KARI as an herbicide target. To this end, we review our results on the kinetic mechanism of a plant KARI. Finally, to better understand the molecular basis of the inhibition process, structural 3-D determination was performed on the plant enzyme co-crystallized with IpOHA.

INTRODUCTION

Today, the enzymes of the branched-chain amino acid pathway have not been yet thoroughly studied in plants. Although acetohydroxy acid synthase (ALS), the first enzyme of the pathway, is the target of several herbicides, effective at low dose rate, the active enzyme was never purified to homogeneity from plant. Surprisingly, most of the biochemical research was carried out on a model enzyme as isolated from *Escherichia coli*. However, even though the branched-chain amino acid pathway seems to be similar in plants and micro-organisms, several million years of evolution may have led to differences between corresponding enzymes from plant and micro-organism enzymes, notably at the level of their structure, regulation and affinity for substrates or inhibitors. Therefore, it may be hazardous to extrapolate to a plant enzyme the inhibition properties of a compound that have been solely determined for a bacterial enzyme.

In order to improve our knowledge of the plant biosynthetic pathway and to raise the question of whether the enzymes following ALS are suitable as herbicide targets, acetohydroxy acid isomeroreductase (KARI) has been extensively studied. KARI, the second common enzyme in this biosynthetic pathway, catalyses an alkyl migration in which the substrate, either (2*S*) 2-acetolactate (AL) (ultimate product, valine and leucine) or (2*S*) 2-aceto-2-hydroxybutyrate (AHB) (ultimate product, isoleucine), is converted to 3-hydroxy-3-methyl-2-oxobutyrate or 3-hydroxy-3-methyl-2-oxopentanoate, respectively, followed by a NADPH-dependent reduction to give (2*R*) 2,3-dihydroxy-3-isovalerate or (2*R*, 3*R*) 2,3-dihydroxy-3-methylvalerate, respectively (Fig. 1). The enzyme catalyzed reaction obeys an ordered mechanism in which NADPH and magnesium bind first and independently, followed by acetohydroxy acid substrate binding (Chunduru *et al.*, 1990; Dumas *et al.*, 1992).

In 1988, Schulz *et al.* showed for the first time that inhibition of KARI by 2-dimethylphosphinoyl-2-hydroxy acetic acid (HOE 704) (Fig. 1) leads to plant death. Working with the *E. coli* enzyme, these authors demonstrated that inhibition by HOE 704 was time-dependent and competitive with the enzyme's substrate (K_i value of 0.82 µM). They observed that plants treated with this compound accumulate massive amounts of 2-acetolactate and acetoin, the decarboxylation product of 2-acetolactate. This increase in substrate concentration follows immediately the addition of inhibitor: thus, after respectively 1, 2 and 4 hours incubation of *Lemna gibba* with 20 µM HOE 704, the concentrations of 2-acetolactate and acetoin were increased by 1.5, 3.6 and 8.2 fold. Furthermore, experiments carried out on 6-week-old corn, showed that 2 weeks after treatment with HOE 704 at a dose equivalent to 1 $kg.ha^{-1}$, the concentration of substrate was dramatically increased by a factor of 1000.

Two years later, Aulabaugh & Schloss (1990) discovered an extremely potent inhibitor of KARI, *N*-hydroxy-*N*-isopropyloxamate (IpOHA) (Fig. 1) which also behaves as a competitive inhibitor with the enzyme's substrate. In addition, Wittenbach *et al.* (1990) showed, in agreement with the results of Schulz *et al.*(1988), that inhibition of black mexican sweet corn cells by IpOHA leads also to a dramatic built up in the level of substrates. IpOHA was found to bind slowly to the *E.coli* enzyme (association rate, $k_0 = 5.9\ 10^4 M^{-1}$) and to dissociate extremely slowly with an half-time for release of 6 days (dissociation rate, $k_{-0} = 1.3\ 10^{-6} s^{-1}$), thus defining a final K_i (k_{-0}/k_0) of 22 pM. Obviously, the complex formed is nearly irreversible and therefore, strictly on the basis of the overall affinity, the affinity of KARI for IpOHA appears much higher (greater than 1000-fold) than any of the inhibitors of ALS (which are considered as reversible inhibitors). In spite of these results, IpOHA behaves as a poor herbicide since its rate of application was approximatively 100-fold higher than those for inhibitors of ALS. This behaviour does not result from a failure of IpOHA to reach its target site, since this compound translocates readily within plants and penetrates plastids where KARI is localized (Wittenbach *et al.*,1990). From these observations, Wittenbach *et al.* (1990) and Schloss & Aulabaugh (1990) concluded that, although inihibition of KARI can be herbicidal, it is not likely to be as effective as inhibition of ALS. This led agrochemical companies to believe that KARI is a poor herbicide target.

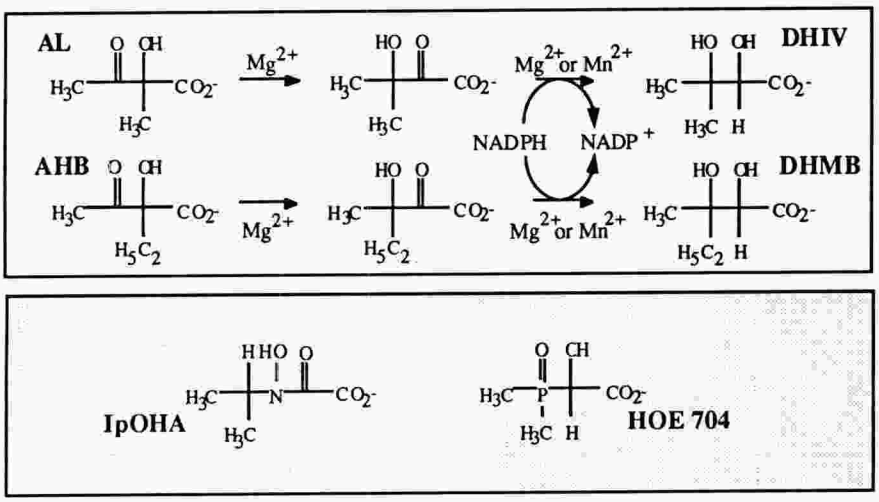

Figure 1: Reactions catalysed by KARI and the structure of IpOHA and HOE 704. Abbreviations: AL, 2-acetolactate; AHB: 2-aceto-2-hydroxy butyrate; DHIV, 2,3-dihydroxy-3-isovalerate; DHMV, 2,3-dihydroxy-3-methylbutyrate.

In order to determine whether the plant enzyme is inhibited by the same mechanism and to the same extent by IpOHA and HOE 704 than the *E. coli* enzyme and to evaluate the influence of parameters such as slow-binding and competitivity in relation to the accumulation of substrate in the treated plants, we carried out biochemical and molecular studies on the true herbicidal target ie the plant enzyme. KARI has been purified to homogeneity from the stroma of spinach (*Spinacia oleracea*) chloroplasts (Dumas *et al.*, 1989) and from barley (*Hordeum vulgare*) (Durner *et al.*, 1993). It is a homodimer of molecular mass 114,000 Da that contains one NADPH-binding site (Dumas *et al.*, 1991) and two magnesium binding sites (Dumas *et al.*, 1995) per monomeric unit. Magnesium is of particular importance for KARI as it is essential for the two-step reaction, ie the isomerization followed by the NADPH-dependent reduction (Fig. 1). It is also essential for the binding of IpOHA or HOE 704 to KARI. Interestingly, the plant enzyme exhibits a much higher affinity for Mg^{2+} (K_m = 6 µM) (Dumas *et al.*, 1992) than its bacterial counterpart (K_m = 450 µM) (Chunduru *et al.*, 1988). The primary structure of KARI has been determined from spinach (Dumas *et al.*, 1991) and *Arabidopsis thaliana* cDNA (Curien *et al.*, 1993) and its gene sequenced from *A. thaliana* (Dumas *et al.*, 1993). The cDNA encoding the mature polypeptide sequence from spinach was further used to overexpress the enzyme in *E. coli* (Dumas *et al.*, 1992). Recently (Dumas *et al.*, 1994b), we have crystallized the overexpressed enzyme as a complex with NADPH, magnesium and either IpOHA or HOE 704 and solved the structure of the complex formed with IpOHA at a resolution of 2.4 Å (Biou *et al.*, in preparation).

INHIBITION STUDIES OF THE PLANT KARI

Herbicidal effects: HOE 704 is a 10-fold stronger herbicide than IpOHA

As respectively described by Schulz *et al.* (1988) and Wittenbach *et al.* (1990), HOE 704 and IpOHA lead to a rapid and complete arrest of growth with symptoms similar to those observed with the inhibitors of ALS. Most importantly, herbicidal effects can be alleviated by addition of the three branched-chain amino acids in the growth medium. These observations essentially confirm that inhibition of KARI is lethal in plants. They are in good agreement with molecular experiments which demonstrate that inactivation of the KARI gene by disruption is also lethal in *S.cereviasiae* (M. Lebrun, pers. comm.) and bacteria (Inui *et al.*, 1993). In order to quantify the respective herbicidal activity of HOE 704 and IpOHA, three plants, *Solanum nigrum*, *Ipomea purpurea* and *Lemna gibba* were grown in the presence of various concentrations of these inhibitors. As shown in Table 1 and Figure 2, these two compounds have very different herbicidal activity since the concentration of HOE 704 needed to achieve growth inhibition is about respectively 12, 15 and 10 times lower than that of IpOHA, for *S. nigrum*, *I. purpurea* and *L. gibba* respectively.

Table 1: Growth inhibition of *Solanum nigrum*, *Ipomea purpurea* and *Lemna gibba* by IpOHA and HOE 704. The values correspond to the amount of inhibitor added in the medium (agar medium for *S. nigrum* and *I. purpurea*, liquid medium for *L. gibba*) to achieve growth inhibition.

	I. purpurea	*S. nigrum*	*L. gibba*
IpOHA	30 $mg.l^{-1}$	60 $mg.l^{-1}$	15 $mg.l^{-1}$
HOE 704	2 $mg.l^{-1}$	5 $mg.l^{-1}$	1.5 $mg.l^{-1}$

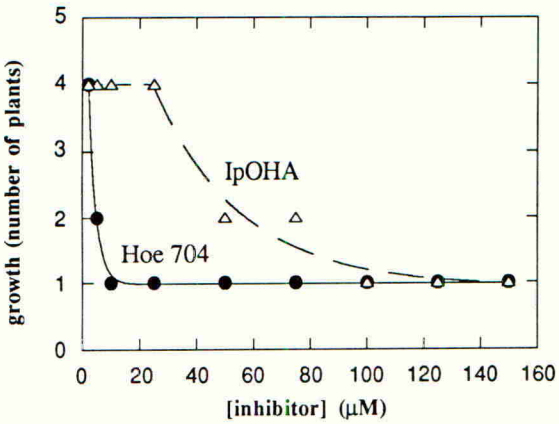

Figure 2: Growth inhibition of *Lemna gibba* by IpOHA and HOE 704. *L. gibba* were grown in Erlen meyer containing 50 ml of nutrient medium and various amounts of HOE 704 or IpOHA. *L. gibba* growth was measured 3 days after addition of the inhibitor.

HOE 704 and IpOHA behave as tight-binding inhibitor of the plant enzyme

In the presence of magnesium and NADPH, IpOHA and HOE 704 behave as tight-binding inhibitors of the plant enzyme (Dumas *et al.*, 1994a). In agreement with the results of Schloss & Aulabaugh (1990), stoichiometric binding occurs between IpOHA and each monomer of the enzyme. However, unlike the *E. coli* enzyme (Schulz *et al.*, 1988), our results indicate that the two enantiomers of HOE 704 (which is a racemic compound) act as inhibitors of the plant enzyme, and bind stoichiometrically to each monomer of the plant enzyme.

HOE 704 and IpOHA behave as competitive inhibitors of the plant enzyme

The mechanism of inhibition was determined by investigating the effect of substrate and inhibitor concentrations on the pseudo first order rate constant for inhibition k_{obs}, under experimental conditions that should prevail *in vivo*, ie in the simultaneous presence of substrate and inhibitor (Tian & Tsou, 1982) (Fig. 3). This analysis showed that IpOHA and HOE 704 behave as competitive inhibitors with respect to acetohydroxy acid substrates (plots of $1/k_{obs}$ versus [S] were linear), as reported (Schulz *et al.*, 1988; Aulabaugh & Schloss, 1990; Hawkes & Edwards, 1990; Durner *et al.*, 1993).

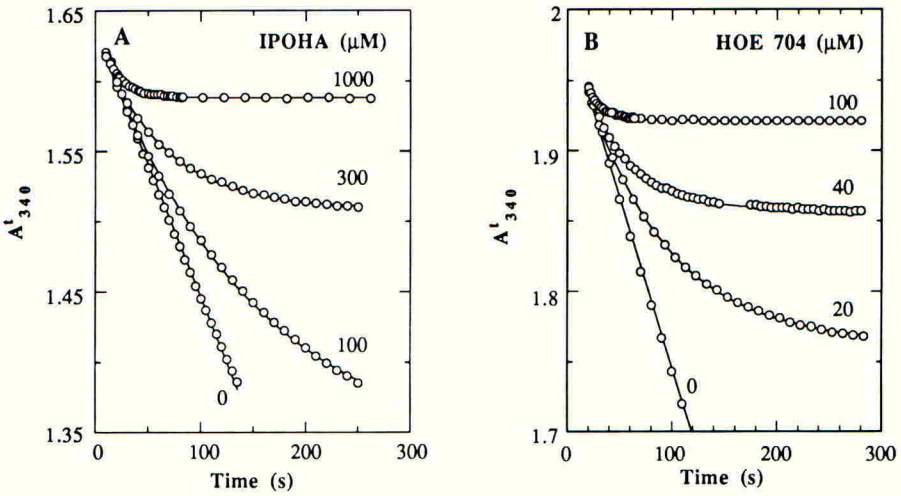

Figure 3: Time course inhibition of spinach KARI in the presence of IpOHA and HOE 704. Reactions were initiated by adding simultaneously 2-aceto-2-hydroxybutyrate (0.6 mM) and various concentrations of IpOHA (A) or HOE 704 (B) designed in µM in the figures in 1 ml of 50 mM Tris/HCl (pH 8.2), containing 3 mM $MgCl_2$, 0.25 mM NADPH, and 61 nM enzyme. In the absence of inhibitor (0) the data were analyzed by linear regression. For the experiments conducted in the presence of inhibitors, the continuous lines were obtained by nonlinear least-square analysis of the data to:

$$A^t_{340} = A^{\infty}_{340} + \left(A^0_{340} - A^{\infty}_{340}\right).e^{-k_{obs}.t}, \text{ equation 1,}$$

where A^t_{340}, A^0_{340}, A^{∞}_{340} are respectively the absorbance at 340 nm at time t, at time zero, and at time approaching infinity. For competitive inhibitors that bind in a single step to the enzyme, the apparent rate constant k_{obs} is related by eq 2 to the association rate constant k_o, the dissociation rate constant k_{-o}, the inhibitor concentration, the substrate concentration, and the apparent K_m of enzyme for substrate, as follows (Liu & Tsou, 1986):

$$k_{obs} = \frac{k_o.[I]}{\left(1 + \frac{[S]}{K^S_m}\right)} + k_{-0}, \text{ equation 2,}$$

Since IpOHA and HOE 704 behave as nearly irreversible inhibitors, k_{-o} is small and can be neglected in the latest equation. (From Dumas *et al.*, 1994a).

HOE 704 and IpOHA bind extremely slowly to the plant enzyme

As shown in Figure 3, the time to reach full inhibition of enzyme activity was dependent on the inhibitor concentration, and at a given inhibitor concentration, the inhibition developed far more slowly in the presence of IpOHA than in that of HOE 704. We have determined (from the eq. of k_{obs} in Figure 3) that HOE 704 (k_o = 2.2 10^4 M^{-1} s^{-1}) binds to the plant enzyme 11-fold faster than IpOHA (k_o = 1.9 10^3 M^{-1} s^{-1}). This result has three important consequences. Firstly, these rate values correspond to very slow association rate constants and thus the time needed to reach full inhibition of enzyme activity at given substrate and inhibitor concentrations can be extremely high (see later in Fig. 4). Secondly, HOE 704 binds faster than IpOHA to the enzyme, which may be related to their respective herbicidal potency (Table 1, Fig. 2). Thirdly, IpOHA binds considerably faster (31-fold) to the *E. coli* enzyme (k_o = 5.9 10^4 M^{-1} s^{-1}; Aulabaugh & Schloss, 1990) than to the plant enzyme. This would help to explain the observation that contrarily to plants, the growth of *E. coli* cells is extremely sensitive to inhibition by this compound (Aulabaugh & Schloss, 1990).

HOE 704 dissociates readily from the plant enzyme releasing KARI in a half-inactive form

Interestingly, the herbicidal potency of IpOHA and HOE 704 was not correlated with the ease of recovery of enzyme activity (experimented by substrate challenge) from the inhibited enzyme complexes, since for inhibition by IpOHA it has not been possible to recover any enzyme activity, even after several days of incubation with an excess of substrate. On the other hand, for HOE 704 about 50% of the initial enzyme activity was recovered in a few minutes (prolonged incubation did not increase the level of recovery of enzyme activity). For the off rate (k_{-o} = 1.6 $10^{-3}s^{-1}$) and on rate previously determined for HOE 704 (k_o = 2.2 10^4 M^{-1} s^{-1}), one can calculate a K_i of 70 nM. Higher values were reported for inhibition of the *S. cerevisiae* (K_i of 300 nM; Hawkes & Edwards, 1990) and the *E. coli* (820 nM; Schulz *et al.*, 1988) enzyme by HOE 704. Strickingly, after HOE 704 dissociates from the enzyme, the KARI activity was not fully restored, as if it behaved as an hysteretic enzyme (Neet & Ainslie, 1980). Such a behaviour was observed after inhibition of ALS by chorsulfuron or imazaquim (Durner *et al.*, 1991). As Hawkes (1993) has pointed out this peculiar behaviour might be important to account for the potency of a good inhibitor, because once dissociated from the enzyme, such compounds release the enzyme in an inactive state and they can be reused to inactivate new enzyme molecules.

Physiological implications of the slow binding and competitive inhihibition of HOE 704 and IpOHA

In planta, the inhibition of KARI has been shown to lead quickly to a dramatic buildup of the substrate concentration (Schulz *et al.*, 1988; Wittenbach *et al.*, 1990). Indeed, a 1000-fold increase in the substrate concentration has been reported in plant cells. In these conditions, slow-binding competitive inhibitors such as IpOHA and HOE 704 bind to the enzyme very slowly (see Fig. 4). For example, from the observed association rate constant (k_{obs}), one can calculate that at 1 µM IpOHA, the time needed to reach 90% of inhibition of activity ($t_{0.9}$) varies from 42 to 1680 min over a substrate concentration increase from 10 µM to 1 mM (Fig. 4A). Figure 4B shows that $t_{0.9}$ with HOE 704 is smaller than that for IpOHA, because of its higher k_o value. In this case, at 10 µM substrate and 1 µM HOE 704, $t_{0.9}$ is only 3.6 min. These calculations suggest that, unless a very high inhibitor concentration is used, a substantial proportion of the acetohydroxy acid isomeroreductase activity would escape *in vivo* to inhibition by IpOHA and HOE 704. This corresponds to the high application rates for herbicidal activity.

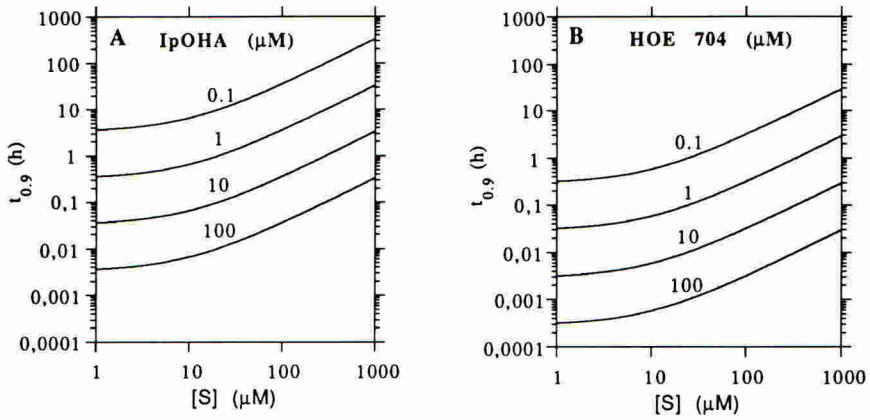

Figure 4: The calculated time to reach 90% inhibition of spinach KARI in the presence of IpOHA (A) or HOE 704 (B). The time to reach 90% inhibition of the enzyme activity ($t_{0.9}$) in the presence of various concentrations of inhibitor (designed in µM in the figure) was calculated as a function of acetohydroxy acid substrate concentration, assuming that the enzyme is assayed in the simultaneous presence of inhibitor and an acetohydroxyacid substrate (see Fig. 3). Continuous lines are computer simulations of the eq: $t_{0.9} = 2.3 / k_{obs}$. The values of the rate constants for association of IpOHA and HOE 704 with the enzyme-NADPH-Mg^{2+} complex were fixed at 1940 M^{-1} s^{-1} and 2.2 10^4 M^{-1} s^{-1}, that of the apparent K_m of enzyme for the *S*-enantiomer of acetohydroxy acid substrates at 10 µM (Dumas *et al.*, 1991, 1992, 1994a).

Which inhibitors for KARI?

The present results support the view that a slow rate of association delays the time required for inhibition at a given concentration *in vivo*. The later feature is of particular importance for KARI and the slow-binding competitive inhibitor compounds such as IpOHA and HOE 704 because the apparent K_m of the plant enzyme for its substrates is quite low, in the order of 10 µM (Dumas *et al*, 1992). Presumably, if the rate constants for association of compounds like IpOHA and HOE 704 with the plant enzyme were near those expected for a diffusion-controlled encounter of enzyme and inhibitor [10^8-10^9 M^{-1} s^{-1}; (Fersht, 1985; Job *et al.*, 1993)], then these compounds would exhibit much better herbicidal activity than experimentally observed. Note that it might be possible to increase the rate of ligand binding within the catalytic pocket by several orders of magnitude since the estimate association rate constant (k_{cat}/K_m) of spinach KARI for acetohydroxy acid substrates is of the order of 10^6 M^{-1} s^{-1} (Dumas *et al.*, 1995).

In a similar way, 1-hydroxy-2-nitrocyclopentane-1-carboxylic acid (Hawkes *et al.*, 1993) and *O*-isobutenyl oxalylhydroxamate (*O*-ibOHA) (Wittenbach *et al.*, 1992), behave as potent inhibitors of isopropylmalate isomerase and isopropylmalate dehydrogenase respectively and lead to plant death. Possibly, the low activity of these compounds (0.4 $kg.ha^{-1}$) can also be explained by the fact that they both act as slow-binding competitive inhibitors.

It must be stressed that the inhibitors of ALS also belong to the class of slow-binding inhibitors. However, in this case, these compounds are not competitive with the enzyme's substrates, but

act as "extraneous" site inhibitors (Wittenbach *et al.*, 1990). Then, contrarily to the reaction intermediate analogues of KARI, the rate of inhibitor binding to ALS is independent of substrate concentration.

In order to discover new inhibitors of KARI, such tight-binding competitive inhibitors with faster association rates than that of HOE 704 or new non- or un-competitive inhibitors, we are carrying out *in vitro* screening associated to drug-design studies on the spinach enzyme.

Structural inhibitor-enzyme interactions studies

Drug-design strategies require structural information on the target enzyme. We have therefore crystallized the spinach enzyme as a complex with NADPH, magnesium and either IpOHA or HOE 704 (Dumas *et al.*, 1994b). The structure of the complex formed with IpOHA was solved at a resolution of 2.4 Å (Biou et *al.*, in preparation). This determination discloses that each monomer of KARI is composed of two structural distinct domains: an amino-terminal domain which interacts with the NADPH and a carboxy-terminal domain almost entirely composed of α-helices which interact with two magnesium atoms (Fig. 5). The interface of these two domains forms a pocket in which the inhibitor binds, interacting with both magnesium atoms, in close proximity of the exchangeable proton of NADPH (Fig. 5).

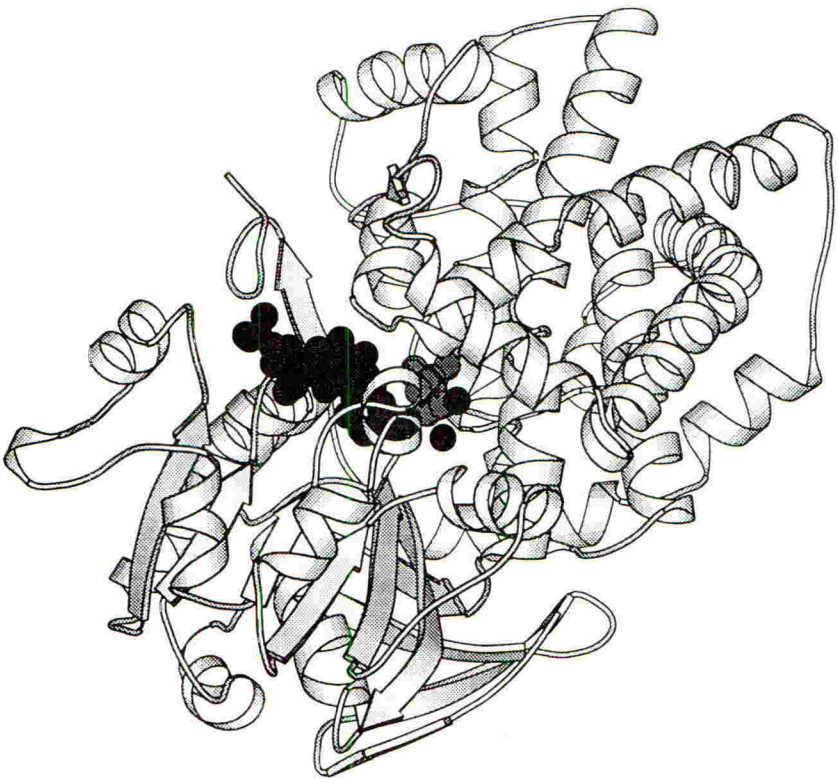

Figure 5: Model of the structure of the spinach enzyme cocrystallized with NADPH (dark grey), IpOHA (light grey) and the two magnesium atoms (black).

CONCLUSION

IpOHA and HOE 704 behave as tight-binding inhibitors of the plant enzyme. Also, they are competitive with the enzyme's substrates and slow binding inhibitors. Despite the extremely high overall affinity of IpOHA, this later compound binds 11-fold slower than HOE 704 to the plant enzyme. Since the amount of IpOHA needed to give an herbicidal effect is about 10-to 15-fold higher than that for HOE 704, it appears that the herbicidal potency of these compounds is not related to their overall affinity ($K_i = k_{-0}/k_0$), but rather to their rate of association (k_0) with the plant enzyme. Furthermore, it is known that inhibition of the plant enzyme by these compounds leads to a dramatic build up of the substrate concentration in leaves (Schulz *et al.*, 1988; Wittenbach *et al.*, 1990). In these conditions, slow-binding competitive inhibitors such as IpOHA and HOE 704, bind to the enzyme considerably slower as the substrate concentration increases. Thus, what seems important is not so the fact that these compounds display an exceptional overall affinity for the enzyme, but that the time needed for the system to attain full inhibition may become excessively long. Under such conditions, the degree of enzyme inhibition that can be obtained at a given inhibitor concentration is not dependent on the concentration of the target enzyme, but instead is a function of enzyme substrate concentration. We propose that this would account for the observation that herbicidal activity is only obtained at high dose rates for HOE 704 and IpOHA.

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EVIDENCE FOR CROSS-PATHWAY REGULATION OF METABOLIC GENE EXPRESSION IN PLANTS

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ABSTRACT

Blocking histidine biosynthesis in *Arabidopsis thaliana* using a specific inhibitor of imidazoleglycerol phosphate dehydratase resulted in increased expression of eight genes from other biosynthetic pathways. In many cases this was accompanied by an increase in the free pools of end products of these pathways. Under the same conditions one gene was repressed, while two additional genes show little or no change in expression. The addition of histidine eliminated changes in gene expression indicating the effects were a result of histidine starvation. These results suggest plants are capable of cross pathway regulation similar to that observed in *Saccharomyces cerevisiae*.

INTRODUCTION

Coordinate regulation of diverse metabolic pathways exists in both prokaryotes and eukaryotes (Gottesman, 1984). In yeast and many other fungi, starvation for a single amino acid leads to derepression of enzymes in many unrelated amino acid biosynthetic pathways (Carsiotis and Lacy, 1965; Carsiotis *et al*, 1974; Wolfner *et al*, 1975; Ebbole *et al*, 1991) Regulation of this phenomenon has been shown to act at the level of transcription (Hinnebusch and Fink, 1983). In *Saccharomyces cerevisiae* this cross pathway regulation, known as general control, affects at least 35 genes encoding enzymes in 12 biosynthetic pathways including aromatic amino acids, branched chain amino acids, lysine, threonine, methionine, glutamine, histidine, arginine, and amino acyl-tRNA synthetases (Hinnebusch, 1992).

In plants, only one report of gene expression following pathway inhibition has appeared in the literature. Blocking the synthesis of aromatic amino acids with glyphosate caused a several-fold increase in activity of 2-keto-3-arabino-heptulosonate 7-phosphate synthase (DHS), the committed step in the shikimate pathway (Pinto *et al*, 1988). Here we show that inhibiting a specific step in one metabolic pathway causes changes in gene expression in several unrelated pathways (Guyer *et al*, 1995).

MATERIALS AND METHODS

Arabidopsis seeds (Ecotype Columbia) were germinated on GM medium (4.3

g/litre MS salts, 0.5 g/litre MES, 1% sucrose, 10 µg/litre thiamine, 5 µg/litre pyridoxine, 5 µg/litre nicotinic acid, 1 mg/litre *myo*-inositol; pH 5.8) containing 8 g/litre agar and transferred to flasks containing liquid GM medium 1 week after germination (5 seedlings/250 ml flask containing 50 ml of medium). Flasks were agitated (100 rpm) at 20 °C in 16 h light (approximately 100 µE m^{-2} s^{-1}), 8 h dark. After 1 week of growth in liquid, inhibitors or supplements were added at the following final concentrations: IRL 1803, 30 ppm (130 µM); glyphosate (Crescent Chemical Co., Hauppauge, NY), 120 ppm (712 µM); primisulfuron (Ciba-Geigy Corp., Greensboro, NC), 10 ppb (21 nM); acifluorfen (Crescent Chemical Co.), 100 nM; histidine, 1 mM. Concentrations of herbicides were chosen that strongly inhibited seedling growth.

Free amino acids were extracted from samples of equal fresh weight (100 mg) in water/$CHCl_3$/methanol as described (Shaul and Galili, 1992). A known amount of the amino acid analog norleucine was spiked into each tissue sample before extraction to correct for recovery. HPLC separation and quantitation was performed after derivitization with phenylisothiocyanate using an Applied Biosystems amino acid analyzer.

Tissue was harvested by freezing in liquid N_2 at the times indicated. RNA preparations and gel blot hybridizations were carried out as described (Ward *et al*, 1991). Each gel lane contained 10 µg total RNA; equal gel loading was confirmed by staining with ethidium bromide incorporated into each sample at gel loading. Hybridization signals were quantitated using a Betascope 603 blot analyzer (Betagen Corp., Waltham, MA).

Using primers directed to known cDNA or genomic sequence, we used the polymerase chain reaction (PCR) to generate nucleic acid probes for the following genes; 5'-phosphoribosyl-5-aminoimidazole synthetase (AIRS) (Senecoff and Meagher, 1993); anthranilate synthase ß subunit (ASB) (Niyogi *et al*, 1993); 2-keto-3-arabino-heptulosonate 7-phosphate synthase (DHS) (Keith *et al*, 1991); enolpyruvyl-shikimate phosphate synthase (EPSPS) (Klee *et al*, 1987); glutamine synthetase (GS) (Peterman and Goodman, 1991) and phosphoribosylanthranilate transferase (PAT) (Rose *et al*, 1992). Full length or partial cDNAs were obtained and used as probes for the following genes: acetohydroxyacid synthase (AHAS) (Mazur *et al*, 1987); chorismate mutase (CM) (Eberhard *et al*, 1993); histidinol dehydrogenase (HDH) (Nagai *et al*, 1991) and imidazoleglycerol phosphate dehydratase (IGPD) (Tada *et al*, 1994).

RESULTS AND DISCUSSION

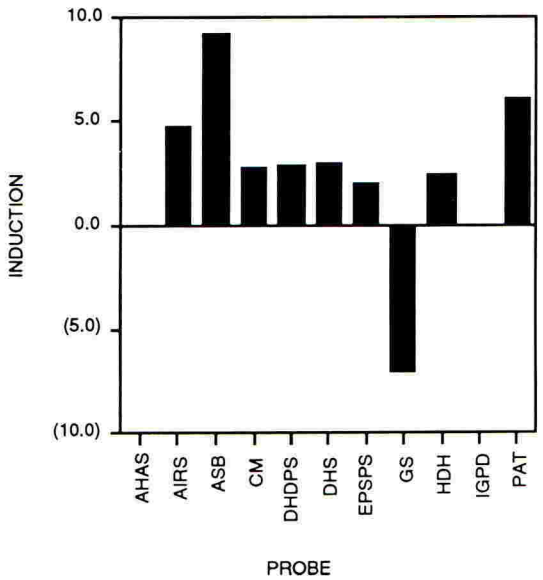
Recently, inhibitors of histidine biosynthesis have been described that have herbicidal activity (Hawkes *et al*, 1993; Mori et al, 1995). IRL 1803 is a potent inhibitor of imidazoleglycerol phosphate dehydratase with an IC_{50} value of 40 nm (Mori *et al*, 1995). In cell cultures, IRL 1803 strongly inhibits growth in a manner that can be specifically reversed by the addition of histidine (Mori *et al*, 1995). Growth inhibition could not be reversed by the addition of aromatic amino acids,

branched chain amino acids or the aspartate family (Mori *et al*, 1995). In addition, treatment of *Xanthium* seedlings with IRL 1803 resulted in a reduction of free histidine (Mori *et al*, 1995).

We tested the effects of IRL 1803 on free amino acids in *Arabidopsis thaliana*. Two week old seedlings transferred to liquid culture with 30 ppm of IRL 1803 had undetectable levels of free histidine within 60 h. Amino acid analysis also revealed the depletion of histidine was accompanied by a 1.5- to 2-fold increase of free pools of some but not all amino acids (alanine, aspartate, glutamate, phenylalanine, proline, threonine, tryptophan, tyrosine and valine).

General amino acid control in *Saccharomyces cerevisiae* was first observed using 3-amino-1,2,4-triazole, a specific inhibitor of IGPD in microbes (Hilton *et al*, 1965). To see if a similar response exists in plants, the expression of genes involved in the biosynthesis of histidine and other amino acids was examined in *Arabidopsis* treated with IRL 1803. As analyzed by RNA gel blot, an increase in steady state levels of HDH RNA was observed 24 h after exposure to IRL 1803. Gene specific probes to IGPD revealed a slight increase in mRNA levels in only one of the two genes. Thus, at least some genes in the histidine biosynthetic pathway can increase their expression in response to starvation for the end product of the pathway.

Figure 1. Alteration of gene expression by IRL 1803. Gel blots of total RNA from plants treated as indicated, hybridized to nucleic acid probes corresponding to described genes. Samples were harvested 60 h after treatment. Fold-induction (or repression) relative to controls is shown.



The steady state levels of RNA from the following genes were analyzed after IRL 1803 treatment (Figure 1): AHAS, the committed step in branched-chain amino acid biosynthesis; AIRS, the imidazole ring-closing step in *de novo* purine biosynthesis; ASB, the committed step in tryptophan biosynthesis; CM, the committed step in tyrosine and phenylalanine biosynthesis; DHPS, the committed step in lysine biosynthesis; DHS; EPSPS, the target for glyphosate in aromatic amino acid biosynthesis; GS, a key enzyme in nitrogen assimilation; HDH; IGPD; PAT, the second step in tryptophan biosynthesis. In addition to HDH, the genes encoding AIRS, ASB, CM, DHPS, DHS, EPSPS, and PAT were significantly induced by treatment with IRL 1803. Expression of AHAS and IGPD showed little change, while GS was markedly repressed. Of these pathways, only purine biosynthesis shares a known metabolic link with histidine biosynthesis (Winkler, 1987).

To ensure the changes in gene expression were a direct result of histidine starvation, plants were treated with a mixture of IRL 1803 and 1 mM histidine. In all cases, histidine completely reversed the effects of the IGPD inhibitor alone (data not shown).

To investigate whether starvation for other amino acids had similar effects, *Arabidopsis* plants were treated with glyphosate or primisulfuron, herbicides that block aromatic and branched chain amino acid biosynthesis respectively (Steinrücken & Amrhein, 1980; Maurer et al, 1987). As with IRL 1803, ASB and PAT were significantly induced following treatment with either compound. GS mRNA was clearly decreased in both treatments but to a lesser extent than in histidine-starved tissue. Other biosynthetic genes for which expression was markedly altered by IRL 1803 treatment showed little response to either glyphosate or primisulfuron (data not shown).

In addition, plants were treated with the photobleaching herbicide acifluorfen, which inhibits heme and chlorophyll biosynthesis but does not directly affect amino acid or purine metabolism (Matringe *et al*, 1989). Over a 60 h time course, during which the tissue became chlorotic and failed to grow, none of the genes examined were induced. Either a constant mRNA level or a slight decrease was seen in each case (data not shown).

The changes in gene expression associated with blocking histidine biosynthesis in plants may represent a mechanism similar to general control in yeast that can sense starvation of many amino acids. Supporting evidence of this is seen with specific inhibitors of leucine biosynthesis. Pea roots treated with inhibitors of isopropylmalate dehydrogenase were shown to have increased pools of amino acids unrelated to the branched chain pathway. (Wittenbach *et al.* 1994). Plants do, however, appear to be more sensitive to the levels of histidine than the other amino acids examined, which may be related to the high energy requirements of its biosynthesis. Indeed, bacterial mutants resistant to histidine feedback inhibition behave essentially as adenine auxotrophs (Johnston and Roth, 1979). Thus, dramatic changes in the intracellular concentrations of ATP could also be involved in the signalling changes in gene expression. It is also possible the system is particularly sensitive to the accumulation of intermediates specific to the

histidine pathway.

In contrast to other genes which were expressed at elevated levels after inhibition of amino acid biosynthesis, GS expression decreased. Because glutamine is already present at very high levels in *Arabidopsis* seedlings (see Fig. 1), its biosynthesis could conceivably be deprioritized under conditions of amino acid starvation. It is not unreasonable to postulate that a reduced demand for glutamine could be reflected in changes in the steady-state levels of GS mRNA.

General control in yeast is a regulatory system that maintains sufficient amino acid levels in coordination with its environment. Plants typically do not grow heterotrophically for amino acids or nucleosides, but may encounter imbalances in amino acid levels. For instance, infection with some pathogenic and parasitic bacteria can specifically inhibit amino acid pathways or deplete specific amino acid pools (Sinden and Durbon, 1968; Patil *et al*, 1970). Phaseolotoxin, a phytotoxin produced by *Pseudomonas phaseolicola*, inhibits ornithine carbamoyltransferase, an enzyme involved in the biosynthesis of arginine (Patil *et al*, 1970). The accumulation of a number of amino acids has shown to be associated with reduced levels of arginine in bean plants infected with *P. phaseolicola* (Patel and Walker, 1963). Physiological stress may lead to the increase of specific amino acids. For instance, proline is known to accumulate in drought tolerant plants (Delauney and Verma, 1993). Increases in intracellular levels of individual amino acids has also been shown to stimulate the general control mechanism in yeast (Niederberger *et al*, 1981).

The results presented here suggest plants are capable of cross-pathway regulation similar to general amino acid control in *Saccharomyces cerevisiae*. Further elucidation of this phenomenon, both through physiological and genetic analysis, are required to understand its role in plant growth and development.

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