SESSION 9B

CURRENT ADVANCES IN HERBICIDE ACTION

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INVITED PAPERS 9B-1 to 9B-6

ROLE OF CYTOCHROME P450 IN HERBICIDE METABOLISM

O. T. G. JONES
Department of Biochemistry, University of Bristol, BS8 1TD.
J. C. CASELEY
Long Ashton Research Station, Bristol, BS18 9AF

ABSTRACT

Cytochrome P450 catalyses the oxygenation of xenobiotics by plants, animals and microbes. Herbicides can be modified by the plant microsomal system, contributing to herbicidal specificity and decreasing pesticide residues in food. Reactions catalysed by plant P450 include aryl hydroxylation, alkyl hydroxylation, sulphoxidation, N-dealkylation, epoxidation of aromatic rings and O-demethylation. The oxidation reaction requires NADPH and O_2 and is sensitive to inhibitors such as CO and 1-aminobenzotriazole. The oxygenase system is inducible by herbicide treatment, which can cause increased microsomal content of cytochrome P450. This induction is blocked by inhibiting biosynthesis of the haem prosthetic group of cytochrome P450. Components of the oxidase system have been purified from plants and active oxygenase complexes can be reconstituted in vitro from purified components.

INTRODUCTION

Herbicide metabolism has been studied for many years and there is considerable knowledge of the breakdown products by plants and soil organisms. There is, however, less certainty concerning the biochemistry of the reactions involved. Since the toxicity to consumers, and the selectivity of herbicides, is influenced by their subsequent metabolism by crops and weeds a knowledge of these metabolic pathways in important. Oxidation is a major reaction in the detoxification of foreign molecules (xenobiotics) by plants (Cole, 1983) and is commonly catalysed by the microsomal cytochrome P450 monooxygenase system. Cytochrome P450 is well characterised in mammalian systems where it has been shown to be a group of isoenzymes, products of a multigene family (Nebert & Gonzales, 1987), which have protohaem as a prosthetic group and which bind with CO to form a complex with a strong absorption maximum at 450nm, giving the pigment its name. Cytochrome P450 binds both substrate xenobiotic and O_2 and catalyses the transfer of one atom of oxygen to oxygenate the xenobiotic, usually forming an -OH group, whilst the second atom of oxygen forms water.

In mammalian systems for detoxifying hydrophobic xenobiotics oxygenation is called Phase 1, or activation step. In Phase 2 a water soluble substituent is added to the newly-formed -OH group, making a water soluble derivative (see review in Gibson & Skett, 1986). Examples of such soluble substituents are glutamic acid, cysteine or glutathione. The conjugate is much more hydrophilic than the parent xenobiotic and can be excreted in urine or bile. In green plants the properties of the P450 system have similarities to those of mammalian systems, although they are less well characterised;

there is no simple route for excretion of oxygenated products, as through bile or urine in mammals, but the products are likely to have lower biological activity and may be sequestered in vacuoles or in structural polymers (Shimabukuro, 1985).

PROPERTIES OF CYTOCHROME P450 MONOOXYGENASE SYSTEMS

Cytochromes P450 in mammalian cells are normally found firmly embedded in the smooth endoplasmic reticulum: they have a molecular weight around 56000 daltons. They are unusual haem proteins since one of the axial haem ligands is the thiolate anion ($-S^-$) of cysteine and this contributes to the remarkably long wavelength of the CO complex of its Fe^{2+} haem form (Loew & Rohmer, 1980). Any compound which binds CO is likely to bind O_2 and this is true of cytochrome P450: the haem prosthetic group of the protein functions to activate the liganded O_2. The reactions catalyses by cytochrome P450 can be simplified as shown below, where RH is a hydrophobic substrate molecule and where there is a requirement for reduced pyridine nucleotide and O_2.

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

The likely mechanism of the reaction has been best shown in the liver microsomal P450 system, illustrated in Fig. 1. (after Estabrook et al., 1971).

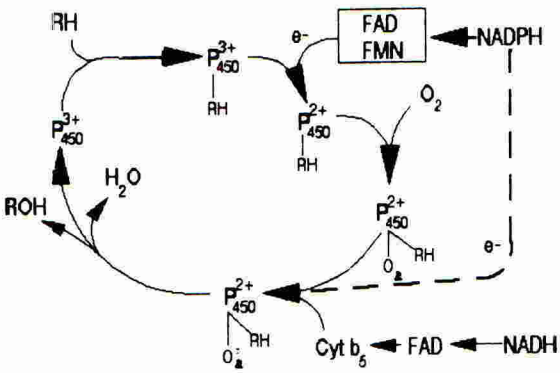

Fig 1 POSSIBLE SCHEME OF THE P450 SYSTEM

Oxidised cytochrome P450 ($P450^{3+}$) can exist in either high or low spin forms, it binds substrate RH to form a complex with altered spectroscopic properties (Type 1 with 390nm max., corresponding to the high spin form of the cytochrome, or Type 2 with max. around 420nm, corresponding to the low spin form) (Jefcoate, 1978). This complex is reduced (to $P450^{2+}$) by an electron transferred from NADPH by the specific flavoprotein NADPH:cytochrome P450 reductase. This membrane-bound flavoprotein has been purified from animal and plant tissues (Madhyastha & Coscia, 1979; Benveniste et al., 1986) and found to contain both FAD and FMN as prosthetic groups. It appears

to function by oscillating between the 2 electron-reduced and 3 electron-reduced states.

The substrate, RH, binds to the periphery of the reduced P450 haem, leaving the Fe^{2+} accessible to O_2 to form a sixth ligand. Binding O_2 causes a spectral shift with an absorption maximum on the difference spectrum at 440nm. A second electron is transferred to the P450, with the formation of a state equivalent to bound superoxide ($-O_2^-$.). This electron is donated from either NADH, via the membrane-bound cytochrome b_5 and the FAD-containing enzyme NADH-cytochrome b_5 reductase, each purified from plants (Jollie et al., 1987); alternatively, the second electron may be donated by NADPH-cytochrome P450 reductase (see Fig. 1). From this reduced oxygenated complex oxygen-oxygen bonds are cleaved, one atom of oxygen is removed to form water, leaving ozone bound to the haem which in turn reacts to oxygenate the substrate RH. The E_{m7} of cytochrome b_5 (around 0mV) is very much higher than that of cytochrome P450 (E_{m7} = -330mV), so cytochrome b_5 can only donate electrons to the P450-xenobiotic-O_2 complex (E_{m7} about +50mV) (Guengerich, 1983).

There is evidence derived from animal and plant microsome studies that cytochrome b_5 and its reductase are not obligatory in the metabolism of all xenobiotics by the cytochrome P450 system (Lu & Levin, 1974): electrons from NADPH cytochrome P450 reductase may donate electrons at two sites, as indicated by the dotted line in Fig. 1. It is commonly found, however, that NADH and NADPH act synergistically in the metabolism of substrates by the monooxygenase of liver microsomes.

NADH-cytochrome b_5 reductase and cytochrome b_5 also have a role, quite separate from their contribution to P450 monooxygenase, in an electron transport system involved in desaturating fatty acyl-CoA derivatives in plants and animals (Stobart et al., 1989).

Some examples of the modifications to xenobiotics catalysed by liver monooxygenase systems are shown in Fig. 2. Less is known about the capacity of plant microsomes but it is likely that they are capable of a similar variety of reactions.

NORMAL FUNCTIONS OF MICROSOMAL OXYGENASES

Some mammalian P450 isozymes are concerned with the normal metabolism of endogenous substrates. Examples are found in oxygenation reactions of the formation of steroid sex hormones and of bile acids and the formation of active forms of vitamin D. In plants the monooxygenase is implicated in the hydroxylation of trans-cinnamic acid at position 4 (Potts et al., 1974), the first step in the synthesis of most coumarins, tannins, lignins and flavenoids; the hydroxylation of geraniol and nerol (Madhyastha et al., 1976) in the formation of alkaloids, and the hydroxylation of kaurene (Hanson & West, 1976), en route to the formation of the giberellin family of plant hormones. P450 is also involved in the hydroxylation of

some fatty acids (Soliday & Kolattukudy, 1977) and in the formation of phytoalexins.

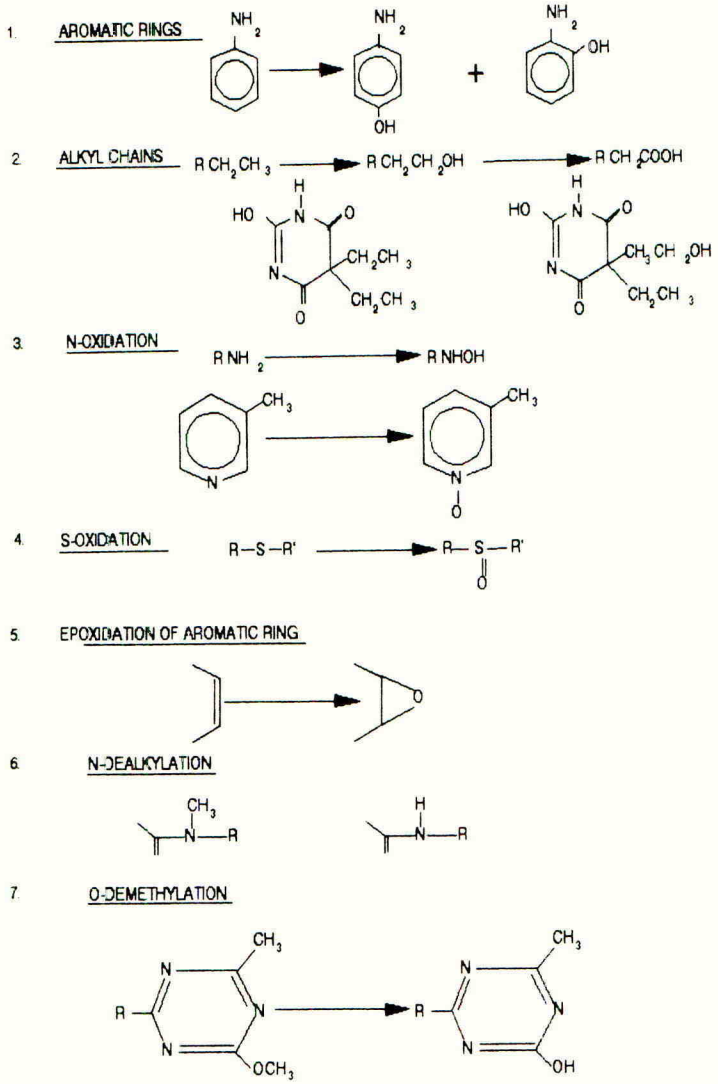

FIG.2 SOME OXYGENASE REACTIONS CATALYSED BY MICROSOMES

INDUCTION

Treatment of rats or other animals with a variety of xenobiotics leads to the formation in the liver of much increased amounts (sometimes several hundred-fold increased) of one or more of the isozymes of cytochrome P450. The mechanism of this induction, when aromatic hydrocarbons are the inducers, has been shown to involve an intracellular receptor protein (the Ah receptor) which binds to the aromatic hydrocarbons to form a complex which regulates the expression of certain P450 genes (see Nebert & Gonzales, 1987 for review). Hepatic

receptors have not been found for other inducers. In higher plants, too, cytochrome P450 and associated oxygenase activity is inducible. Treatments causing increasing cytochrome P450 are varied: slicing or damaging tubers caused substantial increases in P450 concentration as did treatment of the tubers with manganese ions, phenobarbitol (a potent inducer of mammalian P450) and 2,4-D and monuron (Reichhart et al., 1979). P450 was induced in intact mung beans by treatment with geraniol (giving a pigment with a CO-complex absorbing maximally at 449nm) and with cinnamate (yielding P451) (Hendry & Jones, 1984). Endogenous uninduced cytochrome gave a CO complex at 452nm, indicating that multiple forms of P450 are present, or can be formed in higher plants. Indeed, distinct forms of P450 have been extracted and purified from microsomes of storage tissue. We have found that there is increased P450 present in microsomes from biotypes of Alopecurus myosuroides which are resistant to chlorotoluron and are associated with increased rates of degradation of this herbicide (Caseley, Cross, Jones & Kueh, unpublished work).

INHIBITORS OF CYTOCHROME P450-DEPENDENT TRANSFORMATIONS.

The involvement of cytochrome P450 in oxygenation reactions can be tested indirectly with the use of inhibitors. CO combines with the haem prosthetic group and prevents its normal function: this effect is reversible by light of 450nm. Similarly, 1-aminobenotriazole and some of its analogues are suicide substrates of many cytochromes P450 (Ortiz de Montellano et al., 1984) and inhibit the metabolism of several, but not all, herbicides (Cabanne et al., 1987; Gonneau et al., 1988). It is also possible to inhibit the induction of P450 in Jerusalem artichoke tubers by adding gabaculine to the medium to block the synthesis of 5-aminolaevulinic acid, the precursor of the haem of cytochrome P450 (Werck-Reichhart et al., 1988). This causes much decreased activity of the microsomal monooxygenase. Interestingly this inhibitor is not effective in blocking mammalian haem synthesis.

CYTOCHROME P450 MEDIATED HERBICIDE METABOLISM IN PLANTS.

The role of oxidases in xenobiotic and specifically herbicide metabolism have been reviewed by Cole (1983) and O'Keefe (1987) respectively. The most important metabolic reactions of herbicides that are, or may be, mediated by cytochrome P450 include:- aliphatic hydroxylation, O-dealkylation, N-dealkylation, ring hydroxylation, N-oxide and S-oxide formation. Products of these reactions have been characterised and quantified and used as evidence for the involvement of cytochrome P450, but many reactions presumed to be P450 mediated may be catalysed by peroxidases or other oxygenases (see Lamoureux & Frear, 1987). We describe below some examples of oxidative metabolism where there is evidence that cytochrome P450 is involved.

N-demethylation of Monuron.

Frear et al. (1969) used microsomal preparations from etiolated cotton containing a N-demethylase which required

NADPH or NADH as a co-factor to demethylate monuron. The reaction was inhibited by CO and several other inhibitors (Fig. 3a).

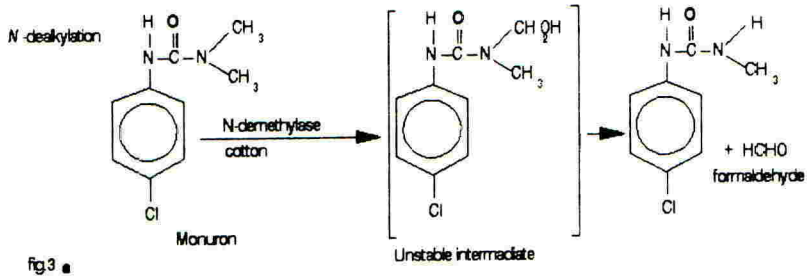

fig.3 a

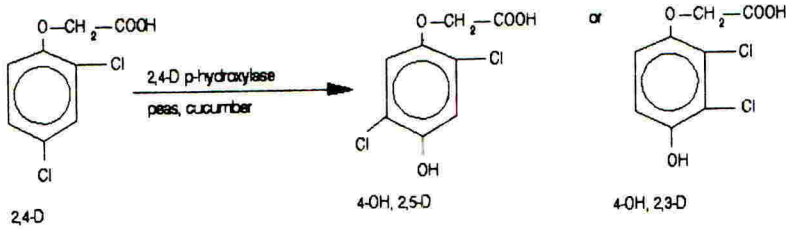

fig.3b Ring Hydroxylation

Aryl hydroxylation of 2,4-D.

Makeev et al. (1977) described the microsomal enzyme responsible for the 4-hydroxylation of 2,4-D. Its synthesis in cucumber was stimulated by treatment with the herbicide suggesting that it can be induced by substrates, as found for hepatic monooxygenase systems. This 2,4-D p-hydroxylase required NADPH as a co-factor and was demonstrated to be CO-inhibited, with light reversal of the inhibition. Aryl hydroxylation was achieved through a 'NIH shift' with the 4-Cl being displaced to the 3 or 5 position (see Fig. 3b).

N-demethylation and ring hydroxylation of chlorotoluron.

Aminobenzotriazole (ABT) is a suicide substrate for cytochrome P450 inactivating cinnamic-4-hydroxylase - a cytochrome P450 monooxygenase found only in plants (Reichhart et al., 1982). Gaillardon et al. (1985) found ABT inhibited chlorotoluron metabolism in wheat and Kemp & Caseley (1987) report that it overcomes chlorotoluron resistance in Alopecurus myosuroides. The nature of the metabolites (Fig. 4) and inhibition of their production by ABT provides circumstantial evidence that cytochrome P450 is involved.

Recent work in our laboratory with suspension cell cultures confirms more rapid demethylation and ring hydroxylation with subsequent conjugation in the resistant biotype which can be reversed with ABT. Microsomal membranes prepared from the suspension cell cultures were resuspended in phosphate buffer and CO-difference spectra obtained. A Type 1

binding spectra was obtained in the presence of chlorotoluron. The rate of metabolism of [^{14}C]-chlorotoluron to the mono-N-demethylated and N-didemethylated metabolites was twice as fast in the resistant biotype. This evidence strongly indicates the involvement of cytochrome P450 in chlorotoluron metabolism in A. myosuroides.

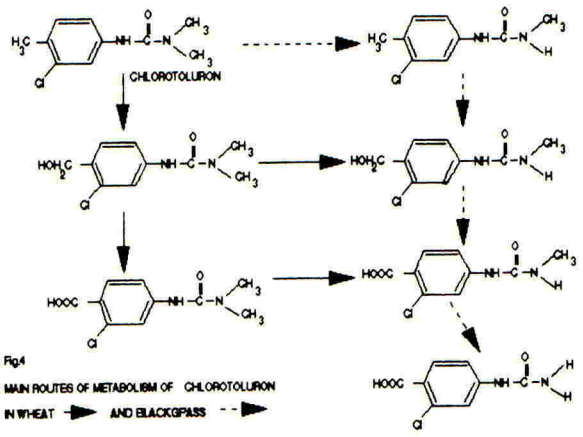

Fig.4
MAIN ROUTES OF METABOLISM OF CHLOROTOLURON
IN WHEAT → AND BLACKGRASS - - →

Aryl hydroxylation of diclofop.

The wild oat herbicide dichlofop-methyl is rapidly de-esterified in susceptible and resistant plants. In tolerant plants, such as wheat, aryl hydroxylation by NIH shift determines selectivity, while in susceptible plants the free acid remains intact (Shimabukuro et al., 1979), see Fig. 5.

Zimmerlin & Durst (1989) have recently shown that a microsomal preparation of wheat catalyses the aryl hydroxylation of diclofop. The reaction required O_2 and NADPH as co-factors and was blocked by inhibitors of cytochrome P450 and CO. The CO inhibition was reversed in light.

In the above cases of oxidative metabolism the involvement of cytochrome P450 was supported by convincing biochemical evidence.

PROSPECT

The discovery, purification and characterisation of the components of plant monooxygenase systems has prepared the ground for future work which is likely to include the transfer of the appropriate genes. The will permit the manipulation of herbicide resistance in crops and diminish hazards from residues.

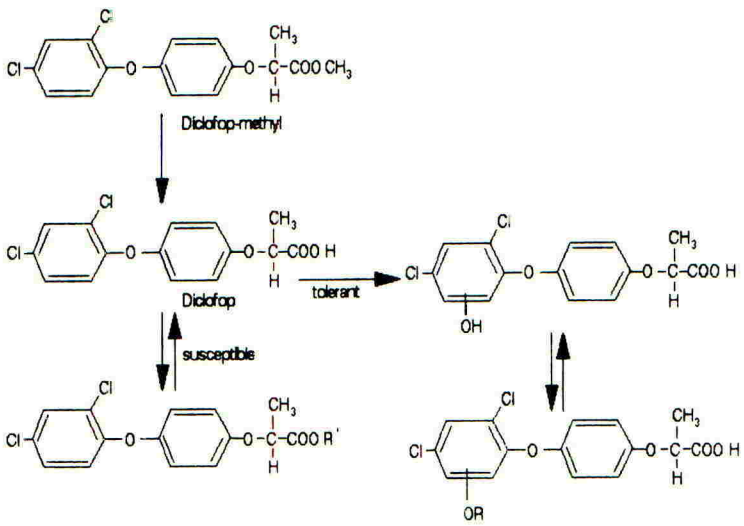

Fig.5 The metabolism of Diclofop-methyl in tolerant and susceptible plants

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RECENT WORK ON PHOTOSYSTEM TWO: STRUCTURE AND SITE OF ACTION OF INHIBITORS OF PHOTOSYNTHETIC ELECTRON TRANSPORT

D.J. CHAPMAN, J. BARBER

A.F.R.C. Photosynthesis Research Group, Department of Biochemistry, Imperial College of Science, Technology & Medicine, London, SW7 2AY, U.K.

ABSTRACT

In the last three years there has been general acceptance of the suggestion that the photosystem two reaction centre consists of the D1 and D2 proteins in an analogous structure to that of the well characterised reaction centre of purple photosynthetic bacteria. This is mainly the result of the isolation of a D1-D2 complex with photosynthetic activity and a pigment composition equivalent to that of the bacterial system. This photosystem two preparation does not contain quinones but reconstitution experiments have been successfully initiated and a model system for studying quinone and herbicide binding is being developed. Recent advances in understanding herbicide interactions with photosystem two have come from mapping amino acid substitutions in herbicide resistant mutants, and comparing the D1 amino acid sequence with both the sequence and the detailed structural information available for the bacterial reaction centre.

INTRODUCTION

Solar energy is converted to an electrochemical potential by the photochemical charge separation reactions which occur in the two types of photosystem in the chloroplast thylakoid membranes of plants. Photosystem two (PS2) acts as a water-plastoquinone oxidoreductase to initiate linear electron flow, and photosystem one completes the overall linear electron transfer process by reducing ferredoxin and $NADP^+$. The oxidation of water is possible because there is sufficient redox energy in the radical pair, $P680^+ Pheo^-$, which is created by the initial photochemical reaction in PS2. The possibility of loss of this state by rapid recombination is avoided by stabilisation of the PS2 charge separation by electron transfer to a plastoquinone-9 (PQ9) molecule, designated Q_A. Further stabilisation occurs with the subsequent transfer of electrons to a second PQ molecule (Q_B) which, when doubly reduced, becomes protonated to form plastoquinol (PQH_2). Unlike Q_A, the Q_B site is occupied by PQ or PQH_2 molecules which are not firmly bound. They can therefore exchange freely with molecules in the lipid matrix of the thylakoid membrane. This can include a wide range of different herbicides which inhibit PS2 activity by blocking electron transfer between Q_A and Q_B. For a detailed review of the PS2 electron transfer reactions see Velthuys (1987). Here we will summarise the rapid progress made during the last three years in the understanding of the structure of PS2, and the central role of the herbicide binding, or D1, protein.

STRUCTURE OF PHOTOSYSTEM TWO

Until recently the structure of the PS2 pigment-protein complex was poorly understood beyond the assignment of a light-harvesting function to some chlorophyll binding proteins, the realisation that three extrinsic membrane proteins on the lumenal surface of thylakoids had a role in regulation of water oxidation and a partial characterisation of the ten or more intrinsic membrane proteins found in the smallest isolated protein complex which was capable of photochemical charge separation. The D1 protein (psbA gene product) was one of these intrinsic proteins and had been shown to function as an apoprotein for Q_B. It was also known to bind

PS2 herbicides and had been shown to be rapidly degraded and re-synthesised in a light-regulated process (Kyle, 1985).

Analogy between reaction centres of PS2 and purple bacteria

In 1983 and 1984 analyses of the amino acid sequences of the D1 and D2 proteins, showed that these two proteins had some close similarities with the L and M subunits of the reaction centre of purple photosynthetic bacteria and this led several authors to suggest that, in contradiction to the then current models of PS2 structure, the D1 and D2 proteins might together form the PS2 reaction centre (Deisenhofer et al, 1984; Trebst & Depka, 1985; Barber & Marder, 1986). This has formed the basis of the present understanding of PS2 structure as indicated in Figure 1. In addition, using hydrophathy plots and the equivalence in the amino acid sequences of the L, M, D1 and D2 proteins, it was possible to obtain a sound projection of the likely structure of much of the PS2 reaction centre especially as X-ray crystallographic studies had given a precise structure for the bacterial reaction centre (Trebst, 1986; Barber, 1987; Michel and Deisenhofer, 1988). In particular, a reliable model could be proposed for the Q_B binding region of D1 (Figure 2).

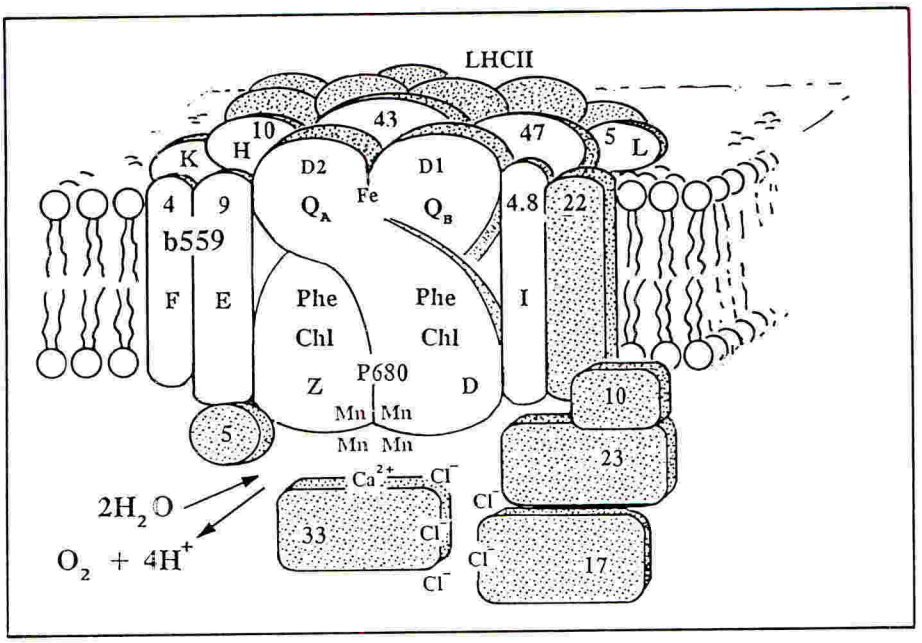

FIGURE 1. Diagrammatic representation of the photosystem two complex embedded in the thylakoid membrane of the chloroplast of higher plants or green algae. Abbreviations are: LHCII for light harvesting complex of PS2; E,F,H,I,K and L are the products of the chloroplast genes denoted by the same letter (e.g. psbE); D1,47,43 and D2 are the products of the psbA, psbB, psbC, psbD genes; 5,10,17,23 and 33 are extrinsic proteins on the lumenal surface; Chl is chlorophyll a; Ph is pheophytin a; Q_A and Q_B are bound plastoquinones which act as secondary electron acceptors; P680 is the primary electron donor; Z and D are tyrosines which can be oxidised by $P680^+$. The numbers are apparent molecular weights given in kilodaltons. Proteins encoded by nuclear genes are denoted by full shading and all others are encoded by the chloroplast genome.

Isolation of the PS2 reaction centre

Until 1987 there was no direct evidence in favour of the D1-D2 model of the PS2 reaction centre, although a few reports had indicated that the D1 protein could have a role in electron donor as well as acceptor reactions (e.g. Carpentier et al, 1985; Hsu et al, 1986). Antibody labelling of intact membranes had also produced results in agreement with the proposed folding pattern (Sayre et al, 1986). Clear cut experimental evidence came from the isolation by Nanba and Satoh (1987) of a pigment-protein complex capable of photochemical charge separation and which contained the D1 and D2 proteins and bound 4 chlorophyll a molecules (Barber et al, 1987; Nanba, and Satoh, 1987; Seibert, et al, 1988). This significant advance, along with subsequent detailed analysis of the complex isolated from several plant species has meant a rapid and general acceptance of the principle features of the D1-D2 PS2 reaction centre model. The pigment composition is directly equivalent to that of the reaction centre of purple bacteria with a molar ratio of 4 chlorophyll a:2 pheophytin a:1 β-carotene (Nanba and Satoh, 1987; Barber et al, 1987). It was immediately apparent that, besides the D1 and D2 polypeptides with molecular masses of about 32 kDa, there was also a 10 kDa component in the isolated complex which corresponded with the presence of reducible cytochrome b559. Two other low molecular mass polypeptides have been detected and analyses of their N-terminal amino acid sequences (Ikeuchi and Inoue, 1988; Webber et al, 1989) have shown them to be the β-subunit of cytochrome b559 and a polypeptide which is the product of the psbI gene of the chloroplast genome but has no known function. The cytochrome b559 content was found to be 1 per 2 pheophytin a and therefore 1:1 with the reaction centre (Miyazaki et al, 1989). Although the pigment composition of the isolated PS2 complex was equivalent to that of the preparations from purple photosynthetic bacteria, a significant difference was the absence of the PQ9 molecules which act as Q_A and Q_B in PS2 (Barber et al, 1987).

Electron transfer activity of the isolated PS2 reaction centre

In the absence of Q_A, Q_B and any added exogenous electron donors and acceptors, the photochemical activity of the isolated reaction centre is restricted to primary charge separation to create the radical pair $P680^+ Pheo^-$. This charge separated state relaxes by recombination with a half-time of about 35 ns giving rise to a fluorescence yield which indicates that the majority of reaction centres in preparations isolated in our laboratory are functional (Crystall et al, 1989). This back reaction also produces a chlorophyll triplet state which can be detected by transient absorption spectroscopy at room temperature (Durrant et al, 1989). At 4°K the triplet state dominates and can be detected by a characteristic epr signal (Okamura et al, 1987; Telfer et al, 1988).

Prevention of the recombination reaction and assay of net secondary electron transfer was found to be possible by adding artificial electron donors and acceptors. When sodium dithionite was used as a donor photoaccumulation of reduced pheophytin was detected (Barber et al, 1987; Nanba and Satoh, 1987). Silicomolybdate was found to act as an electron acceptor giving a reversible light induced absorption charge indicative of $P680^+$ (Barber et al, 1987), a relatively long lived epr signal characteristic for $P680^+$ and loss of the epr signal arising from triplet formation (Nugent et al, 1989; Takahashi et al, 1989). When silicomolybdate was present the oxidised $P680_{2+}$ could be reduced by artificial electron donors such as diphenyl-carbazide, Mn^{2+} or KI, giving net light dependent electron flow from the donor to silicomolybdate (Chapman et al, 1988). Although the quantum efficiency for the net electron transfer is low, this reaction can be monitored conveniently by the absorbance increase at 600 nm due to reduction of silicomolybdate and serves as a useful assay for the activity of preparations.

Temperature and light dependent instability of the isolated PS2 reaction centre

Loss of electron transfer activity on exposure of samples to temperatures above 4°C in the dark was found to be due to instability of preparations prepared in Triton X-100 and concomitant changes included a blue shift in the light absorption and fluorescence emission (4°C) peaks in the red region of the spectra (Chapman et al. 1988, 1989a; Seibert, et al, 1988). A significant stabilisation of these samples was achieved by transfer from Triton X-100 to dodecylmaltoside (Chapman et al. 1988; 1989a) or polyethylene glycol (McTavish et al. 1989). The stabilisation in dodecylmaltoside is particularly successful when the detergent transfer is carried out during the second ion-exchange chromatographic purification (Chapman et al, 1989b).

Illumination was found to damage the isolated reaction centre and besides the changes found on raising the temperature in the dark, there was also a rapid destruction of pigments and modifications of protein conformation (Chapman et al. 1989a). Protection against photodamage could be achieved either by adding silicomolybdate to the samples (Telfer and Barber, 1989) or by removal of oxygen from the incubation media (Crystall et al, 1989; He et al. 1989; McTavish et al, 1989). These results support the suggestion that the photodamage of the isolated reaction centre is caused by singlet oxygen formed by the interaction of the oxygen usually present in media with the chlorophyll triplet formed by charge recombination in the absence of secondary electron acceptors.

Reconstitution with the isolated PS2 reaction centre

Two biochemical approaches are used for determining the components of PS2 essential for full PS2 activity and for regulation of the reactions. Comparison of different types of preparation has proved a valuable method and in some circumstances reconstitution procedures have been useful. The latter approach has advantages when a preparation with a simple composition, such as the reaction centre complex, is available. In our laboratory we are using the reconstitution approach to assess the interaction of specific thylakoid components with the stabilized reaction centre and to measure the recovery of various PS2 functions.

An important question in determining the structure of PS2 is the organisation of the proteins and cofactors involved in the oxidation of water and donation of electrons to P680. An answer can not come from comparisons with the purple bacterial reaction centre because PS2 is unique in this respect. Recent experiments on PS2, including site directed mutagenesis of specific amino acids, have given evidence that the immediate donor to P680 is a tyrosine at position 161 in the D1 protein (Debus et al, 1988; Vermaas et al, 1988) and is therefore present in the isolated reaction centre. Further key elements of the water oxidising region of PS2 are manganese and an extrinsic 33 kDa protein which appears to stabilise the manganese binding. These components are missing in the isolated complex but a direct association with the reaction centre has been indicated by our experiments on the binding of the 33 kDa protein (Gounaris et al, 1988a, 1989).

Reconstitution procedures have been used with some success in our laboratory to give quinone dependent secondary electron transfer reactions in the isolated reaction centre preparations. Addition of an artificial compound, decylplastoquinone (DPQ), to aqueous suspensions of the reaction centre gave a preparation capable of photoreduction of cytochrome b559 (Chapman et al, 1988) and a light dependent net electron flow from diphenylcarbazide to dichlorophenolindophenol (Gounaris et al, 1988b; Chapman et al, 1989a). When the naturally occurring PQ9 was added to the reaction centre in the presence of a diaclyglycerolipid dispersion, rates of electron transfer could be achieved which were comparable with those with DPQ and thermoluminescence measurements have indicated that at least a small fraction of centres can bind a quinone tightly (Chapman et al, 1989b). To-date our results indicate that proteins, other than those present in the preparation, are not needed to give isolated reaction

centres capable of reducing PQ9. Besides quinone, the only addition needed is diacylglycerolipid. However, proteins may be needed to give fully functional binding at the Q_A and Q_B sites and this is currently a main subject of study in our laboratory. With improved binding of the quinones a model system will be produced which should prove valuable in studying not only quinone, but also herbicide, interactions with PS2. It is interesting to note that, as isolated, the reaction centres could bind herbicides including diuron, dinoseb and atrazine although with weak affinities (Giardi et al, 1988). At high concentrations (0.1mM) diuron inhibited quinone dependent electron transfer reactions by up to 50% (Gounaris et al, 1988b) and after reconstitution with diacylglycerolipid, inhibition was almost 100% (D.J. Chapman, unpublished results).

Other PS2 proteins

In addition to reaction centre components there are many intrinsic membrane proteins present in the relatively large PS2 particles isolated by removing the light harvesting chlorophyll proteins. These preparations represent the PS2 core and have bound reducible quinones. Those isolated with the extrinsic 33kDa polypeptide present are capable of water oxidation. Little is known of the full complement of proteins needed for these activities or of the roles of those proteins which are known to be present. Interest is currently focussed on a 10 kDa phosphoprotein as well as proteins of 10,22, 28, 43 and 47 kDa, all of which may have roles in quinone binding. In addition there are several proteins of less than 10 kDa and some of these are the products of genes which have been identified and sequenced. For a review of this area see Barber 1989, and the diagrammatic representation in Figure 1.

INTERACTION OF HERBICIDES WITH PHOTOSYSTEM TWO

Herbicide binding protein

Several different classes of chemicals used as herbicides are known to inhibit photosystem two activity by blocking electron transport between Q_A and Q_B. These include the triazines (e.g. atrazine), substituted ureas (e.g. DCMU, i.e. diuron), uracils (e.g. bromacil) and phenolic derivatives (e.g. ioxynil and dinoseb). Competition experiments demonstrated that these herbicides share common binding determinants with each other and the Q_B site (Vermaas et al, 1984) and using a photoaffinity labelling technique azido-derivatives of some of these herbicides were shown to label the D1 protein (Pfister et al, 1981; Oettmeier et al, 1984). Further conclusive evidence of the dominant role of this protein in herbicide binding came from analysis of mutants in which the protein was found to be the location of a single and specific amino acid substitution which gave herbicide resistance (Hirschberg and McIntosh, 1983). As a result of this work D1 became known as the PS2 herbicide binding protein.

Amino acids involved in herbicide binding

A photoaffinity labelling method was used to identify a region of the D1 protein between residues 214 and 225 which is probably part of the site of atrazine binding (Wolber et al, 1986). In addition, a longer sequence in the same region of the D1 protein has been shown to be involved in the binding of several herbicides by identification of the amino acid substitutions in the psbA gene in a wide range of different herbicide resistant mutants. Many of these results are summarised in Table 1 and a diagrammatic representation of the herbicide binding region of D1 is given in Figure 2. The first amino acid substitution to be reported was glycine for serine-264 in a population of triazine resistant Amaranthus hybridus (Hirschberg & McIntosh, 1983). In this case the decrease in atrazine binding affinity was greater than 1000-fold but the loss in affinity for diuron was much less (Pfister et al, 1979). Subsequently other mutants with substitutions at position 264 have been discovered and those with glycine in place of serine all have similar herbicide sensitivity. However, with

alanine or threonine as the replacement there is a difference, there being an improved resistance to substituted ureas. It has been suggested that in these cases there is a conformational change in the protein as well as loss of the serine hydroxyl group (Sigematsu et al, 1989). Besides these changes at position 264, an increased resistance to atrazine but not diuron is found with substitutions at the nearby residues: 255 and 256 (Erickson et al, 1989). In a further class of mutants the amino acids at positions 219 and 275 are altered to give resistance to diuron without significant change to atrazine resistance (references 3, 10, 11, 12 in Table 1). These

TABLE 1. Characteristics of mutants with alterations in the psbA gene which give different amino acid substitutions in the D1 protein.

substitution from → to			resistance to Atr./DCMU/Bro.			inhibition Q_A to Q_B	organism	reference
phe	211	ser	R	u	u	u	Syn. sp PCC 7002	10
val	219	ile	u	R	u	u	Syn. sp PCC 7002	10
val	219	ile	N	R	N	N	C. reinhardtii Dr2	3
val	219	ile	N	R	N	N	C. reinhardtii Dr18	12
ala	251	val	R	R	u	I	C. reinhardtii	8
phe	255	tyr	R	N	N	N	C. reinhardtii	12
gly	256	asp	R	N	R	I	C. reinhardtii AR204	11
gly	256	asp	R	N	R	I	C. reinhardtii Br 24	11
ser	264	gly	R	N	R	I	A. hybridus	1
ser	264	gly	R	u	u	u	S. nigrum	4
ser	264	gly	R	u	u	I	S. nigrum	5
ser	264	gly	R	u	u	u	Ch. album	7
ser	264	gly	R	u	u	u	P. paradoxa	9
ser	264	gly	R	N	u	u	A. retroflexus	13
ser	264	ala	R	R	R	I	C. reinhardtii	2
ser	264	ala	R	R	u	I	A. nidulans	6
ser	264	thr	R	R	u	u	N. tabacum	13
leu	275	phe	N	R	R	N	C. reinhardtii	11

References:

1 Hirschberg and McIntosh, 1983
2 Erickson et al, 1984
3 Galloway and Metz, 1984
4 Goloubinoff et al, 1984
5 Hirschberg et al, 1984
6 Golden and Haselkorn, 1985
7 Bettini et al, 1987
8 Johanningmeier et al, 1987
9 Schoenfeld, M. et al, 1987
10 Gingrich et al, 1988
11 Rochaix and Erickson, 1988
12 Erickson et al, 1989
13 Sigematsu et al, 1989

Abbreviations: R, resistant (more than 5 x wild type); N, normal; u, unknown; I, inhibited; Syn. Synechocystis; C, Chlamydomonas; A, Anacystis; S, Solanum; N, Nicotiana; Ch, Chenopodium; P, Phalaris.

mutants are particularly interesting because they appear to have normal rates of electron transfer from Q_A to Q_B, as does the triazine resistant mutant with a substitution at 255 (Erickson et al, 1989) and an ioxynil-resistant strain of Synechocystis 6714 with asparagine 266 replaced by threonine (Etienne et al, 1989). They will be of particular interest to those who wish to introduce herbicide resistance into crop species because other substitutions have been found to result in impaired Q_B reduction (Table 1) and this might be related to a loss of growth rate in resistant plants.

Further rapid progress can now be expected in the detailed mapping of the site of herbicide binding by generation and characterisation of new mutants of green and blue-green algae. Comparison can be made with mutants of purple photosynthetic bacteria (Paddock et al, 1988; Sinning et al, 1989) in which it is possible to obtain precise structural details from crystal structures. Furthermore the use of random mutagenesis and selection can now be complemented by site-directed mutagenesis of specific amino acids (Vermaas, 1989).

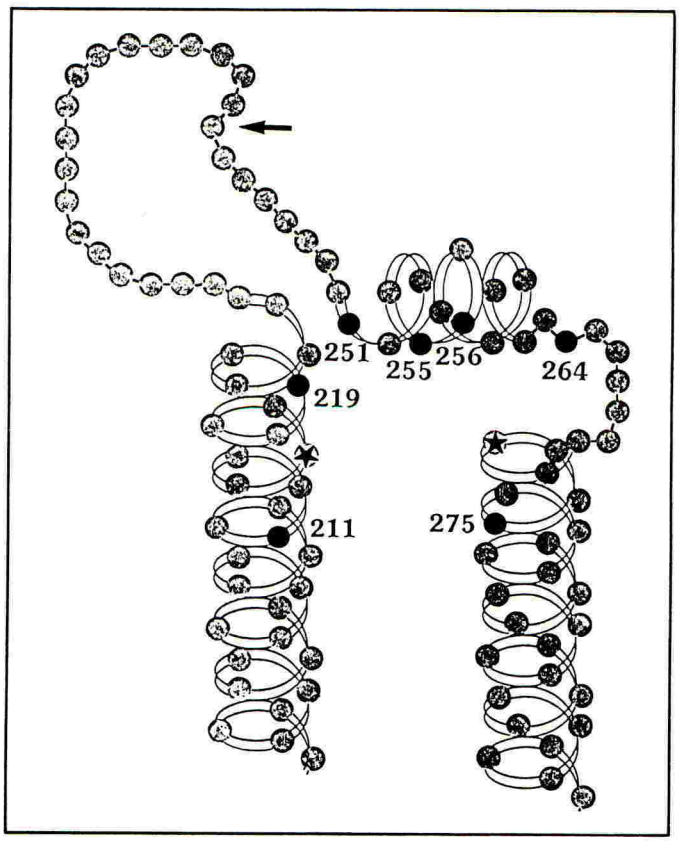

FIGURE 2. Diagrammatic representation of the Q_B binding region of the D1 protein. Amino acids 194 to 294 are represented and the numbers indicate the positions of substitutions in herbicide resistant mutants as described in Table 1. Histidines thought to act as two of the ligands for iron are denoted with a star and the cleavage site involved in D1 turnover indicated by an arrow.

Other PS2 components affecting herbicide interaction

Photoaffinity labelling with herbicide derivatives has suggested that proteins other than D1 and D2 are close to the herbicide binding site (e.g. Donner & Oettmeier, 1989). The possible involvement of at least one additional protein is also emphasised by a comparison of isolated PS2 and bacterial reaction centres. The bacterial H-subunit protein is known to stabilise Q_B binding but an equivalent protein appears to be absent from the PS2 preparation in which both Q_A and Q_B are missing. As mentioned earlier, there are several proteins which could have a crucial role on the reducing side of PS2 and be equivalent to the H-subunit. In addition to the proteins there is particular interest in the iron atom which is present close to the Q_B site in the bacterial reaction centre and can be detected by e.p.r. in thylakoids but is absent in many PS2 reaction centre preparations. Our recent reconstitution work (Chapman et al, 1989) also emphasises the role of lipids, as did previous analyses of the lipid composition of herbicide resistant mutants (Chapman et al, 1985).

A possible second site of herbicide binding to PS2

It has been suggested that, in addition to the electron acceptor reactions, the donor activity of PS2 is inhibited by many herbicides (Carpentier et al, 1985; Hsu et al, 1986). This conclusion has been based largely on detection of herbicide sensitive reduction of an artificial electron acceptor which acts before the Q_B site and the ability of artificial electron donors to overcome inhibition by these herbicides. A clear understanding of these results probably awaits a better appreciation of the close interaction of donors and acceptors. For example, Z and Q_B are now known to be associated, both being located within the D1 protein.

Mode of herbicide action

As mentioned above many herbicides inhibit electron transfer reactions in PS2 by blocking Q_B reduction and possibly by slowing electron donation from water. An inevitable result of the inhibition is a decrease in plant growth rate because of the fall in levels of the essential products of the electron transfer reactions. Another means by which herbicides might damage plants is an increase in the yield of toxic oxygen radicals. An example of the mechanism which could be involved is given by our work on the isolated PS2 reaction centre. As discussed in an earlier section of this paper, much of the damage to the isolated complex caused by light can be explained by the generation of singlet oxygen produced as a consequence of the chlorophyll triplet formed when quinone reduction is blocked. A further role of herbicides is suggested by the results of Ohad et al, (1989). They show that occupancy of the Q_B site by herbicides can inhibit a light induced change in PS2 which is closely related to D1 degradation. At first sight this appears to be a protective effect of the herbicide. However, it is possible that it will lead to an increased long-term loss of PS2 activity in vivo because it is in fact a supression of the normal D1 repair mechanism. Certainly, the involvement of herbicides in the rapid, light-regulated turnover of D1 is an interesting area and an important subject of our own work. One significant result from our laboratory is the finding that D1 turnover involves specific amino acids located within the herbicide binding site of D1 (Shipton et al, 1989).

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USE OF PLANT CELL AND TISSUE CULTURES IN STUDIES OF HERBICIDE MODE OF ACTION AND METABOLISM

W J OWEN

Dow Chemical Company Limited, Letcombe Laboratory, Letcombe Regis, Wantage, Oxfordshire, OX12 9JT

ABSTRACT

In recent years plant cell cultures have been used increasingly as alternatives to *in vivo* plant and algal systems in studies of herbicide action and metabolism and for the isolation of herbicide resistant variants. Cell cultures are ideal axenic systems for such investigations and avoid problems relating to cuticular transfer and translocation within the plant. Concerns about the ability of white heterotrophic cultures to accurately record the phytotoxicity of photosynthesis inhibitors have largely subsided as a result of the development of green fully autotrophic cultures of many species. Suspension-cultured cells provide the added advantages of ease of manipulation, rapid kinetics and allow the opportunity of observing cellular consequences of primary site inhibition. Emphasis is placed on the parallelism between plants and cell cultures for herbicide action and metabolism purposes though discrepancies do arise.

INTRODUCTION

Studies concerning the screening, metabolism, mode of action and resistance of herbicides have traditionally been carried out using whole plants. More recently, however, investigators have been utilising cell-free extracts and enzyme preparations for the study of herbicide phytotoxicity, mechanism of action and metabolism. The gap between isolated enzyme systems and whole plants is being increasingly filled by studies utilising algae, mechanically or enzymatically isolated cells, and undifferentiated tissue or cell cultures (Mumma & Hamilton, 1979). Whilst algae have proved useful for screening and studies on the mode of action of herbicides inhibiting photosynthesis and carotenoid biosynthesis in particular, they do have permeability barriers and can react quite differently from plants to some herbicides. Some particularly important features of cell culture systems are their axenic nature, rapid kinetics (especially for metabolism studies) and ease of manipulation and maintenance for long periods of time. The phenomenon of somaclonal variation associated with cell cultures also renders them a particularly appropriate tool for the selection of herbicide resistant variants. These and other advantages of the use of plant cell cultures in a number of areas of herbicide research will be discussed, though attention will also be drawn to some possible limitations.

ESTIMATION OF GROWTH/PHYTOTOXICITY

Several methods are available to estimate the growth of cell suspension cultures and the relative merits of a number of commonly used procedures have been discussed previously (Zilkah & Gressel, 1978a). Plant cell cultures lend themselves to growth assessments based on techniques such as cell number, packed cell volume and turbidity which are not possible in the case of whole plant tissues. Some of the techniques available for estimating cell viability, such as precursor incorporation into macromolecules, membrane permeability to fluorescein and reduction of triphenyltetrazolium chloride (Zilkah & Gressel, 1978a) are also more convenient to administer in cell suspensions.

HERBICIDE SCREENING/SELECTIVITY

The question as to how closely cell cultures mirror the whole plants from which they are derived is of particular importance to their use in pre-screening programmes and recognition of selective properties.

A herbicide which has received a great deal of attention in the cell culture context, as an indirect consequence of its inclusion in most tissue culture media to meet the requirement for auxin, is 2,4-D. This and related synthetic auxins selectively control broadleaved herbaceous species and monocotyledonous plants such as the Gramineae are generally very tolerant. This difference is usually reflected very well in corresponding cell cultures. Whereas the auxin requirement for optimum growth of dicotyledonous cultures is met by concentrations up to 1 - 2mg per litre, much higher concentrations of 2,4-D (up to 15mg per litre) are frequently necessary to obtain maximum callus growth and to minimise root and shoot outgrowths in the Gramineae. Differences in biotype response to 2,4-D have also been noted in tissue culture. Thus two clones of field bindweed (Convolvulus arvensis L.) which differed in their susceptibility to the auxin exhibited similar differences when stem cells were cultured in both liquid and agar media (Harvey & Muzik, 1973). Differences between tobacco species with respect to their ability to utilise phenoxybutyric acids were also quite pronounced in callus culture indicating that biochemical differences relating to -oxidation were retained (McComb & McComb, 1978).

Since tissue cultures represent a mass of rapidly proliferating cells, they offer considerable potential for studying inhibition of mitosis. Consequently, it was not surprising that trifluralin, an inhibitor of tubulin polymerisation, was considerably phytotoxic to carrot callus tissue (Sloan & Camper, 1981). In an extensive investigation of a number of herbicides of differing biochemical modes of action (including 2,4-D, chlorpropham, mecoprop, napropamide, simazine, trifluralin and diuron), Zilkah and Gressel (1978b) found that green calli (albeit heterotrophic) gave a much better overall correlation with whole plant data than did achlorophyllous calli from the same species. However, the observations that three thiocarbamates and napropamide (see Gressel et al., 1978) were considerably more toxic to white calli led these authors to conclude that in order to develop a universal microscreen for herbicides both green and achlorophyllous systems are required.

Though heterotrophic cultures, whether or not they are green, fail to allow assessment of the full phytotoxicity of photosynthetic inhibitors they may be used to identify secondary sites of action likely to contribute to the overall phytotoxicity of such compounds. Thus in a study of the phytotoxicity and detoxification of metribuzin in dark-grown cell suspensions of resistant and susceptible soybean cultivars (Oswald et al., 1978) it was demonstrated that phytotoxicity was not restricted to photosynthesis. Enzymatic detoxification of metribuzin was inoperative in susceptible cell cultures, this being attributed to the presence of a specific inhibitor. Varietal differences in response to metribuzin were therefore apparent in the soybean cell suspensions also. Although metribuzin is a photosynthetic inhibitor that might not be expected to inhibit growth of dark-grown achlorophyllous suspension cultures, phytotoxicity and detoxification were apparently little affected by the absence of photosynthesis. In contrast, growth of non-photosynthetic cell suspensions of four tomato (Lycopersicon esculentum Mill) cultivars was not affected by metribuzin concentrations up to 150 ppm and there was no evidence of the differential tolerance observed at the seedling level (Ellis, 1978). In general agreement with Oswald et al., (1978), Abusteit et al., (1985) reported that the differing susceptibilities of diploid and tetraploid soybean cultivars to metribuzin extrapolated very well to heterotrophic suspension cultures where differential metabolism appeared to account for cultivar response.

Egli et al., (1985) have utilised [^{14}C] leucine incorporation as a measure of the effects of a range of herbicides on suspension-cultured heterotrophic black nightshade (Solanum nigrum) cells. Most herbicidal compounds could be distinguished from non-herbicidal analogues in a set of 47 compounds based on extent of inhibition of leucine incorporation. Herbicides which failed to inhibit leucine incorporation were photosynthetic inhibitors. Both phytotoxic and non-phytotoxic thiocarbamate analogues, as determined by whole plant studies, tended to inhibit leucine incorporation. These authors concluded that the leucine incorporation screen could detect a majority of compounds which are herbicidal and that it may also be useful to detect compounds which have cellular toxicity not observed in the whole plant.

The ability to observe and detect symptoms characteristic of photosynthesis inhibition, such as pigment bleaching, is improved by the use of photomixotrophic cultures which will photosynthesise but retain a dependency on some sugar (usually 0.2 - 0.3%) in the medium. Under these mixotrophic conditions, Cséplo and Medgyesy (1986) found that a number of photosystem-II herbicides (including triazines, ureas and uracils) at concentrations which inhibit photosynthetic electron transport, effectively inhibited the light-dependent component of callus growth and caused bleaching. Herbicides having no photosystem-II activity displayed total growth inhibition above a threshold concentration under both heterotrophic and photomixotrophic conditions. This latter group included diquat, paraquat, propachlor, amitrole and glyphosate. Bromacil and bentazon showed dual effects in that in addition to the symptom of inhibition of photosynthetic electron transport, higher concentrations caused total growth inhibition and necrotic dying of calli under both heterotrophic and photomixotrophic conditions. These latter results demonstrate that, in photomixotrophic and heterotrophic cultures, the

dual effects of such herbicides (i.e., inhibition of photosynthetic electron transport and uncoupling of oxidative phosphorylation) are clearly separable in a way which would be difficult to realise with intact plants. This line of investigation was extended recently by Sato et al., (1987) who compared the effects of herbicides with different primary modes of action on growth of heterotrophic, photomixotrophic and photoautotrophic suspension cultures of tobacco. In particular, since photoautotrophic cultures appear to be modified in some biochemical aspects of photosynthesis, even though they are fully photosynthetic, this investigation was aimed at assessing their use as a model for mesophyll cells. Herbicides which primarily inhibit or disturb photosynthetic processes (atrazine, diuron, paraquat) did suppress the growth of photoautotrophic cells most strongly. On the other hand compounds (2,4-D, diphenamid, glyphosate, dinoseb, bialaphos) having other primary modes of action inhibited growth of all types of cultured cells at similar concentrations though the photoautotrophic cells were still the most sensitive. With the exception of glyphosate and diphenamid, the photoautotrophic cells responded to herbicides as did treated seedlings. Such results led to the conclusion that photoautotrophic cultured cells are very appropriate tools in herbicide discovery research.

Phytotoxicity of glyphosate (Lee, 1980) has, however, been readily demonstrated in heterotrophic cultures of other species as for the ALS-inhibiting sulphonylureas (Chowdhury et al., 1987), imidazolinones (Anderson & Hibberd, 1985) and triazolopyrimidines (Subramanian & Gerwick, 1989). In the case of the sulphonylurea, 'Classic', species and soybean varietal differences in susceptibility were accurately represented in corresponding callus cultures (Chowdhury et al., 1987).

Studies on the influence of glyphosate on field bindweed (Convolvulus arvensis L.) biotypes in cell culture emphasise the importance of such factors as rate of growth of cultures on interpretation of results. Biotypes with the slowest growth rate were considerably more tolerant of glyphosate (Duncan & Weller, 1984). Differences in sensitivity to the acetyl CoA carboxylase inhibitor haloxyfop have been observed in suspension cultures of proso millet depending on culture age. 1 M haloxyfop was completely lethal to cultures when applied one day after subculture whereas 1mM haloxyfop was required to elicit the same response when applied three days later (Irzyk & Carpita, 1988). Differential inhibition of exponentially growing cells, compared to cells in stationary phase, by the plant growth regulator dikegulac (Zilkah & Gressel, 1978c) was in good agreement with the behaviour of the compound at the plant level where it inactivates the dividing cells of apical meristems. Though the biochemical mode of action of isoxaben (EL107) remains unknown, growth curves of heterotrophic callus cultures of Arabidopsis thaliana mimicked data obtained in vivo indicating that sensitivity to this herbicide also is not dependent on a differentiated function (Heim et al., 1989)

MODE OF ACTION STUDIES

Many of the ways in which cell cultures offer an advantageous tool for pinpointing biochemical target sites of herbicides will already be apparent from the previous section, and it is not intended to elaborate

on these here. Indeed some of the methods used in the assessment of phytotoxicity themselves provide clues as to the area of cellular biochemistry disrupted by particular compounds. The advent of fully autotrophic cell suspensions should now mean that such systems will serve as attractive alternatives to isolated mesophyll cells and plant tissues for studying mode of action of photosynthesis-inhibiting herbicides, also providing the added advantages of rapid kinetics and the opportunity of observing cellular consequences of primary site inhibition. For studies with target site enzymes, cell cultures may offer the further advantage of high specific activities as has been observed for acetolactate synthase (Subramanian & Gerwick, 1989; Subramanian, personal communication) and acetyl CoA carboxylase (Owen, unpublished results).

Worthy of special mention is the particular contribution made by cell culture systems to the elucidation of the biochemical sites of action of herbicides which inhibit amino acid biosynthesis. Suspension cultured cells in particular lend themselves readily to evaluation of the addition of various growth supplements to media as for example in the identification of auxotrophs. The striking alleviation of inhibition of culture growth in the presence of glyphosate (Gresshoff, 1979) and the ALS-inhibiting imidazolinones (Anderson & Hibberd, 1985) and triazolopyrimidines (Subramanian & Gerwick, 1989) by medium supplementation with aromatic acids and valine, leucine and isoleucine respectively, rapidly focused attention on the corresponding amino acid biosynthetic pathways as likely primary targets.

Cell cultures have also proved very beneficial in the elucidation of the mechanism of action of nitrodiphenylethers such as acifluorfen-methyl. Phytotoxicity in the case of these compounds is light-dependent but the interpretation of action spectra obtained using green seedlings or algae was confounded by the presence of chlorophylls and carotenoid pigments. Studies with nonchlorophyllous soybean cultures further depleted of carotenoids by the inclusion of norflurazon, a carotenoid biosynthesis inhibitor, in the culture medium indicated that neither chlorophyll nor carotenoids were required for sensitivity to acifluorfen-methyl. Extension of this study with the nonchlorophyllous soybean suspension culture led to the demonstration that acifluorfen- methyl inhibited porphyrin biosynthesis as indicated by the accumulation of tetrapyrroles, especially protoporphyrin IX, detected by fluorescence measurements (Matringe & Scalla, 1988).

A few studies have also been carried out on the mechanisms of action of herbicide safeners using cell culture systems, providing data which correlates well with whole plant results. One of the effects of diallyl-dichloroacetamide (R-25788) which protects corn from thiocarbamate injury, is to increase the glutathione level (already high in corn) to a concentration enabling glutathione S-transferase to efficiently detoxify these herbicides. Gressel et al., (1978) found that glutathione levels characteristic of plants of a number of species carried through to the respective callus cultures as did enhancement by R-25788. Similar results were obtained in our own laboratory with respect to glutathione levels in plants and corresponding cell cultures of rice and maize. There was also a parallel response to the rice chloracetanilide safener CGA123407 in plants and cell culture. Glutathione levels were elevated by the safener in both plants and

suspension cultures of both species but activity of glutathione S-transferase was increased only in rice plants and cells. (Barton, Edwards & Owen, unpublished observations).

METABOLISM STUDIES

Though herbicide metabolism studies may be carried out using both callus and suspension cultures, most researchers have chosen to use the latter because of their ease of manipulation and the increased possibility for standardisation of techniques between laboratories (Mumma & Hamilton, 1979). Axenic cell suspension cultures represent a means by which observed transformations may be attributed unequivocally to plant, rather than microbial enzymes. In addition, studies made with an increasing selection of herbicides of differing chemistries have concluded that transformations are generally more rapid in cell suspension, which also display the same spectrum of enzymic detoxification reactions as their plant counterparts.

Their capacity for enhanced metabolism is a consequence of a dense and rapidly growing population of cells which presents a large surface area to the added compound, and of the absence of the limiting factors of cuticular penetration and movement within the plant. Thus metabolites representing advanced stages of breakdown can often be identified over periods of days rather than weeks for whole plants. Recovery of the parent molecule and metabolites is often more favourable, since less bound products are likely and volatilisation problems are minimised. Extraction of tissues and clean-up of metabolites is easier, and particularly facilitates the analysis of less significant metabolites difficult to extract in quantity from whole plants. A better understanding of the control of pesticide degradation processes may allow the additional possibility of diverting pathways to produce artificially large amounts of such minor residues for analysis or to alter herbicide selectivity.

Since the most recent reviews on this topic (Mumma & Davidonis, 1983, Swisher, 1987) reports have appeared describing the ability of suspension cultures of various species to metabolise chlortoluron (Owen & Donzel, 1986) triclopyr (Lewer & Owen, 1987), metolachlor (Edwards & Owen, 1986), chlorsulfuron (Swisher & Weiner, 1986), bentazon (Sterling & Balke, 1989) and terbutryn (Edwards & Owen, 1989). These recent studies further confirm the earlier emerging view that suspension-cultured cells constitute a useful and generally reliable system for interspecies comparisons with respect to herbicide metabolism. Indeed, cultivar differences in the metabolism of metribuzin (Abusteit *et al.*, 1985) and bentazon (Sterling & Balke, 1989) observed in soybean plants and of chlortoluron in wheat (Owen, unpublished observations) were also accurately reproduced in corresponding cell suspensions.

Though metabolic profiles of herbicides are therefore qualitatively the same in both plants and cell cultures quantitative differences do exist. Such differences in proportions of metabolites might arise in some cases from rapid export of phase I metabolites into culture media or, alternatively, from a particularly efficient sugar conjugation mechanism, promoted perhaps by the presence in the medium of large

amounts of sucrose. In either case the net result would be the effective removal of phase I metabolites thus rendering them inaccessible to further metabolic attack. Other factors such as stage of growth, explant source, culture age and medium composition have also to be considered. Thus whereas metabolism of propanil (Ray & Still, 1975) by suspension cultures of rice was most rapid in stationary phase cells, transformation of diphenamid by soybean cultures was considerably more pronounced in the early log phase of growth (Davis *et al.*, 1978). The absence of balanced growth in batch cell suspension cultures certainly does not preclude their use in herbicide metabolism studies, but an accurate growth analysis prior to herbicide application and an appreciation of the rapid changes that can occur during the growth cycle are essential.

Influence of explant source on metabolism has been observed previously in studies of the fate of the synthetic auxin, 2,4-D in cultured cells of soybean (Mumma & Hamilton 1979). Although no other herbicide has been studied in such a methodical way, a similar study in our own laboratory using the fungicide metalaxyl revealed very little variation in either rate or qualitative nature of metabolism between lettuce (*Lactuca sativa* L.) cultures newly isolated from cotyledon, hypocotyl and root tissues (Cole & Owen, 1987 a). However, a suspension culture initiated from 4 year old hypocotyl callus produced fewer identifiable metabolites and had an impaired ability to perform alkyl hydroxylation, *O*-dealkylation, and ester hydrolysis. Studies on the fate of chlortoluron in the same hypocotyl-derived suspension cultures of lettuce (Cole & Owen, 1988) indicated that a selective decline in the ability to perform ring methyl hydroxylation of chlortoluron had occurred in the older culture.

Chlortoluron was also the subject of a study designed to assess the influence of different culture media on metabolism (Cole & Owen, 1987 b). Metabolism in both cotton and maize suspensions was qualitatively unperturbed with respect to organic nutrients and 2,4-D concentration. Whereas the rate of metabolism in cotton did not change over a fifty-fold range in 2,4-D concentration, in maize there was a distinct trend towards accelerated metabolism up to a 2,4-D concentration of 20mg litre^{-1}, which was independent of effects on growth. The absence of any significant shift in the relative contribution of *N*-didemethylation or ring-methyl hydroxylation to chlortoluron metabolism in this study was suggestive of strict control being exerted on these transformations and a substantial independence from the cell's environment. Thus age-dependent change in metabolic capabilities may constitute a more important limiting factor than nutrient medium composition or explant souces. Hence the use of long-established tissue cultures for metabolism purposes should probably be avoided and workers need to be aware of the history of cultures acquired from other laboratories.

It would be wrong to suggest that, apart from quantitative variations such as those discussed above, plant cell cultures always transform a particular pesticide in a manner identical to that established for whole plants. Qualitative discrepancies can also occur as illustrated in the case of cisanilide (Frear & Swanson, 1975). Since xenobiotics are thought to be attacked largely by enzymes having specific roles in secondary metabolism, such differences, though very rare, may in part be due to the fact that many such enzymes are not fully expressed in

cultured cells. This was clearly shown to be the case in a cell-suspension culture of maize (Black Mexican Sweet) in which an inability to metabolise atrazine, in contrast to maize plants, was attributed to lack of expression of the atrazine-specific glutathione S-transferase in dedifferentiated tissue (Edwards & Owen, 1986).

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RECENT DEVELOPMENTS IN THE PHYSIOLOGY AND BIOCHEMISTRY OF HERBICIDE SAFENERS

KRITON K. HATZIOS

Laboratory for the Molecular Biology of Plant Stress, Department of Plant Pathology, Physiology and Weed Science, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0330, U.S.A.

ABSTRACT

Herbicide safeners are chemicals used for manipulating the tolerance of large-seeded grass crops to selected soil-applied herbicides. Safeners may act either as "bioregulators" influencing the amount of a given herbicide that reaches its target site in an active form or as "antagonists" of herbicidal effects at a similar site of action. A safener-induced enhancement of herbicide detoxication in protected plants seems to be the most apparent mechanism for the action of the currently marketed safeners. Safeners enhance the conjugation of carbamothioate and chloroacetanilide herbicides with glutathione either by elevating the levels of reduced glutathione (GSH) or by inducing the activity of specific glutathione S-transferases (GST). Enhancement of oxidative reactions may also contribute to the protective action of herbicide safeners. Metabolic processes related to acetyl-CoA metabolism appear to be likely target sites for a competitive antagonism between safeners and chloroacetanilide or carbamothioate herbicides. At the molecular level, the "gene activation" theory offers a likely explanation for the action of safeners.

INTRODUCTION

"Herbicide safeners" (also known as "herbicide antidotes") are chemical substances that selectively protect crop plants against herbicide injury (Hatzios, 1989a). The selectivity of herbicide safeners results either from a selective placement of the safener or is due to biochemical principles. Thus, some safeners are applied directly to crop seeds prior to planting, while others possess true selectivity and are applied to the crop and weeds as prepackaged mixtures with the herbicide (Hatzios, 1989a).

Commercialized safeners are members of diverse chemical groups including naphthopyranones such as naphthalic anhydride (NA, naphthalene-1, 8-dicarboxylic acid anhydride); dichloroacetamides such as dichlormid (2,2-dichloro-N,N-di-2-propenylacetamide) and CGA-154281 [4-(dichloroacetyl) -3,4-dihydro-3-methyl-2H-1,4-benzoxazine]; oxime ether derivatives such as oxabetrinil [α-(1,3-dioxolan-2-yl-methoxy)-iminobenzeneacetonitrile] and CGA-133205 {O-[1,3-dioxolan-2-yl-methyl]-2,2,2-trifluoro-4'-chloroacetophenone-oxime}; substituted thiazoles such as flurazole [phenylmenthyl 2-chloro-4-(trifluoro-methyl)-5-thiazolecarboxylate]; dichloromethyldioxolans such as MG-191 (2-dichloromethyl-2-methyl-1, 3-dioxolane); and phenylpyrimidines such as fenclorim (4,6-dichloro-2-phenyl-pyrimidine). Most of the currently marketed safeners are particularly effective in protecting large-seeded grass crops such as corn (*Zea mays* L.), grain sorghum [*Sorghum bicolor* (L.) Moench,], and rice

(Oryza sativa L.) against soil-applied, shoot-absorbed herbicides such as the carbamothioates and chloroacetanilides. Safeners, however, can also protect grass crops against injury caused by several diverse groups of herbicides such as the sulfonylureas, imidazolinones, aryloxyphenoxypropionates, cyclohexanediones, and isoxazolidinones (Hatzios 1989a).

Investigations into the possible physiological or biochemical mechanisms of action of herbicide safeners were conducted first in the early 1970s, soon after the introduction of naphthalic anhydride and dichlormid as protectants of corn against carbamothioate herbicide injury (Pallos and Casida 1978). Since then, numerous articles on the physiology and biochemistry of the action of herbicide safeners have been published and they have been summarized in several comprehensive reviews (Fedtke and Trebst 1987, Hatzios 1983, Hatzios and Hoagland 1989, Pallos and Casida 1978, Parker 1983, Stephenson and Ezra 1987). The purpose of this report is to summarize recent developments in the physiology and biochemistry of the action of herbicide safeners.

SPECIFICITY OF CROP-HERBICIDE-SAFENER COMBINATIONS

A recent analysis of the literature related to the physiological aspects of the successful crop-herbicide-safener combinations (Hatzios 1989b) showed that the interactions of safeners and antagonized herbicides are characterized by four major facts: a) safeners are most effective when applied prior to or simultaneously with the herbicides whose injury they prevent; b) safeners exhibit a high degree of botanical and chemical specificity protecting only certain grass crops against injury from specific herbicides; c) the emerging shoot (coleoptile) of the protected grass seedlings appears to be the site of uptake and action of both safeners and antagonized herbicides; and d) protected grass crops are moderately tolerant to the antagonized herbicides;

The botanical specificity of the commercialized safeners (only grass crops are protected) and the moderate tolerance of the protected grass crops to the antagonized herbicides suggest that the protective action of the safeners may be related to some physiological or biochemical function which is either unique or highly efficient in these grass crops. The ability to detoxify certain herbicides by specific biochemical reactions is not evenly distributed among various plant species and it has long been recognized as an important process contributing to the selectivity of herbicides as well as to the protective action of herbicide safeners (Hatzios 1989b).

The chemical specificity exhibited by the currently marketed herbicide safeners has been partially attributed to their structural similarity to carbamothioate and chloroacetanilide herbicides. A good example of such a case is the safener dichlormid which is structurally very similar to EPTC or other carbamothioate herbicides (Stephenson et al. 1978). The α-chloroacetamide derivative allidochlor or CDAA, which is chemically almost identical to the safener dichlormid, acts both as a safener and as a herbicide depending on the concentration used (Stephenson and Ezra 1987). In other cases, however, the structural similarity of herbicides and their respective safeners is not so obvious. Nevertheless, studies employing the use of computer-assisted molecular modelling (CAMM) showed that at the molecular level, herbicides and their respective safeners are quite similar (Yenne and Hatzios, 1989a). Comparison of the molecular parameters measured for the

safener/herbicide pairs of flurazole/alachlor and CGA-133205/metolachor, showed that these molecules posses similar degrees of bonding and charge distribution as well as volumes.

Therefore, safeners may act by elevating the levels of substrates or the activity of enzymes involved in the detoxication of their respective herbicides in moderately tolerant plants. According to this mechanism, safeners act as "bioregulators" influencing the amount of a herbicide that reaches its target site in an active form (Hatzios 1989b). Alternatively, it has been proposed that safeners may antagonize the action of chemically similar herbicides by competing with them at a common target site or by eliciting opposite effects at different steps of the same physiological process. This potential mechanism is known as the "antagonist" hypothesis of safener action (Hatzios 1989b).

ENHANCEMENT OF HERBICIDE DETOXICATION BY SAFENERS

A safener-induced enhancement of herbicide detoxication in protected plants seems to be the major mechanism involved in the protective action of the currently developed safeners. Safeners enhance the glutathione conjugation of chloroacetanilide and sulfoxidized carbamothioate herbicides either by elevating the levels of reduced glutathione (GSH) or by inducing the activity of glutathione-dependent enzymes. Enhancement of the activity of oxidative enzymes involved in herbicide detoxication may also contribute to the protective action of herbicide safeners.

Effects of Safeners on Glutathione and Glutathione-Dependent Enzymes

A safener-induced elevation in the levels of GSH provides safened plants with sufficient GSH needed for the normal function of key metabolic processes such as protein synthesis, protection of chloroplast membranes from peroxidative damage and detoxication of the antagonized herbicides (Rennenberg 1982, 1987).

Safeners may elevate GSH levels in protected plants either directly or indirectly. A direct enhancement of GSH synthesis in safener-treated plants may result from a safener-induced activation of key enzymes involved in the biosynthesis of GSH. The GSH biosynthetic pathway in plants is a two-step process involving first the coupling of glutamate and cysteine to form γ-glu-tamyl-L-cysteine and then the addition of glycine to the C-terminal of this dipeptide. The first reaction is catalyzed by the enzyme glutamyl-cysteine synthetase or GSH synthetase I (EC 6.3.2.2). The second step is catalyzed by synthetase II (EC 6.3.2.3) (Rennenberg, 1982, 1987). The overall regulation of GSH synthesis is through feedback inhibition of GSH synthetase I by the end product of the pathway, GSH. Studies with mammalian systems have shown that GS-conjugates of xenobiotics can bind to GSH synthetase I and override the feedback inhibition caused by GSH (Kondo et al. 1984). A similar mechanism has been suggested by Breaux et al. (1989) to explain the enhanced GSH levels observed in corn and grain sorghum seedlings treated with the safener flurazole which conjugates with GSH. Carringer et al. (1978) reported that the safener dichlormid increased the activity of GSH Synthetase II from corn in vitro. GSH synthetase II levels, however, in untreated and dichlormid-treated corn seedlings were similar (Carringer et al. 1978).

Dichlormid and other dichloroacetamide safeners may enhance GSH synthesis in protected grass plants by affecting directly the assimilation of sulfate. Adams et al. (1983) showed that dichlormid enhanced the activity of ATP-sulfurylase (EC 2.7.7.4), the first enzyme in sulfate assimilation, which catalyzes the reaction of between ATP and sulfate to yield adenosine-5'-phosphosulfate. More recently, however, Farago and Brunold (1989) showed that the dichloroacetamide safeners of corn, dichlormid and CGA-154281, were more effective in enhancing the activity of adenosine-5'-phosphosulfate sulfotransferase rather than the activity of ATP-sulfotransferase. The effects of these two safeners were more pronounced on the activity of this enzyme extracted from corn roots rather than shoots (Farago and Brunold 1989).

Safeners may elevate GSH levels in protected plants indirectly by inducing the activity of glutathione reductase (GR, WC 1.6.4.2). GR is a NADPH-dependent enzyme which catalyzes the reduction of oxidized glutathione (GSSG) to GSH. An induction of GR by safeners will maintain a high GSH/GSSG ratio in the cells of protected grasses compensating for GSH used as a reductant in the formation of the GS-conjugates of chloroacetanilide and sulfoxidized carbamothioates or in the ascorbate-dehydroascorbate redox system of the chloroplast. Kömives et al. (1985) showed that dichlormid and MG-191 enhanced GR activity of corn seedlings by 2.5-fold. Significant enhancement of GR activity was also observed in grain sorghum seedlings treated with the oxime ether safener CGA-133205 (Yenne and Hatzios 1989b).

Enhanced metabolism of chloroacetanilide and sulfoxidized carbamothioate herbicides by GSH conjugation could result also from a safener-induced increase of the activity of the respective glutathione-S-transferase enzymes (GSTs, EC 2.5.1.18) which catalyze this reaction in protected grass crops. Plants contain multiple forms of GST enzymes which exhibit a rather high degree of substrate specificity (Timmerman 1989). At present, however, only the GST enzymes from corn have been studied in any detail. Three GST isozymes exhibiting a high specificity for chloroacetanilide herbicides and at least two isozymes with high specificity for s-triazine herbicides are known to exist in corn (Timmerman 1989). GST isozymes are usually dimeric proteins having an approximate molecular weight of 50,000.

A strong correlation between the efficacy of a safener in protecting grain sorghum from chloroacetanilide injury and its ability to increase GST activity has been demonstrated (Gronwald 1989). Flurazole was the most effective sorghum safener eliciting a 30-fold increase in GST activity. Oxabetrinil and NA were also effective safeners of sorghum against metolach-lor injury causing a 20-fold and 17-fold increase of GST activity, whereas dichlormid was the least effective safener of sorghum causing only a 5-fold increase in GST activity. Mozer et al. (1983) showed that flurazole not only enhanced the activity of maize GSTs that are constitutively present, but it also induced a novel GST isozyme with greater activity in conjugating chloroacetanilide herbicides with GSH. The exact mechanism of the safener-induced enhancement of GST activity is not known. It appears likely that safeners act by an enzyme induction process rather than an enzyme activation since in in vitro studies dichlormid and oxabetrinil did not alter the activity of GST enzymes (Gronwald 1989).

Effects of Safeners on Oxidative Enzymes

Safeners may act also by inducing the activity or the de novo synthesis of mixed function oxidases (MFOs, EC 1.14.14.1) involved in the metabolic detoxication of chloroacetanilide and carbamothioate herbicides in protected grass crops (Fedtke and Trebst 1987, Hatzios 1989b). Such a hypothesis is supported by several indirect studies conducted with the use of selected antioxidants or insecticide synergists which act as inhibitors of MFO enzymes. Direct studies on the effects of herbicide safeners on the activity of specific cytochrome P-450 containing MFO enzymes are limited. Dichlormid and NA did not change the levels of cytochrome P-450 and the activity of cinnamic acid 4-hydroxylase (EC 1.14.13.1) of etiolated corn seedlings (Kömives and Dutka 1989). Similarly, pretreatment of grain sorghum coleoptiles did not enhance the activity of an epoxidase (EC 1.14.14.1) which metabolizes the insecticides aldrin and dieldrin (Ketchersid et al. 1985).

Based on the similarities of the reported effects of carbamothioate and chloroacetanilide herbicides as well as the interactive effects of safeners and synergists with these herbicides, Fedtke and Trebst (1987) proposed a model attempting to explain the actions and interactions of these herbicides and their safeners. The central feature of this model is the function of MFO enzymes involved in plant metabolism as well as in the detoxication of herbicides and safeners. Certain herbicides and safeners are suggested as inducers of the activity of these MFO enzymes. Therefore, MFOs that participate in the oxidative metabolism of herbicides are available at high levels and contribute to enhanced detoxication and crop protection.

Other oxidative enzymes that have been studied in relation to the mechanism of action of herbicide safeners include peroxidase (EC 1.11.1.7) and polyphenol oxidase (EC 1.10.3.2). Peroxidases are hemoproteins which catalyze the oxidation of their substrates utilizing hydrogen peroxide. Apart from their association with lignification, peroxidases catalyze the oxidation of indoleacetic acid (IAA), the hydroxylation of proline, and they may participate in the metabolic detoxication of xenobiotics in plants (Butt 1980; Lamoureux and Frear 1979). Harvey et al. (1975) reported that the safener dichlormid reduced peroxidase activity in corn seedlings and counteracted the stimulatory effects of the herbicide EPTC on the activity of this enzyme. Polyphenol oxidase is a chloroplast oxidase with no established function (Vaughn et al. 1988). Wilkinson (1978) reported that the safener NA stimulated the activity of this enzyme in corn.

It is evident from the above discussion that our understanding of the interactions of herbicide safeners with plant oxidative enzymes is far fromcomplete and awaits further investigation.

INTERACTIONS OF HERBICIDES AND SAFENERS AT THE SITE OF ACTION

The chemically diverse groups of herbicides that are antidoted by safeners on grass crops exert their action by a variety of biochemical mechanisms which are well-defined for sulfonylureas, imidazolinones, isoxazolidinones, aryloxyphenoxypropionates, and cyclohexanediones, but still speculative for carbamothioates and chloroacetanilides (Böger and Sandmann 1989).

Sulfonylurea and imidazolinone herbicides are potent inhibitors of the enzyme acetohydoxyacid synthase (AHAS, EC 4.1.3.18) and of the biosynthesis of branched chain amino acids (Mazur and Falco 1989). Rubin and Casida (1985) reported that pretreatment of corn with dichlormid elevated AHAS activity contributing partially to the protection of corn against injury from the herbicide chlorsulfuron. Polge et al. (1987), however, reported that while NA and dichlormid enhanced the activity of AHAS in treated corn seedlings, AHAS extracted from safened plants was more sensitive to chlorsulfuron inhibition. In other studies, Barrett (1989) failed to detect any measurable effects of the safeners NA, oxabetrinil, dichlormid and flurazole on extractable AHAS activity in shoots or roots or corn seedlings.

Aryloxyphenoxypropionate and cyclohexanedione herbicides have been recently identified as potent inhibitors of the enzyme acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) (Harwood 1988). Oxime ether safeners such as oxabetrinil and CGA-133205 did not affect the activity of ACCase (Yenne and Hatzios 1989c). Dichlormid antagonized the effects of sethoxydim on lipid synthesis of isolated grain sorghum protoplasts (Hatzios and Moon 1985), but the effects of this safener of ACCase activity have not been examined.

The symptomology of carbamothioate and chloroacetanilide herbicides is not indicative of an acute blockage of a central metabolic reaction in susceptible plants, but it is rather consistent with a chronic loss of vital metabolic components (Wilkinson, 1978). Shoot deformations and growth inhibitions caused by these herbicides could result from their reported effects on cell division and cell elongation in tissues of treated grass plants (Fedtke 1982). Some of the symptoms (e.g. stunting) caused by carbamothioate and chloroacetanilide herbicides on corn and grain sorghum seedlings are similar to those observed in grasses treated with classical growth retardants (Wilkinson 1978). In fact, the stunting effects of EPTC and alachlor on corn could be prevented by exogenous applications of gibberellin (GA), but the leaf deformations (twisiting and rolling) were not prevented (Wilkinson 1978). At the cellular level, metabolic processes that are affected by chloroacetanilide and/or carbamothicate herbicides include lipid synthesis, terpenoid synthesis, lignin biosynthesis, protein synthesis, nucleic acid synthesis, membrane function and ion transport (Fuerst 1987; LeBaron et al. 1988; Wilkinson 1988). Consequently, antagonistic interactions between these herbicides and safeners at the aforementioned sites of action are possible and they have been reviewed (Hatzios 1989b).

Because many of the plant metabolic products affected by chloroacetanilide and carbamothioate herbicides are synthesized via acetyl-CoA intermediates, it has been proposed that the action of these herbicides may be related to some aspect of acetyl-CoA metabolism (Fuerst 1987). Wilkinson (1988) suggested recently that metolachlor may act by inhibiting the activity of α-amylase (EC 3.2.1.1) and isocitric lyase (EC 4.2.1.3), two key enzymes involved in the breakdown of seed storage products to form acetate and acetyl-CoA. The metolachlor-induced inhibition of α-amylase extracted from grain sorghum was reversed by the safener oxabetrinil (Wilkinson, 1988). Yenne and Hatzios (1989c) have also reported that metolachlor and oxime ether safeners appear to affect the formation of acetyl-CoA rather than the incorporation of acetyl-CoA into fatty acids or other lipids of grain sorghum seedlings.

MOLECULAR ASPECTS OF SAFENER ACTION

Alteration of structural genes, gene regulation, and gene amplification are currently recognized as significant genetic factors conferring herbicide tolerance or resistance on agronomic crops (Mazur and Falco 1989). A number of selected plant enzymes catalyzing the metabolic detoxication of specific herbicides in higher plants have been isolated and partially characterized (Hatzios 1987). In most cases, however, the genes coding for these enzymes as well as their regulation by herbicide safeners have not been studied in detail.

As mentioned earlier, three GST isozymes, designated as GST I, GST II, and GST III, have been purified and characterized from corn (Timmerman 1989). All three isozymes catalyze the conjugation of chloroacetanilide herbicides with GSH, but while GST I and GST III are constitutively present in corn, GST II is seen only in safener-treated corn (Mozer et al. 1983). DNA sequences for the GST I and GST III isozymes from corn have been reported and they show some sequence similarity to each other and to other known GST sequences (Timmerman 1989). Wiegand et al. (1986) showed that the safener flurazole may act at the transcriptional level inducing a 3- to 4-fold increase in the steady state level of mRNA encoding for the GST I gene in corn tissues grown from flurazole-treated seeds. Therefore, it appears that the "gene activation" theory which has been implicated in the action of natural or synthetic plant hormones (Theologis 1986), is also promising for explaining the protective action of herbicide safeners.

If safeners indeed act at a transcriptional level by regulating the expression of selected plant genes then their molecular mechanism of action should include an induction of mRNA, which is rapid, specific and unaffected by protein synthesis inhibitors. Wiegand et al. (1986) showed that the induction of mRNA by flurazole is very specific and rapid, but studies on the effect of protein synthesis inhibitors on this effect of safeners are not available. The potential involvement of positive or negative control models in the regulation of safener-inducible genes for GSTs or other enzymes in protected plants has been postulated (Hatzios 1989b). Undoubtedly, further research is needed to elucidate the molecular mechanisms of action of herbicide safeners.

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SELECTION FOR TOLERANCE TO GRAMINICIDE HERBICIDES IN MAIZE TISSUE CULTURE

J.W. GRONWALD[1], W.B. PARKER[2], D.A. SOMERS[2], D.L. WYSE[2], B.G. GENGENBACH[2]

[1]USDA-Agricultural Research Service and [2]Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108, USA.

ABSTRACT

Embryogenic cell cultures of maize (Zea mays L., A188 X B73) were selected for tolerance to sethoxydim or haloxyfop using an enrichment selection strategy. One cell line (S2), selected in the presence of sethoxydim, was > 40-fold more tolerant to sethoxydim and 20-fold more tolerant to haloxyfop. Another cell line (H2), selected in the presence of haloxyfop, was 3-fold more tolerant to sethoxydim and 2-fold more tolerant to haloxyfop. As compared to the unselected line, acetyl-CoA carboxylase activity in S2 was less sensitive to inhibition by sethoxydim or haloxyfop. In the H2 line, acetyl-CoA carboxylase activity was 5-fold higher than that in unselected lines, but enzyme activity was equally sensitive to inhibition by haloxyfop or sethoxydim. The S2 line was regenerated from tissue culture and exhibited tolerance to foliar applications of haloxyfop or sethoxydim.

INTRODUCTION

Tissue culture systems offer certain advantages when selecting for herbicide tolerance in plants. A large number of cells can be rapidly screened. Furthermore, tissue culture per se spontaneously induces genetic variability in cells (Lee and Phillips, 1988) which increases the probability of selecting for variant cells tolerant to herbicides. Tissue culture systems have been used successfully to select for cells tolerant to glyphosate (Steinrücken et al., 1986; Dyer et al., 1988), phosphinothricin (Donn et al., 1984), sulfonylurea (Chaleff and Ray, 1984), and imidazolinone (Anderson and Georgeson, 1986; Saxena and King, 1988) herbicides. Tolerance has been shown to be due to either the overproduction of the target site enzyme (Donn et al., 1984; Steinrücken, et al., 1986; Dyer et al., 1988) or the presence of an altered enzyme that is less susceptible to inhibition by the herbicide (Chaleff and Ray, 1984; Anderson and Georgeson, 1986; Saxena and King, 1988).

Two classes of herbicides, the aryloxyphenoxypropionic acids and the cyclohexanediones, referred to as graminicides, are used to control grass weeds in broadleaf crops. Recent evidence suggests that the selectivity of these herbicides is primarily based on their differential inhibition of the enzyme acetyl-CoA carboxylase (ACCase) in grasses and dicots (Burton et al., 1987; Focke and Lichtenthaler, 1987; Secor and Cséke, 1988; Rendina and Felts, 1988; Rendina et al., 1988; Walker et al., 1988). Both herbicide classes have little or no effect on the enzyme from dicots but with few exceptions, are potent inhibitors of ACCase from grasses. One grass that is an exception is red fescue where tolerance to the cyclohexanedione, sethoxydim, and the aryloxyphenoxypropionic acid, haloxyfop, is due to the presence of a tolerant form of ACCase (Stoltenberg et al., 1989).

The objectives of this study were to: (1) select for mutants tolerant to sethoxydim and haloxyfop in embryogenic maize tissue culture; (2) determine whether tolerance was due to modified ACCase activity in the selected lines; (3) regenerate plants from the selected tolerant lines and evaluate tolerance at the whole plant level.

MATERIALS AND METHODS

Maize tissue culture

Friable, embryogenic maize (Zea mays L.) callus obtained from the cross (A188 x B73) was used. The callus was maintained in low light (20 $\mu E \cdot m^{-2} \cdot s^{-1}$) on solid N6 medium (Chu et al., 1975) supplemented with 2% sucrose, 25 mM L-proline, 100 mg Casamino acids and 1 mg 2,4-D/litre at pH 5.8 (Armstrong and Green, 1985). Callus was plated as 5 callus pieces (0.5 g) per petri plate and subcultured by dividing callus on each plate 1:5 at two-week intervals.

Selection for herbicide tolerance

To select for herbicide tolerance, callus (five 0.5 g callus pieces/petri plate) was transferred to N6 medium containing specified levels of technical grade sethoxydim or haloxyfop. Sethoxydim was diluted in 95% ethanol, filter-sterilized, and added to cool autoclaved N6 medium prior to pouring. Haloxyfop was added to the N6 medium prior to autoclaving. At two week intervals, the most vigorously growing 0.5 g sector from each callus was subcultured onto fresh medium containing increased levels of sethoxydim (0.5, 1.0, 2.0, 5.0, 10 µM) or haloxyfop (0.01, 0.02, 0.05, 0.1, 0.2 µM). Callus lines exhibiting growth on 10 µM sethoxydim and 0.2 µM haloxyfop were designated S2 and H2, respectively.

Herbicide tolerance

Callus (ca. 20 g fresh wt) of the unselected line, S2 and H2 were grown in the absence of the herbicides for 2 weeks. Callus for each line was mixed to form a uniform cell population for evaluation of herbicide tolerance. Callus (0.5 g) from each line was then spread onto sterile Whatman #1 filter paper disks that were overlayed onto culture medium containing various concentrations of sethoxydim and haloxyfop. Dry weights were obtained after 2 weeks.

Acetyl-CoA carboxylase activity

The callus of the unselected, H2 or S2 lines was homogenized with a mortar and pestle at 4°C in a medium containing 0.1 M tricine-KOH, pH 8.3, 0.3 M glycerol, and 5 mM dithiothreitol. The extract was filtered through 2 layers of Miracloth and centrifuged at 10,000 g for 30 min. The supernatant was desalted on a Sephadex G-25 column which had been equilibrated with the homogenizing medium. ACCase activity was assayed by measuring the incorporation of $H^{14}CO_3^-$ into malonyl-CoA as described by Burton et al. (1989a). ACCase activity was expressed as a percentage of the activity measured in each line in the absence of herbicides. Protein was determined by the procedure of Smith et al. (1985).

Plant regeneration

Plants of S2 and the unselected callus line were regenerated by placing several callus pieces (150 mg) which exhibited embryogenic morphology onto modified N6 medium containing 6% sucrose but no hormones. After 7-10 days, the callus was transferred to a medium containing MS salts (Murashige and Skoog, 1962), 2% sucrose, 150 mg/litre L-asparagine and no hormones at pH 5.8 (MS-OD). Developing plantlets were isolated from the callus surface and transferred to magenta boxes containing 50 ml MS-OD to allow for further root and shoot development. At the 2-3 leaf stage, plants were transferred to peat pots containing potting soil:vermiculite (1:1 v/v) and grown in a culture room for 7 to 14 days. Plants were then transferred to pots containing soil and moved to a greenhouse.

Tolerance of regenerated plants

Regenerated plants from unselected and S2 lines growing in the greenhouse were grouped into blocks based upon plant height. Sethoxydim concentrations of 0, 0.01, 0.05, 0.11, 0.22 or 0.44 kg/ha plus 2.3 litre/ha crop oil concentrate were applied in 187 litre/ha at 207 kPa. To evaluate haloxyfop tolerance, regenerated plants were sprayed with 0, 0.005, 0.01, 0.05, 0.11 or 0.22 kg/ha haloxyfop plus crop oil concentrate. Plant heights were measured 14 DAT. Measurements were made from the soil surface to the top of the whorl.

RESULTS

Tolerance of selected lines

The enrichment selection strategy employed yielded two tolerant cell lines. One tolerant line (S2) was obtained using sethoxydim as the selection agent and another cell line (H2) was obtained using haloxyfop as the selection agent. The degree of tolerance of the selected lines was expressed in terms of GR_{50} values (herbicide concentration required to inhibit callus growth [dry weight increase] by 50%). The GR_{50} values for the unselected line and S2 were 2.6 and >100 µM for sethoxydim, respectively (Fig 1A). For haloxyfop, the GR_{50} values for the unselected and S2 lines were 0.04 and 0.82 µM, respectively (Fig. 1B). Hence, as compared to the unselected control, the S2 line was > 40-fold more tolerant to sethoxydim and 20-fold more tolerant to haloxyfop. For the H2 line, the GR_{50} values for sethoxydim and haloxyfop were 8.4 and 0.08 µM, respectively. As compared to the unselected line, the H2 line was 3-fold more tolerant to sethoxydim and 2-fold more tolerant to haloxyfop.

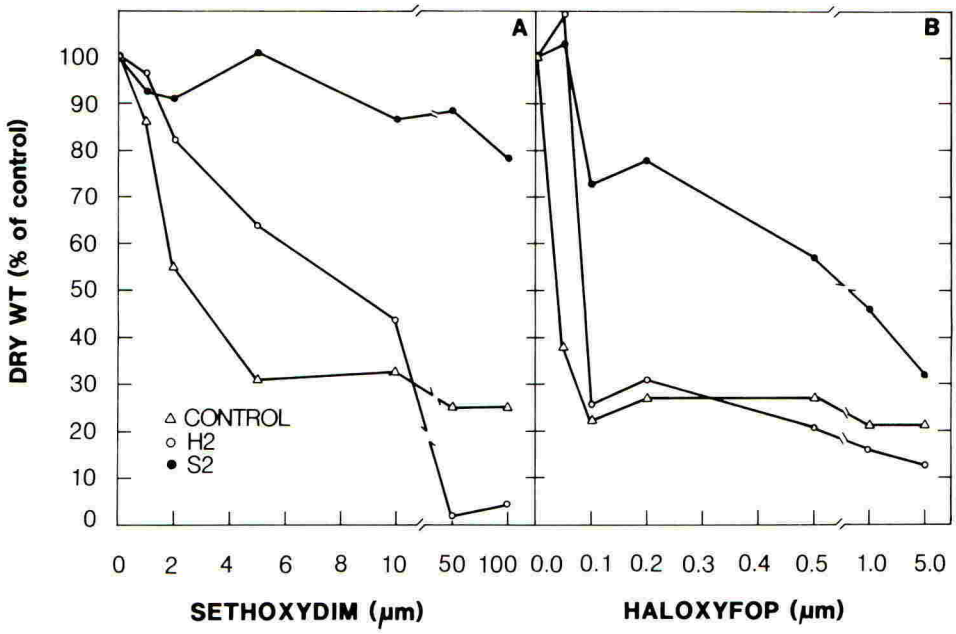

Fig. 1. Effect of sethoxydim (A) and haloxyfop (B) on growth of the unselected control, H2, and S2 cell lines. Dry weights of the untreated control, H2, and S2 were 251, 404, and 285 mg for (A) and 212, 254, and 176 mg for (B), respectively.

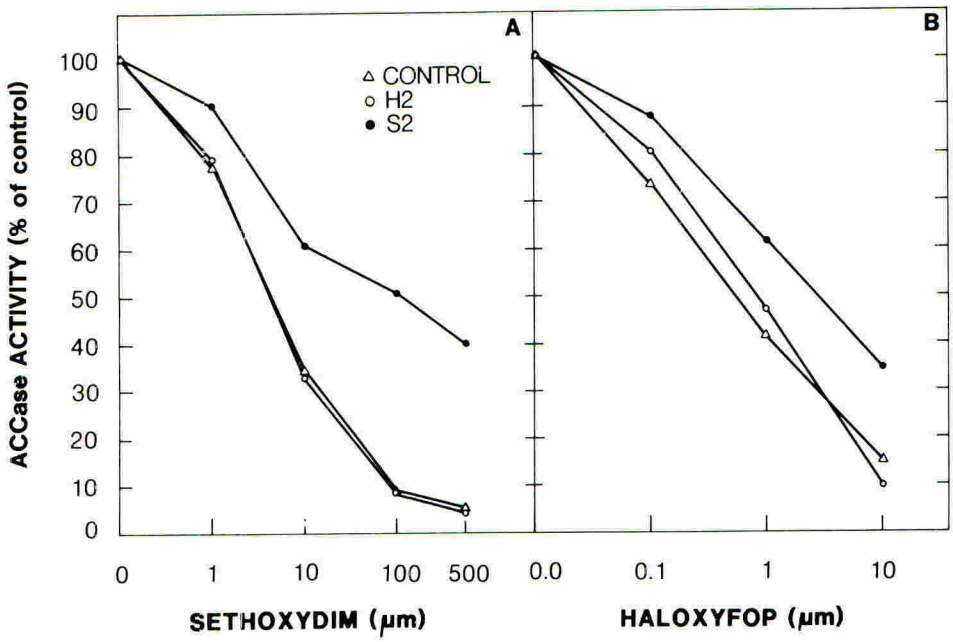

Fig. 2. Effect of sethoxydim (A) and haloxyfop (B) on ACCase activity of the unselected, H2, and S2 cell lines. ACCase activity in the absence of the herbicides was 4.2, 19.8, and 4.5 nmol·mg protein^{-1}·min^{-1} for the unselected, H2 and S2 lines, respectively.

ACCase activity

ACCase activity was assayed in the absence and presence of haloxyfop and sethoxydim in unselected and selected lines. Susceptibility of ACCase to inhibition by these herbicides was evaluated in terms of I_{50} values (concentration of the herbicide required to inhibit enzyme activity by 50%). ACCase activity measured in the absence of the herbicides was similar in the unselected and S2 lines (Fig. 2). For sethoxydim, the I_{50} values for ACCase activity were 6.7 and 77.8 μM in the unselected control and S2 lines, respectively (Fig. 2A). For haloxyfop, the I_{50} values for ACCase activity were 0.7 and 4.1 μM for the unselected control and S2 lines, respectively (Fig. 2B). Compared to the unselected control, ACCase activity in S2 was 11.6-fold and 5.8-fold more tolerant to inhibition by sethoxydim and haloxyfop, respectively. In the H2 line, ACCase activity measured in the absence of herbicides was approximately 5-fold higher than in the unselected control (Fig. 2). For sethoxydim, the I_{50} values for ACCase activity were the same (6.6 μM) in the unselected control and H2 lines. For haloxyfop, the I_{50} values were similar; 0.7 μM in the unselected control and 0.9 μM in the H2 line. These results indicate that there was little or no change in sensitivity of ACCase activity in the H2 line to herbicide inhibition.

Tolerance in regenerated plants

The H2 line could not be regenerated from tissue culture. However, the S2 line was regenerated and the expression of tolerance at the whole plant level was evaluated. Tolerance of the treated plants was evaluated 14 DAT both in terms of plant height and viability. At 14 DAT, all of the unselected control regenerated plants were killed by 0.05

kg/ha sethoxydim (Fig. 3). The small increase in plant height of the unselected control measured 14 DAT occurred during the first couple of days after herbicide application. Regenerated S2 plants were injured by 0.22 and 0.44 kg/ha sethoxydim but all plants survived. The most prominent injury symptom in the S2 line at the higher sethoxydim concentrations was chlorosis in leaf tissue that was developing at the time of herbicide application. All unselected control regenerated plants were killed by 0.01 kg/ha haloxyfop (Fig. 4). The regenerated S2 plants survived 0.01 kg/ha haloxyfop, but were killed by 0.05 kg/ha.

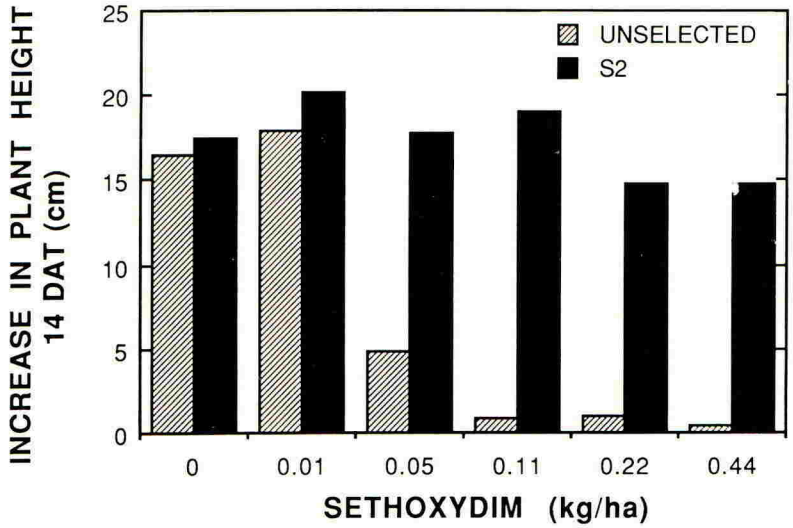

Fig. 3. Effect of sethoxydim on growth of unselected control and S2 regenerated plants. Growth was measured as the increase in plant height 14 DAT. The average plant height of the unselected control and S2 when treated was 16.5 and 17.5 cm, respectively.

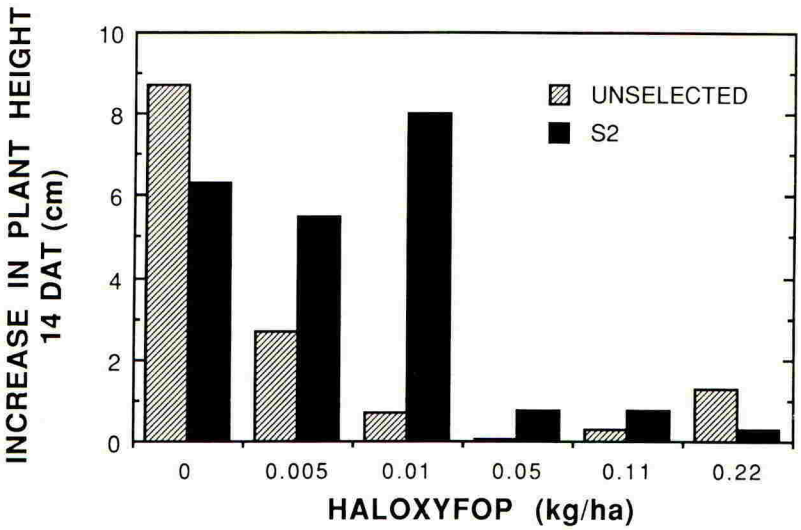

Fig. 4. Effect of haloxyfop on growth of the unselected control and S2 regenerated plants. Growth was measured as the increase in plant height 14 DAT. The average plant height of the unselected control and S2 when treated was 8.8 and 9.1 cm, respectively.

DISCUSSION

Embryogenic maize callus cultures that were tolerant to sethoxydim and haloxyfop were obtained by enrichment selection using stepwise increases in herbicide concentration. The S2 line, selected in the presence of sethoxydim, was > 40-fold more tolerant to sethoxydim and 20-fold more tolerant to haloxyfop. The H2 line, selected in the presence of haloxyfop, exhibited a 3 and 2-fold increase in tolerance to sethoxydim and haloxyfop, respectively. These mutations arose from somaclonal variation since the callus was not mutagenized prior to applying selection pressure.

Tolerance in the S2 line appears to be due to a modification at the site of action. The I_{50} values for inhibition of ACCase activity by sethoxydim and haloxyfop were considerably higher in S2 as compared to the unselected control. In parallel to what was found for tolerance measured in terms of growth, ACCase in the S2 line exhibited greater tolerance to sethoxydim than haloxyfop.

The cross-tolerance of the S2 line indicates that using a cyclohexanedione herbicide as a selection agent can yield cells tolerant to both cyclohexanedione and aryloxyphenoxypropionic acid herbicides. One interpretation of this result is that both herbicide classes bind at a common domain on the enzyme and that a modification in this domain confers cross-tolerance. This interpretation is consistent with the recent report that haloxyfop and sethoxydim are mutually-exclusive inhibitors of maize ACCase (Burton et al., 1989b). While the general domain on ACCase where haloxyfop and sethoxydim interact may be similar, the fact that the ACCase activity of the S2 line exhibited a greater tolerance to sethoxydim than haloxyfop suggests that the specific interaction of each herbicide in this domain may not be the same. This suggests that it may be possible to select for modifications at the binding site that result in different degrees of cross-tolerance to the two herbicides.

It is interesting to note that cross-tolerance to both sulfonylurea and imidazolinone herbicides was obtained when cells of Datura innoxia were selected for tolerance to sulfonylurea herbicides (Saxena and King, 1988). The sulfonylurea and imidazolinone herbicides represent two distinct classes of chemistry that inhibit acetolactate synthase. Tolerance to sulfonylurea and imidazolinone herbicides in the selected lines of Datura was due to an altered form of acetolactate synthase that was less sensitive to herbicide inhibition.

As compared to the S2 line, the H2 line exhibited a relatively small increase in tolerance to both sethoxydim and haloxyfop. In contrast to the S2 line, tolerance was not due to the presence of an altered ACCase that was less sensitive to the herbicides. Rather, the small increase in tolerance was the result of elevated ACCase activity. The elevated ACCase activity may be due to the presence of a modified ACCase exhibiting increased activity, but no change in herbicide tolerance. Alternatively, overproduction of ACCase may be responsible for the 5-fold increase in specific activity. It should also be considered that the more rapid growth rate of the H2 line may have contributed to its tolerance evaluated in terms of growth. Whether there is a casual relationship between the elevated ACCase activity in the H2 line and its more rapid growth rate is unknown.

Plants regenerated from the S2 line exhibited a significant degree of tolerance to sethoxydim and haloxyfop. S2 plants survived greenhouse application of 0.44 kg/ha sethoxydim which is approximately twice the field rate for control of annual grasses. If the tolerance trait in the S2 line is stable, it may be possible to transfer tolerance to the aryloxyphenoxypropionic acid and cyclohexanedione herbicides into elite corn inbreds by backcrossing. The availability of corn hybrids tolerant to these herbicide classes would increase the options available for post-emergence control of grass weeds in corn.

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TRANSFER OF BROMOXYNIL RESISTANCE INTO CROPS

G. FREYSSINET, B. LEROUX, M. LEBRUN, B. PELISSIER, A. SAILLAND

Rhône-Poulenc Agrochimie, Biologie Moléculaire et Cellulaire, BP 9163, 69263 Lyon, Cédex 09, France.

K. E. PALLETT

Rhône-Poulenc Agriculture, Fyfield Road, Ongar, Essex CM5 OHW, U.K.

ABSTRACT

A gene coding for nitrilase has been introduced into the PBD6 cultivar of industrial tobacco. This enzyme converts bromoxynil a broad-leaf herbicide into the non-phytotoxic 3, 5-dibromo-4-hydroxy benzoic acid by hydrolysis removal of its cyano group. Transgenic tobacco plants,transformed with the nitrilase gene are resistant to dose rates of bromoxynil up to 7 kg a.i./ha, more than 20 times the lethal dose for non-transgenic plants. Resistance is obtained when the nitrilase coding region is put under the control of a promoter of the RuBisCo small subunit and thus is probably expressed mainly in leaves.

Photosynthetic electron transport, the main target site for bromoxynil is equally sensitive in vitro. Chloroplasts isolated from transgenic tobacco have an I_{50} for electron transport inhibition similar to that of non-transgenic chloroplasts. However, photosynthesis in sprayed transgenic plants is not affected. Initial metabolism studies have shown that bromoxynil is rapidly metabolised to the acid in transgenic tobacco. These results confirm the fact that an efficient metabolism of bromoxynil by the nitrilase is the sole basis for resistance of transgenic tobacco.

INTRODUCTION

The goal of obtaining selective herbicides with the necessary margin of crop safety has been largely reached to date by differential screening of molecules on crop and weed plants. This empirical approach has shown efficacy and selectivity. However, it is becoming increasingly difficult to obtain new herbicide molecules which meet all the necessary requirements (activity, specificity, cost, non-toxicity etc. Also the number of molecules needed to be screened in order to discover a new commercial herbicide has dramatically increased over the past 10 years. Since the beginning of the 80s several laboratories have been trying to exploit techniques linked to the totipotency of plant material and to genetic engineering in order to introduce herbicide resistance to a given crop. Successes have been obtained e.g. glyphosate (Comai, et al., 1985; Shah, et al., 1986), sulphonylureas (Haughn, et al., 1988), imidazolinones (Anderson and Georgeson, 1986), phosphinothricin (de Block, et al., 1987) and atrazine (Thomzik and Hain, 1988).

We present here work performed to introduce bromoxynil resistance into industrial tobacco. Bromoxynil is a broad-leaved herbicide which has been used for several years in the control of dicotyledonous weeds in cereals. This herbicide mainly acts by inhibiting and uncoupling photosynthetic electron transport (Oettmeier, et al, 1982; Sanders and Pallett, 1985). The strategy used was to isolate a gene coding for nitrilase, an enzyme which hydrolyses bromoxynil to its non-phytotoxic benzoic acid (figure 1) and to introduce this gene into tobacco. Researchers from CALGENE (Davis, California, USA) have isolated a strain of Klebsiella pneumoniae subsp ozanae from soil treated with bromoxynil, which uses the herbicide as the sole nitrogen source (McBride et al, 1986), The enzyme and the corresponding gene were isolated and characterised. (Stalker et al, 1986; Stalker and McBride, 1987).
We have used its coding region to make chimaeric genes able to function in plant cells. These chimaeric genes have been transferred into the PDB6 cultivar of industrial tobacco. Photosynthesis in transgenic tobacco is not effected by bromoxynil and plants are resistant to dose rates of 7 kg a.i./ha, the lethal dose is 0.3 kg/ha for non-transgenic tobacco.

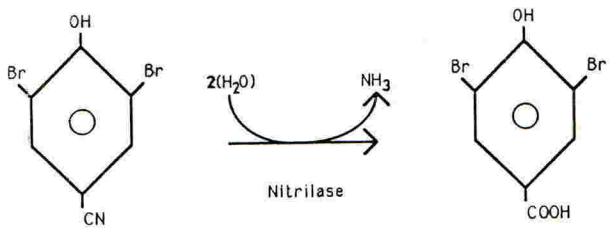

FIGURE 1. Transformation of bromoxynil to 3, 5-dibromo-4-hydroxy benzoic acid by a specific nitrilase.

MATERIALS AND METHODS

1. Gene and tobacco

Plasmids pBRX 25 and pBRX 47, carrying the nitrilase gene were provided by Dr D Stalker from Calgene (Davis, California, USA). Seeds of PBD6 cultivar of industrial tobacco were, obtained from SEITA (Bergerac, France).

2. Chimaeric genes and plant transformation

Two chimaeric genes (figure 2) were constructed according to conventional techniques, pRPA-BL-154 was obtained by placing the nitrilase gene of pRBX 25 behind the 35S CaMV promoter (Odell et al., 1985) and pRPA-BL-221 was constructed by splicing the same nitrilase gene from pBRX 47 to the sunflower RuBisCo small subunit promoter (Waksman et al., 1987). Both chimaeric genes carried in addition the Nos polyadenylation signal (Depicker et al., 1982). These chimaeric genes have been placed in a binary plasmid containing the two borders of the T-DNA from Agrobacterium tumefaciens. This T-DNA contains in addition the neomycin

phosphotransferase gene controlled by the nopaline synthase promoter. This gene confers resistance to kanamycin in plant cells. These plasmids were introduced into Agrobacterium tumefaciens, EHA 101. Leaf discs were cut from tobacco and co-cultivated with Agrobacterium tumefaciens using conventional techniques (Stalker et al, 1986).
Transgenic plants have been obtained, selfed and seeds collected for further analyses.

3. Molecular and phenotypic analyses

DNA, mRNA and protein were analysed using conventional techniques (Maniatis et al., 1982). Resistance to bromoxynil was studied under greenhouse or field conditions. Dose rates used are indicated in the text.

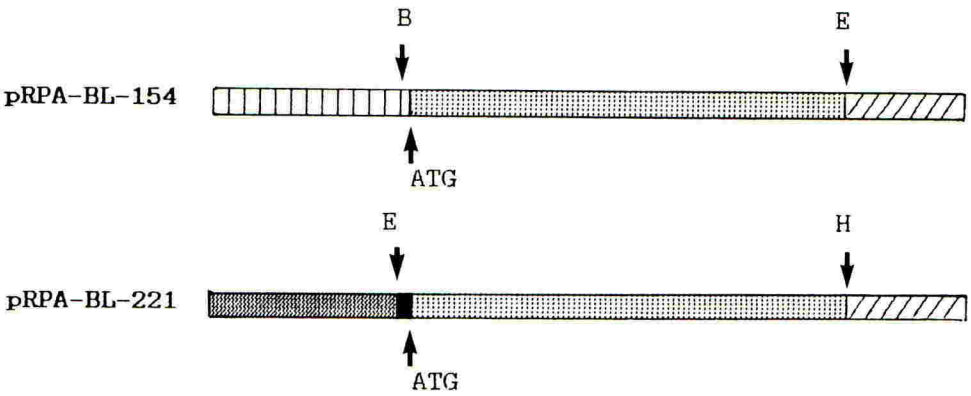

FIGURE 2. Schematic representation of the chimaeric genes coding for a nitrilase and used to introduce resistance to bromoxynil in industrial tobacco.

Promoter region from the sunflower RuBisCO SSU; Promoter region coming from the 35S CaMV gene; linker; Region coding for the nitrilase; Terminator of transcription coming from the nopaline synthase gene; Restriction sites: E = Eco R1; H = Hind III; B = BamH1 ATG = initiation codon.

Biohazards associated with the experiments described in this publication have been examined satisfactorily by the French National Control Committee for the laboratory work and by the French Biomolecular Engineering Committee for the field experiments.

4. Photosynthetic measurements

Bromoxynil was applied as aqueous solutions of the K salt to 4-5 leaf plants using a laboratory pot sprayer. Plants were either non-transgenic or transgenic containing the pRPA-BL-154 gene. They were retained under glasshouse conditions and at various times photosynthesis was measured using two non-destructive methods.

i CO_2 exchange rates were monitored using a portable Infra Red Gas Analyser (ADC Limited, Hoddesdon, Hertfordshire, U.K.).

ii Leaf fluorescence was monitored using a Modulated Fluorescence Measurement System (MFMS) from Hansatech Limited, Kings Lynn, Norfolk, U.K. Further details are given in the figure legends.

Photosynthetic electron transport inhibition by bromoxynil was also determined in chloroplasts isolated from the transgenic and non-transgenic tobacco. Type B chloroplasts were isolated by homogenisation for 10s using an Ultra-Turrax blender. The extraction medium (50 ml with 6 g leaves) contained 50mM tricine, 330mM sorbitol, 3mM $MgCl_2$ and 0.01% bovine serum albumin (BSA) adjusted to pH 7.5 with NaOH. The homogenate was filtered through 8 layers of muslin prior to centrifugation at 500 g for one minute. The supernatant was recentrifuged at 3000 g for ten minutes to sediment the chloroplasts. The pellet was washed in 10ml resuspension medium (5mM tricine, 330mM sorbitol, 2mM EDTA, 3mM $MgCl_2$ and 0.1% BSA adjusted to pH 7.5 with NaOH) and recentrifuged at 3000g to resediment the chloroplasts. Chloroplasts were resuspended in the above medium.

Electron transport was determined by monitoring O_2 evolution in a Hansatech DW1 O_2 electrode with potassium ferricyanide as electron acceptor and chloroplasts equivalent to 50 µg chlorophyll.

5. Determination of Bromoxynil metabolism

^{14}C-Bromoxynil (uniformally ring labelled) formulated as the K salt was applied to plants with the pRPA-BL-154 gene at the two leaf stage, at a dose rate equivalent to 495 g a.i/ha using an air-brush (Humbrol Consumer Products, Hull, Humberside, U.K.). At various time intervals leaves and stems were separated from the roots at soil level and washed in acetone:water (1:1) to remove leaf surface deposits of ^{14}C-bromoxynil. Leaves and stem were then extracted twice with acetone:water (1:1), followed by acetone using an Ultra-Turrax Homogeniser followed by centrifugation. Aliquots of washes and plant extracts were analysed by direct liquid scintillation counting (washes) or combustion followed by LSC (extracts). The remainder was concentrated by rotary evaporation and taken up in acetone for metabolic investigation using thin-layer chromatography, and gas-chromatography-mass spectroscopy, following clean-up and derivatisation.

RESULTS

1. Genes and transgenic tobaccos

Two chimaeric genes (figure 2) have been used for these experiments. pRPA-BL-154 contains the promoter of the 35S CaMV gene which allows expression of the protein in most plant tissues. pRPA-BL-221 contains the promoter of a sunflower RuBisCo (RuBPc/o) SSU gene (Waksman et al., 1987), expressed in leaf tissue (Waksman, unpublished observation). Such type of promoter should allow the expression of the chimaeric gene mainly in the photosynthetic tissues of the transgenic plants (Poulsen et al., 1986). These two genes have been transferred into the industrial tobacco PBD6 using the leaf disc technique and co-cultivation with Agrobacterium tumefaciens EHA 101 containing a binary type plasmid. Transgenic shoots are obtained by in vitro selection with either 100mg kanamycin/1 or 5 to 10 mg bromoxynil/ha to confirm the in vitro selection. Regenerated

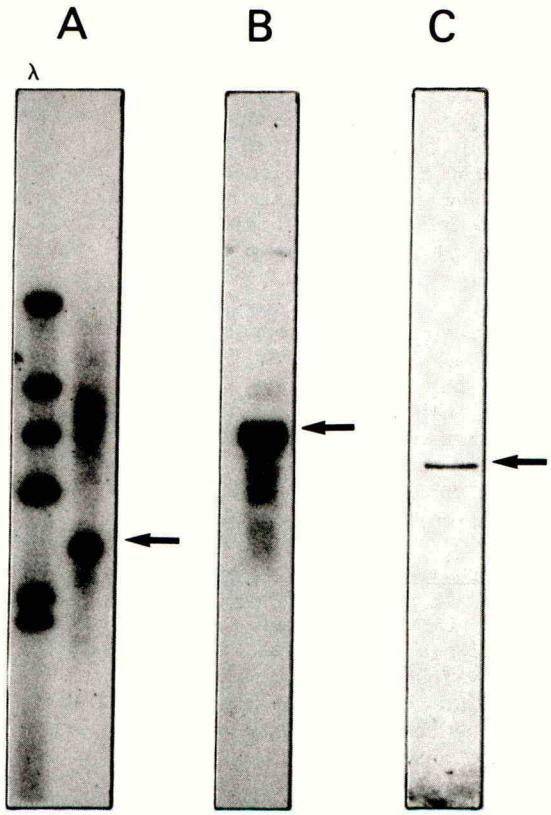

FIGURE 3. Molecular analyses of transgenic tobaccos containing the chimaeric gene pRPA-BL-221.
A. Southern analysis (→ insert markers are 23, 9.4, 6.6, 4.3, 2.3 and 2 kbp)
B. Northern analysis (→ nitrilase mRNA; 1.35 kb)
C. Western analysis (→ nitrilase; 33 kda)

TABLE 1 CO_2 exchange rates of leaf 3 of 4-leaf wild type and transformed tobacco plants 4 and 24 hours after treatment with 0 and 56 g/ha bromoxynil K salt.

	Wild Type		Transformed	
Treatment	4h	24h	4h	24h
0	3.81 + 0.40	5.31 + 0.08	4.04 + 0.63	5.22 + 0.40
56 g/ha	0.88 + 0.21	0.47 + 0.12	4.44 + 0.40	5.66 + 0.17

Data is expressed as μmoles CO_2 uptake/6.25 cm^2 leaf area/min + s.e. measured at 280-300 μmoles/m^2/s (PAR)

plantlets were sprayed with 0.6 kg bromoxynil/ha to identify those which expressed the nitrilase gene.

F1 plants with one locus were selected (segregation of bromoxynil and kanamycin in a 3:1 ratio) and treated with various rates of bromoxynil ranging from 0.6 to 7kg a.i/ha. Transgenic plants were found to be resistant at all concentrations tested. The two types of plants were resistant suggesting that the expression of nitrilase in the leaf (plants with pRPA-BL-221) was enough to confer resistance in agreement with the fact that bromoxynil is a contact herbicide. We did not find differences between homozygote or heterozygote plants for their resistance to bromoxynil.

We have analysed the molecular composition of these plants. Figure 3 shows the results of these analyses for plants with the pRPA-BL-221 gene. At the DNA level the presence of one band after digestion with EcoR1 confirms the fact that only one gene is integrated into the plant genome. The mRNA has the expected size, 1.35 kb; its amount varies from one transgenic event to the other (results not shown). The nitrilase is produced in transgenic tobacco (figure 3C) and has the expected molecular weight. The amount varies with that of the mRNA.

2. Effects of bromoxynil on photosynthesis

Wild type tobacco is very susceptible to bromoxynil when applied at 56g a.i/ha to 4-5 leaf plants. Table 1 shows complete inhibition of CO_2 exchange within twenty-four hours of treatment. The transgenic plants showed no inibition of CO_2 exchange at this dose rate.
Figures 4 and 5 show the leaf fluorescence data in bromoxynil treated wild type and transgenic tobacco plants. MFMS kinetics are seen as a steady-state base fluorescence (F_B) upon leaf illumination with the modulated light source. Upon illumination with white actinic light fluorescence induction occurs and rapidly reaches a maximum (F_P) and reduces to a steady state within two minutes (F_{2min}) (see figure 4A). Photosynthetic inhibition by bromoxynil treatment clearly elevates F_B and although induction to an F_P occurs, the steady state F_{2min} is clearly elevated (see figure 4B). In all MFMS determinations of transgenic plants treated with doses <560g a.i/ha, kinetics closely resembled untreated controls (see figure 4C). Figure 5 shows a time course experiment in which plants were treated with 0, 350, 1400 amd 5600g a.i/ha bromoxynil. Rapid elevation of F_{2min} to a maximum (i.e. complete photosynthetic inhibition) occurs in wild type plants. A small but insignificant initial elevation of F_{2min} occurred 2 to 4 hours after treatment of transgenic plants. However, values returned to those of controls within six hours. Measurements taken after 48 hours showed no deviation of transgenic plant F_{2min} values from those of controls. After 48 to 72 hours all wild type plants treated with 350 and 1400g a.i/ha became chlorotic and died. No symptoms were apparent on transgenic plants.

Photosynthetic electron transport in chloroplasts isolated from transgenic tobacco was similarly sensitive to that of chloroplasts isolated from wild type plants with I_{50}'s of 4.8 and 3.5 μM respectively. This confirms that the target site is sensitive to the chemical in both types of plants.

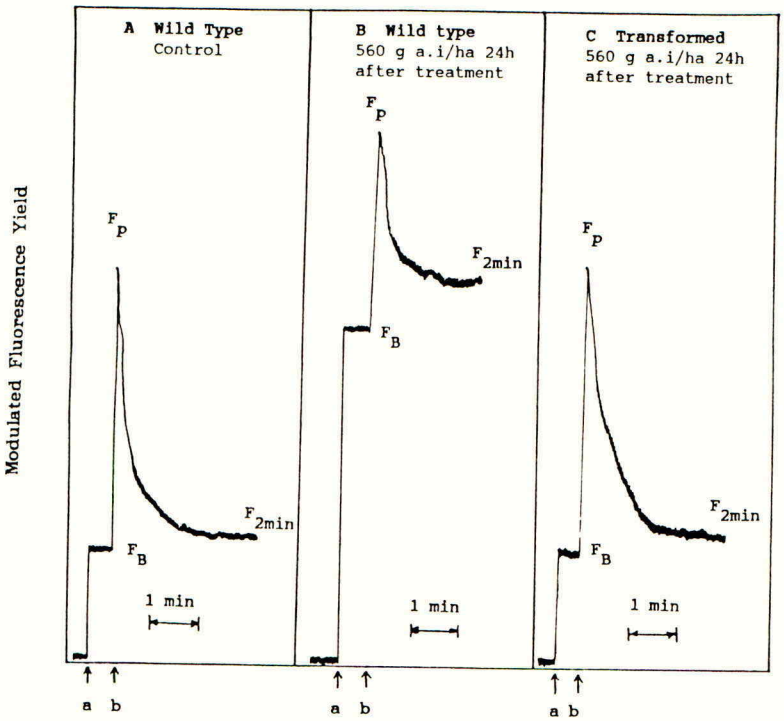

FIGURE 4. Typical MFMS traces from non-transgenic and transgenic tobacco 24 hours after treatment with 560g a.i. bromoxynil/ha; a = modulated light on; b = white actinic light on; see "Materials and Methods" for other details.

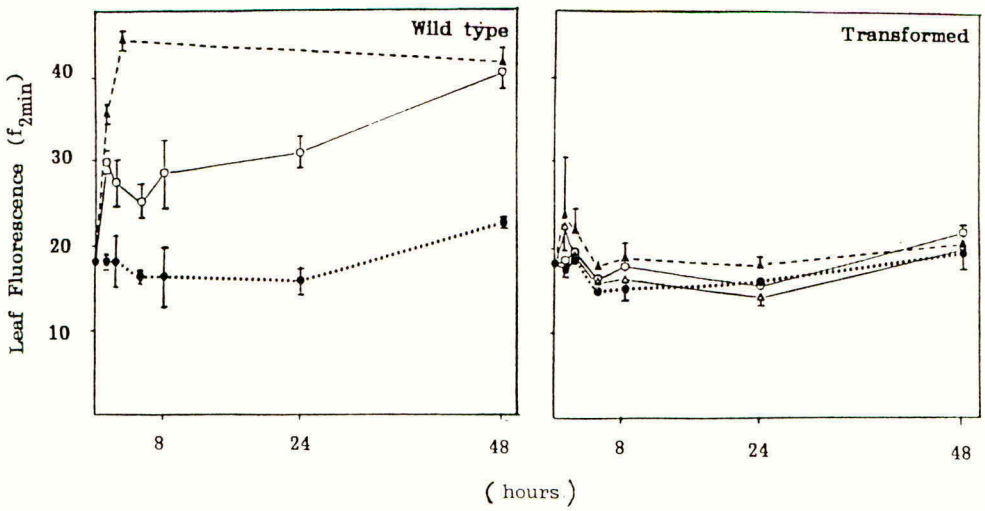

FIGURE 5. The effect of 0(●·····●), 350 (○——○), 1400 (▲- - - -▲) and 5600 (△——△) g a.i/ha bromoxynil on leaf fluorescence of 4-5 leaf plants. Data is a mean of six replicates.

3. Metabolism of Bromoxynil

There were no differences in the uptake of ^{14}C-bromoxynil K salt as determined by analysis of ^{14}C-activity in the surface deposit washes and in that accumulated within the stems and leaves (data not presented). Bromoxynil was the only significant ^{14}C-compound in the plant washes and stem/leaf extracts of non-transgenic plants (data not presented). Bromoxynil was again the only significant ^{14}C-compound in the plant washes of transgenic plants. However, in the extracts obtained from the transgenic plants a major metabolite was observed as early as four hours after treatment, in addition to conjugated compound(s) and bromoxynil. The amount of conjugates increased with time, with an accompanying loss of bromoxynil and of the metabolite. Spectroscopic analysis of the 72 hour transgenic plant samples showed this metabolite to be 3,5-dibromo-4-hydroxybenzoic acid, present both as the free compound and in the form of unknown conjugates. Bromoxynil appeared to be only a minor components by this time. This confirms the reaction presented in figure 1.

4. Field experiments

Transgenic plants have been tested under field conditions. Differences in the growth kinetics were not observed between transgenic and non-transgenic tobacco plants. Transgenic plants treated with up to 4.8kg bromoxynil/ha (highest dose tested) showed no symptoms (figure 6) and developed as the untreated plants. Non-transgenic plants are completely destroyed after a treatment with 1.2kg bromoxynil/ha (lowest dose tested in the field).

FIGURE 6. Analysis of the behaviour of the transgenic and non-transgenic tobacco plants under field conditions. Tobacco plants have been treated with 1.8kg bromoxynil/ha. Pictures were taken two weeks after treatment. A. non-transgenic tobacco B. transgenic tobacco

DISCUSSION

Results presented here clearly show that transgenic tobacco plants containing a gene coding for a nitrilase are resistant to more than twenty times the normal lethal dose of bromoxynil for tobacco. Transgenic tobacco is resistant even when the nitrilase gene is controlled by a type of promoter known to be expressed mainly in the leaves where bromoxynil inhibits photosynthetic electron transport. The amount of nitrilase needed is very low since plants with as low as 10μg nitrilase/mg total protein are resistant.

We have further shown that photosynthetic electron transport in isolated transgenic plant chloroplasts is clearly sensitive to bromoxynil, however whole plant photosynthesis is clearly insensitive even at doses 100 times those that kill wild type plants. A slight reversible inhibition of photosynthesis was detectable within 2-4 hours, presumably due to the uptake of bromoxynil exceeding its rate of degradation.

As chloroplasts isolated from both non-transgenic and transgenic plants were similarly sensistive to bromoxynil, photosynthetic inhibition would be expected in both types of tobacco unless metabolism was occurring in the transgenic plants. The metabolism data clearly shows that the transgenic tobacco is insensitive to bromoxynil treatment because of an ability to metabolise it rapidly to the corresponding acid. This then becomes conjugated and is then gradually further metabolised and eliminated.

In addition, bromoxynil treated transgenic tobacco behaves as untreated non-transgenic tobacco under field coniditions. Further analyses are in progress on these transgenic tobacco plants to more clearly define their behaviour and to be able eventually to bring them to market.

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AUTHOR INDEX

Adamidis, Th. 4C-4
Akinyeniju, O. A. 3D-2
Akobundu, I. O. 3D-2
Alder, M. R. 7D-6
Amrein, J. 2-9, 2-12
Andersen, P. G. 6B-5
Anderson, M. D. 4C-6
Apted, J. E. 3C-1
Armstrong, M. J. 7C-3
Arndt, F. 2-3
Arthur, M. F. 3D-3

Baandrup, M. 6A-2
Baeva, G. 8C-5
Bailey, J. 3A-3
Bain, A. B. 8D-1
Baker, N. R. 4D-11
Ballegaard, T. 6A-2
Banks, M. 7A-4
Barber, J. 9B-2
Bardsley, E. S. 7C-7
Barlow, N. D. 8A-4
Barnwell, P. 3B-5, 4D-3
Barrett, D. 5-3
Bartlett, D. H. 7C-1
Bassi, A. 6C-1
Baudry, J. 7B-2
Bauer, K. 2-10
Bayer, H. O. 2-7
Beaudoin, J. D. 3B-3
Bekker, R. 8D-4
Bell, A. R. 2-8
Bell, G. A. 7A-7
Bell, G. J. 6B-2
Bencivelli, A. 6C-1
Berghaus, R. 4D-6
Bergstrom, L. 9A-3
Beste, C. E. 8C-6
Bier, B. 8C-4
Bieringer, H. 2-10
Blair, A. M. 9A-2
Boatman, N. D. 7B-4, 8D-1
Bond, W. 8C-2
Booth, K. D. 7B-1
Booth, T. C. 4B-1
Botterman, J. 8B-2
Bourdôt, G. W. 4A-5, 8A-4
Breen, M. K. 3B-3
Brown, V. K. 7B-3
Buckley, D. 7C-3
Burch, J. 8C-2
Burden, R. S. 4D-1
Burkhart, D. C. 3D-1
Burow, R. F. 3C-5
Büttner, B. 4D-12, 7A-3

Carter Andree D. 9A-8
Carter, E. 5-1
Cartwright, D. 7A-2
Caseley, J. C. 9B-1
Chaloner, P. A. 7A-1
Chapman, D. J. 9B-2
Chiba, K. 2-7
Chiverton, P. A. 8D-7
Chueca, M. C. 4C-8
Clarke, J. H. 4A-4, 6A-6
Clausen, I. B. 4A-1
Clay, D. V. 4A-6, 4B-3, 4B-4
Cleal, R. A. E. 3C-3
Cobb, A. H. 3B-5, 4D-3, 4D-4
Contos, D. A. 3D-5
Cooke, D. T. 4D-1
Cooper, S. 6B-5
Cornes, D. W. 3C-1, 7A-5
Cossens, C. G. 7D-1
Cotterman, 4A-3
Courtney, A. D. 3A-7
Coutts, J. 3A-3
Cowgill, S. 8D-9
Craig-Kennard, A. C. 3B-3
Crawley, M. J. 8B-1
Crossan, G. S. 7D-1
Cséke, C. 3B-1

Davies, D. B. 3A-3
Davies, D. H. K. 8A-6, 8A-7
Dawson, R. 6B-8
Dent, J. B. 8A-1
Devendra, R. 6C-2
Dixon, F. L. 7C-12
Dombrowski, N. 6B-9
Douchet, J. P. 6C-1
Dover, P. A. 3C-4
Doyle, C. J. 8A-3
Drennan, D. S. H. 9A-4
Drexler, D. M. 4C-6
Du Rieu, A. 6B-8
Dutka, F. 4D-7

Eagle, D. J. 9A-7
Early, C. 4D-3
Easson, D. L. 3A-7
Ebert, E. 2-10, 7A-3
Edward-Jones, G. 6A-3
Edwards, C. A. 6A-7
Efthimiadis, P. 4C-4, 4C-7
Egli, H. 9A-6
Eke, K. R. 7C-5
Ekeland, R. A. 3C-5
Evbuaomwan, F. O. 3D-2
Everest-Todd, M. 7C-9

Fawcett, R. H. 8A-1
Feare, C. 8D-3
Feld, A. 4D-10
Fielder, G. J. 7C-7
Firbank, L. G. 6A-4
Focke, M. 3B-4
Förster, B. 3D-3
Fowler, S. V. 8B-4
Freer, J. B. S. 6B-2, 8A-5
Freyssinet, G. 9B-6
Frost, A. J. 7B-5
Froud-Williams, R. J. 6A-6
Fuesler, T. P. 2-1
Fujikawa, K. 2-2

Garcia-Baudin, J. M. 4C-8
Garrett, H. J. 7C-2
Gengenbach, B. G. 9B-5
Gibbard, M. 3A-3
Gilbert, A. J. 6B-1
Giltrap, N. J. 7C-6
Graham, D. J. 7B-1
Graham-Bryce, I. J. 1-1
Grayson, B. T. 3C-6
Green, J. W. 4D-11
Green, M. 3A-5, 8C-3
Greenfield, A. J. 7A-6, 8C-1
Gregory, L. 7D-6
Gronwald, J. 9B-5
Gussin, E. J. 3C-2

Hacker, E. 2-10
Haga, T. 2-2
Hamada, T. 2-4
Hansen, C. O. 2-7
Harre, M. 2-3
Harrington, K. C. 4A-5
Harris, R. I. 7C-11
Harwood, J. L. 3B-2, 4D-8
Hatzios, K. K. 9B-4
Hayashi, Y. 2-6
Hede-Hauy, L. 2-5
Henderson, E. J. 7A-5
Heppel, V. A. F. 7C-4
Herbert, T. A. 4C-2
Herrington, P. J. 6B-2, 6B-6
Heubach, G. 2-10
Hibbitt, I. 6B-9
Hicks, W. K. 6B-3
Hirase, K. 4D-2
Hislop, E. C. 6B-6
Hobson, G. P. 4C-2
Holloway, P. J. 3C-6
Hopkins, W. L. 3C-7
Howard, C. 8D-6
Huff, H. P. 7A-3, 7A-4
Hutchings, M. J. 7B-1
Hutson, J. L. 9A-1

Ioos, P. 6C-1
Iwanzik, W. 9A-6

Jablonkai, I. 4D-7
James, C. S. 4D-1
Jessop, C. D. 7C-1

Jones, E. I. 6B-6
Jones, O. T. G. 9B-1

Kamoshita, K. 2-4
Karpinski, A. K. 7C-9
Kelley, D. E. 2-7
Kerber, E. 2-11
Kimura, F. 2-2
King, J. M. 7C-10
Knacker, T. 3D-3
Knott, C. M. 7C-5
Kobayashi, S. 2-7
Kobek, K. 3B-4, 4D-9, 4D-10
Köcher, H. 4D-12
Kocur, J. 8C-4
Kouji, H. 2-6
Kowanski, K. A. 3C-8
Krähmer, H. 2-3
Krishnamurthy, K. 6C-2
Krüger, H. 2-3
Kudsk, P. 6A-1

Landell-Mills, J. 8B-5
Lane, P. 8D-3
Langelüddeke, P. 7A-3, 8C-4
Lavers, A. 6B-7
Lawrie, J. 4B-4
Lawson, H. M. 7C-8
Lawton, J. H. 8B-4
Leah, J. M. 4D-4
Leek, G. L. 2-1
Lebrun, M. 9B-6
Leist, K. H. 2-10
Leroux, B. 9B-6
Lewis, T. 4D-8
Lewosz, W. 3C-9
Leypoldt, G. 2-11
Liang, P. H. 2-1
Lichtenthaler, H. K. 3B-4, 4D-9, 4D-10
Longman, D. 8B-5
Lötzsch, K. 4D-12
Lutman, P. J. W. 7C-12

Mabb, L. P. 6B-3
Macdonald, D. W. 8D-2
Maigrot, P. H. 2-5
Makepeace, R. J. 4C-3
Makowski, E. J. 4C-6
Marcinkoswi, A. 3D-3
Marinkov, P. 8C-5
Marrs, R. H. 7B-5
Marshall, J. 7C-11
Martin, T. D. 9A-2
Matsunaka, S. 4D-2
Mawer, C. J. 6B-10
May, M. J. 6A-3, 7C-3
Mayes, A. J. 7C-11
Mayland, P. 4C-6
Meier, B. 7D-3
Merritt, C. R. 6B-4
Meyer, A. M. 4D-5
Miller, P. C. H. 6B-2, 6B-10
Milsom, T. P. 8D-3
Mitchell, R. B. 7D-1
Mohan, S. L. 6C-2
Mortimer, A. M. 4A-2, 8D-6
Moss, S. R. 3A-6, 4A-4
Müller, F. 4D-5
Mumford, J. 6A-3
Muntan, L. 6C-3
Murai, S. 2-2
Murphy, M. 5-4
Murray, A. 2-5
Murray, D. D. 8B-5
Musco, V. A. 2-7

Nagano, E. 2-4
Nikolova, G. 8C-5
Nordh-Christerson Marie 9A-3
Norton, G. 6A-3
Nowicka, B. 4C-5
Nyffeler, A. 2-9

Ogilvy, S. E. 3C-3
Ohyama, H. 2-7
Orson, J. H. 3A-1, 3A-5, 7C-2
Osada, H. 2-6
Oshio, H. 2-4
Ostermeyer, A. 7D-3
Owen, W. J. 3B-1, 9B-3

Pallett, K. E. 9B-6
Palm, H. L. 2-1
Parfitt, R. I. 4B-5
Parker, W. B. 9B-5
Paterson, C. D. 8C-7
Pearson, M. 5-2
Peddie, A. S. 2-8
Pelissier, B. 9B-6
Percival, M. P. 4D-11
Perrot, A. 2-5
Peter, C. J. 3D-1, 9A-5
Plant, R. A. 7B-5
Pollack, R. T. 8C-3
Popay, A. I. 4A-5, 8A-4
Prince, B. 8C-3
Proctor, G. 6A-3
Proven, M. J. 8A-5
Putwain, P. D. 4A-2, 8D-6

Quadranti, M. 2-12

Ramachandra Prasad, T. V. 6C-2
Rea, B. L. 7C-7
Reed, W. T. 4A-3
Regnier, E. 6A-7
Rendina, A. R. 3B-3
Retzlaff, G. 4D-6
Richards, M. C. 6A-5, 8A-7
Ridley, S. M. 4D-8
Robinson, T. 6B-8
Roebuck, J. 7C-6
Rogers-Lewis, D. S. 3C-3
Roggenbuck, F. C. 3C-5
Rola, J. 4C-5
Rook, D. 4B-2
Röttele, M. 8C-4
Rowe, L. 3C-5
Rufener, J. 2-9, 2-12
Rutherford, I. 6B-2
Rule, J. S. 4C-1
Ryan, P. J. 3C-1, 4C-2, 6B-8, 7A-5

Sailand, A. 9B-6
Sakashita, N. 2-2
Saladini, J. L. 4A-3
Salto, T. 4C-8
Sansome, G. 3A-4
Schmidt, E. 4D-12
Schultz, A. 4D-12
Schumacher, H. 7A-4
Scott, T. A. J. 3C-1, 7A-5
Secor, J. 3B-1
Seiler, A. 2-11
Sharp, J. K. 9A-5
Sheppard, B. W. 8A-7
Shrimpton, D. R. 7C-1
Skorda, E. A. 4C-4, 4C-7
Slivon, L. E. 3D-5
Smith, H. 8D-2
Somers, D. A. 9B-5
Speed, C. 8B-4
Speller, C. S. 3C-4
Standell, C. J. 7D-4, 7D-5
Steer, P. M. 3A-5
Stock, D. 3C-6
Strachan, J. R. 6B-9
Strachan, S. D. 2-1, 3D-1
Strahan, J. C. 9A-5
Streibig, J. C. 8A-2
Strek, H. J. 3D-1
Sutton, P. B. 5-3

Taylor, W. A. 6B-5
Thomas, M. B. 8D-8
Thornton, P. K. 8A-1
Tolle, D. A. 3D-3
Tooby, T. 9A-9
Tottman, D. 3A-5
Turner, D. J. 4C-3
Turner, M. G. 4C-3
Turner, M. T. F. 7C-10
Turner, R. 6A-3

Válkóva, O. 4B-6
Van Groenendael, J. M. 8D-4
Van Heijst, M. 8D-4
Von Olfers, H. 7A-4

Wadd, Debra J. 9A-4
Waganet, R. J. 9A-1
Walker, A. 9A-2, 9A-7
Walker, K. A. 4D-8
Walklate, P. J. 6B-10
Warner, H. L. 2-7
Warner, J. S. 3D-4

Watson, A. K. 8B-3
Welch, Sarah J. 9A-2
West, T. M. 7C-12, 7D-2, 7D-4, 7D-5
Western, N. M. 6B-6
Whitehead, R. 3A-2
Whitehouse, P. 3C-6
Whiting, A. J. 8A-6, 8A-7
Whytock, G. 7C-4, 8A-6
Wilson, B. J. 3A-6
Wilson, P. J. 8D-5
Wiseman, J. S. 7C-8
Wittenbach, V. A. 2-1
Worrall, T. L. 4D-4
Wright, B. 8D-3
Wright, G. McN. 7C-8
Wright, H. C. 3A-2
Wright, K. J. 3A-6
Wyse, D. L. 9B-5

Yamamura, H. 2-7
Yancey, M. F. 3D-4
Yoshida, R. 2-4