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Series on Harmonization of Regulatory Oversight in Biotechnology No. 11

CONSENSUS DOCUMENT ON GENERAL INFORMATION CONCERNING THE GENES AND THEIR ENZYMES THAT CONFER TOLERANCE TO PHOSPHINOTHRICIN HERBICIDE

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OECD Environmental Health and Safety Publications

Series on Harmonization of Regulatory Oversight in Biotechnology

No. 11

Consensus Document on General Information Concerning the Genes and Their Enzymes that Confer Tolerance to Phosphinothricin Herbicide

Environment Directorate

Organisation for Economic Co-operation and Development

Paris 1999

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 29 industrialised countries in North America, Europe and the Pacific, as well as the European Commission, meet to co-ordinate and harmonize policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialized Committees and subsidiary groups composed of Member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's Workshops and other meetings. Committees and subsidiary groups are served by the OECD Secretariat, located in Paris, France, which is organised into Directorates and Divisions.

The Environmental Health and Safety Division publishes free-of-charge documents in six different series: **Testing and Assessment**; **Good Laboratory Practice and Compliance Monitoring**; **Pesticides**; **Risk Management**; **Harmonization of Regulatory Oversight in Biotechnology**; **and Chemical Accidents.** More information about the Environmental Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (see below).

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FOREWORD

The OECD's Working[1] Group on Harmonization of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on the development of **Consensus Documents** that are mutually recognised among Member countries. These Consensus Documents contain information for use during the regulatory assessment of a particular product. In the area of plant biosafety, Consensus Documents are being developed on the biology of certain plant species, on specific genes and resulting proteins that, when introduced into a plant, result in the expression of specific traits, and on biosafety issues arising from certain general trait modifications made to plants.

This document, which addresses general information concerning the genes and their enzymes that confer tolerance to the herbicide phosphinothricin, was prepared by the United States as lead country in collaboration with Germany and the Netherlands. It has been revised based on comments received from OECD Member countries and on subsequent comments from National Co-ordinators, following a second round of review in 1998.

The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals has recommended that this document be made available to the public. It is published on the authority of the Secretary-General of the OECD.

1. In August 1998, following a decision by the OECD Council to rationalise the names of Committees and Working Groups across the OECD, the "Expert Group on Harmonization of Regulatory Oversight in Biotechnology" became the "Working Group".

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Preamble

OECD Member countries are now commercialising and marketing agricultural and industrial products of modern biotechnology. They have identified the need for harmonization of regulatory approaches for the assessment of these products, in order to avoid unnecessary trade barriers.

In 1993, **Commercialisation of Agricultural Products Derived through Modern Biotechnology** was instituted as a joint project of the OECD's Environment Policy Committee and its Committee on Agriculture. The objective of this project is to assist countries in their regulatory oversight of agricultural products derived through modern biotechnology – specifically in their efforts to ensure safety, to make oversight policies more transparent and efficient, and to facilitate trade. The project is focused on the review of national policies, with respect to regulatory oversight, that will affect the movement of these products into the marketplace.

The first step of this project was to carry out a survey concentrating on national policies in regard to regulatory oversight of these products. Data requirements for products produced through modern biotechnology, and mechanisms for data assessment, were also surveyed. The results were published in *Commercialisation of Agricultural Products Derived through Modern Biotechnology: Survey Results* (OECD, 1995).

Subsequently, an OECD Workshop was held in June 1994 in Washington, D.C. with the aim of improving awareness and understanding of the various systems of regulatory oversight developed for agricultural products of biotechnology; identifying similarities and differences in various approaches; and identifying the most appropriate role for the OECD in further work towards harmonization of these approaches. Approximately 80 experts in the areas of environmental biosafety, novel food safety and varietal seed certification, representing 16 OECD countries, eight non-member countries, the European Commission and several international organisations, participated in the Workshop. *Report of the OECD Workshop on the Commercialisation of Agricultural Products Derived through Modern Biotechnology* was also published by the OECD in 1995.

As a next step towards harmonization, the Working Group on Harmonization of Regulatory Oversight in Biotechnology instituted the development of **Consensus Documents** that are **mutually recognised** among Member countries. The purpose of these documents is to describe common elements in the safety assessment of a new plant variety developed through modern biotechnology, to encourage information sharing and prevent duplication of effort among countries. These common elements fall into three general categories: the biology of the host plant species, or crop; the introduced genes and gene products conferring the novel trait; and biosafety issues arising from the introduction of certain general trait types into plants.

This Consensus Document is a "snapshot" of current information that may be relevant in a regulatory risk assessment. It is meant to be useful not only to regulatory officials, as a general guide and reference source, but also to industry and others carrying out research and product development.

It is anticipated that the Consensus Documents related to genes and products that confer novel traits, together with the relevant Consensus Documents on plant species biology and those providing information on biosafety issues arising from the use of general trait types in plants, will be of use in the biosafety assessment of genetically modified plants.

Reference to two other OECD publications that have appeared in recent years will also prove useful. *Traditional Crop Breeding Practices: An Historical Review to Serve as a Baseline for Assessing the Role of Modern Biotechnology* presents information concerning 17 different crop plants. It includes sections on phytosanitary considerations in the movement of germplasm and on current end uses of the crop plants. There is also a detailed section on current breeding practices. *Safety Considerations for Biotechnology: Scale-Up of Crop Plants* provides a background on plant breeding, discusses scale dependency effects, and identifies various safety issues related to the release of plants with "novel traits".[1]

To ensure that scientific and technical developments are taken into account, OECD countries have agreed that Consensus Documents will be updated regularly. Additional areas relevant to the subject of each Consensus Document will be considered at the time of updating.

Users are therefore invited to provide relevant new scientific and technical information, and to make proposals concerning additional areas that might be considered in the future. ***A short, pre-addressed questionnaire is included at the end of this document. The information requested should be sent to the OECD at one of the addresses shown.***

1. For more information on these and other OECD publications, contact the OECD Publications Service, 2 rue André-Pascal, 75775 Paris Cedex 16, France. Fax: (33) 01.49.10.42.76; E-mail: PUBSINQ@oecd.org; or consult **http://www.oecd.org**

   Also see the BioTrack Online web page at **http://www.oecd.org/ehs/service.htm**

SUMMARY NOTE

This document summarises the information available on the source of the genes that have been used to construct phosphinothricin tolerant transgenic plants, the nature of the enzymes they encode, and the effects of the enzymes on the plant's metabolism.

Scope of this document: OECD Member countries agreed to limit this document to a discussion of the introduced genes and resulting enzymes that confer phosphinothricin tolerance to plants. The document is not intended to be an encyclopaedic review of all scientific experimentation with phosphinothricin tolerant plants. In addition, this document does not discuss the wealth of information available on the herbicide phosphinothricin itself or the uses of the herbicide in agricultural and other applications. Food safety aspects of the use of phosphinothricin on phosphinothricin tolerant transgenic plants are not discussed. Such information is available from other sources, including the respective governmental organisations which regulate the use of the herbicide.

While the focus of this document is on the genes and enzymes involved in encoding phosphinothricin tolerance, reference is not made to specific plant species into which phosphinothricin tolerance might be introduced. Any issues relating to the cultivation of phosphinothricin tolerant plants or to the potential for, or potential effects of, gene transfer from a phosphinothricin tolerant plant to another crop plant or to a wild relative are outside the agreed scope of this document. It is intended, however, that this document should be used in conjunction with specific plant species biology Consensus Documents (see list of publications at the front of the document) when a biosafety assessment is made of plants with novel phosphinothricin herbicide resistance.

Section I - Herbicide Tolerance

Many herbicides kill plants by interfering with enzyme function in the plant. Enzymes are the proteins which catalyse the diverse reactions which comprise the plant's metabolism. Some herbicides exert their effect on a single enzyme which catalyses a key metabolic reaction in the plant. In general, plants exhibit a range of sensitivities to the herbicides used in agriculture, with some species exhibiting considerable tolerance to a herbicide. There are several mechanisms by which plants can tolerate exposure to herbicide: (1) the plant produces an enzyme which detoxifies the herbicide, (2) the plant produces an altered target enzyme which is not affected by the herbicide, or (3) the plant produces physical or physiological barriers to uptake of the herbicide into the plant tissues and cells (Devine et al. 1993).

Phosphinothricin tolerance has been conferred to a variety of plant species (see Section V) by using recombinant DNA techniques to transfer one of two genes (*pat* or *bar*) from bacteria to enable the plant to produce an enzyme (phosphinothricin acetyl transferase; PAT). Expression of PAT within the plant cell detoxifies L-PPT, a herbicide (the L-isomer of phosphinothricin), and thereby makes the plant tolerant to L-PPT. This document summarises the information available on the source of these genes, the nature of the enzymes they encode, and the consequences of transgene expression in the plant. Finally, it is suggested that the reader visit the OECD BioTrack Online website to see the current status of phosphinothricin tolerant plants that have been released under small-scale experimental field trial conditions (**http://www.olis.oecd.org/biotrack.nsf**) and those that have been approved for commercial release (**http://www.olis.oecd.org/bioprod.nsf**).

Section II - Phosphinothricin as a Herbicide

The herbicide phosphinothricin

Phosphinothricin is the amino acid, 4-[hydroxy-(methyl) phosphinoyl]-D,L-homoalanine. The L-isomer of phosphinothricin (L-PPT) is widely used as a broad-spectrum weed control agent and is registered for use as a herbicide in many countries. The D-isomer, D-PPT, exhibits no herbicidal activity. L-PPT is the active ingredient of the herbicide glufosinate ammonium. Glufosinate ammonium is an equimolar, racemic mixture of the D- and L-isomers of PPT. Although D-PPT is not herbicidal, L-PPT inhibits glutamine synthetase of susceptible plants and results in the accumulation of lethal levels of ammonia. L-PPT is considered a broad-spectrum herbicide because it is herbicidal to a wide range of plant species. Some plant species exhibit greater sensitivities than others. Additional information on the properties and use of the herbicide phosphinothricin can be obtained from the governmental authorities which regulate its use. For example, the United States Environmental Protection Agency regulates herbicide use and maintains health assessment information concerning phosphinothricin (glufosinate ammonium) available on the Internet (**http://www.epa.gov/ngispgm3/subst/irisbak/0247.htm**).

Production of L-PPT by micro-organisms

Species of the genera *Streptomyces* and *Kitasatosporia* are the only organisms reported to synthesise the amino acid L-PPT. Species of these genera are Gram-positive, sporulating soil micro-organisms, commonly referred to as actinomycetes (Cross 1989, Locci 1989).

. L-PPT has been reported as a component of only two tripeptides, bialaphos and phosalacine (Wild and Ziegler 1989, Omura et al. 1984). Bialaphos is a tripeptide (phosphinothricyl-L-alanyl-L-alanine) produced naturally by *Streptomyces hygroscopicus* and *S. viridochromogenes*. Each molecule of bialaphos comprises L-PPT and two residues of alanine. Phosalacine is a tripeptide (phosphinothricyl-L-alanyl-L-leucine) produced by *Kitasatosporia phosalacinea* (Takahashi et al. 1984). Peptidase activity readily breaks the peptide bonds, liberating the L-PPT moiety from either bialaphos or phosalacine (Thompson et al. 1987, Wild and Ziegler 1989, Omura et al. 1984).

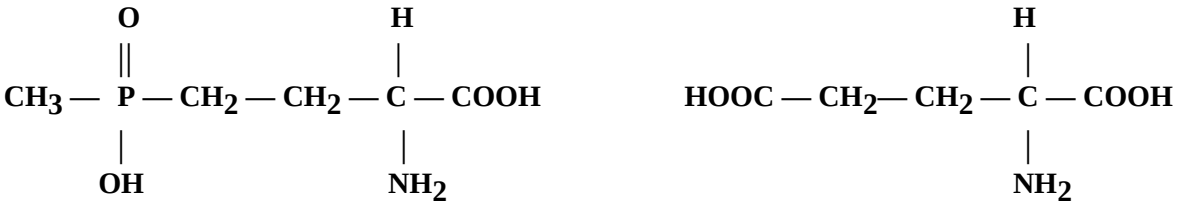
L-PPT is the active ingredient in a number of commercial herbicide formulations. The L-PPT can be derived either from fermentation cultures that yield bialaphos, or from chemical synthesis of glufosinate ammonium. Glufosinate ammonium is an equimolar racemic mixture of L-PPT and D-PPT. There are presently no commercial herbicides which use phosalacine.

Mode of action of L-PPT herbicides

Herbicides based on L-PPT are active against a broad spectrum of plant species. L-PPT is a structural analogue of glutamate, the substrate of glutamine synthetase (see the side-by-side comparison of L-PPT and glutamate in Figure 1). L-PPT exerts its herbicidal effect through the inhibition of glutamine synthetase (Bayer

et al. 1972). In the presence of ATP, L-PPT inhibits glutamine synthetase irreversibly (Devine et al. 1993). When L-PPT inhibits glutamine synthetase, phytotoxic levels of ammonia accumulate in the plant (Miflin and Lea 1976, Tachibana et al. 1986).

Figure 1 L-isomer of phosphinothricin (left) compared to glutamate (right)



Glutamine synthetase is the enzyme responsible for the synthesis of the amino acid glutamine from glutamic acid and ammonia in both eukaryotes and prokaryotes. This is the first reaction in the pathway that assimilates inorganic nitrogen into organic compounds. In plants, glutamine synthetase exists in multiple isozymic forms that can be localised within the cell in the cytosol and plastids. In addition, various isozymic forms are predominately found in certain plant tissues or organs (McNally et al. 1983). In plant roots, the primary role of glutamine synthetase is to assimilate ammonia. However, the glutamine synthetase in leaves is primarily responsible for the reassimilation and detoxification of ammonia (Shah et al. 1986, Kishore and Shah 1988). Glutamine synthetase is the only enzyme in plants that can detoxify the ammonia released by photorespiration, nitrate reduction and amino acid degradation.

As scientists have increased their understanding of the mode of action of L-PPT, several strategies have emerged for developing plants that are tolerant of exposure to the herbicide. The two most prominent strategies are (1) to identify a variant of glutamine synthetase that is insensitive to inhibition by L-PPT, and (2) to introduce a gene that encodes an enzyme designed to inactivate the herbicidal activity of L-PPT. Despite attempts to utilise the first strategy (AgrEvo 1994), to date only the second strategy has been successful in conferring tolerance to L- PPT.

Section III - The Development of L-PPT Tolerant Plants

"Traditional" plant breeding techniques. To date, plant breeders have not been successful in using so-called "traditional" plant techniques to develop L-PPT tolerant crop plants. Historically, plant breeders have tried to identify desirable attributes in the germplasm collection of the crop itself or among closely related plant species. The desirable trait(s) would then be bred into the crop via sexual hybridizations, some of which might require some human intervention to achieve success.

Alternatively, in the absence of finding the desired trait in germplasm collections, breeders have used chemical or radiation induced mutagenesis to create variants that would then be evaluated for efficacy and agronomic performance. This technique relies on slightly modifying the plant enzyme which is the "target" of the herbicide (i.e. the enzyme(s) which the herbicide inhibits). Thus, the mutagenesis results in a target enzyme that still functions but has lost its sensitivity to a herbicide. This approach has been successful in developing maize and soybean varieties which produce a form of acetolactate synthase that is no longer sensitive to imidazolinone and sulfonylurea herbicides (Saari and Mauvais 1996, Shaner et al. 1996). Readers interested in an overview of techniques for producing herbicide tolerant plants may consult Dyer (1996).

Attempts to use such mutagenesis and selection techniques have also failed to produce a useful level of L-PPT tolerance in crop plant species. Included in these efforts has been a decade of failed attempts to obtain maize plants which have a glutamine synthetase that is not inhibited by L-PPT (AgrEvo 1994).

Recombinant DNA techniques. Over the past decade, recombinant DNA techniques have been successfully employed to confer L-PPT tolerance to a variety of crop plant species (see below). Using this approach, plants have been transformed with one of two bacterial genes (*pat* or *bar*) which encode an enzyme, phosphinothricin acetyl transferase (PAT), that detoxifies L-PPT. The expression of the PAT enzyme in the transgenic plants has been used in three different ways: (1) to confer agronomically useful levels of L-PPT tolerance for crop production, (2) to provide a selectable genetic trait (marker) that can be used in the laboratory or field, or (3) to provide a selectable genetic trait in conjunction with a genetic male sterility system.

L-PPT tolerance for agronomic use. In some plants modified to express PAT, the tolerance to L-PPT will be used agronomically in the cultivation of the crop by the grower. An example of such a transgenic L-PPT tolerant plant is the oilseed rape/canola (*Brassica napus* L.) line HCN92, which was the first L-PPT tolerant plant cleared by governmental authorities. Line HCN92 was authorised by Canadian agencies for unconfined release, food and livestock feed use in Canada in 1995 (Agriculture and Agri-Food Canada 1995a, 1995b, 1996a, 1996b). Since then, other transgenic L-PPT tolerant crop plant lines have been cleared through relevant governmental regulatory authorities.

The OECD "Biotrack On-line" database (**http://www.olis.oecd.org/bioprod.nsf**) maintains an updated listing of such approvals.

L-PPT tolerance as a selectable marker. In some of the plants engineered with the *pat* or *bar* gene, the gene serves as a selectable marker gene. Such plants may not necessarily express agronomically useful levels of tolerance to L-PPT. Marker genes are routinely used in developing transgenic plants because they enable the researches to select successful transformants in the laboratory. In addition, tolerance to L-PPT can be used as a selectable marker in the field. Vasil (1996) states that, in some plant species, expression of L-PPT tolerance has been a more useful selectable marker that the kanamycin resistance that has been used since the inception of recombinant DNA research with plants. Final clearances were granted in the United States in 1995 (USDA 1995) for the first transgenic plant which utilised L-PPT tolerance (conferred by the *bar* gene) as a selectable marker trait.

L-PPT tolerance for selection as part of a male-sterility system. Transformation with L-PPT can be used alone or in conjunction with other genes. An example of this is when PAT expression is also part of a genetically engineered male sterility system that can be used in the production of F. hybrid plant varieties (Mariani et al. 1990). In this system, plants are transformed with a genetic construct that couples genes that block pollen production, together with the selectable marker gene which confers expression of PAT. Therefore, the PAT expression in the transformed plants makes it possible to use L-PPT as part of a practical system for plant breeders to produce hybrid seed. In 1996, a maize line engineered with this male sterility system was cleared in the United States prior to commercial release (U.S. Department of Agriculture information found at **http://www.aphis.usda.gov/biotech**). Such transgenic male sterility systems are currently being employed for variety development and seed production in canola, chicory and maize.

A variety of plant species have been engineered with either the *pat* or *bar* genes, and many of these plants have been grown in small-scale field tests to evaluate performance under field conditions. As of 1997, these include: *Agrostis palustris* (creeping bentgrass), *Avena sativa* (barley), *Arachis hypogaea* (peanut), *Beta vulgaris* (sugarbeet), *Brassica oleracea* (wild cabbage), *Chichorium intybus* (chicory), *Daucus carota* (carrot), *Festuca arundinacea* (tall fescue), *Gossypium hirsutum* (cotton), *Hordeum vulgare* (barley), *Lycopersicon esculentum* (tomato), *Medicago sativa* (alfalfa), *Gladiolus* sp. (gladiolus), *Cucumis melo* (melon), *Populus* spp. (poplar), *Solanum tuberosum* (potato), *Brassica napus* (rapeseed), *Oryza sativa* (rice), *Glycine max* (soybean), *Sorghum bicolor* (sorghum), *Saccharum officinarum* (sugarcane), *Nicotiana tabacum* (tobacco), *Triticum aestivum* (wheat) and *Zea mays* (maize).

A number of countries have governmental organisations which regulate the field testing and unrestricted release of genetically engineered plants. Information about these plants in OECD Member countries is available to anyone interested. The database, available on the Internet, is periodically updated to provide information that is both current and accurate (**http//www.oecd.org/ehs/service.htm**).

Section IV - Genes and Enzymes that Confer L-PPT Tolerance

Donor organisms for the genes

Two species of actinomycetes, *Streptomyces viridochromogenes* and *S. hygroscopicus*, have been the source of the genes which have been transferred to plants to confer tolerance to L-PPT (Thompson et al. 1987, Kumada et al. 1988, Hara et al. 1991). These species of *Streptomyces* are saprophytic, soil-borne microbes and are not considered pathogens of plants, humans, or other animals (Locci 1989, Cross 1989).

Genes encoding PAT enzymes (PATs) have been isolated from *S. viridochromogenes* and *S. hygroscopicus*. In *S. hygroscopicus*, a PAT is encoded by the *bar* (*b*ial*a*phos-*r*esistance) gene, whereas in *S. viridochromogenes* a PAT is encoded by the *pat* gene (some researchers refer to the PAT encoded by *bar* as BAR). The *pat* and *bar* genes are very similar, sharing 87 per cent homology at the nucleotide sequence level (Wohlleben et al. 1988, 1992). The respective PAT enzymes encoded by *pat* and *bar* are also very similar, and share 85 per cent homology at the amino acid level (Wohlleben et al. 1988, 1992). Wehrmann and co-workers (1996) recently published results of extensive characterisation of the PATs encoded by *bar* and *pat*. They conclude that the PATs encoded by *pat* and *bar* are so similar as to be functionally equivalent for the purpose of conferring tolerance to L-PPT.

Modification of the native gene to enable expression in plants

In order to achieve efficient expression of the *pat* and *bar* genes within plants, it has been common for researchers to modify the codon usage pattern of genes of bacterial origin prior to introducing them into plants. The *bar* and *pat* genes isolated from *Streptomyces* spp. have relatively high G:C content when compared to plant genes, and as a consequence the native microbial genes are inefficiently expressed in plants. In this case, the codon usage pattern of the native *Streptomyces* genes have been modified prior to introduction into the plant. This resulted in increased expression levels. The amino acid sequence of the resultant PAT is not changed (Eckes et al. 1989, USDA 1995).

Genes of bacterial origin require modification with appropriate plant-expressible regulatory sequences such as promoters, enhancers, intron and terminators. These regulatory sequences do not encode amino acids and therefore do not affect the coding region of the PAT enzyme. Further discussion on the use of regulatory sequences to achieve expression of transgenes in plants is beyond the scope of this document.

Specificity of PAT enzymatic activity

Both PAT enzymes encoded by *bar* and *pat* appear to be: (1) functionally equivalent for the purpose of conferring tolerance to L-PPT, and (2) highly specific for their substrate (Wehrmann et al. 1996). In the presence of acetyl-CoA as a co-substrate, PAT catalyses the acetylation of the free amino group of L-PPT to yield *N*-acetyl-L-PPT, a compound that does not inactivate glutamine synthetase. Both of the PAT enzymes are highly specific for L-PPT and do not acetylate other L-amino acids, nor do they acetylate D-PPT (Wehrmann et al. 1996, AgrEvo 1994). In the presence of excess concentrations of L-amino acids, both PATs also are unaffected in their ability to acetylate L-PPT (Wehrmann et al. 1996).

In L-PPT tolerant plants which express relatively high levels of PAT, the main residue metabolite of L-PPT catabolism is N-acetyl-phosphinothricin (Droege-Laser et al. 1994). When PAT expression is low, the degradation pathways of L-PPT can result in the residue metabolites found in L-PPT sensitive plants, namely 4-methyl-phosphinico-2-hydroxy-butanoic acid and 3-methylphosphinico-propionic acid (Droege-Laser et al. 1994).

Section V - Effects of Transgene Expression in Plants

During the life cycle of any herbicide tolerant plant, the plant is only rarely exposed to the herbicide. When the active herbicide L-PPT is applied to the herbicide tolerant plants, the PAT activity will enable the plant to render L-PPT non-toxic to the plant. The PAT enzyme detoxifies phosphinothricin (L-PPT) by acetylation into an inactive compound. Metabolism studies on genetically modified oilseed rape (*Brassica napus* L.) showed a rapid conversion of L-PPT to the non-toxic metabolite, N-acetyl-glufosinate (European Commission 1998). It has also been reported that PAT has extremely high substrate specificity for L-PPT and demethylphosphinothricin (DMPT) (Thompson et al. 1987), but experimental data have shown it cannot acetylate L-PPTs analogues L-glutamic acid, D-PPT, nor any protein or amino acid (Wehrmann et al. 1996, Agriculture and Agri-Food Canada 1995a, 1995b).

Expression of PAT is not detrimental to plant growth, since such crops have agronomic performance similar to their parents when engineered with either *pat* or *bar* genes. These conclusions have been described in decision documents published by regulatory authorities in Canada, the European Union and the United States prior to the commercialisation of L-PPT tolerant *Chichorium intybus* (chicory), *Brassica napus* (rapeseed, oilseed rape, canola) and *Zea mays* (maize). Information on decisions concerning phosphinotrhicin herbicide tolerant plants can be found at:

http://www.olis.oecd.org/bioprod.nsf
http://www.cfia-acia.agr.ca/english/plant/pbo/home_e.html (Canada)
http://ss.s.affrc.go.jp/docs/sentan/eguide/commerc.htm (Japan)
http://www.aphis.usda.gov/biotech/petday.html (United States)
http://europa.eu.int/comm/dg24/health/sc/scp/outcome_en.html (European Commission)

In recent years, a number of allergenic constituents of plants have been characterised. Allergens usually share a number of characteristics, including the following: (1) they are proteins, (2) they range between 10-70 kiloDaltons in molecular weight, (3) they typically, but not absolutely, are glycosylated, (4) they are stable to digestion (peptic and tryptic conditions of the mammalian digestive system), (5) they are stable to processing, and (6) they are present as the major protein component in the specific food (Metcalfe et al. 1996, FAO/WHO 1996, Fuchs and Atwood 1996). The PAT protein is not a known allergen. SDS-PAGE shows a molecular mass of 22-23 kD for *pat* and *bar* gene products, slightly higher than the calculated mass of 20.6 kD. Gel filtration chromatography shows activity at the 43 kD peak (homodimer) (Wehrmann et al. 1996). The same authors reported that when PAT and BAR proteins, produced from the *pat* and *bar* genes respectively, were subjected to simulated gastric conditions with pepsin, both proteins were degraded within seconds, and the enzymatic activity dropped to zero within a 5-15 second timeframe.

Other reported studies have shown that the enzyme was inactivated within one minute when subjected to typical mammalian stomach conditions and was inactivated during processing of canola seed (from transgenic *Brassica napus* expressing the PAT enzyme) into feed ingredients (European Commission 1998). The USEPA (1997) reported that experimental data indicated that the PAT protein is rapidly degraded in the gastric environment and is also readily denatured by heat or low pH. Many food

allergens have been biochemically characterised, and databases make it possible to compare the amino acid sequence of a protein to those proteins in the database which are known to elicit allergenic responses. The nucleotide sequence of the gene was provided. When subjected to comparative analyses using the GENEBANK DNA database (Agriculture and Agri-Food Canada 1995a) and the FASTDB algorithm of Intelligenetics with three databases of polypeptide sequences (Agriculture and Agri-Food Canada 1995b), the PAT enzyme amino acid sequence did not show significant homology with other proteins present in the databases, except with other phosphinothricin acetyltransferases originating from different organisms. No resemblance with potential toxins or allergens was observed. USEPA (1997a) concluded that "the potential for the PAT protein to be a food allergen is minimal."

When proteins are toxic, they are known to act via acute mechanisms and at very low dose levels (Sjobald et al, 1992). There is no evidence available indicating that the PAT protein is toxic to either humans or other animals. In a 14-day feeding study using bacterially produced purified PAT enzyme, mice gavaged with high levels of the protein (5,050 milligram/kilogram bodyweight) showed no treatment-related significant toxic effects (USEPA 1995). It has also been reported that an avian dietary test was performed with the seed-eating canary bird (*Serinus canaria domestica*), and that a feeding study was performed with the domesticated rabbit (*Oryctolagus cuniculus*); these studies showed no differences in food consumption, behaviour and body weight between birds or rabbits fed with the transgenic PAT producing *Brassica napus* L. (rapeseed, oilseed rape, canola) or non-transgenic counterparts (Agriculture and Agri-Food Canada 1995b).

With respect to the toxicity of PAT, USEPA concludes that "the acute oral toxicity data submitted support the prediction that the PAT protein would be non-toxic to humans." In the United States the EPA, based on submitted toxological data, established an exemption from the requirement of a tolerance for residues of the plant-pesticide ingredients phosphinothricin acetyltransferase (PAT) and the genetic material necessary for its production in all plants (USEPA 1997b).

Governmental regulatory authorities in the United States, Canada, Japan and European Union have made decisions that the presence of the PAT protein in plants does not render them unsafe for consumption as food or feed (see above). Further information on the food safety criteria can be found in published regulations, guidelines and policy statements of various governmental agencies.

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