SECTION 2

GENERAL INFORMATION CONCERNING THE GENES AND THEIR ENZYMES THAT CONFER TOLERANCE TO GLYPHOSATE HERBICIDE

Summary Note

This document summarises the information available on the source of the genes that have been used to construct glyphosate-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 glyphosate tolerance to plants. The document is not intended to be an encyclopaedic review of all scientific experimentation with glyphosate-tolerant plants. In addition, this document does not discuss the wealth of information available on the herbicide glyphosate itself or the uses of the herbicide in agricultural and other applications. Food safety aspects of the use of glyphosate on glyphosate-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 glyphosate tolerance, reference is not made to specific plant species into which glyphosate tolerance might be introduced. Any issues relating to the cultivation of glyphosate-tolerant plants or to the potential for, or potential effects of, gene transfer from a glyphosate-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 glyphosate herbicide resistance.

1. Herbicide Tolerance

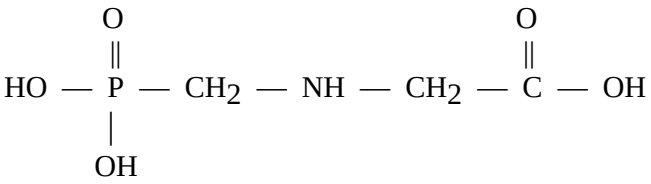
Many herbicides kill plants by interfering with enzyme function in the plant. Most of these herbicides exert their effect on a single enzyme which catalyses a key metabolic reaction in the plant. In general, plants exhibit a range of sensitivities to the herbicides used in agriculture, with some species exhibiting considerable tolerance to a single 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).

2. Glyphosate as a Herbicide

Glyphosate is widely used as a broad-spectrum weed control agent and is registered in many countries (Duke 1996, Shah *et al*. 1986). Even though glyphosate is a reversible competitive inhibitor of the enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS) with respect to phosphoenolpyruvic acid (PEP), it does not inhibit any other PEP-dependent enzymatic reactions. It is a non-competitive inhibitor of EPSPS with respect to 3-phosphoshikimic acid (Steinrucken *et al*. 1984). Glyphosate is produced by

chemical synthesis. It is not a natural product. Chemically, glyphosate is N-phosphonomethyl-glycine (see *Figure 2*.1). Glyphosate is the active ingredient of the herbicide Roundup® (Monsanto).

Figure 2.1 Glyphosate Structure



The high sensitivity of crop plants to glyphosate has limited its use as a pre-crop emergence herbicide in no-till management strategies, and as a herbicide and crop desiccant when applied shortly before crop harvest. With the development of genetically engineered crop plants that are resistant to glyphosate, this herbicide can instead be applied after both crops and weeds have emerged, with little or no damage to the crop.

Glyphosate interferes with normal plant metabolism through inhibiting the enzyme 5-enolpyruvyl-3-phosphoshikimic acid synthase (EPSPS). In plants and micro-organisms, EPSPS is involved in the biosynthesis of aromatic amino acids, vitamins, and many secondary metabolites. It is not present in animals (Levin and Sprinson 1964, Steinrucken and Amrhein 1980). In plants, EPSPS is localised within plastids. This enzyme condenses phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid. As a consequence of the inhibition of aromatic amino acid biosynthesis, protein synthesis is disrupted, resulting in the plant's death (Kishore and Shah 1988). While some of the downstream products of the EPSPS reaction, amino acids and vitamins, are strictly essential for the growth of all living organisms, some secondary metabolites derived from the shikimate pathway may have specific survival value for the producing organism (Malik 1986). The enzyme has rigid specificity towards its substrates, which are shikimate-3-phosphate and phosphoenolpyruvate (Anderson and Johnson 1990). The reaction product, 5-enolpyruvylshikimate-3-phosphate (EPSP), is further acted upon by other enzymes to yield chorismic acid, which gives anthranilic acid (a precursor of tryptophan) and, on rearrangement, prephenic acid (a precursor of phenylalanine and tyrosine).

Based on the knowledge of the mode of action of glyphosate, several strategies have emerged for developing plants that are tolerant of exposure to the herbicide. The two successful strategies to produce glyphosate-tolerant plants are introduction of glyphosate-tolerant EPSPS and introduction of an enzyme that inactivates glyphosate, glyphosate oxidoreductase (GOX). Recombinant DNA techniques have been used to express genes that encode glyphosate-tolerant EPSPS enzyme alone or a combination of EPSPS and GOX genes in susceptible plants (Nida *et al*. 1996, Padgette *et al*. 1995, 1996).

3. The Development of Glyphosate-Tolerance Plants

Scientists have been unsuccessful in producing glyphosate-tolerant plants using classical techniques. Traditional mutagenesis and selection techniques have to date failed to produce a useful level of tolerance in crop plant species, although such an approach could yield a mutant form of the target enzyme that is tolerant of the herbicide but retains its desirable enzymatic function. Plant breeders also have been unable to develop glyphosate-tolerant crops using the standard techniques in which chemical or radiation exposure of seeds generates mutations in the plant genome. In cases where the desired phenotype is herbicide tolerance, spraying seedlings in the growth chamber or field can sometimes be used with success to select tolerant individual plants from millions of mutagenised individuals. Even though this approach has been used in the commercial development of imidazolinone-tolerant maize and soybean cultivars, it has not been successful in producing glyphosate-tolerant plants. This is because all mutant EPSPS, in parallel to

glyphosate tolerance, has decreased affinity for phosphoenolpyruvate. This has resulted in glyphosate-tolerant plants that have invariably shown reduced biosynthesis of aromatic amino acids.

Recombinant DNA techniques have been used to confer glyphosate tolerance to a variety of crop plant species. In this approach, plants have been transformed with genes that encode a glyphosate-tolerant enzyme that is not inhibited by glyphosate but provides substrates for the biosynthesis of amino acids. In some cases, the tolerance imparted by this gene has been further augmented by expressing a second gene that encodes the enzyme glyphosate oxidoreductase (GOX) to detoxify glyphosate (Padgette *et al*. 1996, Shah *et al*. 1986).

4. Genes and Enzymes that Confer Glyphosate Tolerance

Three genes which provide field-level tolerance to glyphosate, the active ingredient in Roundup® herbicide, have been introduced into commercial cultivars. The first glyphosate-tolerant EPSPS gene was isolated from a soil bacterium, *Agrobacterium* (Barry *et al*. 1994, Duke 1996). The EPSPS synthase from this *Agrobacterium* was highly tolerant to glyphosate. When it is expressed in transgenic plants, the EPSPS encoded by this *Agrobacterium* gene fulfills the aromatic amino acid needs of the plant in the presence of glyphosate, whereas the plant version of this enzyme (ubiquitous in nature) is sensitive to glyphosate. *Agrobacterium* spp. are not human or animal pathogens, but some species are pathogenic to plants (Croon 1996, Holt 1984).

Recently, the EPSPS gene from corn (*Zea mays*) has been mutagenized *in vitro* to obtain a glyphosate-tolerant enzyme. The tolerant version of the enzyme produced by the modified maize gene is 99.3% identical to the parent enzyme (Monsanto 1997).

Also, a gene that encodes for a glyphosate-degrading enzyme called glyphosate oxidoreductase (GOX) was isolated from *Achromobacter* strain LBAA, a soil bacterium ubiquitous in nature (Barry *et al*. 1994). The encoded enzyme deactivates the herbicidal effect of glyphosate. Glyphosate oxidoreductase catalyses the conversion of glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate. GOX requires flavin adenine dinucleotide (FAD) and magnesium for activity; therefore, it is more appropriately designated an apoenzyme.

EPSPS enzyme, the target of glyphosate action, is synthesised in the cytoplasm and then transported to the chloroplast (Kishore and Shah 1988). The translocation of the protein to the chloroplast is carried out by an N-terminal protein sequence called the chloroplast transit peptide (CTP). CTPs are typically cleaved from a mature protein and degraded following delivery to the plastid (Della-Cioppa *et al*. 1986). A plant-derived coding sequence expressing a chloroplast transit peptide is often linked with each of the genes imparting glyphosate tolerance. This peptide facilitates the import of the newly translated enzymes into the chloroplasts, the site of both the shikimate pathway and glyphosate mode of action.

Use of the technology achieving transgene expression in plants is now routine. In order to achieve efficient expression of bacterial 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. In this case, the codon usage pattern of the *Agrobacterium* glyphosate-tolerant EPSPS gene and glyphosate oxidase genes of *Achromobacter* have been chemically synthesised for codon optimisation for efficient expression in the plant. The amino acid sequence of the resulting enzymes is not changed. The genes associated with their transit peptide coding sequence are usually linked to other regulatory sequences like promoters, terminators, enhancers and introns. These regulatory sequences do not usually encode for a protein (Croon 1996).

These genes have been engineered (singly or in combination) into many plant species for the development of glyphosate tolerance and for use as selectable markers for identification of transformed plants. Plants field-tested with these genes include: *Beta vulgaris* (beet), *Zea mays* (corn), *Gossypium hirsutum* (cotton), *Lactuca sativa* (lettuce), *Populus* (poplar), *Solanum tuberosum* (potato), *Brassica napus* (oilseed rape, rapeseed, canola), *Glycine max* (soybean), *Nicotiana tabacum* (tobacco), *Lycopersicon esculentum* (tomato), *Triticum aestivum* (wheat).

OECD Member countries have governmental organisations which regulate the field-testing and unrestricted release of genetically engineered plants. Information about these plants is shared among various Member countries. The OECD sponsors an electronic database format for the exchange of this information. The database information is periodically updated to provide information that is both current and accurate (**www.oecd.org/ehs/service.htm**).

5. Effect of Transgene Expression in Plants

During the life cycle of any herbicide-tolerant plant, the plant is exposed only rarely to the herbicide. Except for the production of the enzyme(s) encoding glyphosate tolerance, there should be no other changes in plant metabolism. After glyphosate application, the enzyme activities expressed by the transgenes enable the plant to survive herbicide exposure. In the case of introduced EPSPS, no new metabolic products are formed since the only difference from the native enzyme is its insensitivity to glyphosate. However, if very high expression levels result from the insertion, the levels of downstream metabolites might change. In contrast, GOX will convert glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate when glyphosate herbicide is applied (Torstensson 1985). Since glyoxylate is a naturally occurring plant metabolite involved in carbon cycling, it will be further metabolised to provide intermediates for the Krebs cycle. Since GOX is highly specific for its substrate, glyphosate, in the absence of glyphosate no metabolites are expected. The United States Environmental Protection Agency has decided that only glyphosate residues are to be regulated in plant and animal commodities, and that the major metabolite AMPA is not of toxicological concern regardless of its level in food (US EPA 1997). Information regarding decisions concerning glyphosate 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 (USA)
http://europa.eu.int/comm/dg24/health/sc/scp/outcome_en.html (European Commission)

Western blot and enzymatic activity assays indicate that EPSPS protein from *Agrobacterium* strain CP4 is readily degraded in less than two minutes by incubation in simulated gastric fluid. In simulated intestinal fluid the enzyme activity and immunoreactivity lasts longer, being still detectable at ten minutes but undetectable at 270 minutes. The GOX protein is rapidly degraded in simulated gastric fluid and simulated intestinal fluid. After a 15 second incubation in gastric fluid, GOX has less than 90% of its initial protein epitopes as assayed by Western blot analysis, and enzyme activity loss is also greater than 90% when assayed after one minute incubation in gastric fluid. Similar results are seen in simulated intestinal fluid (US EPA 1996 and 1997).

Expression of GOX and glyphosate-tolerant EPSPS is not detrimental to plant growth, since such crops have agronomic performance similar to their parents. Governmental regulatory agencies in the United States (US Department of Agriculture 1994, 1995, 1997), Canada (Agriculture and Agrifood Canada 1995, 1996), Japan (Ministry of Agriculture, Forestry and Fisheries 1996) and European Union (European Commission 1998a, b) have made decisions that the presence of the EPSPS and GOX proteins in plants does not result in plants that are unsafe in their environments. Several lines of evidence support

the conclusion that these enzymes show low mammalian toxicity: (1) Neither enzyme shows amino acid homology to known allergens or mammalian toxins (Burke and Fuchs 1996); (2) Data from acute oral toxicity tests at high concentration of enzymes showed no toxicity (Harrison *et al*. 1996). In acute oral toxicity tests of bacterially derived CP4 EPSPS protein, no test substance adverse effects occurred at a dose of 572 milligrams per kilogram body weight (mg/kg) of the test animals. The acute toxicity of bacterially derived GOX protein showed no test substance adverse effects at doses of 91.3 mg/kg of the test animals; (3) Both enzymes are readily inactivated by heat or mild acidic conditions and are readily degraded in an *in vitro* digestibility assay which is consistent with the lack of oral toxicity (US EPA 1996, 1997). That the two enzymes show little if any toxicity is consistent with the observation that most enzymes are not considered toxic to vertebrates (Kessler *et al*. 1992). Notable exceptions are diphtheria toxin and certain enzymes in the venom of snakes, with very different exposure scenarios.

Governmental regulatory agencies in the United States (US Food and Drug Administration 1996), Canada (Agriculture and Agrifood Canada 1995, 1996), Japan and the European Union have made decisions that the presence of the EPSPS and GOX proteins in plants released into the environment do not pose a significant allergenicity risk. Two independent lines of evidence support the decision that these enzymes are not potential allergens: (1) Current scientific knowledge suggests that common food allergens tend to be resistant to degradation by heat, acid and proteases, are glycosylated, and are present at high concentrations in food. The EPSPS and GOX proteins are rapidly degraded by gastric fluid *in vitro* and are non-glycosylated. Thus, the potential for these proteins to be food allergens is minimal (Astwood *et al*. 1996, Burke and Fuchs 1996); (2) It is possible to utilise international gene databases to compare the gene sequences of a protein with other genes that encode known allergens. None of the amino acid sequences of known allergens or proteins involved in disease were shown to have similarity to the EPSPS or GOX proteins, as defined by eight identical and contiguous amino acids in a sequence. Likewise, none of the amino acid sequences of known allergens or proteins involved in coleiac disease were shown to have similarity to the GOX protein as defined by eight contiguous amino acids in a sequence (US EPA 1997).

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