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A transgene-centered approach to the biosafety of transgenic phosphinothricin-tolerant plants

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Abstract

The microbial *bar* and *pat* genes confer tolerance to the non-selective herbicide phosphinothricin (PPT; sold as Basta or Finale). This tolerance in plants could provide an environmental gain compared to current-day herbicide cocktails, but the safety of such a transgene approach is questioned by many. The biosafety of the presence of these herbicide tolerance genes in plants is evaluated in a 'transgene-centered approach'. Potentially, the introduction of transgenic PPT-tolerant crops could result in acquired PPT tolerance in weedy relatives of these crops. Assuming responsible use of this trait in agronomy, the ecological consequences with respect to weediness or spread of the transgenic PPT tolerance are concluded to be negligible. The key issue for the toxicological evaluation is whether or not the plant has actually been sprayed with PPT. Consumption of the gene and/or gene product from unsprayed transgenic plant material will not have adverse effects. In case of PPT-sprayed material, PPT or its derivatives could be present in food and feed and crop-specific metabolites might be formed. To date, the toxicological impact of such a putative exposure is not sufficiently clear, and further premarket testing is recommended.

Introduction

With the introduction of transgenic plants, various aspects of such plants have been studied to evaluate their biosafety and admission. Many 'transgene independent' studies have concluded that transgenic plants should be evaluated on a case-by-case basis with attention being given to the ecological and toxicological impact of the introduced genes and gene products. These aspects are the motivation for the alternative, 'transgene-centered' evaluation, in which all characteristics of a particular transgene are evaluated [28, 33]. Such an approach could help to generalize outcomes irrespective of the plant species into which the transgenic trait is introduced. The combination of plant and transgene characteristics will contribute to informed decision making. Furthermore, concentrating on the presence of gene *x* and the gene product X allows definite questions to be evaluated and may identify open issues more readily. Biochemical, ecological and toxicological data on the gene, its product, substrates and degradation products will help to streamline the discussions about the ongoing commercialization of transgenic crops. Here, the *bar* and *pat* genes whose gene products confer tolerance to the herbicide phosphinothricin (PPT) are reviewed as an illustration of the transgene-centered approach. The starting-point question is: given current agricultural practice, what could be the consequences of introducing PPT tolerance into agronomic crops?

Phosphinothricin and phosphinothricin tolerance

Phosphinothricin: properties and applications

PPT originates from the actinomycetes *Streptomyces viridochromogenes* and *S. hygroscopicus* [4, 26]. Industrially it is synthesized as a DL-racemic mixture, of which only the naturally occurring L-PPT is herbicidal. PPT, or glufosinate, is sold under the brand names Basta, Finale and Radicale. It is widely used as

broad-spectrum, pre-emergence herbicide and also for pre-harvest desiccation in potato, legumes and oilseed rape through application to the leaves. PPT has no margin for discrimination between crop and weed: it is a so-called non-selective herbicide [8].

PPT interferes with amino acid synthesis through inhibition of glutamine synthetase (GS) [24]. GS is the key enzyme in nitrogen metabolism that assimilates ammonia produced by nitrate reduction, and recycles ammonia produced by processes such as photorespiration and deamination [24]. As a structural analogue of the GS substrate glutamate, PPT inhibits GS irreversibly. This inhibition triggers ammonia accumulation to levels up to 100-fold higher than in control plants, resulting in cessation of photosynthesis and disruption of the chloroplast structure [12, 37]. In common agricultural practice, two to four hours after application photosynthesis slows down and the plants yellow and die in two to five days [20]. Over 40 monocotyledonous and more than 150 dicotyledonous weeds are sensitive to PPT [20]. Weeds generally require 0.6–2.0 kg/ha, but, for example, *Cassia obtusifolia* requires 8.5 kg/ha, whereas *Setaria viridis* is killed by 0.2 kg/ha. There is no example of absolute PPT resistance, rather it is a matter of PPT tolerance.

PPT tolerance

A successful strategy for obtaining PPT-tolerant crops is based on the mechanism used by the PPT-producing actinomycetes, which protect themselves against the autotoxic action by metabolizing the compound. They produce phosphinothricin-N-acetyltransferase (PAT) that acetylates the free NH_2 group of PPT, causing its inactivation.

The PAT-encoding *bar* and *pat* genes were isolated from *S. hygroscopicus* and *S. viridochromogenes* Tü494, respectively [32, 36]. Both genes code for PAT proteins of 183 amino acids, which show 85% homology, variations of the genes being confined to the 5′-non-coding regions [44].

For expression in plants, the PAT-encoding genes driven by promoters active in plants have been successfully introduced in crops using standard transformation technology [6]. Transgenic plants prove to be tolerant to 4–10 times the dose of PPT required to kill control plants. PAT levels of no more than 0.001% of total soluble protein proved sufficient to confer tolerance at field dose applications of the herbicide [6]. In large-scale field trials, transgenic PAT-containing plants showed similar agronomic performance as controls [18, 19].

Currently, there are three applications of transgenic PPT tolerance in the development and use of plant material: as selectable marker during genetic transformation, as agronomic character, and in hybrid seed production.

Biosafety issues

Transgenic PPT tolerance raises ecological and toxicological concerns: it might transform a crop into an uncontrollable weed; it may spread from the crop to wild relatives or other organisms, which consequently become uncontrollable; or it might disturb ecological relationships of the crop in another way. Furthermore, the presence of the PPT tolerance gene or its gene product may directly or indirectly render the plant unsuitable for consumption or industrial processing. Also, the use of PPT in transgenic crops may challenge consumers with the herbicide or its metabolites. Finally, there may be unexpected pleiotropic effects associated with transgenic PPT tolerance.

Environmental impact of PPT and its metabolites

As a chemical compound, PPT is stable, but in the soil it is rapidly degraded by microbiological activity to 3-methylphosphinicopropionic acid (MPP) and ultimately to CO_2 [38, 45]. MPP is non-phytotoxic [13] and has no residual herbicidal activity [20]. After 13 to 14 weeks 30–60% of deposited radioactive MPP could be detected as $^{14}CO_2$ [14, 20]. In metabolism studies, no residues of the active compound could be detected in plants or in animal tissue, indicating rapid secretion.

Another important issue in the characteristics of transgenic PPT-tolerant plants is the relative environmental load of PPT. The use of PPT and PPT-tolerant crops could imply a considerable reduction in the amounts of herbicides used compared to current practice [25, 31]. Generally, the environmental impact of PPT is considered to be less than for currently used cocktails of herbicides [34]. Due to the high solubility in water, accumulation in the food chain will not occur [27]. PPT is considered to be safe for both water and soil life. Aquatic invertebrates showed no mortality with a test dosage of 1000 mg/l and for fish lethal dose (LD_{50}) values were as high as 580 mg/kg [27, 45]. No mortality in earthworms and honey bees could be observed [27, 45]. PPT will not leach into groundwater reserves in spring [34]. Although it is considered

less safe with respect to leaching into groundwater in autumn [34], PPT has not been detected to a greater depth than 15 cm in field studies [45], presumably because of rapid microbial degradation. Therefore, the likelihood of accumulation in soil or groundwater is low.

Weediness

The weediness of a PPT-tolerant plant largely depends on the interplay between the intrinsic characters of the plant, in combination with the specific habitat the plant occupies [23, 40]. The scenario relevant to biosafety is enhancement of fitness. Spraying with PPT creates a clear selective advantage for PPT-tolerant crop plants in production areas. Except perhaps occasionally in verges adjacent to production fields, it is unlikely that such selective conditions will be found outside agricultural production fields. In the absence of spraying with PPT, PPT tolerance does not contribute to weediness.

Analysis of the competitiveness of transgenic PPT-tolerant oilseed rape under non-selective PPT conditions revealed no significant differences between transgenic and non-transgenic lines. Inclusion of the more competitive crucifer *Sinapis alba* as reference species in the experimental design indicated that any putative change in competitiveness would not exceed the competitive ability of this crucifer [18].

No increase in invasive potential conveyed by PPT tolerance was observed for oilseed rape in a variety of habitats and under a range of climatic conditions in which there were no selective concentrations of PPT present [11]. Whenever there was any significant difference, transgenic lines were less invasive and less persistent than their non-transgenic counterparts. In the absence of selective conditions, there is no advantage for PPT-tolerant crops and there will be no increased weediness of these crops. The paper by Crawley et al. [11] on the impact of transgenic plants on the ecology of natural habitats has been called a 'landmark paper in ecology' [22] and resulted in lots of discussions among ecologists and others about its scientific merits, its experimental designs as well as the validity and generality of the conclusions drawn [9, 10, 22, 29, 30, 43]. Miller et al. [29, 30] consider the Crawley et al.'s experiment as 'an example of a well-executed but poorly conceived risk assessment experiment' of which 'the result was largely predictable' not worth the magnitude of the experiment. Crawley reacted with: 'The negative result made the important (if predictable) point that the act of genetic engineering does not, of itself, make any measurable difference to the ecological performance of oilseed rape. The limitations of extrapolating the result do not mean that the work was no worth doing. It was one step in a step-by-step process' [10]. The debate between Crawley et al. and Miller et al. does not seem to contribute a lot to the issue at hand. The application of molecular techniques and markers might be useful for further development of 'molecular ecology' [43] which will yield valuable insights into the dynamics and plasticity of ecosystems and contribute to the biosafety assessment of transgenic plants. Molecular techniques and markers broaden the possibilities to follow the putative spread and persistence of a certain trait.

Spread of the transgene

The spread of the PPT tolerance transgene to wild relatives depends on a myriad of ecological situations, genetic factors and stochastic events [40]. In view of current large-scale agriculture, it is prudent to assume that the PPT tolerance transgene will spread by cross-pollination to wild relatives in some conditions and at some locations. For various crop-weed combinations the likelihood of hybridization in relation to distance has been analyzed [1, 3]. Such studies have demonstrated gene flow at varying distances (reviewed by [2]). It is important, therefore, to assess the effect of such a spread. Outcrossing to a weedy wild relative may result in a PPT-tolerant weed that moves back into the field and cannot be controlled any more with PPT. The field itself can be, therefore, a site of selection for PPT-tolerant hybrids. Outside agricultural fields, a weedy wild relative will only be able to go out of control in case of selective PPT conditions, which are unlikely to exist or build up. If the introduction of transgenic PPT tolerance could result in the occurrence of acquired PPT tolerance in weeds, it is difficult to predict how fast and how complete such a putative loss of PPT sensitivity might be. The impact, however, is likely to be of an economic rather than an ecological nature.

Horizontal gene transfer to another organism requires a chain of events, each step having little likelihood [35]. The final outcome, irrespective of the time it will take to happen, will be an organism that is tolerant to PPT. The consequence of this tolerance will depend on the presence of selective conditions, which only occur in agricultural production fields. It

is unlikely, therefore, that such an organism, may it develop, will have any selective advantage in natural ecosystems. The use of PPT and PPT-tolerant crops in the production of hybrid seeds and as selection marker during transformation is fully biosafe. In these applications, PPT tolerance is either only applied as dominant selective marker under conditions for seed production or under controlled laboratory conditions.

Consumption

The introduction of the *bar* or *pat* transgene in crops, and subsequent use of PPT during crop cultivation, imply that three additional classes of molecules could be present: the transgene, *bar* or *pat*, and it metabolites; the transgene product, PAT, and its metabolites; and the herbicide PPT and its metabolites. Each of these should be evaluated for undesirable effects on consumers. The large amount of DNA that passes the digestive tract daily indicates that foreign DNA is not intrinsically toxic to man and all other organisms. DNA is efficiently degraded and no functional genes are assumed to remain present [5]. In this respect, *bar* and *pat* DNA will not differ from any other DNA and will not pose any adverse effects. In the unlikely case that intestinal cells or micro-organisms acquire the *bar* or *pat* DNA, it is comparable to putative horizontal gene transfer in ecosystems. The absence of any positive selective pressure for PAT-containing cells or organisms in the digestive tract of consumers will preclude any conceivable harm.

PAT and its metabolites

Undesirable effects of the presence of the PAT protein could result from enzymatic activity of PAT in either the transgenic plant or the digestive tract, the presence of PAT itself and/or the degradation products of PAT.

The PAT enzyme has a high substrate specificity for L-PPT [13, 39]. Glutamate and analogues are poor substrates, having affinities at least 500 times lower than PPT. The overall high substrate specificity suggests that enzymatic activity of PAT in the transgenic plant will not result in the establishment of pools of unfamiliar secondary metabolites. In the human digestive tract, no substrate is likely to be available and the gastric conditions (pH 2 to 4) preclude catalytic activity. The pH optimum for the enzyme is 7.5 and rapid thermo-inactivation is observed at temperatures exceeding 35 °C [7, 42]. PAT loses all enzymatic activity within one minute of exposure to gastric pH (E. Kok, pers. commun.). In addition, the required co-factor acetyl-CoA is not stable in such acidic conditions. Enzymatic activity of PAT in the human digestive tract can, therefore, be excluded.

Without enzymatic activity, the PAT protein molecule could prove toxic or allergenic upon consumption. Generally, proteins are non-toxic [21]. The OECD has summarized the criteria which indicate allergenicity of a protein [17]: relative abundance; glycosylation; resistance to proteolytic degradation and resistance to heat denaturation. The amount of PAT protein is not likely to exceed 0.1% of the total soluble protein content of the transgenic plant material and the protein has no glycosylation sites. Database comparisons with known protein sequences gave no hint of any allergenic or toxic potential of the PAT protein [41]. All these criteria indicate that no allergenicity or toxicity of the PAT protein or its degradation products are to be expected.

PPT and its metabolites

The agronomic use of PPT-tolerant crops will imply a shift from the current pre-emergence applications to post-emergence applications of PPT. The food and feed safety of transgenic PPT-tolerant plants will depend on the additional metabolites present. This, in turn, depends on whether or not and when the plant was sprayed with PPT prior to consumption. Without spraying with PPT, the additional metabolites that occur in transgenic PPT-tolerant crops are the *bar* and *pat* transgenes and the PAT enzyme. As indicated above, these metabolites do not have any adverse effects. All cases in which the PPT tolerance is only used as marker for transformation in the laboratory are covered by this scenario.

In the majority of cases, however, PPT-tolerant crops are likely to be used in combination with PPT. The additional metabolites present in transgenic PPT-tolerant plants upon PPT spraying are PPT itself, its metabolites and the metabolites formed through PAT activity. Commercial PPT is a racemic mixture of D- and L-PPT and requires the evaluation of both enantiomers. For a proper evaluation, two scenarios must be considered, depending on the amount of PAT protein present in the transgenic plants. In plants with relatively high PAT amounts, L-PPT is quantitatively acetylated giving acetyl-PPT, while D-PPT remains stably present [13, 15]. If commercialized transgenic crops contain sufficient amounts of PAT protein to establish the quantitative acetylation of PPT, only the metabolites acetyl-PPT and D-PPT need to

be evaluated for consumption. Acetyl-PPT is a stable compound that may accumulate in the plant and some transport via the xylem into the fruits or seeds may occur [15]. Upon oral administration, acetyl-PPT, which is also formed in the gut of animals via the normal detoxification pathway [41], is excreted rapidly, mainly via faeces and some via urine. There is no deacetylation in the stomach to yield PPT. Mammalian toxicity studies yielded LD_{50} values for oral and dermal administration larger than 2.8 g/kg body weight, indicating that acetyl-PPT is essentially non-toxic. Acetyl-PPT, therefore, poses no concern for fresh human consumption, but no toxicological data are available for consumption by non-mammalian organisms. The amount of acetyl-PPT that could be present in foodstuff is unclear, also the fate of acetyl-PPT upon food processing is unknown. These issues seem to deserve more attention.

The toxicity of D-PPT has only been determined in combination with L-PPT. Although DL-PPT inhibits mammalian GS [16], it is generally not toxic to mammals because of its rapid clearance by the kidneys [24] (LD_{50} 1.5 to 4 g/kg body weight). The commercial formulation of PPT, which includes DL-PPT and a wetting agent, must according to EU directive 83/467/EEC be classified as 'harmful' on the basis of the acute oral toxicity tests. It induced slight dermal toxicity and eye irritation and was slightly toxic following oral exposure to laboratory animals [16]. It is unclear whether these effects are due to the D-enantiomer, or to the wetting agent. No genotoxic, teratogenic or carcinogenic potential was observed [16]. There was no toxicity for bees, earthworms or soil micro-organisms [27]. A daily intake of 0.02 mg DL-PPT per kg body weight per day is proposed as acceptable [16]. It would seem highly unlikely that sprayed transgenic plants will ever accumulate such amounts of D-PPT, so the putative presence of D-PPT in transgenic plant material is no cause for concern.

In plants with relatively low PAT activity, the L-PPT will not be fully converted to acetyl-PPT. In addition to substantial amounts of L-PPT, D-PPT and acetyl-PPT, the metabolites 4-methylphosphinico-2-oxobutanoic acid (PPO), 4-methyl-phosphinico-2-hydroxybutanoic acid (MHB) and MPP were observed [15]. Similar to non-transgenic, PPT-sensitive plants, in plants with relatively low PAT activity, deamination of L-PPT results in PPO and subsequent decarboxylation yields MPP [38]. No further decarboxylation of MPP was detected [13, 15]. In plants, PPO can alternatively be reduced to MHB. In addition, 4-methylphosphinicobutyric acid (MB) was a PPT metabolite found so far only in monocots [15]. The possibility of crop or species-specific PPT metabolites has therefore to be taken into account in the analyses of PPT metabolites in transgenic plants.

The presence of MHB and MPP in PPT-tolerant plants depends on the amount of PAT present, indicating competition between the PPO-MPP/MHB and the PAT metabolic routes [15]. Both MHB and MPP were found to be final and stable products of the plant's metabolic pathways [13, 15]. Transport of these metabolites via the xylem to the upper regions of the plant was observed. No toxicological data concerning MHB and MPP or other putative metabolites are available. The putative accumulation and exposure to metabolites such as MPP or MHB deserves attention. It is currently insufficiently clear to what levels of PPT and/or its metabolites consumers are exposed. As long as there is not sufficient familiarity with the trait, it would seem to be advisable to develop a protocol to evaluate the levels of PPT metabolites in PPT-tolerant plant food. This will indicate if, and if so which, further toxicological data are neccessary.

Pleiotropic effects

The presence of the *bar* or *pat* transgene or its product, or any of its metabolites, may in some unexpected way alter any of the manifold ecological relationships or toxicological characteristics of the crop. The same applies to any wild relative derived from outcrossing, or any organism derived from horizontal gene transfer and any product derived from it. For example, the tabtoxin resistance gene (*ttr*) from *Pseudomonas syringae* encodes an acetyltransferase, which inactivates tabtoxin but not bialaphos [46]. If the PAT enzyme were to inactivate the tabtoxin, *Pseudomonas* resistance could be a pleiotropic effect of PPT tolerance. Although there are no reports of PPT-tolerant plants tested for tabtoxin resistance, the high substrate specificity of PAT makes the occurence of this particular pleiotropic effect highly unlikely. In general, it is currently unclear whether pleiotropic effects do occur to the extent that any effect can be measured. If any effect can be measured, it might be unclear whether such an effect has any relevance for the ecological relationships or toxicological characteristics of the crop. And, if an effect has any relevance, it is unclear whether the outcome should be considered an adverse effect. The relatively minor and well documented changes brought about by the *bar* and *pat* transgenes suggest

there is little need for concern. It could be argued that the dynamics and self-regulatory properties of ecosystems and consumers are likely to create sufficient 'noise' to allow the conclusion that pleiotropic effects will be of no or very minor importance, but such views are highly debated.

The evaluation of the transgenic PPT-tolerant phenotype based on data on the transgene, its product, substrates and degradation products establishes the file for this particular transgene. Ideally, for every individual transgene present in plants such file of a 'transgene-centered' analysis should become publicly available.

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