Regulatory Toxicology and Pharmacology 97 (2018) 170–185

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Characterization and safety evaluation of HPPD W336, a modified 4-hydroxyphenylpyruvate dioxygenase protein, and the impact of its expression on plant metabolism in herbicide-tolerant MST-FGØ72-2 soybean

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ARTICLE INFO

Keywords:
4-Hydroxyphenylpyruvate dioxygenase
HPPD W336
Genetically modified crop
Characterization
Safety assessment
Allergenicity assessment
Functional and structural equivalence
Overexpression
MST-FGØ72-2 soybean

ABSTRACT

By transgenic expression technology, a modified 4-hydroxyphenylpyruvate dioxygenase enzyme (HPPD W336) originating from *Pseudomonas fluorescens* is expressed in MST-FGØ72-2 soybean to confer tolerance to 4-benzoyl isoxazole and triketone type of herbicides. Characterization and safety assessment of HPPD W336 were performed. No relevant sequence homologies were found with known allergens or toxins. Although sequence identity to known toxins showed identity to HPPD proteins annotated as hemolysins, the absence of hemolytic activity of HPPD W336 was demonstrated *in vitro*. HPPD W336 degrades rapidly in simulated gastric fluid. The absence of toxicity and hemolytic potential of HPPD W336 was confirmed by *in vivo* studies. The substrate spectrum of HPPD W336 was compared with wild type HPPD proteins, demonstrating that its expression is unlikely to induce any metabolic shifts in soybean. The potential effect of expression of HPPD W336 on metabolic pathways related to tyrosine was investigated by comparing seed composition of MST-FGØ72-2 soybean with non-genetically modified varieties, demonstrating that expression of HPPD W336 does not change aromatic amino acid, homogentisate and tocochromanol levels. In conclusion, HPPD W336 was demonstrated to be as safe as other food proteins. No adverse metabolic effects were identified related to HPPD W336 expression in MST-FGØ72-2 soybean.

1. Introduction

Genetically modified (GM) crops which are tolerant to the non-selective herbicides glyphosate and glufosinate have proven their value for modern agriculture (Green, 2014). However, current issues with weed resistance and weed population shifts show that today's growers would benefit hugely if they could use different herbicide treatments to avoid over reliance on a single herbicide (Green, 2018). Hence, the development of alternative weed control technologies has become essential. This publication introduces a modified protein to generate a novel generation of herbicide-tolerant GM crops.

4-Hydroxyphenylpyruvate dioxygenase (HPPD; EC 1.13.11.27, EC 1.14.2.2) catalyzes the oxidative decarboxylation of 4-hydroxyphenylpyruvate (4-HPP) to form homogentisate (HGA). HPPD is a ubiquitous enzyme present in virtually all aerobic organisms and it performs a key step in the catabolism of tyrosine which results in the formation of fumarate and acetoacetate (Fig. 1).

In plants, HGA also has a key anabolic role as a precursor in the biosynthesis of tocochromanols (tocopherols and tocotrienols) and prenylquinones, such as plastoquinone. The former have an essential anti-oxidant function, whereas plastoquinone is an essential cofactor for phytoene desaturase, a key enzyme in the biosynthesis of carotenoid pigments (Norris et al., 1995). In addition, plastoquinone is a key component of the photosynthetic electron transport chain in photosystem II. Inhibition of HPPD has severe consequences for the plant (Fig. 1), causing phototoxicity because the reduction of plastoquinone levels leads to impaired electron transport. Furthermore, the reduction of phytoene desaturase activity prevents carotenoid synthesis, essential for chloroplast development and photosynthesis (Pallett et al., 1998). The impact of inhibition of HPPD leads to a 'bleached' phenotype of

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https://doi.org/10.1016/j.yrtph.2018.06.002
Received 12 February 2018; Received in revised form 9 May 2018; Accepted 5 June 2018
Available online 15 June 2018

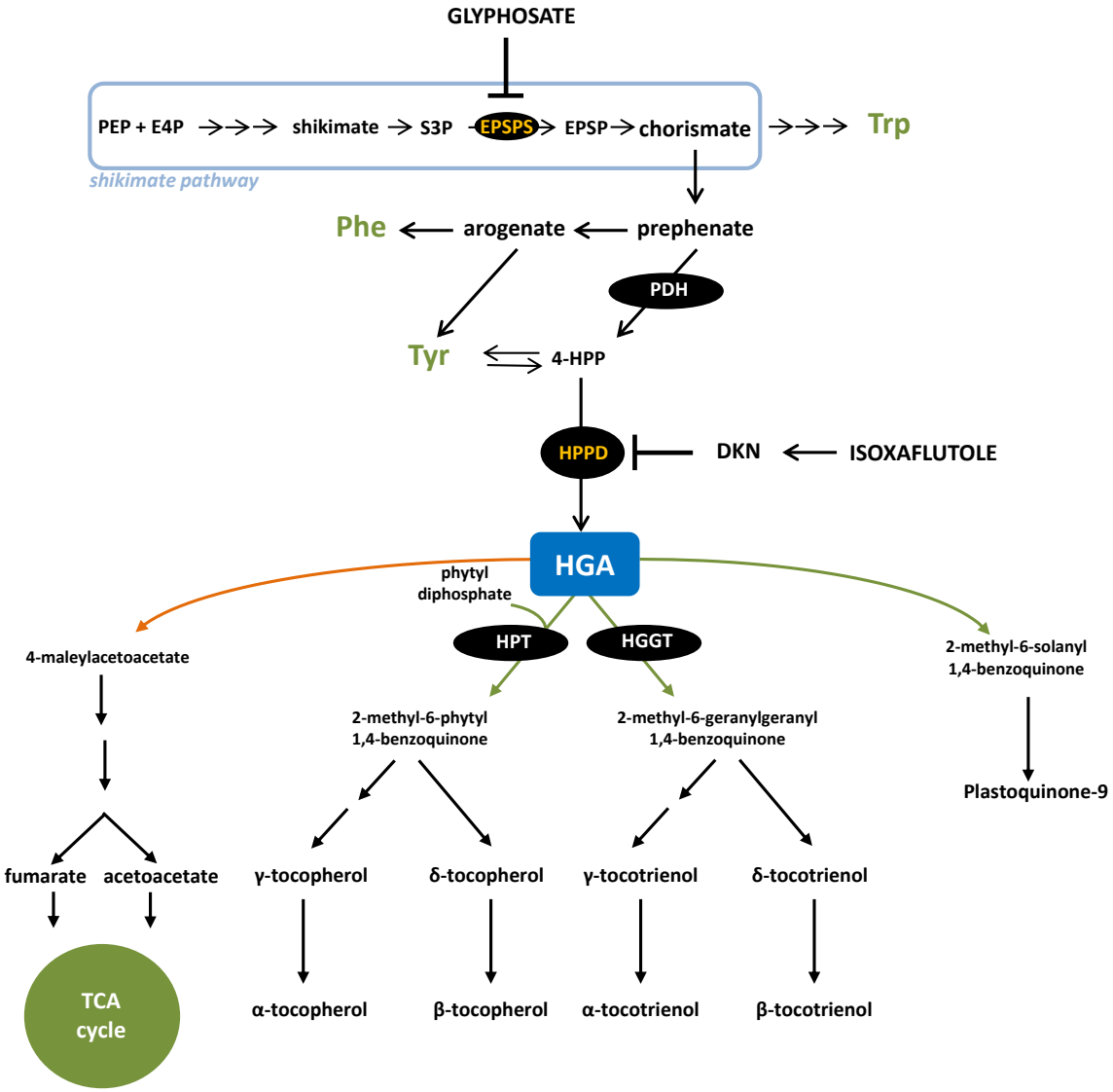

Fig. 1. Biosynthesis of aromatic amino acids and degradation of tyrosine in plants related to the anabolic and catabolic function of homogentisate, the reaction product of HPPD enzymatic activity.

Chorismate is the precursor of the aromatic amino acids tyrosine (Tyr), phenylalanine (Phe) and Tryptophan (Trp) and is the resulting compound from the shikimate pathway. One of the key enzymes of this pathway is 5-enoyl-pyruvylshikimate 3-phosphate synthase (EPSPS), which is the target of the herbicide glyphosate. Tyrosine is degraded into homogentisate (HGA) in two enzymatic steps, of which the second step is catalyzed by 4-hydroxyphenylpyruvate dioxygenase (HPPD), the target of the herbicide isoxaflutole. HGA is a central compound in the degradation pathway of tyrosine since it is a substrate of many enzymes. Within the catabolic part of the pathway (indicated by the orange arrow) HGA is degraded into fumarate and acetoacetate, which are ultimately recycled within the tricarboxylic acid (TCA) cycle. The anabolic pathways starting from HGA are indicated by the green arrows. The action of the enzymes in this anabolic pathway lead to the formation of tocopherols, tocotrienols and plastoquinone-9.

Legend: 4-HPP: 4-hydroxyphenylpyruvate; DKN: diketonitrile; E4P: D-erythrose 4-phosphate; HPT: homogentisate prenyl transferase; HGGT: homogentisate geranyl geranyl transferase; PEP: phosphoenolpyruvate; PDH: prephenate dehydrogenase; S3P: shikimate-3-phosphate.

susceptible species, because of the dual effects of reduced pigment biosynthesis and impaired chloroplast development (Fig. 2, panel A2). The anabolic role of HPPD in plants makes this enzyme a highly effective target site for herbicides such as 4-benzoyl isoxazoles, *e.g.* isoxaflutole and the triketones, *e.g.* mesotrione[1] (Luscombe et al., 1993; Mitchell et al., 2001), and the expression of modified HPPD in plants has led to herbicide tolerance (Matringe et al., 2005; Clarke et al., 2013; Kramer et al., 2014; Siehl et al., 2014).

MST-FGØ72-2 is a GM soybean line developed jointly by Bayer CropScience and MS Technologies, expressing the HPPD W336 protein, which has a single amino acid (AA) substitution at position 336 and is derived from the *Pseudomonas fluorescens* strain A32 *hppd* gene (Genbank Acc N° AAE74448). When treated with isoxaflutole, MST-FGØ72-2 soybean retains the normal 'green' phenotype (Fig. 2, panel B2). The replacement of the glycine residue at position 336 by a tryptophan leads to reduced sensitivity to diketonitrile (DKN), which is the herbicidal principle of isoxaflutole (Pallett et al., 1998; Matringe et al., 2005). The point mutation has a strong effect on DKN sensitivity but has a moderate impact on the normal enzyme kinetics of HPPD (Matringe et al., 2005). In addition to isoxaflutole, MST-FGØ72-2 soybean also shows tolerance to the herbicide glyphosate due to the expression of a modified 5-enoyl-pyruvylshikimate 3-phosphate synthase (2mEPSPS) derived from maize. The 2mEPSPS protein was previously demonstrated as being safe for food and feed consumption (Herouet-Guicheney et al., 2009).

[1] The use of these types of herbicides is pending regulatory approval.