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Food and feed safety of DAS-444Ø6-6 herbicide-tolerant soybean

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ABSTRACT

DAS-444Ø6-6 soybean was genetically engineered (GE) to withstand applications of three different herbicides. Tolerance to glufosinate and glyphosate is achieved through expression of the phosphinothricin acetyltransferase (PAT) and double-mutated maize 5-enolpyruvyl shikimate-3-phosphate synthase (2mEPSPS) enzymes, respectively. These proteins are expressed in currently commercialized crops and represent no novel risk. Tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) is achieved through expression of the aryloxyalkanoate dioxygenase 12 (AAD-12) enzyme, which is novel in crops. The safety of the AAD-12 protein and DAS-444Ø6-6 event was assessed for food and feed safety based on the weight of evidence and found to be as safe as non-GE soybean.

1. Introduction

DAS-444Ø6-6 soybean was genetically engineered (GE) to withstand applications of three different herbicides. Expression of the aryloxyalkanoate dioxygenase 12 (AAD-12) enzyme provides tolerance to 2,4-dichlorophenoxyacetic acid (2,4-D) by catalyzing its degradation, expression of the phosphinothricin acetyltransferase (PAT) enzyme inactivates glufosinate ammonium by acetylation of the l-isomer into *N*-acetyl-l-glufosinate ammonium, and expression of the double-mutated maize 5-enolpyruvyl shikimate-3-phosphate synthase (2mEPSPS) enzyme provides tolerance to glyphosate by replacing the function of the native soybean EPSPS enzyme (which is inactivated by glyphosate to control weeds/plants) (Lepping et al., 2013). Together, these traits offer growers multiple additional options for weed control in soybean.

The food and feed safety of a GE crop is determined from the weight of evidence (OECD, 1993). This evidence includes any history of safety for the expressed proteins and/or the source organism from which it was identified. In addition, certain properties of the expressed protein provide important evidence to consider when assessing safety. These properties include mode of action, amino acid sequence similarity to proteins with a history of safe use and to those known to be toxic or allergenic, stability to normal food processing and cooking procedures, and digestive stability. For many proteins, these factors are sufficient to robustly evaluate safety (Delaney et al., 2008; Hammond et al., 2013). Toxicity can also be empirically assessed *in vivo* via high-dose animal studies with purified protein.

When GE proteins remain intact in a feedstuff, whole-food animal-feeding studies might be useful in the safety assessment for proteins that cannot be purified in an active form, and for which high margins of exposure can be obtained relative to actual exposure under normal use (Herman and Ekmay, 2014). In practice, whole-food feeding studies are typically used to investigate unintended compositional changes within the GE crop that might not be detected in studies that directly analyze the composition of the edible portion of the crop (Herman and Ekmay, 2014). While a large body of information indicates that adverse unintended compositional changes in GE crops are a lower risk compared with traditionally bred crops, composition studies are almost universally required for all new GE events, and whole-food animal studies are required by some regulatory authorities that assess GE crop safety (Herman and Ekmay, 2014; Herman and Price, 2013).

Here, we review the weight of evidence for the safety of DAS-444Ø6-6 soybean based on: 1) the intended GE traits and 2) potential unintended adverse effects of the GE proteins (or the transgenesis process) on endogenous plant metabolic pathways. The PAT and 2mEPSPS proteins are expressed in approved GE crops that have been assessed globally for safety. For the 2mEPSPS protein, these crops include GA21 maize, FG72 soybean, and Glytol cotton; for the PAT protein, these crops include T25 maize, A5547-127 soybean, and 281-24-236 cotton (CERA - ILSI Research Foundation, 2016a; CERA - ILSI Research Foundation, 2016b). Therefore, the PAT and 2mEPSPS proteins do not represent novel food or feed risks (Herouet-Guicheney et al., 2009; Hérouet et al., 2005). For these reasons, the present safety assessment focuses on the AAD-12 protein and any potential unexpected effects in the DAS-444Ø6-6 soybean event originating from transgenesis or the AAD-12 enzyme. It is noteworthy that the AAD-12 protein is also expressed by event DAS-68416-4 soybean (Herman et al.,

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2011).

2. History of AAD-12 safety

In the context of safety, it is important to recognize that food and feed contain thousands of proteins, many of which are enzymes like AAD-12. As such, there is a history of safe consumption for a great many proteins and enzymes (Delaney et al., 2008; Hammond et al., 2013). Similar to the *Bacillus thuringiensis* (Bt) bacterium, which is the source organism for transgenic proteins in all currently commercialized insect-protected GE crops (Roh et al., 2007), the source organism for the AAD-12 enzyme (*Delftia acidovorans*: formerly *Pseudomonas acidovorans*) is found widely in soil and water (Bergogne-Bérézin, 2004). *D. acidovorans* has also been used to transform ferulic acid into vanillin in the food processing industry (Toms and Wood, 1970). Due to the source organism being widespread in the environment and to its use in the food processing industry, there is some history of safe use for the source organism from which the AAD-12 protein originated; however, previous exposure to the AAD-12 protein in food and feed has not been documented.

3. Intended effects

3.1. AAD-12 mode of action

The AAD-12 enzyme catalyzes the conversion of the herbicide 2,4-D to 2,4-dichlorophenol (DCP), a herbicidally inactive compound (Wright et al., 2010). AAD-12 is also able to catalyze the degradation of related achiral phenoxyacetate herbicides such as MCPA ((4-chloro-2-methylphenoxy) acetic acid) and pyridyloxyacetate herbicides such as triclopyr and fluroxypyr to their corresponding inactive phenols and pyridinols, respectively (Fig. 1). The AAD-12 enzyme has selectivity for *S*-enantiomers of the chiral phenoxy acid herbicides (*e.g.*, dichlorprop and mecoprop). Since the *R*-enantiomer of phenoxy acid herbicides is the herbicidally active form, AAD-12 does not confer tolerance to commercially available chiral phenoxy-acid herbicides.

The enzymatic substrate specificity of AAD-12 was assessed extensively using endogenous plant compounds including all twenty L-amino acids, four natural plant hormones, and three common phenylpropanoid and flavonoid secondary metabolites (Griffin et al., 2013). Negligible activity was detected for all compounds examined. Only *trans*-cinnamate and indole-3-acetic acid (IAA) showed a barely detectable oxidation when excessive amounts of enzyme were used. AAD-12 kinetic parameters were determined using an enzyme-coupled assay with *trans*-cinnamic acid and IAA as substrates. The k_{cat} and K_m for AAD-12 using *trans*-cinnamic acid were found to be 0.1 s^{-1} and 645.2 μM, respectively. The K_m for IAA was significantly elevated at ~3.4 mM and the k_{cat} was 0.03 s^{-1}. Therefore, although AAD-12 is capable of catalyzing the oxidation of both *trans*-cinnamic acid and IAA, the extremely poor kinetics indicate that these transformations are unlikely to have a metabolic impact within DAS-444Ø6-6 soybean (Griffin et al., 2013).

3.2. AAD-12 bioinformatic analyses

For food crops, the potential allergenicity of a newly expressed protein is evaluated using a weight-of-evidence approach. This includes bioinformatic analysis of the amino acid sequences to identify homology and potential cross reactivity with known allergens (Ladics et al., 2011). The AAD-12 amino acid sequence was compared with known allergens in an up-to-date allergen database (COMPARE, 2017; http://comparedatabase.org/) using the methods recommended by FAO/WHO and CODEX (Codex Alimentarius Commission, 2003; Codex Alimentarius Commission, 2009; FAO/WHO, 2002). A search of sequentially overlapping 80-mers against the aforementioned allergen database did not generate any alignments with > 35% identity over ≥80 amino acids. In addition, no matches were detected when comparing overlapping 8-mers from the AAD-12 protein sequence with known allergens, although short amino acid segment matches are now known to be of low value in predicting allergen cross reactivity (Herman et al., 2009; Silvanovich et al., 2005) and this query is no longer endorsed by some regulatory agencies (EFSA, 2010).

Recent findings have also called into question the use of percent amino acid identity as a conservative threshold for potential allergen cross-reactivity (Herman et al., 2015). The use of a stable E-value threshold ($\leq 10^{-9}$) based on a 1:1 FASTA algorithm has been shown to be superior in selectively identifying cross-reactive allergens (Song et al., 2014, 2015). The minimum E-value from a 1:1 FASTA query of the AAD-12 amino acid sequence against the 2017 COMPARE database was 3.1 x 10^{-4}, which predicts no cross reactivity with known allergens.

The safety assessment of a protein newly expressed in transgenic plants also includes an evaluation of whether the protein can function as a potential toxin when present in the human diet or livestock feed. It has been suggested that assessing the potential toxicity of a protein should include comparison of the protein sequence with the sequences of known protein toxins (Codex Alimentarius Commission, 2009). Since there is no commonly recognized definition of a protein toxin based on its sequence, a comparison should be made to a database of all available protein sequences as a conservative approach. The AAD-12 sequence was queried against the NCBI non-redundant protein sequences (up to date as of March 4, 2016) using BLASTp (Altschul et al., 1997). The majority of the sequence alignments (E-value < 1) returned by the BLASTp search were with enzymes from the dioxygenase family, followed by alpha-ketoglutarate related proteins, dehydrogenases, pyoverdine biosynthesis proteins, and hypothetical or unnamed proteins. AAD-12 is an alpha-ketoglutarate dependent dioxygenase. Hypothetical and unnamed proteins are derived from conceptual translation of DNA

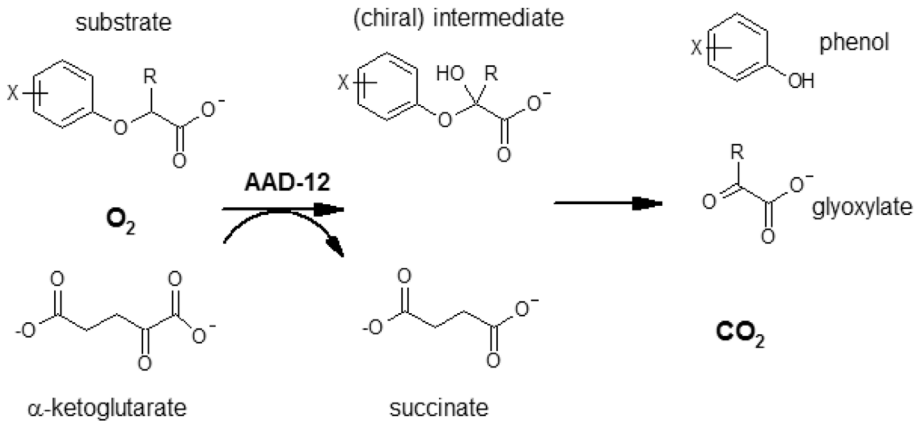

Fig. 1. Mode of action of the AAD-12 protein (R = H or CH_3).