EXECUTIVE SUMMARY
to
Application to Food Standards Australia New Zealand for the Inclusion of Herbicide-Tolerant KWS20-1 Sugar Beet in *Standard 1.5.2 - Food Derived from Gene Technology*

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Food/Feed Safety and Nutritional Assessment of KWS20-1 Sugar Beet

Bayer and KWS have jointly developed biotechnology-derived sugar beet KWS20-1 that is tolerant to in-crop applications of dicamba, glufosinate and glyphosate herbicides. Herbicide-tolerant sugar beet KWS20-1 will provide sugar beet growers with the option to use multiple herbicide modes-of-action to control a wide spectrum of weeds, including herbicide-resistant biotypes.

Herbicide tolerance was conferred to KWS20-1 sugar beet *via Agrobacterium*-mediated insertion of a single T-DNA containing a demethylase gene from *Stenotrophomonas maltophilia* that expresses a dicamba mono-oxygenase (DMO) protein to confer tolerance to dicamba herbicide, a gene from *Streptomyces viridochromogenes* that expresses the phosphinothricin N-acetyltransferase (PAT) protein to confer tolerance to glufosinate-ammonium herbicide, and the *cp4 epsps* coding sequence isolated from *Agrobacterium* sp. strain CP4 that expresses the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein to confer tolerance to glyphosate herbicide.

Molecular Characterization of KWS20-1 Sugar Beet Verifies the Integrity and Stability of the Inserted DNA

KWS20-1 sugar beet was produced by *Agrobacterium*-mediated insertion of the transfer DNA (T-DNA) contained in the transformation plasmid vector PV-BVHT527462 into the sugar beet genome. This plasmid vector contains one T-DNA, which is delineated by Right and Left Border regions. The T-DNA contains the *dmo*, *pat* and *cp4 epsps* expression cassettes and the vector backbone contains the *aadA* expression cassette. During transformation, the T-DNA was inserted into the sugar beet genome. Subsequently, traditional breeding, segregation, selection and screening were used to isolate those plants that contain the T-DNA.

Characterization of the DNA insert in KWS20-1 sugar beet was conducted using a combination of Southern blotting, sequencing and polymerase chain reaction (PCR). The results of this characterization demonstrate that KWS20-1 sugar beet contains one copy of the intended T-DNA containing the *dmo*, *pat* and *cp4 epsps* expression cassettes integrated at a single locus that is stably inherited over multiple generations and segregates in multiple generations according to Mendelian principles. These conclusions are based on several lines of evidence:

- Molecular characterization of KWS20-1 sugar beet by Southern blot analyses demonstrated that KWS20-1 sugar beet contains a single, intended T-DNA insert. These Southern blot analyses provided a comprehensive assessment of KWS20-1 sugar beet to determine the presence and identity of sequences derived from plasmid vector PV-BVHT527462. These analyses demonstrate that KWS20-1 sugar beet contains a single T-DNA insert with no detectable backbone.

- Directed sequencing (i.e., locus-specific PCR, DNA sequencing and analyses) performed on KWS20-1 sugar beet was used to determine the complete sequence of the single T-DNA insert from plasmid vector PV-BVHT527462, the adjacent flanking genomic DNA, and the 5' and 3' insert-to-flank junctions. This analysis confirms that the sequence and organization of the T-DNA is identical to the corresponding region in the plasmid vector PV-BVHT527462 T-DNA.

- Furthermore, the genomic organization at the insertion site in KWS20-1 sugar beet was assessed by comparing the sequences flanking the T-DNA insert in KWS20-1 sugar beet to the sequence of the insertion site in conventional sugar beet. This analysis determined that no major DNA rearrangement occurred at the insertion site in KWS20-1 sugar beet upon DNA integration; although, a seven (7) bp deletion was observed at the site of T-DNA integration in KWS20-1 sugar beet.

- Generational stability analysis by Southern blot demonstrated that the single plasmid vector PV-BVHT527462 T-DNA insert in KWS20-1 sugar beet has been maintained through five (5) breeding generations, thereby confirming the stability of the T-DNA insert in KWS20-1 sugar beet.

- Segregation analysis in three (3) generations segregating population corroborates the insert stability demonstrated by Southern blot analysis and independently establishes the nature of the T-DNA as a single chromosomal locus that shows an expected pattern of inheritance.

Taken together, the characterization of the genetic modification in KWS20-1 sugar beet demonstrates that a single copy of the intended TDNA is stably integrated at a single locus of the sugar beet genome and that no plasmid vector PV-BVHT527462 backbone sequences are present in KWS20-1 sugar beet.

DMO, PAT and CP4 EPSPS are Safe for Consumption in Food or Feed

KWS20-1 sugar beet contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses a dicamba mono-oxygenase (DMO) protein. As a mono-oxygenase protein, the DMO protein is part of the larger oxygenase family of enzymes that incorporate one or two oxygen atoms into substrates and are widely distributed in many universal metabolic pathways (Harayama *et al.*, 1992). The DMO protein enzymatically catalyzes the demethylation of the broadleaf herbicide dicamba to the non-herbicidal compound 3,6-dichlorosalicylic acid (DCSA) and formaldehyde, thus conferring dicamba tolerance (Chakraborty *et al.*, 2005). Expression of the DMO protein in KWS20-1 sugar beet is targeted to the chloroplast by a chloroplast transit peptide (CTP), which facilitates its co-localization with endogenous reductase and ferredoxin enzymes required to supply electrons for the DMO demethylation reaction (Behrens *et al.*, 2007).

The DMO protein is specific for the oxidative demethylation of dicamba, forming DCSA. Dicamba interacts with amino acids in the catalytic site of DMO through both the carboxylate moiety and the chlorine atoms of dicamba, which are primarily involved in orienting the substrate in the catalytic site. These chlorine atoms are required for catalysis (D'Ordine *et al.*, 2009; Dumitru *et al.*, 2009). Given the limited existence of chlorinated compounds with structures similar to dicamba in plants and other eukaryotes (Wishart *et al.*, 2009; Wishart, 2010), it is unlikely that KWS20-1 sugar beet-produced DMO will catalyze the metabolism of endogenous compounds. A previous assessment of MON 87429 maize DMO that contains a variant identical to the KWS20-1 sugar beet-produced DMO, aside from the residual 27 amino acids from the CTP, evaluated the potential for DMO to catabolize dicamba and *o*-anisic acid. *O*-anisic acid was the natural plant metabolite chosen for the MON 87429 corn DMO substrate specificity assessment since it is the plant metabolite most structurally similar to dicamba (i.e., identical to dicamba, except for absence of chlorine atoms) (Dumitru *et al.*, 2009). A confirmatory assessment demonstrated the minor differences in amino acid sequences present in the KWS20-1 sugar beet-produced DMO protein relative to other DMO proteins expressed in previous biotechnology-derived crops do not impact the activity or selectivity for dicamba herbicide as compared to potential endogenous substrates, thereby confirming the specificity of the DMO protein expressed by KWS20-1 sugar beet.

KWS20-1 sugar beet contains an acetyltransferase gene from *Streptomyces viridochromogenes* that expresses phosphinothricin N-acetyltransferase (PAT) protein. The molecular mechanism of the PAT protein, which acetylates glufosinate in the presence of acetyl CoA to form N-acetyl glufosinate, is well understood (Thompson *et al.*, 1987). Glufosinate is a racemic mixture of the D- and L-forms of the amino acid phosphinothricin. The herbicidal activity of glufosinate results from the binding of L-phosphinothricin to glutamine synthetase in plants (OECD, 1999; OECD, 2002a). Expression of the PAT protein in KWS20-1 sugar beet results in the ability to convert L-phosphinothricin to the non-herbicidal N-acetyl-L-phosphinothricin, thus conferring glufosinate tolerance to the crop.

Phosphinothricin N-acetyltransferase (PAT) proteins have been isolated from two separate species of *Streptomyces*, *S. hygroscopicus* (Thompson *et al.*, 1987) and *S. viridochromogenes* (Wohlleben *et al.*, 1988). The PAT protein isolated from *S. hygroscopicus* is encoded by the *bar* gene, whereas the PAT protein isolated from *S. viridochromogenes* is encoded by the *pat* gene. These PAT proteins are made up of 183 amino acids with 85% identity to each other at the amino acid level (Wohlleben *et al.*, 1988). Based on previous studies (Wehrmann *et al.*, 1996) that have extensively characterized PAT proteins produced from *bar* and *pat* genes, it has been noted that both proteins are so similar as to be functionally equivalent (OECD, 1999).

Expression of the *pat* gene in KWS20-1 sugar beet results in a single polypeptide of 182 amino acids with an apparent molecular weight of ~22.3 kDa. Data from N-terminal sequencing analysis of the KWS20-1 sugar beet-produced PAT protein indicate that it is identical to the wild type PAT protein encoded by *S. viridochromogenes* and to the PAT proteins produced in glufosinate-tolerant crops previously submitted to FSANZ (A2704-12 and A5547-127 soybean [A481], MON 87419 maize [A1118] and MON 87429 maize [A1192]) (Hérouet *et al.,* 2005; ILSI-CERA, 2011), except for the first methionine that is removed due to cotranslational processing in KWS20-1 sugar beet. N-terminal methionine cleavage is common and naturally occurs in the vast majority of proteins (Meinnel and Giglione, 2008).

The PAT protein expressed in KWS20-1 sugar beet is highly specific for glufosinate. Enzyme assays indicated that the PAT protein does not acetylate other common L-amino acids that are structurally similar to L-phosphinothricin, and substrate competition assays showed no inhibition of glufosinate acetylation in the presence of high concentrations of L-amino acids that are structurally similar to L-phosphinothricin (including the glufosinate analog L-glutamate) (Wehrmann *et al.,* 1996). Recent metabolic profiling reported some non-specific PAT (*bar*)-mediated acetylation of two amino acids (aminoadipate and tryptophan) in senescent leaf extracts from *A. thaliana* and also in PAT (*pat*)-expressing soybean (Christ *et al.,* 2017). However, the activity level for these two amino acids was very low relative to the activity for L-phosphinothricin, indicating that PAT (*pat*) has a very high level of specificity for the herbicidal glufosinate molecule.

KWS20-1 sugar beet contains a codon optimized coding sequence of the *aroA* gene from the soil bacterium *Agrobacterium* sp. strain CP4 that expresses the CP4 EPSPS protein (Barry *et al.*, 2001; Padgette *et al.*, 1996). 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) is an enzyme of the shikimate pathway of aromatic amino acid biosynthesis, and is present in plants, bacteria and fungi. The molecular mechanism of the CP4 EPSPS protein is well understood, where EPSPS catalyzes a reversible reaction that produces EPSP and inorganic phosphate (Pi) from phosphoenolpyruvate (PEP) and shikimate-3-phosphate (S3P) (Levin and Sprinson, 1964), known to specifically react with these substrates (Gruys *et al.*, 1992). In most plants, the endogenous EPSPS is inhibited by the herbicide glyphosate that causes cell death (Franz *et al.*, 1997). The CP4 EPSPS protein is structurally similar and functionally equivalent to endogenous plant EPSPS enzymes, but has a much-reduced affinity for glyphosate relative to endogenous plant EPSPS (Barry *et al.,* 2001; Padgette *et al.,* 1996). The presence of this protein renders the plant tolerant to glyphosate. Expression of the CP4 EPSPS protein in KWS20-1 sugar beet is targeted to the chloroplast by a CTP, where the plant EPSPS resides and is the site of aromatic amino acid biosynthesis (Klee *et al.,* 1987; Kishore *et al.,* 1988).

The *cp4 epsps* expression cassette contains the *cp4 epsps* gene encoding a precursor protein of 531 amino acids (i.e., 455 amino acids encoded by the *cp4 epsps* gene and 76 amino acids encoded by the *CTP2* gene for targeting the CP4 EPSPS protein into chloroplasts). KWS20-1 sugar beet expresses an ~43.5 kDa CP4 EPSPS protein, consisting of a single polypeptide of 455 amino acids starting at the methionine position 77 (Padgette *et al.*, 1996) after a complete cleavage of the chloroplast transit peptide (CTP2).

The KWS20-1 sugar beet-produced CP4 EPSPS protein is similar to EPSPS proteins consumed in a variety of food and feed sources. CP4 EPSPS protein is homologous to EPSPS proteins naturally present in plants, including food and feed crops (e.g., maize and soybean) and fungal and microbial food sources such as baker's yeast (*Saccharomyces cerevisiae*), all of which have a history of safe consumption (Padgette *et al.*, 1996; Harrison *et al.*, 1996). The CP4 EPSPS protein in KWS20-1 sugar beet is also produced in several glyphosate-tolerant

crops previously reviewed by the FSANZ (MON 89788 soybean [A592], MON 88302 canola [A1071], NK603 maize [A416], MON 88017 maize [A548], MON 87411 maize [A1097], H7-1 sugar beet [A525], J101/163 alfalfa [A575], and MON 88913 cotton [A553]).

The weight-of-evidence of analyses conducted in accordance with guidelines established by the Codex Alimentarius Commission and OECD, and the principles and guidance of the FDA's 1992 policy on foods from new plant varieties indicates that the DMO, PAT and CP4 EPSPS proteins poses no meaningful risk to human or animal health. The assessment includes: 1) documenting the history of safe consumption of the expressed protein or its structural and functional homology to proteins that lack adverse effects on human or mammalian health; 2) characterization of the physicochemical and functional properties of each expressed protein; 3) quantification of each expressed protein in plant tissues; 4) examination of the similarity of each expressed protein to known allergens, toxins or other biologically-active proteins known to have adverse effects on humans and other mammals; 5) evaluation of the susceptibility of each expressed protein to the digestive enzymes pepsin and pancreatin; 6) evaluation of the stability of the expressed protein after heat treatment; 7) investigation of potential animal toxicity of the expressed proteins through mouse acute oral toxicity testing. The safety assessment completed for KWS20-1 sugar beet supports the conclusion that exposure to the DMO, PAT and CP4 EPSPS proteins produced by KWS20-1 sugar beet would not pose a dietary risk to human or mammalian health.

Compositional Analysis of KWS20-1 Sugar Beet Demonstrates Equivalence to the Conventional Sugar Beet

Safety assessments of biotechnology-derived crops follow the comparative safety assessment process in which the composition of sugar beet and/or other raw agricultural commodities of the biotechnology-derived crop are compared to the appropriate conventional control that has a history of safe use.

Compositional analysis was conducted on tops and root of KWS20-1 sugar beet grown in 2020 at five sites representative of typical agricultural regions for sugar beet production in the U.S. The compositional analysis provided a comprehensive comparative assessment of the levels of key nutrients and a secondary metabolite of KWS20-1 sugar beet and the near-isogenic control and conventional commercial reference varieties following considerations relevant to the compositional quality of sugar beet as defined by the OECD consensus document (OECD, 2002b). Tops samples were analyzed for moisture and levels of key nutrients, including: proximates (protein, total fat and ash), carbohydrates by calculation and crude fiber. Root samples were analyzed for moisture and levels of key nutrients, including: proximates (protein, total fat and ash), amino acids (18 components), carbohydrates by calculation, sucrose, fiber (crude fiber and pectin) and minerals (phosphorus, potassium and sodium). Root samples were also analyzed for the secondary metabolite oleanolic acid. In all, 36 different components were analyzed. Of the 36 measured components, one component (sodium in root) had more than 50% of the observations below the assay limit of quantitation (LOQ) and was excluded from statistical analysis. Moisture values for tops and root were measured for conversion of components from fresh to dry weight but were not statistically analyzed. Therefore, 33 components were statistically analyzed (five in tops and 28 in root).

The results of the compositional assessment found that there were no compositional differences that were biologically-meaningful between KWS20-1 sugar beet and the near-isogenic control; supporting the conclusion that KWS20-1 sugar beet is compositionally equivalent to the near

isogenic control. These results support the overall food and feed safety of KWS20-1 sugar beet.

Conclusion

The data and information presented in this safety summary provide a weight of evidence that supports the conclusion that the food and feed derived from KWS20-1 sugar beet and its progeny are as safe and nutritious as food and feed derived from conventional sugar beet. The food and feed safety of KWS20-1 sugar beet is based on the following lines of evidence:

1. A detailed molecular characterization of the inserted DNA demonstrates a single, intact copy of the expected T-DNA inserted at a single locus within the sugar beet genome. The genetic elements are present in the expected order and are inherited following Mendelian principles,

2. Extensive evaluation of the DMO, PAT and CP4 EPSPS proteins demonstrates that it does not pose any meaningful risk to food or feed safety, and

3. The comprehensive compositional assessment demonstrates that KWS20-1 sugar beet tops and roots are compositionally equivalent to tops and roots from conventional sugar beet.

These data herein demonstrate that the food and feed derived from KWS20-1 sugar beet and its progeny are as safe and nutritious as food and feed derived from conventional sugar beet.

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