Dow AgroSciences LLC Petition (13-262-01p) for Determination of Nonregulated Status of Event DAS-8191Ø-7 Cotton

OECD Unique Identifier: DAS-8191Ø-7

Plant Pest Risk Assessment

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A. Introduction

Dow AgroSciences LLC (hereafter referred to as DAS) has petitioned the Animal and Plant Health Inspection Service (APHIS) of the United States Department Agriculture (USDA) for a determination that genetically engineered (GE) glufosinate and 2,4-dichlorophenoxyacetic acid (2,4-D) herbicide-resistant[1] cotton event DAS-8191Ø-7 and OECD Unique Identifier DAS-8191Ø-7 (hereinafter referred to as DAS-8191Ø-7) is unlikely to pose a plant pest risk and, therefore, should no longer be a regulated article under the APHIS' 7 Code of Federal Regulations (CFR) part 340. This petition was assigned the number 13-262-01p, and is hereafter referenced as DAS 2013. APHIS administers 7 CFR part 340 under the authority of the plant pest provisions of the Plant Protection Act (PPA) of 2000 (7 U.S.C. 7701 *et seq*.)[2]. This plant pest risk assessment was conducted to determine if DAS-8191Ø-7 cotton is unlikely to pose a plant pest risk.

APHIS regulations in 7 CFR part 340 regulate the introduction (importation, interstate movement, or release into the environment) of certain GE organisms and products. A GE organism is no longer subject to the plant pest provisions of the PPA or to the regulatory requirements of part 340 when APHIS determines that it is unlikely to pose a plant pest risk. A GE organism is considered a regulated article under part 340 if the donor organism, recipient organism, or vector, or vector agent used in engineering the organism belongs to any genera or taxa designated in 7 CFR 340.2 and meets the definition of plant pest, or is an unclassified organism and/or an organism whose classification is unknown, or any product which contains such an organism, or any other organism or product altered or produced through genetic engineering which the APHIS Administrator determines is a plant pest or has reason to believe is a plant pest[3].

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[1] DAS has described the phenotype of DAS-8191Ø-7 cotton as "herbicide tolerant" and historically APHIS has also referred to GE plants with reduced herbicide sensitivity as herbicide tolerant. However, the phenotype would fall under the Weed Science Society of America (WSSA) definition of "herbicide resistance" since DAS-8191Ø-7 cotton has an "inherited ability to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type". By the WSSA, (1998) definition, "resistance (to an herbicide) may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis." Herbicide tolerance, by the WSSA definition, only applies to plant species with an "inherent ability to survive and reproduce after herbicide treatment. This implies that there was no selection or genetic manipulation to make the plant tolerant; it is naturally tolerant."

[2] Plant Protection Act in 7 U.S.C. 7702 § 403(14) defines plant pest as: "Plant Pest - The term "plant pest" means any living stage of any of the following that can directly or indirectly injure, cause damage to, or cause disease in any plant or plant product: (A) A protozoan. (B) A nonhuman animal. (C) A parasitic plant. (D) A bacterium. (E) A fungus. (F) A virus or viroid. (G) An infectious agent or other pathogen. (H) Any article similar to or allied with any of the articles specified in the preceding subparagraphs."

[3] Limited exclusions or exemptions apply for certain engineered microorganisms and for interstate movement of some organisms, as in 7 CFR 340.1 and 340.2(b).

DAS-8191Ø-7 cotton was produced by transformation of cotton hypocotyl segments with *Agrobacterium tumefaciens.* In addition, some of the regulatory sequences were derived from plant pests including, promoter and 5' untranslated region (UTR) from cassava vein mosaic virus, and 3' UTR and T-DNA border and intervening sequence from *A. tumefaciens* (DAS 2013, pp. 25-36). Therefore, DAS-8191Ø-7 cotton is considered a regulated article under APHIS regulations at 7 CFR part 340. DAS has conducted releases into the environment of DAS-8191Ø-7 cotton as a regulated article under APHIS-authorized notifications since 2010 (DAS 2013, Appendix 9, p. 202), in part, to gather information to support that DAS-8191Ø-7 is unlikely to pose a plant pest risk.

Potential impacts in this Plant Pest Risk Assessment are those that pertain to plant pest risk associated with DAS-8191Ø-7 cotton and its progeny and their use in the absence of confinement relative to the unmodified recipient and/or other appropriate comparators. APHIS utilizes data and information submitted by the applicant, in addition to current literature, to determine if DAS-8191Ø-7 cotton is unlikely to pose a plant pest risk. APHIS regulations in 7 CFR 340.6(c) specifies the information needed for consideration in a petition for nonregulated status. APHIS will assess information submitted by the applicant about DAS-8191Ø-7 cotton related to: plant pest risk characteristics; expression of the gene product, new enzymes, or changes to plant metabolism; disease and pest susceptibilities and indirect plant pest effects on other agricultural products; effects of the regulated article on nontarget organisms; weediness of the regulated article; impact on the weediness of any other plant with which it can interbreed; changes to agricultural or cultivation practices that may impact diseases and pests of plants; and transfer of genetic information to organisms with which it cannot interbreed.

APHIS may also consider information relevant to reviews conducted by other agencies that are part of the 'Coordinated Framework for the Regulation of Biotechnology' (51 FR 23302, 1986; 57 FR 22984, 1992). Under the Coordinated Framework, the oversight of biotechnology-derived plants rests with APHIS, the Food and Drug Administration (FDA), and the Office of Pesticide Programs of the U.S. Environmental Protection Agency (EPA). Depending on its characteristics, certain biotechnology-derived products are subjected to review by one or more of these agencies.

EPA regulates under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) (7 U.S.C. 136 *et seq.)* the distribution, sale, use and testing of pesticidal substances produced in plants and microbes, including those pesticides that are produced by an organism through techniques of modern biotechnology. EPA also sets tolerance limits for residues of pesticides on and in food and animal feed, or establishes an exemption from the requirement for a tolerance, under the Federal Food, Drug and Cosmetic Act (FFDCA) (21 U.S.C. Chapter 9). Prior to registration for a new use for a new or previously registered pesticide, EPA must determine through testing that the pesticide does not cause unreasonable adverse effects on humans, the environment, and nontarget species when used in accordance with label

instructions. EPA must also approve the language used on the pesticide label in accordance with 40 CFR part 158. Other applicable EPA regulations include 40 CFR part 152 - Pesticide Registration and Classification Procedures, part 174 - Procedures and Requirements for Plant Incorporated Protectants (PIPs) and part 172 - Experimental Use Permits.

The FDA under the FFDCA is responsible for ensuring the safety and proper labeling of all plant-derived foods and feeds, including those developed through modern biotechnology. To help sponsors of foods and feeds derived from genetically engineered crops comply with their obligations, the FDA encourages them to participate in its voluntary early food safety evaluation for new non-pesticidal proteins produced by new plant varieties intended to be used as food (FDA 2006) and a more comprehensive voluntary consultation process prior to commercial distribution of food or feed (57 FR 22984).

DAS submitted a 2,4-D formulation (Enlist Duo) registration for DAS-8191Ø-7 cotton and a tolerance petition to EPA on July 30, 2014. DAS submitted data on the food and feed safety of DAS-8191Ø-7 in Premarket Biotechnology Notice (PBN) to FDA (Biotechnology Notification File (BNF) 000142) and completed consultation on November 14, 2014 (DAS 2013, p. 19).

B. Development of DAS-8191Ø-7 cotton

The United States is a major producer of cotton with exports alone in 2013 amounting to $5.7 billion (USDA ERS FAS 2013). Cotton belongs to the genus *Gossypium*, which consists of 49 species, four of which are generally cultivated in tropical and subtropical regions around the world (Fryxell 1979; Fryxell 1984; OECD 2008; Percival 1999; Wendel 2003; Wendel 2010). Cotton is a perennial cultivated as an annual, and is more limited geographically than other major crops in the United States because it can be grown only in regions with more than 180-200 frost-free days per year (Fryxell 1979; OECD 2008). The most commonly cultivated species in the United States, *G. hirsutum* (Upland cotton), comprising 98% of the 2013 cotton crop (USDA NASS 2013) and is the subject of this risk assessment. Over the last decade, Upland cotton has been grown on an average of 11.9 million acres annually in 17 states from Virginia southward and westward to California, an area often referred to as the Cotton Belt (Figure 1). Smaller amounts of *G. barbadense* (Pima or Egyptian cotton) are cultivated in Arizona, California, New Mexico, and Texas (USDA NASS 2013). Two other cultivated species, *G. arboretum* and *G. herbaceum*, are not grown in the United States or its territories (USDA NRCS 2013, USDA NASS 2013).

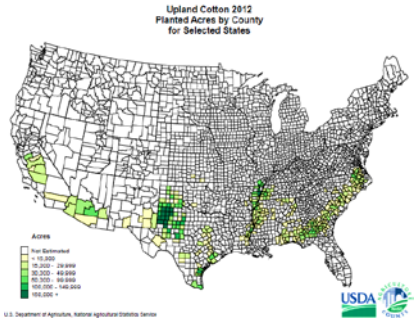

Figure 1. Upland cotton planted in the United States (USDA NASS 2013).

Weed control in cotton is essential to maximize cotton fiber yield and quality. Prior to the introduction of herbicides, mechanical tillage and hand hoeing were the primary means of controlling weeds in cotton (Buchannan 1992). As herbicide use began to develop in the 1940s, and rapidly accelerated in the 1960s, a series of herbicides active against broadleaf plants were introduced into the market. By 1968, 22 herbicides and herbicide combinations were being used in cotton (Buchanan 1992). With the commercial introduction of genetically engineered glyphosate-resistant cotton in 1997, glyphosate use increased while the use of other herbicides declined (Webster 2012). In 2010 approximately 78 percent of Upland cotton produced in the United States were glyphosate-tolerant varieties (USDA ARS 2013).

Widespread adoption of glyphosates in cotton, soybean and corn cultivation in the United States and the common practice of using it exclusively, has led to the emergence of glyphosate-resistant weeds (USDA ARS 2013). Repeated and intensive use of herbicides with the same mechanisms of action can rapidly select for tolerant, difficult-to-control weeds and for herbicide-resistant weeds, especially in the absence of the concurrent use of herbicides with different mechanisms of action and/or use of different mechanical or cultural practices for weed control (Vencill 2012). As a result of the common use of glyphosates to control weeds, fourteen confirmed glyphosate-resistant weeds are reported in the United States, including Palmer amaranth (*Amaranthus palmeri*) now considered the most troublesome weed in cotton growing regions and ranked as a troublesome weed in nine states (Heap 2013; Webster 2012).

DAS-8191Ø-7 was generated using a recipient line (Coker 310) of Upland cotton (*Glycine max* L.) that has been genetically engineered to be resistant to the herbicide 2,4-D and contains a marker gene for transformation selection with glufosinate. According to DAS, DAS8191Ø-7 was developed to provide expanded weed management options in cotton (DAS 2013, p. 130). The herbicide 2,4-D has been used post-emergence in many monocot crops (such as corn, wheat and rice) for the selective control of broadleaf weeds (EPA 2005; Wright 2013). 2,4-D is a synthetic auxin herbicide affecting plant growth in a manner similar to the plant-made auxin-type hormone 3-indoleacetic acid (IAA) by binding to IAA receptors in plants. Although the specific binding site relevant to the action of auxin-mimicking herbicides has not been identified, endogenous auxins have been shown to be essential for plant development and facilitate a diverse array of responses in plants,

such as the control of senescence, response to pathogens, fruit formation, leaf abscission, apical dominance, polarity and tropic responses to light and gravity (Grossman 2009; Sauer 2013; WSSA 2007). At the cellular level, auxin controls cell division and cell elongation and is involved in cross-talk responses with other phytohormones. When auxin-type herbicides are used in weed control they typically cause twisting of stems and petioles, stem swelling and elongation and leaf cupping and curling. This is followed by chlorosis at the growing points, growth inhibition, wilting and necrosis. Death of susceptible plants usually occurs within 3 -5 weeks (WSSA 2007).

DAS-8191Ø-7 cotton expresses the AAD-12 (aryloxyalkanoate dioxygenase-12) and PAT (phosphinothricin acetyltransferase) proteins. AAD-12 is able to degrade the pyridyloxyacetates auxins (e.g., triclopyr, fluoroxypyr) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid)(Wright 2013). AAD-12 is enantioselective[4], selectively cleaving only (S) enantiomers of chiral aryloxyphenoxypropionate (AOPP) herbicides (Müller 2006; Wright 2010). Because only (R) enantiomers are effective as herbicides, AAD-12 is not of utility for providing resistance to chiral AOPP herbicides (Wright 2010).

DAS-8191Ø-7 cotton also expresses the PAT protein that inactivates glufosinate (DAS 2013, p. 30). Glufosinate is a non-selective foliar herbicide used for pre-plant and post-emergence control of broadleaf plants and annual and perennial grasses (DAS 2013, p. 17; EPA 2008). Glufosinate was first registered by EPA for use in crops in 2000 as a non-selective foliar herbicide used for pre-plant and post-emergence control of broadleaf weeds (EPA 2008). It is currently registered for use on many crops including apples, berries, cotton, corn, cotton, currants, grapes, grass grown for seed, potatoes, rice, soybeans, sugar beets, and tree nuts and for use in non-crop areas including lawns and residential areas (EPA 2008). Numerous glufosinate resistant crops containing the PAT protein have undergone regulatory review and approval in the United States and several other countries (see list in ILSI CERA 2011). DAS stated in communications with APHIS, that "In DAS-8191Ø -7, pat is a selectable marker and is not intended to be commercialized as a stand-alone trait providing commercial field tolerance to glufosinate" (Rudgers 2014).

The *aad-12* and *pat* expression cassettes introduced into DAS-8191Ø-7 cotton are the same as those introduced into DAS-68416-4 (09-349-01p) and DAS-444Ø6-6 (11-234-01p) soybean for which APHIS reached a Determination of nonregulated status on September 22, 2014, following completion of a Final Environmental Impact Statement (DAS, 2010; DAS and MS Tech 2011; DAS 2013, p. 4).

[4] Chiral (not superimposable) molecules come in pairs of enantiomers, labeled R and S, such as diclorprop and mecoprop. Achiral (not chiral) molecules are superimposable on their mirror images, such as 2,4-D and MCPA.

C. Description of Inserted Genetic Material, Its Inheritance and Expression, Gene Products, and Changes to Plant Metabolism

To inform the potential hazards resulting from the genetic modification and potential routes of exposure related to the inserted DNA and its expression products, APHIS assessed data and information presented in the petition related to: the transformation process; the source of the inserted genetic material and its function in both the donor organism and DAS-8191Ø-7; and the integrity, stability and mode of inheritance of the inserted genetic material through sexual or asexual reproduction based on the location of the insertion (e.g. nucleus or organelle) and the number of loci inserted.

APHIS also assessed data presented in the petition on whether the genetic modification results in expression of new genes, proteins, or enzymes or changes in plant metabolism or composition in DAS-8191Ø-7 cotton relative to the nontransgenic counterparts. The assessment encompasses a consideration of the expressed proteins and any observed or anticipated effects on plant metabolism including, any relevant changes in levels of metabolites, antinutrients, or nutrients in harvested seed and fiber derived from DAS-8191Ø-7 compared to those in the conventional counterpart or to other comparators.

This information is used later in this risk assessment to inform whether there is any potential for plant pest vectors or sequences to cause disease or greater plant pest risks in DAS-8191Ø-7; or for expression of inserted DNA, new proteins or enzymes, or changes in metabolism to affect plant pest or diseases, nontarget beneficial organisms, weediness, agricultural practices that impact pest or diseases or their management, or plant pest risks through horizontal gene flow.

Description of the genetic modification and inheritance of inserted DNA

Cotton DAS-8191Ø-7 was produced by transformation using disarmed *Agrobacterium tumefaciens* (DAS 2013, pp. 21-24). Cotton cultivar Coker 310 hypocotyl segments were infected with *A. tumefaciens* strain LBA4404 carrying the binary vector pDAB4468 (Ooms 1982). Infected hypocotyl segments were cultured on media containing carbenicillin to inhibit the growth of *A. tumefaciens* and glufosinate to select for transformed tissue followed by *add-12* and *pat* specific PCR analysis. Putative transformants were treated with plant growth regulators to stimulate root generation, painted with 1.5% w/v glufosinate to screen for transformants and screened for the absence of plasmid backbone sequence and the presence of the *add-12* and *pat* genes.

The introduced DNA included a partial T-DNA border sequence from *A. tumefaciens* and intervening sequences to facilitate cloning (DAS 2013, pp. 25-32). The pDAB4468 plasmid generated a T-DNA with two gene expression cassettes, as follows:

Aryloxyalkanoate dioxygenase-12 (AAD-12)

- RB7 Matrix attachment region[5] (MAR) from the *Nicotiana tabacum rb-7-5A* gene (Hall 1991).
- Polyubiquitin promoter (AtUbi10) from *Arabidopsis thaliana* (Norris 1993).
- *aad-12* gene from *Delftia acidovorans* (den Dooren de Jon) strain MC1 encoding an aryloxyalkanoate dioxygenase-12 enzyme (AAD-12), formerly *spdA* (Westendorf 2002; Westendorf 2003; Wright 2010; Wright 2013).
- 3′ untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 23 (ORF23) of *Agrobacterium tumefacien*s pTi15955 (Barker 1983)

Phosphinothricin N-acetyltransferase (PAT)

- Promoter and the 5'UTR from the cassava vein mosaic virus (CsVMV) (Verdagurer 1996).
- *pat* (phosphinothricin N-acetyltransferase) gene from *Streptomyces viridochromogenes* inactivates the herbicide glufosinate (Wohlleben 1988).
- 3′ untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of open reading frame 1 (ORF1) of *Agrobacterium tumefaciens* pTi15955 (Barker 1983).

DAS provided evidence demonstrating that:

- The DNA inserted into the DAS-8191Ø-7 genome is present at a single locus and contains one copy of the AAD-12 and PAT expression cassettes, but does not contain any of the backbone sequences from pDAB4468 (DAS 2013, pp. 29-66).
- The inserted DNA was stably inherited across five generations (DAS 2013, pp. 67-68).

Expression of inserted DNA, changes in gene expression, new proteins or metabolism

Event DAS-8191Ø-7 cotton expresses the AAD-12 protein, comprised of 293 amino acids with a molecular weight of 32 kDal (DAS 2013, p. 69). The native *aad-12* gene was derived from *Delftia acidovorans* (Wright 2007; Wright 2013). *D. acidovorans* (previously designated *Pseudomonas acidovorans* prior to 1987, and *Comanonas acidovorans* from 1987-1999) is a chemoorganotrophic, non-spore-forming, strictly aerobic, gram negative bacterium widely distributed in soil and water and also found infrequently in clinical settings (Tamaoka 1987; von Graeventiz 1985; Wen 1999). The native AAD-12 protein was altered for optimal

[5] Chromosomal DNA is organized into looped domains through interactions with and attachment to a proteinaceous structure, called the nuclear scaffold or matrix. Specific chromosomal DNA sequences are thought to contain attachment regions that anchor the DNA to the matrix. Attachment regions may increase levels of expression, reduce transformant-to-transformant variation of expression, stabilize transgene expression and minimize transgene silencing (Allen 2000).

expression in cotton by modifying the G+C content bias to better match the bias in plants (DAS 2013, p. 25). The native and plant optimized DNA sequences of the *aad-12* gene are 80% identical, and produce an AAD-12 protein that is greater than 99% identical, differing only by the addition of a single amino acid, alanine, at position number two in DAS-8191Ø-7 (DAS 2013, p. 25 & 69). The additional alanine encodes part of a *Nco*I restriction enzyme recognition site (CCATGG) spanning the ATG translation start codon. The additional codon serves the dual purpose of facilitating subsequent cloning and improving the sequence context surrounding the ATG start codon, thus optimizing translation initiation (DAS 2013, p. 69). The alanine should not have an effect on the structure, activity, or specificity, because the N-terminus is sterically distant from the catalytic site of the AAD-12 protein (Hogan 2000). The AAD-12 protein expressed in DAS-8191Ø-7 is identical to that produced in DAS-68416-4 and in DAS 444Ø6-6 soybean for which APHIS has reached a Determination of nonregulated status (petition numbers 09-349-01p and 11-234-01p, respectively), (DAS 2010; DAS and MS Tech 2011; DAS 2013).

Functional assays demonstrated that the AAD-12 protein was active and protected DAS-8191Ø-7 from damage following 2,4-D application (DAS 2013, Table 19 and Fig. 58, pp. 115 and 158). The AAD-12 protein was found to be expressed throughout the life-cycle and tissues of the plant, including roots and seeds, with highest expression in leaves (DAS 2013, Table 8, p. 73). This is expected since expression of the *add-12* gene in DAS-8191Ø-7 is driven by the constitutive polyubiquitin UBQ10 promoter from *A. thaliana* (DAS 2013, Table 2, p. 30; Norris 1993).

The AAD-12 protein is highly specific for 2,4-D (Griffin 2013; DAS 2013, pp. 70-71). *In vitro* assays using plant endogenous compound, including natural plant hormones, phenylpropanoid and flavonoid secondary metabolites and all 20 amino acids, demonstrated that none of these served as a substrate for AAD-12. Therefore, because of the apparent high substrate specificity, the AAD-12 protein is unlikely to catalyze the conversion of other endogenous substrates, thereby affecting the metabolic system of DAS-8191Ø-7.

The AAD-12 enzyme confers herbicidal resistance by cleaving of 2,4-D into non-herbicidal 2,4-dichlorophenol (2,4-DCP) (Wesendorf 2002; Wesendorf 2003; Wright 2013). The degradation products of 2,4-D are 1,2,4 benzenetriol, 2,4-dichlorophenol (2,4-DCP), 2,4-dichloroanisole (2,4-DCA), 4-chlorophenol, chlorohydroquinone (CHQ), volatile organics, bound residues, and carbon dioxide (EPA 2005). EPA has determined that residues other than 2,4-D are not of risk concern due to low occurrence under environmental conditions, comparatively low toxicity, or a combination thereof (EPA 2005).

The PAT protein expressed in DAS-8191Ø-7 is comprised of 183 amino acids with a molecular weight of 20 kDal (DAS 2013, p. 78, Fig. 39). The native *pat* gene was derived from *Streptomyces viridochromogenes* (Wohlleben 1988), a gram-positive soil bacterium that is widely distributed in nature (Trejo 1963). The PAT protein is

identical to the native *S. hygroscopicus* protein and that produced in DAS-68416-4 soybean and DAS 444Ø6-6 (DAS 2010, DAS and MS Tech 2011, DAS 2013) with an APHIS Determination of nonregulated status. However, both the DAS-68416-4 and DAS 444Ø6-6 soybean lines are intended to be commercialized for use with the herbicide glufosinate (and the herbicide 2,4-D), whereas in DAS-81910-7, pat is a selectable marker and is not intended to be commercialized as a stand-alone trait providing commercial field tolerance to glufosinate (Rudgers 2014). The *pat* gene is also expressed in other previously deregulated GE crops, including soybean and corn (USDA APHIS 1996, USDA APHIS 2001, USDA APHIS 2004, USDA APHIS 2005) and the PAT protein has been the subject of numerous safety reviews (EPA 2008; Hérouet 2005; OECD 1999).

Functional assays demonstrated that the PAT protein was active and protected DAS-8191Ø-7 from damage following glufosinate application (DAS 2013, Table 19 and Fig. 65, pp. 115 and 172). The PAT protein in DAS-8191Ø-7, driven by the constitutive promoter from the cassava vein mosaic virus (DAS 2013, Table 2, p. 30; Verdaguer 1996) was found to be expressed throughout the life-cycle and tissues of the plant, including roots and seeds, with the highest expression in leaves (DAS 2013, Table 10, p. 80).

PAT is an enzyme that confers glufosinate resistance by catalyzing the acetylation of the free amino group of the L-isomer of phosphinothricin (L-PPT) to yield the herbicidally inactive compound N-acetyl-L-PPT (EPA 2008; OECD 1999). L-PPT is the active ingredient of the herbicide glufosinate ammonium, because only the L-isomer inhibits glutamine synthetase and is, therefore, herbicidally active. PAT proteins are highly specific for L-phosphinothricin (Wehrmann 1996). Other L-amino acids, including the L-phosphinothricin analogue L-glutamate, are unable to be acetylated by PAT and do not inhibit acetylation of L-phosphinothricin in competition assays (Wehrmann1996). Therefore, the PAT protein is unlikely to affect the metabolic system of DAS-8191Ø-7.

DAS carried out a compositional assessment of DAS-8191Ø-7 by comparing DAS-8191Ø-7 seed to seed from conventional control varieties using the principles outlined in the OECD consensus document of the compositional considerations for cotton (Herman 2013; OECD 2009). The samples for compositional assessment were collected from eight locations in 2012, chosen to represent typical cotton growing regions in the United States (DAS 2013, pp. 83 & 202). To provide a range of values of the normal variability of commercial cotton lines when grown in a variety of locations, the compositional assessement was obtained from planting six commercial nontransgenic cotton reference lines as reference varieties, along with values provided from published literature ranges (DAS 2013, p. 83). Compositional analyses of cotton seed samples included: nine proximates and fiber, twelve minerals, eighteen amino acids, twenty-two fatty acids, seven vitamins and five anti-nutrients (DAS 2013, pp. 83-111). Of the 73 analytes tested, 14 were excluded from the combined site analysis because more than 50% of the results were below the limit of quantification (LOQ). Overall, a comprehensive evaluation of event DAS-

8191Ø-7 cottonseed and the controls showed no biologically meaningful differences for seed composition for either major nutrients or key anti-nutrients in cotton seed. The few detected differences were small in number, and were less than the values found in the reference varieties and/or the literature ranges. Based on these results, it can be concluded that cottonseed from DAS-8191Ø-7 can be considered compositionally equivalent to those derived from convention cotton.

D. Potential Plant Pest and Disease Impacts

APHIS assessed whether potential plant pest or disease impacts are likely to result from the transformation process, from DNA sequences from plant pests, or from any other expression products, new enzymes, proteins or changes in plant metabolism or composition in DAS-8191Ø-7 cotton that are known or anticipated to cause disease symptoms, or to affect plant pests or diseases or plant defense responses (as identified from the previous section). APHIS also assessed or whether DAS-8191Ø-7 is likely to have significantly increased disease and pest susceptibility based on data and observations from field trials on specific pest and disease damage or incidence and any agronomic data that might relate to such damage. Impacts or changes are assessed to determine if they would (1) affect the new DAS-8191Ø-7 and/or result in significant introduction or spread of a damaging pest or disease to other plants; (2) result in the introduction, spread, and/or creation of a new disease; and/or (3) result in a significant exacerbation of a pest or disease for which APHIS has a control program. Any increase in pest or disease susceptibility is evaluated with respect to the context of currently cultivated varieties, the ability to manage the pest or disease, and the potential impact on agriculture.

Cotton itself is not considered a plant pest in the United States (7 CFR part 340.2). The *A. tumefaciens*-transformed plants used in the generation of DAS-8191Ø-7 were treated with the antibiotic carbenicillin to kill *A. tumefaciens* cells (DAS 2013, p. 21), so DAS-8191Ø-7 is not infected with *A. tumefaciens* (a plant pest). Furthermore, sequences derived from plant pests (promoter from cassava vein mosaic virus, terminator and intervening sequences from *Agrobacterium* Ti plasmids, and T- DNA border sequences, summarized above) incorporated into DAS-8191Ø-7 do not result in the production of infectious agents or disease symptoms in plants (DAS 2013, pp. 26-27); therefore, it is unlikely that DAS-8191Ø-7 cotton could pose a plant pest risk based upon these sequences.

A number of insects and diseases affect cotton. In 2012, the most important insect pests in terms of cotton yield loss were, in descending order of economic loss, *Lygus* species, stink bugs (Pentatomidae), thrips (Thripidae), spider mites, bollworm/budworm complex (*Helicoverpa zea* and *Heliothis virescens*), and the cotton fleahopper (*Pseudatomoscelis seriatus*) (Williams 2012). Of the various diseases affecting cotton, the most troublesome in 2011, in descending order of percent lost, were nematodes (*Meloidogyne* spp., *Reniform* spp. and other spp.), seedling diseases (*Rhizotonia,* etc.), boll rots (*Rhizopus,* etc.), root rot

(*Phymatotrichopsis omnivora* (=*Phymatotrichum omnivorum*)), leaf spots and bacterial blight (*Xanthomonas malvacearum*) (National Cotton Council 2011).

DAS collected data relevant to cotton diseases and insect pests from field experiments conducted at eight sites in 2012 (DAS 2013, pp. 83, 84, 116-117, 202, Figure 4, Tables 19 and 20). These locations cover a diverse range of environmental conditions where commercial cotton is grown in the United States. Personnel familiar with cotton cultivation practices (field station managers, field agronomists, and field associates) collected data on disease and pest damage ratings, and arthropod abundance for DAS-8191Ø-7, control line 98M-2983 (near isogenic nontransgenic isoline) and six unique reference lines.

The introduced genes did not significantly alter the observed insect pest infestation and disease occurrence or resulting damage on DAS-8191Ø-7 over the control lines (DAS 2013, pp. 115-117, Tables 19 and 20). The insect and disease pressure was typical of that found in those growing locations. Insect damage and disease was limited and when present, was found in the test and control plots.

Host plant quality (including such components as carbon, nitrogen, amino acid sources, trace elements, and defensive metabolites) is known to affect herbivore performance and fecundity; and higher-trophic level interactions, such as the performance of predators and parasitoids, may also be affected (Arimura 2009; Awmack 2002). Similarly a wide array of secondary metabolites in plants is known to provide defense against microbes (Dixon 2001). Thus, changes in host plant quality and secondary metabolites may have the potential to affect the ability of DAS-8191Ø-7 to withstand attack by pests and diseases or have impacts on nontarget organisms including beneficial organisms. However, as discussed earlier there were no significant changes in DAS-8191Ø-7 composition that would render DAS-8191Ø-7 more susceptible to pests and diseases over its control or reference cotton varieties. Likewise, the observed agronomic traits did not reveal any significant changes that would indirectly indicate that DAS-8191Ø-7 is or could be relatively more susceptible to pests and diseases over control or reference cotton varieties. These observations further support the conclusion that DAS-8191Ø-7 is unlikely to be more susceptible to plant pathogens and insect pests than conventional cotton.

In response to the cotton boll weevil (*Anthonomus grandis*) having caused more than $23 billion in economic losses, in 1978 the Boll Weevil Eradication Program was established as a cooperative effort between USDA and State officials to work with cotton growers to eradicate the boll weevil from the United States (USDA APHIS 2013). To date, the boll weevil has been eradicated from more than 98 percent of the U.S. cotton acreage in 15 Southeastern and Southwestern States, as well as significant portions of 3 others. Nearly all 17 cotton states have local regulations concerning the boll weevil. A proposed Federal quarantine for boll weevil was drafted and published for public comment in early 2007, and the final rule may be published in 2014.

Current boll weevil control measures include recommendations for cotton stalk destruction (Texas Department of Agriculture 2013; USDA APHIS 1998; USDA APHIS 2009). Accordingly, volunteer cotton plants are of special concern where the boll weevil is present and can serve as host plants and negatively influence the control of these pests (Texas Department of Agriculture 2013). Specifically Louisiana and Texas provide that 2,4-D is a key and preferred method to kill regrowth (Miller 2011; USDA APHIS 2012). APHIS considered whether 2,4-D tolerant cotton might impact the ability to control boll weevil in areas where 2,4-D is used as part of a control program. APHIS concludes that this is unlikely to result in an increased plant pest risk, because a number of methods for control of cotton regrowth are available to growers (Charles 2013; Dow 2013, p. 132; Keeling 2009; Morgan 2011a & 2011b; Thompson 2008). In addition, DAS recognizes the utility of 2,4-D in the eradication of the boll weevil and has been actively engaged in research focused on identifying alternative herbicides that can be used for cotton stalk control (DAS 2013, pp. 131-133).

For all of the reasons above, APHIS concludes that DAS-8191Ø-7 is unlikely to differ from conventional cotton in its ability to harbor or transmit plant pathogens or pests and cause indirect plant pest effects on other agricultural products.

E. Potential Impacts on Nontarget Organisms Beneficial to Agriculture

DAS-8191Ø-7 cotton is not engineered for pest resistance, thus there are no 'target' or 'nontarget' species. APHIS assessed whether exposure or consumption of DAS-8191Ø-7 would have a direct or indirect adverse impact on species beneficial to agriculture**.** Organisms considered were representatives of the species associated with production of the regulated crop in the agricultural environment. This assessment includes an analysis of data and information on the DAS-8191Ø-7 compared to the non-GE counterpart (or other comparators) for any biologically relevant changes in the phenotype or substances (e.g. proteins, nutrients, antinutrients, metabolites, etc.) produced which may be novel or expressed at significantly altered amounts that are associated with impacts on organisms beneficial to agriculture, and/or any observations of beneficial organisms associated with the plants.

Although many of the invertebrate organisms found in cotton-producing areas are considered pests, such as the cotton bollworm and tobacco budworm, most invertebrates are considered beneficial (University of Arkansas 2014). Beneficial insects include a wide variety of predators which catch and eat smaller insects and parasitic insects that live on or in the body of other insects during at least one stage of their life cycle. Other beneficial insects are pollinators. In the United States, bumble bees (*Bombus* spp.), honeybees (*Apis dorsata, A.indica* and *A. mellifera*) and long-horned bees (*Melissodes* spp.) are considered the most important pollinators of cotton (McGregor 1976). Other beneficial organisms include soil macrofauna

(earthworms, termites, ants, beetles, and millipedes, among others) which play a major role in the breakdown of surface dead plant material (Ruiz 2008).

The petitioner assessed the potential for DAS-8191Ø-7 to impact nontarget organisms, including those considered beneficial to agriculture, and determined that there would be no effect (DAS 2013, p. 131). DAS-8191Ø-7 is compositionally similar to other cotton varieties and will not adversely affect other organisms compared to other cotton varieties. Furthermore, APHIS has found no evidence or reason to believe that the presence of the *add-12* and *pat* genes or the expressed AAD-12 and PAT proteins in DAS-8191Ø-7 would have any impact on other organisms, including organisms beneficial to agriculture. The AAD-12 protein is expressed in a variety of plant tissues in DAS-8191Ø-7 with average expression values ranging from 10.74 ng/mg dry weight in roots at plant maturity to 71.17 ng/mg dry weight in leaves at the 4-leaf stage of growth (DAS 2013, p. 73; Oosterhuis 1990). Similarly, the PAT protein is expressed throughout the plant during multiple growing stages with average values from 0.11 ng/mg dry weight in pollen at the early bloom stage to 13.29 ng/mg in leaves at the 4-leaf stage of growth (DAS 2013, p. 80). AAD-12 and PAT expression values were similar for sprayed treatments as well as for plots sprayed and unsprayed with 2,4-D.

The inserted genetic material is not toxic and does not produce any substance that would be considered toxic. The AAD-12 protein does not share any meaningful amino acid sequence similarities with known toxins (DAS 2013, p. 75). AAD-12 amino acid sequence similarities were evaluated using BLASTp search algorithm against the GenBank non-redundant protein dataset. The only significant similarities identified were grouped into 10 categories: 2,4-D/alpha-ketoglutarate dioxygenase, putative alkylsulfatase, alpha-ketoglutarate (dependent) dioxygenase, alphaketoglutarate-dependent sulfonate dioxygenase, taurine catabolism dioxygenase, taurine dioxygenase, dioxygenase, oxidoreductase, pyoverdine biosynthesis protein, and hypothetical (putative) or unnamed proteins. DAS reported that none of the similar proteins returned by the search identified any safety concerns that might arise from the expression of AAD-12 protein in cotton. Bioinformatic analyses demonstrated that the PAT protein does not share amino acid sequence similarity with known protein toxins that would present any safety concerns. In addition, acute oral toxicity studies have indicated that the AAD-12 and PAT proteins have no adverse effects in mice at the highest dose tested (DAS 2013, pp. 75 & 82). The lack of known toxicity of ADD-12 and PAT suggests no potential for deleterious effects on beneficial organisms such as bees and earthworms.

The donor organism for the *aad-12* gene, *Delftia acidovorans* (formerly designated as *Pseudomonas acidovorans* (1926-1987) and *Comamonas acidovorans* (1987-1999)) is widely distributed in nature (soil, water) and has been infrequently isolated from humans and animals (von Graevenitz 1985; Wen 1999). The PAT protein was derived from *Streptomyces viridochromogenes*, a gram-positive soil bacterium (OECD, 1999). *D. acidovorans* and *S. viridochromogenes* are neither plant pests nor known pests of organisms beneficial to agriculture.

The use of the 2,4-D herbicide in the cultivation of DAS-8191Ø-7 or its offspring is regulated by EPA under its existing regulations for the registration of pesticide use. EPA considers the impacts on the environment, including effects on nontarget organisms in establishing application rates and residue tolerances for herbicides, including 2,4-D (EPA 1997; EPA 2005). APHIS has not identified any other potential mechanisms for deleterious effects on nontarget organisms.

Therefore, based on the above analysis of protein sequence analysis, acute toxicity studies, and the natural occurrence of the proteins in nature, APHIS concludes that exposure to and/or consumption of DAS-8191Ø-7 are unlikely to have any adverse impacts to organisms beneficial to agriculture.

F. Potential for Enhanced Weediness of DAS-8191Ø-7

APHIS assessed whether DAS-8191Ø-7 cotton is likely to become more weedy (i.e. more prevalent, competitive, damaging or difficult-to-control in situations where it is not wanted) than the nontransgenic progenitor from which it was derived, or other varieties of the crop currently under cultivation. The assessment considers the basic biology of the crop, the situations in which crop volunteers or feral populations are considered weeds, and an evaluation of the DAS-8191Ø-7 compared to the nontrangsenic progenitor or other appropriate counterpart evaluated under field (and/or lab) conditions characteristic for the regions of the United States where the DAS-8191Ø-7 cotton is intended to be grown for characteristics related to establishment, competiveness, reproduction, survival, persistence and/or spread that could influence weediness and the ability to manage the crop as a weed. For cotton, such characteristics include seed dormancy and germination, vigor, rate of growth and development, flowering, seed yield and propagule dispersal. The assessment also considers whether the engineered trait affects methods of control for cotton in situations where it is managed as a weed or volunteer in subsequent crops or in feral populations.

Upland cotton (*G. hirsutum*) possesses few of the characteristics common to plants that are successful weeds (Baker 1965; Keeler 1989) and is not considered to be a serious or common weed in the United States. It is not listed as a weed in the major weed references (Crocket 1977; Holm 1979; Muenscher 1980), nor is it present on Federal or State lists of noxious weed species (USDA APHIS 2010; USDA NRCS 2014). Cotton can become locally feral or naturalized in suitable areas, such as southern Florida, Hawaii, and Puerto Rico (Coile 2003; Fyxell 1979; USDA NRCS 2014; University of South Florida 2014). Upland cotton is a domesticated perennial grown as an annual crop that is not generally persistent in unmanaged or undisturbed environments without human intervention. Modern cultivars are not frost tolerant and do not survive freezing winter conditions, do not produce abundant or long-lived seeds that can persist or lie dormant in soil, do not exhibit vegetative propagation or rapid vegetative growth, and do not compete effectively with other cultivated plants (OECD 2008). In areas where winter temperatures are mild and freezing does not

occur, cotton plants can occur as volunteers in the following growing season (Baughman 2011; Charles 2013; Fromme 2011; Keeling 2009; Morgan 2011a & 2011b; Thompson 2008). However, these volunteers can be easily controlled by herbicides or mechanical means.

This assessment includes an evaluation of the unique characteristics of DAS-8191Ø-7 when grown under field conditions. DAS conducted field trials during the 2012 growing season across eight locations representative of the major cotton-growing areas of the United States to evaluate phenotypic, agronomic and ecological characteristics (DAS 2013, p. 202). DAS-8191Ø-7, seven near isogenic nontransgenic lines and reference variety plants were grown under conditions of no herbicide applications and sprayed with 2,4-D plus glufosinate (DAS 2013, pp. 112-119). DAS evaluated seven agronomic characteristics: early population, seedling vigor, flower initiation, nodes above first white flower, plant height, percent open bolls and lint yield; and disease and insect pressure (DAS 2013, p115, Table 19). Analyses of these field data revealed no statistically significant differences between non-sprayed DAS-8191Ø-7 cotton and the isoline (control). Seed dormancy is a characteristic that is often associated with plants that are considered weeds. Lab studies found no significant differences in germination (as an indicator of dormancy) of DAS-8191Ø-7 cottonseed compared with nontransgenic control cottonseed (98M-2983XCoker310) under warm (30º C) and cool conditions (18º C).

Based on the agronomic field data and literature survey about cotton weediness potential, the herbicide-resistant traits conferred by the *aad-12* gene is very unlikely to provide DAS-8191Ø-7 with a selective advantage in unmanaged ecosystems and allow it to persist as a troublesome or invasive weed. To control various cotton pests, including nematodes, verticillium wilt, seedling diseases, and pink bollworm, cotton is often rotated with other crops. As described above, cotton plants can volunteer under appropriate environmental condition in the following growing season but typically do not reduce crop yield (York 2004). Still, the herbicide-resistance traits could complicate efforts to control volunteer cotton in settings where 2,4-D is being applied for weed control where DAS-8191Ø-7 would be considered a weed (Charles 2013; Fromme 2011; Keeling 2009; Morgan 2011a & 2011b; Thompson 2008). In such circumstances cotton volunteers can be controlled by mechanical means (tillage), use of an herbicide with a different mode of action, appropriate variety selection and crop rotation (DAS 2013, p. 135; Keeling 2009; Morgan 2010; Morgan 2011a; Morgan 2011b; Thompson 2008; York 2004).

Based on the agronomic field and laboratory data, and literature survey concerning weediness potential of cotton, DAS-8191Ø-7 is unlikely to persist as a troublesome weed or to have a significant impact on current weed management practices. DAS-8191Ø-7 volunteers and feral populations can be managed using a variety of currently available methods and alternative herbicides. Furthermore, extensive post-harvest monitoring of field trial plots planted with DAS-8191Ø-7 under USDA-APHIS notifications and permits and field data reports did not reveal any differences in survivability or persistence relative to other varieties of the same crop currently

being grown. These data suggest that DAS-8191Ø-7 is no more likely to become a weed than conventional varieties of the crop.

G. Potential Impacts on the Weediness of Any Other Plants with which DAS-8191Ø-7 Can Interbreed

Gene flow is a natural biological process with significant evolutionary importance. A number of angiosperm taxa are believed to be derived from hybridization or introgression between closely related taxa (Ellstrand 2003; Harlan 1975; Rieseberg 1993; Stace 1987; van Tienderen 2004). It has been a common practice by plant breeders to artificially introgress traits from wild relatives into crop plants to develop new cultivars (Khoury 2013). However, gene flow from crops to wild relatives is also thought of as having a potential to enhance the weediness of wild relatives, as observed in rice, sorghum, sunflower and a few other crops (Ellstrand 1999). This topic is covered in two sections: 1) the potential for gene flow and introgression and 2) if so, the potential impact of introgression.

1) Potential for hybridization and gene introgression

Two cultivated and two wild species of cotton grow in the United States and its territories. *G. hirsutum* (Upland cotton) is the most widely cultivated species, comprising 98% of the U.S. cotton planted in 2013 (USDA NASS 2013). The vast majority of Upland cotton is cultivated in the Cotton Belt, which stretches across the southern United States from Virginia to California (USDA NASS 2013). Small amounts are also grown in Puerto Rico for breeding and seed production purposes (Information Systems for Biotechnology 2013). In addition to cultivated varieties, naturalized or native[6] populations of *G. hirsutum* grow in Florida, Puerto Rico, and the Virgin Islands, while naturalized populations grow in some of the Hawaiian Islands (Coile 2003; Fryxell 1979; Lee 1984; USDA NRCS 2014; Wagner 1990; Wagner 2012; Wunderlin 2008).

The second cultivated species, *G. barbadense* (Pima or Egyptian cotton), is grown in Arizona, California, New Mexico, and Texas, but no longer widely grown as an agricultural commodity in Hawaii (Pleasants 2005; USDA NASS 2013). Naturalized populations of *G. barbadense* grow in Puerto Rico, the Virgin Islands and most of the major Hawaiian Islands (Wagner 1990; USDA NRCS 2014). Two wild species of cotton are native to the United States, *G. thurberi* and *G. tomentosum*, which grow in Arizona and Hawaii respectively (USDA NRCS 2014).

G. hirsutum is tetraploid and thus effectively incompatible with diploid species such as *G. thurberi.* Plants from these two groups do not normally hybridize in natural settings and produce fertile offspring, and experimental crosses are difficult (OECD 2008). In contrast, *G. hirsutum* is sexually compatible with the tetraploids *G.*

[6] A "native" plant is one that has grown in a particular region or ecosystem for hundreds or thousands of years. A "naturalized" plant is one that does not need human help to reproduce and maintain itself over time in an area where it is not native (USDA NRCS 2014).

barbadense and *G. tomentosum* and can form viable and fertile progeny with both species (Brubaker 1993; OECD 2008; Saha 2006). Thus, unassisted outcrossing and gene introgression could potentially occur in areas where these species are co-located.

Wind dispersal of cotton pollen is negligible because of its large size and self-adherent properties (Jenkins 1993; McGregor 1976; OECD 2008). However, cross-pollination between cotton species can occur through the activity of pollinating insects (McGregor 1976; OECD 2008; Van Deynze 2005). For transgene introgression from DAS-8191Ø-7 to occur there would have to be spatial proximity between DAS-8191Ø-7 and the recipient variety or species, overlap in their flowering phenology, and overlap in their pollinators (Pleasants 2005). In general, the extent of transgene introgression will depend on the species pool, preferences, and abundance of pollinators, which can vary according to region, location, season, time of day, and use of insecticides. In addition, transgene introgression will decrease with increasing geographic distance between the source and receiver populations and physical barriers; and intermediate pollinator-attractive plants can reduce the potential for pollen movement (Green 1953; Llewellyn 2007; McGregor 1976; OECD 2008; Umbeck 1991; Van Deynze 2005; Zhang 2005). Additional information on the biology of cotton can be found within the OECD cotton consensus document (OECD 2008).

Because of eradication intended to control the pink bollworm, native and feral populations of *G. hirsutum* have become very rare and it has been listed as endangered by the state of Florida (USDA FS 2013). Although the remaining naturalized populations of *G. hirsutum* grow in Southern and Central Florida, their northernmost reported location (Gilchrest County, FL) is separated by over 120 miles from the nearest commercial cotton production areas in the Florida panhandle (Calhoun County, FL) (Wunderlin 2008; USDA NASS 2014). Thus, outcrossing from DAS-8191Ø-7 to naturalized *G. hirsutum* in Florida is highly unlikely.

In contrast, *G. hirsutum* is cultivated in many areas where *G. barbadense* is also grown (USDA NASS 2013). In addition, as noted above, native and/or naturalized populations of both species are present in Hawaii, Puerto Rico, and the Virgin Islands. Although cultivated varieties of both species are largely self-pollinated, insect-mediated cross-pollination can occur both within and between the species (Brubaker 1993; Llewellyn 2007; OECD 2008; Van Deynze 2005; Van Deynze 2011). Bumble bees (*Bombus* spp.), *Melissodes* and *Halictus* bees, honey bees (*Apis mellifera*), and *Scolia* wasps are the primary pollinators (McGregor 1976).

Published studies report that there has been relatively little gene introgression from *G. hirsutum* into native or naturalized *G. barbadense* in Central America and the Caribbean, despite the fact that *G. barbadense* has been grown in the presence of the predominant *G. hirsutum* since prehistoric times (Fryxell 1979). In contrast, introgression from *G. barbadense* to native or naturalized *G. hirsutum* in these areas has been relatively common (Brubaker 1993; Wendel 1992). Various mechanisms

have been suggested to account for this asymmetry (Brubaker 1993; Jiang 2000; OGTR 2008; Percy 1990). While none of these mechanisms leads to complete isolation between the two species, the reported asymmetry in gene flow suggests that gene introgression from cultivated *G. hirsutum* varieties such as DAS-8191Ø-7 to native or naturalized *G. barbadense* should be rare.

However, gene introgression from cultivated *G. hirsutum* to cultivated *G. barbadense* may be more likely, since gene flow between cultivated varieties of these species appears to occur with the opposite asymmetry from that observed between native or naturalized varieties (Brubaker 1993; Van Deynze 2011; Wendel 1992). The mechanism underlying this reversal in the directionality of gene flow accessions is not known. Nonetheless, outcrossing rates from DAS-8191Ø-7 to cultivated *G. barbadense* are still likely to be low. For instance, Van Deynze (2005) reported that Upland/Pima hybrid plants have been observed at a rate of 0.01% in fields sown with seeds of cultivated varieties that were obtained from production fields separated by at least 800 meters.

With regard to *G. tomentosum*, natural populations of this species are found on all Hawaiian Islands except Hawaii; the species is dominant on Kohoolawe and several sizable populations are found on Oahu and Maui. Populations are located on the drier, leeward coastal plains of the islands at low elevations, which are also the areas that are primarily used for agriculture (Pleasants 2005). *G. hirsutum* has not been grown as an agricultural commodity in Hawaii for decades, and APHIS has no information suggesting that seed companies use the Hawaiian Islands as a winter nursery.

The flowering period for *G. tomentosum* corresponds to the end of the rainy season; it may begin as early as January, with peak flowering occurring in April and May, and may extend through August in a very wet year (Pleasants 2010). Thus, any cultivated cotton that blooms between January and August could potentially overlap with *G. tomentosum*. Previously, it was thought that peak anthesis and receptivity in *G. tomentosum* occurs at dusk, whereas in *G. hirsutum* the flowers open in the morning and whither by evening (OECD 2008). However, Pleasants (2010) found that *G. tomentosum* flowers also open in the morning, dehisce rapidly, and begin to senesce by late afternoon. These results suggest that there is substantial overlap in flowering phenology between *G. hirsutum* and *G. tomentosum*.

Spontaneous self-pollination is rare in *G. tomentosum*, perhaps due to the structure of its flowers. Instead, the species appears to rely on the action of pollinators (Münster 2007; Pleasants 2005). It was previously thought that moths were the only insects that pollinated *G. tomentosum*, and thus that there was little overlap with pollinators of *G. hirsutum* (OECD 2008; Pleasants 2005). However, more recent studies have shown that *G. tomentosum* is pollinated by honeybees and carpenter bees, which are among the species that also pollinate commercially grown *G. hirsutum*. In addition, both of these pollinators are long-distance foragers; for instance, honeybees may forage up to 6 – 10 miles from their nest (Pleasants 2010).

Thus, in addition to overlap in flowering phenology, there is overlap in pollinators between *G. tomentosum* and *G. hirsutum*. However, no hybrids between *G. hirsutum* and *G. tomentosum* have been identified to date, although only a relatively small number of accessions and marker loci have been examined (DeJoode 1992).

Expression of the AAD-12 and PAT proteins do not cause any major changes in the phenotype of cotton plants other than to confer resistance to the herbicide 2,4-D and expression of the marker protein PAT. Thus, the introduced genetic material is unlikely to cause an increased rate of outcrossing of DAS-8191Ø-7 relative to non-transgenic varieties. Should outcrossing from DAS-8191Ø-7 to *G. barbadense* or *G. tomentosum* occur, transgene introgression would still require the establishment of hybrid progeny followed by persistence of the transgene through self-crossing or back-crossing into the recipient species in subsequent generations.

The low level of introgression from *G. hirsutum* to native or naturalized *G. barbadense* observed in the Caribbean and the phenomenon of hybrid breakdown[7] suggests that transgene introgression from DAS-8191Ø-7 to native or naturalized *G. barbadense* can occur but is likely to be rare (Fryxell 1979; Fang 2013; Jiang 2000; OGTR 2008). In the absence of herbicide treatment, the transgenic material in DAS-8191Ø-7 is unlikely to confer a selective advantage on any hybrid progeny that may result from outcrossing. Thus, the transgenes present in DAS-8191Ø-7 are unlikely to increase the rate of successful transgene introgression from DAS-8191Ø-7 into native or naturalized *G. barbadense* populations relative to the rate of gene introgression from conventional cultivars.

Transgene introgression from DAS-8191Ø-7 to cultivated *G. barbadense* can also occur but is also likely to be rare since cultivated *G. barbadense* is regularly harvested. While the likelihood of transgene movements to *G. barbadense* is likely greater with cultivated varieties than with native or naturalized *G. barbadense*, such movements would tend to involve plants producing seeds intended for processing rather than planting because seed production fields are isolated from commercial fields. Seed production isolation standards will help ensure that any movement of transgenes into seed production fields will remain at very low levels (AOSCA 2012; Van Deynze 2005). The transgenes present in DAS-8191Ø-7 unlikely to increase the rate of successful transgene introgression from DAS-8191Ø-7 into cultivated *G. barbadense* relative to the rate of gene introgression from conventional cultivars.

Finally, introgression into *G. tomentosum* in Hawaii is also likely to be rare, both because of barriers to introgression (Brubaker 1993; Jiang 2000; OGTR 2008; Percy 1990), and because there is no commercial cotton production on these islands (USDA NASS 2013). If any Upland cotton is grown in the Hawaiian Islands, it is grown at a very small scale and outcrossing to *G. tomentosum* is unlikely to occur. Should outcrossing nonetheless occur, transfer of the transgenes present in DAS-8191Ø-7 would not be expected to confer a selective advantage on the hybrid progeny or to reduce hybrid breakdown, which would be expected to eliminate

[7] "Hybrid breakdown" is the poor viability or lethality in F_1 hybrids between species.

introgressed genes from the *G. tomentosum* population. Thus, the transgenes present in DAS-8191Ø-7 are unlikely to increase the rate of successful transgene introgression from DAS-8191Ø-7 to *G. tomentosum*.

In summary the available evidence indicates that there is a low potential for introgression of transgenic material from DAS-8191Ø-7 cotton to *G. tomentosum* or to native or naturalized *G. barbadense*. There is no evidence that any of the genetic elements used in DAS-8191Ø-7 would increase the rate of outcrossing or gene introgression of DAS-8191Ø-7 relative to non-transformed cotton.

2) Relative fitness/weediness of recipients after introgression

As discussed in the previous section, the genetic material introduced into DAS-8191Ø-7 does not confer or enhance weedy characteristics of cultivated Upland cotton. There is no reason to believe that it would do so in naturalized or native *G. hirsutum*, in *G. tomentosum*, or in cultivated, naturalized, or native *G. barbadense*. Thus, in the unlikely event that transgene introgression from DAS-8191Ø-7 to one of these other types of cotton were to occur, the herbicide resistance traits would provide a selective advantage only when the resulting hybrids were in contact with the herbicide (i.e., in an agricultural field or treated rights of way). However, APHIS could find no reports that any of these potential recipient populations are actively controlled by herbicides. Therefore, transgene introgression from DAS-8191Ø-7 would not be expected to adversely impact recipient plants or increase their fitness or weediness any more than would gene flow from cultivated non-transgenic Upland cotton. Nor would it affect efforts to remove wild populations, as no such efforts exist.

Therefore, it is highly unlikely that Upland cotton plants in the United States and its territories will be found outside of an agricultural setting, except along roadsides along seed transportation routes. It is also highly unlikely that gene flow and introgression will occur between DAS-8191Ø-7 plants and wild or weedy species in a natural environment. Herbicides are available to control volunteer glufosinate and 2,4-D resistant cotton and weedy relatives. USDA has therefore determined that any adverse consequences of gene flow from DAS-8191Ø-7 to wild or weedy species in the United States and its territories are highly unlikely.

Based on the information presented in the petition and in relevant literature, APHIS has reached the following conclusions. The genetic modification in the DAS-8191Ø-7 cotton is not expected to increase the potential for gene flow, hybridization and/or introgression to occur to sexually compatible taxa compared to the nontransgenic recipient or other varieties of the crop commonly grown. Gene flow, hybridization and/or introgression of genes from DAS-8191Ø-7 to other sexually compatible relatives, including wild, weedy, feral or cultivated species in the United States and its territories is not likely to occur. Therefore, DAS-8191Ø-7 is not expected to increase the weed risk potential of other species with which it can interbreed in the United States and its territories.

H. Potential Changes to Agriculture or Cultivation Practices

APHIS assessed whether significant changes to agricultural or cultivation practices from the adoption of DAS-8191Ø-7 cotton are likely to impact plant diseases or pests or their management, including any APHIS control programs. This includes consideration of any changes in pesticide applications, tillage, irrigation, harvesting, etc. as they relate to plant pests and diseases.

Other than the use of 2,4-D to control weeds, none of the management practices currently employed for conventional cotton cultivation is expected to change if DAS-8191Ø-7 is determined to be no longer subject to the regulatory requirements of 7 CFR part 340 or the plant pest provisions of the Plant Protection Act. DAS studies demonstrate that the cultivation practices needed for growing DAS-8191Ø-7 are essentially indistinguishable from practices used to grow conventional cotton varieties with the exception of the 2,4-D based weed-control regime (DAS 2013, pp. 121-140).

Herbicides can impact pests or pathogens directly or indirectly through effects on the control of the crop or weeds associated with the crop. As noted in Section D. "Potential Plant Pest and Disease Impacts" above, field studies demonstrated that neither the herbicide resistance traits nor the herbicide treatments appear to alter the response of DAS-8191Ø-7 cotton to abiotic stress, diseases, or arthropod pests under natural levels of these stressors, nor were pest arthropods more abundant around DAS-8191Ø-7.

No differences in insect or disease damage were observed in field trials with DAS-8191Ø-7 (DAS 2013, pp. 112-117). Because agricultural and cultivation practices would not be significantly different than that of conventional cotton, APHIS does not foresee changes in either insects or disease damage or control measures employed due to agricultural or cultivation practices with DAS-8191Ø-7.

The current and proposed uses of 2,4-D in cotton are described in the petition (DAS 2013, pp. 133-135). Although 2,4-D is not currently registered by EPA for use on cotton, it is used for burndown in no-till cotton fields (Greenberg 2007; Lalli 2011; Miller 2011; University of Delaware 2008; USDA APHIS 2012). Because 2,4-D has approximately three weeks of residual soil activity, a 30 day waiting period is generally required before planting cotton (DAS 2013 pp. 33-34; Penn State 2014). If EPA approves DAS's submitted application to register 2,4-D for use on cotton, growers would be authorized to apply 2,4-D on cotton (or in mixtures of other herbicides if *aad-12* is stacked with other herbicide tolerant genes) for preplant applications without restrictions and for in-crop post-emergence applications on DAS-8191Ø-7. The proposed use will allow application of 2,4-D at burndown or pre-emergence (1 lb. acid equivalent/acre), followed by one or two post-emergence (0.5 – 1.0 lb. acid equivalent/acre) at least 12 days apart through the mid-bloom stage of cotton. The total maximum seasonal application rate is estimated to be 3.0

lbs acid equivalent/acre. Issues related to herbicide drift and volatilization will be further addressed in the USDA APHIS NEPA document for this petition.

Upon EPA registration of DAS-8191Ø-7 DAS expects that growers will have the ability to continue to use established cotton production practices including crop rotation, tillage systems, labeled herbicides, and planting and harvesting machinery currently being utilized (DAS 2013, pp. 121-129 & 133-140). The anticipated registration changes would facilitate a wider window of application for 2,4-D in cotton, which is expected to provide a tool for improved control of broadleaf weeds (including some with resistance to other herbicides such as glyphosate and ALS) that can be integrated into weed management programs using no-till or reduced tillage or conventional tillage. The impacts of this system for reducing or managing weeds and the evolution of herbicide-resistant weeds is outside the scope of this analysis will be examined as part of APHIS analysis under the National Environmental Policy Act. Greater weed control could potentially reduce disease and pest pressure in cotton if the diseases and pests of the weeds also use cotton as a host.

Crop rotations (successive planting of different crops on the same land) has been recommended for cotton cultivation to optimize soil nutrition, prevent soil erosion, improve pest control and alternating herbicides with altered modes of action (Waddle 1984). Cotton is often rotated with other crops to control various cotton pests including nematodes and various soil-borne seedling diseases (University of California 2013). Cover crops may be planted between periods of regular crop production to prevent soil erosion and provide humus or nitrogen may include guar, annual sweet-clover, biennial sweet clover, cowpeas, mung bean, crimson clover, sespedeza and *Crotalaria* and some non-legumes (Waddle 1984). Crop rotation practices in cotton were analyzed in the petition (DAS 2013, pp. 121-129 & 133-140). 2,4-D has short-lived soil activity, with a half-life of 6.2 days for 2,4-D under aerobic soil conditions (EPA 2005). Crop rotation restrictions of a maximum of 30 days and should be adequate for rotation to other crops (DAS 2013, p. 133; Penn State 2014). Therefore, crop rotation practices are not expected to be adversely impacted by the use of 2,4-D on fields planted to DAS-8191Ø-7.

None of the management practices currently employed for cotton production is expected to change if DAS-8191Ø-7 is determined to be no longer subject to the regulatory requirements of 7 CFR part 340 or the plant pest provisions of the Plant Protection Act. DAS's studies demonstrate that the agronomic characteristics and cultivation practices employed when growing DAS-8191Ø-7 are essentially indistinguishable from practices used to grow other cotton varieties. The geographic range or seasonality of cotton cultivation is not expected to change to accommodate the cultivation of DAS-8191Ø-7. DAS-8191Ø-7 is comparable to currently available cotton varieties in terms of resistance to insects and disease.

APHIS could not identify any significant changes to agricultural or cultivation practices (e.g. pesticide applications, tillage, irrigation, harvesting, etc.) from

adoption of DAS-8191Ø-7; therefore, no impact on plant diseases or pests or their management is likely to occur.

I. Potential Impacts from Transfer of Genetic Information to Organisms with which DAS-8191Ø-7 Cannot Interbreed

APHIS examined the potential for the new genetic material inserted into DAS-8191Ø-7 cotton to be horizontally transferred without sexual reproduction to other organisms and whether such an event could lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants. The gene transfer between unrelated organisms (horizontal gene transfer) is one of the most intensively studied fields in the biosciences since 1940, and the issue gained extra attention with the release of transgenic plants into the environment (Dröge 1998). Potential risks from stable horizontal gene transfer (HGT) from genetically engineered organisms to another organism without reproduction or human intervention were recently reviewed (Keese 2008). Mechanisms of HGT include conjugation, transformation and transduction, and other diverse mechanisms of DNA and RNA uptake and recombination and rearrangement, most notably through viruses and mobile genetic elements. HGT has been a major contributor to the spread of antibiotic resistance amongst pathogenic bacteria; emergence of increased virulence in bacteria, eukaryotes and viruses; and, in the long run, to major transitions in evolution.

1) Potential for horizontal gene transfer to bacteria, fungi, or invertebrates

DAS-8191Ø-7 has the *aad-12* and *pat* genes derived from bacteria. Horizontal gene transfer and expression of DNA from a plant species to bacterial, fungal or invertebrate species is unlikely to occur based on the following observations. Although there are many opportunities for plants to directly interact with fungi and bacteria (e.g. as commensals, symbionts, parasites, pathogens, decomposers, or in the guts of herbivores) and with invertebrates as plant pests, there are almost no evolutionary examples of HGT from eukaryotes to bacteria or from plants to fungi or invertebrates (Keese 2008). Examples of HGT between eukaryotes and fungi primarily involve gene acquisition or transfer by fungi to or from other distantly related fungi or bacteria (Keeling 2008; Keese, 2008) and HGT between plants and fungi is extremely rare (Richards 2009). Examples of HGT between plants and invertebrates are also extremely rare, and most examples of HGT in insects involve acquisition of genes from their pathogens or endosymbionts (Acuna 2012; Keese 2008; Zhu 2011).

Horizontal transfer from and expression in bacteria of the foreign DNA inserted into the nuclear genome of the GE plant is unlikely to occur. First, many genomes (or parts thereof) have been sequenced from bacteria that are closely associated with plants including *Agrobacterium* and *Rhizobium* (Wood 2001; Kaneko 2002). There is no evidence that these organisms contain genes derived from plants. HGT from plants to bacteria is a very low frequency event, primarily because functional and

selective barriers to HGT increase with genetic distance (Keese 2008). Second, in cases where review of sequence data implied that horizontal gene transfer occurred, these events are inferred to occur on an evolutionary time scale on the order of millions of years (Brown 2003; EFSA 2009; Koonin 2001). Third, transgene DNA promoters and coding sequences are optimized for plant expression, not prokaryotic bacterial expression. Thus even if horizontal gene transfer occurred, proteins corresponding to the transgenes are not likely to be produced. Fourth, both the FDA (1998) and the European Food Safety Authority (EFSA 2009) have evaluated horizontal gene transfer from the use of antibiotic resistance marker genes and concluded that the likelihood of transfer of antibiotic resistance genes from plant genomes to microorganisms in the gastrointestinal tract of humans or animals, or in the environment, is very rare or remote.

2) Potential for horizontal gene transfer to viruses

DAS-8191Ø-7contains the promoter and the 5'UTR from the cassava vein mosaic virus (CsVMV) (DAS 2013, p. 30; Verdaguer 1996). APHIS also considered whether horizontal transfer of DNA from the GE plant to plant viruses was likely to occur and would lead to the creation or selection of plant viruses that are more virulent or have a broader host range. This issue has been considered before by other science review panels and government regulatory bodies (EPA 2006; Keese, 2008). HGT is not unusual among plant viruses; however this is generally limited to exchange between viruses present in the same host organism in mixed infections, and most commonly involves homologous recombination, relying on sequence similarity at the point of crossover (Keese 2008). HGT from virus sequences engineered into plants has been demonstrated with infecting or challenge viruses, including both DNA viruses (e.g. Caulimoviridae and Geminiveridae, which replicate in the nucleus) (Frischmuth 1998) and RNA viruses (which typically replicate in the cytoplasm); however most have been under conditions that favor recombination to restore a defective virus (Fuchs 2007; Keese 2008; Thompson 2010). Populations of recombinants between virus transgenes expressed in transgenic plants infected with related viruses are similar to recombinants found in mixed infections of the same viruses in nontransgenic plants, indicating that there was no novel recombination mechanism in the transgenic plants and no increased risk is expected over what is expected from mixed infections (Keese 2008; Turturo 2008). Nonhomologous recombination in HGT among viruses or between virus transgenes and infecting viruses can occur, but frequently results in gene deletions which can result in nonviable viruses (Morroni 2013). Depending on the particular virus and sequences involved, various hot-spots for recombination have been found in both coding and noncoding regions, and strategies implemented in design of transgenes to avoid recombination have been suggested. No recombinant or undesirable viruses with new properties have been detected for over at least 8-10 years in field tests or during commercial growth of deregulated virus resistant plum, squash, or papaya engineered with genes from viruses that have been deregulated in the United States (Fuchs 2007).

3*) Potential for horizontal gene transfer to parasitic plants*

Evidence for HGT from plants to other plants is limited to two specific scenarios: (1) exchange of genes between a parasitic plant and its host; and (2) exchange of genes between cells of two plants living in close proximity, such as in a graft junction. In both cases, this type of HGT requires physical contacts between the two plants. Most cases of HGT in plants involve transfer of mitochondrial genomes, which are primarily maternally inherited in plants (Barr 2005), to other mitochondria genomes, and mostly involve parasitic plants and their hosts (Richardson 2007). Recently, (Yoshida 2010) through a comparative genomics analysis implicated HGT for the incorporation of a specific genetic sequence in the parasitic plant purple witchweed (*Striga hermonthica*) from its monocot host plant. According to this study, the incorporation of the specific genetic sequence (with an unknown function) occurred between sorghum and purple witchweed. However, this HGT occurred before speciation of purple witchweed and related cowpea witchweed (*S. gesnerioides*) from their common ancestor. Furthermore, *S. hermonthica* is not found in the United States and *S. asiatica*, another related parasite of cereal crops, is only present in North Carolina and South Carolina (USDA NRCS 2014). More recent studies demonstrated that in a few parasitic species of the Rafflesiaceae family, out of several genetic sequences examined, about 2.1% of nuclear (Xi 2012) and 24 –41% of mitochondrial (Xi 2013) gene transcripts appeared to be acquired from their obligate host species. However, all the above-mentioned instances of HGT between parasitic plants and their hosts were reported to be of ancient origins, on an evolutionary time scale spanning thousands to millions of years ago and as recently reviewed by Huang (2013). Furthermore in DAS-8191Ø-7, the DNA sequences were inserted into the nuclear genome, not the mitochondrial genome.

If the GE plant becomes infected by a parasitic plant or is naturally grafted to another plant, there is a very low probability that HGT could result in the other plant acquiring DNA from the GE plant. However, in both scenarios this newly introduced DNA would likely reside in somatic cells, and with little chance of reaching the germ cells, this introduced DNA could not persist in subsequent generations unless the recipient plant reproduced asexually from the affected cells.

Based on the above analysis APHIS therefore concludes that HGT of the new genetic material inserted into the GE plant to other organisms is highly unlikely, and is not expected to lead directly or indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

J. Conclusion

APHIS has reviewed the information submitted in the petition, supporting documents, and other relevant information to assess the plant pest risk of DAS-8191Ø-7 compared to the unmodified variety from which it was derived. APHIS concludes that DAS-8191Ø-7 is unlikely to pose a plant pest risk based on the following findings:

- No plant pest risk was identified from the transformation process or the insertion of new genetic material because DAS-8191Ø-7 was produced by transformation using disarmed *Agrobacterium tumefaciens*; and the plants were treated with an antibiotic to devitalize *A. tumefaciens*. The inserted genetic material which was derived from plant pests does not result in the production of infectious agents or disease symptoms in plants.
- No increase in plant pest risk was identified in DAS-8191Ø-7 from the expression of the inserted genetic material of new proteins, because DAS-8191Ø-7 can be considered compositionally and nutritionally equivalent to those derived from convention cotton.
- Disease and pest incidence and/or damage were not observed to be significantly increased or atypical in DAS-8191Ø-7 compared to the nontransgenic counterpart or other comparators in field trials conducted in growing regions representative of where DAS-8191Ø-7 is expected to be grown and greenhouse and laboratory studies. Observed agronomic traits also did not reveal any significant differences that would indirectly indicate that the DAS-8191Ø-7 is more susceptible to pests or diseases.
- Exposure to and/or consumption of DAS-8191Ø-7 are unlikely to have any adverse impacts on organisms beneficial to agriculture based on the analysis of the potential toxicity based on lack of amino acid sequence similarities with known toxins and mice acute toxicity studies and the donor organisms are widely distributed in nature.
- DAS-8191Ø-7 is no more likely to become a weed or be weedier than conventional varieties of the crop based on its observed agronomic characteristics, weediness potential of the crop and current management practices available to control DAS-8191Ø-7 as a weed. Volunteers and feral populations of the herbicide resistant DAS-8191Ø-7 can be managed to include, mechanical means (tillage) and the use of an herbicide with a different mode of action.
- DAS-8191Ø-7 is not expected to increase the weed risk potential of other species with which it can interbreed in the United States or its territories. Gene flow, hybridization and/or introgression of inserted genes from DAS-8191Ø-7 to other sexually compatible relatives with which it can interbreed is not likely to occur. These compatible relatives are not considered weedy or invasive. The new phenotypes conferred by genetic engineering are not likely to increase the weediness of these compatible relatives or affect the current ability to control these relatives in situations where they are considered weedy or invasive. The following measures are still available for their control such as tillage practices and the use of alternative herbicides.
- Significant changes to agricultural or cultivation practices (e.g. pesticide applications, tillage, irrigation, harvesting, etc.) from adoption of DAS-8191Ø-7 were not identified and not likely to increase plant diseases or pests or compromise their management.
- Horizontal gene transfer of the new genetic material inserted into DAS-8191Ø-7 to other organisms is highly unlikely, and is not expected to lead directly or

indirectly to disease, damage, injury or harm to plants, including the creation of new or more virulent pests, pathogens, or parasitic plants.

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