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Regulatory Status Review Submission for corn (*Zea mays,* L.) expressing brazzein from *Pentadiplandra brazzeana*, a South African fruit.

Submitted by GreenLab, Inc.; Jonesboro, AR 72404

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This document contains no confidential business information.

Introduction:

GreenLab, Inc. (GL) is submitting this document to USDA-APHIS Biotechnology Regulatory Services for a Regulatory Status Review as described in 7 CFR part 340.4. GL has been growing this corn for many years under USDA-APHIS permits and intends to scale up production to increase sales of its brazzein protein sweetener product for a variety of potential sugar replacement uses. The brazzein of note here has never been produced in large enough quantities or at low enough cost for the intended applications.

Corn as a plant and crop

Zea mays, L., is a food, feed, fiber, and fuel crop plant. It is grown on approximately 85-95 million acres in the U.S. every year. Roughly forty percent of the crop goes to ethanol production as a gasoline additive. Another 30-40% goes to animal feed for cattle, hogs, and poultry. A small percentage is exported, and small amounts are used in human food products.

Corn is an open pollinated crop with separate male and female flowers. It is produced as a hybrid, and thus the male and female cross to produce the hybrid must be controlled. This is fortunate for the GL team in that when doing back crosses to generate inbred elite lines for the hybrid, selection for higher expression of the transgene can be accomplished over the several generations required for breeding.

Genes inserted into corn

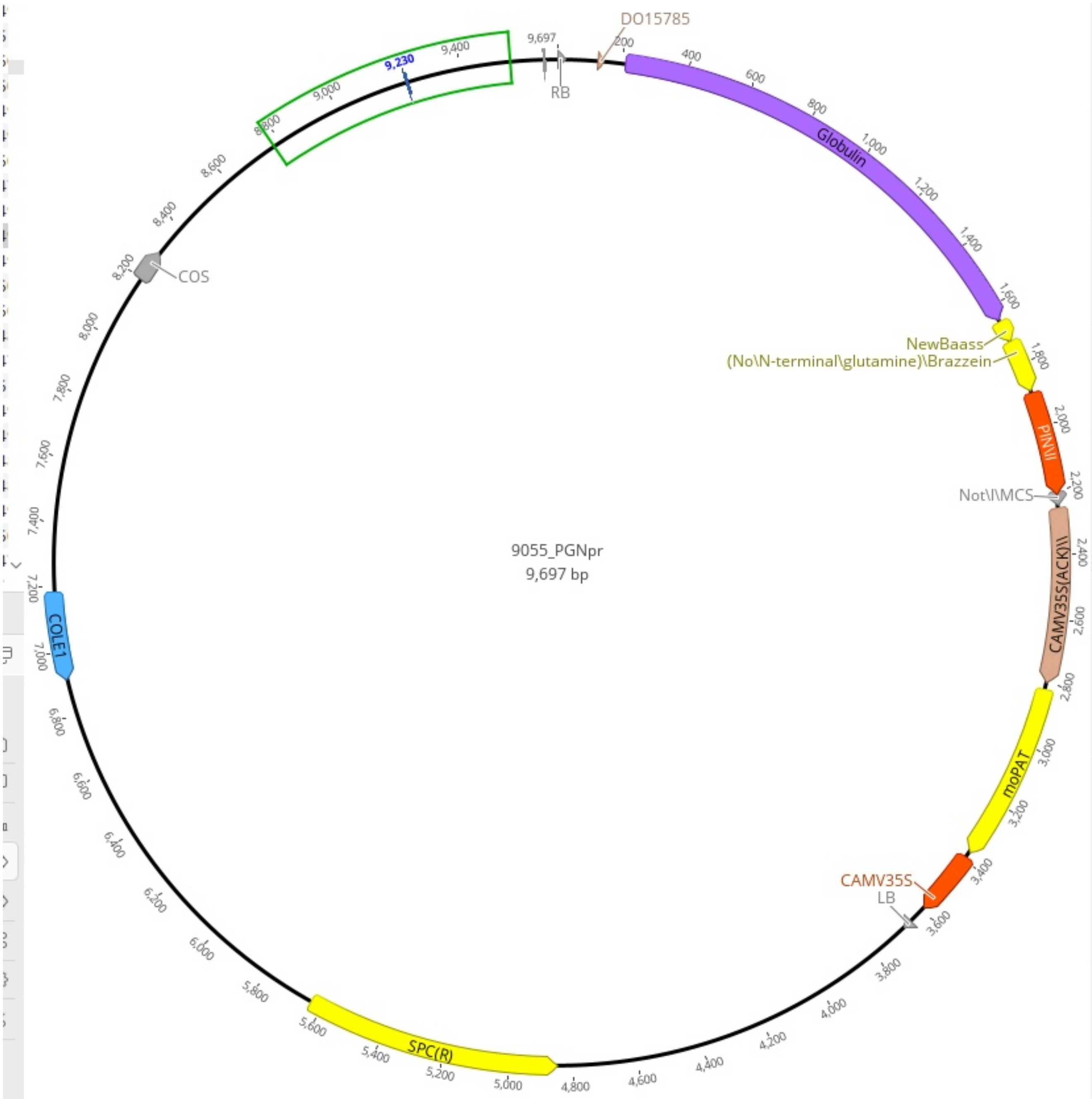
The brazzein gene inserted into this corn is for the sweet protein identified from *Pentadiplandra brazzeana,* a fruit from southern Africa. Transformation was achieved using the disarmed *A. tumefaciens* strain, EHA101 with the super binary plasmid from Ishida et al. (Ishida et al., 1996). The construct used is described in the following table.

Table 1: Genetic Elements and Their Functions in GreenLab, Inc. brazzein-producing corn.

Genetic Element	Location in Plasmid	Function (Reference)
T-DNA regions		
Right Border Region	1-25	DNA region from *Agrobacterium tumefaciens* containing the Right Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Intervening sequence	26-210	Sequence used in DNA cloning
Globulin-1 promoter	211-1652	DNA region from *Zea mays* globulin-1 gene (1.4 kb) (Belanger and Kriz, 1991) AH001354.2
Intervening Sequence	1653	Sequence used in DNA cloning
BAASS	1654-1725	Alpha amylase signal sequence from barley, *Hordeum vulgare* (Rogers, 1985). ABBO1247.1
Brazzein gene	1726-1887	From *Pentadiplanda brazzeana* (Ming and Hellekant, 1994)
Intervening Sequence	1888-1892	Sequence used in DNA cloning
Pin II terminator	1893-2203	Protease inhibitor II gene terminator from potato, *Solanum tuberosum* (An et al., 1989) X04118.1
Intervening Sequence	2204-2256	Sequence used in DNA cloning
35S promoter	2257-2798	Cauliflower mosaic virus promoter for the 35S rna. (Franck et al., 1980) NC_001497.2
Intervening Sequence	2799-2818	Sequence used in DNA cloning
Maize optimized PAT	2819-3370	Phosphinothricin acetyl transferase from *Streptomyces viridochromogenes* (Wohlleben et al., 1988) WP_003988626.1
Intervening sequence	3371-3388	Sequence used in DNA cloning
35S terminator	3389-3591	Cauliflower mosaic virus terminator for the 35S rna. (Franck et al., 1980) NC_001497.2
Intervening Sequence	3592-3650	Sequence used in DNA cloning
Left Border Region	3651-3675	DNA region from *Agrobacterium tumefaciens* containing the Left Border sequence used for transfer of the T-DNA (Depicker et al., 1982; Zambryski et al., 1982)
Vector	3676-9697	Sequence used in DNA cloning; spectinomycin resistance; origin of replication;

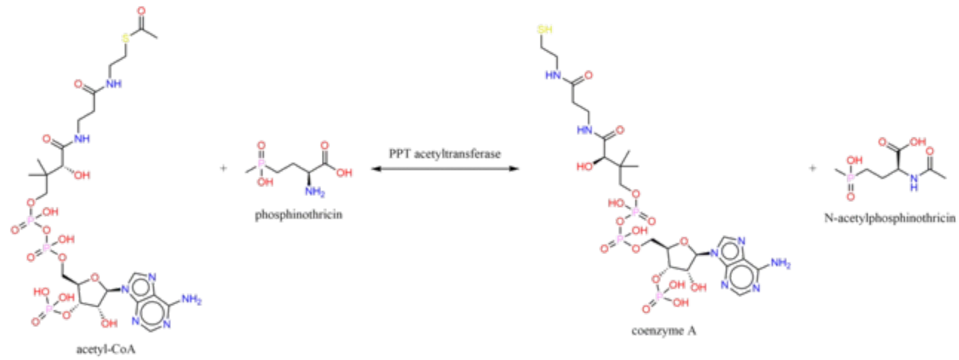
The plasmid map used for the transformation is found on the following page and the intended sequence insertion (between the right and left borders) is found in Appendix I.

Figure 1: Plasmid map of brazzein expression plasmid: PGN9055



The PAT gene encodes **Phosphinothricin acetyltransferase** (*PAT*). This confers resistance to the herbicide phosphinothricin, or Liberty™. The enzyme catalyzes the following chemical reaction.

acetyl-CoA + phosphinothricin CoA + N-acetylphosphinothricin



Reaction mechanism of the PAT enzyme (taken from Wikipedia, (Wohlleben et al., 1988)).

GL has grown the brazzein event in the field for a number of years. GL spent years breeding brazzein in the backcross program putting the transgene into two inbred lines to produce a production hybrid. The lines are Stine male and female elite lines MBS411 and 16038-025. Two self-pollinated generations made them essentially homozygous. All growing of the brazzein lines to date has been at 1 location in California and 1 in Chile.

Intended Use

As intended, the trait expressed in these plants is identified as embryo-preferred production of brazzein. This is accomplished by use of the *Zea mays* globulin-1 promoter in combination with the barley alpha amylase signal sequence which further targets protein to the cell walls of embryo tissues. This construct has been shown to be highly effective for this purpose (Lamphear et al., 2005). Expression of brazzein in other tissues or times of plant development, other than embryo growth and development, is not expected. Secondarily, constitutive production of PAT using the cauliflower mosaic virus 35S promoter results in plant tolerance to glufosinate ammonium herbicides.

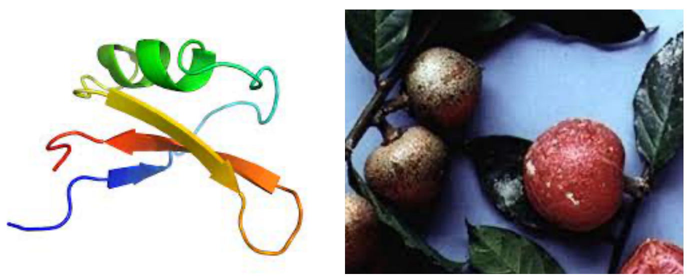
Characteristics of recombinant brazzein

The existence of naturally derived proteins that possess intrinsic sweetness has been known for many years (reviewed in (Faus, 2000)). Interest in these proteins has increased with increasing demand for 'low-calorie' sweeteners and food products described as 'natural' and 'healthy' to address the needs of millions of individuals conscious of carbohydrate intake for dietetic and diabetic reasons. This has led to the commercialization of only one member of this family, thaumatin, as a sweetener and flavor enhancer (Faus, 2000; Witty and Higginbotham, 1994). Unfortunately, commercial production of thaumatin, as well as all other sweet proteins, has been limited because the natural sources for all of these proteins are tropical plant species that are difficult to cultivate, and repeated attempts to produce recombinant sweet proteins in microorganisms and transgenic plant systems have failed to yield these proteins at sufficiently high levels to make widespread commercialization economically feasible (Faus, 2000; Witty and Higginbotham, 1994; Zemanek and Wasserman, 1995).

Brazzein is a recently identified protein derived from the African plant, *Pentadiplandra brazzeana* Baillon, that has an intrinsic sweetness 500–2000 times that of sucrose (Ming and Hellenkant, 1994). The brazzein-containing fruit from *P. brazzeana* has been consumed in native regions of tropical Africa because of its sweet properties, where it has been associated with the French name 'l'oubli ', meaning 'forgetting' (Hladik, 1993). This is due to the propensity of native children to become so focused on obtaining more of the delicious ripe fruit that they 'forget' their mothers whilst looking for them. However, limited availability of the fruit and complications associated with large-scale production of the native plant have rendered large-scale production of brazzein from natural sources uneconomical. Therefore, widespread commercial production of brazzein requires the transfer of protein expression to a heterologous system by means of recombinant DNA technology. The suitability of brazzein for recombinant expression has already been demonstrated in *Escherichia coli*, enabling further characterization of the protein's biochemical properties (Assadi-Porter et al., 2000a; Assadi-Porter et al., 2000b).

Brazzein is a 6.5-kDa, single-chain polypeptide with four intramolecular disulfide bridges that enable it to maintain its sweetness profile even after incubation at 80^{o}C for 4 h (Ming and Hellekant, 1994). Three forms of the protein differ only at the N-terminal amino acid residue. Type 2 brazzein corresponds to the predicted 54-amino acid translation product containing a glutamine at its N-terminus. This form appears to be short lived as the N-terminal glutamine undergoes natural conversion to pyroglutamate, resulting in type 1 brazzein, and the loss of the N-terminal glutamine (or pyroglutamate) yields the 53-amino acid type 3 brazzein. Only the last two species are detected in the ripe fruit. The sweetness intensity varies between forms, and it has been reported that the type 3 form is twice as sweet as the type 1 form (Izawa et al., 1996).

Figure 2: Three-D structure of brazzein protein (6.5 kDa) and the fruit of *P. brazzeana*.



Conclusion

GreenLab, Inc. has been growing its brazzein-producing corn lines under USDA-APHIS permit since 2000. GreenLab, Inc. is requesting that USDA-APHIS-BRS determine that the brazzein-producing corn described in this document is not subject to its regulations at 7 CFR part 340.

Respectfully submitted,

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Appendix I: Sequence of intended insert in GreenLab, Inc. brazzein corn

Right border sequence
Sequences used in DNA cloning (throughout the construct)
Maize globulin-1 promoter
BAASS—barley alpha amylase signal sequence
Brazzein gene from *Pentadiplandra brazzeana*
Pin II terminator from potato
Cauliflower mosaic virus 35S promoter sequences
Maize optimized phosphinothricin N-acetyl transferase [*Streptomyces viridochromogenes*]
Cauliflower Mosaic Virus 35S terminator sequence
Left border sequence

Right border

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CCACAATATATCCTGCCA **Left Border**

Vector sequence

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