CBI-deleted Copy

Request for Determination
Whether TraitUP™-FB100 meets the Definition
of a
Regulated Article per 7 CFR §340

---

1. Summary:

TraitUP™-FB100:

TraitUP™-FB100 is a plasmid DNA based product designed to transiently express the [**CBI**] tomato plants. TraitUP™-FB100 is being developed as a Biopesticide for the control of Fusarium crown rot on tomatoes.

A meeting was held on November 1, 2012 with the APHIS Biotechnology Regulatory Services (BRS) and, a similar meeting was held with the EPA Biopesticide and Pollution Prevention Division (BPPD) that same day. The primary purpose of the APHIS BRS and the EPA BPPD meetings was to introduce the TraitUP™-FB100 technology and specifically the first product, TraitUP™-FB100. During the BRS meeting submission of a letter to determine if TraitUP™-FB100 meets the definition of a regulated article under 7 CFR §340 was discussed. This overview includes the supporting background data.

The TraitUP™ Technology

The transient expression is obtained by using the TraitUP™ technology, which is based on [

CBI] resulting in the phenotype of resistance against Fusarium crown rot in Tomato.

Each of the plasmids composed of partial sequences of common commercial bacterial plasmid vectors and parts of [**CBI.**] Although those plasmids contain parts from the [**CBI**], therefore there are no longer any pathogenic aspect related to this plasmid source.

[

CBI]

contains the following components:

[

– **CBI**]

[

CBI] is composed of the following:

[

– **CBI]**

[**CBI**]

TraitUP-FB100 is introduced to the tomato plants via [

CBI]

Key Characteristics of TraitUP™ FB-100 Plants

The plants treated with TraitUP™ FB-100 have the following characteristics:

[

– **CBI**]

Tomato plants developed from TraitUP™-FB100 treated seeds have the phenotype of resistance to Fusarium crown rot.

2. Introduction:

Introduction and expression of foreign genes in plants was first based on *Agrobacterium* mediated transformation (Horsch *et al.,* 1985). Later, bombardment of DNA-coated inert beads was adopted for introducing and expressing foreign genes into the plant (Klein *et al*., 1987). An additional approach made use of plant [

CBI] as vectors were their restricted host range and the relatively small size of the foreign gene insert to be introduced. In addition, for larger inserts, replication and movement were affected by expression abilities and remained only in affected cells regions (Carrillo-Trip *et al*., 2006). [

CBI] and expression of foreign genes are considered as non-transgenic application due to their non integration behavior.

The TraitUP™ plant plasmid vector system described herein is a

CBI]

[

CBI] the host machinery.

[**CBI**] infection of tomato (*Solanum lycopersicum*) is harmful and causes major agricultural and economic destruction (Czosnek and Laterrot, 1997; Czosnek et al., 2001). [**CBI**] is transmitted by the [**CBI]** (Brown and Czosnek, 2002) and cannot be mechanically inoculated.

[

CBI]

[

CBI]

Further on, the researchers constructed [

CBI].

The introduction of these unique plasmid constructs into plants was demonstrated via biolistic or root uptake applications (Sela, *et al,* 2007; Sela,*et al,* 2009). Recently, the researchers developed a simple and efficient protocol to introduce [
CBI]

The described TraitUP^{TM} plasmid vectors were used to express the natural anti-fungal [
CBI] is a secondary metabolic produced by a number of rhizospheric bacteria known to serve as biocontrol agents of soil-borne plant pathogens (Weller *et al*., 2002; Spadaro and Gullino, 2005; Lugtenberg and Kamilova, 2009). [**CBI**] has been found long ago, to be active against a wide range of pathogens [

CBI]

This full operon was cloned into [

CBI] result in a product called **TraitUP^{TM}-FB100**. The TraitUP^{TM}-FB100 mediates the [

CBI]

The obtained resistance to root rot disease presents an obvious potential advantage of [
CBI], which have been tested in the past by others for biological control of phytopathogens. In their work; despite their potential antifungal activity, control has been inconsistent, probably due to the diversity of

environmental niches and sensitivity of [**CBI**] to environmental factors such as UV light (Compant *et al*, 2005).

Based on the results above we carried out a series of experiments to test the TraitUP™-FB100 ability to confer resistance against soil borne pathogens. The bioassays performed for *Fusarium crown rot* resistance in tomato plants grown from [**CBI**] have clearly demonstrated the ability of this [**CBI**] TraitUP™ system to confer resistance to treated plants in a short, simple and efficient procedure.

Hence, the **TraitUP™-FB100** product is composed of the [**CBI**] (Figure 4). This product is capable of introducing the entire [**CBI**] protocols following its expression in tomato plants while displaying the trait of conferring resistance against *Fusarium crown rot* in tomato.

Figure 1: Schematic description [**CBI**]

Figure 2: Description of the [**CBI**] operon and its biosynthetic pathway:

[**CBI**] is a secondary metabolite produced by various species of soil bacteria with a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens. The biosynthetic pathway of [**CBI**] has been identified by Kirner *et al*., 1998, and found to be encoded by one operon.

- [

CBI]

3. Description of the TraitUP™-FB100 components:

The product contains [**CBI]**

Their detailed description is as followed:

A. Structure of the [

CBI]

Figure 3: Schematic description of [_

_

CBI]

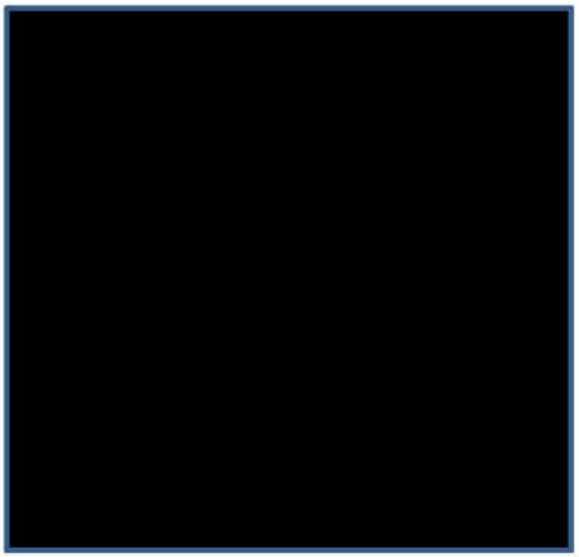
The numbers in bracket represent the size in base pairs.

B. Structure of the [

CBI]

Figure 4: Schematic description of the [

CBI]



[**CBI]**

C. TraitUP™ FB-100 Characteristics:

Comparison of the TraitUP™ FB-100 to its origin [**CBI]**

The general replication and spreading mode of action of TraitUP™ FB-100 system differs from its origin [**CBI]** The TraitUP™ FB-100 plasmids replication is depends solely on the[**CBI]**. The characteristic activity of the major plasmid in comparison to its [**CBI]** origin is summarized in the following table:

Comparison of the TraitUP™ FB-100 to other [**CBI**] systems:

Several [**CBI**] for transient expression have been developed and commercialized. Most of them are for pharmaceutical application. Still, a few [**CBI**] systems are registered and approved for field tests.

TraitUP™ technology differs from the known [**CBI**] for expressing foreign genes in plants. Unlike other [**CBI**], the TraitUP™ FB-100 [**CBI**]

The characteristics of the TraitUP™ FB-100 in comparison to other [**CBI**] systems are summarized in the following table:

Comparison of the TraitUP™ technology to genetically modified (GM) approach:

The **TraitUP™** technology differs from the GM approach as, unlike in GM plant, its effect is [

CBI] These facts may demonstrate the regulatory advantage over modifying plants through GM approach as summarized in the following table:

4. Efficacy report:

Several experiments to test TraitUP™-FB100 efficacy were carried out using tomato seeds of two varieties, [**CBI**] . After treatment with TraitUP™-FB100 the [**CBI**] on 7 or 21 days post germination. The resistance to Crown rot was verified 2-3 weeks post challenging.

The following are the results of two experiments out of several:

Experiment 1: [**CBI**]

One week old seedlings were exposed to [**CBI**] of Fusarium crown rot and diseased plants were detected after 3 weeks. A control was also included which were plants grown from seeds that were primed without the addition of TraitUP-FB100. At concentration of up to [**CBI**] the treated plants were fully resistant while in the control 67% of the plants have the disease symptoms. At [**CBI**] the TraitUP treatment provided resistance to more than 60% of the plants while in control almost 90% of the plants wilted.

Experiment 2: cv. Moneymaker

One week or 21 day old seedlings were exposed to two doses of Fusarium crown rot concentration [**CBI**]. The diseased plants were evaluated after 4 weeks. In both concentrations there was almost 100% resistance in treated plants, while in the control -21 day at [**CBI**] almost 90% of the plants were affected.(figure 5).

Figure 5: Challenging TraitUP-FB100 seed treated Tomato plants with crown rot.

Tomato plants, [**CBI**], were seed treated with TraitUP™-FB-100. 21 days post-sowing the plantlets were challenged with [**CBI**] of Fusarium crown rot. Picture was taken 1 month later:

Left side: control plants (seed primed without TraitUP-FB100).
Right side: plants treated with TraitUP-FB100 [**CBI**]
The plants that exposed to [**CBI**] are shown in the front flat, whereas the one that were exposed to [**CBI**] are seen in the back.

5. Safety

a. Potential of Transmissibility by Insects:

In order to attain [**CBI**], two phenomena are expected to occur:

1. Expression in plants of an [**CBI**]
2. Recognition between [**CBI**]

As indicated above, the [**CBI]** gene encoding for the [**CBI**] is truncated and incomplete. Therefore, it does not represent the native [**CBI**] addition, the TraitUP™ FB-100 plasmids are composed of sections of [**CBI**] rendering its size different and larger than native [**CBI**] Therefore, [**CBI**] is not likely to occur, and indeed no [**CBI**] particles were detected in treated plants expressing these plasmids.
Although the scientific knowledge implies that natural transmission of TraitUP™ by [**CBI**] is not likely to occur, several experiments were carried out to ascertain transmissibility with the [**CBI**]. In these experiments the insects were released for 3 days of acquisition process, onto plants treated by the TraitUP™ and verified by PCR to harbor the TraitUP™ plasmids. Then, they were released onto untreated plantlets for 3 additional days. On average, the number of [**CBI**]per plantlet ranged from 15 to 20. The presence of the TraitUP™ plasmids in the challenged plants was PCR tested 3 weeks after treatment (Figure 6). More than 100 plants were tested and in all those, no evidence for plasmid presence in the plant leaves was found.

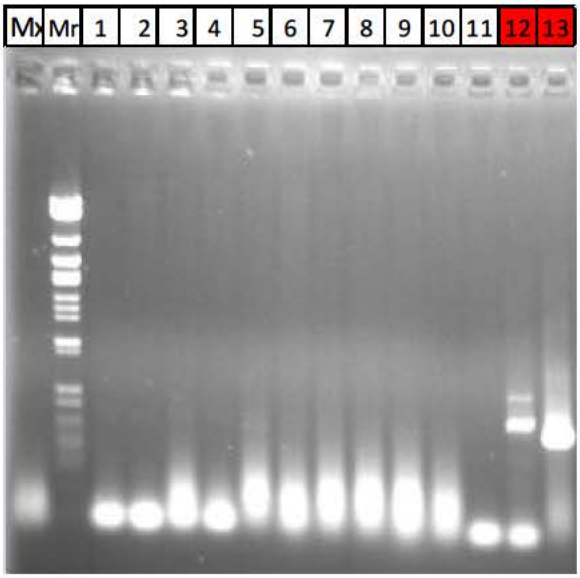
Based on these results and several preliminary results we concluded:

TraitUP™ treatment is most likely NOT Transmitted by [**CBI**]

[**CBI**] is transmitted solely by [**CBI**]. However, there exists a remote possibility that sucking insects, which are not the [**CBI**] natural insect vectors, may acquire the TraitUP™ while feeding on a treated host and then release when feeding on another host plant. This possibility was tested using [**CBI**] were released on TraitUP™ potato treated plants, one week post treatment. The presence of TraitUP™ was PCR tested and verified in 100% of the treated plants (30 plants) but was absent in the [**CBI**]. This experiment was carried out in cooperation with [**CBI**]

Figure 6: PCR results of insect transmissibility experiment.

PCR analysis was performed on tomato seedlings exposed to [**CBI**]pre-fed on plants treated by the TraitUP™ and verified by PCR to harbor the TraitUP™ plasmids. In this experiment the insects were released for 3 days of acquisition process, then, they were released onto untreated plantlets for 3 additional days. In average, the number of [**CBI**] per plantlet ranged from 15 to 20. The PCR analysis was performed 3 weeks after treatment. The PCR amplification was performed with primers for the p1470 plasmid component.



MX: Mix PCR with no DNA
Mr: Marker size [**CBI**]
Lane 1-11: DNA from tomato plants exposed to [**CBI**].
Lane 12: [CBI] infected plant
Lane 13: [CBI] plasmid.

b. Potential of Transmissibility by Mechanical Contact:

To assess the possibility of mechanical transmission, a set of experiments was conducted. In the first set, the ability of transmission by simple contact of leaves was examined. The test was carried out either by contacts between leaves of positively treated plant and untreated plants, or by using scissors to cut leaves (imitating growers handling). In all cases, [**CBI**] (as verified by PCR).

In addition, several experiments to study mechanical delivery of [**CBI**], were conducted as follows: Tomato seedlings were rubbed with solution containing [**CBI**]. PCR analysis reveals no evidence of [**CBI**] presence in all acceptor plants tested (>50).

Furthermore, a set of experiments were conducted using [**CBI**] (not usually used by growers in agricultural practice) added to the mechanical delivery protocol. In these experiments several plasmid solutions were tested as follows:

1. [

5. **CBI**]

Results negate transfer of the TraitUP™ plasmids into plant tissues via mechanical means when no abrasive material was added (Figure 7). However, when abrasive material was added the results were inconclusive and may indicate possible transfer in low efficiency.

Based on these results we concluded:

TraitUP™ treatment is most likely NOT Transmitted by mechanical means.

c. Potential Transmissibility by Plant Residues:

A possible risk may arise from soils contaminated with residues of plants grown from [**CBI**] with TraitUP™. In order to ascertain the risk, the following experiment was performed: Leaves of plants tested positive for presences of TraitUP™ plasmids were fragmented with scissors, then mixed with soil and left to decompose for two days. Young untreated tomato seedlings were planted in the treated soil and samples were taken 4 weeks after planting, for PCR analysis.
PCR tests, performed on 20 plants, did not reveal uptake of TraitUP™ plasmids from the treated soil. Therefore, we concluded that the possibility of unintended passage of TraitUP™ to planted seedlings from soil containing plant residues is low.

Figure 7: PCR results of Mechanical delivery experiment.

PCR analysis was performed on tomato plants which leaves were:

A) [**CBI**] of a plant treated by the TraitUP™ and verified by PCR to harbor the TraitUP™ plasmid (positive) and [**CBI**].

B) cut with [**CBI**] that were used to cut leaves of the positive plant.

The PCR analysis was performed 4 weeks after treatment. The PCR amplification was performed with primers for the p1470 plasmid component.

Mx: Mix PCR with no DNA.
Mr: Size marker (MassRuler DNA ladder-Fermentas).
Lane 2-13: DNA from treated plants rubbed with [**CBI**].
Lane 14: DNA extracted from the TraitUP-FB100 treated plant.
Lane 15: Control plant.
Lane 16: [**CBI**]

B

Mx: Mix PCR with no DNA. **Mr**: Size marker [**CBI**]Lane 2: control untreated plant. **Lane 3-22:** DNA from treated plants cut with scissors. **Lane 23:** DNA extracted [**CBI**] infected plant. **Lane 24:** [**CBI**]

d. Heritability via Seeds

Seeds were collected from various self pollinated treated plants, verified to carry TraitUP™ plasmids (Verification done by PCR analysis on leaves), and the progeny (S1) plants were grown and tested for **TraitUP™** plasmids presence.

In 110 tomato and 22 pepper progeny plants (S1) tested so far, no evidence for TraitUP™ plasmids presence was found, by mean of PCR analysis (Parent plants were harboring the following plasmids: [
CBI]

Figure 8: PCR results of heritability via seeds.

PCR analysis was performed on S1 seedlings emerged from seeds collected from plants that were seed-treated with TraitUP™-FB100 or by [
CBI], and verified to be positive by PCR.
The list of tested S1 seedlings is as follows:

Source plant	Treatment	Number of S1 seedlings	Positive PCR results
Tomato #1	[	20	0/20
Tomato #2		20	0/20
Tomato #3		20	0/20
Tomato #4		20	0/20
Tomato #5	**CBI]**	20	0/20

The following is a PCR analysis result performed on 3 weeks old seedlings of S1 progeny of tomato plant No #3. The PCR amplification was performed with primers for the [**CBI**] component.

MX: Mix PCR with no DNA
Mr: Marker [**CBI]**
Lane 1-10: DNA from S1 seedlings of Tomato plant #3
Lane 11: parent plant
Lane 12: [**CBI]**
Lane 13: [**CBI]**

Based on these results we concluded:

TraitUP™ treatment is most likely not heritable via seeds.

e. Presence in Pollen

Even though the data obtained so far negate passage of TraitUP™ through progeny seeds, we decided to ascertain the possible presence of the TraitUP™ plasmids in pollen of positive treated plants.

DNA was extracted from pollen taken from flowers of 4 different plants that have been shown to harbor [**CBI**] (the component of the TraitUP™-FB100) by PCR and were tested biologically using the *Fusarium Crown Rot* bioassay.

DNA was assayed using PCR with primers targeting both genomic and [**CBI**] sequence. Results reveal the absence of the TraitUP™ plasmids in the tested samples, as can be seen in figure 9:

Figure 9: PCR analysis of pollen from TraitUP™-FB100 seed-treated plants.
PCR analysis was performed on pollen collected from plants that were seed-treated with TraitUP™-FB100 and verified to be positive by PCR and bioassay tests.
The DNA extracted from the pollen was amplified using specific primers for the [**CBI**] introduced by the TraitUP-FB100 treatment (A). As a control assay, PCR of native gene was performed on the same DNA extract using primers to amplify the endogenous tomato gene (B).

Lane 1: size markers (1kb Ladder).
Lanes 2-5: DNA extracted from pollen of the [**CBI**] carrying plants.
Lane6: spacer
Lanes 7 & 8: DNA extracted from leaves of [**CBI**] carrying plants.
Lane 9: PCR mix.
Lane 10: Positive control (plasmid).

Lane 1: Size markers (1kb Ladder).
Lanes 2-5: DNA was extracted from pollen of [**CBI**] positive plants.
Lane 6: DNA was extracted from pollen of untreated tomato.
Lane 7: [**CBI**].

Based on these results we concluded:
TraitUP™ plasmids are most likely NOT present in pollen.

f. Allergy & Toxicological data

Each of the **TraitUP^{TM}-FB100** components were searched by bioinformatics means for potential allergy risk. The analysis was done by the Bioinformatics Core Facility at Ben-Gurion University of the Negev, Israel (BGU).

The [**CBI**] which have no known allergy effect. Nevertheless, a bioinformatics test was performed on the full sequence of this construct to disproof possible risk as a result of unintended protein formation.

The [**CBI**.] In the process, [CBI] that act in sequence to catalyze the bio-active secondary metabolite called [**CBI.]**

The sequences of the [**CBI**] were translated to possible ORF (Sense and antisense) and the deduced proteins were submitted to sequence similarity search against "Allergen Database for Food Safety" (ADFS). ADSF is a web-based database of allergenic proteins relevant to food safety, which is as a project of the Division of Novel Foods and Immunochemistry of the National Institute of Health Sciences.

The results summarized, in the BGU reports, negates the possibility of similarity with high probability to known allergenic proteins or peptides (Appendix A).

Hence, we concluded the low probability to allergic risks.

Furthermore, as [**CBI**] is expected to be produced by the plants we detected the expressed quantities of [**CBI**] in roots, leaf and fruit organs by HPLC means (Figure 10). No [**CBI**] was detected in the fruit flesh, however [**CBI**] was detected in roots and leaves as well.

From the HPLC results we calculated the [**CBI**] quantities in tomato leaves to be: **1ug** [**CBI**] **per 1 gram** fresh tissue leaves.

To estimate the potential risk of the [**CBI**] expression in plant tissues for animal risk we compared it to LD50 value of [**CBI**]
Literature search reveal studies on [**CBI**] LD_{50} in animals which reported to have the following values: (according to Arima *et al.*,US patent No. 3,597,325, (1971)):

- LD_{50} value for mice: **500 mg/kg**
- No effect to mice fed **30 mg/kg**, daily for 3 months.

Based on these calculations and assumptions we consider the TraitUP^{TM}-FB100 treated plants do not pose any environmental risk to field animals.

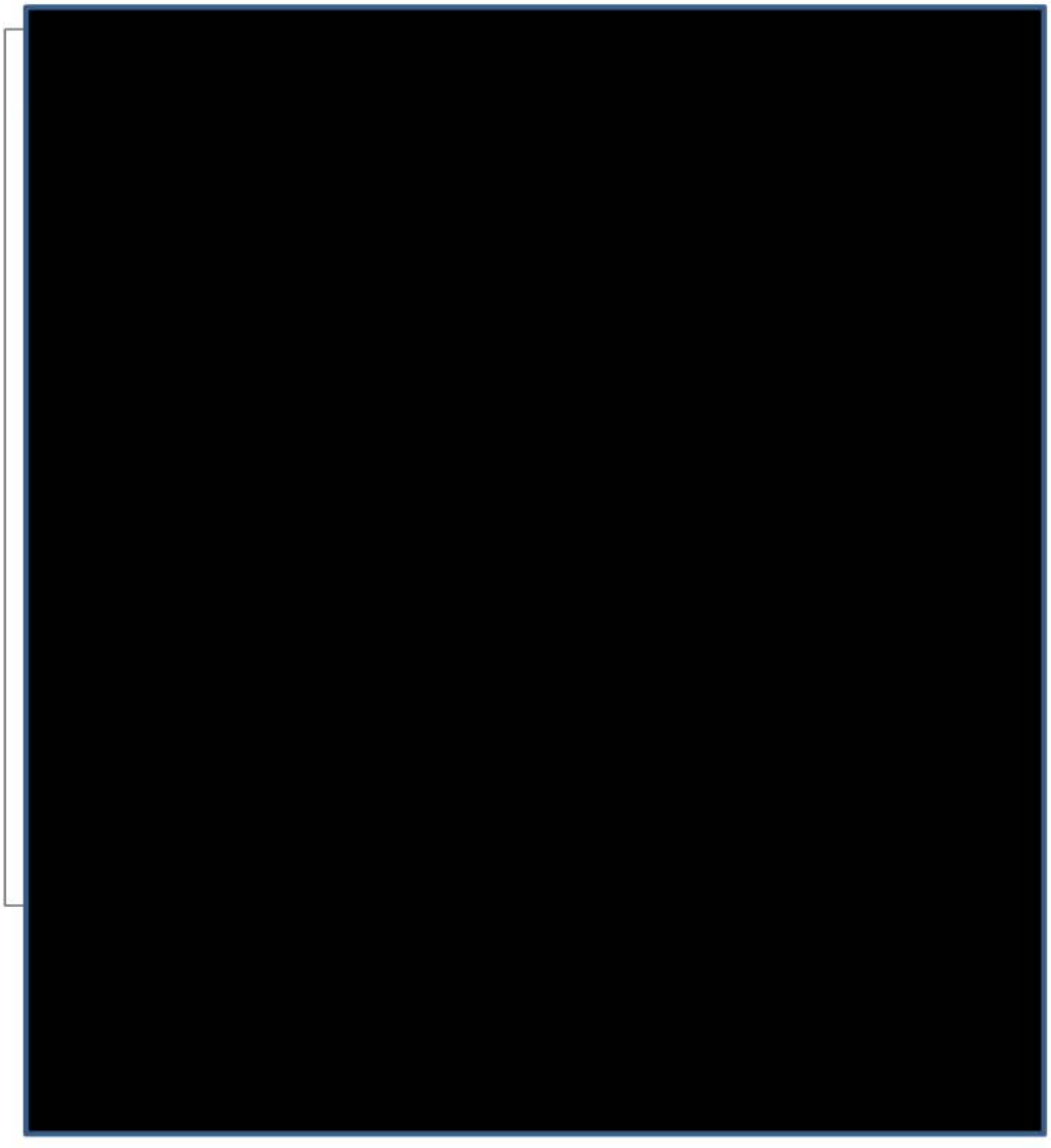
Figure 10: HPLC analyses of [**CBI**] produced in TraitUP-FB100 treated tomato plants.

[**CBI]**

Plant tissue was extracted and analysis was performed by HPLC equipped with a [**CBI]**. Each plant extract sample is equivalent to [**CBI]** in the initial plant (starting from 4 g plant tissue).

HPLC elution profiles of: **[**

CBI]



6. References:

[

CBI]

[

CBI]

[

CBI]

Carrillo-Tripp J, Shimada-Beltran H, Riviera-Bustamante R (2006) Use of geminiviral vectors for functional genomics. Curr Opin Plant Biol 9:209–215

Chapman S, Kavanagh T, Baulcomb D (1992) Potato virus X as a vector for gene expression in plants. Plant J 2: 549–557

[

CBI]

Compant S, Duffy B, Nowak J, Cle´ment C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl Environ Microbiol 71:4951–4959

[

CBI]

[

CBI]

[

CBI]

[

CBI]

Gleba Y, Marillonnet S, Klimyuk V (2004) Engineering viral expression vectors for plants: the ‘full virus’ and the ‘deconstructed virus’ strategies. Curr Opin Plant Biol 7: 182–188

[

CBI]

Hanley-Bowdoin L, Settlage SB, Orozco BM, Nager S, Robertson D (2000) Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit Rev Biochem Mol Biol 35:105–140

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science 227: 1229–1231

[

CBI]

Klein TM, Fromm M, Weissinger A, Thomas D, Schaaf S, Sletten M, Sanford JC (1987). High velocity microprojectiles for delivering nucleic acids into living cells. Nature 327: 70–73

Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63: 541–556

Mozes-Koch R, Gover O, Tanne E, Peretz Y, Maori E, Chernin L, and Sela I (2012). Expression of an Entire Bacterial Operon in Plants. Plant Physiology158:1883–1892

Peretz Y, Mozes-Koch R,Akad F, Tanne E, Czosnek H, Sela I (2007) A universal expression/silencing vector in plants. Plant Physiol 145: 1251–1263

Sela, I, Mozes-Koch R, Peretz Y, Huet H. PLANT EXPRESSION CONSTRUCTS AND METHODS OF UTILIZING SAME Patent application WO2007/141790. **06-07-2007**

Sela, I, Peretz Y, Mozes-Koch R. PLANT EXPRESSION CONSTRUCTS COMPRISING AND USES THEREOF. Patent application WO2010/004561**. 08-07-2009**

Sela, I, Rabinowitch, H.D., Gover, O. Introducing DNA into plant cells. Patent application WO2011/001434_A1. **2011**

Spadaro D, Gullino ML (2005). Improving the efficacy of biocontrol agents against soilborne pathogens. Crop Prot 24: 601–613 Stanley, 1985

Weller DM, Raaijmakers JM, Gardener BBM, Thomashow LS (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev Phytopathol 40: 309–348

Stanley J (1985). The molecular biology of geminiviruses. Adv Virus Res 30:139–177