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(54) **ALTERNATIVE TRANSIT PEPTIDES TO INCREASE PLANT TRANSFORMATION EFFICIENCY**

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CPC ***C12N 15/8274*** (2013.01); ***C07K 14/415*** (2013.01); *C07K 2319/60* (2013.01)

(57) **ABSTRACT**

The present invention provides DNA constructs for producing transgenic plants. The DNA constructs encode a transit peptide that is targeted to the mitochondria and the chloroplasts within a cell. Also provided are plant cells and vectors comprising the DNA constructs. Methods of generating a transgenic plant using the vectors and constructs provided herein and methods of targeting a protein to mitochondria or chloroplasts in a cell.

Specification includes a Sequence Listing.

Figure 1

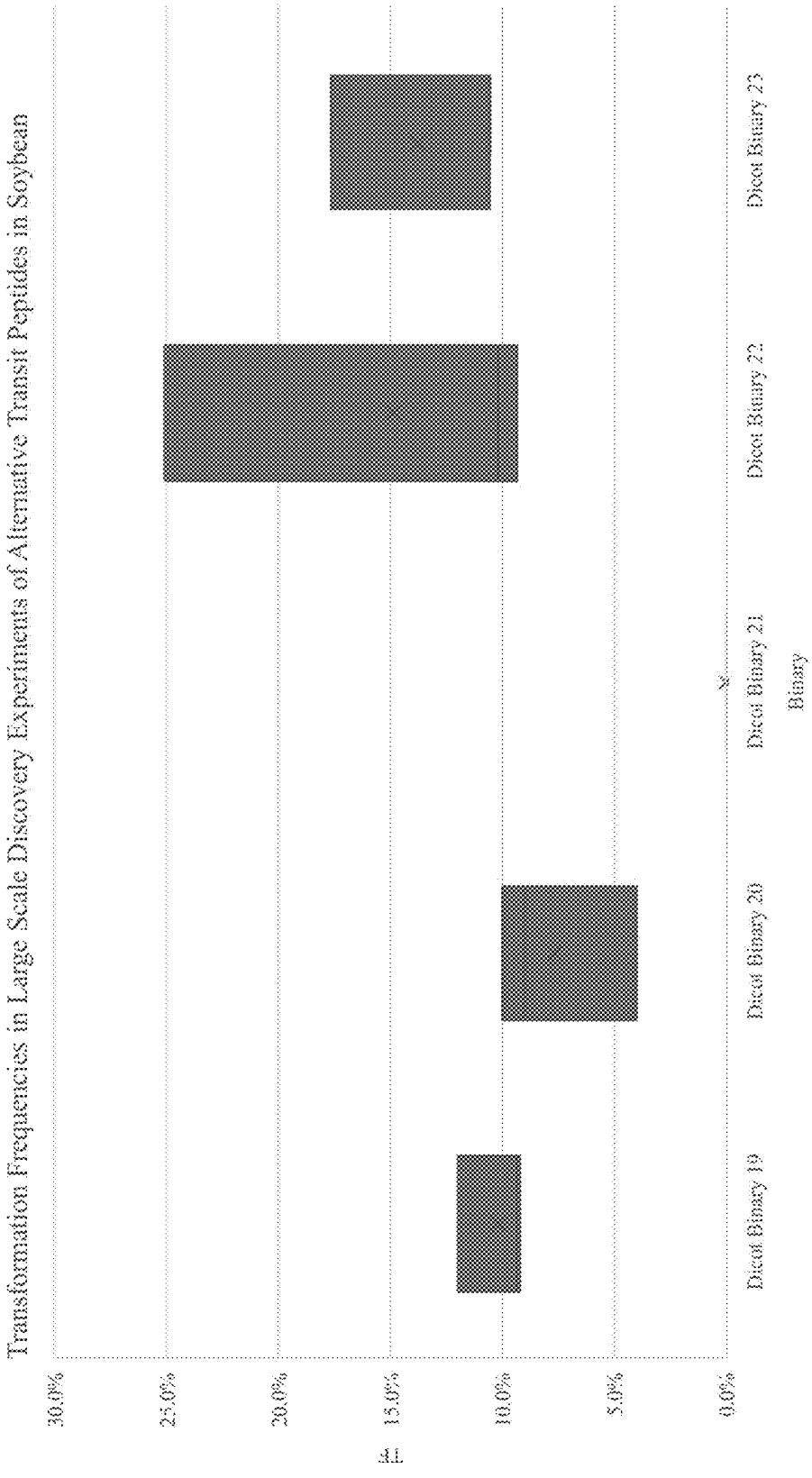
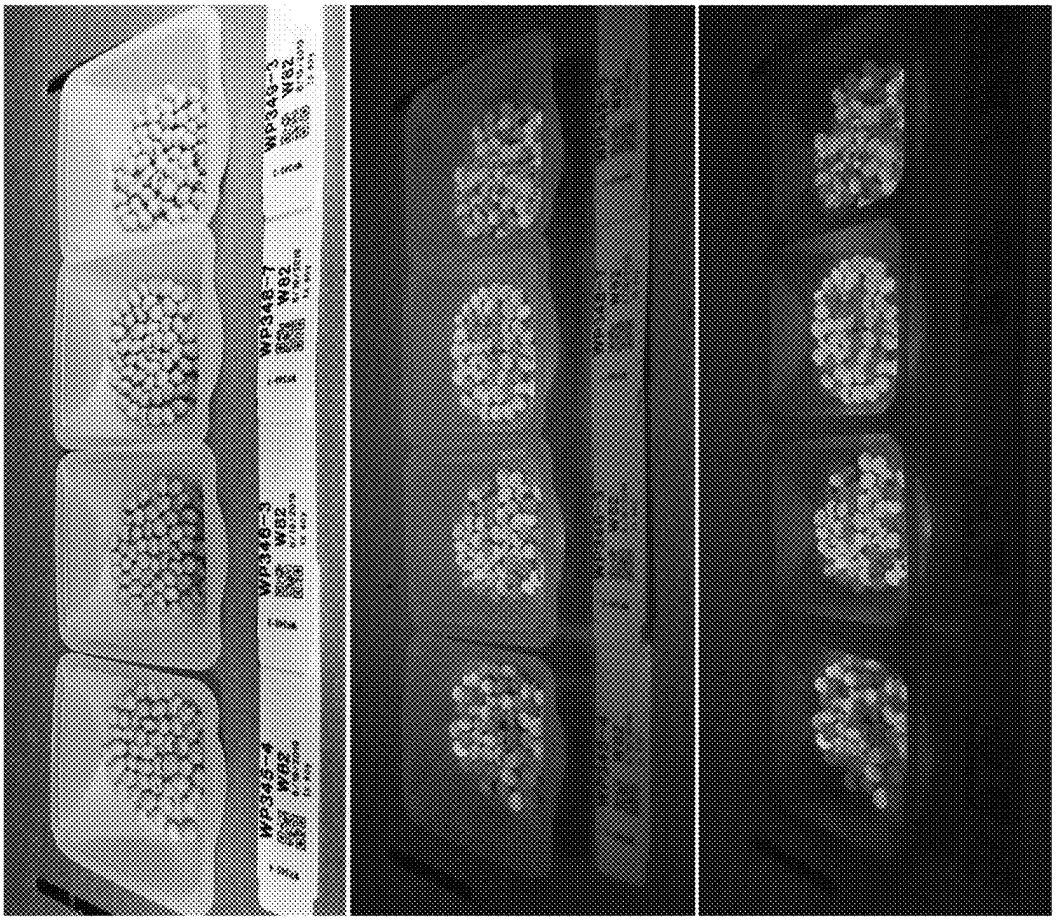

Transformation Frequencies in Large Scale Discovery Experiments of Alternative Transit Peptides in Soybean
30.0%
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
TF
Dicot Binary 19
Dicot Binary 20
Dicot Binary 21
Dicot Binary 22
Dicot Binary 23
Binary

Figure 2



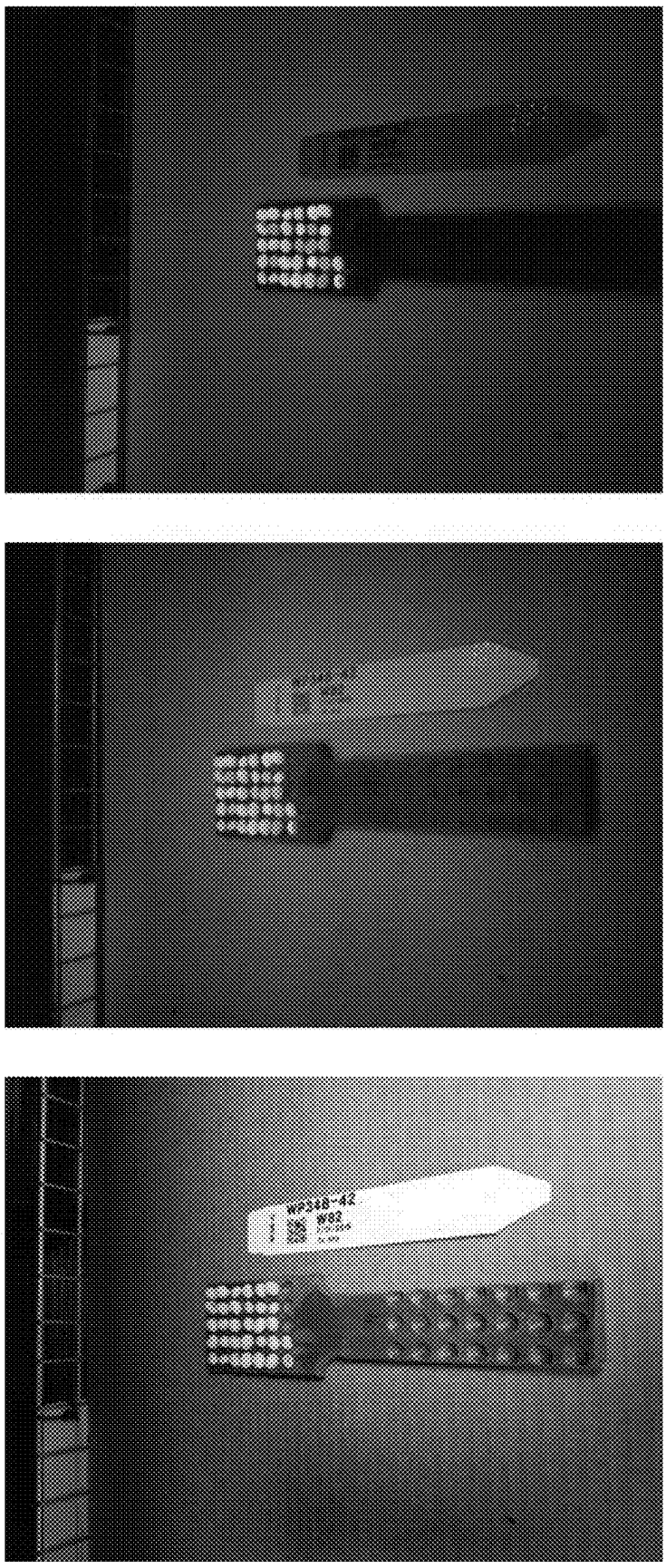


Figure 3

Figure 4

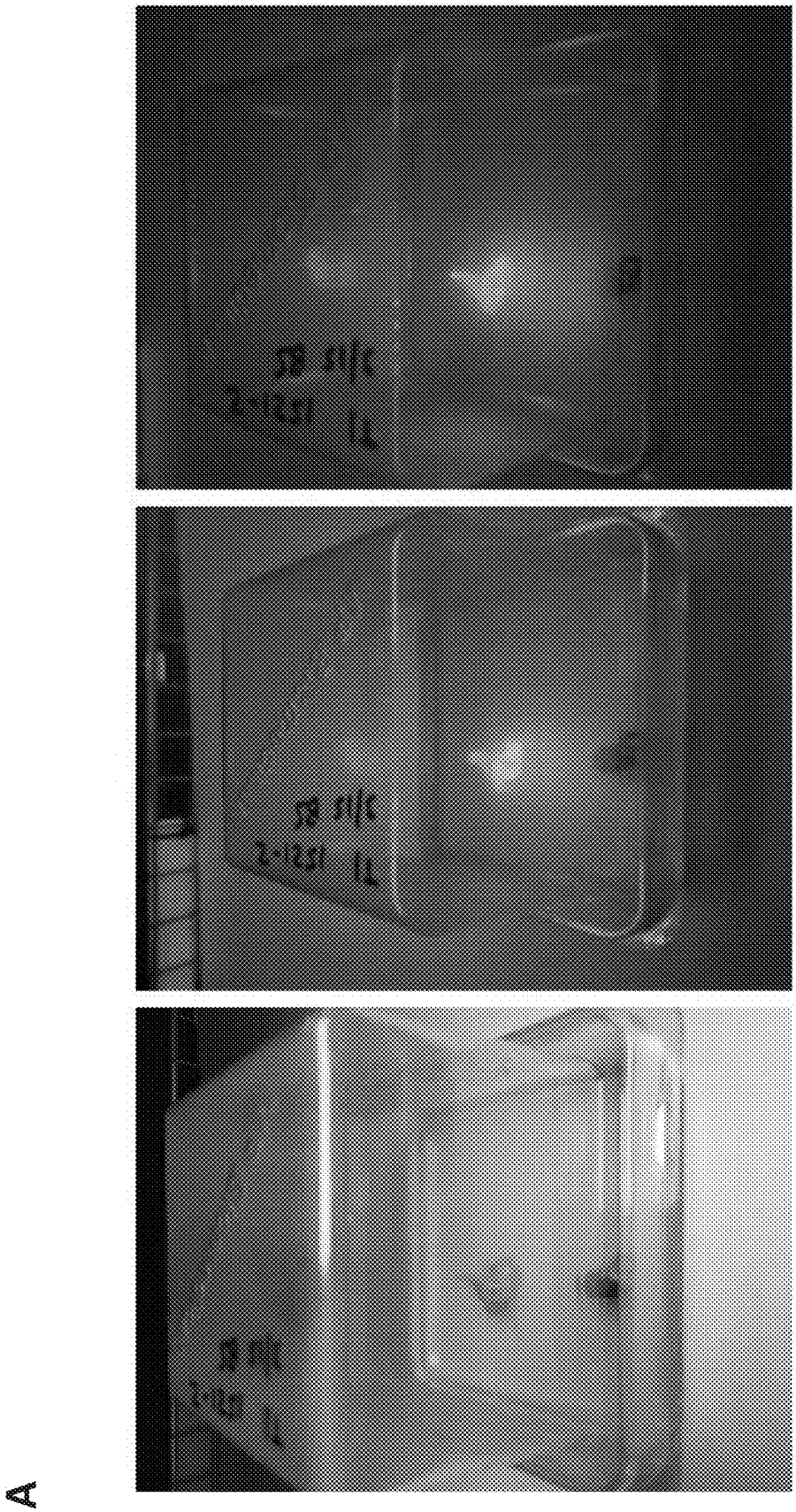


Figure 4 (continued)

B

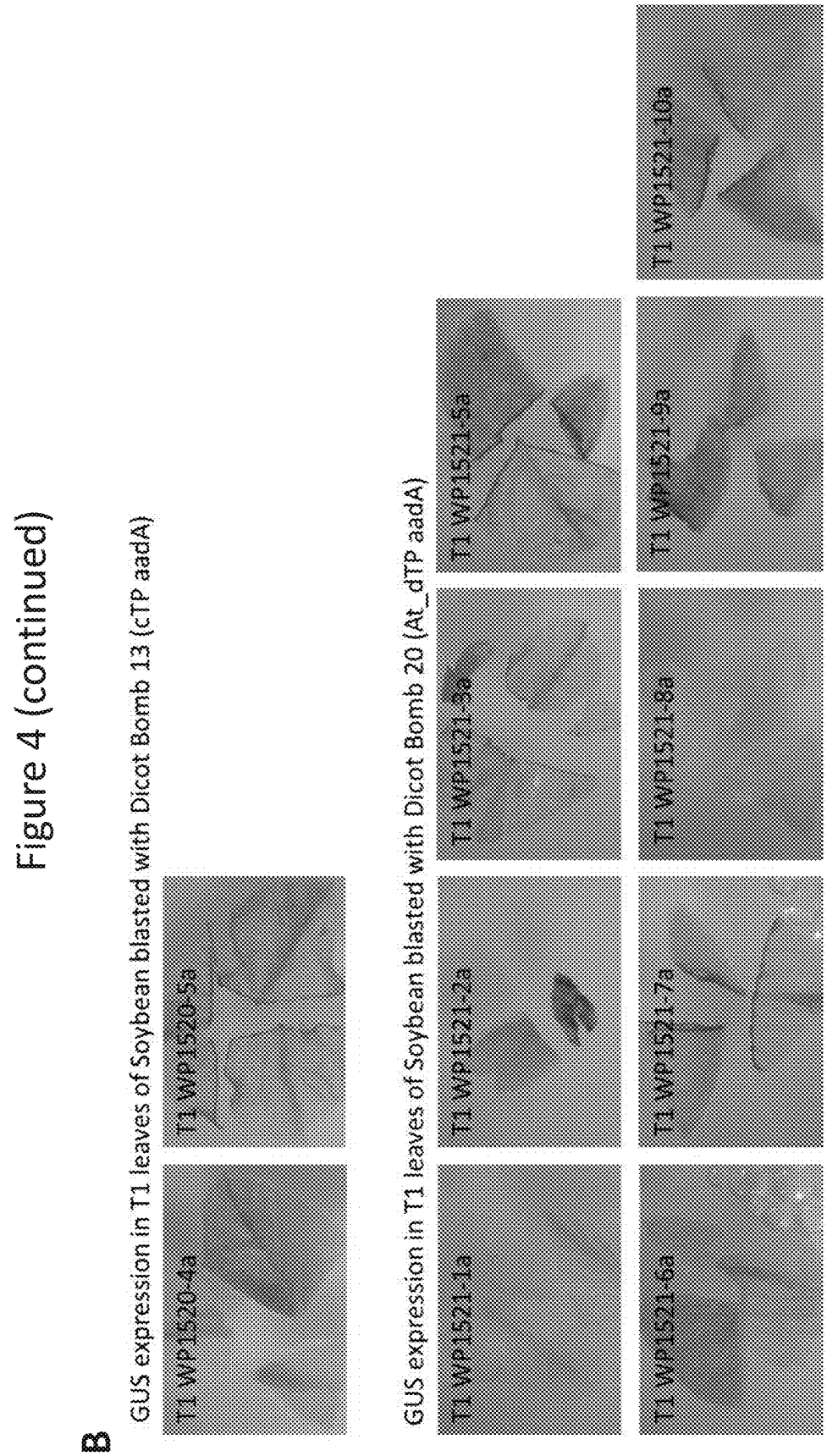


Figure 5

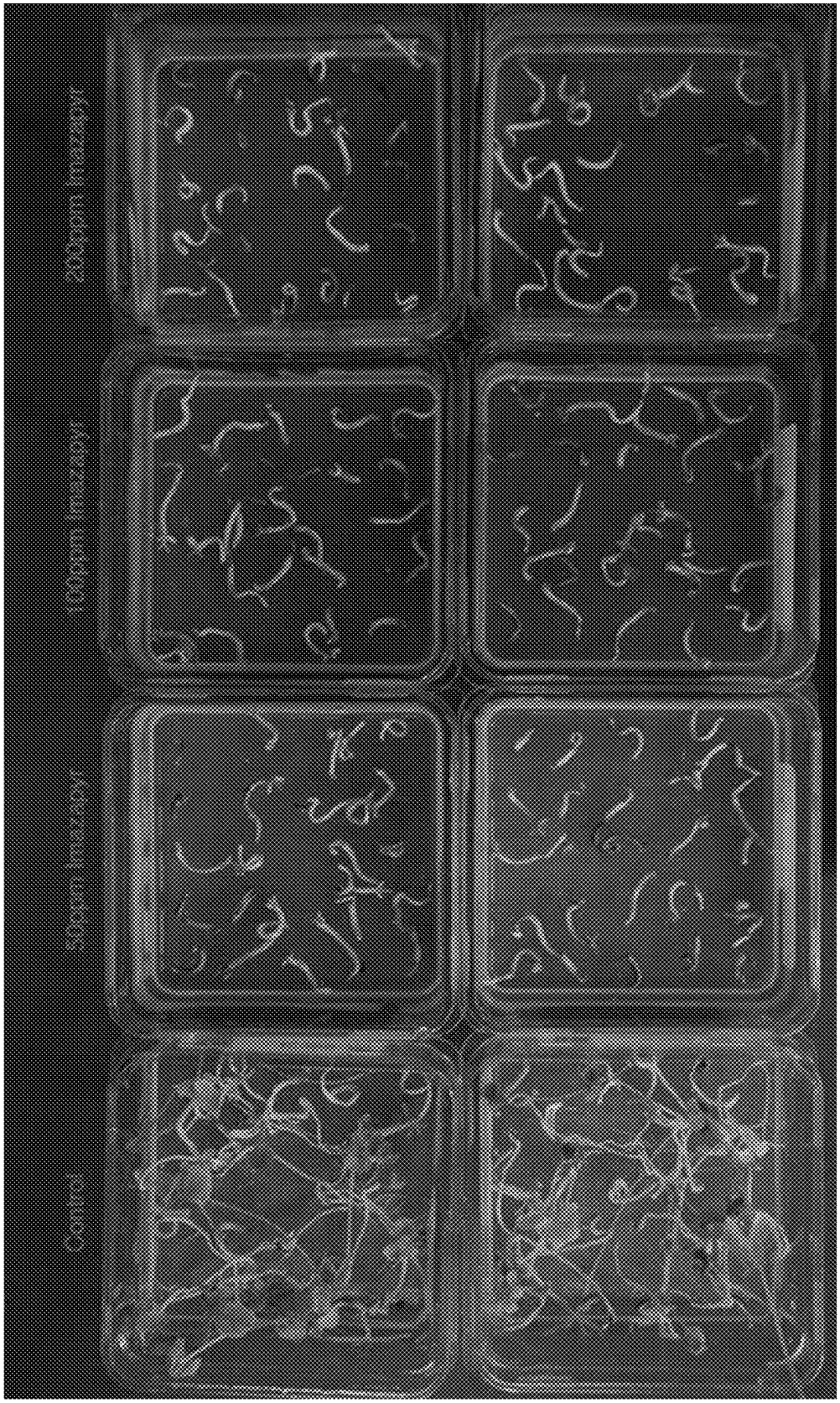
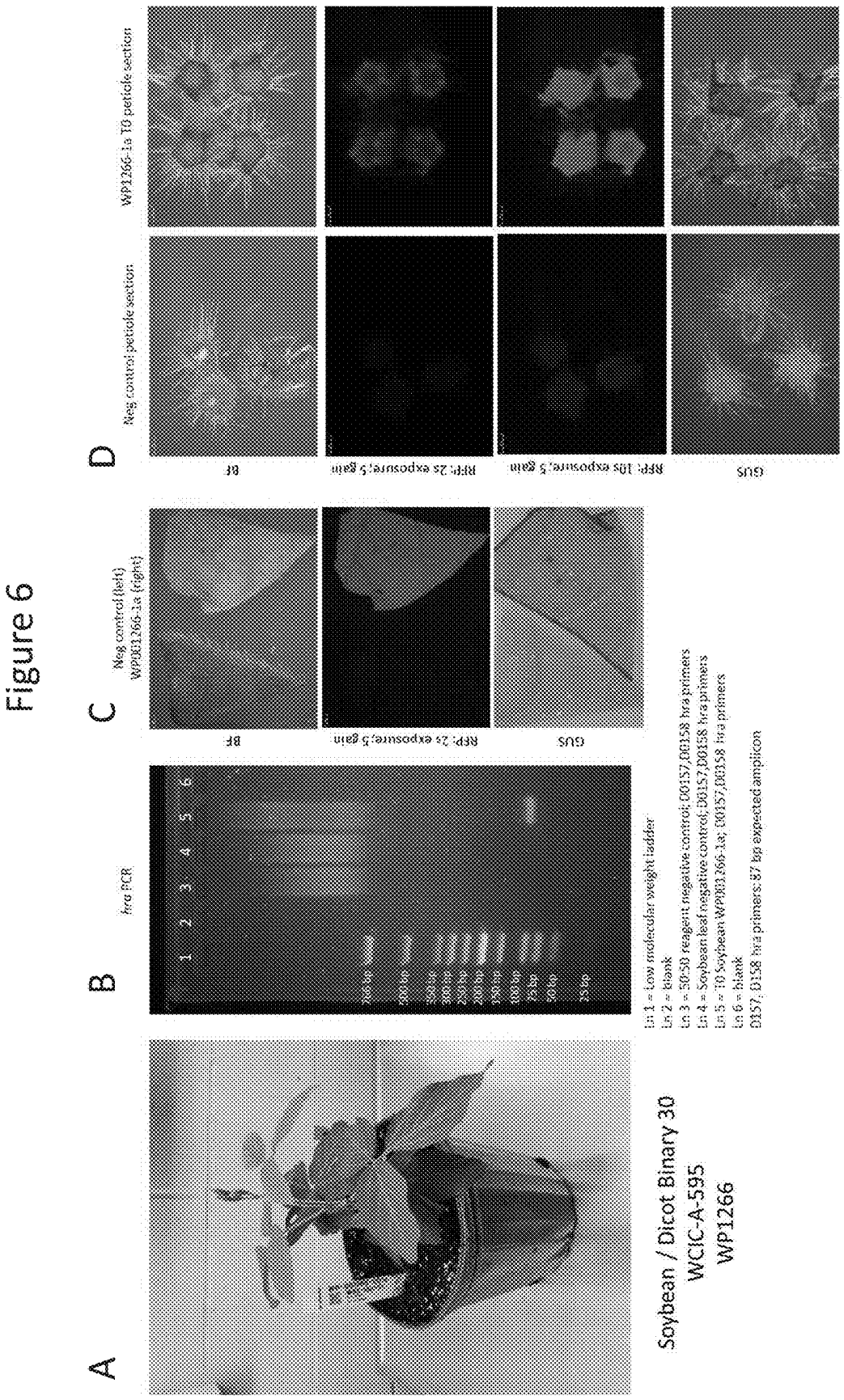


Figure 6


A
Soybean / Dicot Binary 30
WCIC-A-595
WP1266
B
hra PCR
1 2 3 4 5 6
750 bp
500 bp
400 bp
300 bp
200 bp
100 bp
75 bp
50 bp
25 bp
Ln 1 = Low molecular weight ladder
Ln 2 = blank
Ln 3 = 50-50 reagent negative control; D0157,D0158 hra primers
Ln 4 = Soybean leaf negative control; D0157,D0158 hra primers
Ln 5 = T0 Soybean WP003266-1a; D0157,D0158 hra primers
Ln 6 = blank
D157, D158 hra primers: 87 bp expected amplicon
C
Neg control (left)
WP003266-1a (right)
BF
RFP: 2s exposure; 5 gain
GUS
D
Neg control petiole section
WP1266-1a T0 petiole section
BF
RFP: 2s exposure; 5 gain
RFP: 10s exposure; 5 gain
GUS

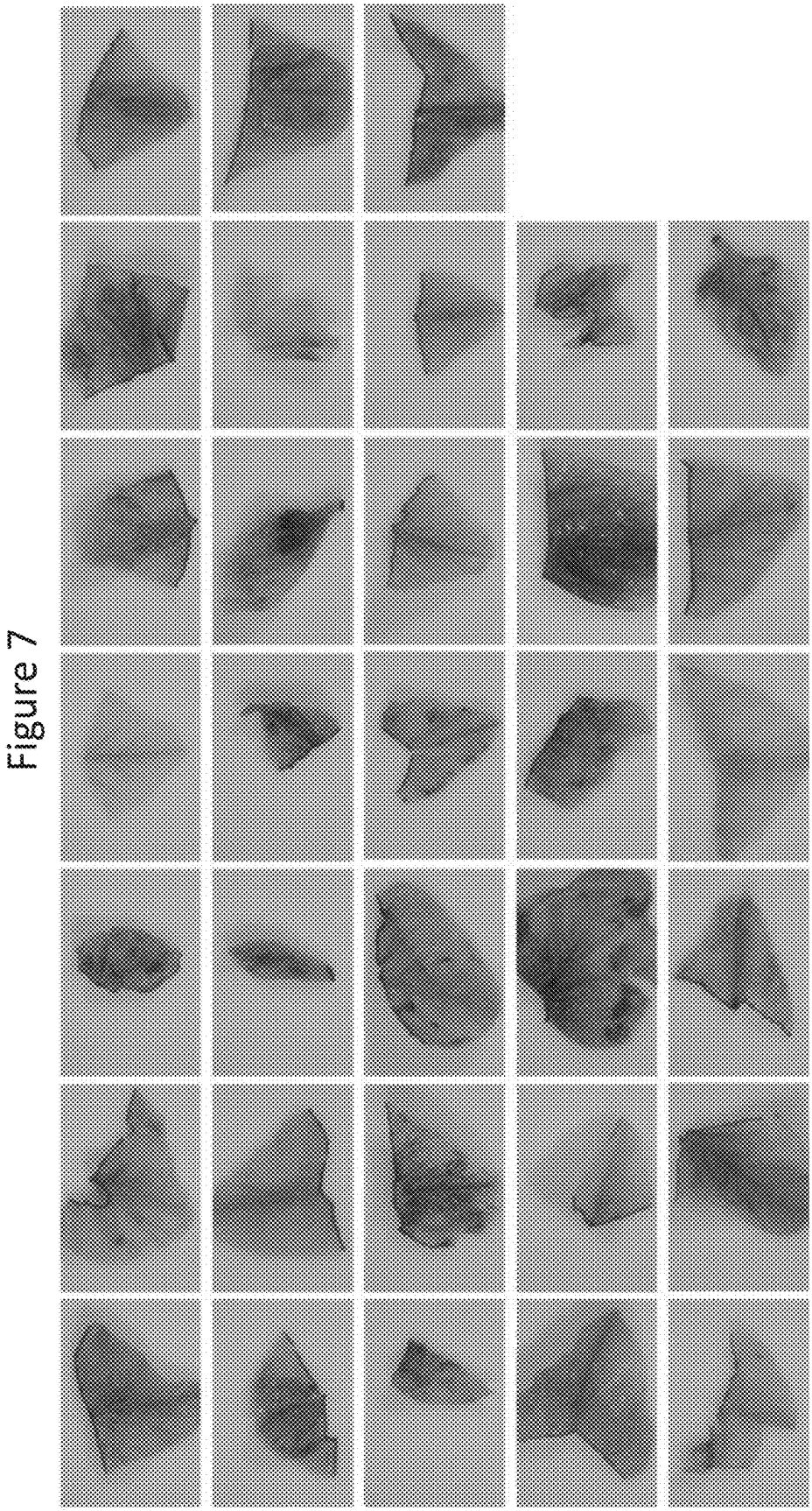
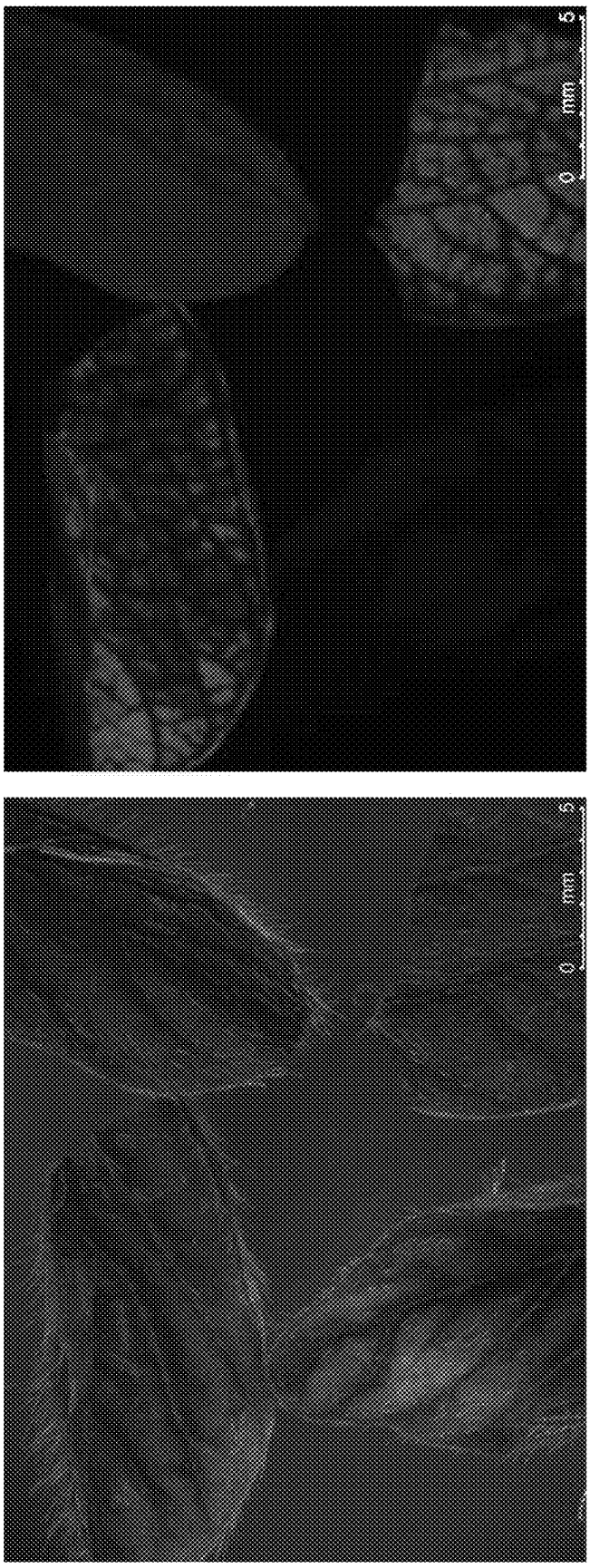


Figure 7

Figure 8



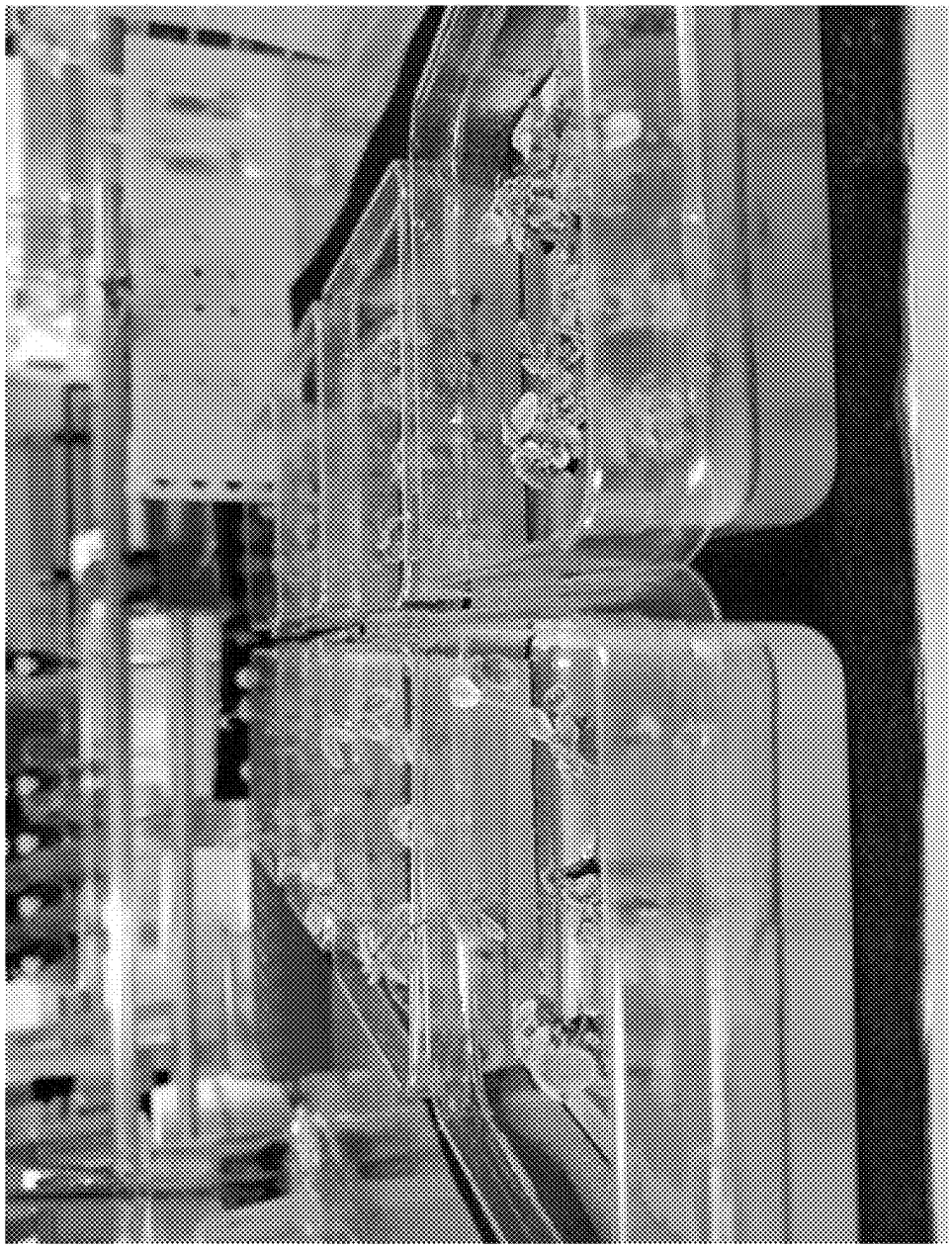


Figure 9

A

Figure 9 (continued)

B

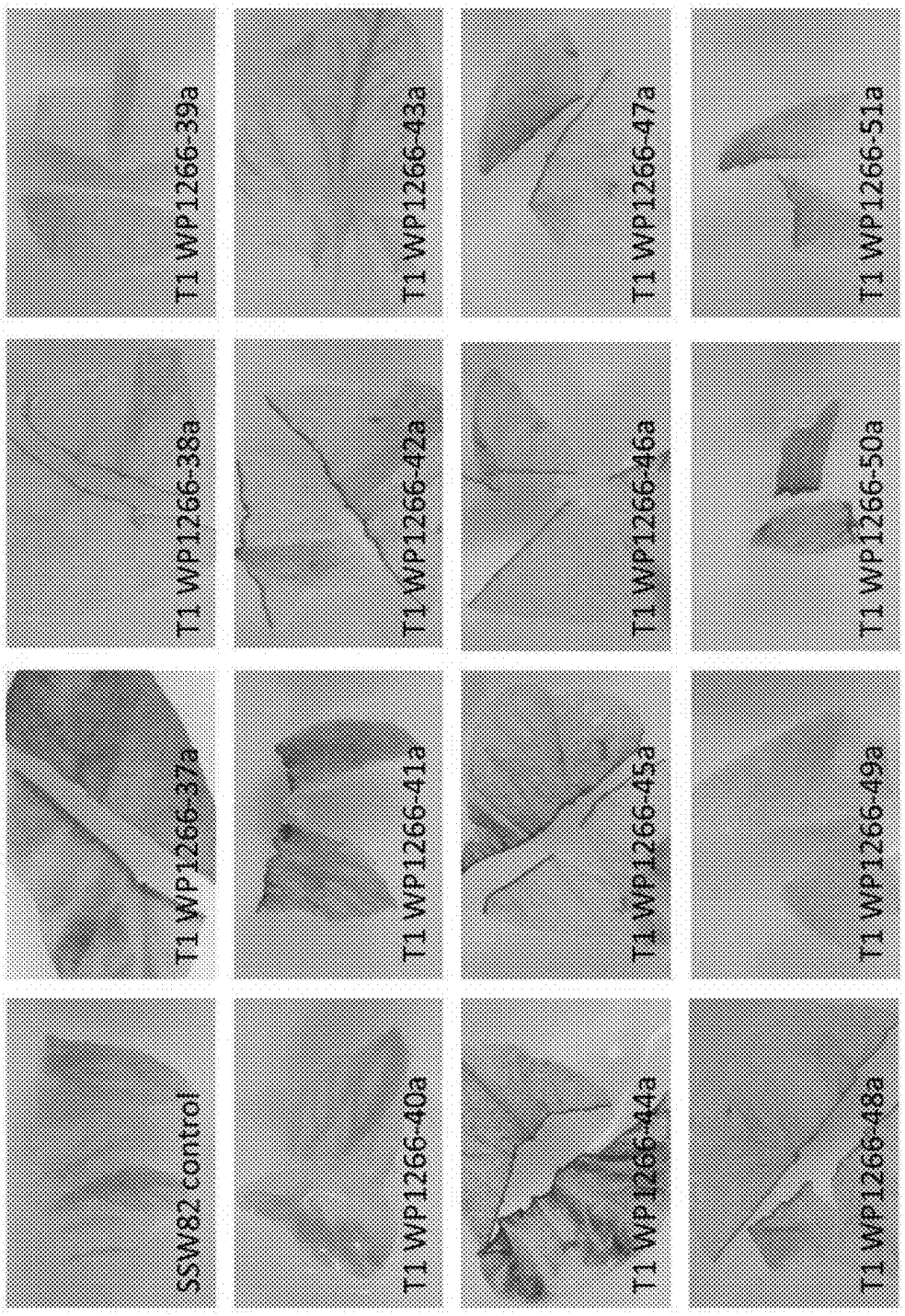
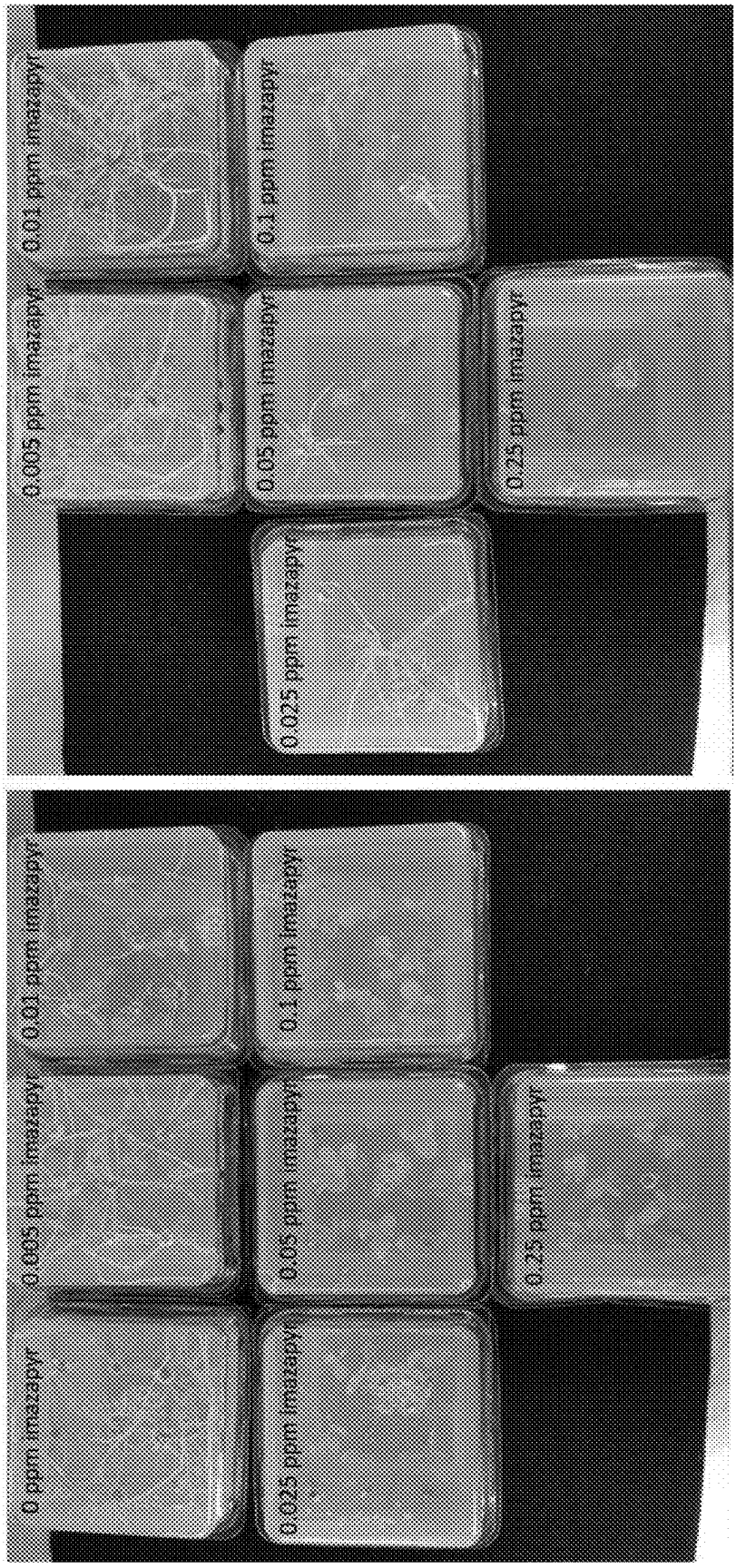


Figure 10


Positive control (segregating) T1 WP1266-1a Soybean
0 ppm imazapyr
0.005 ppm imazapyr
0.01 ppm imazapyr
0.025 ppm imazapyr
0.05 ppm imazapyr
0.1 ppm imazapyr
0.25 ppm imazapyr
0.5 ppm imazapyr
Negative control SW82 Soybean
0 ppm imazapyr
0.005 ppm imazapyr
0.01 ppm imazapyr
0.025 ppm imazapyr
0.05 ppm imazapyr
0.1 ppm imazapyr
0.25 ppm imazapyr
0.5 ppm imazapyr

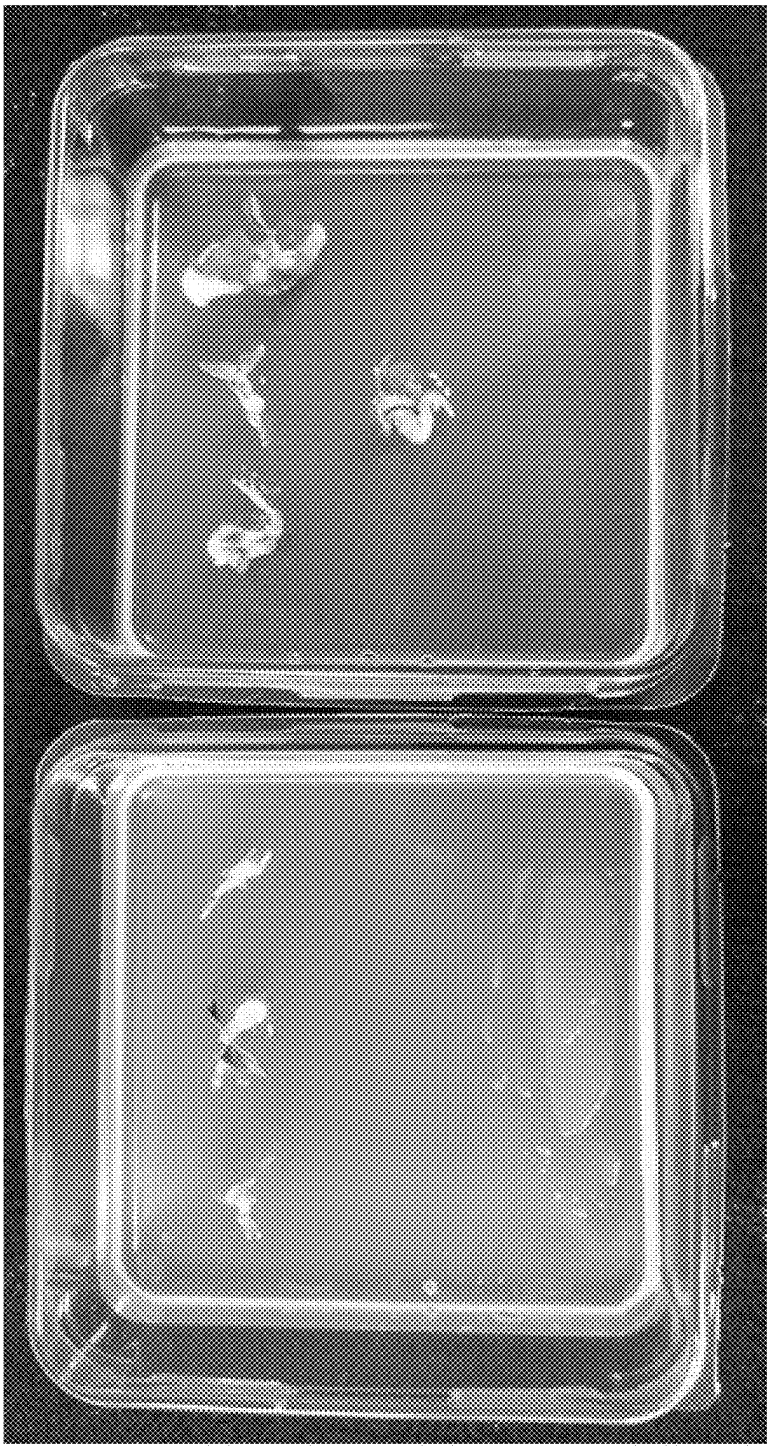


Figure 11

Figure 12

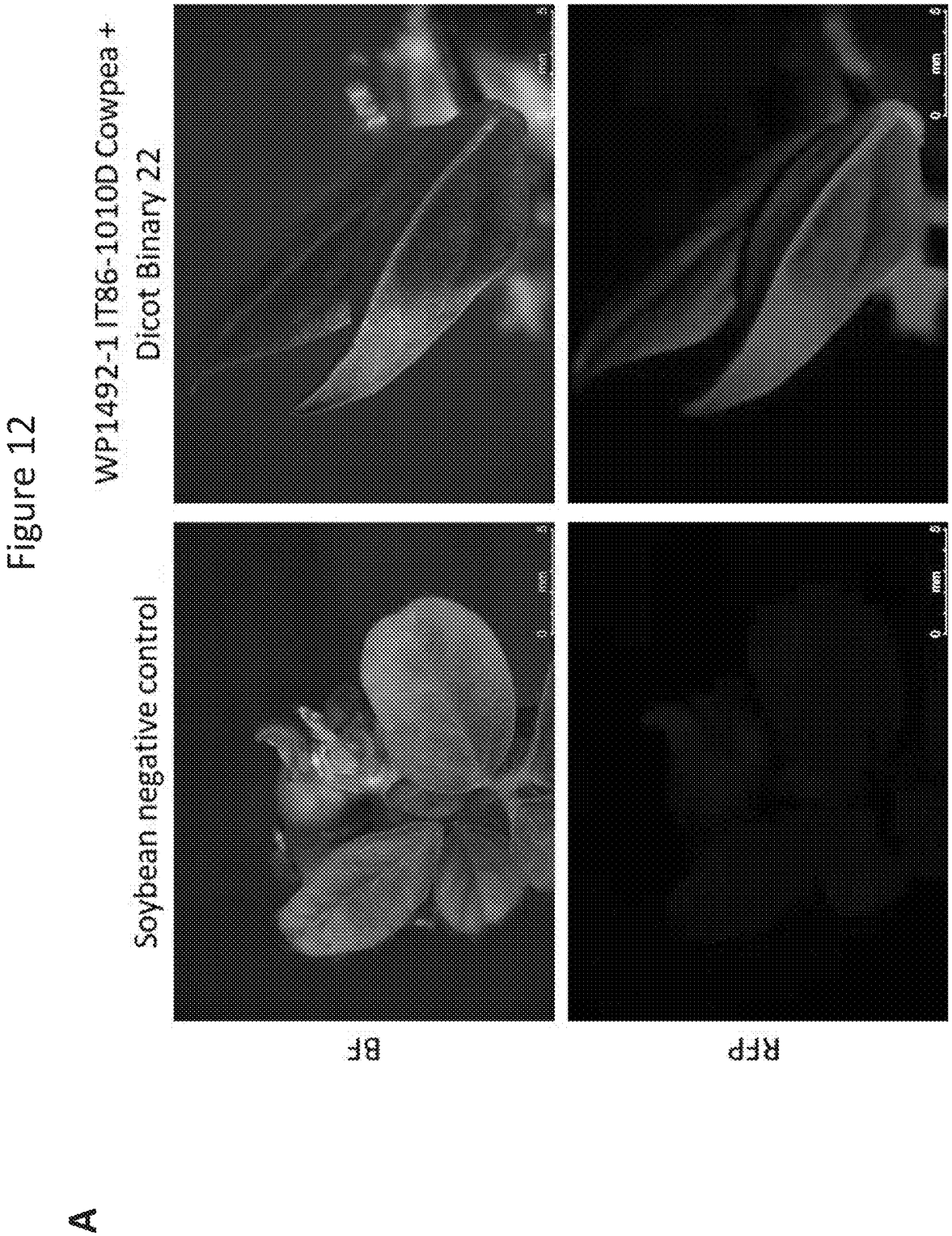
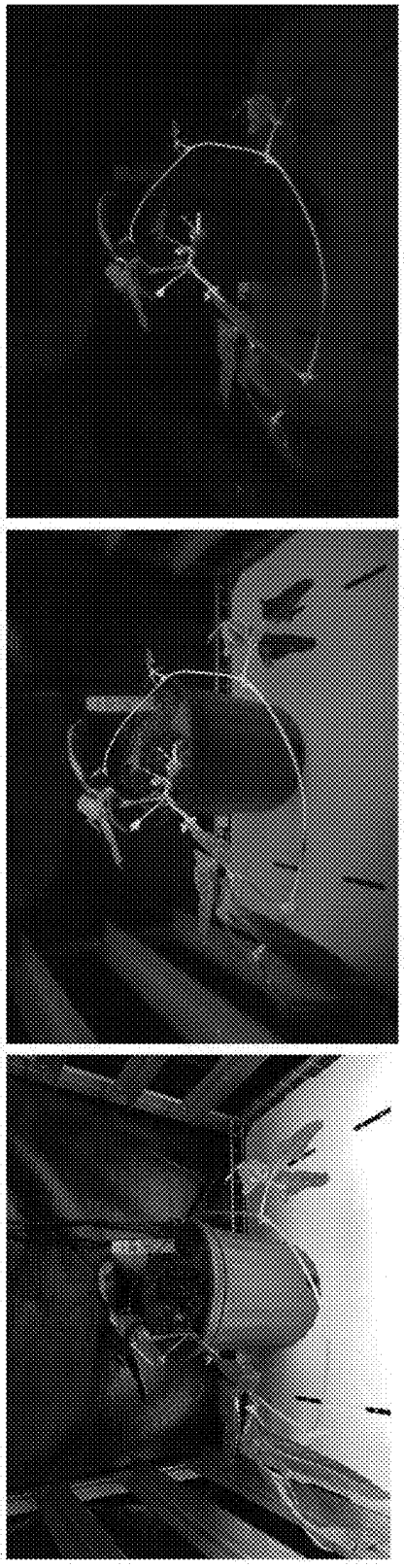

A
WP1492-1 IT86-1010D Cowpea +
Dicot Binary 22
Soybean negative control
BF
RFP

Figure 12 (continued)

B

T0 WP1492-1a whole plant imager: plant appears uniformly tdTomato positive (little chimerism)



T0 WP1492-2a whole plant imager: plant appears chimeric tdTomato positive in sectors of plant

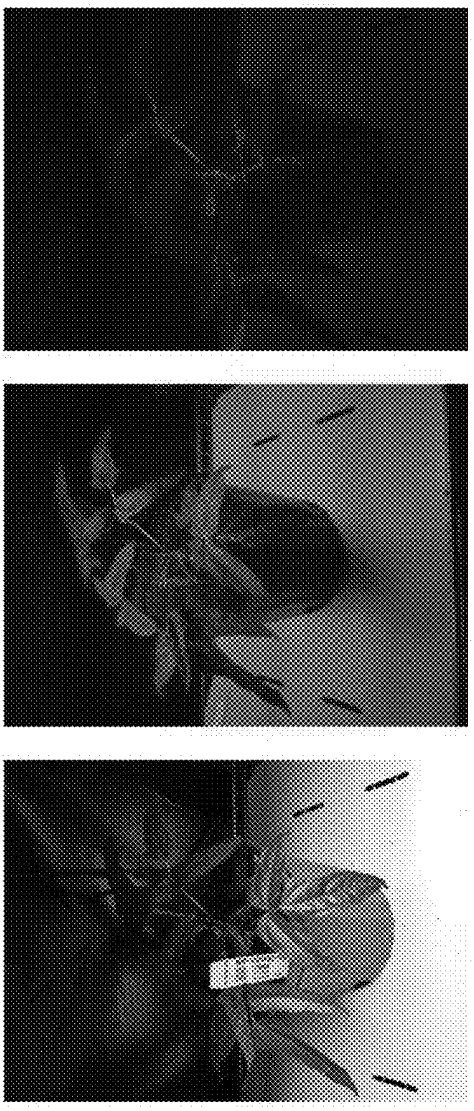


Figure 13

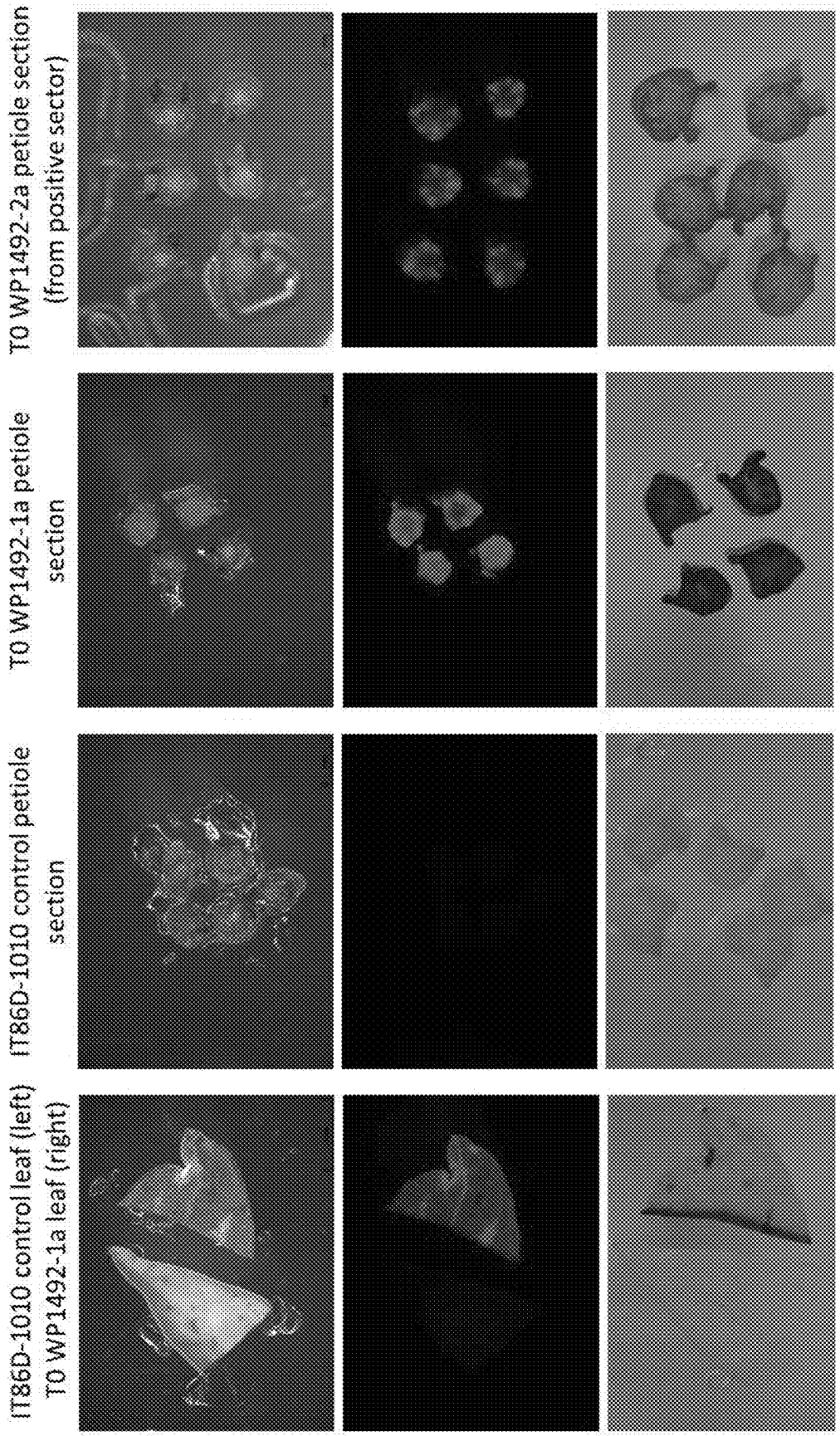

IT86D-1010 control leaf (left)
T0 WP1492-1a leaf (right)
IT86D-1010 control petiole section
T0 WP1492-1a petiole section
T0 WP1492-2a petiole section (from positive sector)

Figure 14

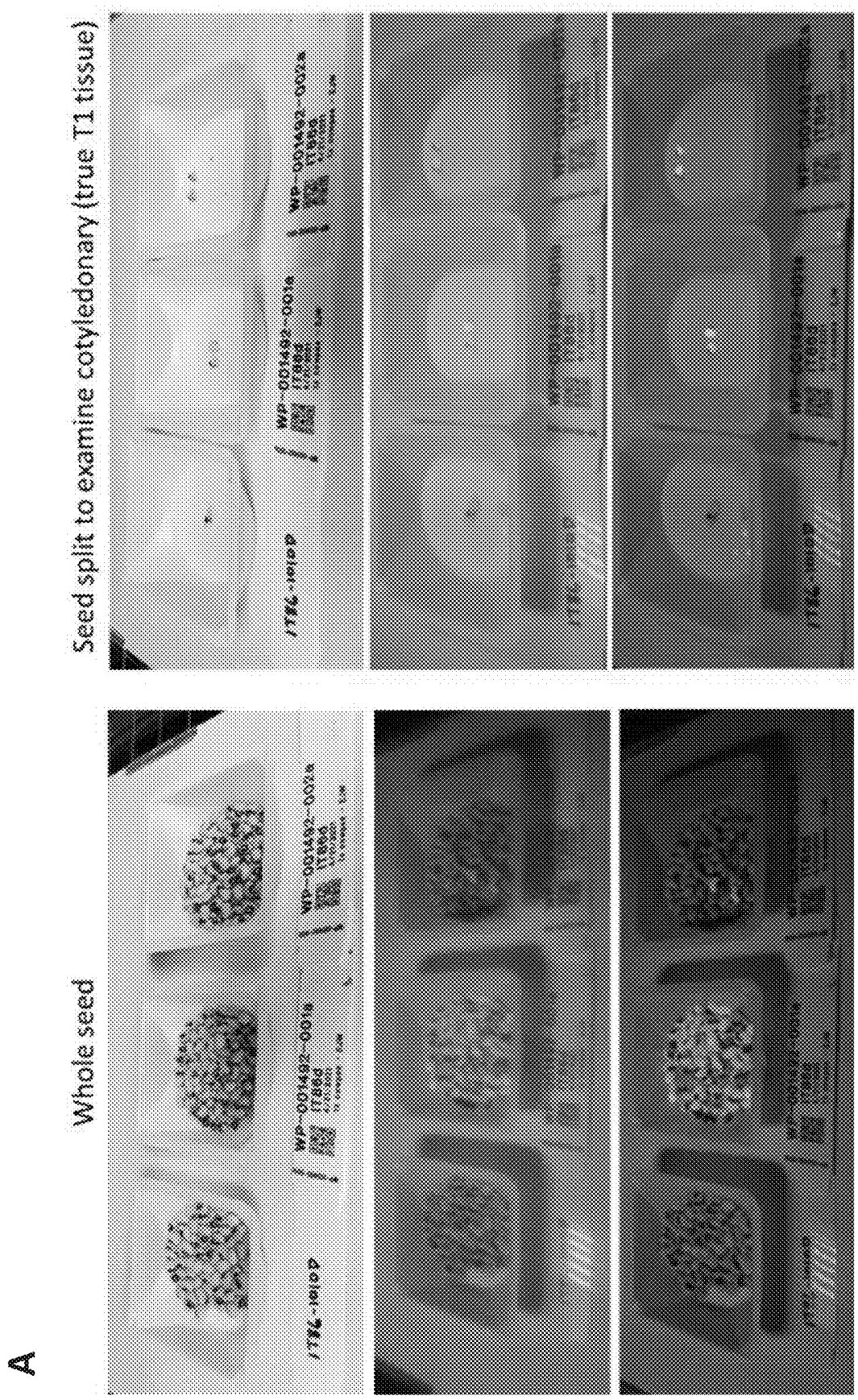
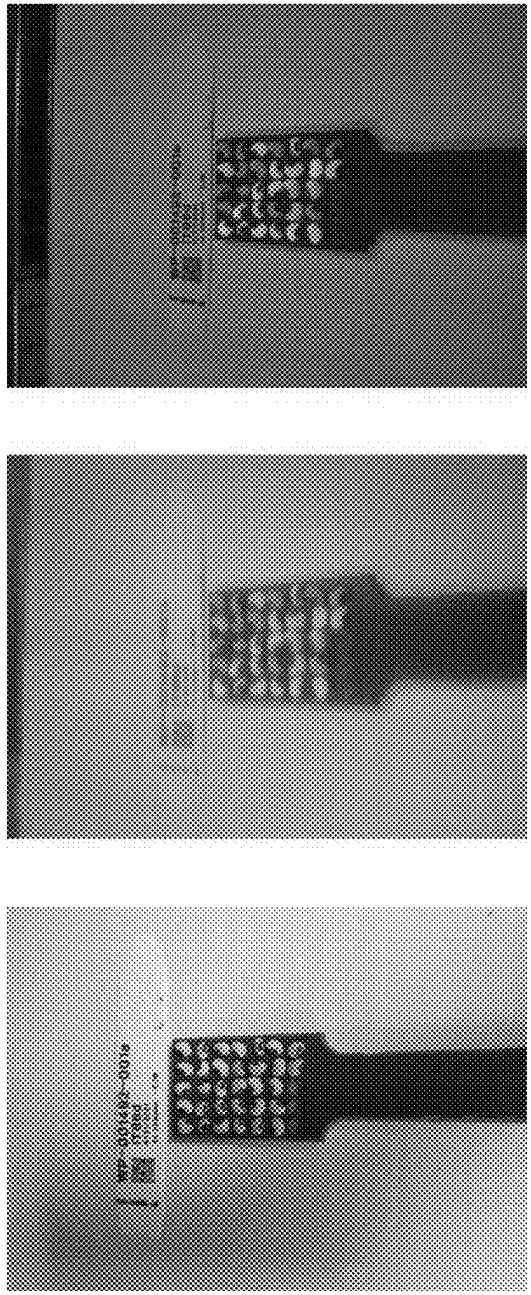

A
Whole seed
Seed split to examine cotyledonary (true T1 tissue)

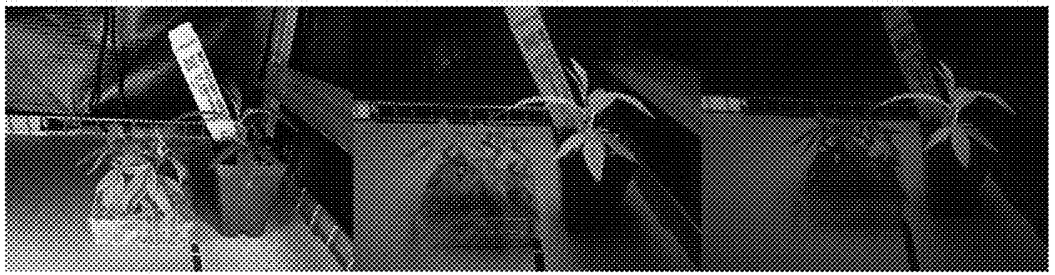
Figure 14 (continued)



B

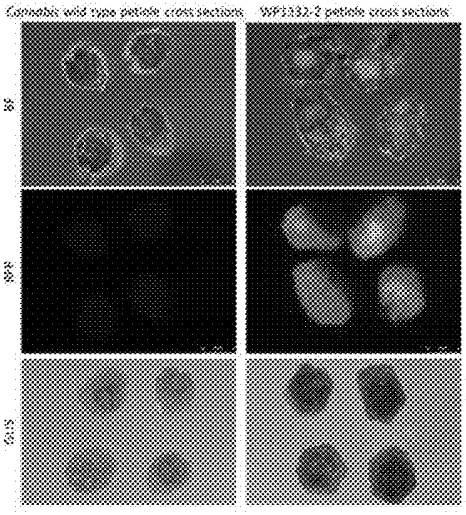
Figure 15

A

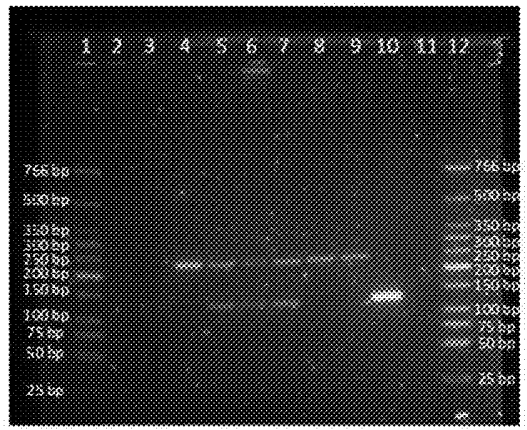


- Whole Plant RFP images of wild-type Cannabis (right) and WP1332-2 (left)
- T0 plant appears to be expressing tdTomato and GUS in vascular bundles of petiole sections (possible early indicator of germline)

B



C



Ln 1 = Low molecular weight ladder
Ln 2 = blank

Ln 3 = 50:50 reagent negative control; P140, P141 endogenous + D80,D81 aadA primers
Ln 4 = Cannabis leaf negative control; P140, P141 endogenous + D80,D81 aadA primers
Ln 5 = T0 Cannabis WP001331-1a leaf 1; P140, P141 endogenous + D80,D81 aadA primers
Ln 6 = T0 Cannabis WP001331-1a leaf 2; P140, P141 endogenous + D80,D81 aadA primers
Ln 7 = T0 Cannabis WP001331-1a leaf 3; P140, P141 endogenous + D80,D81 aadA primers
Ln 8 = T0 Cannabis WP001331-1a leaf 4; P140, P141 endogenous + D80,D81 aadA primers
Ln 9 = T0 Cannabis WP001331-1a leaf 5; P140, P141 endogenous + D80,D81 aadA primers
Ln 10 = 2 ng plasmid DNA; P140, P141 endogenous + D80,D81 aadA primers

Ln 11 = blank
Ln 12 = Low molecular weight ladder

P140, P141 Cannabis ubiquitin endogenous primers: 233 bp expected amplicon
D80, D81 aadA primers: 129 bp amplicon

Figure 16

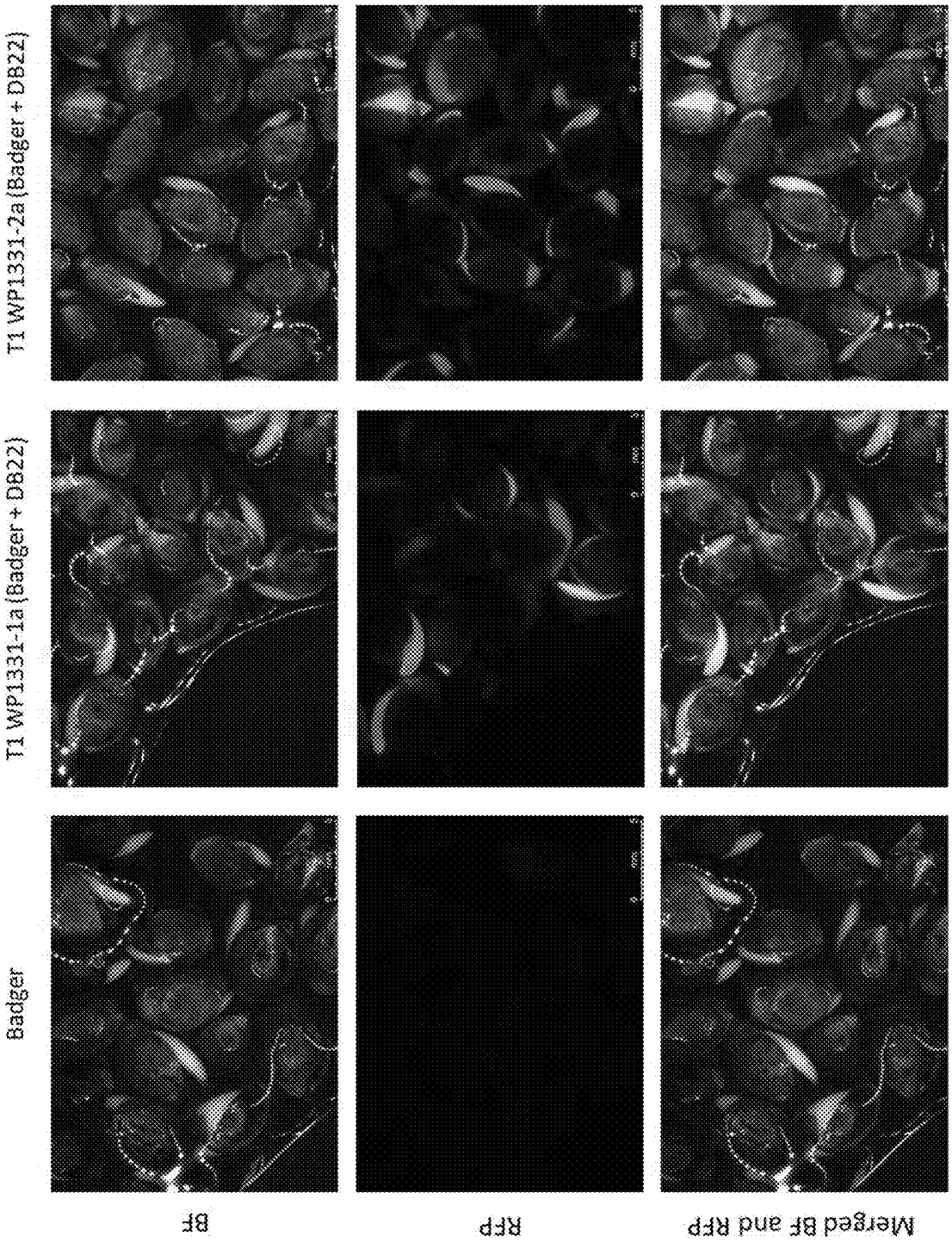

A
BF
RFP
Merged BF and RFP
Badger
T1 WP1331-1a (Badger + DB22)
T1 WP1331-2a (Badger + DB22)

Figure 16 (continued)

B

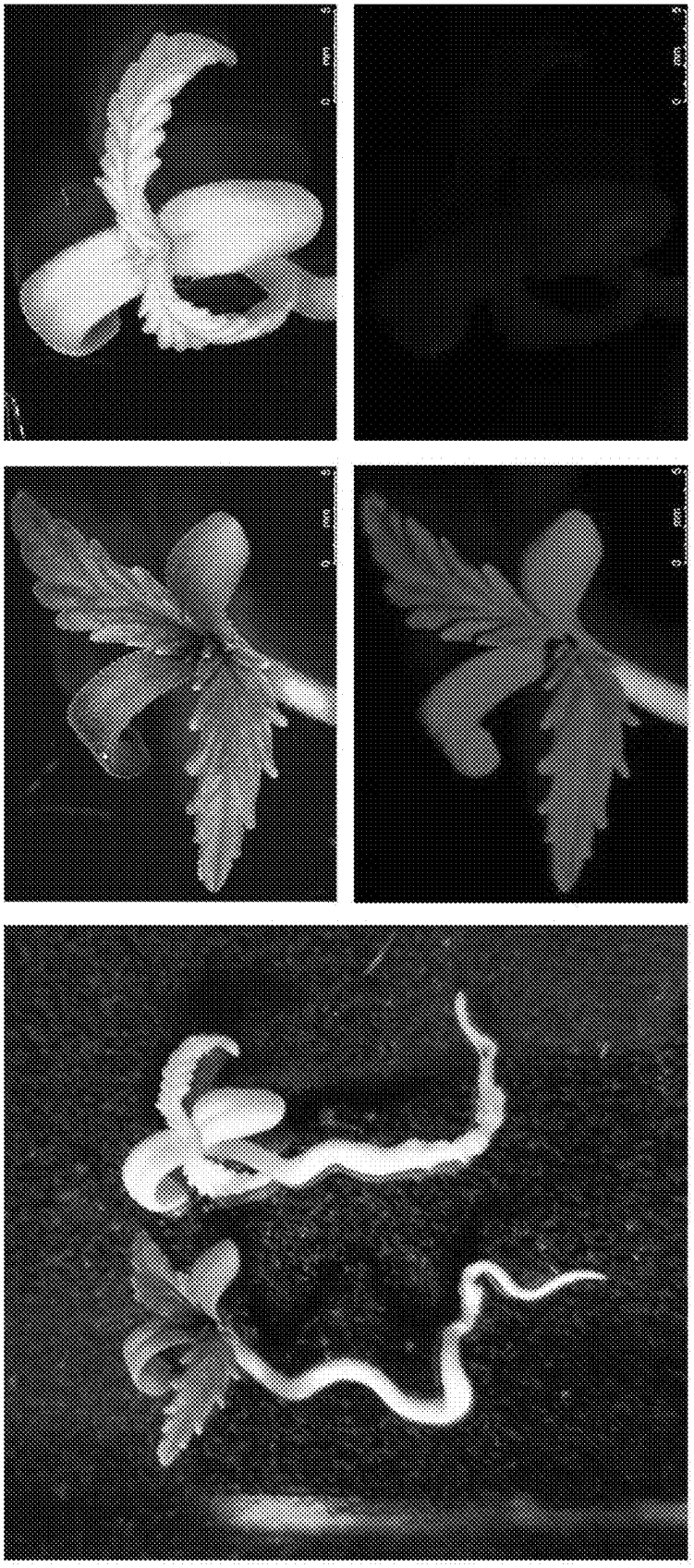


Figure 17

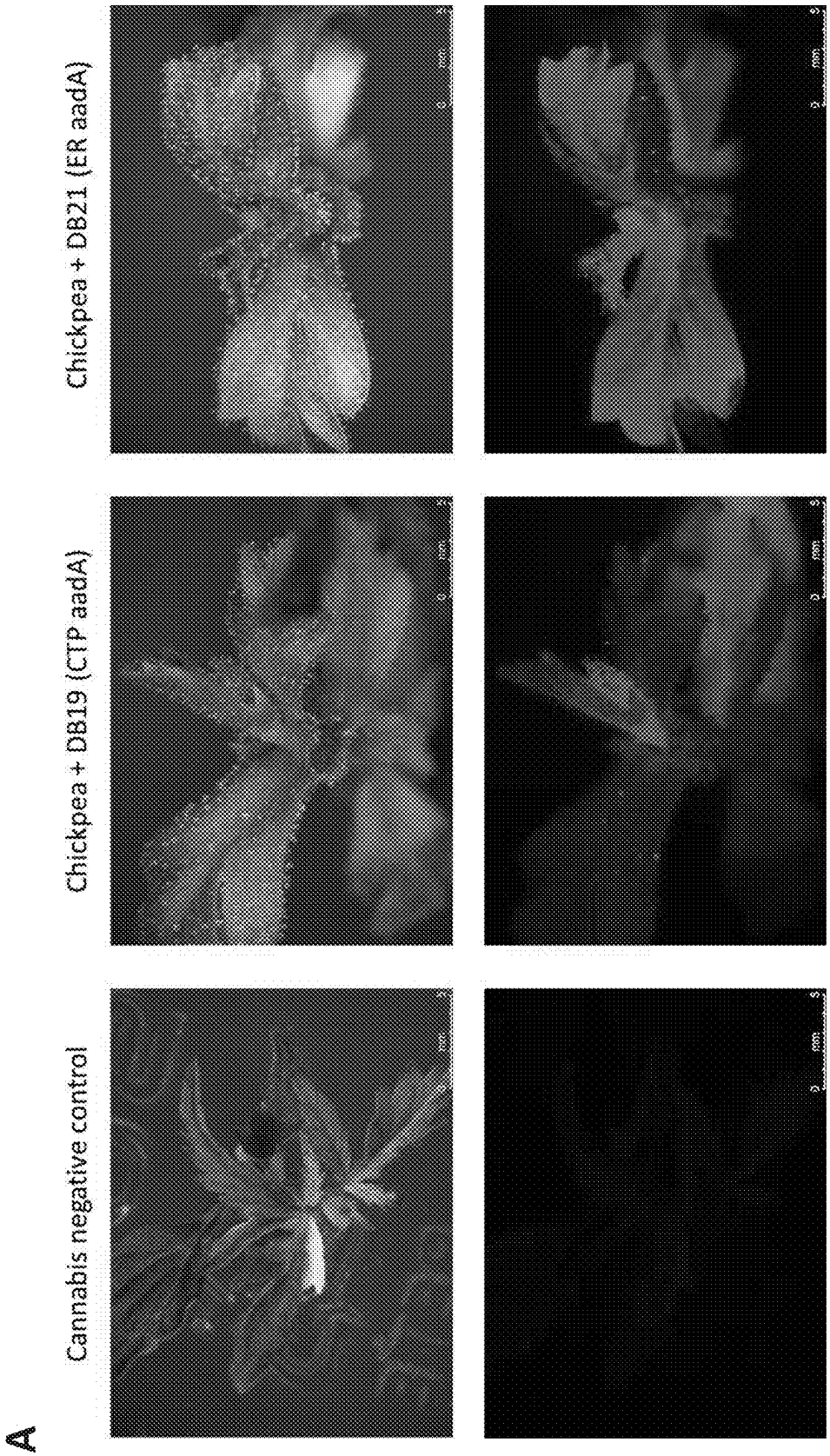

A
Cannabis negative control
Chickpea + DB19 (CTP aadA)
Chickpea + DB21 (ER aadA)

Figure 17 (continued)

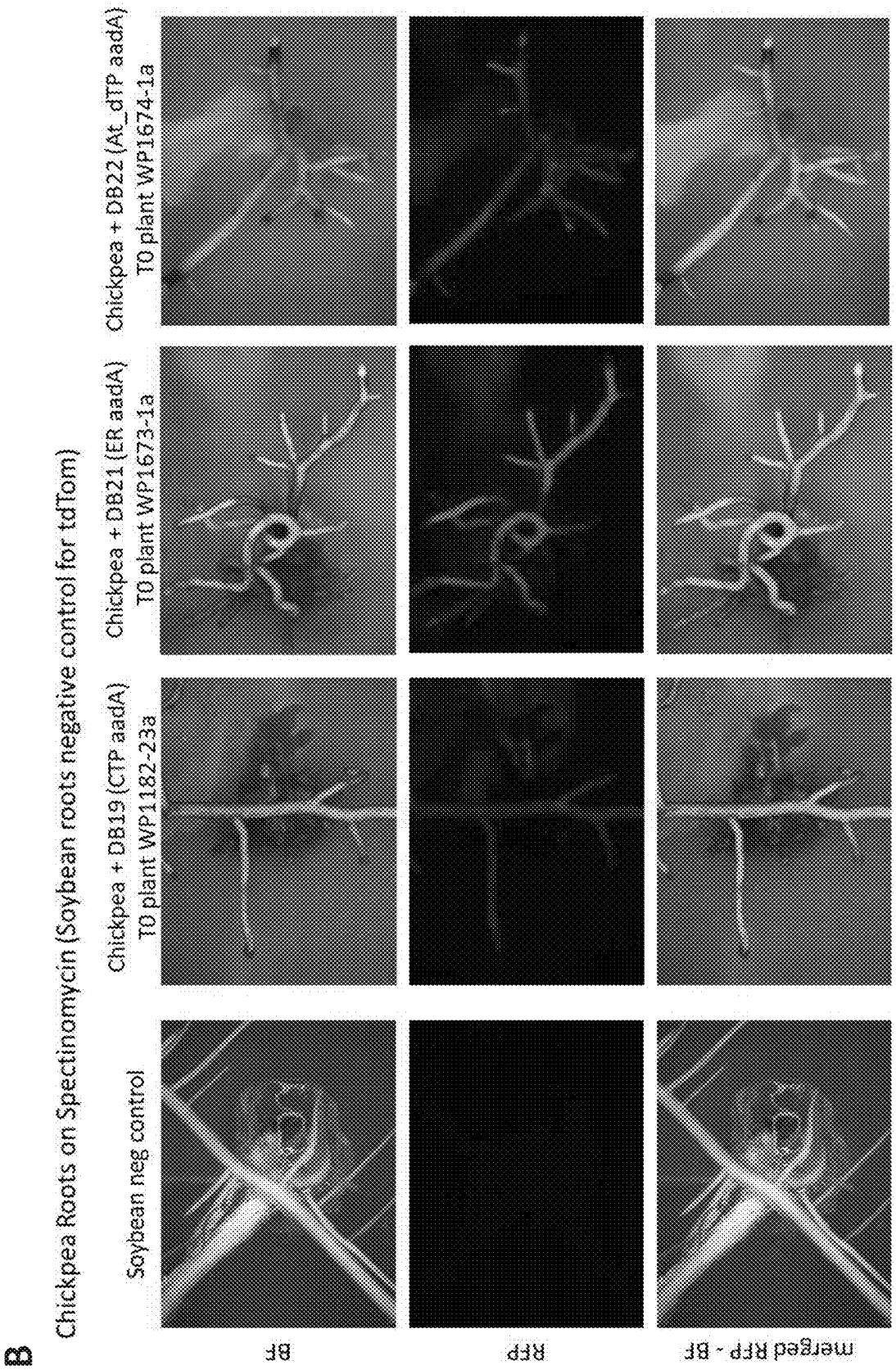


Figure 18

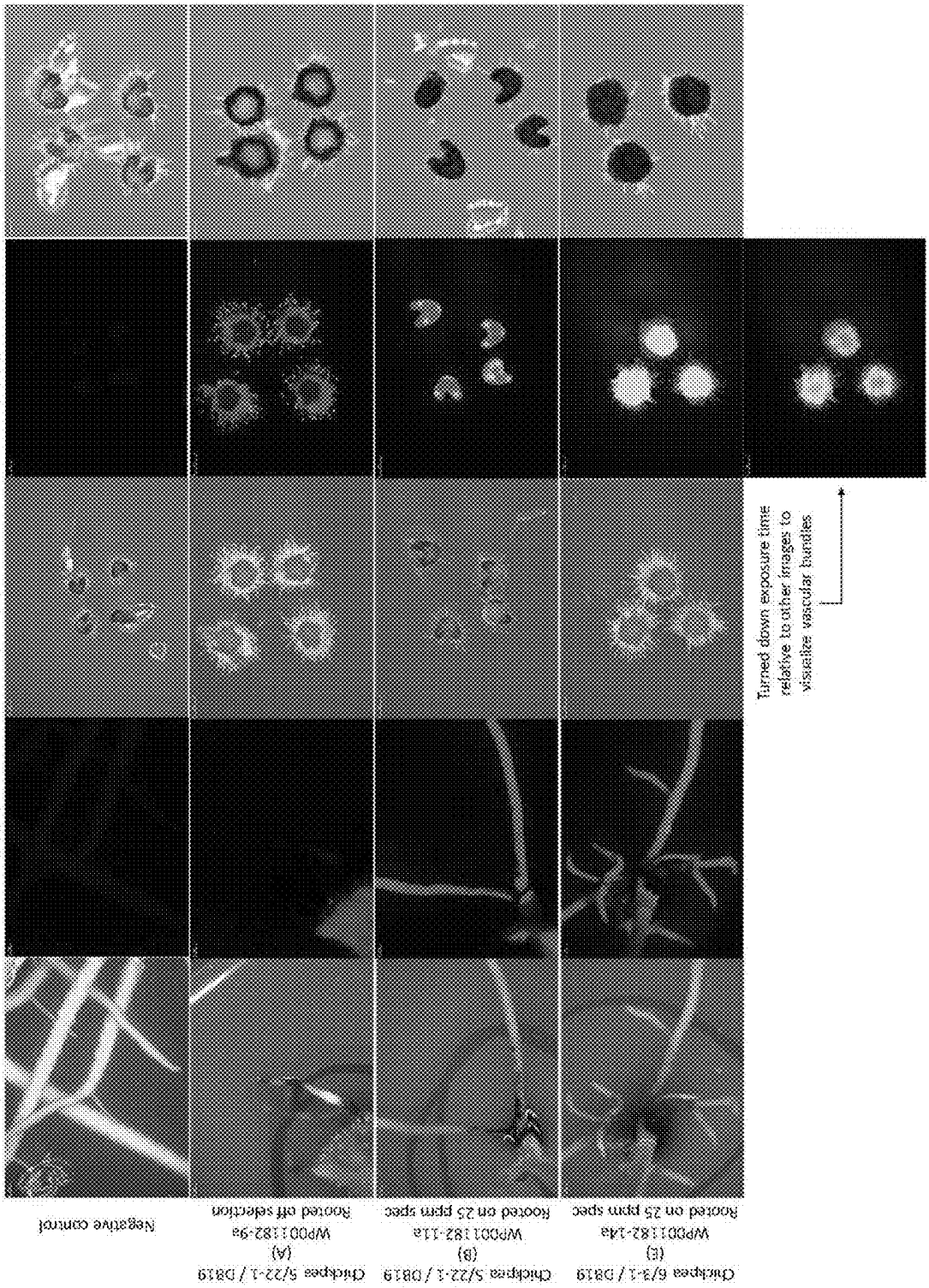

GUS petiole section
RFP petiole section
BF petiole section
RFP roots
BF roots
Turned down exposure time relative to other images to visualize vascular bundles
Negative control
Rooted off selection
Chickpea 5/22-1 / DB19 (A) WP00118 2-9a
Chickpea 5/22-1 / DB19 (B) WP00118 2-11a
Chickpea 6/3-1 / DB19 (E) WP00118 2-14a
Rooted on 25 ppm spec
Rooted on 25 ppm spec

Figure 19

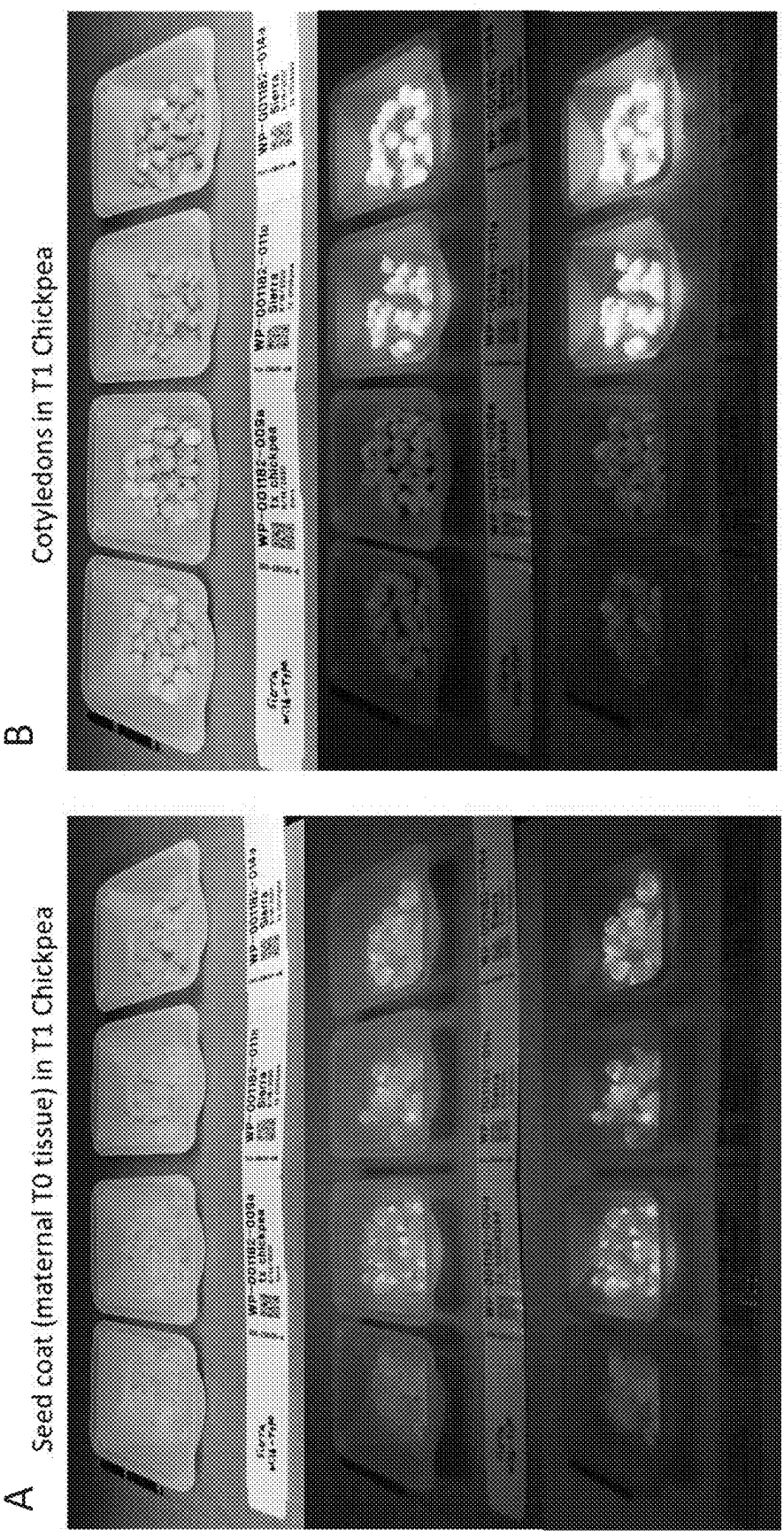

A
Seed coat (maternal T0 tissue) in T1 Chickpea
B
Cotyledons in T1 Chickpea

Figure 20

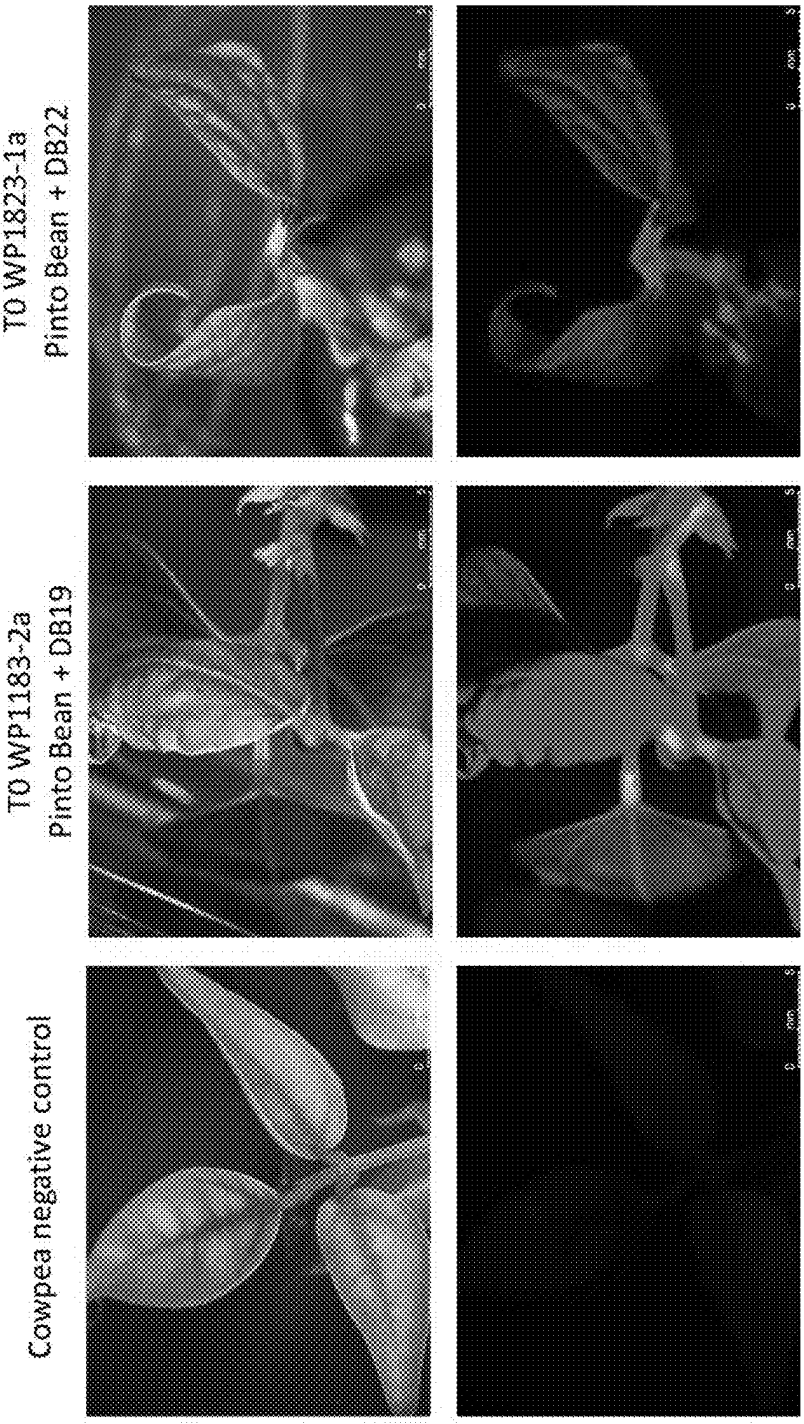

Cowpea negative control
T0 WP1183-2a
Pinto Bean + DB19
T0 WP1823-1a
Pinto Bean + DB22

Figure 21

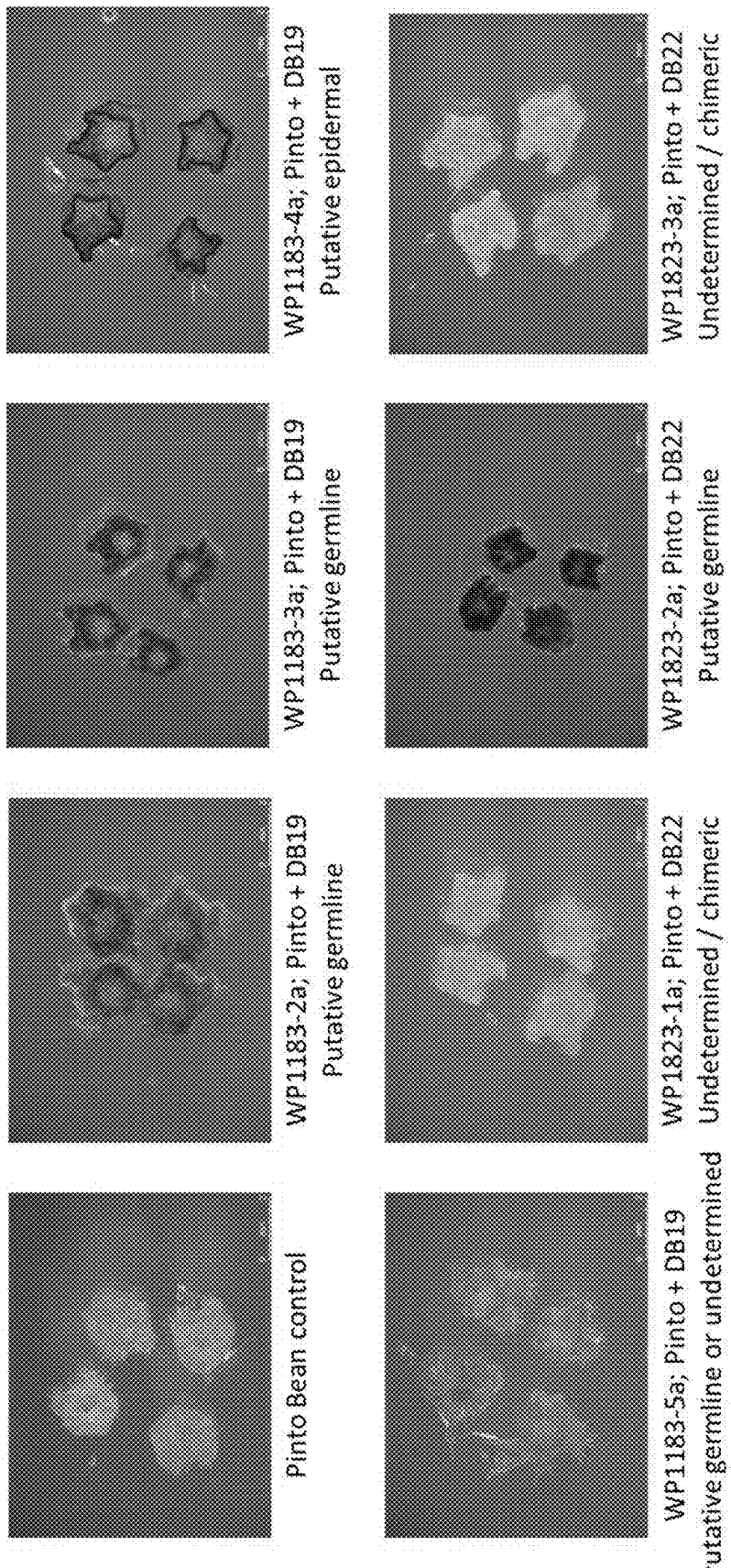

Pinto Bean control
WP1183-2a; Pinto + DB19
Putative germline
WP1183-3a; Pinto + DB19
Putative germline
WP1183-4a; Pinto + DB19
Putative epidermal
WP1183-5a; Pinto + DB19
Putative germline or undetermined / weak expression
WP1823-1a; Pinto + DB22
Undetermined / chimeric
WP1823-2a; Pinto + DB22
Putative germline
WP1823-3a; Pinto + DB22
Undetermined / chimeric

Figure 22


Wild-type Pea from meristem explant
Pea + D822 from meristem explant transformation
BF
RFP

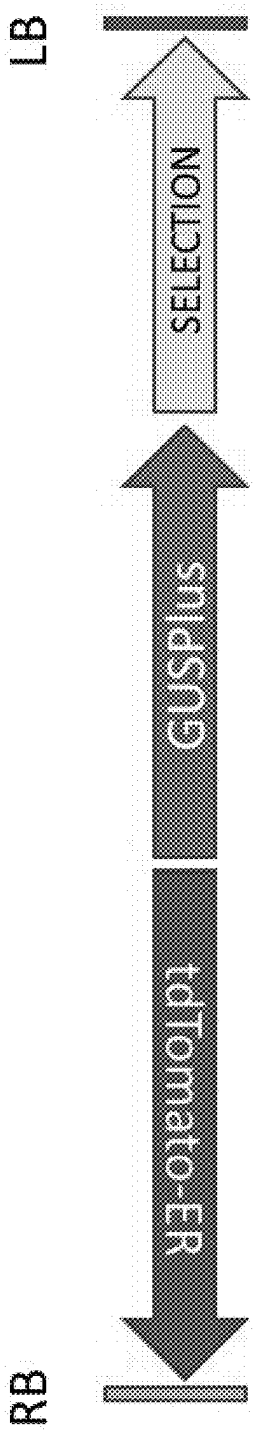


Figure 23

ALTERNATIVE TRANSIT PEPTIDES TO INCREASE PLANT TRANSFORMATION EFFICIENCY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 63/208,090 filed on Jun. 8, 2021, the contents of which are incorporated by reference in their entireties.

SEQUENCE LISTING

[0002] A Sequence Listing accompanies this application and is submitted as an ASCII text file of the sequence listing named "960296_04292_ST25.txt" which is 23,916 bytes in size and was created on May 13, 2022. The sequence listing is electronically submitted via EFS-Web with the application and is incorporated herein by reference in its entirety.

BACKGROUND

[0003] Transit peptides are commonly used in genetic engineering to deliver proteins to specific organelles within cells. For example, transit peptides are used to target selectable markers to specific organelles, such as the chloroplasts, to enable plant transformation and allow delivery of engineered proteins to the chloroplasts to allow these engineered proteins to avoid degradation in the cytoplasm of the cell and effect changes in plant photosynthesis, stress resistance, grain production and other changes to the metabolic functions of the plant. One commonly used selectable marker for dicot transformation is ANT(3")(9), an adenylyltransferase that is encoded by the aadA1a gene, which confers resistance to streptomycin and spectinomycin. To achieve higher transformation rates, a chloroplast transit peptide (cTP) may be used in combination with this selectable marker. Alternative target peptides that produce even higher transformation rates or target peptides to different organelles are needed in the art.

SUMMARY

[0004] In a first aspect, the present invention provides DNA constructs comprising a first expression cassette comprising a first heterologous promoter operably linked to a sequence encoding a transit peptide and a target protein. The transit peptide is at least 90% identical to SEQ ID NO:3 or SEQ ID NO:9.

[0005] In a second aspect, the present invention provides vectors comprising the DNA constructs described herein.

[0006] In a third aspect, the present invention provides plant cells comprising the DNA constructs described herein.

[0007] In a fourth aspect, the present invention provides methods of targeting a target protein to the chloroplasts and mitochondria of a plant cell. The methods comprise transforming the plant cell with a DNA construct described herein.

[0008] In a fifth aspect, the present invention provides methods of producing a transgenic plant. The methods comprise: (a) transforming an explant with a construct comprising a heterologous promoter operably linked to a sequence encoding a transit peptide described herein and a selectable marker; (b) contacting the explant with a selective agent; and (c) regenerating a transformed plant from the transgenic cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] This patent or application contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0010] FIG. **1** shows a boxplot of transformation frequencies produced using alternative transit peptides and the optimized transformation protocol for soybean.

[0011] FIG. **2** shows tandem Tomato, targeted to the endoplasmic reticulum (tdTomato-ER), expression in T1 soybean seeds, demonstrating germline transmission. The seeds are visualized under LED spotlights. A roscolux 90 dark yellow-green LED is used to visualize tdTomato-ER. To reduce undesirable wavelengths of light, a colored filter is placed underneath the LED source. To visualize fluorescence, a second, excitation filter is affixed to the front of the cart. Here, Gamcolor245 is used to visualize tdTomato-ER. The top row shows seeds under ambient light (i.e., brightfield), the middle row shows seeds under green light with a red excitation filter (Gamcolor 245), and the bottom row shows seeds under a green light with a separate red excitation filter. From left to right, the seed samples were treated with DICOTBINARY19 (cTP aadA1a; labeled as WP345), DICOTBINARY20 (cytosolic aadA1a; labeled as WP346), DICOTBINARY22 (At_dTP aadA1a; labeled as WP348), and DICOTBINARY23 (Gm_dTP aadA1a; labeled as WP349). The photographs show T1 seeds segregating for tdTomato-ER (the null seeds are not glowing, while the positive seeds glow).

[0012] FIG. **3** shows PVC seed paddles containing 32 randomly sampled T1 seeds for segregation analysis of tdTomato-ER expression in T1 soybean seeds. The photograph on the left shows seeds under ambient light (i.e., brightfield), the photograph in the middle shows seeds under green light with a red excitation filter (Gamcolor 245), and the photograph on the right shows seeds under a green light with a separate red excitation filter.

[0013] FIG. **4** shows the results of transforming soybean using particle bombardment. (A) tdTomato-ER expression and segregation in T1 seedlings of soybean line WP1521-2a, which was generated using DICOTBOMB20 (At_dTP aadA1a). (B) GUS expression and segregation in T1 leaves of soybean lines generated using DICOTBOMB13 (cTP aadA1a; top) or DICOTBOMB20 (At_dTP aadA1a; bottom).

[0014] FIG. **5** shows the results of a pilot study using various amounts of imazapyr for selection at 5 weeks in W82 soybean value added explants (VAEs).

[0015] FIG. **6** shows the T0 plant phenotype of the first soybean VAE transformed with using At_dTP hra and imazapyr selection. (A) Photograph showing the T0 phenotype. (B) Image of a PCR gel showing a band of the expected size (87 bp) in lane 5, demonstrating that the T0 soybean plant expresses hra. (C) Images of leaf samples (left: negative control; right: T0 plant), demonstrating that the T0 plant expresses tdTomato-ER and GUS in its leaves. (D) Images of petiole sections (left: negative control; right: T0 plant), demonstrating that the T0 plant expresses tdTomato-ER and GUS in its vascular bundles, which indicates that the transfection likely penetrated into the L2/L3 layers of the meristem and that T1 positive seeds will likely be recovered. tdTomato-ER was visualized in the petiole sections and leaf samples using a Leica M165FC microscope and Leica

Application Suite X (LAS X) software. For brightfield images, the iris was set to 64, and for tdTomato-ER images the iris was set to 100. For tdTomato-ER images, the lamp intensity was adjusted to the top setting, the exposure to approximately 500-2000 ms, and the gain to approximately 5. These settings appeared to be adequate to remove autoflorescence from the control samples and capture tdTomato-ER expression in transgenic samples.

[0016] FIG. **7** shows GUS expression and segregation in leaves of T1 progeny of the first soybean VAE transformed using At_dTP hra and imazapyr selection.

[0017] FIG. **8** shows tdTomato-ER expression and segregation in leaves of T1 progeny of the first soybean VAE transformed using At_dTP hra and imazapyr selection.

[0018] FIG. **9** shows soybean transformation using the optimized imazapyr selection protocol. (A) Photograph of soybean VAEs selected on 1 ppm imazapyr to 0.5 ppm imazapyr with elongated shoots (left), and on 2.5 ppm imazapyr to 0.5 ppm imazapyr without elongated shoots (right). (B) GUS expression and segregation in T1 leaves of soybean lines produced using the optimized imazapyr selection protocol.

[0019] FIG. **10** shows an imazapyr rooting assay for and segregating soybean after 2 weeks on rooting media with varying amounts of imazapyr.

[0020] FIG. **11** shows cowpea greening explants on spectinomycin media ~4 weeks after inoculation with Ar18r12v/DICOTBINARY19 (cTP aadA1a; left) or Ar18r12v/DICOTBINARY22 (At_dTP aadA1a; right).

[0021] FIG. **12** shows tdTomato-ER expression in T0 cowpea plants produced using DICOTBINARY22 (At_dTP aadA1a). (A) Comparison of event WP1492-1a to a soybean negative control. (B) Comparison of the uniform tdTomato-ER expression in WP1492-1a (top) to the chimeric expression in WP1492-2a (bottom).

[0022] FIG. **13** shows tdTomato-ER and GUS expression in vascular bundles of petiole sections of T0 cowpea plants produced using DICOTBINARY22 (At_dTP aadA1a).

[0023] FIG. **14** demonstrates tdTomato-ER expression and segregation in T1 cowpea seeds. (A) Comparison of expression in various seeds produced using DICOTBINARY22 (At_dTP aadA1a). (B) Seeds from the WP1492-1a event.

[0024] FIG. **15** shows tdTomato-ER and GUS expression in *Cannabis* T0 events WP1331-2a and WP1331-1a, which were both transformed with DICOTBINARY22 (At_dTP aadA1a). (A) Images of whole plants, including a control *cannabis* plant (right) and T0 event WP1331-2a (left). The image on the left shows the plants under ambient light (brightfield), the image in the middle shows the plants under green light with a red excitation filter (Gamcolor 245), and the image on the right shows the plants under green light with a separate red excitation filter. (B) Images of petiole sections of WP1331-2a, demonstrating that the T0 plant expressed tdTomato-ER and GUS in its vascular bundles. These images were taken on a Leica M165FC microscope. (C) Image of a PCR gel in which aadA1a was amplified from 5 leaves of WP1331-1a. Three of the five leaves show the expected aadA1a amplicon (129 bp).

[0025] FIG. **16** shows tdTomato-ER expression in *Cannabis* lines produced using DICOTBINARY22 (At_dTP aadA1a). (A) tdTomato-ER expression and segregation in T1 seeds. (B) tdTomato-ER expression in T1 seedlings of *Cannabis* line WP1331-2a. A T1 positive seedling is shown on the left, and a T1 null is show on the right.

[0026] FIG. **17** shows tdTomato-ER expression in chickpeas. tdTomato-ER expression in T0 chickpea leaves (A) and roots (B) transformed with the constructs DICOTBINARY19 (cTP aadA1a), DICOTBINARY21 (ER aadA1a), and DICOTBINARY22 (At_dTP aadA1a).

[0027] FIG. **18** shows tdTomato-ER and GUS expression in roots and vascular bundles of petiole sections of chickpea T0 events WP1182-11a and -14a (DICOTBINARY19, cTP aadA1a) as compared to that of the L1 epidermal chimeric event WP1182-9a.

[0028] FIG. **19** shows tdTomato-ER expression in seed coats and cotyledons in T1 seed of germline chickpea events WP1182-11a and -14a and L1 epidermal chimeric event WP1182-9a (DICOTBINARY19, cTP aadA1a). The top row shows seeds under ambient light (i.e., brightfield), the middle row shows seeds under green light with a red excitation filter (Gamcolor 245), and the bottom row shows seeds under a green light with a separate red excitation filter. (A) Images of seed coats. From left to right: wild-type Sierra Chickpea, WP1182-9a, WP1182-11a, and WP1182-14a. Seed coats are maternally inherited T0 tissue, so even the L1 epidermal event (WP1182-9a) has glowing seed coats. (B) Images of cotyledons, from left to right: wild-type Sierra Chickpea, WP1182-9a, WP1182-11a, and WP1182-14a. Cotyledons are true T1 tissue, so L1 epidermal event WP1182-9a does not express tdTomato-ER in T1 cotyledons, but T1 cotyledons of germline events WP1182-11a and WP1182-14a express tdTomato-ER (as predicted by root/vascular bundle expression in FIG. **18**).

[0029] FIG. **20** shows tdTomato-ER expression in T0 pinto bean plants transformed with the constructs DICOTBINARY19 (cTP aadA1a) and DICOTBINARY22 (At_dTP aadA1a).

[0030] FIG. **21** shows GUS expression in petiole sections of T0 pinto bean plants transformed with the constructs DICOTBINARY19 (cTP aadA1a) and DICOTBINARY22 (At_dTP aadA1a).

[0031] FIG. **22** shows tdTomato-ER expression in a T0 pea plant transformed with DICOTBINARY22 (At_dTP aadA1a).

[0032] FIG. **23** shows a schematic depicting the construct design described in Example 8, in which the plant selection marker gene is oriented such that its terminator is proximal to the T-DNA left border, and that transcription of the reporter gene is directed towards the T-DNA left border. In the schematic, each arrow represents a transcriptional unit comprising from 5' to 3': a promoter, a gene, and a terminator (including a polyadenylation signal). The arrowhead points in the direction of transcription. The T-DNA borders (RB=right border, LB=left border) delimit the T-DNA, with initiation occurring at the RB and termination occurring at the LB.

DETAILED DESCRIPTION

[0033] The present invention provides DNA constructs for producing transgenic plants. The DNA constructs encode a transit peptide that is targeted to the mitochondria and the chloroplasts within a cell. Also provided are plant cells and vectors comprising the DNA constructs.

[0034] In the present application, the inventors tested the ability of two transit peptides to serve as alternatives to the proprietary chloroplast transit peptide (cTP). The first of these peptides is a "dual targeting peptide" (dTP) that targets both the mitochondria and the chloroplasts, and the second

is an ER-targeting peptide/retention signal. DNA sequences encoding these transit peptides were cloned upstream of the aadA1a gene, which encodes an adenylyltransferase that confers resistance to streptomycin and spectinomycin, and these constructs were transformed into plants. The inventors tested two versions of the dual targeting peptide, one derived from *Arabidopsis thaliana* (At_dTP) and one derived from *Glycine max* (Gm_dTP). However, Gm_dTP failed to increase transformation frequency. Thus, only At_dTP was used in subsequent experiments.

[0035] While the inventors found that the ER-targeting peptide did not increase transformation frequencies in soybean plants, At_dTP produced greater transformation frequencies and recombinant protein expression than either of two control constructs, which contained either no transit peptide (i.e., the cytosolic construct) or a *petunia*-derived cTP. In fact, At_dTP produced transformation frequencies that were 1.4 to 3 times greater than those produced with cTP (see Example 1). Further, At_dTP enabled transformation of soybean with a particular resistance gene, highly resistant acetolactate synthase (hra), under conditions in which no transformed plants were recovered using the two control constructs (see Example 2). At_dTP also enabled the transformation of cowpea and chickpea, which were not successfully transformed using cTP under the same conditions (see Examples 3 and 5). Thus, the inventors have shown that this novel transit peptide is more effective at promoting transformation and allowing protein expression than the industry standard cTP.

[0036] The dual targeting peptide of the present invention, At_dTP, represents a promising means to increase plant transformation efficiencies and subsequent recombinant protein expression for genetic engineering applications. Importantly, this increase in transformation efficiency allows for more rapid regeneration of candidate transformed plant tissues, increased efficiency in identifying and growing transformed shoots and plants, reduced labor requirements, and reduced costs and ergonomic burden.

DNA Constructs:

[0037] In a first aspect, the present invention provides DNA constructs comprising a first expression cassette comprising a first heterologous promoter operably linked to a sequence encoding a transit peptide and a target protein. As used herein, the term "DNA construct" refers a to recombinant polynucleotide, i.e., a polynucleotide that was formed by combining at least two polynucleotide components from different sources, natural or synthetic. For example, a construct may comprise the coding region of one gene operably linked to a promoter that is (1) associated with another gene found within the same genome, (2) from the genome of a different species, or (3) synthetic. Constructs can be generated using conventional recombinant DNA methods.

[0038] The DNA constructs of the present invention comprise an expression cassette. An "expression cassette" is a polynucleotide comprising a sequence encoding a polypeptide or a functional RNA as well as elements needed to express the encoded polypeptide or RNA (e.g., a promoter). The sequences controlling the expression of the gene are commonly referred to as a regulatory unit. Most parts of the regulatory unit are located upstream of coding sequence of the gene and are operably linked thereto. The expression cassette may also contain a downstream 3' untranslated region comprising a polyadenylation site. The regulatory unit may be directly linked to the sequence to be expressed or separated therefrom by intervening DNA, e.g., by the 5'-untranslated region of a gene.

[0039] As used herein, the term "promoter" refers to a DNA sequence that regulates the expression of a polypeptide or functional RNA. Typically, a promoter is a regulatory region that is capable of binding RNA polymerase and initiating transcription of a downstream (3' direction) sequence. However, a promoter may be located at the 5' or 3' end, within a coding region, or within an intron of a gene that it regulates. Promoters may be derived in their entirety from a native gene, may be composed of elements derived from multiple regulatory sequences found in nature, or may comprise synthetic DNA. A promoter is "operably linked" to a polynucleotide if the promoter is connected to the polynucleotide such that it can affect transcription of the polynucleotide. The promoters of the present invention are "heterologous", meaning that they are not naturally associated with the host genome. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. A number of promoters that are active in plant cells have been described in the literature. Such promoters include, but are not limited to, the nopaline synthase (NOS) and octopine synthase (OCS) promoters that are carried on T1 plasmids of *Agrobacterium tumefaciens*, caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S and 35S promoters and the Figwort mosaic virus (FMV) 35S promoter, and the enhanced CaMV35S promoter (e35S). In some embodiments, the first heterologous promoter is a CaMV 35S promoter or a ubiquitin promoter.

[0040] The terms "transit peptide" and "targeting peptide" are used interchangeably herein to refer to a peptide that mediates post-translational translocation of a protein into a particular organelle (e.g., chloroplast, mitochondria, microbody, cyanelle). Transit peptides are commonly included in proteins as an N-terminal presequence. The transit peptides of the present invention are "dual targeting" peptides that direct translocation into both the mitochondria and the chloroplasts.

[0041] In the Examples, the inventors tested two versions of the dual targeting peptide (dTP): one derived from the *Arabidopsis thaliana* threonyl-tRNA synthetase (At2g04842.1; SEQ ID NO:1) and one derived from the *Glycine max* homolog of that protein (Glyma.19G191200.1; SEQ ID NO:7). The tested dTPs comprised the first 65 amino acids of these full-length peptides, i.e., SEQ ID NO:3 from *Arabidopsis thaliana* or SEQ ID NO:9 from *Glycine max*. Thus, the transit peptides used in the constructs of the present invention are at least 90% identical to SEQ ID NO:3 or SEQ ID NO:9. In some embodiments, the transit peptide is SEQ ID NO:3 or SEQ ID NO:9. Notably, the transit peptide of SEQ ID NO: 3 generated the highest transformation frequencies.

[0042] "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at

which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Protein and nucleic acid sequence identities are evaluated using the Basic Local Alignment Search Tool ("BLAST"), which is well known in the art (Karlin and Altschul, 1990, *Proc. Natl. Acad. Sci. USA* 87: 2267-2268; Altschul et al., 1997, *Nucl. Acids Res.* 25: 3389-3402). The BLAST programs identify homologous sequences by identifying similar segments, which are referred to herein as "high-scoring segment pairs," between a query amino or nucleic acid sequence and a test sequence which is preferably obtained from a protein or nucleic acid sequence database. Preferably, the statistical significance of a high-scoring segment pair is evaluated using the statistical significance formula (Karlin and Altschul, 1990), the disclosure of which is incorporated by reference in its entirety. The BLAST programs can be used with the default parameters or with modified parameters provided by the user.

[0043] The "target protein" used with the present invention can be any protein that one wishes to express in a plant. In some embodiments, the target protein confers a desirable trait, such as a trait of agronomic interest or a trait of industrial interest. Examples of traits of agronomic interest include, but are not limited to, disease, insect, or pest tolerance; herbicide tolerance; quality improvements, such as yield, fiber quality, nutritional enhancements (e.g., oil modification), or improved flavor; environmental or stress tolerances; any desirable changes in plant physiology, growth, development, morphology; and plant product production (e.g., starch, biopolymers, pharmaceutical peptides, etc.). Examples of traits of industrial interest include, but are not limited to, traits that are useful for carbon sequestration, biosensor development, or chemical/soil remediation.

[0044] In other embodiments, the target protein is a reporter protein or a selectable marker. A "reporter protein" is a protein that confers a characteristic that is easily identified or measured to an organism. A reporter protein is often used as an indication of whether a transgene has been taken up by or expressed in the cell or organism. Exemplary reporter proteins are known in the art and include, for example, β-glucuronidase (GUS), R-locus proteins, β-lactamase, luciferase, xy1E protein, α-amylase, tyrosinase, green fluorescence protein, other fluorescent proteins and α-galactosidase. A "selectable marker" is a protein that protects an organism from a selective agent that would normally kill it or prevent its growth. For example, in some embodiments, the selectable marker confers resistance to an antibiotic or an herbicide that would otherwise be toxic to the organism. In the Examples, the inventors demonstrate that the transit peptides described herein can be used to increase the efficiency of transforming a soybean plant with two genes that encode selectable markers: (1) aadA1a, which confers resistance to the antibiotic spectinomycin, and (2) hra, which confers resistance to the herbicide imazapyr. Thus, in some embodiments, the selectable marker is encoded by an aadA1a gene or a hra gene. In some embodiments, the selectable marker is encoded by SEQ ID NO:12, which is the soybean codon optimized aadA1a gene. In some embodiments, the selectable marker is encoded by SEQ ID NO:13, which is a cloning construct comprising SEQ ID NO:12.

[0045] In some embodiments, the DNA constructs further comprise a second expression cassette comprising a second heterologous promoter. In these embodiments, the DNA constructs may encode two target proteins: (1) a first target protein that is encoded by the first expression cassette and is expressed as a fusion protein with the transit peptide, and (2) a second target protein that is encoded by the second expression cassette such that it is expressed independently from the first target protein. For example, a construct may encode a first target protein that is a selectable marker (i.e., to allow for easy identification of cells that are successfully transformed with the construct) and a second target protein that confers a desirable trait that one wishes to transform into a plant. The second target protein may also be fused to a transit peptide if expression of the target requires trafficking to the chloroplast or mitochondria for effective expression. Alternatively, in these embodiments, the second heterologous promoter may be operably linked to a sequence that encodes an RNA molecule (e.g., antisense RNA, siRNA) that reduces the expression of an endogenous gene via gene silencing.

[0046] The constructs of the present invention are designed to express the transit peptide as a fusion protein with the target protein. A "fusion protein" is a protein consisting of at least two domains that are encoded by separate genes that have been joined such that they are transcribed and translated as a single unit, producing a single polypeptide. Thus, in some embodiments, the sequence encoding the transit peptide is translationally fused to the sequence encoding the target protein. The term "translationally fused" refers to two protein coding sequences that have been joined in a manner that retains the open reading frame of both protein coding sequences.

[0047] In some embodiments, the transit peptide is linked to the target protein by a linker peptide. As used herein, the term "linker peptide" refers to a peptide sequence that bridges two protein domains in a fusion protein. The linker may "flexible" such that it has no required fixed structure in solution and the adjacent protein segments are free to move relative to one another. The linker comprises 1 or more amino acid residues, preferably 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 or more residues. The linker may be an existing sequence provided by a protein domain included in the fusion protein or it may be provided by insertion of one or more amino acid residues between the protein domains of the fusion protein. The linker may comprise any amino acid sequence that does not substantially hinder the interaction of the polypeptides with their corresponding target molecules. The fusion proteins tested in the Examples comprise a simple two amino acid glycine-serine linker between the transit peptide and the target protein. Thus, in some embodiments, the linker comprises a glycine-serine linker.

[0048] The DNA construct used in the Examples comprises SEQ ID NO:5, which is a DNA sequence encoding the *Arabidopsis thaliana* dTP of SEQ ID NO:3 (At_dTP) that was codon-optimized for expression in soybean. Thus, in some embodiments, the DNA construct comprises SEQ ID NO:5. In some embodiments, the DNA construct comprises SEQ ID NO:6, which is a cloning construct comprising SEQ ID NO:5. In other embodiments, the DNA construct comprises SEQ ID NO:10, which is a DNA sequence encoding the *Glycine max* dTP (Gm_dTP) of SEQ ID NO:9. In some

embodiments, the DNA construct comprises SEQ ID NO:11, which is a cloning construct comprising SEQ ID NO:10.

[0049] In a second aspect, the present invention provides vectors comprising the DNA constructs described herein. The term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it is linked. Some vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors that include a bacterial origin of replication and episomal mammalian vectors). Other vectors can be integrated into the genome of a host cell such that they are replicated along with the host genome (e.g., viral vectors and transposons). Vectors suitable for use with the present invention comprise a DNA construct described herein and heterologous sequences necessary for proper propagation of the vector and expression of the encoded polypeptide. For example, the vector may contain genetic components that facilitate transformation of a plant cell or regulate the expression of the encoded proteins.

[0050] In some embodiments, the vector is a transfer DNA (T-DNA) vector for *Agrobacterium*-mediated transformation. A "T-DNA vector" is an artificial vector that is designed based on the naturally occurring T1 plasmid found in bacterial species of the genus *Agrobacterium*.

[0051] In some embodiments, the DNA constructs and vectors described herein are formulated for gene gun transformation. In gene gun transformation, a biolistic particle delivery system is used to deliver exogenous DNA into cells. Often, particles of a heavy metal (e.g., tungsten or gold) are coated with a DNA construct, and the particles are fired as micro-projectiles into cells using mechanical force. Thus, in some embodiments, the DNA constructs and vectors are coated onto heavy metal particles. For a description of gene gun transformation, see McCabe et al. (Bio/Technology (1988), 6:923-926), which is incorporated by reference in its entirety.

Plants:

[0052] In a third aspect, the present invention provides plant cells comprising the constructs described herein. A "plant cell" is the basic structural unit of the plant, comprising a protoplast and a cell wall. A plant cell can be in the form of an isolated single cell or aggregate of cells (e.g., a friable callus or a cultured cell) or can be part of a higher organized unit. In the Examples, the inventors demonstrate that constructs encoding the transit peptide At_dTP can be used to transform soybean (see Examples 1 and 2), cowpea (see Example 3), hemp (see Example 4), chickpea (see Example 5), pinto bean (see Example 6), and pea (see Example 7). Thus, in some embodiments, the plant cell is a soybean, cowpea, hemp, chickpea, pinto bean, or pea plant cell. In some embodiments, the plant cells express the transit peptide and the target protein as a fusion protein. The transit peptides of the present invention are designed to target the fusion protein to the mitochondria and the chloroplasts. Thus, in some embodiments, the fusion protein is targeted to the mitochondria and the chloroplasts within the plant cell.

Methods:

[0053] The transit peptides of the present invention are "dual targeting" peptides that simultaneously direct translocation of the target protein to both the mitochondria and the chloroplasts. Thus, in a fourth aspect, the present invention provides methods of targeting a target protein to the chloroplasts and mitochondria of a plant cell. The methods comprise transforming the plant cell with a DNA construct described herein.

[0054] The plant cell may be transformed using any method of plant transformation known in the art. Suitable methods for transforming nucleic acids into plant tissue include, without limitation, high velocity microprojection, microinjection, electroporation, direct DNA uptake, and bacterially mediated transformation. Bacteria known to mediate plant cell transformation include a number of species of Rhizobiaceae, including, but not limited to, *Agrobacterium* sp., *Sinorhizobium* sp., *Mesorhizobium* sp., and *Bradyrhizobium* sp. In some embodiments, the plant cells are transformed using *Agrobacterium*-mediated transformation. In this method, the DNA constructs are delivered into the plant cells using transferred (T)-DNA from bacterial cells through a bacterial type IV secretion system (T4SS).

[0055] Methods by which a plant is regenerated from tissue culture are known in the art and depend on the type of tissue that was used as an explant. See, e.g., Zambryski et al. (EMBO J. (1983) 2(12):2143-2150) for a description of such methods. See also U.S. Patent Publication No. US2019/0211347 and U.S. Patent Publication No. US2019/0208723, both of which are incorporated herein by reference in their entireties.

[0056] A variety of tissue culture media that support plant tissue growth and development when supplemented appropriately are known. These tissue culture media can be purchased commercially or can be prepared in-house. Those of skill in the art are aware that media and media supplements, such as nutrients and growth regulators, are usually optimized for the particular target crop or variety of interest. Tissue culture media may be supplemented with carbohydrates such as glucose, sucrose, maltose, mannose, fructose, lactose, galactose, dextrose, and combinations thereof. Suitable media include those utilized in the Examples, i.e., media based on Murashige and Skoog (MS) medium, Gamborg (B5) medium, and woody plant medium (WPM).

[0057] A "transgenic plant" is a plant into which one or more genes from another species have been introduced. A transgenic plant formed via *Agrobacterium*-mediated transformation typically contains a single recombinant DNA sequence inserted into one chromosome, which is referred to as a transgenic event. Such transgenic plants are hemizygous for the transgene. A transgenic plant that is homozygous for the transgene can be obtained by selfing an independent segregant transgenic plant that contains a single copy of the transgene (e.g., an T0 plant) to produce T1 seed. One fourth of the T1 seed produced will be homozygous with respect to the transgene. Germinating T1 seed produces plants that can be tested for zygosity, typically using a single-nucleotide polymorphism (SNP) assay or a thermal amplification assay that allows for the distinction between heterozygotes and homozygotes. To confirm the presence of the transgene in the transgenic plant, a variety of assays may be performed. Such assays include, for example, Southern blotting, northern blotting, western blotting, polymerase chain reaction (PCR)-based assays, Invader® assays, enzyme-linked immunoassays (ELISAs). Alternatively, if the transgene produces an observable phenotype, its presence may be confirmed by analyzing the phenotype of the whole regenerated plant.

[0058] Once a transgene has been introduced into a plant, that gene can be introduced into any plant that is sexually compatible with the first plant via crossing, i.e., without the need to directly transform the second plant. Therefore, the plants of the present invention include the progeny of any generation of a parent plant produced by the present methods, so long as the progeny comprise the transgene. Progeny can be produced by crossing a donor plant line that comprises the transgene with another plant line of interest. To accomplish this, one could, for example, perform the following steps: (a) plant seeds of a first (donor line) and second (line of interest) parent plant; (b) grow the seeds of the first and second parent plants into plants that bear flowers; (c) pollinate a flower from the first parent plant with pollen from the second parent plant; and (d) harvest seeds produced on the parent plant bearing the fertilized flower.

[0059] In the Examples, the inventors demonstrate that the use of a dual targeting peptide increases the efficiency of transformation in plants. Thus, in a fifth aspect, the present invention provides methods of producing a transgenic plant. The methods comprise: (a) transforming an explant with a construct comprising a heterologous promoter operably linked to a sequence encoding a transit peptide described herein and a selectable marker; (b) contacting the explant with a selective agent; and (c) regenerating a transformed plant from the transgenic cells.

[0060] The term "plant" is used herein to refer to a plant at any stage of development or to any part of a plant, including a plant cutting, a plant cell, a plant cell culture, a plant organ, a plant tissue, a plant seed, or a plantlet. Particularly useful parts of a plant include harvestable parts and parts that can be used for propagation of progeny plants. A harvestable part of a plant can be any useful part of a plant, for example, flowers, pollen, seedlings, tubers, leaves, stems, fruit, seeds, roots, and the like. A part of a plant useful for propagation includes, for example, seeds, fruits, cuttings, seedlings, tubers, rootstocks, and the like. The tissue culture will preferably be capable of regenerating plants.

[0061] Any species of plant may be used with the present invention, including monocot and dicot species. The term "monocot" is an abbreviation for monocotyledon and refers to plants that will have only one cotyledon, whereas the term "dicot" is an abbreviation for dicotyledon and refers to plants that will have two cotyledons. These two groups represent two divisions of the flowering plants. Suitable plants for use with the present invention include, for example, soybean, chickpea, wheat, barley, corn, cotton, pea, *cannabis*, cucurbits, tomato, potato, alfalfa, cowpea, dry beans, canola, and rice. In some embodiments, the plant is selected from a species that was transformed using the transit peptide At_dTP in the Examples, i.e., soybean, cowpea, *cannabis*, chickpea, pinto bean, and pea.

[0062] As used herein, the term "explant" refers to a cell, organ, or piece of tissue from a plant that has been transferred to a nutrient medium. Explants are the target material for transformation. They comprise meristematic tissue, which consists of undifferentiated cells that give rise to multiple plant structures (e.g., stems, roots, leaves, germline tissue, and seeds). Plant tissues that can be used as explants include, without limitation, embryos, cotyledons, hypocotyls, leaf bases, mesocotyls, plumules, protoplasts, and embryonic axes.

[0063] After the explants are transformed with the DNA construct, they are cultured in the presence of a selective agent. As used herein, the term "selective agent" refers to a compound that terminates or retards the growth of most of the plant cells into which the construct has not been delivered. Thus, a selective agent permits differentiation between cells that were successfully transformed with the construct and cells that were not transformed. Exemplary selective agents include, but are not limited to, kanamycin, hygromycin, streptomycin, chloramphenicol, ampicillin, erythromycin, spectinomycin, tetracycline, bialaphos, glyphosate, glufosinate, rifampicin, or dicamba, and the like. For example, in some embodiments, the selectable marker is encoded by the aadA1a gene and the selective agent is spectinomycin. In other embodiments, the selectable marker is encoded by the hra gene and the selective agent is imazapyr.

[0064] In some embodiments, the construct used in the methods further comprises a second heterologous promoter operably linked to a sequence that encodes a target protein, for example, a protein that confers a trait of agronomic interest. Alternatively, the construct may further comprise a second heterologous promoter operably linked to a sequence that encodes an RNA molecule (e.g., antisense RNA, siRNA) that reduces the expression of an endogenous gene via gene silencing.

[0065] The present disclosure is not limited to the specific details of construction, arrangement of components, or method steps set forth herein. The compositions and methods disclosed herein are capable of being made, practiced, used, carried out and/or formed in various ways that will be apparent to one of skill in the art in light of the disclosure that follows. The phraseology and terminology used herein is for the purpose of description only and should not be regarded as limiting to the scope of the claims. Ordinal indicators, such as first, second, and third, as used in the description and the claims to refer to various structures or method steps, are not meant to be construed to indicate any specific structures or steps, or any particular order or configuration to such structures or steps. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to facilitate the disclosure and does not imply any limitation on the scope of the disclosure unless otherwise claimed. No language in the specification, and no structures shown in the drawings, should be construed as indicating that any non-claimed element is essential to the practice of the disclosed subject matter. The use herein of the terms "including," "comprising," or "having," and variations thereof, is meant to encompass the elements listed thereafter and equivalents thereof, as well as additional elements. Embodiments recited as "including," "comprising," or "having" certain elements are also contemplated as "consisting essentially of" and "consisting of" those certain elements.

[0066] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is

incorporated into the specification as if it were individually recited herein. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended, and all possible combinations of numerical values between and including the lowest value and the highest value enumerated are to be considered to be expressly stated in this disclosure. Use of the word "about" to describe a particular recited amount or range of amounts is meant to indicate that values very near to the recited amount are included in that amount, such as values that could or naturally would be accounted for due to manufacturing tolerances, instrument and human error in forming measurements, and the like. All percentages referring to amounts are by weight unless indicated otherwise.

[0067] No admission is made that any reference, including any non-patent or patent document cited in this specification, constitutes prior art. In particular, it will be understood that, unless otherwise stated, reference to any document herein does not constitute an admission that any of these documents forms part of the common general knowledge in the art in the United States or in any other country. Any discussion of the references states what their authors assert, and the applicant reserves the right to challenge the accuracy and pertinence of any of the documents cited herein. All references cited herein are fully incorporated by reference, unless explicitly indicated otherwise. The present disclosure shall control in the event there are any disparities between any definitions and/or description found in the cited references.

[0068] The following examples are meant only to be illustrative and are not meant as limitations on the scope of the invention or of the appended claims.

EXAMPLES

Example 1—Alternative Transit Peptides in Soybean (*Glycine max*, L.) Transformation with aadA1a/Spectinomycin Selection

[0069] We first attempted transformation of soybean meristem explants (of variety Williams82) that had been machine excised from imbibed seed and dried down to allow storage prior to transformation. For a description of soybean explants, see US Patent Publication No. US2019/0208723, which is incorporated by reference in its entirety. For this experiment, we used the following soybean transformation protocol:

[0070] Surface plate explants onto selective B5 media (without orientation) after co-culture

[0071] Overlay media at week 3 to delay *Agrobacterium* overgrowth and minimize transfers

[0072] Perform selection with 150 mg/L spectinomycin (corrected for activity) post co-culture

[0073] Transfer phenotype positive explants to 50 mg/L spectinomycin (active) at week 4-6 and then to non-selective B5 medium at week 8 for 2 weeks

[0074] Harvest shoots onto 150 mg/L spectinomycin rooting medium (BRM) at each stage if they are sufficiently elongated

[0075] In this pilot study, we compared the proprietary, *petunia*-derived chloroplast transit peptide (cTP) to two types of alternative transit peptides: "dual targeting peptides" (dTPs) that target both the mitochondria and the chloroplasts and an endoplasmic reticulum (ER)-targeting peptide. Two different dTPs were tested: one derived from the *Arabidopsis thaliana* threonyl-tRNA synthetase (At_dTP) and one derived from the *Glycine max* homolog of that protein (Gm_dTP). We also included a no transit peptide control that localizes to the cytosol in this experiment. DNA sequences encoding these transit peptides were cloned upstream of the aadA1a gene, which encodes an adenylyltransferase that confers resistance to streptomycin and spectinomycin, and were transformed into plants. We found that the construct comprising the *Arabidopsis thaliana* version of the dual targeting peptide (At_dTP) yielded the highest transformation rate, while the construct comprising the ER-targeting peptide yielded the lowest transformation rate (Table 1).

TABLE 1

Transformation metrics for the pilot study

Binary	Promoter driving aadA1a	Subcellular location of aadA1a	Visible Explants	Regenerated Explants	T0 Plants	TF (vis)	TF (regen)
ST2	GmUbi3	cTP (chlor)	530	364	10	1.9%	2.8%
DB15	2X enhanced	cytosol	526	361	10	1.9%	2.8%
DB16	CaMV35S + TMV Ω	endoplasmic reticulum	533	366	2	0.4%	0.5%
DB17		At_dTP (mito & chlor)	528	362	30	5.7%	8.3%
DB18		Gm_dTP (mito & chlor)	404	276	12	3.0%	4.3%

ST = SOYTEST,
DB = DICOTBINARY

[0076] In this pilot study, the promoter used in the chloroplast transit peptide (cTP) construct was different from the promoter used in the other constructs, so we repeated the study using identical promoters in all the constructs and obtained similar results (Table 2). In this follow-up experiment, we used the same soybean transformation protocol that was used in the pilot test, but we embedded the explants (orienting apical meristems upward and radicals in media) directly after co-culture.

TABLE 2

Transformation metrics for the follow-up study

Binary	Promoter driving aadA1a	Subcellular location of aadA1a	Visible Explants	Regenerated Explants	T0 Plants	TF % (vis)	TF % (regen)
DB19	2X enhanced	cTP (chlor)	707	476	22	3.1	4.6
DB20	CaMV35S +	cytosol	719	464	23	3.2	5.0
DB21	TMV Ω	endoplasmic reticulum	541	380	0	0.0	0.0
DB22		At_dTP (mito & chlor)	623	419	43	6.9	10.3
DB23		Gm_dTP (mito & chlor)	685	455	19	2.8	4.2

DB = DICOTBINARY

[0077] We ran further tests to optimize the soybean value added explant (VAE) transformation protocol using the cTP construct (DICOTBINARY19). We found that embedding the explants in B5 media with their apical meristems oriented upward directly after co-culture (as opposed to surface plating without orientation) produced a large increase in transformation frequency. Based on this result, we eliminated the liquid overlay steps and reduced the number of regeneration transfers to three, producing the following optimized soybean transformation protocol:

[0078] Co-culture for 4-5 days in 2.5 ml INO medium with 1 ppm thidiazuron (TDZ)/plantcon

[0079] Embed explants (oriented apical meristems upward and radicals in media) directly after co-culture and for subsequent transfers for greening, phenotype positive explants on spectinomycin

[0080] Perform selection on 150 mg/L spectinomycin (corrected for activity) post co-culture for 3-4 weeks

[0081] Transfer phenotype positive explants to 50 mg/L spectinomycin (active) for 3-4 weeks and then to non-selective B5 medium for 2 weeks

[0082] Harvest shoots onto 150 mg/L spectinomycin in dicot basal rooting medium at each stage if sufficiently elongated

The media used in this optimized protocol are detailed in Tables 3-5, below.

TABLE 3

Dicot INO medium composition

Ingredients and notes	Amount to add per liter (grams)
Phytotechnology Laboratories B5 salts G398	1.284
Glucose	30
MES hydrate (Alfa Aesar CAS 4432-31-9)	2.8
pH to 5.4 with 1N KOH	
Autoclave	

TABLE 4

Gamborg True B5 medium composition

Ingredients and notes	Amount to add per liter (grams)
Phytotechnology Laboratories B5 salts G398	3.21
Sucrose	20
Cleary's 3336 (50WP)	0.06
Ca Gluconate	1.29
pH to 5.8 with 1N KOH	
Phytagel	3.50
Autoclave	
Add the following fresh before use:	
Timetin (150 mg/ml stock)	Use 1 mL per Liter (150 mg/L)
Cefotaximine (100 mg/ml stock)	Use at 2 ml per Liter (200 mg/L)
Carbenicillin (100 mg/ml stock)	Use at 4 ml per Liter (400 mg/L)
Selective agent	as needed

TABLE 5

Dicot basal rooting medium (BRM) composition

Ingredients and notes	Amount to add per liter (grams)
MS Salts no vitamins M524	2.15
Myo-inositol	0.1
Sucrose	30
pH 5.8 with KOH	
Agar, Sigma A7921	8
Autoclave	
Add after autoclaving:	
Cysteine (100 mg/ml) - make stock fresh	Use at 1.0 ml per Liter (100 mg/L)
Cefotaxime (100 mg/ml)	Use at 2.0 ml per Liter (200 mg/L)
IAA (1 mg/ml)	Use at 0.1 ml per Liter (0.1 mg/L)
MS Vitamins (1000X)	Use at 1.0 ml per Liter
Selection	as needed

[0083] We tested the optimized protocol against the original surface plating protocol with a construct encoding cTP-aadA1a and found that it produced higher transformation frequencies. Specifically, the surface plating method produced a transformation frequency of 0.1%, while the optimized protocol produced a transformation frequency of 3-5% (Table 6). The soybean plants used in this experiment were single seed descent of the Williams82 and 3613N varieties.

[0084] Using the optimized soybean transformation protocol, we tested the alternative transit peptides in a larger experiment with three replicates and produced similar results as in the initial tests (Table 7). As is shown in FIG. **1**, the DICOTBINARY22 construct comprising the bi-or-

ganellar transit peptide (At_dTP) yielded the highest transformation rate (TF). The soybean plants used in this experiment were single seed descent of the Williams82 variety.

[0085] In Tables 6 and 7, the number of visible explants is based on a sample taken of the VAE batch after excision. A mass is measured, and a technician counts the number of explants under a microscope, giving the batch a visible explant metric of "visible explants/gram." This metric is used to estimate how many visible explants are used in a given experiment. However, not all visible explants have intact meristematic tissue due to mechanical damage. Thus, we also count the subset of "regenerable explants" after transferring the explants off co-culture. The term "greening explants" refers to explants that stay green through spectinomycin selection, which bleaches non-transgenic tissues. Thus, greening explants, which are counted at the different transfer steps, are an early indicator of "phenotype positive" explants. We also harvest shoots and obtain rooted shoots (i.e., T0 plants) at each transfer step. An elongated greening shoot is a good sign that a plant is transgenic, and a shoot that roots in the presence of selection suggests that the event is germline (i.e., will give rise to T1 positive seed). "TF (vis)" is the transformation frequency based on visible explants, which is calculated by dividing the total number of T0 events by the number of initial visible explants. "TF (regen)" is the transformation frequency based on regenerable explants, which is calculated by dividing the total number of T0 events by the number of initial regenerable explants. The abbreviation "DB" stands for dicot binary.

TABLE 6

Transformation metrics for the optimized soybean transformation protocol

Cultivar	Treatment	Binary	Visible explants to 150 ppm spec B5	Regenerable explants to 150 ppm spec B5	Shoots from 150 ppm spec B5	T0 plants from 150 ppm spec (rooted on 150 ppm spec)	# Greening explants at wk 4 transferred to 50 ppm spec B5
SSW82	explants + debris surface plated	DB19	65	50	0	0	4
SSW82	pure singulated explants	DB19	100	100	2	2	26
SSW82	singulated explants	DB19	126	93	8	8	32
3613N	singulated explants	36222	600	450	2	2	83

Cultivar	Shoots from 50 ppm spec B5	T0 plants from 50 ppm spec (rooted on 150 ppm spec)	# Greening explants at wk 8 transferred to non-selective B5	Shoots from non-selective B5	T0 plants from non-selective B5 (rooted on 150 ppm spec)	TF (vis)	TF (regen)
SSW82	0	0	1	1	1	1.5%	2.0%
SSW82	5	5	5	5	5	12.0%	12.0%
SSW82	5	5	17	4	4	13.5%	18.3%
3613N	11	11	55	8	7	3.3%	4.4%

TABLE 7

Transformation metrics for the larger scale experiment with the optimized soybean transformation protocol. Data for individual replicates are shown in the top portion of the table, and data for the combined replicates are shown in the bottom portion of the table.

Binary	Ex-plants	Shoots from 150 ppm spec	T0 plants (rooting on 150 spec BRM)	Shoots from 50 ppm spec	T0 plants (rooting on 150 spec BRM)	Shoots from B5	T0 plants (rooting on 150 spec BRM)	Total Shoots	Total T0 plants (rooting on 150 spec BRM)	Shooting %	Rooting %	TF
DB19	425			36	33	23	18	59	51	13.9%	86.4%	12.0%
DB20	300			22	6	10	6	32	12	10.7%	37.5%	4.0%
DB21	300			0	0	0	0	0	0	0.0%	n/a	0.0%
DB22	275			46	45	28	24	74	69	26.9%	93.2%	25.1%
DB23	300			49	40	24	13	73	53	24.3%	72.6%	17.7%
DB19	300	12	10	9	9	9	9	30	28	10.0%	93.3%	9.3%
DB20	300	18	11	22	11	8	5	48	27	16.0%	56.3%	9.0%
DB21	250	0	0	0	0	0	0	0	0	0.0%	n/a	0.0%
DB22	275	4	4	18	17	7	7	29	28	10.5%	96.6%	10.2%
DB23	275	11	11	15	14	7	4	33	29	12.0%	87.9%	10.5%
DB19	250			13	11	15	12	28	23	11.2%	82.1%	9.2%
DB20	250			25	10	28	15	53	25	21.2%	47.2%	10.0%
DB21	250			0	0	0	0	0	0	0.0%	n/a	0.0%
DB22	225			12	10	15	11	27	21	12.0%	77.8%	9.3%
DB23	250			29	23	16	10	45	33	18.0%	73.3%	13.2%
DB19	975	12	10	58	53	47	39	117	102	12.0%	87.2%	10.5%

TABLE 7-continued

Transformation metrics for the larger scale experiment with the optimized soybean transformation protocol. Data for individual replicates are shown in the top portion of the table, and data for the combined replicates are shown in the bottom portion of the table.

Binary	Ex-plants	Shoots from 150 ppm spec	T0 plants (rooting on 150 spec BRM)	Shoots from 50 ppm spec	T0 plants (rooting on 150 spec BRM)	Shoots from B5	T0 plants (rooting on 150 spec BRM)	Total Shoots	Total T0 plants (rooting on 150 spec BRM)	Shooting %	Rooting %	TF
DB20	850	18	11	69	27	46	26	133	64	15.6%	48.1%	7.5%
DB21	800	0	0	0	0	0	0	0	0	0.0%	n/a	0.0%
DB22	775	4	4	76	72	50	42	130	118	16.8%	90.8%	15.2%
DB23	825	11	11	93	77	47	27	151	115	18.3%	76.2%	13.9%

[0086] In addition to the aadA1a reporter gene, most of our test constructs (e.g., DICOTBINARY22) contain transcription units encoding a non-lethal scorable marker (i.e., tdTomato-ER) and a lethal scorable marker (i.e., GUS). Inclusion of these additional markers allows corroborative data to be collected during the course of the experiment. For example, the fluorescent signal of tdTomato-ER (which is a tandem Tomato marker that is localized to the endoplasmic reticulum for enhanced brightness) can be detected without harming the plants prior their use in the lethal GUS reporter assay. Thus, we next confirmed germline transmission in the plants generated in the second, follow-up experiment (Table 2) using non-destructive whole seed imaging of tdTomato-ER in T1 seed (FIG. **2**, Table 8).

[0087] We then assessed segregation by randomly sampling about 32 T1 seeds per line using a PVC block bored with indentions to accommodate a soybean seed and imaging these singulated seeds for tdTomato-ER expression (FIG. **3**). tdTomato-ER-positive seeds were counted and ratio compared. A 3:1 ratio was expected based on Mendelian segregation of a single copy transgene in the parental hemizygotic event (Table 9). Germline transmission was high (greater than 80%) with all of the alternative transit peptides, but the cTP and At_dTP peptides had the highest percentage of lines possibly segregating 3:1 by chi-square.

TABLE 8

Summary of germline transmission and segregation of tdTomato-ER in T1 seed from the follow-up experiment

Species	Genotype	Binary	Subcellular location of aadA1a	T0 plants	Workplan	# Lines examined for T1 tdTom	# Lines with tdTom+ T1 seed	# Lines possible segregating 3:1 by chi-square	% Lines with tdTom+ T1 seed (% germline)	% Lines possible segregating 3:1 by chi-square
Soybean	W82	DB19	cTP	22	WP345	16	14	7	88%	44%
Soybean	W82	DB20	cytosol	23	WP346	18	15	4	83%	22%
Soybean	W82	DB22	At_dTP	43	WP348	35	32	14	91%	40%
Soybean	W82	DB23	Gm_dTP	19	WP349	16	14	3	88%	19%

TABLE 9

Raw data used to generate Table 8.

Line	Subcellular location of aadA1a	# T1 seed	# RFP pos	# RFP neg	Expected RFP pos (3:1)	Expected RFP neg (3:1)	Chi square p
WP345-3	cTP	32	11	21	24	8	0.0000
WP345-4	cTP	32	20	12	24	8	0.1025
WP345-5	cTP	32	0	32	24	8	0.0000
WP345-6	cTP	32	11	21	24	8	0.0000
WP345-7	cTP	32	22	10	24	8	0.4142
WP345-8	cTP	32	20	12	24	8	0.1025
WP345-9	cTP	32	0	32	24	8	0.0000
WP345-10	cTP	32	8	24	24	8	0.0000
WP345-11	cTP	32	27	5	24	8	0.2207
WP345-12	cTP	32	13	19	24	8	0.0000
WP345-14	cTP	32	13	19	24	8	0.0000
WP345-15	cTP	32	12	20	24	8	0.0000
WP345-16	cTP	32	20	12	24	8	0.1025
WP345-17	cTP	32	25	7	24	8	0.6831
WP345-18	cTP	32	16	16	24	8	0.0011
WP345-19	cTP	32	27	5	24	8	0.2207
WP346-3	cytosol	32	22	10	24	8	0.4142

TABLE 9-continued

Raw data used to generate Table 8.

Line	Subcellular location of aadA1a	# T1 seed	# RFP pos	# RFP neg	Expected RFP pos (3:1)	Expected RFP neg (3:1)	Chi square p
WP346-5	cytosol	32	2	30	24	8	0.0000
WP346-6	cytosol	32	25	7	24	8	0.6831
WP346-7	cytosol	32	4	28	24	8	0.0000
WP346-8	cytosol	32	0	32	24	8	0.0000
WP346-10	cytosol	32	26	6	24	8	0.4142
WP346-11	cytosol	32	2	30	24	8	0.0000
WP346-12	cytosol	32	13	19	24	8	0.0000
WP346-13	cytosol	32	16	16	24	8	0.0011
WP346-14	cytosol	32	0	32	24	8	0.0000
WP346-15	cytosol	32	19	13	24	8	0.0412
WP346-16	cytosol	32	16	16	24	8	0.0011
WP346-17	cytosol	32	15	17	24	8	0.0002
WP346-18	cytosol	32	15	17	24	8	0.0002
WP346-19	cytosol	32	6	26	24	8	0.0000
WP346-21	cytosol	32	17	15	24	8	0.0043
WP346-22	cytosol	32	0	32	24	8	0.0000
WP346-23	cytosol	16	11	5	12	4	0.5637
WP348-6	At_dTP	32	5	27	24	8	0.0000
WP348-7	At_dTP	32	23	9	24	8	0.6831
WP348-8	At_dTP	32	16	16	24	8	0.0011
WP348-10	At_dTP	32	17	15	24	8	0.0043
WP348-11	At_dTP	32	16	16	24	8	0.0011
WP348-12	At_dTP	32	18	14	24	8	0.0143
WP348-13	At_dTP	32	10	22	24	8	0.0000
WP348-15	At_dTP	32	5	27	24	8	0.0000
WP348-16	At_dTP	32	5	27	24	8	0.0000
WP348-17	At_dTP	32	20	12	24	8	0.1025
WP348-18	At_dTP	32	21	11	24	8	0.2207
WP348-19	At_dTP	32	22	10	24	8	0.4142
WP348-20	At_dTP	32	0	32	24	8	0.0000
WP348-21	At_dTP	32	27	5	24	8	0.2207
WP348-22	At_dTP	32	17	15	24	8	0.0043
WP348-23	At_dTP	32	24	8	24	8	1.0000
WP348-24	At_dTP	32	21	11	24	8	0.2207
WP348-25	At_dTP	32	17	15	24	8	0.0043
WP348-26	At_dTP	32	18	14	24	8	0.0143
WP348-27	At_dTP	32	0	32	24	8	0.0000
WP348-28	At_dTP	32	18	14	24	8	0.0143
WP348-29	At_dTP	32	12	20	24	8	0.0000
WP348-30	At_dTP	32	23	9	24	8	0.6831
WP348-31	At_dTP	32	23	9	24	8	0.6831
WP348-32	At_dTP	32	22	10	24	8	0.4142
WP348-33	At_dTP	32	28	4	24	8	0.1025
WP348-34	At_dTP	32	12	20	24	8	0.0000
WP348-36	At_dTP	32	29	3	24	8	0.0412
WP348-37	At_dTP	32	25	7	24	8	0.6831
WP348-38	At_dTP	32	18	14	24	8	0.0143
WP348-39	At_dTP	32	19	13	24	8	0.0412
WP348-40	At_dTP	32	0	32	24	8	0.0000
WP348-41	At_dTP	32	14	18	24	8	0.0000
WP348-42	At_dTP	32	24	8	24	8	1.0000
WP348-43	At_dTP	32	24	8	24	8	1.0000
WP349-2	Gm_dTP	32	12	20	24	8	0.0000
WP349-3	Gm_dTP	32	19	13	24	8	0.0412
WP349-4	Gm_dTP	32	17	15	24	8	0.0043
WP349-5	Gm_dTP	32	12	20	24	8	0.0000
WP349-6	Gm_dTP	32	0	32	24	8	0.0000
WP349-7	Gm_dTP	32	27	5	24	8	0.2207
WP349-9	Gm_dTP	32	5	27	24	8	0.0000
WP349-10	Gm_dTP	32	13	19	24	8	0.0000
WP349-11	Gm_dTP	32	0	32	24	8	0.0000
WP349-12	Gm_dTP	32	24	8	24	8	1.0000
WP349-14	Gm_dTP	32	13	19	24	8	0.0000
WP349-15	Gm_dTP	32	8	24	24	8	0.0000
WP349-16	Gm_dTP	32	15	17	24	8	0.0002
WP349-17	Gm_dTP	32	28	4	24	8	0.1025
WP349-18	Gm_dTP	32	14	18	24	8	0.0000
WP349-19	Gm_dTP	32	19	13	24	8	0.0412

[0088] We also tested the At_dTP transit peptide in particle-mediated transformation of soybean VAEs (single seed descent variety of Williams82) using the PDS-1000 Helium gun. In this experiment, we used the constructs DICOTBOMB13 (cTP aadA1a) and DICOTBOMB20 (At_dTP aadA1a). A 6 cm distance from the microcarrier (which contained a 5 μl drop of the DNA-gold prep) to the soybean explants was used. The T1 metrics (germline frequencies and segregation data) for this experiment are presented in Tables 10-13 and in FIG. **4**. These data demonstrate germline transmission of GUS in soybean using At_dTP aadA with particle bombardment.

TABLE 10

Transformation metrics for particle-mediated transformation with DICOTBOMB20 (At_dTP aadA1a). The bead prep metric gives the amount of DNA precipitated on a given mass of gold. 1350 psi refers to the rupture disk in the PDS-1000 Helium gun.

Experiment	Preculture	Bead prep	Blasting conditions	Rest	# Explants to 150 mg/L spec B5 (active)	# Greening to 50 ppm spec B5 (active) 3 wks post-blast	# Shoots	# Rooted shoots	TF	% Germline
Soybean VAE 3/17-3	EJW1 with 8 g/L agarose I; 28 C. 16/8 photoperiod	1.2 ng DNA/ug 0.6 um gold BOMB 20	1X blast; 1 cm gap; 1350 psi; 6 cm distance	EJW1 with 8 g/L agarose I; 28 C. 16/8 photoperiod	95	18	7	3	3.2%	67%
Soybean VAE 3/17-3B	EJW1 with 8 g/L agarose I; 28 C. 16/8 photoperiod	1.2 ng DNA/ug 0.6 um gold BOMB 20	1X blast; 1 cm gap; 1100 psi; 6 cm distance	EJW1 with 8 g/L agarose I; 28 C. 16/8 photoperiod	13	1	1	1	7.7%	100%
Soybean VAE 3/17-4	INO TDZ with 8 g/L agarose I; 28 C. 16/8 photoperiod	1.2 ng DNA/ug 0.6 um gold BOMB 20	1X blast; 1 cm gap; 1350 psi; 6 cm distance	INO TDZ with 8 g/L agarose I; 28 C. 16/8 photoperiod	71	5	3	3	4.2%	100%
Soybean VAE 3/17-5	Mod TDZ OR v33; 28 C. 16/8 photoperiod	1.2 ng DNA/ug 0.6 um gold BOMB 20	1X blast; 1 cm gap; 1100 psi; 6 cm distance	Mod TDZ OR v33; 28 C. 16/8 photoperiod	27	4	0	0	0.0%	n/a

TABLE 11

Transformation metrics for particle-mediated transformation with DICOTBOMB20 (At_dTP aadA1a) or DICOTBOMB13 (cTP aadA1a).

Experiment	Preculture conditions	Construct	Rest conditions	Initial Explants on 150 ppm spec B5	Shoots harvested	T0 plants	TF	% Germline
Soybean VAE 2/25-1	VAE, precultured on EJW1 o/n at 15 C. dark	Dicot Bomb 13	EJW1 o/n at 28 C. 16/8 photoperiod	48	2	1	2.1%	n/a (no T1 seed set)
Soybean VAE 2/25-2	VAE precultured on EJW1 with 8 g/L agarose I o/n at 15 C. dark	Dicot Bomb 13	EJW1 with 8 g/L agarose I o/n at 28 C. 16/8 photoperiod	43	2	2	4.7%	50%
Soybean VAE 2/25-3	VAE, precultured on EJW1 o/n at 15 C. dark	Dicot Bomb 20	EJW1 o/n at 28 C. 16/8 photoperiod	15	1	0	0.0%	n/a
Soybean VAE 2/25-4	VAE precultured on EJW1 with 8 g/L agarose I o/n at 15 C. dark	Dicot Bomb 20	EJW1 with 8 g/L agarose I o/n at 28 C. 16/8 photoperiod	15	1	1	6.7%	0%

TABLE 12

Transformation metrics for particle-mediated transformation with DICOTBOMB20 (At_dTP aadA1a) with 2X concentrated bead prep

Experiment	Preculture conditions	Construct	Rest conditions	Initial Explants on 150 ppm spec B5	Shoots harvested	T0 plants	TF	% Germline
Soybean VAE 2/25-5	VAE, precultured on EJW1 o/n at 15 C. dark	Dicot Bomb 20 "2X" (pellet resuspended in 18 uL EtOH)	EJW1 o/n at 28 C. 16/8 photoperiod	35	1	1	2.9%	100%

TABLE 12-continued

Transformation metrics for particle-mediated transformation with DICOTBOMB20 (At_dTP aadA1a) with 2X concentrated bead prep

Experiment	Preculture conditions	Construct	Rest conditions	Initial Explants on 150 ppm spec B5	Shoots harvested	T0 plants	TF	% Germline
Soybean VAE 2/25-6	VAE, precultured on EJW1 with 8 g/L agarose I o/n at 15 C. dark	Dicot Bomb 20 "2X" (pellet resuspended in 18 uL EtOH)	EJW1 with 8 g/L agarose I o/n at 28 C. 16/8 photoperiod	34	1	1	2.9%	100%

TABLE 13

GUS segregation data in T1 seedlings produced via particle bombardment using DICOTBOMB20 (At_dTP aadA1a) or DICOTBOMB 13 (cTP aadA1a)

Experiment	Line	Germ-plasm	Bomb	Germline	Observed T1 GUS+	Observed T1 GUS−	Expected T1 GUS+	Expected T1 GUS−	Probability of chi square value
Soybean VAE 3/17-3	WP1521-1a	SSW82	Dicot Bomb 20	POS	1	10	8.25	2.75	0.0000
Soybean VAE 3/17-3	WP1521-2a	SSW82	Dicot Bomb 20	POS	1	1	1.5	0.5	0.4142
Soybean VAE 3/17-3	WP1521-8a	SSW82	Dicot Bomb 20	neg	0	14	10.5	3.5	0.0000
Soybean VAE 3/17-3B	WP1521-9a	SSW82	Dicot Bomb 20	POS	13	1	10.5	3.5	0.1228
Soybean VAE 3/17-4	WP1521-5a	SSW82	Dicot Bomb 20	POS	6	6	9	3	0.0455
Soybean VAE 3/17-4	WP1521-10a	SSW82	Dicot Bomb 20	POS	5	9	10.5	3.5	0.0007
Soybean VAE 3/17-4	WP1521-13a	SSW82	Dicot Bomb 20	no T1 seed set	n/a	n/a	n/a	n/a	n/a
Soybean VAE 2/25-1	WP1520-3a	SSW82	Dicot Bomb 13	no T1 seed set	n/a	n/a	n/a	n/a	n/a
Soybean VAE 2/25-2	WP1520-4a	SSW82	Dicot Bomb 13	neg	0	11	8.25	2.75	0.0000
Soybean VAE 2/25-2	WP1520-5a	SSW82	Dicot Bomb 13	POS	7	2	6.75	2.25	0.8474
Soybean VAE 2/25-4	WP1521-6a	SSW82	Dicot Bomb 20	neg	0	16	12	4	0.0000
Soybean VAE 2/25-5	WP1521-3a	SSW82	Dicot Bomb 20 (2X)	POS	12	1	9.75	3.25	0.1495
Soybean VAE 2/25-6	WP1521-7a	SSW82	Dicot Bomb 20 (2X)	POS	11	5	12	4	0.5637

Example 2—Alternative Transit Peptides in Soybean (*Glycine max*, L.) Transformation with Hra/Imazapyr Selection

[0089] Successful transformation using imazapyr selection has been reported in soybean meristems using particle bombardment (*Theon Appl Genet* (2000) 101:1-6). We had previously run a selection kill curve on non-transformed soybean VAEs that indicated that imazapyr could be used as a relatively efficient selection agent. Thus, we tested whether we could transform our re-dried soybean VAE meristem explants with *Agrobacterium* using imazapyr selection with the alternative transit peptide At_dTP. In a pilot study, we tested a large range of imazapyr concentrations, and we found that it was advantageous to use lower concentrations of this herbicide (FIG. **5**). Table 14 presents a summary of the data generated in the pilot study, and Table 15 presents the data generated in a follow-up study using lower imazapyr concentrations. Consistent with the aadA1a/spectinomycin selection experiments described in Example 1, only the At_dTP targeting peptide enhances hra/imazapyr selection.

TABLE 14

Transformation metrics for the pilot study

Binary	Subcellular location of HRA	Imazapyr Selection Levels (mg/L)	Regenerated Explants	RFP+ shoots	T0 plants to GH	Putative TF
DB28	cytosol	0.2-20	887	0	0	0
DB29	Ph_cTP (chlor)		878	0	0	0
DB30	At_dTP (chlor + mito)		867	10	7	0.8%

TABLE 15

Transformation metrics for the follow-up study

Binary	Subcellular location of HRA	Imazapyr Selection Levels (mg/L)	Regenerated Explants	RFP+ shoots	T0 plants to GH	Putative TF
DB28	cytosol	1-2.5	257	0	0	0
DB29	Ph_cTP (chlor)		269	0	0	0
DB30	At_dTP (chlor + mito)		270	8	6	2.2%

[0090] For the first T0 plant we recovered from transforming soybean VAE with imazapyr selection, 5 mg/L imazapyr was used during regeneration. We designated this plant WP1266-1a. WP1266-1a has tested positive for hra by PCR and is both tdTomato-ER-positive and GUS-positive in leaf samples and in vascular bundles of a petiole section, which indicates germline transmission (FIG. **6**). We confirmed germline transmission of this event by germinating several T1 seeds and examining T1 leaves for both GUS and tdTomato-ER expression (FIG. **7**, FIG. **8**).

[0091] In ongoing work, we are optimizing the imazapyr selection protocol using tdTomato-ER to allow for shoot elongation in positive shoots and to suppress shooting in negative explants. The following protocol appears to promote shooting in tdTomato-ER-positive explants (FIG. **9**A) and rooting in the presence of imazapyr appears to be an effective screen for selecting germline transformants.

[0092] 1 ppm imazapyr B5 for 3-4 weeks

[0093] 1 ppm imazapyr B5 for 3-4 weeks

[0094] 0.5 ppm imazapyr B5 for 2-4 weeks

[0095] Rooting on 0.025 ppm imazapyr BRM

[0096] We tested this optimized imazapyr selection protocol, with and without TDZ, in co-culture using Dicot Binary 30 (At_dTP hra). The results are provided in Table 16. Twelve T1 seed from each line generated from this lead imazapyr protocol were surface sanitized in 20% Clorox for 5 minutes, rinsed, then plated on B5 media. T1 leaves were assayed for GUS expression to determine germline status (Table 17, FIG. **9**B). Events WP1266-37 through 44, and WP1266-48, -50, and -51 were generated from the 0 ppm TDZ co-culture treatment, while events WP1266-45 through 47, and WP1266-49 were generated from the 1 ppm TDZ co-culture treatment.

TABLE 16

Transformation and T1 metrics for generated using the optimized imazapyr selection protocol and DICOTBINARY30 (At_dTP hra)

Treatment	Binary	Explants inoculated w/optimized protocol	T0 plants to GH with GUS+ roots	TF	% Germline
0 ppm TDZ in co-culture	DICOT BINARY 30	250	11	4.4%	73%
1 ppm TDZ in co-culture	DICOT BINARY 30	225	4	1.8%	75%

TABLE 17

GUS segregation data for T1 seedlings produced using the optimized imazapyr selection protocol

Line	Germplasm	Strain	Binary	Germline	Observed T1 GUS+	Observed T1 GUS−	Expected T1 GUS+	Expected T1 GUS−	Probability of chi square value
WP1266-37	SSW82	Ar18r12v	Dicot Binary 30	POS	5	2	5.25	1.8125	0.8596
WP1266-38	SSW82	Ar18r12v	Dicot Binary 30	POS	12	0	9	2.25	0.0714
WP1266-39	SSW82	Ar18r12v	Dicot Binary 30	neg	0	9	6.75	3.9375	0.0003
WP1266-40	SSW82	Ar18r12v	Dicot Binary 30	neg	0	7	5.25	3.0625	0.0013
WP1266-41	SSW82	Ar18r12v	Dicot Binary 30	POS	9	3	9	3	1.0000
WP1266-42	SSW82	Ar18r12v	Dicot Binary 30	POS	5	3	6	2.25	0.5186
WP1266-43	SSW82	Ar18r12v	Dicot Binary 30	neg	0	8	6	3.5	0.0006
WP1266-44	SSW82	Ar18r12v	Dicot Binary 30	POS	9	1	7.5	2.125	0.3440
WP1266-48	SSW82	Ar18r12v	Dicot Binary 30	POS	9	3	9	3	1.0000
WP1266-50	SSW82	Ar18r12v	Dicot Binary 30	POS	8	4	9	3.25	0.5940
WP1266-51	SSW82	Ar18r12v	Dicot Binary 30	POS	8	1	6.75	1.9375	0.4078
WP1266-45	SSW82	Ar18r12v	Dicot Binary 30	POS	10	2	9	2.75	0.5742
WP1266-46	SSW82	Ar18r12v	Dicot Binary 30	POS	8	3	8.25	2.8125	0.8873

TABLE 17-continued

GUS segregation data for T1 seedlings produced using the optimized imazapyr selection protocol

Line	Germplasm	Strain	Binary	Germline	Observed T1 GUS+	Observed T1 GUS−	Expected T1 GUS+	Expected T1 GUS−	Probability of chi square value
WP1266-47	SSW82	Ar18r12v	Dicot Binary 30	POS	7	2	6.75	2.1875	0.8735
WP1266-49	SSW82	Ar18r12v	Dicot Binary 30	neg	0	12	9	5.25	0.0000

[0097] Ongoing experiments also include screening shoots by rooting them in the presence of selection to avoid selecting L1 chimeras/plants transformed in only epidermal layers that will not give rise to T1 positive seed. We are using the confirmed germline event WP1266-1a as a positive control in this effort (FIG. **10**). Based on the negative control results, concentrations of imazapyr less than or equal to 0.01 ppm appeared to be insufficient to suppress rooting in non-transformed soybean, while in the positive control we were able to obtain rooting in the 0.025-0.1 ppm imazapyr range. To account for segregation, we assayed leaves from these plants to ensure that rooting events were GUS-positive and that non-rooting plants consisted of nulls. We found a good correlation between robust rooting and GUS expression (Table 18).

TABLE 18

Rooting in presence of various concentrations of imazapyr and leaf GUS expression in segregating T1 progeny of WP1266-1a

Imazapyr concentration in rooting media	Plant	Presence of roots	Leaf GUS expression
0.025 ppm	A	very small roots	GUS negative
0.025 ppm	B	robust roots	GUS positive
0.025 ppm	C	no roots	GUS negative
0.05 ppm	D	no root	GUS negative
0.05 ppm	E	no roots	GUS negative
0.05 ppm	F	robust roots	GUS positive
0.1 ppm	G	robust roots	GUS positive
0.1 ppm	H	no roots	GUS negative
0.1 ppm	I	robust roots	GUS positive

[0098] The DICOTBINARY30 construct comprises the At_dTP targeting peptide fused to tdTomato-ER. Thus, using the confirmed germline event WP1266-1a, we should be able to image tdTomato-ER to confirm its expression in the mitochondria and chloroplasts using confocal microscopy.

Example 3—Alternative Transit Peptides in Cowpea (*Vigna unguiculata*, L.) Transformation with aadA1a/Spectinomycin Selection

[0099] We ran a single experiment comparing the ability of DICOTBINARY19 (cTP aadA) and DICOTBINARY22 (At_dTP aadA) to increase transformation efficiencies in cowpea meristem explants of the variety IT86-1010D. Cowpea transformation was performed as described in US Patent Publication No. US2019/0211347, which is hereby incorporated by reference in its entirety. We used the following protocol for cowpea meristem transformation:

[0100] Co-culture for 5 days with 1.75 ml INO with 1 ppm TDZ per plantcon

[0101] Embed explants (orienting apical meristems upward and radicals in media) directly after co-culture

[0102] Select using 150 mg/L spectinomycin (corrected for activity) post co-culture for 3-4 weeks

[0103] Transfer phenotype positive explants to 50 mg/L spectinomycin (active) for 3-4 weeks and then to non-selective B5

The results of this experiment are consistent with the soybean results. They demonstrate that At_dTP results in greater transformation frequency in cowpea as compared to cTP (Table 19, FIG. **11**).

TABLE 19

Transformation metrics for the cowpea study

Species	Genotype/Line	Strain	Binary	Subcellular location of aadA1a	Explants to 150 ppi spec B5 after co-culture	Greening explants transferred to 50 ppm spec B5 selection media	Greening explants transferred to B5	T0 plants to GH	TF
Cowpea	IT86D-1010	Ar18r12v	DB19	cTP	145	3	1	0	0.0%
Cowpea	IT86D-1010	Ar18r12v	DB22	At_dTP	155	4	3	2	1.3%

[0104] We imaged tdTomato-ER expression in the T0 cowpea events generated using DICOTBINARY22 (At_dTP aadA) (FIG. **12**). Both identified events, i.e., WP1492-1a and WP1492-2a, were tdTomato-ER positive. However, WP1492-1a showed more uniform expression and WP1492-2a showed chimeric expression. We also took petiole sections of these events and found that both tdTomato-ER and GUS are expressed in the vascular bundles (FIG. **13**), which is an early indicator of germline status. This result was confirmed by tdTomato-ER expression in T1 cowpea seeds (FIG. **14**, Table 20). These data demonstrate germline transformation of cowpea meristems using At_dTP aadA.

TABLE 20

tdTomato-ER segregation data in T1 seedlings produced using DICOTBINARY22 (At_dTP aadA)

Line	Germplasm	Strain	Binary	Germline	Observed T1 aadA+	Observed T1 aadA−	Expected T1 aadA+	Expected T1 aadA−	Probability of chi square value
WP1492-1	IT86D-1010	Ar18r12v	Dicot Binary 22	POS	20	12	24	8	0.1025
WP1492-2	IT86D-1010	Ar18r12v	Dicot Binary 22	POS*	0	32	24	8	0.0000

*2 T1 seeds identified positive for tdTomato-ER

Example 4—Alternative Transit Peptides in Hemp (*Cannabis sativa*, L.) Transformation with aadA1a/Spectinomycin Selection

[0105] We ran an experiment using *Cannabis* meristem explants of the variety Badger inoculated with Ar18r12v/DICOTBINARY22 (At_dTP aadA1a). *Cannabis* transformation was performed as described in US Patent Publication No. US2021/0071186, which is hereby incorporated by reference in its entirety. We used the following transformation protocol:

[0106] Imbibe seeds at 37° C. in bean germinating medium in the dark

[0107] Co-culture for 5 days in the dark with 1.25-1.5 ml INO with 1 ppm TDZ per plantcon

[0108] Select using 50 ppm spectinomycin (active) hemp node medium with 0.5 ppm meta-topolin

[0109] Root on WPM-based bean rooting media (WP1331-1a) or hemp rooting media (WP1331-2a)

The media used in this protocol are detailed in Tables 21-24, below.

TABLE 21

Bean germinating medium composition

Ingredients and Notes	Amount to add per liter (grams)
Phytotechnology Laboratories WPM L499	2.41
Sucrose	20
pH to 5.8 with 1N KOH and autoclave	
Add the following prior to use:	
Captan fungicide (50WP)	0.06
Bravo fungicide (Daconil) (82DP)	0.03
Cefotaxime (100 mg/ml)	1.25 ml

TABLE 22

Hemp node medium composition

Ingredients and Notes	Amount to add per liter (grams)
MS Salts complete with vitamins (PhytoTech M519)	4.43
Sucrose	30
Cleary's 3336	0.06
pH to 5.7 with 1N KOH	
Agar (Sigma A7921)	8
Autoclave	

TABLE 22-continued

Hemp node medium composition

Ingredients and Notes	Amount to add per liter (grams)
Meta-topolin (mT) (1 mg/ml)	0.5 ml
Carbenicillin (100 mg/ml)	4 ml
Cefotaxime (100 mg/ml)	2 ml
Timetin (150 mg/ml)	1 ml
Selection	as needed

TABLE 23

WPM-based bean rooting medium composition

Ingredients and Notes	Amount to add per liter (grams)
Phytotechnology Laboratories WPM L499	2.41
Myo-inositol	0.1
Sucrose	30
pH 5.8 with KOH	
Agar, Sigma A7921	8
Autoclave	
Add after autoclaving:	
Cysteine (100 mg/ml) - make stock fresh	Use at 1.0 ml per Liter (100 mg/L)
Cefotaxime (100 mg/ml)	Use at 2.0 ml per Liter (200 mg/L)
IAA (1 mg/ml)	Use at 0.1 ml per Liter (0.1 mg/L)
MS Vitamins (1000X)	Use at 1.0 ml per Liter
Selection	as needed

TABLE 24

Hemp rooting medium composition

Ingredients and Notes	Amount to add per liter (grams)
MS Salts complete with vitamins (PhytoTech M519)	2.215

TABLE 24-continued

Hemp rooting medium composition

Ingredients and Notes	Amount to add per liter (grams)
Sucrose	15
pH to 5.6 with KOH	
Agar (Sigma A7921)	8
Autoclave	
IBA stock (1 mg/ml - Ed has stock)	0.51 ml = 510 uL
Carbenicillin (100 mg/ml stock)	2.5 ml
Cefotaxime (100 mg/ml stock)	2 ml
Timentin (150 mg/ml stock)	1 ml
Selection	as needed

[0110] 47 explants were inoculated with 1.25 ml of the co-culture treatment, and this produced 1 T0 plant, WP1331-2a (TF 2.1%). 55 explants were inoculated with 1.5 ml of the co-culture treatment, and this also produced 1 T0 plant, WP1331-1a (TF 1.8%). The WP1331-1a T0 plant tested positive for aadA1a by PCR in ⅗ leaves. The WP1331-2a T0 plant expressed tdTomato-ER throughout the plant and was both tdTomato-ER-positive and GUS-positive in the vascular bundles of a petiole section (FIG. **15**).

[0111] We confirmed germline transmission of these events (FIG. **16**A). We then germinated the T1 seeds of these events on a liquid formulation of Hemp Node Media containing 25 mg/L spectinomycin to determine segregation frequencies (Table 25). Within 2 weeks, null T1 seedlings were bleached while transgenic T1 seedlings remained greening and expressing tdTomato-ER (FIG. **16**B).

TABLE 25

Segregation data for T1 seedlings of *Cannabis* lines produced using DICOTBINARY22 (At_dTP aadA1a)

Line	Germplasm	Strain	Binary	Germline	Observed T1 aadA+	Observed T1 aadA−	Expected T1 aadA+	Expected T1 aadA−	Probability of chi square value
WP1331-1	Badger	Ar18r12v	Dicot Binary 22	POS	3	8	5.5	5.5	0.1317
WP1331-2	Badger	Ar18r12v	Dicot Binary 22	POS	9	6	7.5	7.5	0.4386

Example 5—Alternative Transit Peptides in Chickpea (*Cicer arietinum*, L.) Transformation with aadA1a/Spectinomycin Selection

[0112] We ran experiments on chickpea meristem explants with DICOTBINARY19 (cTP aadA1a), DICOTBINARY21 (ER aadA1a), and DICOTBINARY22 (At_dTP aadA1a) using the following transformation protocol:

[0113] Remove shoot apical meristem (SAM) after excision prior to inoculation and co-culture

[0114] Co-culture for 5 days with 2.5 ml INO with 1 ppm TDZ per plantcon

[0115] Embed explants (orienting apical meristems upward and radicals in media) directly after co-culture and for subsequent transfers for greening, phenotype positive explants on spectinomycin

[0116] Select using 150 mg/L streptomycin post co-culture for 3-4 weeks

[0117] Transfer phenotype positive explants to 50 mg/L spectinomycin (active) for 3-4 weeks and then to non-selective B5 medium for 2 weeks

[0118] Harvest shoots onto 25 mg/L spectinomycin WPM-based rooting media at each stage if sufficiently elongated

[0119] Notably, we included ER-targeted aadA1a (DICOTBINARY21) in this study even though it did not increase transformation efficiency in soybean because the endoplasmic reticulum is associated with plastids in plant cells, and we hypothesized that placement of the spectinomycin degrading activity within the ER might protect to the associated plastids.

[0120] We obtained T0 plants that express tdTomato-ER in both leaves and roots that were generated using DICOTBINARY19, DICOTBINARY21, and DICOTBINARY22 and selected on 25 mg/L spectinomycin (FIG. **17**B,C; Table 26). Notably, the T0 plants obtained with DICOTBINARY22 were derived from later tests. These later tests involved varying the amount of TDZ used in the co-culture (Table 27) and using PEG as an osmotic agent when the chickpea shoot apical meristem (SAM) is removed prior to inoculation (Table 28). The expression of tdTomato-ER in chickpea roots is an early indicator of germline transformation using these three binaries.

TABLE 26

Transformation metrics for the initial chickpea experiment. 50 mg/L spectinomycin was used for the second selection and 25 mg/L spectinomycin was used in the rooting media.

Binary	Subcellular localization of aadA	Explants	RFP positive shoots	Shooting frequency (SF)	T0 plants	Transformation frequency (TF)
Dicot Binary 19	Chloroplast (cTP)	457	15	3.3%	6	1.3%
Dicot Binary 21	Endoplasmic reticulum (ER)	358	8	2.2%	2	0.6%
Dicot Binary 22	Mitochondria and Chloroplast (At_dTP)	469	21	4.5%	0	0.0%

TABLE 27

Transformation metrics for chickpea experiment in which the amount of TDZ used in co-culture was varied

Strain	Binary	Co-culture conditions	Explants	Shoots	Shooting frequency (SF)	T0 plants	TF
Ar18r12v	Dicot Binary 22	0 ppm TDZ	183	0	0.0%	0	0.0%
Ar18r12v	Dicot Binary 22	1 ppm TDZ	168	15	8.9%	4	2.4%
Ar18r12v	Dicot Binary 22	0 ppm TDZ	57	0	0.0%	0	0.0%
Ar18r12v	Dicot Binary 22	0.25 ppm TDZ	63	8	12.7%	2	3.2%
Ar18r12v	Dicot Binary 22	0.5 ppm TDZ	50	3	6.0%	1	2.0%
Ar18r12v	Dicot Binary 22	1 ppm TDZ	33	3	9.1%	0	0.0%

TABLE 28

Transformation metrics for chickpea experiment in which PEG was added as an osmotic agent after SAM removal

Strain	Binary	Pre-inoculation conditions	Explants	Shoots	Shooting frequency (SF)	T0 plants	TF
Ar18r12v	Dicot Binary 22	PEG	216	15	6.9%	3	1.4%
Ar18r12v	Dicot Binary 22	H2O	208	11	5.3%	1	0.5%

[0121] Using the optimized chickpea protocol, we observed an enrichment of T0 plants expressing tdTomato-ER and GUS in roots and in vascular tissue within petiole sections of chickpea (FIG. **18**). Expression of tdTomato-ER in roots and in vascular bundles of petiole section appears to be an indicator of germline status, as the T1 seed of WP1182-11a and -14a expressed tdTomato-ER in cotyledons (true T1 tissue) and maternal seed coats (T0 tissue), whereas the T1 seed of WP-1182-9a did not express tdTomato-ER in cotyledons and expression was limited to seed coat T0 tissue (FIG. **19**).

Example 6—Alternative Transit Peptides in Pinto Bean (*Phaseolus vulgaris*, L.) Transformation with aadA1a/Spectinomycin Selection

[0122] We ran experiments with DICOTBINARY19 (cTP aadA1a) and DICOTBINARY22 (At_dTP aadA1a) in pinto bean using the protocol provided in US Patent Publication No. 2019/0211347, which is hereby incorporated by reference in its entirety. We modified this protocol to add a vacuum infiltration step after the sonication step based on promising transient results in another variety of pinto bean. The results of this experiment are presented in Table 29 and FIG. **20**. These results demonstrate that both DICOTBINARY19 (cTP aadA1a) and DICOTBINARY22 (At_dTP aadA1a) were able to produce germline events in pinto bean.

TABLE 29

Transformation metrics for pinto bean experiment

Species	Genotype/Line	Explants to 25 mg/L spectinomycin B5	Strain	Binary	tdTom+ phenotypes to B5	Plants to GH	Putative TF
Dry Bean	Pinto	173	18r12v	Dicot Binary 19	7	4	2.3%
Dry Bean	Pinto	182	18r12v	Dicot Binary 22	11	3	1.6%

[0123] We took petiole sections of the transgenic pinto bean events to determine if we could detect GUS in the vascular bundles, an early indicator of germline transformation in our mature meristem-based system (FIG. **21**). The events WP1183-2a (DB19), WP1183-3a (DB19), and WP1823-2a (DB22) showed clear GUS expression in the vascular bundles. Weaker GUS expression, possibly in the vascular bundles, was detected in WP1183-5a (DB19). These events demonstrate that alternate transit peptides can be used to transform pinto bean with aadA. Notably, the event WP1183-4a appears to be epidermal, and WP1823-1a and -3a are likely chimeric, as the sampled petioles did not appear to express GUS.

Example 7—Alternative Transit Peptides in Pea (*Pisum sativum*, L.) Transformation with aadA1a/Spectinomycin Selection

[0124] We ran a transformation test with DICOTBINARY22 (At_dTP aadA1a) in pea meristems using a modified chickpea protocol. We recovered a tdTomato-ER positive shoot (FIG. **22**). Further tests are ongoing.

Example 8—Construct Design

[0125] T-DNA generation in *Agrobacterium* starts with a single stranded nick at the T-DNA right border, followed by covalent attachment of the virD2 protein to the nascent single-stranded T-DNA molecule. The full-length T-DNA is generated with a second nick at the T-DNA left border. The virD2::T-DNA is trafficked from the *Agrobacterium* cell into the plant cell, where the T-DNA is further trafficked to the plant cell nucleus, where the T-DNA is integrated into the plant genome. In a final genomic sequence resulting from a transgenic event, it is common to lose part of the transgene sequence or have some run-on (i.e., gain extra sequence) from the left gene border (see, e.g., Plant J (2018) doi: 10.1111/tpj.13992). Thus, the inventors have designed their constructs with the plant selection marker gene oriented such that its terminator is proximal to the T-DNA left border, and that transcription of the reporter gene is directed towards the T-DNA left border, as depicted schematically in FIG. **23**. With this orientation, even small truncations of the T-DNA at the left border are likely to result in an obvious phenotype (i.e., loss of selection marker gene expression) since the polyadenylation signal in the terminator might be lost. In the opposite scenario in which the promoter is located proximal to the left border, sequence truncations could produce a wide array of reporter gene phenotypes without fully eliminating selection marker gene expression. Further, when a promoter is located at the end of a T-DNA, its activity can be influenced by nearby genomic sequences. These variable results are undesirable in a transgenic plant production facility, which seeks to generate plants that express consistent levels of a reporter gene. By orienting the selection marker such that the terminator is proximal to the T-DNA left border, the chemical selection will eliminate those events in which truncations have rendered the selection marker transcriptional unit less than optimally functional. Pragmatically, this means that the transgenic plants which are produced will be those with robust expression of the selection marker gene. Since T-DNA truncation is mainly a left border phenomenon, one can infer, from robust selection marker gene expression, that the other transcriptional units included in the T-DNA will also be present.

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```
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        35                  40                  45

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    50                  55                  60

Pro Ser Thr Val Val Leu Pro Ser Asn Glu Ser Ser Asp Lys Leu Leu
65                  70                  75                  80

Lys Ile Arg His Thr Cys Ala His Val Met Ala Met Ala Val Gln Lys
                85                  90                  95

Leu Phe Pro Asp Ala Lys Val Thr Ile Gly Pro Trp Ile Asp Asn Gly
            100                 105                 110

Phe Tyr Tyr Asp Phe Asp Met Glu Pro Leu Thr Asp Lys Asp Leu Lys
        115                 120                 125

Arg Ile Lys Lys Glu Met Asp Arg Ile Ile Ser Arg Asn Leu Pro Leu
    130                 135                 140

Leu Arg Glu Glu Val Ser Arg Glu Glu Ala Lys Lys Arg Ile Met Ala
145                 150                 155                 160

Ile Asn Glu Pro Tyr Lys Met Glu Ile Leu Asp Gly Ile Lys Glu Glu
                165                 170                 175

Pro Ile Thr Val Tyr His Ile Gly Asn Glu Trp Trp Asp Leu Cys Ala
            180                 185                 190

Gly Pro His Val Glu Thr Thr Gly Lys Ile Asn Lys Lys Ala Val Glu
        195                 200                 205

Leu Glu Ser Val Ala Gly Ala Tyr Trp Arg Gly Asp Glu Lys Arg Gln
    210                 215                 220

Met Leu Gln Arg Ile Tyr Gly Thr Ala Trp Glu Ser Glu Glu Gln Leu
225                 230                 235                 240

Lys Ala Tyr Leu His Phe Lys Glu Glu Ala Lys Arg Arg Asp His Arg
                245                 250                 255

Arg Ile Gly Gln Asp Leu Asp Leu Phe Ser Ile Gln Asp Glu Ala Gly
            260                 265                 270

Gly Gly Leu Val Phe Trp His Pro Lys Gly Ala Ile Val Arg Asn Ile
        275                 280                 285

Ile Glu Glu Ser Trp Lys Lys Met His Val Glu His Gly Tyr Asp Leu
    290                 295                 300

Ile Tyr Thr Pro His Val Ala Lys Ala Asp Leu Trp Lys Ile Ser Gly
305                 310                 315                 320

His Leu Asp Phe Tyr Arg Glu Asn Met Tyr Asp Gln Met Glu Ile Glu
                325                 330                 335

Asp Glu Leu Tyr Gln Leu Arg Pro Met Asn Cys Pro Tyr His Ile Leu
            340                 345                 350

Leu Tyr Gln Arg Lys Arg Gln Ser Tyr Arg Asp Leu Pro Ile Arg Val
        355                 360                 365

Ala Glu Leu Gly Thr Val Tyr Arg Tyr Glu Leu Ser Gly Ser Leu His
    370                 375                 380

Gly Leu Phe Arg Val Arg Gly Phe Thr Gln Asp Asp Ala His Ile Phe
385                 390                 395                 400

Cys Leu Glu Asp Gln Ile Lys Asp Glu Ile Arg Gly Val Leu Asp Leu
                405                 410                 415

Thr Glu Glu Ile Leu Ser Arg Phe Gly Phe Asn Lys Tyr Glu Val Asn
            420                 425                 430
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Leu Ser Thr Arg Pro Glu Lys Ser Val Gly Gly Asp Asp Ile Trp Glu
        435                 440                 445

Lys Ala Thr Cys Ala Leu Arg Asp Ala Leu Asp Asp Lys Gly Trp Ser
    450                 455                 460

Tyr Glu Val Asp Glu Gly Gly Gly Ala Phe Tyr Gly Pro Lys Ile Asp
465                 470                 475                 480

Leu Lys Ile Glu Asp Ala Leu Gly Arg Lys Trp Gln Cys Ser Thr Ile
                485                 490                 495

Gln Val Asp Phe Asn Leu Pro Gln Arg Phe Asp Ile Thr Tyr Val Asp
            500                 505                 510

Thr Asn Ser Asp Lys Lys Arg Pro Ile Met Ile His Arg Ala Val Leu
        515                 520                 525

Gly Ser Leu Glu Arg Phe Phe Gly Val Leu Ile Glu His Tyr Ala Gly
    530                 535                 540

Asp Phe Pro Leu Trp Leu Ser Pro Val Gln Val Arg Val Leu Pro Val
545                 550                 555                 560

Thr Asp Asn Gln Leu Glu Phe Cys Lys Glu Val Ser Lys Lys Leu Arg
                565                 570                 575

Ala Cys Gly Val Arg Ala Glu Leu Cys His Gly Glu Arg Leu Pro Lys
            580                 585                 590

Leu Ile Arg Asn Ala Glu Thr Gln Lys Ile Pro Leu Met Ala Val Val
        595                 600                 605

Gly Pro Lys Glu Val Glu Thr Gly Thr Val Thr Val Arg Ser Arg Phe
    610                 615                 620

Gly Gly Glu Leu Gly Thr Ile Pro Val Asp Asp Leu Ile Asn Lys Ile
625                 630                 635                 640

Asn Ile Ala Val Glu Thr Arg Thr Ala Leu
                645                 650


<210> SEQ ID NO 2
<211> LENGTH: 1953
<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(1953)
<223> OTHER INFORMATION: DNA sequence encoding At2g04842.1 (SEQ ID NO:1)

<400> SEQUENCE: 2

atggcctctt ctcattctct tctcttttct tcttccttcc tctctaaacc ctcttctttc     60

acttcttctc tgagacggtt tgtttacctt cccactcgcc aattttggcc aaggcagcga    120

catggtttct ctaccgtctt cgccgtcgcc accgagccag ctatctccag cagcggtcct    180

aagaaagcag agccatcgac ggtcgtgctt cctagtaatg aatcatctga taagcttctc    240

aaaattcgac acacgtgtgc gcatgtaatg gcaatggcag ttcagaaact cttcccggat    300

gcaaaagtga cgataggtcc ttggattgat aatggtttct actatgattt tgatatggag    360

cctttaacag ataaagacct taagaggatc aagaaagaga tggatcgtat cattagtcgg    420

aatttaccac ttttaagaga agaagtatct agagaagaag cgaagaaaag gatcatggcg    480

ataaatgagc cttataagat ggagattttg gatggtatta aggaagaacc catcaccgtt    540

tatcatatcg gtaatgaatg gtgggatctt tgtgcgggtc ctcatgtcga gaccacaggg    600

aagataaaca agaaagcagt tgaactagaa tctgttgctg gtgcatactg gagaggagat    660
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gaaaagaggc aaatgcttca gaggatatac ggaacagctt gggagagcga agagcaactg 720

aaagcatatc ttcattttaa agaagaagct aaacgcaggg atcatagacg catcggccaa 780

gaccttgatc tgttttctat ccaggatgaa gctggaggag ggttagtgtt ttggcatccg 840

aaaggtgcaa ttgttagaaa cataattgaa gaatcgtgga agaaaatgca tgttgaacat 900

ggttatgatt tgatctatac tcctcatgtt gccaaagctg acctgtggaa gatcagtggt 960

cacttggact tctacagaga gaatatgtat gatcaaatgg agatcgagga tgagctttat 1020

caactccgac caatgaattg cccttaccac atcttgcttt accaaagaaa gcgtcaatca 1080

tatcgagatt tgcccataag agttgcagag ctcggtacag tttacagata tgagttgtct 1140

ggtagcttgc acggtctctt ccgtgtcaga gggttcacac aggacgatgc acacatattt 1200

tgtttagagg atcagatcaa agatgagatc agaggtgttc tagatctcac agaagagata 1260

ttgtcgaggt ttggattcaa caagtatgag gttaacttat caactagacc tgaaaaatca 1320

gttggaggtg atgatatatg ggagaaggcg acctgtgccc ttagagatgc tttagatgat 1380

aagggatgga gctatgaggt tgacgaagga ggtggcgcct tttacggccc aaagatcgac 1440

cttaagattg aagatgcatt gggtagaaaa tggcagtgct caactattca ggtcgacttc 1500

aatcttcctc agagatttga cattacctat gtagacacaa actcagataa gaaacgtcct 1560

ataatgatcc acagagcggt tttaggatca ttagaaagat ttttcggtgt tctcatagaa 1620

cactacgcag gggatttccc gttatggctc tctcctgttc aagtccgggt cttacctgtc 1680

acagacaatc agttggagtt ttgtaaggaa gtgagtaaga aactgagagc ttgtggagtg 1740

agggctgagt tgtgccatgg ggagagactg cctaagctca taaggaacgc agagacgcag 1800

aagatcccgt taatggcggt tgttggaccc aaagaagtcg agaccggaac tgttaccgtt 1860

agatcgaggt ttggaggaga gcttggaacc attcctgttg atgacctcat caacaaaatc 1920

aacattgcag ttgaaaccag aactgctctg taa 1953

<210> SEQ ID NO 3
<211> LENGTH: 65
<212> TYPE: PRT
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (1)..(65)
<223> OTHER INFORMATION: At_dTP - Dual targeting peptide comprising the first 65 amino acids of SEQ ID NO:1

<400> SEQUENCE: 3

Met Ala Ser Ser His Ser Leu Leu Phe Ser Ser Ser Phe Leu Ser Lys
1 5 10 15

Pro Ser Ser Phe Thr Ser Ser Leu Arg Arg Phe Val Tyr Leu Pro Thr
20 25 30

Arg Gln Phe Trp Pro Arg Gln Arg His Gly Phe Ser Thr Val Phe Ala
35 40 45

Val Ala Thr Glu Pro Ala Ile Ser Ser Ser Gly Pro Lys Lys Ala Glu
50 55 60

Pro
65

<210> SEQ ID NO 4
<211> LENGTH: 195

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<212> TYPE: DNA
<213> ORGANISM: Arabidopsis thaliana
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(195)
<223> OTHER INFORMATION: DNA sequence encoding At_dTP (SEQ ID NO:3)

<400> SEQUENCE: 4

atggcctctt ctcattctct tctcttttct tcttccttcc tctctaaacc ctcttctttc 60

acttcttctc tgagacggtt tgtttacctt cccactcgcc aattttggcc aaggcagcga 120

catggtttct ctaccgtctt cgccgtcgcc accgagccag ctatctccag cagcggtcct 180

aagaaagcag agcca 195

<210> SEQ ID NO 5
<211> LENGTH: 195
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic - Soybean codon optimized DNA sequence encoding At_dTP (SEQ ID NO:3)

<400> SEQUENCE: 5

atggcttcca gtcattcact gctttttttct tcttctttcc tgtcaaaacc tagctccttc 60

acttccagcc ttcgccgttt tgtgtatctt cctacaagac aattttggcc caggcaacgt 120

catggcttta gtaccgtttt cgccgtcgca accgaacccg ccatatcatc atccgggcca 180

aagaaagccg aacca 195

<210> SEQ ID NO 6
<211> LENGTH: 235
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic - Cloning construct comprising the codon optimized DNA sequence of SEQ ID NO:5

<400> SEQUENCE: 6

ttcgaattcg gtctcaccat ggcttccagt cattcactgc ttttttcttc ttctttcctg 60

tcaaaaccta gctccttcac ttccagcctt cgccgttttg tgtatcttcc tacaagacaa 120

ttttggccca ggcaacgtca tggctttagt accgttttcg ccgtcgcaac cgaacccgcc 180

atatcatcat ccgggccaaa gaaagccgaa ccaggaatga gagaccaagc ttgaa 235

<210> SEQ ID NO 7
<211> LENGTH: 652
<212> TYPE: PRT
<213> ORGANISM: Glycine max
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (1)..(652)
<223> OTHER INFORMATION: Glyma.19G191200.1 - Soybean homolog of At2g04842.1 threonyl-tRNA synthetase

<400> SEQUENCE: 7

Met Ala Thr Phe Ala Val Ala Thr Thr Leu Ser Thr Ser Ser Ile Ala
1 5 10 15

Pro Leu Asn Ala Arg Arg Gly Phe Gly Phe Gly Phe Gly Ser Phe Leu
20 25 30

Ser Pro Thr Thr Lys Pro Lys Arg Pro Ser Ile Thr Thr Thr Leu Ala
35 40 45

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Val Ala Thr Asp Ser Ser Val Cys Phe Asn Pro Asn Pro Asn Pro Asn
    50                  55                  60

Pro Asn Pro Asn Gln Leu Val Leu Pro Ser Asn Glu Ser Ser Glu Met
65                  70                  75                  80

Leu Leu Arg Ile Arg His Thr Ser Ala His Val Met Ala Met Ala Val
                85                  90                  95

Gln Lys Leu Tyr Pro Asp Ala Lys Val Thr Ile Gly Pro Trp Ile Glu
            100                 105                 110

Asn Gly Phe Tyr Tyr Asp Phe Asp Met Glu Pro Leu Thr Asp Lys Asp
        115                 120                 125

Leu Lys Arg Ile Lys Lys Glu Met Asp Arg Ile Ile Gly Lys Asn Leu
    130                 135                 140

Pro Leu Val Arg Glu Glu Val Ser Arg Gly Glu Ala His Arg Arg Ile
145                 150                 155                 160

Ser Ala Leu Asn Glu Pro Tyr Lys Leu Glu Ile Leu Glu Ser Ile Lys
                165                 170                 175

Asp Asp Pro Ile Thr Ile Tyr His Ile Gly Asp Glu Trp Trp Asp Leu
            180                 185                 190

Cys Ala Gly Pro His Val Asp Ser Thr Gly His Ile Asn Lys Lys Ala
        195                 200                 205

Val Glu Leu Glu Ser Ile Ala Gly Ala Tyr Trp Arg Gly Asp Glu Arg
    210                 215                 220

Lys Pro Met Leu Gln Arg Ile Tyr Gly Thr Ala Trp Glu Asn Glu Glu
225                 230                 235                 240

Gln Leu Lys Ala Tyr Leu His Phe Lys Glu Glu Ala Lys Arg Arg Asp
                245                 250                 255

His Arg Arg Leu Gly Gln Asp Leu Asp Leu Phe Ser Ile Gln Asp Asp
            260                 265                 270

Ala Gly Gly Gly Leu Val Phe Trp His Pro Lys Gly Ala Met Val Arg
        275                 280                 285

His Ile Ile Glu Asp Phe Trp Lys Lys Ile His Met Lys Arg Gly Tyr
    290                 295                 300

Asp Leu Leu Tyr Thr Pro His Val Ala Lys Ala Asp Leu Trp Lys Ile
305                 310                 315                 320

Ser Gly His Leu Asp Phe Tyr Lys Glu Asn Met Tyr Asp Gln Met Ser
                325                 330                 335

Val Glu Asp Glu Leu Tyr Gln Leu Arg Pro Met Asn Cys Pro Tyr His
            340                 345                 350

Ile Leu Val Tyr Lys Ser Lys Leu His Ser Tyr Arg Asp Phe Pro Ile
        355                 360                 365

Arg Val Ala Glu Leu Gly Thr Val Tyr Arg Tyr Glu Leu Ser Gly Ser
    370                 375                 380

Leu His Gly Leu Phe Arg Val Arg Gly Phe Thr Gln Asp Asp Ala His
385                 390                 395                 400

Ile Phe Cys Leu Asp Asp Gln Ile Lys Asp Glu Ile Arg Gly Val Leu
                405                 410                 415

Asp Leu Thr Glu Glu Ile Leu Leu Gln Phe Gly Phe Glu Lys Tyr Glu
            420                 425                 430

Val Asn Leu Ser Thr Arg Pro Glu Lys Ala Val Gly Asp Asp Asp Ile
        435                 440                 445
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Trp Glu Lys Ala Thr Ser Ala Leu Glu Asp Ala Leu Asp Asp Lys Gly
    450                 455                 460

Trp Thr Tyr Gln Ile Asp Asp Gly Gly Gly Ala Phe Tyr Gly Pro Lys
465                 470                 475                 480

Val Asp Ile Lys Ile Glu Asp Ala Leu Gly Arg Lys Trp Gln Cys Ser
                485                 490                 495

Thr Ile Gln Val Asp Phe Asn Leu Pro Gln Arg Phe Asp Ile Thr Tyr
            500                 505                 510

Val Asp Ser Asn Thr Glu Lys Arg Arg Pro Ile Met Ile His Arg Ala
        515                 520                 525

Val Leu Gly Ser Leu Glu Arg Phe Phe Gly Ile Leu Ile Glu His Tyr
    530                 535                 540

Ala Gly Asp Phe Pro Leu Trp Leu Ser Pro Ile Gln Ala Arg Val Leu
545                 550                 555                 560

Pro Val Thr Asp Ala Gln Leu Glu Tyr Cys Asn Val Val Thr Asn Lys
                565                 570                 575

Leu Lys Met Tyr Gly Ile Arg Ala Glu Val Cys His Gly Glu Arg Leu
            580                 585                 590

Pro Lys Leu Ile Arg Asn Ala Glu Lys Gln Lys Ile Pro Leu Met Val
        595                 600                 605

Val Val Gly Ser Lys Glu Val Glu Thr Glu Thr Val Thr Val Arg Ser
    610                 615                 620

Arg Phe Gly Gly Glu Leu Gly Thr Met Pro Val Asp Asp Phe Ile Asp
625                 630                 635                 640

Arg Ile Lys Leu Gly Thr Glu Asn Pro Ile Ser Leu
                645                 650


<210> SEQ ID NO 8
<211> LENGTH: 1959
<212> TYPE: DNA
<213> ORGANISM: Glycine max
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(1959)
<223> OTHER INFORMATION: DNA sequence encoding Glyma.19G191200.1 (SEQ ID
      NO:7)

<400> SEQUENCE: 8

atggctacct tcgcggttgc aacaacactt tcgacatctt ctatcgctcc ccttaatgca     60

cgacgtggtt ttggttttgg ttttggctcc ttcctttctc caaccacaaa accaaaacgc    120

ccctcaatca ccactaccct cgctgtcgcc accgactctt ccgtttgttt caatcccaac    180

cccaacccta accctaatcc caatcagctt gttctccctt ccaacgaatc atcggagatg    240

cttctcagaa tccgccacac gagtgcgcat gtgatggcaa tggctgttca aaagctttac    300

cctgatgcaa aagtcacaat tgggccttgg atagaaaatg ggttttatta tgacttcgac    360

atggagcctt tgactgataa agatttgaag agaattaaga aagagatgga tcgaattatt    420

ggaaaaaatt tgccacttgt aagagaagaa gtttcaagag gcgaagctca tagaagaata    480

tcggctctga atgaaccata caagttggag attttggaaa gcattaagga tgatcccatc    540

accatttatc acatcggtga tgagtggtgg gacctctgtg ctggacctca tgttgattct    600

actggacata tcaataagaa agcagttgaa cttgagtcta ttgctggtgc atactggaga    660

ggagatgaaa ggaagccaat gttgcaacgg atatacggta ctgcatggga gaatgaagag    720
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cagttgaaag cttaccttca cttcaaagag gaagccaagc gtcgagatca caggcgccta 780

gggcaagatc ttgatttgtt ttctatacag gatgatgctg gtgggggtct agttttctgg 840

cacccaaagg gtgctatggt cagacacata attgaagatt tctggaaaaa aattcacatg 900

aaacgtggct atgatctact gtacacaccg catgtggcaa aagctgatct ttggaagatc 960

agtggtcatt tggatttcta caaagagaat atgtatgatc agatgagcgt tgaggatgag 1020

ctttatcagc ttcggccaat gaattgtcct tatcatattt tggtgtacaa aagcaagctt 1080

cattcctatc gtgatttccc tatcagggta gcagaattag gaactgttta tagatatgaa 1140

ctatctggaa gtttacatgg ccttttccgt gttagaggtt tcacacagga tgacgcacat 1200

atattttgtt tagatgacca gattaaagat gagatcaggg gtgtcctaga tcttactgaa 1260

gaaatactat tgcaatttgg ttttgaaaag tatgaagtaa atttatctac aaggcctgaa 1320

aaggctgtgg gagacgatga tatatgggaa aaagctacct ctgctttaga agatgctttg 1380

gatgataaag gttggaccta tcaaattgat gatggaggag gtgcctttta tgggccaaag 1440

gttgatatca agattgagga tgctcttggt aggaagtggc aatgctcaac catacaggtt 1500

gatttcaatc taccacagcg ttttgacatc acatatgttg actcaaatac tgagaaaagg 1560

aggcctataa tgatccatag agcagtgctt ggatcattgg aaagattctt tggcattctc 1620

attgagcatt atgctggtga ttttccatta tggctttctc caatacaagc tcgagtttta 1680

cctgttactg atgcccagct tgaatattgc aatgttgtga ccaacaaact taaaatgtat 1740

ggcatccgag ctgaagtttg ccatggtgag cgcctaccta aactaataag aaatgcagag 1800

aagcagaaaa ttccattaat ggttgttgtg ggctcaaagg aagttgaaac agaaacagtc 1860

actgttagat ctaggtttgg tggggagctt ggaaccatgc cagttgatga ttttattgac 1920

agaataaagt tgggaacaga aaacccaata tcattatga 1959

<210> SEQ ID NO 9
<211> LENGTH: 65
<212> TYPE: PRT
<213> ORGANISM: Glycine max
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<222> LOCATION: (1)..(65)
<223> OTHER INFORMATION: Gm_dTP - Dual targeting peptide comprising the first 65 amino acids of SEQ ID NO:7

<400> SEQUENCE: 9

Met Ala Thr Phe Ala Val Ala Thr Thr Leu Ser Thr Ser Ser Ile Ala
1 5 10 15

Pro Leu Asn Ala Arg Arg Gly Phe Gly Phe Gly Phe Gly Ser Phe Leu
20 25 30

Ser Pro Thr Thr Lys Pro Lys Arg Pro Ser Ile Thr Thr Thr Leu Ala
35 40 45

Val Ala Thr Asp Ser Ser Val Cys Phe Asn Pro Asn Pro Asn Pro Asn
50 55 60

Pro
65

<210> SEQ ID NO 10
<211> LENGTH: 195
<212> TYPE: DNA
<213> ORGANISM: Glycine max
<220> FEATURE:

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<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(195)
<223> OTHER INFORMATION: DNA sequence encoding Gm_dTP (SEQ ID NO:9)

<400> SEQUENCE: 10

atggctacct tcgcggttgc aacaacactt tcgacatctt ctatcgctcc ccttaatgca 60

cgacgtggtt ttggttttgg ttttggctcc ttcctttctc caaccacaaa accaaaacgc 120

ccctcaatca ccactaccct cgctgtcgcc accgactctt ccgtttgttt caatcccaac 180

cccaacccta accct 195

<210> SEQ ID NO 11
<211> LENGTH: 235
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic - Cloning construct comprising the codon optimized DNA sequence of SEQ ID NO:10

<400> SEQUENCE: 11

ttcgaattcg gtctcaccat ggctaccttc gcggttgcaa caacactttc gacatcttct 60

atcgctcccc ttaatgcacg acgtggtttt ggttttggtt ttggctcctt cctttctcca 120

accacaaaac caaaacgccc ctcaatcacc actaccctcg ctgtcgccac cgactcttcc 180

gtttgtttca atcccaaccc caaccctaac cctggaatga gagaccaagc ttgaa 235

<210> SEQ ID NO 12
<211> LENGTH: 792
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic - Soybean codon optimized sequence of the aadA1 gene

<400> SEQUENCE: 12

atgagagaag ctgttattgc tgaagtttct actcaacttt ctgaagttgt tggagttatt 60

gaaagacatc ttgaacctac tcttcttgct gttcatcttt atggatctgc tgttgatgga 120

ggacttaagc cacattctga tattgatctt cttgttactg ttactgttag acttgatgaa 180

actactagaa gagctcttat taatgatctt cttgaaactt ctgcttctcc aggagaatct 240

gaaattctta gagctgttga agttactatt gttgttcatg atgatattat tccatggaga 300

tatccagcta agagagaact tcaatttgga gaatggcaaa gaaatgatat tcttgctgga 360

atttttgaac cagctactat tgatattgat cttgctattc ttcttactaa ggctagagaa 420

cattctgttg ctcttgttgg accagctgct gaagaacttt ttgatccagt tccagaacaa 480

gatctttttg aagctcttaa tgaaactctt actctttgga attctccacc agattgggct 540

ggagatgaaa gaaatgttgt tcttactctt tctagaattt ggtattctgc tgttactgga 600

aagattgctc caaaggatgt tgctgctgat tgggctatgg aaagacttcc agctcaatat 660

caaccagtta ttcttgaagc tagacaagct tatcttggac aagaagaaga tagacttgct 720

tctagagctg atcaacttga agaatttgtt cattatgtta agggagaaat tactaaggtt 780

gttggaaagt ga 792

<210> SEQ ID NO 13
<211> LENGTH: 829
<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic - Cloning construct comprising the codon optimized sequence of SEQ ID NO:12

<400> SEQUENCE: 13

ttcggtaccg gtctcaaatg agagaagctg ttattgctga agtttctact caactttctg 60

aagttgttgg agttattgaa agacatcttg aacctactct tcttgctgtt catctttatg 120

gatctgctgt tgatggagga cttaagccac attctgatat tgatcttctt gttactgtta 180

ctgttagact tgatgaaact actagaagag ctcttattaa tgatcttctt gaaacttctg 240

cttctccagg agaatctgaa attcttagag ctgttgaagt tactattgtt gttcatgatg 300

atattattcc atggagatat ccagctaaga gagaacttca atttggagaa tggcaaagaa 360

atgatattct tgctggaatt tttgaaccag ctactattga tattgatctt gctattcttc 420

ttactaaggc tagagaacat tctgttgctc ttgttggacc agctgctgaa gaacttttg 480

atccagttcc agaacaagat ctttttgaag ctcttaatga aactcttact ctttggaatt 540

ctccaccaga ttgggctgga gatgaaagaa atgttgttct tactctttct agaatttggt 600

attctgctgt tactggaaag attgctccaa aggatgttgc tgctgattgg gctatggaaa 660

gacttccagc tcaatatcaa ccagttattc ttgaagctag acaagcttat cttggacaag 720

aagaagatag acttgcttct agagctgatc aacttgaaga atttgttcat tatgttaagg 780

gagaaattac taaggttgtt ggaaagtgag cttagagacc gcatgcctt 829

What is claimed:

1. A DNA construct comprising a first expression cassette comprising a first heterologous promoter operably linked to a sequence encoding a transit peptide and a target protein, wherein the transit peptide is at least 90% identical to SEQ ID NO:3 or SEQ ID NO:9.

2. The DNA construct of claim **1**, wherein the sequence encoding the transit peptide is translationally fused to the sequence encoding the target protein.

3. The DNA construct of claim **1**, wherein the transit peptide is SEQ ID NO:3 or SEQ ID NO:9.

4. The DNA construct of claim **1**, wherein the target protein is a selectable marker or a reporter gene.

5. The DNA construct of claim **4**, wherein the selectable marker confers resistance to an antibiotic or an herbicide.

6. The DNA construct of claim **5**, wherein the selectable marker is encoded by SEQ ID NO:12 or SEQ ID NO:13.

7. The DNA construct of claim **1**, further comprising a second expression cassette comprising a second heterologous promoter.

8. The DNA construct of claim **1**, wherein the DNA construct comprises SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:10, or SEQ ID NO:11.

9. A vector comprising the DNA construct of claim **1**.

10. The vector of claim **9**, wherein the vector is a T-DNA vector for *agrobacterium*-mediated transformation.

11. The vector of claim **9** formulated for gene gun transformation.

12. A plant cell comprising the DNA construct of claim **1**.

13. The plant cell of claim **12**, wherein the plant cell expresses the transit peptide and the target protein as a fusion protein.

14. The plant cell of claim **13**, wherein the fusion protein is targeted to the mitochondria and the chloroplasts within the plant cell.

15. The plant cell of claim **12**, wherein the plant cell is a soybean, cowpea, hemp, chickpea, pinto bean, or pea plant cell.

16. A method of targeting a target protein to the chloroplasts and mitochondria of a plant cell, the method comprising: transforming the plant cell with a construct comprising a heterologous promoter operably linked to a sequence encoding a transit peptide and the target protein, wherein the transit peptide is at least 90% identical to SEQ ID NO:3 or SEQ ID NO:9.

17. A method of producing a transgenic plant, the method comprising:

a) transforming an explant with a construct comprising a heterologous promoter operably linked to a sequence encoding a transit peptide and a selectable marker, wherein the transit peptide is at least 90% identical to SEQ ID NO:3 or SEQ ID NO:9;

b) contacting the explant with a selective agent; and

c) regenerating a transformed plant from the transgenic cells;

wherein the selectable marker confers an ability to grow in the presence of the selective agent.

18. The method of claim **17**, wherein the selectable marker is encoded by the aadA1a gene and the selective agent is spectinomycin or wherein the selectable marker is encoded by the hra gene and the selective agent is imazapyr.

19. The method of claim **17**, wherein the plant is a dicot.

20. The method of claim **17**, wherein the plant is a monocot.

21. The method of claim **17**, wherein the construct further comprises a second heterologous promoter operably linked to a sequence that encodes a target protein.

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