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(54) Title: SITES FOR PLASTID TRANSFORMATION

(57) Abstract: Compositions and methods for plastid transformation and regeneration or development of transplastomic plants, plant cells, plant parts, and seeds are provided. Target sequences for plastid transformation, together with defined sequences of a first homology arm and a second homology arm are provided for the production of recombinant plastid transformation constructs that target specific sites within the plastid genome for transformation, together with methods of using such constructs.



WO 2018/125636 A1

TITLE OF THE INVENTION**SITES FOR PLASTID TRANSFORMATION****REFERENCE TO RELATED APPLICATION**

[0001] This application claims the benefit of United States Provisional Application No. 62/440,855, filed December 30, 2016, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present invention relates to the field of agricultural biotechnology, and more specifically to methods and compositions for genetic transformation of plastids.

INCORPORATION OF SEQUENCE LISTING

[0003] A sequence listing contained in the file named "MONS391WO_ST25.txt" which is 396 kilo bytes (measured in MS-Windows®) and created on December 18, 2017, is filed electronically herewith and incorporated by reference in its entirety.

BACKGROUND

[0004] Plastid transformation can provide significant advantages over conventional nuclear transformation methods for creating transgenic plants, including more abundant and reliable transgene expression, maternal inheritance, and lack of silencing mechanisms. Improved compositions and methods are needed, however, for selecting sites within the plastid genome that are suitable for transformation and unlikely to interfere with expression of plastid genes.

SUMMARY

[0005] In one aspect, the invention provides a recombinant plastid transformation construct, said construct comprising: (i) a first homology arm comprising a sequence that is at least 95% identical to at least 100 contiguous nucleotides of any one of SEQ ID NOs: 3, 7, 11, 15, 19, 22, 26, 30, 33, 37, 41, 45, 49, 53, 57, 61, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 123, 127, 131, 135, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261, 265, 269, 273, 277, 281, 285, 289, 293, 297, 301, 305, 309, or 313; and (ii) a second homology arm comprising a sequence that is at least 95% identical to at least 100 contiguous nucleotides of any

one of SEQ ID NOs: 4, 8, 12, 16, 20, 23, 27, 31, 34, 38, 42, 46, 50, 54, 58, 62, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 109, 113, 117, 121, 124, 128, 132, 136, 139, 143, 147, 151, 155, 159, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 214, 218, 222, 226, 230, 234, 238, 242, 246, 250, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, or 314. In certain embodiments, the first homology arm comprises at least 100 contiguous nucleotides of any one of SEQ ID NOs: 3, 7, 11, 15, 19, 22, 26, 30, 33, 37, 41, 45, 49, 53, 57, 61, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 123, 127, 131, 135, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261, 265, 269, 273, 277, 281, 285, 289, 293, 297, 301, 305, 309, or 313. In further embodiments, the second homology arm comprises at least 100 contiguous nucleotides of any one of SEQ ID NOs: 4, 8, 12, 16, 20, 23, 27, 31, 34, 38, 42, 46, 50, 54, 58, 62, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 109, 113, 117, 121, 124, 128, 132, 136, 139, 143, 147, 151, 155, 159, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 214, 218, 222, 226, 230, 234, 238, 242, 246, 250, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, or 314. In some embodiments, the first or second homology arm is between 0.1 and 2.0 kilobases in length.

[0006] Recombinant plastid transformation constructs of the invention may further comprise an insertion sequence positioned between the first homology arm and the second homology arm, which may comprise a transcribable nucleic acid sequence. Transcribable nucleic acid sequences may be genes of agronomic interest, for example a gene of agronomic interest that confers an agronomically beneficial trait selected from the group consisting of modified carbon fixation, modified nitrogen fixation, herbicide tolerance, insect resistance or control, modified or increased yield, fungal disease tolerance or resistance, virus tolerance or resistance, nematode tolerance or resistance, bacterial disease tolerance or resistance, modified starch production, modified oil production, modified fatty acid content, modified protein production, enhanced animal and human nutrition, environmental stress tolerance, drought tolerance, improved processing traits or fruit ripening, improved digestibility, improved taste and flavor characteristics, modified enzyme production, modified fiber production, synthesis of other biopolymers, peptides or proteins, and enhanced biofuel production. In further embodiments, recombinant plastid transformation constructs of the invention comprise a promoter active in plant plastids, for example a promoter selected from the group consisting of Prn, psbA, and

rbcL. In yet further embodiments, recombinant plastid transformation constructs of the invention comprise a selectable marker, for example a selectable marker is selected from the group consisting of *aadA*, *nptII*, *aph IV*, *aac3*, *aacC4*, *CAT*, *EPSPS*, *bar*, *GOX*, *GAT*, and *β -glucuronidase*. Recombinant plastid transformation constructs of the invention may also comprise a screenable marker.

[0007] In another aspect, the invention provides a DNA molecule or vector comprising a recombinant plastid transformation construct as described herein, or a plastid comprising a recombinant plastid transformation construct as described herein. The invention further provides a plant, plant cell, plant part, or seed comprising a plastid comprising a recombinant plastid transformation construct as described herein, for example a crop plant. Plants provided by the invention may be monocotyledonous plants, including corn (maize), wheat, rice, millet, barley, sorghum, sugarcane, oat, rye, and other plants within the *Poaceae* or *Gramineae* family, or dicotyledonous plants, including cotton, canola, and sugar beets, soybean, alfalfa and other *Fabaceae* or leguminous plants.

[0008] In yet another aspect, the invention provides a method of producing a transformed plant cell plastid, comprising the step of transforming at least one plastid of a plant cell with a recombinant plastid transformation construct provided herein to produce plant cell comprising a transformed plastid; wherein the insertion sequence is incorporated into a genome of said plastid flanked by plastid sequences corresponding to the first and second homology arms of the recombinant plastid transformation construct. In certain embodiments, the insertion sequence is incorporated into the genome of said transformed plastid by homologous recombination. The invention further provides transformed plastids produced by the methods provided herein. In further embodiments, methods of the invention further comprise a step of selecting for development or regeneration of a plastid transformed plant cell by contacting said plant cell with a selection agent. Plastid transformed plant cells produced by the methods disclosed herein are further provided by the invention. In yet further embodiments, methods of the invention further comprise regenerating a plastid transformed plant from a plastid transformed plant cell disclosed herein, or obtaining a plastid transformed seed from such a plastid transformed plant. The plastid transformed plant may be developed or regenerated from said plant cell under selection pressure by contacting the developing plant with a selection agent. Plastid transformed plants

and plastid transformed seeds produced by the methods disclosed herein are further provided by the invention.

[0009] In another aspect, the invention provides a method of selecting a target site for plastid transformation comprising identifying a plastid target sequence, wherein said target sequence is located: (1) between two neighboring plastid genes in the plastid genome of a plant cell, (2) at least 20 base pairs from the 5' terminus of the coding sequence of a tRNA or small RNA encoding plastid gene, (3) at least 100 base pairs from the 5' terminus of the coding sequence of a structural protein-encoding plastid gene, (4) at least 20 base pairs from the 3' terminus of the coding sequence of a tRNA or small RNA encoding plastid gene, and (5) at least 150 base pairs from the 3' terminus of the coding sequence of a structural protein-encoding plastid gene.

[0010] In yet a further aspect, the invention provides a recombinant plastid transformation construct, said construct comprising a homology arm comprising a sequence that is at least 95% identical to at least 100 contiguous nucleotides of any one of SEQ ID NO: 3, 7, 11, 15, 19, 22, 26, 30, 33, 37, 41, 45, 49, 53, 57, 61, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 123, 127, 131, 135, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261, 265, 269, 273, 277, 281, 285, 289, 293, 297, 301, 305, 309, or 313; or at least 95% identical to at least 100 contiguous nucleotides of any one of SEQ ID NO: 4, 8, 12, 16, 20, 23, 27, 31, 34, 38, 42, 46, 50, 54, 58, 62, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 109, 113, 117, 121, 124, 128, 132, 136, 139, 143, 147, 151, 155, 159, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 214, 218, 222, 226, 230, 234, 238, 242, 246, 250, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, or 314.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. 1: Schematic illustration of selection of plastid target loci.

DESCRIPTION OF THE SEQUENCES

[0012] SEQ ID NOs: 1, 5, 9, 13, 17, 24, 28, 35, 39, 43, 47, 51, 55, 59, 66, 70, 74, 78, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 125, 129, 133, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 215, 219, 223, 227, 231, 235, 239, 243, 247, 251, 255, 259, 263, 267, 271, 275, 279, 283, 287, 291, 295, 299, 303, 307, and 311 represent plastomic regions comprising target sites for plastid transformation in corn (*Zea mays*), soybean (*Glycine max*), and cotton (*Gossypium hirsutum*) as detailed in Table 1.

[0013] SEQ ID NOs: 2, 6, 10, 14, 18, 21, 25, 29, 32, 36, 40, 44, 48, 52, 56, 60, 63, 67, 71, 75, 79, 83, 87, 91, 95, 99, 103, 107, 111, 115, 119, 122, 126, 130, 134, 137, 141, 145, 149, 153, 157, 161, 165, 169, 173, 177, 181, 185, 189, 193, 197, 201, 205, 209, 212, 216, 220, 224, 228, 232, 236, 240, 244, 248, 252, 256, 260, 264, 268, 272, 276, 280, 284, 288, 292, 296, 300, 304, 308, and 312 represent target sites for plastid transformation as detailed in Table 1.

[0014] SEQ ID NOs: 3, 7, 11, 15, 19, 22, 26, 30, 33, 37, 41, 45, 49, 53, 57, 61, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 123, 127, 131, 135, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261, 265, 269, 273, 277, 281, 285, 289, 293, 297, 301, 305, 309, and 313 comprise sequences useful in designing a first homology arm of plastid transformation constructs.

[0015] SEQ ID NOs: 4, 8, 12, 16, 20, 23, 27, 31, 34, 38, 42, 46, 50, 54, 58, 62, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 109, 113, 117, 121, 124, 128, 132, 136, 139, 143, 147, 151, 155, 159, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 214, 218, 222, 226, 230, 234, 238, 242, 246, 250, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, and 314 comprise sequences useful in designing a second homology arm of plastid transformation constructs.

Table 1. Sequences associated with target sites for plastid transformation.

Organism	Plastomic Junction Interval	Plastomic Junction Sequence SEQ ID NO	Target Sequence SEQ ID NO	First Arm SEQ ID NO	Second Arm SEQ ID NO
<i>Glycine max</i>	rbcL/atpB junction	1	2	3	4
<i>Glycine max</i>	trnL_UAA and trnT-UGU junction	5	6	7	8
<i>Glycine max</i>	psbD and trnT-GGU junction	9	10	11	12
<i>Glycine max</i>	trnT-GGU and trnE-UUC junction	13	14	15	16
<i>Glycine max</i>	petN and rpoB junction	17	18	19	20
<i>Glycine max</i>	petN and rpoB junction	17	21	22	23
<i>Glycine max</i>	trnG-UCC and trnS_GCU junction	24	25	26	27
<i>Glycine max</i>	psbK and rps16 junction	28	29	30	31
<i>Glycine max</i>	psbK and rps16 junction	28	32	33	34
<i>Glycine max</i>	psbE and petL junction	35	36	37	38
<i>Glycine max</i>	rpl20 and rps12 junction	39	40	41	42
<i>Glycine max</i>	Rpl23 and ycf2 junction (repeat region)	43	44	45	46
<i>Glycine max</i>	Rpsrps12 and trnV-GAC junction (repeat region)	47	48	49	50
<i>Glycine max</i>	Rpl32 and ndhF junction	51	52	53	54
<i>Glycine max</i>	rbcL downstream	55	56	57	58
<i>Glycine max</i>	ndhJ and trnL-UAA junction	59	60	61	62
<i>Glycine max</i>	ndhJ and trnL-UAA junction	59	63	64	65
<i>Glycine max</i>	Ycf3 downstream	66	67	68	69
<i>Glycine max</i>	psbZ downstream	70	71	72	73
<i>Glycine max</i>	psbM downstream	74	75	76	77
<i>Glycine max</i>	atpI downstream	78	79	80	81
<i>Glycine max</i>	Rps16 downstream	82	83	84	85
<i>Glycine max</i>	psaI downstream	86	87	88	89
<i>Glycine max</i>	petA downstream	90	91	92	93
<i>Glycine max</i>	psaJ downstream	94	95	96	97
<i>Glycine max</i>	ndhB downstream	98	99	100	101
<i>Glycine max</i>	trnR-ACG downstream	102	103	104	105
<i>Glycine max</i>	trnN-GUU downstream	106	107	108	109
<i>Gossypium hirsutum</i>	Rps16/psbK junction	110	111	112	113
<i>Gossypium hirsutum</i>	trnS-GCU and trnG-GCC junction	114	115	116	117

<i>Gossypium hirsutum</i>	rpoB and petN junction	118	119	120	121
<i>Gossypium hirsutum</i>	rpoB and petN junction	118	122	123	124
<i>Gossypium hirsutum</i>	trnE-UUC and trnT-GGU junction	125	126	127	128
<i>Gossypium hirsutum</i>	trnT-GGU and psbD upstream	129	130	131	132
<i>Gossypium hirsutum</i>	Rps4 and trnL-UAA junction	133	134	135	136
<i>Gossypium hirsutum</i>	Rps4 and trnL-UAA junction	133	137	138	139
<i>Gossypium hirsutum</i>	atpB and rbcL junction	140	141	142	143
<i>Gossypium hirsutum</i>	psbE and petL junction	144	145	146	147
<i>Gossypium hirsutum</i>	Rps18 and rps12 junction	148	149	150	151
<i>Gossypium hirsutum</i>	Rps12 and trnV-GVC junction	152	153	154	155
<i>Gossypium hirsutum</i>	Rpl32 and ndhF junction	156	157	158	159
<i>Gossypium hirsutum</i>	trnK-UUU and rps16 junction	160	161	162	163
<i>Gossypium hirsutum</i>	Rps16 and trnQ-UUG junction	164	165	166	167
<i>Gossypium hirsutum</i>	atpF and atpH junction	168	169	170	171
<i>Gossypium hirsutum</i>	atpH and atpI junction	172	173	174	175
<i>Gossypium hirsutum</i>	petN and psbM junction	176	177	178	179
<i>Gossypium hirsutum</i>	psaA and ycf3 junction	180	181	182	183
<i>Gossypium hirsutum</i>	ndhC and trnV-UAC junction	184	185	186	187
<i>Gossypium hirsutum</i>	rbcL and accD junction	188	189	190	191
<i>Gossypium hirsutum</i>	accD and psaI junction	192	193	194	195
<i>Gossypium hirsutum</i>	ycf4 and cemA junction	196	197	198	199
<i>Gossypium hirsutum</i>	petA and psbJ junction	200	201	202	203
<i>Gossypium hirsutum</i>	Rpl20 and clpP junction	204	205	206	207
<i>Gossypium hirsutum</i>	ycf2 and ndhB junction	208	209	210	211
<i>Gossypium hirsutum</i>	ycf2 and ndhB junction	208	212	213	214

<i>Gossypium hirsutum</i>	trnR-ACG-trnN-GUU junction	215	216	217	218
<i>Gossypium hirsutum</i>	trnL-UAG and rpl32 junction	219	220	221	222
<i>Zea mays</i>	Rps16-psbK junction	223	224	225	226
<i>Zea mays</i>	trnS-GCU and psbD junction	227	228	229	230
<i>Zea mays</i>	trnS-UGA and trnG-UCC junction	231	232	233	234
<i>Zea mays</i>	trnM-CAU-trnG-GGU junction	235	236	237	238
<i>Zea mays</i>	trnD-GUC and psbM junction	239	240	241	242
<i>Zea mays</i>	psbM and petN junction	243	244	245	246
<i>Zea mays</i>	petN and trnC-GCA junction	247	248	249	250
<i>Zea mays</i>	trnC-GCA and rpoB junction	251	252	253	254
<i>Zea mays</i>	atpI and atpH junction	255	256	257	258
<i>Zea mays</i>	trnT-UGU and trnL-UAA junction	259	260	261	262
<i>Zea mays</i>	ndhC and trnV-UAC junction	263	264	265	266
<i>Zea mays</i>	atpB and rbcL junction	267	268	269	270
<i>Zea mays</i>	rbcL and psaI junction	271	272	273	274
<i>Zea mays</i>	petA and psbJ junction	275	276	277	278
<i>Zea mays</i>	psbE and petL junction	279	280	281	282
<i>Zea mays</i>	Rpl20 and rps12 junction	283	284	285	286
<i>Zea mays</i>	trnI-CAU and trnL-CAA junction	287	288	289	290
<i>Zea mays</i>	Rps7 and trnV-GAC junction (duplicate region)	291	292	293	294
<i>Zea mays</i>	trnN-GUU and rps15 junction (duplicate region)	295	296	297	298
<i>Zea mays</i>	ndhF and rpl32	299	300	301	302
<i>Zea mays</i>	Rps15 and trnN-GUU junction (duplicate region)	303	304	305	306
<i>Zea mays</i>	trnV-GAC-rps7 junction (duplicate region)	307	308	309	310
<i>Zea mays</i>	trnL-CAA and trnI-CAU region	311	312	313	314

DETAILED DESCRIPTION

[0016] Plastid transformation provides a number of potential advantages over conventional nuclear transformation methods for generating transgenic plants, including generally higher levels of protein expression from transplastomic events due largely to multiple plastids being present in each cell and the presence of multiple copies of plastomic DNA molecules per plastid. Such a higher level of expression may be used to provide, for example, improved agronomic traits or increased biosynthesis of useful products. Plastids can also transcribe genes as operons, allowing for multiple transgenes or even entire pathways to be expressed together from a single expression cassette. In addition, integration of transgenes into the plastome is site-specific and generally less prone to silencing mechanisms, which may provide more consistent and reliable transgene expression levels among events for a given construct. Such consistency may thus reduce development costs in generating successful transplastomic events. Since plastids are generally maternally inherited, all R_1 seed from a plant homoplastomic for a given transgene would have the integrated transgene, unlike plants hemizygous for a nuclear transgenic event that require additional crosses to achieve stable transmission of the transgene due to chromosomal segregation. Transplastomic events may further target or sequester transgenic protein expression to plastids (or chloroplasts) which may direct or contain their function within these organelles without the need for additional target peptide sequences. As a result, plastid expression of a transgene may reduce cytotoxicity in some cases.

[0017] However, improved methods of plastid transformation are needed to efficiently and consistently produce transplastomic events within the plastid genome at sites that are less likely to interfere with endogenous structural and RNA encoding genes within the plastid genome. The present invention provides a list of plastid transformation target sites within plant plastid genomes of several crop plant species, useful in generating effective transformation events. Due to the conservation of plastid DNA sequences between related plant species, the polynucleotide sequences provided herein can be further used to identify and define similar plastid target sequences of additional plant species. Constructs for plastid transformation of the target sites provided herein are further disclosed, in addition to plants, plant parts, seeds, etc., transformed with DNA molecules or vectors comprising these constructs and having an insertion of a DNA

sequence into their plastid genome. Methods of identifying and transforming useful target sequences within a plant plastid genome are further provided.

I. Constructs for Plastid Transformation

A. Transformation Vectors and Molecules

[0018] An exogenous DNA molecule is provided for plastid transformation according to embodiments of the present invention, which may also be a recombinant DNA molecule. The exogenous DNA molecule may be a linear or circular DNA molecule, although circular DNA plasmids, vectors or constructs may be preferred. Vectors and constructs for plastid transformation according to methods of the present invention may comprise one or more genetic elements and/or transgenes to be introduced into a plant cell or tissue, which may include a selectable marker gene and/or a gene of agronomic interest. These genetic element(s) and/or transgene(s) may be incorporated into a recombinant, double-stranded plasmid or vector DNA molecule that may generally comprise at least the following components: (a) an insertion sequence comprising at least one transgene or expression cassette; and (b) two homology arms (derived from, and corresponding to, target plastid genome sequences of the plant species to be transformed) flanking the insertion sequence. Each of the at least one transgene(s) and/or expression cassette(s) of the insertion DNA sequence may further comprise (i) at least one promoter or regulatory element that functions in plant cells, and more particularly in plant plastids, to cause or drive expression of a transcribable nucleic acid sequence operably linked to the promoter, and (ii) a transcribable DNA sequence encoding a selectable marker or a gene product of agronomic interest (i.e., a selectable marker gene or gene of agronomic interest). Each transgene or expression cassette of the insertion sequence may further comprise a 5' and a 3' untranslated sequences, intron sequences, additional regulatory or expression elements, etc., for transgene expression from a plant cell plastid transformation event.

[0001] According to embodiments of the present invention, the term “recombinant” in reference to a DNA molecule, construct, vector, etc., refers to a DNA molecule or sequence that is not found in nature and/or is present in a context in which it is not found in nature, including a DNA molecule, construct, etc., comprising a combination of DNA sequences that would not naturally occur contiguously or in close proximity together without human intervention, and/or a DNA molecule, construct, etc., comprising at least two DNA sequences that are heterologous with

respect to each other. A recombinant DNA molecule, construct, etc., may comprise DNA sequence(s) that is/are separated from other polynucleotide sequence(s) that exist in proximity to such DNA sequence(s) in nature, and/or a DNA sequence that is adjacent to (or contiguous with) other polynucleotide sequence(s) that are not naturally in proximity with each other. A recombinant DNA molecule, construct, etc., may also refer to a DNA molecule or sequence that has been genetically engineered and constructed outside of a cell. For example, a recombinant DNA molecule may comprise any suitable plasmid, vector, etc., and may include a linear or circular DNA molecule. Such plasmids, vectors, etc., may contain various maintenance elements including a prokaryotic origin of replication and selectable marker, as well as a transgene or expression cassette perhaps in addition to a plant selectable marker gene, etc.

[0019] According to many embodiments, the insertion sequence between the homology arms may at least comprise a plant selectable marker transgene since selection pressure with a corresponding selection agent may be needed for successful generation of plastid transformants. However, additional transgene(s) and/or transcribable DNA sequence(s) may also be present within the insertion sequence and inserted into the target site of the plastid DNA molecule or genome along with the selectable marker gene, which may include one or more transgenes of agronomic interest conferring one or more agronomically or industrially desirable traits. For example, a transgene of agronomic interest may confer one or more of the following traits: modified carbon fixation, modified nitrogen fixation, herbicide tolerance, insect resistance, improved or increased yield, fungal disease tolerance, virus tolerance, nematode tolerance, bacterial disease tolerance, modified starch production, modified oil production, modified fatty acid content, modified protein production, enhanced animal and human nutrition, environmental stress or drought tolerance, improved processing traits, improved digestibility, modified enzyme production, modified fiber production, etc. An exogenous plasmid or DNA molecule may further comprise other sequence elements required for maintenance of the exogenous DNA molecule or vector, such as a bacterial replication origin, bacterial selection marker, etc., such as in the vector backbone (e.g., outside the homology arms and insertion sequence). Means for preparing DNA plasmids, constructs or vectors containing desired genetic components and sequences are well known in the art.

B. Homology Arms

[0020] An exogenous DNA molecule of the present invention may comprise at least two homology arms for homologous recombination at a particular target site or locus within a plasmid or plasmidic DNA molecule or genome of a target explant cell. The exogenous DNA molecule may comprise a first homology arm (or left homology arm) and a second homology arm (or right homology arm) flanking an insertion sequence between the left and right homology arms. Each of these homology arms may typically have a base pair (bp) length of up to about 5 kilobases (kb), such as in a range from about 0.1 kb to about 5 kb in length (i.e., about 100 to about 5000 nucleotides in length), or in a range from about 0.5 kb to about 2 kb in length, or in a range from about 1 kb to about 1.5 kb in length. The homology arms are positioned on either side of an insertion sequence comprising one or more transgene(s) for insertion into a plasmid or plasmidic DNA molecule or genome. Each of the homology arms may generally be highly homologous, nearly identical or identical to a corresponding target plasmid DNA sequence present in a plasmid genome. For example, each homology arm may be at least 80% identical, at least 90% identical, at least 91% identical, at least 92% identical, at least 93% identical, at least 94% identical, at least 95% identical, at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical, at least 99% identical or 100% identical to at least 50, at least 100, at least 150, at least 200, at least 250, at least 300, at least 400 or at least 500 contiguous nucleotides of a sequence selected from the group consisting of SEQ ID NOs: 3, 7, 11, 15, 19, 22, 26, 30, 33, 37, 41, 45, 49, 53, 57, 61, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 123, 127, 131, 135, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261, 265, 269, 273, 277, 281, 285, 289, 293, 297, 301, 305, 309, and 313, or a sequence selected from the group consisting of SEQ ID NOs: 4, 8, 12, 16, 20, 23, 27, 31, 34, 38, 42, 46, 50, 54, 58, 62, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 109, 113, 117, 121, 124, 128, 132, 136, 139, 143, 147, 151, 155, 159, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 214, 218, 222, 226, 230, 234, 238, 242, 246, 250, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, and 314, although lower percentages of identity are also possible.

[0021] According to some embodiments, each homology arm may comprise at least 50 contiguous nucleotides, or at least 100 contiguous nucleotides, or at least 150 contiguous nucleotides, or at least 200 contiguous nucleotides, or at least 250 contiguous nucleotides, or at

least 300 contiguous nucleotides, or at least 400 contiguous nucleotides, or at least 500 contiguous nucleotides, or at least 1000 contiguous nucleotides of a sequence selected from the group consisting of SEQ ID NOs: 3, 7, 11, 15, 19, 22, 26, 30, 33, 37, 41, 45, 49, 53, 57, 61, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 123, 127, 131, 135, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261, 265, 269, 273, 277, 281, 285, 289, 293, 297, 301, 305, 309, and 313, or a sequence selected from the group consisting of SEQ ID NOs: 4, 8, 12, 16, 20, 23, 27, 31, 34, 38, 42, 46, 50, 54, 58, 62, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 109, 113, 117, 121, 124, 128, 132, 136, 139, 143, 147, 151, 155, 159, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 214, 218, 222, 226, 230, 234, 238, 242, 246, 250, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, and 314. However, except in cases where a targeted mutation or editing of the plastid genome sequence is desired, the homology arms will generally be perfectly or 100% identical to the corresponding target plastid DNA sequences to improve plastid transformation efficiency and avoid introduction of additional mutations.

[0022] In addition to the homology arms often being identical or highly homologous to corresponding target plastid DNA sequences, the corresponding target plastid DNA sequences may also be perfectly or almost perfectly continuous with each other prior to the transformation and insertion event (i.e., prior to the insertion sequence of the exogenous DNA molecule becoming inserted into the plastid genome) to avoid making any additional changes or mutations to the plastid DNA sequence as a result of the plastid transformation event – e.g., the deletion of one or more base pairs between the corresponding target plastid DNA sequences. The target site and junction of the corresponding target plastid DNA sequences will also generally or preferably be within an intergenic region or sequence of the plastid DNA to avoid insertion of a transgene into a plastid gene or coding sequence. However, each of the homology arms may comprise or encompass one or more plastid genes, or a portion(s) thereof, within their sequence. According to alternative embodiments, it is further contemplated that the target plastid DNA sequences corresponding to the homology arms may not be continuous with each other (prior to the transformation event), such that the intervening sequence will be deleted by the transformation event and replaced with the exogenous insertion sequence. This approach could thus be used to

delete a portion(s) of the plastid genome and/or knockout gene(s) by the transformation event in addition to inserting the exogenous insertion sequence.

[0023] An exogenous DNA molecule used for plastid transformation according to present method embodiments may potentially comprise only one homology arm immediately adjacent or next to an insertion sequence comprising one or more transgene(s), such as a plant selectable marker gene and/or a transgene of agronomic interest. However, having only one homology arm may lead to further integration of the vector backbone and/or variable event quality. Even if a linear exogenous DNA molecule is used that lacks additional unwanted vector sequences, such as a bacterial replication origin, selectable marker, etc., such an exogenous DNA molecule may have a much lower transformation frequency and variable event quality. Accordingly, two homology arms flanking the insertion sequence comprising one or more transgene(s) and/or selectable marker gene(s) will generally be preferred for an exogenous DNA molecule or construct to provide a higher transformation efficiency and greater fidelity among transformation events.

[0024] According to some embodiments of the present invention, constructs and methods may be further used to engineer, create or introduce one or more mutations (e.g., point mutations or SNPs, deletions, additions, etc.) in the targeted plastid DNA molecule (with or without the additional insertion a gene of agronomic interest). In such a case, the one or more desired mutations relative to the target plastid DNA sequence may be incorporated into one or both of the homology arm(s) of the exogenous DNA molecule such that those mutation(s) may become introduced into the plastid DNA molecule via the homologous recombination event. Despite the possible absence of a gene of agronomic interest within exogenous DNA molecules used for sequence editing or creation of targeted mutations, a plant selectable marker gene may still be present between the two homology arms of the exogenous DNA sequence to allow for selection of transformed cells, tissues and plants with a selection agent. According to other embodiments, a targeted deletion or knockout of an endogenous plastid genome sequence, which may include one or more plastid gene(s), or one or more portion(s) thereof, may also be carried out by the two homologous arms having corresponding plastid target DNA sequences that are not continuous and are separated from each other in the non-transformed plastid genome.

[0025] In certain embodiments, the invention provides plastid transformation target sites within junction sequences or regions between transcribable sequences or genes within the plastid genome. Junction sequences may be between any transcribable sequences or genes, for example protein-coding (structural) sequences or genes and/or tRNA-encoding sequences or genes. For example, junction sequences provided by the present invention include SEQ ID NOs: 1, 5, 9, 13, 17, 24, 28, 35, 39, 43, 47, 51, 55, 59, 66, 70, 74, 78, 82, 86, 90, 94, 98, 102, 106, 110, 114, 118, 125, 129, 133, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 215, 219, 223, 227, 231, 235, 239, 243, 247, 251, 255, 259, 263, 267, 271, 275, 279, 283, 287, 291, 295, 299, 303, 307, and 311. Target sites for plastid transformation are present within the intergenic regions of the junction sequences. Thus, the homology arms of an exogenous DNA molecule of the present invention are preferably designed such that the insertion sequence of the exogenous DNA molecule becomes inserted or transformed within the target site through recombination. Without being bound by theory, it is believed that target sites for plastid transformation may be closer to tRNA coding sequences than protein coding structural genes in the plastid genome. In some embodiments, a target site may be at least 100 base pairs (bp), at least 150 base pairs, at least 200 base pairs, at least 250 base pairs, at least 300 base pairs or at least 350 base pairs away from the 5' terminus of a plastid structural (protein-coding) gene; at least 20 base pairs, at least 50 base pairs, at least 100 base pairs, at least 150 base pairs, at least 200 base pairs, at least 250 base pairs, at least 300 base pairs, or at least 350 base pairs away from the 5' terminus of a transcribable tRNA encoding sequence; at least 100 base pairs, at least 150 base pairs, at least 200 base pairs, at least 250 base pairs, at least 300 base pairs, at least 350 base pairs or at least 400 base pairs away from the 3' terminus of a plastid structural (protein-coding) gene; and/or at least 20 base pairs, at least 50 base pairs, at least 100 base pairs, at least 150 base pairs, at least 200 base pairs, at least 250 base pairs, at least 300 base pairs, or at least 350 base pairs away from the 3' terminus of a transcribable tRNA encoding sequence. Target sites for plastid transformation in corn, soybean and cotton may include one of SEQ ID NOs: 2, 6, 10, 14, 18, 21, 25, 29, 32, 36, 40, 44, 48, 52, 56, 60, 63, 67, 71, 75, 79, 83, 87, 91, 95, 99, 103, 107, 111, 115, 119, 122, 126, 130, 134, 137, 141, 145, 149, 153, 157, 161, 165, 169, 173, 177, 181, 185, 189, 193, 197, 201, 205, 209, 212, 216, 220, 224, 228, 232, 236, 240, 244, 248, 252, 256, 260, 264, 268, 272, 276, 280, 284, 288, 292, 296, 300, 304, 308, and 312.

C. Transgene Expression Cassettes

[0026] According to embodiments of the present invention, recombinant DNA molecule for plastid transformation may generally comprise an insertion sequence comprising one or more transgene(s), transcribable nucleic acid sequence(s), and/or expression cassette(s) that is/are introduced into a plastid DNA molecule (i.e., a plastid genome or plastome) of a plant or plant cell. Each transgene, expression cassette, etc., will generally comprise a sequence encoding a gene product of agronomic interest and/or a plant selectable marker gene, which may each be operably linked to one or more regulatory element(s), such as promoters, enhancers, leaders, introns, linkers, untranslated regions, termination regions, etc., that are suitable for regulating plastid expression of the transgene or expression cassette. Known examples of plastid regulatory elements suitable for expression in plant plastids, such as from the ribosomal RNA operon plastid gene (*Prn*; Staub and Maliga, *Plant Cell* 4:39-45, 1992), the *psbA* promoter (Staub and Maliga, *EMBO Journal* 12:601-606 1993) or the *rbcL* ribosome binding site region (Svab and Maliga, *PNAS* 90:913-917, 1993), may be operably linked to a transgene or plant selectable marker gene. See, e.g., Kung, SD, *et al.*, “Chloroplast promoters from higher plants”, *Nucleic Acids Res.*, 13(21): 7543-9 (1985); and Liere, K, *et al.*, “The transcription machineries of plant mitochondria and chloroplasts: Composition, function, and regulation”, *Journal of Plant Physiology*, 168: 1345-1360 (2011), the entire contents and disclosures of which are hereby incorporated by reference. Plastid regulatory elements or promoters may include those naturally occurring in plastids of the plant species to be transformed, or possibly DNA sequences homologous to those plastid regulatory elements or promoters, or possibly even heterologous plastid regulatory elements or promoters from other closely, or even distantly, related species in plastids of transformed cells. Plastid regulatory elements and promoters may further include synthetic or engineered promoters, as well as promoters altered or derived from other regulatory elements or promoter sequences.

[0027] For purposes of the present invention, the term “heterologous” means that the plastid promoter, regulatory element, transgene, selectable marker gene, etc., is from a different species than the plant species to be transformed. Thus, a plastid promoter or regulatory element in exogenous DNA molecules of the present invention may include a homologous, heterologous, or even disparate or divergent plastid or regulatory element sequence(s), in addition to nucleotide sequence(s) identical to a plastid promoter or regulatory element sequence(s) from the plant

species to be transformed. A plastid regulatory element or promoter may functionally include any nucleotide sequence element that drives, or at least affects, expression of a transgene operably linked to the regulatory element or promoter (at least transiently) when the plastid regulatory element or promoter and transgene are inserted or integrated into the plastid genome of the plant species to be transformed. Even if a plasmid promoter or regulatory element from the plant species to be transformed is used, the plasmid promoter or regulatory element may be operably linked to a transgene, transcribable nucleotide sequence, selectable marker gene, etc., in a manner, form or combination that (in terms of its exact nucleotide sequence) does not naturally exist in nature, or at least does not naturally exist in the plant species to be transformed.

D. Transcribable Nucleic Acid Sequences

[0028] The transcribable nucleic acid or DNA sequence of a transgene or expression cassette within the insertion sequence of an exogenous DNA molecule to be inserted into the plastid genome or plastomic DNA of target explant cells may include a gene of agronomic interest to be expressed in a transplastomic cell or plant. As used herein, the term “gene of agronomic interest” refers to any transgene or expression cassette comprising a transcribable nucleic acid or DNA sequence operably to one or more plastid regulatory element(s) that, when expressed in a plastid of a transgenic plant tissue or cell, provides or confers an agronomically beneficial trait or phenotype, such as a desirable product or characteristic associated with plant morphology, physiology, growth, development, yield, nutritional profile, disease or pest resistance, and/or environmental or chemical tolerance. In some embodiments, a trait of agronomic interest may be modified carbon fixation, modified nitrogen fixation, herbicide tolerance, insect resistance or control, modified or increased yield, fungal disease tolerance or resistance, virus tolerance or resistance, nematode tolerance or resistance, bacterial disease tolerance or resistance, modified starch production, modified oil production, modified fatty acid content, modified protein production, enhanced animal and human nutrition, environmental stress tolerance, drought tolerance, improved processing traits or fruit ripening, improved digestibility, improved taste and flavor characteristics, modified enzyme production, modified fiber production, synthesis of other biopolymers, peptides or proteins, biofuel production, etc.

[0029] A gene or transgene of agronomic interest may further include a gene or transcribable DNA sequence of interest that may have unknown characteristics but may be in testing or

proposed or theorized for providing a desirable trait of agronomic interest to a plant. Indeed, a transgene of agronomic interest may include any known gene (or any putative or annotated gene sequence) believed, or tested or screened for its ability, to cause, confer, or create a trait or phenotype of agronomic or industrial interest in the transplastomic plant. A transgene of agronomic interest may further include any transcribable DNA sequence that produces a desirable effect in a plant, such as RNA molecule(s) used to confer insect resistance, etc.

[0030] Examples of genes of agronomic interest known in the art may include any known or later discovered genes, coding regions or transcribable DNA sequences providing herbicide resistance or tolerance, increased yield, insect resistance or control, fungal disease resistance, virus resistance, nematode resistance, bacterial disease resistance, plant growth and development, starch production, modified oils production, high oil production, modified fatty acid content, high protein production, fruit ripening, enhanced animal or human nutrition, biopolymers, environmental stress resistance, pharmaceutical peptides and secretable peptides, improved processing traits, improved digestibility, low raffinose, industrial enzyme production, improved flavor, nitrogen fixation, hybrid seed production, fiber production, biofuel production, etc.

[0031] Plastids can be transformed with polycistronic operons, and can effectively integrate and express large transgenic inserts, thereby enabling stacking of genes and/or simultaneous expression of genes from the same insertion sequence inserted into the plastid DNA by methods of the present invention. As mentioned above, transgenes integrated in plastomes are also generally not susceptible to gene silencing, which often occurs with multi-copy nuclear events. Thus, plastid transformation according to methods of the present invention may be particularly useful in cases in which high levels of transgene expression are desirable and/or where multiple genes or possibly even entire pathways (or portions of a biochemical pathway) need to be expressed. Accordingly, the insertion sequence of an exogenous DNA molecule may comprise (i) multiple transgenes or cassettes under the control of separate regulatory element(s), and/or (ii) a single transgene or cassette that simultaneously encodes a polycistronic RNA molecule under the control of a common set of regulatory element(s). Such insertion sequences may thus be used to produce multiple gene products from a single plastid DNA insertion event.

E. Selectable markers

[0032] According to embodiments of the present invention, the insertion sequence of an exogenous DNA molecule for plastid transformation will generally comprise at least a plant selectable marker gene to allow for successful selection for, and production of, transplastomic R₀ plants. A plant selectable marker gene or transgene may include any gene conferring tolerance to a corresponding selection agent, such that plant cells transformed with the plant selectable marker transgene may tolerate and withstand the selection pressure imposed by the selection agent. As a result, transplastomic cells are favored to grow, proliferate, develop, etc., under selection. Although a plant selectable marker gene is generally used to confer tolerance to a selection agent, additional screenable marker gene(s) may also be used in addition to the selectable marker, perhaps also along with a gene of agronomic interest. Such screenable marker genes may include, for example, β -glucuronidase (GUS; e.g., as described in U.S. Pat. No. 5,599,670, which is hereby incorporated by reference) or green fluorescent protein and variants thereof (GFP described in U.S. Pat. Nos. 5,491,084 and 6,146,826, each of which is hereby incorporated by reference), or any other screenable marker gene known in the art. Additional examples of screenable markers may include secretable markers whose expression causes secretion of a molecule(s) that can be detected as a means for identifying transformed cells.

[0033] A plant selectable marker gene may comprise a gene encoding a protein that provides or confers tolerance or resistance to an herbicide, such as glyphosate and glufosinate. Useful plant selectable marker genes known in the art may include those encoding proteins that confer resistance or tolerance to streptomycin or spectinomycin (e.g., *aadA*, *spec/strep*), kanamycin (e.g., *nptII*), hygromycin B (e.g., *aph IV*), gentamycin (e.g., *aac3* and *aacC4*), and chloramphenicol (e.g., *CAT*). Additional examples of known plant selectable marker genes encoding proteins that confer resistance or tolerance to an herbicide or other selection agent. For example, a transcribable DNA molecule encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS for glyphosate tolerance; e.g., as described in U.S. Pat. Nos. 5,627,061; 5,633,435; 6,040,497; and 5,094,945, all of which are hereby incorporated by reference); a transcribable DNA molecule encoding a glyphosate oxidoreductase and a glyphosate-N-acetyl transferase (GOX; e.g., as described in U.S. Pat. No. 5,463,175; GAT described in U.S. Patent Publication No. 20030083480); a transcribable DNA molecule encoding phytoene desaturase (*crtI*; e.g., as described in Misawa, *et al.*, *Plant Journal*, 4:833-840 (1993) and Misawa, *et al.*, *Plant Journal*,

6:481-489 (1994) for norflurazon tolerance, incorporated herein by reference); and the *bar* gene (e.g., as described in DeBlock, *et al.*, EMBO Journal, 6:2513-2519 (1987) for glufosinate and bialaphos tolerance, incorporated herein by reference). See also, e.g., Bock, R., "Engineering Plastid Genomes: Methods, Tools, and Applications in Basic Research and Biotechnology," *Annu. Rev. Plant Biol.*, 66: 3.1-3.31 (2015), and Ziemienowicz, A., "Plant selectable markers and reporter genes," *Acta Physiologiae Plantarum* 23(3): 363-374 (2001), the entire contents and disclosure of which are hereby incorporated by reference.

[0034] The insertion sequence of an exogenous DNA molecule may further comprise sequences for removal of one or more transgene(s) or expression cassette(s), such as a plant selectable marker transgene, or any portion or sequence thereof, after successful production and/or confirmation of a transplastomic plant(s), especially after the transgene or expression cassette is no longer needed. In some embodiments, this may be accomplished by flanking the transgene sequence to be removed, with known or later developed recombination sites (e.g., LoxP sites, FRT sites, etc.) that can be recognized and removed by an endogenous or exogenously provided recombinase enzyme (e.g., Cre, Flp, etc.). The recombinase enzyme may be introduced and expressed in trans, such as by crossing the transplastomic plant to another plant having the recombinase transgene, to accomplish excision of the transgene. Accordingly, the unwanted sequence element or transgene can be removed once its use or purpose has expired, thus preventing its further expression or transmission in the germ line.

F. 3' Untranslated Regions

[0035] An insertion sequence according to the present invention may further comprise a 3'-untranslated region to facilitate mRNA stability in the plastid. Transgene transcripts may be stabilized, for example, by inclusion of the 3'-untranslated region of the plastid *rps16* gene (Trps16; US Patent Number 5,877,402) or the 3'-untranslated region of the plastid *petD* gene (TpetD) downstream of a transcribable nucleic acid sequence.

II. Methods of Plastid Transformation

[0036] Embodiments of the present invention provide methods of transforming plant plastids comprising introducing an exogenous DNA molecule into at least one cell of a plant tissue to produce a transplastomic event in at least one plastid of that cell. A "plastid" refers to a class of organelles in the cytoplasm of a plant cell which contain one or more small circular double-

stranded DNA molecules (i.e., the plastome, plastomic DNA, or plastid DNA). Examples of plastids include, but are not limited to, proplastids, chloroplasts, chromoplasts, gerontoplasts, leucoplasts, elaioplasts, proteinoplasts, and tannosomes. As used herein, plant plastid refers to a plastid in higher plants (*i.e.*, a dicot or a monocot).

[0037] Methods of the present invention employ homologous recombination to achieve site-specific insertion of a transgene from an exogenous DNA molecule into the plastid genome DNA (i.e., the “plastome”) of at least one cell of the explant target tissue. As described above, the exogenous DNA molecule may generally comprise two arm regions flanking an insertion sequence with each of the two arm regions being homologous to respective target plastid genome sequences to drive recombination and insertion of the transgene into the target site of the plastome. Site-directed integration of the transgene into the plastid genome of a plant cell via homologous recombination reduces event variability commonly associated with nuclear transformation events having transgene insertions at different locations throughout the nuclear genome. As a result, transgene expression levels in plastids should generally be consistent between transplastomic events of the same quality (unlike nuclear transformation events that exhibit variable and unpredictable levels of transgene expression depending on their insertion site). Such consistent and predictable transgene expression reduces development costs for producing transplastomic events.

[0038] Various methods have been developed for transferring genes into plant tissue cells including high velocity microprojection or particle bombardment, microinjection, electroporation, PEG-mediated transformation, direct DNA uptake, and bacterially-mediated transformation. According to embodiments of the present invention, an exogenous DNA molecule may preferably be introduced into at least one cell of a target plant tissue via particle-mediated bombardment of the explant using particles carrying one or more copies of the exogenous DNA molecule. Such particle-mediated bombardment may utilize any suitable particle gun device known in the art, such as a helium particle gun, electric particle gun, etc. Prior to bombardment, particles may be loaded or coated with copies of the exogenous DNA molecule. The particles themselves may include any suitable type of particle or bead known in the art, such as gold or tungsten beads, etc. According to embodiments of the present invention, a ratio in a range of approximately 0.5 – 2.0 µg of exogenous DNA molecules per mg of beads, such as about 1.2 µg of exogenous DNA per mg of beads, may be combined together for bead

preparation and coating. Methods for coating beads with an exogenous DNA molecule are known in the art. Blasting conditions for the particle gun are also well known in the art, and various conventional screens, rupture disks, etc., may be used, such as for a helium particle gun. The electric gun may provide some advantages in reducing the amount of time required for transformation and by using fewer consumables in the process.

[0039] For particle bombardment, plant tissue may be plated onto a target medium or substrate that is able to hold the plant tissue in place and properly oriented for blasting. Such a target medium or substrate may contain, for example, a gelling agent, such as agar, and carboxymethylcellulose (CMC) to control the viscosity of the medium or substrate. Plant tissue may also be blasted with coated particles at various pressures, forces, and/or once or multiple times. Although particle mediated bombardment may be preferred for plastid transformation of explants according to embodiments of the present invention, other non-conventional methods are contemplated for use potentially in plastid transformation.

[0040] After transformation or bombardment, the plant tissue may be contacted with one or more selection media containing a selection agent to bias the survival, growth, proliferation and/or development of transplastomic cells having expression of a selectable marker gene integrated into the plastome from the exogenous DNA molecule used for transformation. The selectable marker gene will generally be paired to the selection agent used for selection such that the selectable marker gene confers tolerance to selection with the selection agent. For example, the selectable marker gene may be an adenyltransferase gene (*aadA*) conferring tolerance to spectinomycin or streptomycin as the selection agent.

[0041] The methods of the present invention allow for identification and selection of sites for plastid transformation that will minimize or avoid interference with endogenous plastid genes. Candidate transplastomic plants from one or more transformed explant(s) may be identified and plastid transformed shoots and plants may be grown or developed to produce transplastomic plants. For example, after putative plastid transformants have been identified using selectable markers, plantlets may be subcultured and/or placed in soil or on a soil substitute such as on a rooting medium, in the presence or absence of the selection agent. Shoots elongating from explants may be assayed to determine whether they are transgenic. Transgenic R₁ seed may be collected from R₀ plants to produce progeny plants that are also transplastomic. Selection

pressure with the appropriate selection agent may be maintained over one or more subsequent generations from the R_0 plant to produce a homoplastomic or nearly homoplastomic plant, which may be defined as being fixed with respect to inheritance of the plastome-integrated transgene (i.e., without segregation of the transgene among progeny and/or with stable maintenance of homoplastomy in progeny with self-crossing). Growth, survival, development, etc., of transplastomic cells in the R_0 plant may also be selectively achieved or favored by exerting a selection pressure with a selection agent during culturing, sub-culturing, shoot elongation and/or rooting step(s) of the explant to produce a homoplastomic or nearly homoplastomic R_0 plant, or at least a transplastomic R_0 plant having a uniform, ubiquitous or more widespread presence of transgenic plastids throughout the R_0 plant, although selection pressure may alternatively be continued (e.g., periodically, etc.) during the remaining life of the R_0 plant (e.g., as a topical spray, soil or seed application, etc.). Selection pressure may also be continued or maintained over subsequent generation(s) to produce a progeny plant that is homoplastomic or nearly homoplastomic, or at least has a more uniform, widespread and/or ubiquitous presence of transgenic plastids throughout the plant.

[0042] A variety of tissue culture media are known that, when supplemented appropriately, support plant tissue growth and development, including formation of mature plants from excised plant tissue. These tissue culture media can either be purchased as a commercial preparation or custom prepared and modified by those of skill in the art. Examples of such media include, but are not limited to those described by Murashige and Skoog, (1962); Chu *et al.*, (1975); Linsmaier and Skoog, (1965); Uchimiya and Murashige, (1962); Gamborg *et al.*, (1968); Duncan *et al.*, (1985); McCown and Lloyd, (1981); Nitsch and Nitsch (1969); and Schenk and Hildebrandt, (1972), or derivations of these media supplemented accordingly. Those of skill in the art are aware that media and media supplements, such as nutrients and plant growth regulators for use in transformation and regeneration are usually optimized for the particular target crop or variety of interest. Tissue culture media may be supplemented with carbohydrates such as, but not limited to, glucose, sucrose, maltose, mannose, fructose, lactose, galactose, and/or dextrose, or ratios of carbohydrates. Reagents are commercially available and can be purchased from a number of suppliers (see, for example Sigma Chemical Co., St. Louis, MO; and PhytoTechnology Laboratories, Shawnee Mission, KS). These tissue culture media may be used as a resting media or as a selection media with the further addition of a selection agent.

[0043] A variety of assays may be performed to confirm the presence of an exogenous DNA and/or insertion sequence in transplastomic plants. Such assays include, for example, molecular biological assays, such as Southern and Northern blotting, sequencing, PCR, *in situ* hybridization, etc.; biochemical assays, such as detecting the presence of a protein product, e.g., by immunological means (ELISAs and Western blots) or by enzymatic function; visual determination with a screenable marker plant part assays, such as leaf or root assays; or by analyzing the phenotype of a whole regenerated plant or plant part.

[0044] Embodiments of the present invention also provide transplastomic plants and/or plant parts produced by the plastid transformation methods of the present invention as disclosed herein. Plant parts, without limitation, include fruit, seed, endosperm, ovule, pollen, leaf, stem, and roots. In certain embodiments of the present invention, the plant or plant part is a seed.

[0045] Plants for use with the method embodiments provided herein may include a wide variety of dicotyledonous (dicot) or monocotyledonous (monocot) plants. Examples of dicot plants may include various agricultural crop species, such as soybean, alfalfa and other Fabaceae or leguminous plants, and cotton, canola, and sugar beets. Other examples of dicot plants include a member of the *Brassica* sp. (e.g., *B. napus*, *B. rapa*, *B. juncea*), particularly those *Brassica* species useful as sources of seed oil, alfalfa (*Medicago sativa*), sunflower (*Helianthus annuus*), safflower (*Carthamus tinctorius*), oil palm (*Elaeis* spp.), sesame (*Sesamum* spp.), coconut (*Cocos* spp.), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium barbadense*, *Gossypium hirsutum*), sweet potato (*Ipomoea batatas*), cassava (*Manihot esculenta*), coffee (*Coffea* spp.), tea (*Camellia* spp.), fruit trees, such as apple (*Malus* spp.), *Prunus* spp., such as plum, apricot, peach, cherry, etc., pear (*Pyrus* spp.), fig (*Ficus casica*), banana (*Musa* spp.), etc., citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), avocado (*Persea americana*), olive (*Olea europaea*), almond (*Prunus amygdalus*), walnut (*Juglans* spp.), strawberry (*Fragaria* spp.), watermelon (*Citrullus lanatus*), pepper (*Capsicum* spp.), sugar beet (*Beta vulgaris*), grape (*Vitis*, *Muscadinia*), tomato (*Lycopersicon esculentum*, *Solanum lycopersicum*), and cucumber (*Cucumis sativis*). Examples of monocotyledonous (monocot) plants various agricultural crop species, such as corn (maize), wheat, rice, millet, barley, sorghum, sugarcane, oat, rye, and other *Poaceae* or *Gramineae* family of plants that are typically harvested for their seed. Although examples of target and flanking sequences for plastid transformation are provided for a few plant species, one skilled in the art

would be able to use the sequences provided herein to determine analogous plastid sequences in other plant species through sequence alignment and comparison.

[0046] Having described the present disclosure in detail, it will be apparent that modifications, variations, and equivalent embodiments are possible without departing from the spirit and scope of the present disclosure as further defined in the appended claims. Furthermore, it should be appreciated that all examples in the present disclosure are provided as non-limiting examples.

EXAMPLES

Example 1: Identification of Target Sequences for Plastid Transformation

[0047] Specific sequences from soybean, cotton, and corn plastid genomes useful as plastid transformation target sites are provided herein. Target sequences may include SEQ ID NOs: 2, 6, 10, 14, 18, 21, 25, 29, 32, 36, 40, 44, 48, 52, 56, 60, 63, 67, 71, 75, 79, 83, 87, 91, 95, 99, 103, 107, 111, 115, 119, 122, 126, 130, 134, 137, 141, 145, 149, 153, 157, 161, 165, 169, 173, 177, 181, 185, 189, 193, 197, 201, 205, 209, 212, 216, 220, 224, 228, 232, 236, 240, 244, 248, 252, 256, 260, 264, 268, 272, 276, 280, 284, 288, 292, 296, 300, 304, 308, and 312. Eighty target sites were evaluated for distance to the nearest 5' end and distance to the nearest 3' end of a structural gene, and/or distance to the nearest 5' end and distance to the nearest 3' end of a tRNA-encoding sequence, depending on the neighboring genes of the target sequence.

[0048] For corn, soybean and cotton, target sequences were determined to be approximately 100 bp to 450 bp away from the 5' terminus of plastid structural (protein-coding) genes, and approximately 150 bp to 450 bp away from the 3' terminus of plastid structural (protein-coding) genes. In certain examples, target sequences were determined to be approximately 140 bp to 400 bp from the 5' terminus of structural gene sequences (e.g., 143-399 bp from the 5' terminus), and approximately 170 bp to 420 bp from the 3' terminus of structural gene sequences (e.g., 173-419 bp from the 3' terminus).

[0049] For corn, soybean and cotton, target sequences were also determined to be approximately 20 bp to 400 bp away from the 5' terminus and 3' terminus of tRNA gene sequences. In certain examples, target sequences were determined to be approximately 25 bp to 350 bp from a 5' terminus of tRNA gene sequences (e.g., 27-350 bp from the 5' terminus), and

approximately 20 bp to 400 bp from a 3' terminus of tRNA gene sequences (e.g., 24-383 bp from the 3' terminus).

[0050] Exogenous DNA insertion sequences may be specifically integrated into the identified target sites, or portions of the target sites, or completely or partially replace the identified target sites, as a result of a plastid transformation event.

[0051] An exemplary method of selecting a target site for plastid transformation includes identifying a sequence within a plastid genome which has the following characteristics:

- The sequence is located between two neighboring plastid genes.
- The sequence is at least 20 bp away from the 5' terminus of a tRNA (trn) or other small RNA encoding gene within a plastid genome;
- The sequence is at least 20 bp away from the 3' terminus of a tRNA (trn) or other small RNA encoding gene within a plastid genome;
- The sequence is at least 100 bp away from the 5' terminus of a structural (protein encoding) gene within a plastid genome; and
- The sequence is at least 150 bp away from the 3' terminus of a structural (protein encoding) gene within a plastid genome.

Three configurations of neighboring or flanking plastid genes next to target sites for targeted integration and/or recombination of exogenous DNA molecules in plant plastid genomes are shown schematically in FIG. 1 with distance ranges provided relative to neighboring structural or tRNA genes.

Example 2: Target Sequences for Soybean Plastid Transformation

[0052] Target sites for plastid transformation were identified in the soybean chloroplast genome (NCBI, Accession No. NC_007942).

[0053] Junction regions of neighboring structural and/or tRNA gene sequences comprising target sites for soybean plastid transformation are provided as SEQ ID NOs: 1, 5, 9, 13, 17, 24, 28, 35, 39, 43, 47, 51, 55, 59, 66, 70, 74, 78, 82, 86, 90, 94, 98, 102, and 106. Target sites for soybean plastid transformation within the junction regions are provided as SEQ ID NOs: 2, 6, 10, 14, 18, 21, 25, 29, 32, 36, 40, 44, 48, 52, 56, 60, 63, 67, 71, 75, 79, 83, 87, 91, 95, 99, 103, and

107. Transgenic DNA inserted during transformation may be integrated between any two base pairs, and may partially or completely replace the target sequences by homologous recombination. The sequences identified and constructs provided are useful for introducing transgenic DNA into a specific site of a soybean chloroplast genome via transformation.

Example 3: Target Sequences for Cotton Plastid Transformation

[0054] Target sites for plastid transformation were identified in the cotton chloroplast genome (NCBI, Accession No. NC_007944).

[0055] Junction regions of neighboring structural and/or tRNA gene sequences comprising target sites for cotton plastid transformation are provided as SEQ ID NOs: 110, 114, 118, 125, 129, 133, 140, 144, 148, 152, 156, 160, 164, 168, 172, 176, 180, 184, 188, 192, 196, 200, 204, 208, 215, and 219. Target sites for cotton plastid transformation within the junction regions are provided as SEQ ID NOs: 111, 115, 119, 122, 126, 130, 134, 137, 141, 145, 149, 153, 157, 161, 165, 169, 173, 177, 181, 185, 189, 193, 197, 201, 205, 209, 212, 216, and 220. Transgenic DNA inserted during transformation may be integrated between any two base pairs, and may partially or completely replace the target sequences by homologous recombination. The sequences identified and constructs provided are useful for introducing transgenic DNA into a specific site of a cotton chloroplast genome via transformation.

Example 4: Target Sequences for Corn Plastid Transformation

[0056] Target sites for plastid transformation were identified in the corn chloroplast genome (NCBI, Accession No. KF241981).

[0057] Junction regions of neighboring structural and/or tRNA gene sequences comprising target sites for corn plastid transformation are provided as SEQ ID NOs: 223, 227, 231, 235, 239, 243, 247, 251, 255, 259, 263, 267, 271, 275, 279, 283, 287, 291, 295, 299, 303, 307, and 311. Target sites for corn plastid transformation within the junction regions are provided as SEQ ID NOs: 224, 228, 232, 236, 240, 244, 248, 252, 256, 260, 264, 268, 272, 276, 280, 284, 288, 292, 296, 300, 304, 308, and 312. Transgenic DNA inserted during transformation may be integrated between any two base pairs, and may partially or completely replace the target sequences by homologous recombination. The sequences identified and constructs provided are useful for introducing transgenic DNA into a specific site of a corn chloroplast genome via transformation.

Example 5: Plastid Transformation Constructs

[0058] Plastid transformation constructs are designed to include a first homology arm region and a second homology arm region capable of directing insertion of an insertion sequence into a target site within a plastid DNA molecule or genome. Depending on the plant species, plastid transformation constructs are designed to insert heterologous sequences into plastid target sites including sequences selected from the group consisting of any one of SEQ ID NOs: 2, 6, 10, 14, 18, 21, 25, 29, 32, 36, 40, 44, 48, 52, 56, 60, 63, 67, 71, 75, 79, 83, 87, 91, 95, 99, 103, 107, 111, 115, 119, 122, 126, 130, 134, 137, 141, 145, 149, 153, 157, 161, 165, 169, 173, 177, 181, 185, 189, 193, 197, 201, 205, 209, 212, 216, 220, 224, 228, 232, 236, 240, 244, 248, 252, 256, 260, 264, 268, 272, 276, 280, 284, 288, 292, 296, 300, 304, 308, and 312.

[0059] Plastid transformation constructs are designed to include a first homology arm including a sequence corresponding (e.g., identical or similar) to a plastid target site or flanking region. Plastid transformation constructs may be designed to include, for example, a first homology arm sequence comprising at least a portion of a sequence selected from the group consisting of SEQ ID NOs: 3, 7, 11, 15, 19, 22, 26, 30, 33, 37, 41, 45, 49, 53, 57, 61, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 123, 127, 131, 135, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261, 265, 269, 273, 277, 281, 285, 289, 293, 297, 301, 305, 309, and 313, or a variant or fragment thereof.

[0060] Plastid transformation constructs are designed to include a second homology arm including a sequence corresponding (e.g., identical or similar) to a plastid target site or flanking region. Plastid transformation constructs may be designed to include, for example, a second homology arm sequence comprising at least a portion of a sequence selected from the group consisting of SEQ ID NOs: 4, 8, 12, 16, 20, 23, 27, 31, 34, 38, 42, 46, 50, 54, 58, 62, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 109, 113, 117, 121, 124, 128, 132, 136, 139, 143, 147, 151, 155, 159, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 214, 218, 222, 226, 230, 234, 238, 242, 246, 250, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, and 314, or a variant or fragment thereof.

[0061] Plastid transformation constructs comprising a first homology arm and a second homology arm as described are capable of inserting an insertion sequence into a particular target site or locus within a plastid genome via homologous recombination.

CLAIMS

1. A recombinant plastid transformation construct, said construct comprising:
 - (i) a first homology arm comprising a sequence that is at least 95% identical to at least 100 contiguous nucleotides of any one of SEQ ID NOs: 3, 7, 11, 15, 19, 22, 26, 30, 33, 37, 41, 45, 49, 53, 57, 61, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 123, 127, 131, 135, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261, 265, 269, 273, 277, 281, 285, 289, 293, 297, 301, 305, 309, or 313;
 - and
 - (ii) a second homology arm comprising a sequence that is at least 95% identical to at least 100 contiguous nucleotides of any one of SEQ ID NOs: 4, 8, 12, 16, 20, 23, 27, 31, 34, 38, 42, 46, 50, 54, 58, 62, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 109, 113, 117, 121, 124, 128, 132, 136, 139, 143, 147, 151, 155, 159, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 214, 218, 222, 226, 230, 234, 238, 242, 246, 250, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, or 314.
2. The recombinant plastid transformation construct of claim 1, wherein the first homology arm comprises at least 100 contiguous nucleotides of any one of SEQ ID NOs: 3, 7, 11, 15, 19, 22, 26, 30, 33, 37, 41, 45, 49, 53, 57, 61, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 123, 127, 131, 135, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261, 265, 269, 273, 277, 281, 285, 289, 293, 297, 301, 305, 309, or 313.
3. The recombinant plastid transformation construct of claim 1, wherein the second homology arm comprises at least 100 contiguous nucleotides of any one of SEQ ID NOs: 4, 8, 12, 16, 20, 23, 27, 31, 34, 38, 42, 46, 50, 54, 58, 62, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 109, 113, 117, 121, 124, 128, 132, 136, 139, 143, 147, 151, 155, 159, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 214, 218, 222, 226, 230, 234, 238, 242, 246, 250, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, or 314.

4. The recombinant plastid transformation construct of claim 1, wherein the first homology arm is between 0.1 and 2.0 kilobases in length.
5. The recombinant plastid transformation construct of claim 1, wherein the second homology arm is between 0.1 and 2.0 kilobases in length.
6. The recombinant plastid transformation construct of claim 1, further comprising an insertion sequence positioned between the first homology arm and the second homology arm.
7. The recombinant plastid transformation construct of claim 6, wherein said insertion sequence comprises a transcribable nucleic acid sequence.
8. The recombinant plastid transformation construct of claim 7, wherein said transcribable nucleic acid sequence is a gene of agronomic interest.
9. The recombinant plastid transformation construct of claim 8, wherein said gene of agronomic interest confers an agronomically beneficial trait selected from the group consisting of modified carbon fixation, modified nitrogen fixation, herbicide tolerance, insect resistance or control, modified or increased yield, fungal disease tolerance or resistance, virus tolerance or resistance, nematode tolerance or resistance, bacterial disease tolerance or resistance, modified starch production, modified oil production, modified fatty acid content, modified protein production, enhanced animal and human nutrition, environmental stress tolerance, drought tolerance, improved processing traits or fruit ripening, improved digestibility, improved taste and flavor characteristics, modified enzyme production, modified fiber production, synthesis of other biopolymers, peptides or proteins, and enhanced biofuel production.
10. The recombinant plastid transformation construct of claim 6, wherein said insertion sequence comprises a promoter active in plant plastids.
11. The recombinant plastid transformation construct of claim 10, wherein said promoter is selected from the group consisting of *Prn*, *psbA*, and *rbcL*.
12. The recombinant plastid transformation construct of claim 6, wherein said insertion sequence comprises a selectable marker.
13. The recombinant plastid transformation construct of claim 12, wherein said selectable marker is selected from the group consisting of *aadA*, *nptII*, *aph IV*, *aac3*, *aacC4*, *CAT*, *EPSPS*, *bar*, *GOX*, *GAT*, and β -glucuronidase.

14. The recombinant plastid transformation construct of claim 6, wherein said insertion sequence comprises a screenable marker.
15. A DNA molecule or vector comprising the recombinant plastid transformation construct of claim 1.
16. A plastid comprising the recombinant plastid transformation construct of claim 1.
17. A plant, plant cell, plant part, or seed comprising the plastid of claim 16.
18. The plant, plant cell, plant part, or seed of claim 17, wherein said plant is a crop plant.
19. The plant, plant cell, plant part, or seed of claim 18, wherein said plant is a monocotyledonous plant.
20. The plant of claim 19, wherein said monocotyledonous plant is selected from the group consisting of corn (maize), wheat, rice, millet, barley, sorghum, sugarcane, oat, rye, and other plants within the *Poaceae* or *Gramineae* family.
21. The plant, plant cell, plant part, or seed of claim 18, wherein said plant is a dicotyledonous plant.
22. The plant of claim 21, wherein said dicotyledonous plant is selected from the group consisting of cotton, canola, and sugar beets, soybean, alfalfa and other *Fabaceae* or leguminous plants.
23. A method of producing a transformed plant cell plastid, comprising the step of:
transforming at least one plastid of a plant cell with the recombinant plastid transformation construct of claim 6 to produce plant cell comprising a transformed plastid; wherein the insertion sequence is incorporated into a genome of said plastid flanked by plastid sequences corresponding to the first and second homology arms of the recombinant plastid transformation construct.
24. The method of claim 23, wherein the insertion sequence is incorporated into the genome of said transformed plastid by homologous recombination.
25. A transformed plastid produced by the method of claim 23.
26. The method of claim 23, further comprising the step of:

- selecting for development or regeneration of a plastid transformed plant cell by contacting said plant cell with a selection agent.
27. A plastid transformed plant cell produced by the method of claim 23.
28. The method of claim 23, further comprising the step of:
developing or regenerating a plastid transformed plant from said plant cell.
29. The method of claim 28, wherein said plastid transformed plant is developed or regenerated from said plant cell under selection pressure by contacting the developing plant with a selection agent.
30. A plastid transformed plant produced by the method of claim 23.
31. The method of claim 28, further comprising the step of:
obtaining a plastid transformed seed from said plastid transformed plant.
32. A plastid transformed seed produced by the method of claim 31.
33. A method of selecting a target site for plastid transformation, comprising
identifying a plastid target sequence, wherein said target sequence is located: (1) between two neighboring plastid genes in the plastid genome of a plant cell, (2) at least 20 base pairs from the 5' terminus of the coding sequence of a tRNA or small RNA encoding plastid gene, (3) at least 100 base pairs from the 5' terminus of the coding sequence of a structural protein-encoding plastid gene, (4) at least 20 base pairs from the 3' terminus of the coding sequence of a tRNA or small RNA encoding plastid gene, and (5) at least 150 base pairs from the 3' terminus of the coding sequence of a structural protein-encoding plastid gene.
34. A recombinant plastid transformation construct, said construct comprising a homology arm comprising a sequence that is at least 95% identical to at least 100 contiguous nucleotides of any one of SEQ ID NO: 3, 7, 11, 15, 19, 22, 26, 30, 33, 37, 41, 45, 49, 53, 57, 61, 64, 68, 72, 76, 80, 84, 88, 92, 96, 100, 104, 108, 112, 116, 120, 123, 127, 131, 135, 138, 142, 146, 150, 154, 158, 162, 166, 170, 174, 178, 182, 186, 190, 194, 198, 202, 206, 210, 213, 217, 221, 225, 229, 233, 237, 241, 245, 249, 253, 257, 261, 265, 269, 273, 277, 281, 285, 289, 293, 297, 301, 305, 309, or 313; or at least 95% identical to at least 100 contiguous nucleotides of any one of SEQ

ID NO: 4, 8, 12, 16, 20, 23, 27, 31, 34, 38, 42, 46, 50, 54, 58, 62, 65, 69, 73, 77, 81, 85, 89, 93, 97, 101, 105, 109, 113, 117, 121, 124, 128, 132, 136, 139, 143, 147, 151, 155, 159, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 211, 214, 218, 222, 226, 230, 234, 238, 242, 246, 250, 254, 258, 262, 266, 270, 274, 278, 282, 286, 290, 294, 298, 302, 306, 310, or 314.

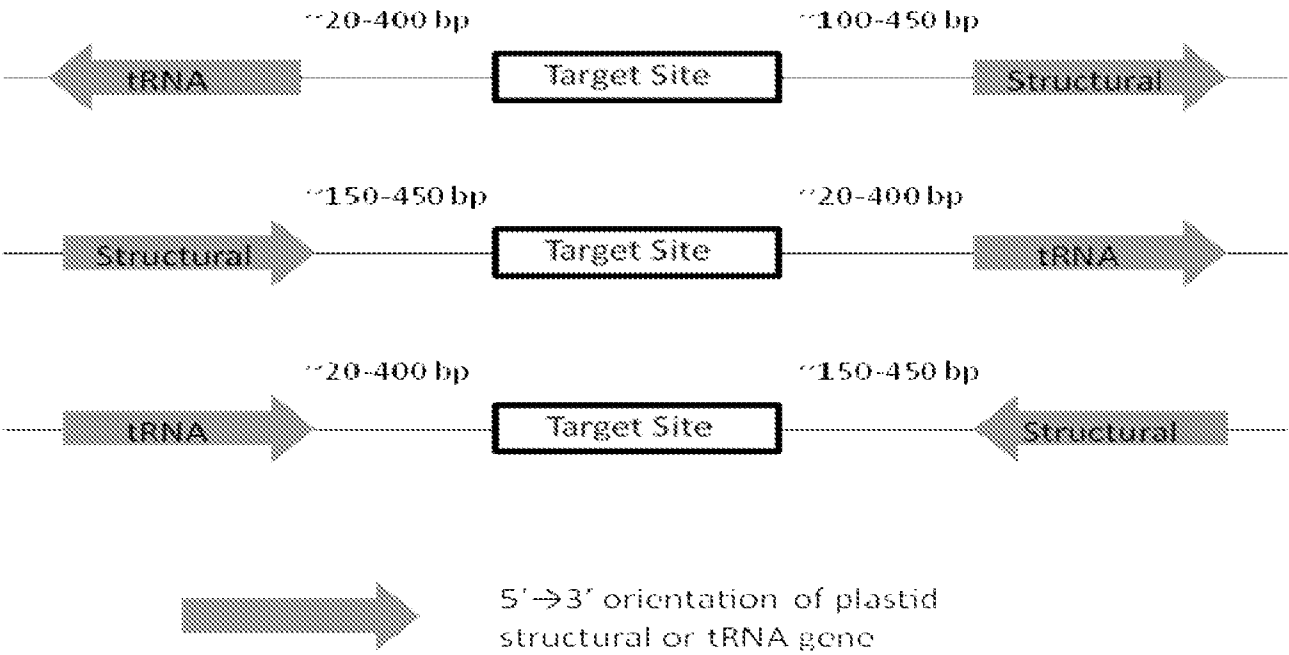


FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/67014

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/67014

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-22, 25, 27, 30, 32, 34 drawn to a recombinant plasmid transformation construct. The construct will be searched to the extent that the first homology arm comprises a sequence that is at least 95% identical to at least 100 contiguous nucleotides of SEQ ID NO: 3, and a second homology arm comprises a sequence that is at least 95% identical to at least 100 contiguous nucleotides of SEQ ID NO: 4. It is believed that claims 1-22, 25, 27, 30, 32, 34 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NOs: 3 and 4. Additional first and second homology arms will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional homology arms. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: a construct comprises a first homology arm SEQ ID NO: 7 and a second homology arm SEQ ID NO: 8 (claims 1-22, 25, 27, 30, 32, 34). - Please see extra sheet for continuation -

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-22, 25, 27, 30, 32, 34 limited to SEQ ID NOs: 3, 4

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/67014

A. CLASSIFICATION OF SUBJECT MATTER
 IPC(8) - C12N 15/82 (2018.01)
 CPC - C12N 15/8214, C12N 15/8207, C12N 15/8213

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2016/0264983 A1 (Monsanto Technology LLC) 15 September 2016 (15.09.2016) Claim 1, para [0005]-[0007], [0009], [0011], [0032], [0034], [0046], [0067], [0068], [0070], [0075], [0083],	1-22, 25, 27, 30, 32, 34
Y	GenBank KC779227 "UNVERIFIED: Glycine soja chloroplast sequence" (21 October 2014) [retrieved 26 April 2018m https://www.ncbi.nlm.nih.gov/nucore/KC779227.1/] pg 1-2, 13-14	1-22, 25, 27, 30, 32, 34
Y	US 2011/0203013 A1 (Peterson et al.) 18 August 2011 (18.08.2011) abstract, claims 48, 49	19, 20

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 April 2018

Date of mailing of the international search report

07 MAY 2018

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/67014

Continuation of:

Box NO III. Observations where unity of invention is lacking

Group II: Claims 23, 24, 26, 28, 29, 31 drawn to a method of producing a transformed plant cell plastid.

Group III: Claim 33, drawn to a method of selecting a target site for plastid transformation.

The inventions listed as Groups I+, II, III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Technical Features:

Group I+ has the special technical feature a recombinant plastid construct with comprising a first and a second homology arms, not required by Groups II or III.

Group II has the special technical feature of a method comprising transforming a plant cell with the recombinant plastid transformation construct, not required by Groups I+ or III.

Group III has the special technical feature of a method of identifying a plastid target sequence that is located in between two neighboring plastid genes in the plastid genome of a plant cell, not required by Groups I+ or II.

No technical features are shared among nucleic acid sequences of homology arm of Groups I+, and accordingly, this groups lacks unity a priori.

Additionally, even if Groups I+ inventions were considered to share the technical features of:

1. a recombinant plastid transformation construct comprising a first homology arm and a second homology arm.
2. the recombinant plastid transformation construct further comprises an insertion sequence positioned between the first homology arm and the second homology arm.

these shared technical features are previously disclosed by US 2016/0264983 A1 to Monsanto Technology, LLC (hereinafter "Monsanto") [published 16 September 2016].

As to shared technical features #1 and #2, Monsanto teaches (claim 1, A method of transforming a plant plastid, comprising the steps of: (a) preparing an explant from a seed of a plant, the explant comprising meristematic tissue of an embryo of the seed; and (b) transforming at least one plastid of a cell of the explant with an exogenous DNA molecule, the exogenous DNA molecule comprising: (i) a first arm region homologous to a first plastid genome sequence; (ii) a second arm region homologous to a second plastid genome sequence; and (iii) an insertion sequence positioned between the first arm region and the second arm region of the exogenous DNA molecule, wherein the cells of the explant do not form a callus tissue prior to the transforming step (b), and wherein the insertion sequence is incorporated into a plastid genome of the plant cell between the first plastid genome sequence and the second plastid genome sequence"; para [0070]: "Each of these homology arms may typically have a base pair (bp) length of up to about 5 kilobases (kb), such as in a range from about 0.1 kb to about 5 kb in length, or in a range from about 0.5 kb to about 2 kb in length, or in a range from about 1 kb to about 1.5 kb in length. The homology arms are positioned on either side of an insertion sequence comprising one or more transgene(s) for insertion into a plastid or plastomic DNA molecule. Each of the homology arms may generally be highly homologous, nearly identical or identical to a corresponding target plastid DNA sequence present in an explant cell, which may be known or determined empirically. For example, each homology arm may be at least 80% identical to the corresponding target plastid DNA sequence, or at least 90% identical, or at least 95% identical").

As the shared technical features were known in the art at the time of the invention, they cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+, II, III lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.