



(51) International Patent Classification:

A01H 1/04 (2006.01) C12N 15/82 (2006.01)  
A01H 1/08 (2006.01) C12Q 1/68 (2018.01)  
A01H 5/10 (2018.01) C12Q 1/6895 (2018.01)  
C12N 9/18 (2006.01)

(21) International Application Number:

PCT/US2020/019499

(22) International Filing Date:

24 February 2020 (24.02.2020)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

PCT/CN2019/076062

25 February 2019 (25.02.2019) CN

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(54) Title: COMPOSITIONS AND METHODS FOR DRIVING T1 EVENT DIVERSITY

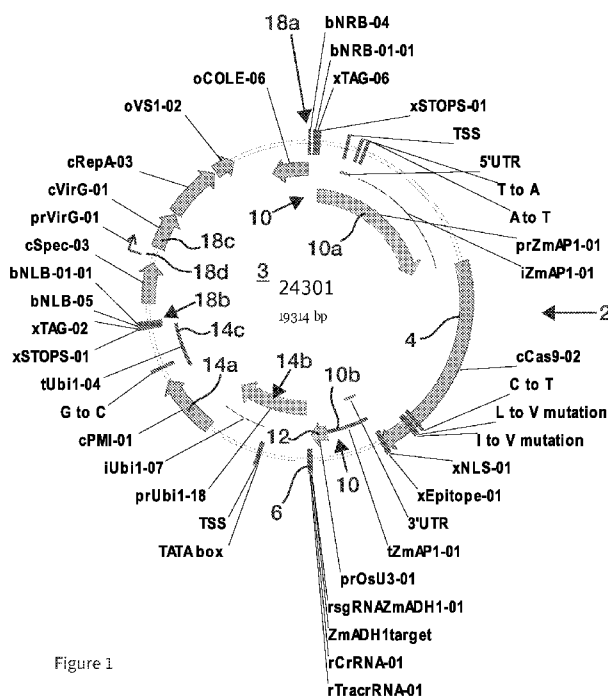


Figure 1

(57) Abstract: Systems and methods for producing a plurality of unique edits in a plant's T1 seed. In one example, a method comprises transforming at least one expression cassette into a plant cell or a plant tissue. The at least one expression cassette may comprise a nucleic acid that encodes a DNA modification enzyme; optionally, a nucleic acid that encodes at least one guide RNA (gRNA); and a floral mosaic (FMOS) regulatory sequence, wherein the FMOS regulatory sequence (i) mediates expression of the DNA modification enzyme in at least one of a floral primordia cell and a floral reproductive organ, and (ii) mediates a plurality of edits in the at least one of the floral primordia and the floral reproductive organ. The method may also include regenerating the plant cell or plant tissue into a TO plant having a plurality of T1 seed, wherein the T1 seed contain a plurality of unique edits.



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(81) **Designated States** (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) **Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Declarations under Rule 4.17:**

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

**Published:**

— *with international search report (Art. 21(3))*  
 — *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*  
 — *with sequence listing part of description (Rule 5.2(a))*

(88) **Date of publication of the international search report:**  
 01 October 2020 (01.10.2020)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/019499

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01H 1/04; A01H 1/08; A01H 5/10; C12N 9/18; C12N 15/82; C12Q 1/68; C12Q 1/6895 (2020.01)  
 CPC - A01H 5/10; C12N 9/18; C12N 15/8216; C12N 15/8218; C12Q 1/686 (2020.05)

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.
A	US 2018/0223295 A1 (KWS SAAT SE) 09 August 2018 (09.08.2018) entire document	1-22
A	US 2018/0092316 A1 (SYNGENTA PARTICIPATIONS AG) 05 April 2018 (05.04.2018) entire document	1-22
A	WO 2017/087682 A1 (SYNGENTA PARTICIPATIONS AG et al) 26 May 2017 (26.05.2017) entire document	1-22
A	US 2018/0273963 A1 (SYNGENTA PARTICIPATIONS AG) 27 September 2018 (27.09.2018) entire document	1-22
A	TANG et al. "Development and Validation of an Effective CRISPR/Cas9 Vector for Efficiently Isolating Positive Transformants and Transgene-Free Mutants in a Wide Range of Plant Species," <i>Frontiers in Plant Science</i> , 23 October 2018 (23.10.2018), Vol. 9, Art. 1533, Pgs. 1-14. entire document	1-22

 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

"D" document cited by the applicant in the international application

"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

"&amp;" document member of the same patent family

Date of the actual completion of the international search

03 August 2020

Date of mailing of the international search report

14 AUG 2020

Name and mailing address of the ISA/US

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P.O. Box 1450, Alexandria, VA 22313-1450

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/019499

**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:

SEQ ID NOs: 2, 3, 20, and 21 were searched.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2020/019499

**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:  
See extra sheet(s).

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 to the extent that they read on an expression cassette of SEQ ID NO:1, a FMOS promotor of SEQ ID NO:2, and a FMOS terminator of SEQ ID NO:3.

**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.

Continued from Box No. III Observations where unity of invention is lacking

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 need to be paid.

Group I+: claims 1-22 are drawn to expression cassettes comprising floral mosaic (FMOS) regulatory sequences, and methods comprising the same.

The first invention of Group I+ is restricted to an expression cassette comprising floral mosaic (FMOS) regulatory sequences, and methods comprising the same, wherein the expression cassette is selected to be SEQ ID NO:1; and the floral mosaic regulatory sequences are a FMOS promoter of SEQ ID NO:2 and a FMOS terminator of SEQ ID NO:3. It is believed that claims 1-22 read on this first named invention and thus these claims will be searched without fee to the extent that they read on an expression cassette comprising SEQ ID NOs:1, 2, or 3.

Applicant is invited to elect additional expression cassettes and/or FMOS regulatory sequences, each with specified SEQ ID NO, to be searched in a specific combination by paying an additional fee for each set of election. An exemplary election would be an expression cassette comprising floral mosaic (FMOS) regulatory sequences, and methods comprising the same, wherein the expression cassette is selected to be SEQ ID NO:19; and the floral mosaic regulatory sequences are a FMOS promoter of SEQ ID NO:20 and a FMOS terminator of SEQ ID NO:21. Additional expression cassettes and/or FMOS regulatory sequences will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. 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.

The inventions listed in Groups I+ 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:

The Groups I+ formulas do not share a significant structural element for producing a plurality of unique edits in a plant, requiring the selection of alternatives for the expression cassettes and/or FMOS regulatory sequences, where "the FMOS promoter is selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 20, SEQ ID NO: 26, SEQ ID NO: 29, SEQ ID NO: 35, SEQ ID NO: 47, SEQ ID NO: 50, SEQ ID NO: 53, SEQ ID NO: 56, SEQ ID NO: 62, SEQ ID NO: 65, SEQ ID NO: 71, SEQ ID NO: 74, SEQ ID NO: 77, SEQ ID NO: 80, SEQ ID NO: 83, SEQ ID NO: 86, SEQ ID NO: 514, SEQ ID NO: 518, SEQ ID NO: 614, SEQ ID NO: 615, SEQ ID NO: 616, SEQ ID NO: 617, SEQ ID NO: 618, SEQ ID NO: 619, SEQ ID NO: 620, SEQ ID NO: 621, SEQ ID NO: 622, SEQ ID NO: 698, SEQ ID NO: 700, SEQ ID NO: 702, SEQ ID NO: 704, SEQ ID NO: 706, SEQ ID NO: 708, SEQ ID NO: 710, SEQ ID NO: 717, SEQ ID NO: 719, SEQ ID NO: 721, and SEQ ID NO: 723 or a sequence with at least one of at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 90% homology thereto" and "the FMOS terminator is selected from the group consisting of at least one of SEQ ID NO: 3, SEQ ID NO: 21, SEQ ID NO: 24, SEQ ID NO: 27, SEQ ID NO: 30, SEQ ID NO: 36, SEQ ID NO: 48, SEQ ID NO: 51, SEQ ID NO: 54, SEQ ID NO: 57, SEQ ID NO: 63, SEQ ID NO: 66, SEQ ID NO: 72, SEQ ID NO: 75, SEQ ID NO: 78, SEQ ID NO: 81, SEQ ID NO: 84, SEQ ID NO: 87, SEQ ID NO: 515, SEQ ID NO: 519, SEQ ID NO: 699, SEQ ID NO: 701, SEQ ID NO: 703, SEQ ID NO: 705, SEQ ID NO: 707, SEQ ID NO: 709, SEQ ID NO: 718, SEQ ID NO: 720, SEQ ID NO: 722, and SEQ ID NO: 724 or a sequence with at least one of at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 90% homology thereto".

Additionally, even if Groups I+ were considered to share the technical features of a method for producing a plurality of unique edits in a plant's T1 seed, the method comprising: a) transforming at least one expression cassette into a plant cell or a plant tissue, wherein the at least one expression cassette comprises a nucleic acid that encodes a DNA modification enzyme, optionally, a nucleic acid that encodes at least one guide RNA (gRNA), and a floral mosaic (FMOS) regulatory sequence, wherein the FMOS regulatory sequence (i) mediates expression of the DNA modification enzyme in at least one of a floral primordia cell and a floral reproductive organ, and (ii) mediates a plurality of edits in the at least one of the floral primordia and the floral reproductive organ; and b) regenerating the plant cell or plant tissue into a T0 plant having a plurality of T1 seed, wherein the T1 seed contain a plurality of unique edits; at least one expression cassette for producing at least 10 unique edits in a plant's T1 seed, the expression cassette comprising: a nucleic acid that encodes a DNA modification enzyme, optionally, a nucleic acid that encodes at least one guide RNA (gRNA), and a floral mosaic (FMOS) promoter, wherein the FMOS promoter (i) mediates expression of the DNA modification enzyme in at least one of a floral primordia cell and a floral reproductive organ, and (ii) mediates a plurality of edits in the at least one of the floral primordia and the floral reproductive organ, and (iii) mediates at least 2x more expression of the DNA modification enzyme in the at least one of the floral primordia and the floral reproductive organ than in a vegetative tissue, and at least 2x more expression of the DNA modification enzyme in the at least one of the floral primordia and the floral reproductive organ than in a seed; these shared technical features do not represent a contribution over the prior art.

Specifically, US 2018/0273963 A1 to Syngenta Participations AG discloses a method for producing a plurality of unique edits in a plant's T1 seed (the invention provides a method of editing a plant's genomic DNA, Para. [0016]; The result is a haploid embryo or plant or seed that contains the chromosome set only from the non-inducer parent, where that chromosome set contains DNA sequences that have been edited, Para. [0015]; T1 progeny that retained two copies of the Cas9 and guide RNA editing machinery, Para. [0143]), the method comprising: a) transforming at least one expression cassette into a plant cell or a plant tissue (maize inbred line NP2222 is transformed with VLHP Cas9-sgRNA vectors, Para. [0172]), wherein the at least one expression cassette comprises a nucleic acid that encodes a DNA modification enzyme (GW2 Cas9-sgRNA vectors, Para. [0172]), optionally, a nucleic acid that encodes at least one guide RNA (gRNA) (GW2 Cas9-sgRNA vectors, Para. [0172]), and b) regenerating the plant cell or plant tissue into a T0 plant having a plurality of T1 seed (transgenic maize T0 plants carrying T-DNA of vector 24038, Para. [0075]; [w]e produced several events and self-pollinated them to make T1 seed. We grew up T1 individuals from event MZET152408A042A. We recovered five T1 progeny that retained two copies of the Cas9 and guide RNA editing machinery, Para. [0143]), wherein the T1 seed contain a plurality of unique edits (Haploids containing the desired edits is chromosomally doubled using standard procedures, Para. [0160]; T1 progeny that retained two copies of

the Cas9 and guide RNA editing machinery, Para. [0143]); and a floral mosaic (FMOS) regulatory sequence (paternal haploid editing (using a maternal haploid inducer line might be increased through the use of a promoter that drives the expression of Cas9 and/or the guide RNA to a higher level in the egg cell before fertilization, Para. [0224]), wherein the FMOS regulatory sequence (i) mediates expression of the DNA modification enzyme in at least one of a floral primordia cell and a floral reproductive organ (paternal haploid editing (using a maternal haploid inducer line might be increased through the use of a promoter that drives the expression of Cas9 and/or the guide RNA to a higher level in the egg cell before fertilization, Para. [0224]), and (ii) mediates a plurality of edits in the at least one of the floral primordia and the floral reproductive organ (These tests showed that all of the seed that UC Davis sent were homozygous for the mutant allele Atcenh3-1, and that there were multiple copies of the ZmCENH3 transgene present, Para. [0209]; a promoter that drives the expression of Cas9 and/or the guide RNA to a higher level in the egg cell before fertilization, Para. [0224]), at least one expression cassette for producing at least 10 unique edits in a plant's T1 seed (extant editing machinery (comprising a site directed nuclease protein and optionally at least one guide RNA) may be introduced to a haploid embryo upon application of appropriate cell-penetrating peptides, Para. [0099]; "at least one", when employed herein to refer to an entity, refers to, for example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100, or more of that entity, Para. [0085]) and (iii) mediates at least one at least one of at least 2x more expression of the DNA modification enzyme in the floral reproductive organ than in a vegetative tissue (Pollen was also used for a qRT experiment, in which the expression of the Cas9 was measured at the RNA level and compared to Cas9 expression in leaf samples when the Cas9 was driven by a sugar cane ubiquitin promoter used in many of the corn and wheat examples given above. As you can see in FIG. 17, the expression was high in the pollen, averaging about 100 fold higher in plants carrying the T-DNA vectors 24038, Para. [0189]), and at least one at least one of at least 2x more expression of the DNA modification enzyme in the at least one of the floral primordia and the floral reproductive organ than in a seed (paternal haploid editing (using a maternal haploid inducer line) might be increased through the use of a promoter that drives the expression of Cas9 and/or the guide RNA to a higher level in the egg cell before fertilization, Para. [0224]; this haploid seed will only contain the chromosomes of the second plant; the first plant's chromosomes have vanished (having been eliminated, lost or degraded), but before doing so, the first plant's chromosomes permitted the gene-editing machinery to be expressed, Para. [0016]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.