Research Article

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Role of MS1 homolog Ntms1 gene of tobacco infertility

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Abstract: The sterile line is the basis of crop heterosis utilization. To broaden the sources of male sterility in tobacco, the Ntms1 (Nicotiana tabacum L. ms1) gene was cloned from the tobacco variety K326 by homologous cloning based on the Cams1 (Capsicum annuum L. ms1) gene sequence of male-sterility genes in pepper. The protein structure and physicochemical properties of the two genes were determined by bioinformatics analysis, and the function of the Ntms1 gene was verified by the CRISPR/Cas9 system. The results showed that the sequences of Ntms1 and Cams1 were 85.25% similar, and plant homeodomains were found in both genes; the physical and chemical properties were also very similar. It is speculated that the Ntms1 gene had the same function as the Cams1 gene in controlling male sterility. Compared to the wild-type plants, the filaments of the Ntms1 knockout mutant plants were shorter, and the stamen was shorter than the pistil. The anthers did not develop fully and had few viable pollen grains; the tapetum and the anther wall had developed abnormally, and the anther chamber was severely squeezed. The malondialdehyde content in the mutant plants was significantly higher than that in the wild-type plants, while self-fertility was significantly lower in the mutant plants. The results showed that the Ntms1 gene plays an important role in regulating fertility in tobacco.

Keywords: MS1 gene, tobacco, fertility, homologous cloning

1 Introduction

Male sterility is an important agronomic trait in studies of crop heterosis utilization. It is a genetic phenomenon defined by the inability of plants to produce normal anthers, pollens, or male gametes during sexual reproduction, while the pistil functions normally [1]. The sterile males are used to produce hybrids that can save artificial castration, reduce seed production costs, and improve seed purity. To date, 13 genes associated with male sterility have been cloned from Arabidopsis and maize. Among them, the MS1 gene, which has been proven to be a key gene regulating pollen formation, is mainly expressed when microspores are released from the tetrad and encodes a plant homeodomain (PHD)-containing nuclear protein [2]. PHD is closely related to the composition of the pollen wall and the tapetum development and does not affect other floral organs. They cause pollen abortion, and thus, can be used in production [3]. The PTC1 gene in rice was found to be homologous to the MS1 gene in Arabidopsis, which also encodes a PHD domain and shows similar functions in regulating the development of villus cells and the pollen wall [4]. The homologous Zmms1 gene in maize was cloned from the Osm1 gene in rice by homologous cloning, and the results of the bioinformatics analysis showed that both Zmms1 and Osm1 were very similar to the members of the PHD domain family. It is speculated that the Zmms1 gene, along with Osm1, may control fertility [5]. The CRISPR/Cas9 system was used to develop male-sterile lines without transgenic maize and obtain stable genetic lines [6]. Using the MS1 gene in Arabidopsis thaliana as a reference sequence, bioinformatic methods were applied to predict and analyze the amino acid sequences encoded by Brassica crops such as rape, Chinese cabbage, and cabbage. The results showed that the MS1 gene in Brassica crops belonged to the PHD finger family, and its highly conserved sequence was involved in the regulation of pollen development and maturation [7]. Using the CRISPR/Cas9 system to create targeted mutations in MS1,
a double displacement code mutation was introduced into MS1, which resulted in complete male sterility in wheat varieties Fielder and Gladius [8]. Three homologous sequences of the MS1 gene modified by the CRISPR/Cas9 system could rapidly generate male-sterile wheat lines [9]. The CRISPR/Cas9 system was used to mediate the knockout of the MS1 gene, causing extremely low expression levels of this gene in the anther, which had missing spikelets; the self-fertilization rate was also significantly reduced [10]. The Cams1 gene in pepper is highly homologous to the MS1 gene in Arabidopsis thaliana. It contains the PHD domain and two low complexity domains, which control pollen fertility. When compared with fertile plants, the number of pollens in sterile pepper plants was significantly reduced [11]. In summary, the role of the MS1 gene in the regulation of fertility has been confirmed in major crops such as rice, maize, rape, wheat, and pepper. However, it has not yet been reported whether the MS1 gene regulates male sterility in tobacco.

The tobacco leaves are the most economically important part of the plant. For controlling the various layouts and areas of tobacco production, in addition to the direct use of sterile hybrids, pure line varieties bred by hybridization are often transformed into sterile lines for tobacco production. The proportion of sterile lines in tobacco production is as high as 80%. However, the sterile lines used in tobacco production are derived only from the cytoplasmic sterile genes of N. suaveolens, and there is a single source of sterility in genes; the cytoplasm is easily infected by pathogenic bacteria, which affects the production and application of tobacco hybrids and sterile lines [12,13]. Therefore, broadening the source of male sterility in tobacco is very important for stable tobacco production. While studying male sterility in tobacco, previous studies had mostly focused on cytoplasmic male sterility and found Atp6, Atp9, Arf25, and Arfb sterile cytoplasmic genes [14]. Tobacco and pepper belong to the Solanaceae family and have high homology. Therefore, in this study, the Ntms1 gene was cloned from tobacco by the homologous cloning method based on the Cams1 genome sequence of pepper, and the knockout vector was constructed by the CRISPR/Cas9 technology. The function of the Ntms1 gene was identified, and sterile tobacco plants were produced, which have immense scientific significance and practical value for broadening the source of male sterility in tobacco.

2 Materials and methods

2.1 Tobacco plant varieties

The tobacco variety used in the experiment was the main variety K326, usually used in tobacco production and preserved and provided by the Guizhou Tobacco Quality Key Research Laboratory.

2.2 Carriers and strains

Escherichia coli DH5α and Agrobacterium tumefaciens EHA105 were preserved and provided by the Guizhou Tobacco Quality Research Key Laboratory. The TA cloning vector pMD18-T was provided by the Shaanxi Border Biotechnology Co., Ltd. The target design and comparison of the CRISPR/Cas9 recombinant vector MSG2 were performed in the Guizhou Key Laboratory of Tobacco Quality Research, and the synthesis of the CRISPR/Cas9 recombinant vector was performed by Baige Gene Technology (Jiangsu Co., Ltd).

2.3 Total RNA extraction and cDNA synthesis

Total RNA was extracted using the RNAiso Plus Tri Zol (Ta Ka Ra). The cDNA was synthesized using the Poly Attract® mRNA Isolation System III with Magnetic Stand kit from Promega, which was purchased from Bao Bioengineering (Dalian) Co., Ltd. The reverse transcription system and reaction procedure are listed in Table S2.

2.4 The creation of mutants

2.4.1 Cloning and vector construction of target genes

The amino acid sequence Cams1 (LOC107852993) of the MS1 protein in pepper was obtained from the NCBI website (https://www.ncbi.nlm.nih.gov/). The amino acid sequence was compared to that in common tobacco to find the protein that had the highest homology with MS1.
The ProtParam system and chemical properties of the isoelectric point in NCBI was used to identify the open reading frames of the extinction and instability coefficients of proteins. The ProtScale online software was used for analyzing the protein domain. The NetPhos InterPro online software was used to estimate the hydrophilicity of the proteins. The TMHMM online software was used to analyze and predict the secondary and tertiary structures of the proteins, respectively. The SOPMA and Phyre2 were used online to analyze the protein phosphorylation sites. The SOPMA and Phyre2 were used online to analyze the protein phosphorylation sites. The SOPMA and Phyre2 were used online to analyze the protein phosphorylation sites. The SOPMA and Phyre2 were used online to analyze the protein phosphorylation sites.

2.4.2 Bioinformatics analysis

The ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) in NCBI was used to identify the open reading frames of the *Cams1* gene in pepper and the *Ntms1* gene in tobacco. The ProtParam system (https://web.expasy.org/protparam/) in the ExPASy database was used to analyze the physical and chemical properties of the isoelectric point (pI) and the extinction and instability coefficients of proteins. The ProtScale online software (https://web.expasy.org/protscale/) was used to estimate the hydrophilicity of the proteins. The InterPro online software (http://www.ebi.ac.uk/interpro/) was used for analyzing the protein domain. The NetPhos 3.1 online website (http://www.cbs.dtu.dk/services/NetPhos/) was used to analyze the protein phosphorylation sites. The SOPMA and Phyre2 were used online to analyze and predict the secondary and tertiary structures of the proteins, respectively. The TMHMM online software (http://www.cbs.dtu.dk/services/TMHMM/) was used to analyze the transmembrane domains in proteins. The Signal P4.1 online software was used to determine whether the proteins contained signal peptides. The DNAMAN software was used to perform multiple alignments of protein sequences and observe the similarity between them. The MEGAl7.0 software was used to construct a molecular evolutionary phylogenetic tree for the species selected from the NCBI database, and the phylogenetic relationship between species was observed using the evolutionary tree.

2.4.3 Genetic transformation and screening for positive strains

The genetic transformation technique was applied based on the CRISPR/Cas9 gene-editing technology established by Tian [16]. The identification of positive mutants was based on the screening method developed by Chen et al. [17].

2.4.4 Gene expression analysis of the *Ntms1* gene in mutant plants

The RT-PCR primers PHD_F (ATGTTGCGCTTGTGATGTCTG)/PHD_R (TTCCGCTTGTATTGTGATGT) were designed based on the *Ntms1* sequence, and the expression of the *Ntms1* gene was analyzed using the tobacco Actin internal reference gene primers, Actin-F (CATGAAGATTAGGCCG GAGTG)/Actin-R (AACAGTTTGTTGGAGTTCTGG).

2.5 Analysis of the fertility of the mutants

2.5.1 Observation of the floral organ in mutant strains

Wild-type tobacco plants were considered as control. The upcoming opening buds were taken; the anther fullness, stigma, and filament length were observed, and photographs were taken.

2.5.2 Identification of mutant strains associated with drug development

The flower buds of the mutant strains were fixed in a formalin-acetic acid-alcohol solution, and the drug delivery sections were observed according to the method developed by Wang et al. [18].

2.5.3 Identification of pollen grains in mutant plants

At the full bloom stage, plants were cut using a dissecting needle to remove the anther wall before it cracked. All the pollens were picked up after dyeing and mixed in a 200 µL I-KI staining solution. The pollen morphology was observed under the microscope (XHC_L2, 40×) after dyeing. Five fields were randomly selected for each plant, and the number of dyed pollen grains was calculated. The active pollens were the fully developed jacinth, while the inactive and less active pollens were buff.

2.5.4 Detection of the MDA content in mutant strains

The mature leaves from the first (bottom) to the 14th leaf (top) in each mutant were transferred to the laboratory in
a wet gauze and measured by the methods developed by Zhang et al. [19].

2.5.5 The estimation of self-fertility rate in mutant strains

After the central flower in the mutant plants opened, ten flowers with slightly red corollas that had been bagged and self-pollinated were randomly selected from each mutant; the seeds were harvested at maturity, and the fruit rate was recorded. The number of seeds per fruit was then calculated, and the relative seed setting rate of the mutant plants was estimated.

2.6 Statistical analysis

The Excel 2013 (Microsoft, USA), DPS 14.10 (Hangzhou Ruifeng Information Technology Co., Ltd., China), and Origin (OriginLab, the US) software were used for statistical data analysis. All the data used were the average of three biological replicates; the error bar represents standard deviation (±SD). A "*" sign represents \( P < 0.05 \), with significant difference; a "**" sign represents \( P < 0.01 \), with highly significant difference.

3 Results

3.1 The function of the Ntms1 gene in controlling fertility

The Ntms1 gene, which was obtained by homologous cloning of the Cams1 gene, controls fertility. The results of the bioinformatics analysis showed that the start codon of Ntms1 is located at the 36th nucleotide, and the stop codon is located at the 2,174th nucleotide, which encodes a total of 712 amino acids. The number of exons is 2,137 bp, and the number of introns is 817 bp. The start codon of Cams1 is located at the first nucleotide, and the stop codon is located at the 2,100th nucleotide. It encodes a total of 699 amino acids; the number of exons is 2,111 bp, and the number of introns is 584 bp. The coding regions of the two genes overlap, and the difference in the number of encoded amino acids is very small, indicating that the proteins encoded by the two genes are not significantly different (Figure S1). The similarity between Ntms1 and Cams1 proteins was 85.25%. Both were non-secretory and non-transmembrane proteins, and their receptors had protein kinase activity (Figures S2–S6). The hydrophilicity indices of these two proteins were −0.238 and −0.299, respectively, which made them hydrophilic proteins. Additionally, the physical and chemical properties such as molecular weight, isoelectric point, and the number of positively and negatively charged amino acids were very similar (Table 1), and they all had the same PHD domain (Figure S5). The spatial similarity of the secondary structure was also very high (Table S1), and the result of the phylogenetic tree analysis (Figure S8) showed that the homology between the two was 100%. It is speculated that the Ntms1 gene has the same function as Cams1 in controlling fertility.

3.2 The effect of the transformant Ntms1 gene editing

The results showed that the Cas9 gene could be detected by the CRISPR/Cas9 gene-editing technology in six genetically transformed plants (Figure S7), and 4–6 base substitutions occurred at the targeted gene sites of the transformed plants (Figure 1). The expression of the Ntms1 gene in the six mutant strains was significantly lower (48–69%) than that in the wild-type plants (Figure 2). The results showed that the CRISPR/Cas9 vector played an important role in editing the target gene in the transformed strain, resulting in significant changes in the sequence and expression level of the target gene. The target gene of the transformed strain was mutated.

Table 1: Physical and chemical properties of Cams1/Ntms1 protein

| Protein | Molecular weight (kDa) | Isoelectric point | Negatively charged amino acid (Asp+Glu) | Positively charged amino acid (Arg+Lys) | Unstable coefficient | Hydrophilic index |
|---------|------------------------|------------------|----------------------------------------|----------------------------------------|---------------------|------------------|
| Cams1   | 78.16                  | 8.22             | 81                                     | 87                                     | 37.95               | −0.238           |
| Ntms1   | 79.62                  | 8.48             | 82                                     | 91                                     | 35.25               | −0.299           |
3.3 Significant changes in fertility-related traits of Ntms1 knockout mutants

3.3.1 Changes in the structure of floral organs in mutant strains

Observations of the morphology of the floral organ of the mutant and wild-type plants (Figure 3) showed that although both the plants could flower normally, the anther and filament length of the mutant plants changed significantly when compared with those of the wild-type plants. The anthers of the wild-type plants were full and larger than the stigma, and pollen grains were scattered on the surface of the hair. However, the filaments of the mutant were shorter, the anther height was lower than that of the stigma, and self-pollination could not be performed. The anthers were shrunk, and there were few mature pollen grains. The knockout of the Ntms1 gene led to the shortening of the filament and pollen abortion.

3.3.2 Abnormal development of the anther wall and tapetum in mutant strains

Observations of the anther slices (Figure 4) showed that the edges of the anther wall of the wild-type tobacco were clear, and multiple, nearly spherical pollen grains were evenly distributed in the anther chamber. The anthers in the mutant plants developed abnormally, and the chambers were severely squeezed; death of the tapetum occurred, and the pollen grains were abnormal. This indicates that the deletion of the Ntms1 gene resulted in the abnormal development of the tapetum, which could not provide the necessary nutrients for microspore development.

3.3.3 Membrane damage in mutants

In a study on male sterility in cotton, a high MDA content caused abnormal metabolism, accumulation and peroxidation of membrane lipids, and pollen abortion [20]. Figure 5 shows that the MDA content of the mutant plants was significantly higher than that of the wild-type plants, indicating that the knockout of the Ntms1 gene had caused a certain degree of damage to the membrane system, which might have led to infertility.

3.3.4 A significant decrease in the number of viable pollen grains

The number of viable pollen grains and pollen viability was the most direct indicators of fertility. The results of the determination of the number of pollen grains and pollen viability in the mutant plants (Table 2, Figure 6) showed that viable pollen grains accounted for 6.4–10.8% of the total pollen grains and were significantly lower than that in the wild-type plants. The color of the viable pollen grains in the mutant plants was pale yellow after staining, which was lighter than that in the wild-type plants, indicating that the vitality of viable pollen grains in the mutant plants was lower than that in the wild-type plants. The knockout of the Ntms1 gene resulted in a significant reduction in the number of viable pollen grains and pollen viability.

3.3.5 A significant decrease in self-pollinated mutants

Self-pollination is the most direct indicator of fertility. The results of the effect of self-pollination on each
Figure 3: Observation on flower morphology of the mutants and WT. (a) On the left is the wild type, the anther is higher than the stigma. On the right is the mutant, the anther is lower than the stigma. (b and c) Mutant, anther atrophy, growth short and below the stigma.

Figure 4: Paraffin section observation of pollen. (a, c, and e) WT. (b, d, and f) Mutant. Msp, microspores. T, tapetum. (a and b), Bars = 200 μm. (c–f), Bars = 20 μm.
represents highly significant difference. Bioinformatics analysis showed that both Zmms1 and Osms1 were very similar and members of the PHD domain family. It is speculated that Zmms1 may have the same function as Osms1 in controlling fertility [5], and stable male-sterile lines in maize were obtained by the CRISPR/Cas9 system [6]. In this study, the open reading frame (ORF) of the Camsl gene in pepper and the Ntms1 gene in tobacco were identified using the ORF from the NCBI database. Due to similar physicochemical properties such as the protein isoelectric point, protein domain, and extinction and instability coefficients, it was predicted that the Ntms1 gene might have the same function as the Camsl gene in controlling fertility. The results of the CRISPR/Cas9 gene-editing technology showed that the bioinformatics analysis was a reliable method for predicting the function of homologous genes and can also improve the efficiency of the identification of gene functions.

The Msi1 gene was proven to be a key regulator of pollen development in major crops such as rice, maize, rape, wheat, and pepper. Studies have found that the Msi1 gene encodes a PHD-containing nuclear protein [2]. PHD was closely related to pollen wall composition and tapetum development and did not affect other floral organs but caused pollen abortion [3]. The knockout of the Msi1 gene resulted in atrophy of the anther in rice, lowering the number of anthers compared to the stigma, thus preventing self-pollination [22]. The Msi1 gene knockout also caused the pollen number and pollen viability in Cucurbita pepo to decrease [23]. Additionally, due to the knockout, anthers were found to be missing, and pollen sac cells in maize flowers remained undifferentiated [24]. Moreover, the anthers of rice were white and short, with no mature pollen grains, and the seed setting rate decreased significantly [25]. The MDA content in cotton increased significantly, causing abnormal metabolism and accumulation and peroxidation of membrane lipids, thus resulting in pollen abortion [20]. In this study, the Ntms1 gene of the tobacco variety K326 was knocked out by the CRISPR/Cas9 gene-editing technology. It was found that the expression of the Ntms1 gene in mutant plants was significantly reduced. The filaments of the floral organs were shortened, and anthers had shrunk; the development of the pollen wall and tapetum were abnormal, and the chamber was severely squeezed. Moreover, the number of viable pollen grains was significantly reduced, and there was also a reduction in the pollen viability; the self-fertility rate was extremely low, and some mutant flowers were even self-infertile, indicating that the Ntms1 gene regulated fertility in tobacco.
The CRISPR/Cas9 gene-editing technology was used to identify the gene functions. If the gene affects quantitative traits, the T₁ generation strain should be used, and the test should be performed according to the unique difference principle to obtain accurate identification results. In this study, since the fertility of tobacco plants was the research interest and self-sterility in pollen could not be achieved, the obtained T₁ generation strain eliminated sterile pollen by natural selection, which no longer provided a strict random population sample. Therefore, the direct identification of sterile mutant traits by using mutant strains of the T₀ generation was highly reliable in this study.

5 Conclusion

In this study, the Ntms₁ gene was cloned from tobacco, and bioinformatics analysis was performed to determine whether the Ntms₁ gene in tobacco and the sterile gene Cams₁ in pepper control fertility similarly. Sterile mutant plants were obtained by the CRISPR/Cas9 gene-editing technology. It was found that the expression level of the mutant Ntms₁ gene was significantly reduced. The filaments of the floral organ were shortened, the anthers were shrunk, and the development of the pollen wall and the tapetum was abnormal. Furthermore, the chamber was severely squeezed, there was a significant reduction in the number of viable pollen grains, and the pollen vitality was also reduced. The self-fertilization rate was extremely low, and some plants were even self-infertile, proving that the Ntms₁ gene regulates fertility in tobacco plants. The results of this research have immense scientific significance and practical value as they provide a

### Table 3: Statistics of self-fertilization of mutants

| Lines | Fruit rate (%) | Number of seeds per fruit (seeds) | Relative seed setting rate (%) |
|-------|----------------|-----------------------------------|-------------------------------|
| WT    | 100            | 3074.30                           | 100.00                        |
| C129  | 80**           | 1319.00**                         | 42.90**                       |
| C60   | 80**           | 1170.20**                         | 38.06**                       |
| B23   | 70**           | 1015.50**                         | 33.03**                       |
| B34   | 70**           | 885.50**                          | 28.80**                       |
| B21   | 70**           | 770.60**                          | 25.07**                       |
| C125  | 60**           | 677.30**                          | 22.03**                       |

Note: All data are the average of three biological repetitions, a "*" sign represents $P < 0.05$, with a significant difference; a "**" sign represents $P < 0.01$, with a highly significant difference.

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basis for further research on the mechanism of the induction and development of new sources of male sterility in tobacco.

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Author contributions: C.Q. and Z.T.T. performed bioinformatics analysis, cloning, and vector construction of the manuscript written by C.Q. C.Q. and T.M.Z. were genetically transformed. C.Q. performed phenotypic observation, pollen vigor measurement, M.D.A. content measurement, and paraffin section. L.R.X. designed and supervised the study, M.Z.J. and D.L.L. performed RNA extraction, and L.Z.H. and L.Y. performed expression data analysis. All authors read and approved the final manuscript.

Conflict of interest: The authors state no conflict of interest.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

References

[1] Kley FKVD. Male sterility and its importance in breeding heterosis varieties. Euphytica. 1954;3:117–24.
[2] Yu S. Identification and characterisation of MS1 putative interacting proteins and regulatory targets in Arabidopsis. PhD thesis. Britain: University of Nottingham; 2015.
[3] Zhang D, Wu S, An X, Xie K, Dong Z, Zhou Y, et al. Construction of a multicontrol sterility system for a maize male-sterile line and hybrid seed production based on the ZmMs7 gene encoding a PHD-finger transcription factor. Plant Biotechnol J. 2018;16:459–71.
[4] Chen J, Jiao Y, Laza H, Payton P, Xin Z. Identification of the first nuclear male sterility gene (male-sterile9) in Sorghum. Plant Genome. 2019;12:1–12.
[5] Qing Xu L, Gao S, Sun L, Zhang ZJ, Zhang JX, Wang M, et al. Bioinformatics analysis of male sterile gene ms1 homologs in Maize. Maize Sci. 2018;26:44–52.
[6] Chen R, Xu Q, Liu Y, Zheng J, Liu Y. Generation of transgene-free maize male sterile lines using the CRISPR/Cas9 system. Front Plant Sci. 2018;9:1180.
[7] Tang WW, Wu XL, Li GH. Bioinformatics analysis of pollen development related gene MS1 in Brassica. Acta Sci Sin. 2017;48:2122–8.
[8] Okada A, Andell T, Borisjuk N, Sharma N, Watson-Haigh NS, Tucker EJ, et al. CRISPR/Cas9-mediated knockout of Ms1 enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. Plant Biotechnol J. 2019;17:1905–13.
[9] Singh M, Kumar M, Albertsen MC, Young JK, Cigan AM. Concurrent modifications in the three homeologs of Ms45 gene with CRISPR/Cas9 lead to rapid generation of male sterile bread wheat (Triticum aestivum L.). Plant Mol Biol. 2018;97:371–83.
[10] Liu H, Wang J, Li C, Qiao L, Wang X, Li J, et al. Phenotype characterisation and analysis of expression patterns of genes related mainly to carbohydrate metabolism and sporopollenin in male-sterile anthers induced by high temperature in wheat (Triticum aestivum). Crop Pasture Sci. 2018;69:469–78.
[11] Kyumi J, Doil C, Jundae L. Fine mapping of the genic male-sterile ms1 gene in Capsicum annuum L. Theor Appl Genet. 2018;131:183–91.
[12] Fitter JT, Thomas MR, Chen N, Rose RJ. Investigation of Nicotiana tabacum (plus) N suaveolens cybrids with carpellod stamens | NOVA. The University of Newcastle’s digital repository. J Plant Physiol. 2005;162:225–35.
[13] Berbec A, Laskowska D. Agronomic performance of flue-cured tobacco F1 hybrids obtained with different sources of male sterile cytoplasm. Btreg Zür Tabakforschung. 2014;21:235–9.
[14] Tao Y, Wang Y, Zhong SR, Wu LM, Xie L, Nie YP, et al. Tobacco ATP synthase F–α analysis of transcriptional editing sites of four subunits in part 0. Acta Crops Sin. 2016;42:1743–53.
[15] Sun GH, Yuan HH, Fan XC, Gu HK, Song MF, Xiao Y, et al. Cloning of Brassica oleracea phytchrome B gene and functional verification of heterologous transformation in Arabidopsis. Chin Agric Sci. 2015;48:4417–27.
[16] Tian MZ. Study on directional mutation of tobacco cyp71d16 gene by CRISPR/cas9 technology. China: Guizhou University; 2019.
[17] Chen HJ, Li F, Yan Y, Ji X, Sun HZ, Zhang J, et al. Using CRISPR/cas9 gene editing technology to create rice phytchrome phyB gene mutant. Mol Plant Breed. 2018;16:7667–75.
[18] Wang XR, Hui ZM, Xu JF, Duan SG, Bian CS, Jin LP, et al. Histocytological study on potato shape development. Chin Veg. 2020;4:67–73.
[19] Zhang QP, Wu XH, Zheng JH, Sun MW. Research progress of determination methods of malondialdehyde in biological samples. Phys Chem Exam (Chem). 2016;52:979–85.
[20] Li YQ, Wang QL, Wei CY, Dong T, Chen JJ, Zhou KY. Study on morphological observation and physiological and biochemical characteristics of cytoplasmic male sterility in cotton. J Southwest Agric. 2020;33:58–63.
[21] Mieck M, Hamish MW, Li M, Franck V, Silvano S, Juri P, et al. A new bioinformatics analysis tools framework at EMBL–EBI. Nucleic Acids Res. 2010;38(Web Server Issue):695–9.
[22] Kusz K, Tomczyk L, Spik A, Latos-Bielenska A, Jedrzejczak P, Pawelczyk L, et al. NANOS3 gene mutations in men with isolated sterility phenotype. Mol Reprod Dev. 2010;76:804.

[23] Shantasov AM, Sokolov SD, Bocharnikov AV, Maletina VA. Determination of fertility and pollen viability of breeding line of pattypan squash with male sterility of functional type. Veg Crops of Russ. 2014;3:8–10.

[24] Rong L, Liu D, Pedersen EF, Zhang G. The effect of wind speed and direction and surrounding maize on hybrid ventilation in a dairy cow building in Denmark. Energy Build. 2015;86:25–34.

[25] Yang Z. Map based cloning and functional analysis of rice male sterile gene TIP3 and molecular mechanism of its homologous gene osms1. China: Huazhong Agricultural University; 2019.
Appendix: Construction of CRISPR/cas9 vector

(1) Select the target.
(2) Construction of intermediate vector gRNA. The gRNA fragments are obtained by the PCR reaction, and the reaction system and procedures are listed in Tables A1 and A2.
(3) Enzyme digestion connection, connection system, and reaction procedure are listed in Tables A3 and A4.
(4) The recombinant plasmid was transformed.
(5) The plasmid was extracted.
(6) Sequencing.
(7) The ligation system and reaction procedure are listed in Tables A5 and A6, respectively.
(8) The system of transformation and bacteria detection is listed in Tables A7 and A8.
(9) Pick out the correct clone, extract the plasmid and send it to the company for sequencing.

| Table A1: gRNA reaction system |
|--------------------------------|
| **Composition of reaction solution** | **Reaction liquid capacity (µL)** |
| Forward primer | 5 |
| Reverse primer | 5 |
| ddH₂O | 40 |
| Total volume | 50 |

| Table A2: Reaction procedure |
|------------------------------|
| **Step** | **Temperature (°C)** | **Time (min)** |
| Denaturation | 95 | 10 |
| Annealing | 55 | 10 |
| Cooling | 14 | 5 |

| Table A3: Enzyme digestion system |
|-----------------------------------|
| **Composition of reaction solution** | **Reaction liquid capacity (µL)** |
| gRNA fragment | 2 |
| Empty carrier1 (pBWA(V)) | 1.5 |
| HS-zmpl | 1.5 |
| ECO31I | 0.5 |
| T4-ligase | 0.5 |
| T4-buffer | 1 |
| ddH₂O | 4.5 |
| Total volume | 10 |

| Table A4: Reaction procedure |
|------------------------------|
| **Step** | **Temperature (°C)** | **Time (min)** |
| Denaturation | 95 | 10 |
| Annealing | 55 | 10 |
| Cooling | 14 | 5 |

| Table A5: Double enzyme digestion system |
|------------------------------------------|
| **Composition of reaction solution** | **Reaction liquid capacity (µL)** |
| Target 1 (plasmid) | 1 |
| Target 2 (plasmid) | 1.5 |
| Lgul | 0.5 |
| T4-ligase | 0.5 |
| T4-buffer | 1 |
| ddH₂O | 5.5 |
| Total volume | 10 |

| Table A6: Reaction procedure |
|------------------------------|
| **Step** | **Temperature (°C)** | **Time (min)** |
| Denaturation | 95 | 10 |
| Annealing | 55 | 10 |
| Cooling | 14 | 5 |
### Table A7: Bacterial test system

| Composition of reaction solution | Reaction liquid capacity (µL) |
|---------------------------------|-------------------------------|
| 2x Mix                          | 10                            |
| M13F-chen (Forward detection primer) | 1                             |
| zmpl-chen (Reverse detection primer) | 1                             |
| ddH₂O                            | 8                             |
| Total volume                     | 20                            |

### Table A8: Reaction procedure

| Step        | Temperature (°C) | Time (min) |
|-------------|------------------|------------|
| Denaturation| 95               | 10         |
| Annealing   | 55               | 10         |
| Cooling     | 14               | 5          |