A novel herbicide-inducible male sterility system

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Abstract

BACKGROUND: Heterosis is a phenomenon that first-generation offspring perform better than their parents. Conventional breeding methods have their shortcomings. It would be optimal to construct inducible male sterile plants.

RESULTS: We developed a novel system for creating male sterile transgenic plants by downregulating the specific expression of the glyphosate tolerance CP4 EPSPS gene in male reproductive tissues. Transcriptional repression was achieved by manipulating DNA binding proteins with their specific corresponding sites. We transferred the CP4 EPSPS gene driven by a modified CaMV 35S promoter with three tetracycline operator copies in the vicinity of the TATA box and tetracycline repressor gene under the control of an anther-specific promoter Osg6B to Arabidopsis thaliana. As a result, we successfully obtained controllable transgenic plants: the whole plant could tolerate exposure of glyphosate but the male tissue was sensitive.

CONCLUSION: The novel inducible male sterility system is applied and easy to handle, so it might be applicable to a wide range of crop plants.
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Keywords: male sterility; tapetum-specific promoter; tetracycline repressor gene; tetracycline operator; glyphosate tolerance gene

INTRODUCTION

Heterosis, also known as hybrid vigor, is a phenomenon wherein first-generation offspring perform better than their parents. Heterosis has been successfully utilized in large-scale production of major crops such as corn, rice, and sorghum. A key step in the application of heterosis is to control cross-pollination so that residue self-pollination is negligible. This step is usually achieved by chemical or genetic control of male sterility in the maternal line. The chemical control is easy to use and requires little time- and labor-consuming pre-breeding work. However, its application in large-scale hybrid production is often limited by low efficiency, poor gamete selectivity, and, most importantly, various environmental pollutions. A genetic male sterility system utilizes nuclear genes to induce and/or restore male sterility of the maternal line; it is often accompanied by an incompatible cytoplasm. While a genetic male sterility system is often effective and specific, it requires great pre-breeding efforts to create two or three different lines to induce, maintain or restore male sterility. Moreover, some genetic male sterility systems are environmentally dependent. Thus it would be optimal if the simplicity and convenience of the chemical approach could be combined with the effectiveness and specificity of the genetic approach in an environmentally independent manner.

For this purpose, genetic engineering may play a significant role. Recent developments in bioengineering have broadened the genetic resource of inducing anther malformation and male sterility in plants. Genetic induction of anther malformation can be achieved by anther-specific expression of RNase1 or a gene that either helps to release a cytotoxin2 or distorts a metabolic pathway.3 In such a system, male fertility can usually be restored in any of three procedures: by expressing another transferred gene or an antisense RNA to neutralize the male sterility gene;4 by changing the growth conditions of hybrids;5 or by not spraying the pre-herbicide on the hybrids.2

For genetic engineering to create male sterility, the tapetum is a very important target.6 The tapetum is a layer of nursing cells surrounding locules in the stamen, which contains developing microspores. Normal tapetum is essential for microspore development. Therefore, it is not surprising that an engineered male sterility system often utilizes a tapetum-specific promoter to achieve tapetum-specific tissue destruction. Tsuchiya et al.7 isolated a tapetum-specific promoter, Osg6B, from rice and showed that this rice promoter also works well in dicotyledonous tobacco species. Further studies show that the Osg6B functions in a narrow time window between the tetrad stage and the uninucleate microspore stage during anther development,8,9 making
it an excellent tapetum-specific promoter for both monocots and dicots.

As a regulated gene expression system, the tetracycline-regulated expression system uses the regulatory elements from the E. coli Tn10-encoded tetracycline resistance operon. Based on the binding of tetracycline repressor to the tetracycline operator, this expression system represses the promoter that controls expression of the target gene. The tetracycline operator can be inserted into the CaMV 35S promoter. The tetracycline-regulated expression system has been successfully used in plant genetic engineering.

In this study, we reported a developmental two-line male sterility system that requires just a single transgenic female line with tetracycline-regulated expression. This system constitutively expresses a herbicide tolerance gene in all tissues except for tapetum. We used an anther-specific promoter, Osg6B, to control tapetum-specific silencing. The female line is completely male sterile if sprayed with the corresponding herbicide during meiosis. Both the female line and its hybrids with any male line will be naturally fertile if no herbicide is sprayed. To demonstrate the feasibility of this system, we used glyphosate and glyphosate tolerance gene.

MATERIALS AND METHODS

Plant materials and herbicide application
The rice (Oryza sativa L.) cultivar Nipponbare was grown in a dark area at 23°C, and leaves were harvested after 2 weeks for genomic DNA isolation. The tobacco (Nicotiana tabacum L.) cultivar G28 was grown in a greenhouse at 23°C with a photoperiod of 8 h night and 16 h day. Seeds of Arabidopsis thaliana (L.) Heynh ecotype N42 were soaked in water at 4°C for 2 days before they were sown into the soil. The Arabidopsis plants were grown in a greenhouse with a photoperiod of 8 h night at 21°C and 16 h day at 23°C. Transgenic Arabidopsis seedlings were sprayed with 0.4 mL L−1 Roundup® aqua (active ingredient glyphosate) to select glyphosate-tolerant plants. Survivors were still sprayed every 3 or 5 days in order to maintain glyphosate stability in the plant. Male sterility was first induced with 0.4 mL L−1 Roundup® aqua when the flower buds appeared; this step was repeated the following day. Inflorescences were then isolated with bags and phenotypic data were collected 7 days later.

Expression vector construction
A new binary expression vector, pCAMOSG, was constructed to induce male sterility. This was based on two established expression vectors: pCAMBIA1304 and pcDNA6/TR (Invitrogen, Carlsbad, CA, USA). To construct this vector, three copies of the tetracycline operator sequence (19nt-synthesized TCCCTATCAGTGTAGAGA) were inserted in the vicinity of the TATA box of a CaMV 35S promoter in the pCAMBIA1304 vector as described by Gatz et al. and Kim et al. To construct the CP4 EPSPS expression cassette, the mgfp5 gene in the pCAMBIA1304 was replaced by a polymerase chain reaction (PCR)-cloned complete encoding fragment of CP4 EPSPS gene, which has been shown to exhibit high tolerance to the broad-spectrum and nonselective herbicide glyphosate. The CP4 EPSPS fragment was inserted between the restriction enzyme sites SpeI and BstEII downstream of the modified promoter. This vector was named pCAM-EPSPS. The plasmid pcDNA6/TR originally contained a CMV (the CMV promoter originates from the cytomegalo virus)-directed expression cassette of the tetracycline resistance repressor gene (tetr). Tetr encodes the tetracycline resistance repressor protein that can repress the expression of the tetracycline resistance gene by binding to its operator. The CMV promoter in the pcDNA6/TR plasmid was replaced by the Osg6B promoter. The entire expression cassette of tetr with the Osg6B control was then inserted between the EcoRI and Xhol site in pCAM-EPSPS. This plant expression vector, consisting of the tetr cassette driven by anther-specific promoter, Osg6B, and the CP4 EPSPS cassette driven by CaMV 35S promoter was named pCAMOSG (Fig. 1).

Genetic transformation
Following the floral-dip method, Arabidopsis plants were transformed with pCAMOSG containing Agrobacterium tumefaciens strain GV3101. The progeny seedlings of putative transgenic plants were grown in soil pots in the greenhouse and selected with 0.4 mL L−1 Roundup® aqua spray.

Genomic DNA PCR detection and Southern blot analysis
Genomic DNA was isolated from young leaves of tobacco and Arabidopsis using the cetyltrimethyl ammonium bromide (CTAB) method. For PCR analysis, the primers for transferred genes were as follows: 5′-CCCATACCCAGCCACCTTTCTAT-3′ and 5′-TCTGCTGCTTAATCTAGG-3′ for Osg6B promoter; 5′-CGCCTCAGAAAGACAG-3′ and 5′-TGGAGCGCGACGCACTCCGAC-3′ for tetracycline repressor gene; 5′-CCCTCTGGAGTTTCCATTG-3′ and 5′-TGGAGCTGCTGCTGATCCC-3′ for CaMV 35S promoter; and 5′-AAATCCCTGGCTTCCGCGG-3′ and 5′-AAATCCCTGGCTTCCGCGG-3′ for the CP4 EPSPS gene.

For Southern blot analysis, genomic DNA (20 μg) was digested with the following restriction enzymes: BglII, EcoRI, BstEII, and HindIII. These were then separated in a 12 g agarose gel L−1 Tris-acetate-EDTA (TAE) at 60 V until the bromophenol blue reached the gel end, after which they were transferred to a nylon membrane. The Osg6B promoter probe was labeled with digoxigenin-11-dUTP supplied by DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany). The membranes were hybridized with 20–30 ng mL−1 of labeled DNA probes and then exposed to X-ray film for 20 min.
Reverse-transcribed PCR and real-time PCR

Total RNA was extracted from Arabidopsis leaves and inflorescence using RNA extraction kit (Tiangen, Beijing, China) according to the manufacturer’s instructions. RNA was reverse-transcribed to synthesize the first-strand cDNA using First-Strand cDNA synthesis kit (TaKaRa, Japan). Reverse transcriptase PCR (RT-PCR) amplification was processed according to the manufacturer’s instructions. RNA was reverse-transcribed to synthesize the first-strand cDNA using RNA extraction kit (Tiangen, Beijing, China) for tetracycline repressor (tetr).

Real-time PCR assay was performed with the SYBR Green RT-PCR kit (TaKaRa) according to the manufacturer’s protocol. The primers for a housekeeping gene, actin 2, were 5'-ATTACAGATGCCAGAATGCTTGTTCC-3' and 5'-ACCACCGATCCAGACACTGTACTTCC-3'. Three technical repeats were performed for each assay.

Paraffin section microscopy

Immature inflorescences of Arabidopsis sprayed with Roundup® were embedded in paraffin as previously described by Sun and Gui. The paraffin sections were mounted onto microscopic slides with water and observed with a multipurpose microscope (Olympus, Tokyo, Japan). For comparison, transgenic plants that were untreated with Roundup® and a wild-type plant were used as controls.

Confirmation of transferred gene transmission

To test the female fertility of transformed plants, the glyphosate-induced male sterile plant was pollinated by a wild-type plant. Further, to study the transmissibility of transgenic gene, 59 T2 plants of the No. 3 T1 plant were examined. The PCR assay was performed for Osg6B promoter, tetr gene and CP4 EPSPS gene. All 59 T2 plants were sprayed with Roundup®. The PCR-positive and Roundup®-resistant plants were calculated and compared.

RESULTS

Cloning and functional confirmation of the Osg6B promoter

Using the PCR-based method, we isolated the anther-specific Osg6B promoter from rice and confirmed its tapetum-specific gene expression in transgenic tobacco (data not shown).

DNA PCR detection and Southern blot analysis of transformed Arabidopsis

The pCAMOSG vector was transformed into Arabidopsis via A. tumefaciens. Among ~3000 T1 seeds obtained, five T1 seedlings survived after being sprayed with 0.4 ml L⁻¹ Roundup® aqua. The PCR assay showed that two of the five survivors (No. 3 and No. 4) had all four inserted sequences (Osg6B promoter, tetr gene, CaMV 35S promoter and CP4 EPSPS gene) (Fig. 2(I)). The plants were subjected to Southern blot verification. Hybridization with the Osg6B probe suggested that single-copy transferred genes had been successfully integrated into the genome of this plant (Fig. 2(II)).

Reverse-transcribed PCR and real-time PCR detection

Total RNA was isolated from leaf and inflorescence samples of the two transgenic plants and a wild-type plant, respectively. As shown in Fig. 3(I), the tetr gene was specifically expressed in the inflorescence; moreover, the CP4 EPSPS gene was expressed in both the leaf and inflorescence. CP4 EPSPS expression appeared to be lower in inflorescences than in the leaves (Fig. 3(II)).

Anther slice and phenotype of transgenic plants detection

No pollen grains were observed in the anthers of treated transgenic plants, whereas many pollen grains were found in the anthers of control plants (Fig. 4(I)). The glyphosate-induced male sterility was also supported by undeveloped siliques and seeds in the transformed transgenic plants (Fig. 4(II)). Furthermore, this inducible male sterility could be maintained by withholding glyphosate application to the transgenic plants during the critical period of anther development.

Figure 2. Detection of DNA levels in the transgenic Arabidopsis. Genomic DNA was isolated from young leaves of transformed Arabidopsis. PCR assay showed two (I: 3, 4) of the five survivors (I: 1–5) and positive control of plasmid pCAMOSG (7) had all four inserted sequences, while the negative control of wild-type Arabidopsis (I: 6) had none of them. Group A indicates Osg6B promoter of 1353 bp; Group B indicates 619 bp fragment of tetr gene; Group C indicates 518 bp fragment of CaMV 35S promoter; Group D indicates 1225 bp fragment of CP4 EPSPS gene. Genomic DNA of transgenic Arabidopsis, digested with restriction enzymes BglII (II: 1), EcoRI (II: 2), BstEII (II: 3), HindIII (II: 5) and hybridized with Osg6B probe showed single band. As controls, wild-type Arabidopsis total DNA digested by BstEII (II: 4) had no band; Osg6B promoter hybridized (II: 6) showed a 1353 bp band; plasmid pCAMOSG hybridized (II: 7) showed a 12 136 bp band.
Inducible male sterility

Figure 3. Detection of RNA levels in transgenic Arabidopsis. Total RNA was isolated from two transgenic Arabidopsis leaves (I: 1, 2) and a wild-type Arabidopsis leaf (I: 3), from transgenic inflorescence (I: 4, 5) and a wild-type inflorescence (I: 6). The 350 bp band of tet gene (Group A) appeared in inflorescence (I Group A: 4, 5) and plasmid pCAMOSG control (I Group A: 7). The CP4 EPSPS gene band of 408 bp was expressed in both the leaf and inflorescence (I Group B: 1, 2, 4, 5) and plasmid pCAMOSG control (I Group B: 7). The 268 bp band of housekeeping gene actin 2 was expressed normally in both transgenic and wild-type Arabidopsis (I Group A: 1–6; GROUP B: 1–6). The results of real-time PCR revealed that the tet gene was highly expressed in inflorescences (II: gray bar of No. 3F and No. 4F) but with almost no expression in leaves (II: gray bar of No. 3L and No. 4L). CP4 EPSPS expression appeared to be lower in inflorescences (II: black bar of No. 3F and No. 4F) than in the leaves (II: black bar of No. 3L and No. 4L). Wild-type Arabidopsis inflorescences had almost no expression of either tet gene or CP4 EPSPS gene (II: Wild F). Vertical coordinates indicate the expression quantity ratios of a gene of interest versus the housekeeping gene.

Confirmation of transferred gene transmission
The glyphosate-induced male sterile plant pollinated by a wild-type plant could seed normally (Fig. 4(II)). The PCR assay for Osg6B, tet and CP4 EPSPS revealed that 14 of the 59 T2 plants (23.7%) were negative for all three markers, as expected from the single-spraying (data not shown).

DISCUSSION AND CONCLUSION
In this study, we reported a developmental inducible male sterility system in Arabidopsis. This male sterility system uses a binary expression vector containing an expression cassette of a herbicide tolerance gene and an expression cassette of a regulating gene with a tapetum-specific promoter. This tapetum-specific promoter allows the expression of the regulating gene, which, in turn, represses the expression of the herbicide tolerance gene in the tapetum. When the transgenic plant is sprayed with herbicide the tapetum is destroyed, leading to anther malformation. As a result, male sterility is achieved, but female fertility remains unaffected. The transgenic line functions in both presenting and maintaining lines for male sterility. Hybrids of this induced male sterile line with any male fertile plant will be male fertile as long as herbicide spray is avoided during the critical period of anther development. This simple system can employ any pair of herbicide and herbicide tolerance gene, which can be easily applied to monocot or dicot crops in any type of large-scale field production.

Kriete et al.\textsuperscript{18} reported a conditional male sterility system based on transferred gene-controlled tapetum-specific conversion from protoxin to toxin. The conditional female sterility system patented by Syngenta Participations AG used the same mechanism but with a female-preferential promoter (US Patent 6 392 123). These two systems are similar that reported here, since the sterility is achieved by inducible destruction of plant tissues with a transferred gene. Nevertheless, our system combined male sterility with herbicide tolerance, thereby identifying a more practical technique for crop production.

Meanwhile, the CP4 EPSPS gene from the Agrobacterium sp. strain CP4 exhibits a high tolerance to glyphosate, a potent herbicide that inhibits the activity of endogenous 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the shikimic acid pathway.\textsuperscript{19} Glyphosate is widely recognized as a broad-spectrum and post-emergent herbicide which is environmentally safe and has a desirably short half-life in soil and groundwater. Therefore glyphosate should be a suitable inducer substance for male sterility. On the other hand, herbicide application for weed management combined with glyphosate tolerance crop varieties have been widely used in producing major crops, such as corn, cotton, and soybean.\textsuperscript{20,21} Our glyphosate-conditioned male sterility system can therefore be easily adapted to such a crop production practice, which is a great advantage that no previous system has ever had. Our system would also make hybrid seed production easier, since it requires just a single line to maintain and uses any race or inbred line as the male line. In addition, the induced male sterility is conditional and will not affect hybrid fertility when the simple condition is avoided. Given that the herbicide application makes the female line completely male sterile, the herbicide-tolerant female and male parents can be interplanted if the conditional sterility mechanism is used to induce female sterility in the male parent. This feature would make hybrid seed production much more efficient and cheaper.

The glyphosate tolerance RR1445 transgenic cotton line showed male sterility with high glyphosate application at developmental stages later than the four-leaf stage.\textsuperscript{22} The main reason for this is that the transgenic CP4 EPSPS gene expression is directed by the wigfowt mosaic virus (FMV) 35S promoter, whose activity is lower in anthers than in other plant organs.\textsuperscript{21,23} Pollen viability of RR1445 plants treated with glyphosate application is affected such that the higher the level of glyphosate application, the less pollen is viable. Our male sterility system inhibits CP4 EPSPS gene expression specifically in the male organ through an indirect effect of the Osg6B promoter. Since the tapetum is destroyed by glyphosate between the tetrad stage and the uninucleate microspore stage, there is not longer any pollen present in the clinandrum. Therefore, the inducible sterility in our system is complete.

The Osg6B promoter used in our study was cloned from the rice genome. Our data showed that the Osg6B promoter worked normally in Arabidopsis. The male sterility system presented here is therefore likely to work well in all dicot and monocot crops. More importantly, the Osg6B promoter is known to have a very short functional window before anthesis.\textsuperscript{9} This makes our male sterility system even more manageable in a large-scale hybrid seed production since herbicide application can be avoided easily during this short period.

In addition to hybrid seed production, the system we developed is also useful in basic science. The glyphosate-induced male sterility could rapidly build the crossbreeding system for crops that could
not easily achieve hybrid seed production. Uncommon crossbreed characters can therefore be investigated by future researchers.

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REFERENCES