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ORIGINAL PAPER

Induction of Male Sterility in Tobacco by Anther-Specific Expression of the Gene for Ricin Enzymatic Subunit A Chain RTA

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Abstract
Targeted gene expression in plants allows us to further study biological traits of interest, such as reproductive and developmental processes. Here, the tobacco TA29 anther-specific promoter was used to direct the expression of the ricin enzymatic subunit A (RTA) in transgenic tobacco plants, phenotypic analysis of the resulting positive transgenic tobacco (*Nicotiana tabacum* L.) plants demonstrated that RTA expression led to a reduction in pistil length and shriveling of anthers, as well as the grayish-brown color of anthers, the reduced pollen viability and male sterility. For the first time, a plant-derived ricin gene enzymatic subunit A (RTA) expression system under the tissue-specific promoter was demonstrated to be sensitive and efficient in controlling plant sterility and creating male-sterile materials. Consequently, it could be used to control other agronomic traits and produce hybrid seeds in plants in the future.

Keywords RTA · Ricin · Male sterility · Anther-specific gene expression

Introduction

The use of tissue-specific promoters to control the expression of toxin genes is effective in regulating fertility and resistance, as well as in evaluating the immunoprotective capabilities, of plants [1–3], In Table 1, we provide a list of the representative literature and relevant information on toxin genes and specific promoters used in plants. Toxin genes that are widely used in plants include diphtheria toxin A chain (*DTA*) [4] and *Barnase* [5], both of which are derived from bacteria. In addition to the listed toxin genes, many others, including those encoding ricin, microtoxin and marine biotoxin, have not been used in this kind of research. Ricin is a toxic substance extracted from the seeds of the perennial castor bean plant. Ricin has a ricin protein, ricinine, ricin allergen and hemagglutinin [6], and one ricin molecule is bound by subunits A and B through disulfide bonds. The enzymatic subunit A chain (*RTA*) is the active toxin chain, while the subunit B chain plays a decisive role in the hemagglutination activity [7]. The toxic effects of ricin on plant pests have been confirmed and studied [8]; however, plant-derived ricin has not been proven to have a toxic effect on plant cells [9]. Here, the toxin gene *RTA* from ricin, driven by an anther-specific promoter TA29, was expressed [10] in tobacco (*Nicotiana tabacum* L.). The corresponding transgenic plants showed male sterility, which provides an application prospect for ricin in controlling other agronomic traits and plant phenotypes in the future.

Materials and Methods

Plant Materials and DNA Extraction

Nicotiana tabacum L. cv. 'Honghuadajinyuan' was grown in the greenhouse in Qingdao city. Total DNA from leaves was extracted using a Plant DNA Isolation Reagent (TaKaRa, Japan). Samples were electrophoresed on 1% agarose gels, and their purity levels were checked using a NanoDrop2000 spectrophotometer (Thermo, USA).

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Table 1 Representative literature on toxin genes driven by specific promoters in plants

Promoter	Species	Toxin gene	Expression tissue	Literature
Napin	*Brassica napus*	Barnase	Seed	Chen et al. [11]
A9	*Arabidopsis thaliana*	DTA	Anther	Guerineau et al. [12]
TA29	*Brassica napus*	Barnase	Pollen	Mariani et al. [13]
	Nicotiana tabacum			
lat 52	*Nicotiana tabacum*	DTA	Anther	Twell [4]
SLG13	*Arabidopsis thaliana*	DTA	Anther	Thorsness et al. [14]
Pvicilin	*Nicotiana tabacum*	DTA	Seed	Czakó et al. [15]
	Arabidopsis thaliana			
TA29	*Nicotiana tabacum*	RIP	Pollen	Czako et al. [16, 17]
Napin	*Brassica napus*	exotoxin	Seed	Koning et al. [18]
STIG1	*Nicotiana tabacum*	barnase	Stigma	Goldman et al. [19]
PNZIP	*Gossypium spp*	Cry9C	Green tissue	Wang et al. [1]
RSs1 and RolC	*Nicotiana tabacum*	Hvt	Phloem	Shah et al. [2]
α 1-tub	*Nicotiana tabacum*	DTA	Anther	Kim and An [20]
BcA9	*Brassica campestris*	DTA	Anther	Lee et al. [21]
GBAN21S-6	*Nicotiana tabacum*	DTA	pollen	Kim et al. [22]
Chickpea legumin promoter	*Nicotiana tabacum*	Cholera toxin B and rabies virus	Seed	Tiwari et al. [3]

Table 2 Information for primers used in this study

Primer	Primer sequence
PNtTA29-F	ACACAAATAGCCGGCTATAT
PNtTA29-R	GCTTCGAATAGCTAATTT CTTTAAGTAAAAAC
TA29-F	TAAGGTGGGTGGCTGGACTA
RTA-R	GCCGACCACATATGCATTGG
TA29-RF	GTGGATTTGGCATTGGTGGT
TA29-RR	ATGATGCGCTTCCTCCTCAT

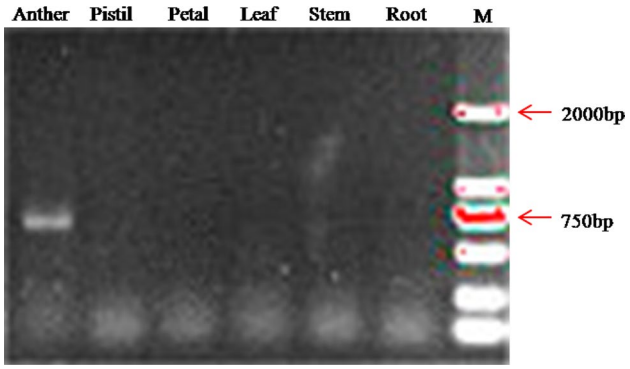

Fig. 1 RT-PCR of TA29 in tobacco

RNA Extraction and RT-PCR

Total RNA from each sample (roots, stems, leaves anthers, pistils and petals) was extracted using the GeneJET™ Plant RNA Purification Mini Kit (MBI Fermentas, Canada). Samples were run on 1% agarose gels, and the purity was checked using a NanoDrop2000 spectrophotometer. Total RNA was reverse transcribed using the RevertAid™ First-Strand cDNA Synthesis Kit (MBI Fermentas, Canada), and cDNA was used for RT-PCR analyses with primers specific to the TA29 genes (TA29-RF/ TA29-RR) (Table 2).

Amplification of the Tobacco TA29 Promoter

The promoter region of TA29 was amplified from tobacco cv. 'Honghuadajinyuan' genomic DNA by PCR using the specific primers P_{NtTA29}-F and P_{NtTA29}-R (Table 2).

Construction of P_{NtTA29}::RTA Recombinant Vector and Genetic Transformation

The 1489-bp promoter was amplified by PCR and inserted in-frame in front of the *GUS* reporter gene using the restriction enzyme restriction sites HindIII and BamHI in the PBI121 vector [23] (provided by our laboratory) (Fig. 1). The CDS sequence of the *Ricinus communis* ricin gene *RTA* [24] (GenBank: X52908.1) was synthesized by BGI and inserted into the P_{NtTA29}::GUS vector using restriction enzymes BamHI and SacI. This construct, named P_{NtTA29}::RTA, was then transformed into *Agrobacterium tumefaciens* strain EHA105 and introduced into cultivated tobacco 'Honghuadajinyuan' plants using the leaf disc

method [25]. Transgenic plants were selected on MS culture medium [26] containing 0.1 mg/ml kanamycin.

Pollen Viability Experiment and Microscopic Observations of the Anthers

Positive transgenic plants were selected by PCR using the specific primers TA29-F and RTA-R (Table 2). The floral organs of transgenic T_0 plants were observed and photographed. Anthers were isolated from flowers and observed under a Leica S8AP0 microscope (Leica, Germany). Iodine and potassium iodide were prepared by dissolving 80 g potassium iodide and 10 g iodine in 100 ml distilled water [27]. After staining with the iodine and potassium solution for 5 min, pollen was viewed using a Nikon Eclipse 80i fluorescence microscope (Nikon, Japan).

Results

Tissue Expression Pattern of TA29 in Tobacco

RT-PCR was conducted in anther, pistil, petal, leaf, stem and root of cultivated tobacco to verify the expression specificity of TA29. The product length is 758 bp according to the primer TA29-RF/TA29-RR, the examination result showed that TA29 can express specifically in anthers (Fig. 1), therefore it can be used for the following construction of recombinant vector.

Construction of the P_{NtTA29}::RTA Recombinant Vector

The recombinant vector containing *RTA* driven by the anther-specific promoter TA29 was constructed. A 1489-bp TA29 promoter region (P_{NtTA29}) was isolated from tobacco variety 'Honghuadajinyuan'. A recombinant vector that contains ricin (*RTA*) driven by P_{NtTA29} was constructed based on the P_{NtTA29}::GUS recombinant vector (Fig. 2). A PCR examination of eight putative transgenic plants was conducted (Fig. 3).

Effects of RTA on the Creation of Male-Sterile Tobacco

In total, eight positive transgenic T_0 plants and four cultivated tobacco plants of variety 'Honghuadajinyuan' were regenerated and grown to maturity in the greenhouse. No significant phenotypic differences were noted between T_0 and control plants before flowering. The floral organs of the

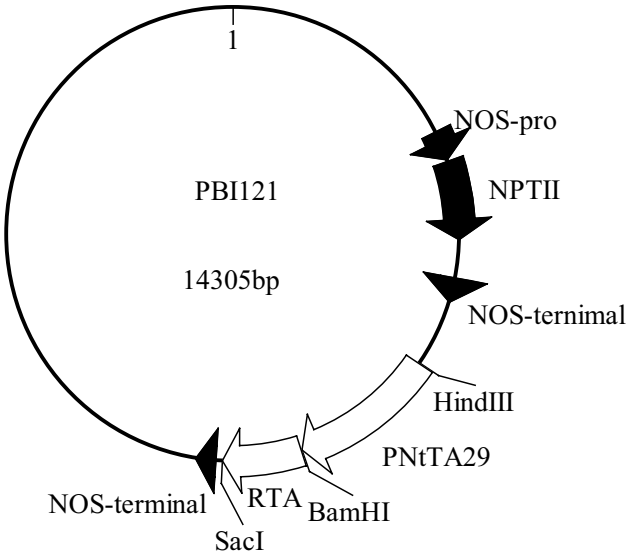

Fig. 2 Schematic diagram of the recombinant vector P_{NtTA29}::RTA

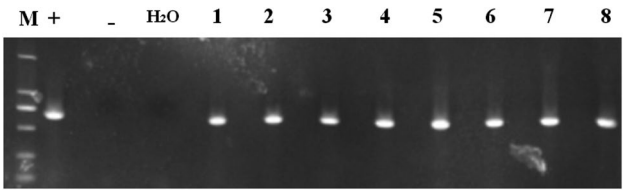

Fig. 3 PCR assay confirming P_{NtTA29}::RTA-transformed tobacco plants

P_{NtTA29}::RTA transgenic tobacco plants were observed and compared with those of untransformed plants. A reduction in pistil length and the shriveling of anthers were clearly observed in transgenic plants (Fig. 4a–d). In addition, the anthers of transgenic plants were grayish-brown compared with red-brown of control plants (Fig. 5c–d), the detection of pollen viability includes pollen staining, pollen germination in vitro and pollen pollination and fruiting detection [28], among which iodine-potassium iodide staining is a kind of simple and rapid method, according to the characteristic of starch turning blue with iodine, the content of starch in pollen grains and then the activity level of pollen was determined according to the depth of blue [29]. From the observation under the microscope the living pollens become reddish-brown because of the deep staining, while the non-living pollens are grayish-brown and have an irregular shape. The proportion of active pollens in five fields was randomly counted and the percentage of pollen viability was

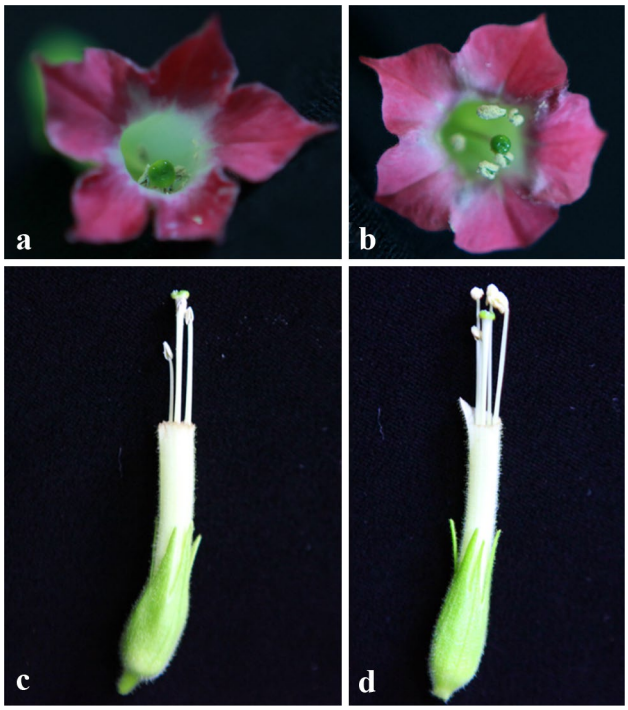

Fig. 4 Morphological differences in flowers between positive transgenic tobacco and control plants. **a** Flower of a positive transgenic plant; **b** flower of an untransformed plant; **c** pistils and stamens of a positive transgenic plant; **d** pistils and stamens of an untransformed plant

calculated, after staining, 95% of the control pollens were reddish-brown, and only 2% of the transgenic pollens were active. The results indicate the unusual phenotype of transgenic T_0 flowers. Pollen viability experiments further demonstrated that not only was less pollen released from anthers of transformants but the pollen viability was also greatly reduced (Fig. 5a–d). Compared with the control plants, the transgenic tobacco can flower normally, but can not produce seeds and show sterility (Fig. 6a–b). The stability of its fertility remains to be further observed.

Discussion

Male sterility is an important method to produce hybrid seeds, and currently male-sterile materials can be artificially created by genetic engineering, including utilizing the expression of toxin genes driven by anther development-associated promoters, which is an effective method. Some of the available toxin genes that have been successfully studied are from bacteria, such as *DTA* and *Barnase*, and some are from animals, such as *RIP* and *Pseudomonas aeruginosa* exotoxin A. However, there is rare report on utilizing the toxin genes from plants to obtain male-sterile materials. Here, an *RTA* gene from castor bean plants was exploited to obtain male-sterile materials for the first time. Ricin is

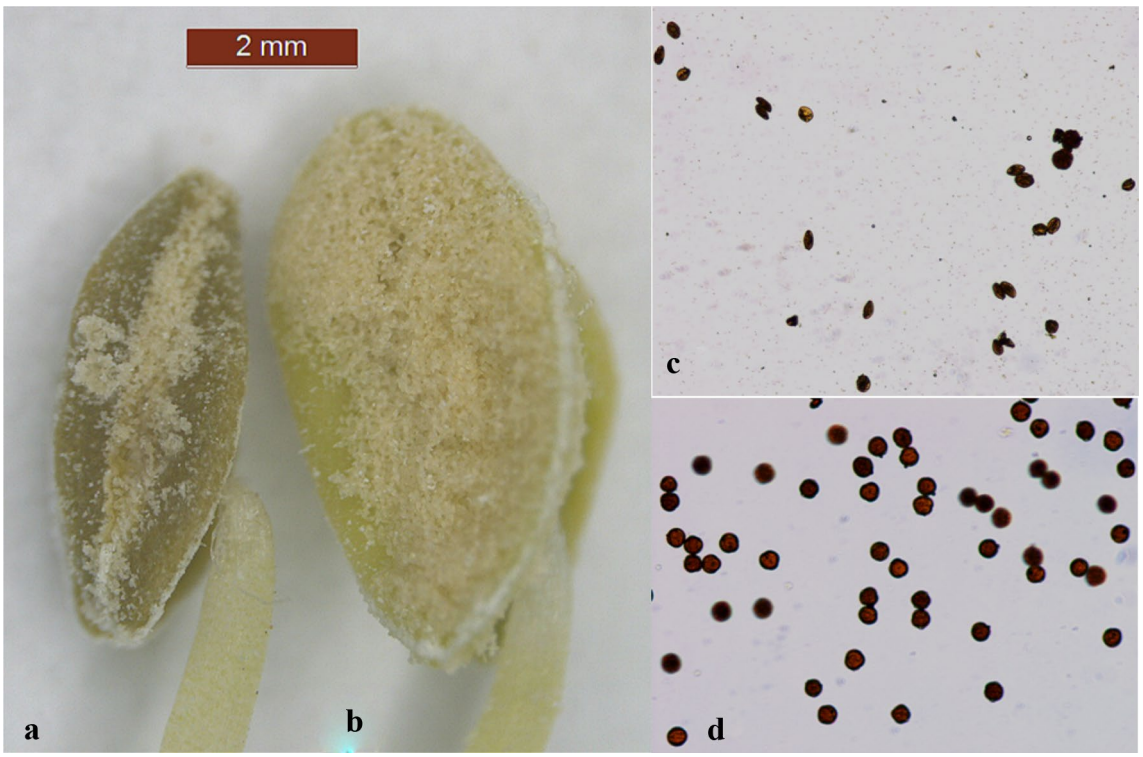

Fig. 5 Differences in the morphology and viability of anthers between positive transgenic and control plants. **a** Anthers from a positive transgenic plant; **b** anthers from an untransformed plant; **c** staining of pollen from a positive transgenic plant; **d** staining of pollen from an untransformed plant

Fig. 6 Differences in the flowering and fruiting between positive transgenic and control plants. **a** Flowers and seeds from a positive transgenic plant; **b** flowers and seeds from an untransformed plant

isolated from the seeds of castor beans and toxic to all mammalian cells. It is usually used to kill tumor cells in medicine and to develop biological insecticides in botany. The ability of the *RTA* gene to induce male sterility represents a new strategy for the production of hybrid crop plants and the optimization of other agronomic traits, including branching and stress resistance.

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