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T1243, an alternative transcript of the mitochondrial *T* gene in *Brassica juncea* var. *tumida*, causes pollen abortion in *Arabidopsis thaliana*

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ABSTRACT

An earlier report revealed that the mitochondrial *T* gene of tumorous stem mustard (*Brassica juncea* var. *tumida*) is a cytoplasmic male sterility (CMS)-related gene. Two alternative transcripts of the *T* gene, *T1170* and *T1243*, showed different expression patterns at different developmental stages. In this work, the potential roles of these two transcripts were studied in *Arabidopsis thaliana* through over-expression under the control of the cauliflower mosaic virus (CaMV) *35S* promoter. Transgenic plants over-expressing the *T1243* transcript produced anthers that appeared devoid of pollen and almost fully collapsed siliques, showing a severe male sterility phenotype, while transgenic lines over-expressing *T1170* were normal. To confirm the above results, we constructed T1243-RNAi lines in *A. thaliana*, and *T1243* was over-expressed using these lines as recipients. Pollen was normal in the *T1243* knocked-out lines. In the control wild type lines expressing *T1243*, plants showed pollen abortion. We observed a positive correlation between the sterility phenotypes and the expression levels of *T1243*. Test-crossing was done by pollinating T1243 transgenic lines with wild type pollen. About half of the progeny showed pollen abortion, indicating that *T1243*, one of the *T* gene transcripts, can cause a male sterility phenotype.

1. Introduction

Alternative splicing is the process whereby different introns/exons are combined in pre-mRNA to produce distinct messenger RNAs and thus to produce different proteins. This mechanism, which is used extensively in higher eukaryotic organisms, causes asymmetry of the number of protein-coding genes in an organism and its overall cellular complexity [1]. DNA microarray analysis has shown that about 74% of all human genes are alternatively spliced [2]. Alternative splicing has been studied extensively in mammalian systems but much less in plants, where it also has an important role in gene regulation. Approximately 20% of expressed genes are alternatively spliced in both Arabidopsis and rice (*Oryza sativa*) [3]. So far, only a few alternative splicing genes in higher plants have been studied to reveal the functions of different transcripts; for example, *FCA* [4], *RCA* [5,6].

Plant cytoplasmic male sterility (CMS) is a mitochondrially inherited trait wherein plants fail to produce functional pollen. CMS lines provide a convenient method to produce hybrid seeds and thereby exploit heterosis. Therefore, uncovering the mechanism of CMS is important from both basic and applied viewpoint. The mitochondria have been focused to investigate CMS because in all cases examined so far, CMS has been found to be associated with changes in the mitochondrial genome. It has been found that CMS occurs by diverse mechanisms. Usually, it is associated with unusual open reading frames (ORFs) found in mitochondrial genomes [7]. Until now, a number of genetic loci associated with CMS have been found in various plant species, such as petunia (*Petunia* sp.), sunflower (*Helianthus annuus*), maize (*Zea mays*), and the common bean (*Phaseolus vulgaris* L.). At least 14 mitochondrial genes that determine CMS have been identified as ORFs composed of segments derived from mitochondrial gene-coding and gene-flanking sequences, and from sequences of unknown origin [8]. The key evidence, a functional test for candidate sequences, has been

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Abbreviations: FCA, flowering locus C; RCA, rubisco activase; ORF, opening reading frame; CMS, cytoplasmic male sterility.

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proved in very few cases [9]. *ORF239*, a CMS-related gene from the common bean) was introduced into the tobacco nuclear genome. Male sterility phenotype was observed in transgenic plants [10]. Recently, an abnormal mitochondrial ORF, *orf79*, in rice with Boro II cytoplasm was reported to encode a cytotoxic peptide that caused gametophytic male sterility [11]. *Msh1*, a nuclear gene involved in controlling stability of the mitochondrial genome, was introduced into tobacco and tomato, resulting in reproducible mitochondrial DNA rearrangements and a condition of maternal male (pollen) sterility [12]. These studies have shed light on the molecular mechanisms of CMS in higher plants.

Earlier, we showed that the *T* gene cloned from tumorous stem mustard mitochondria (*Brassica juncea* var. *tumida*) produced two alternative transcripts, *T1170* (GenBank accession no. AF298550) and *T1243*. They were identified by comparison of cDNA of the CMS and the maintainer line (MF). *T1243* was transcribed with an uncleaved intron that has the partial basic characteristics of a type II intron. At the seedling stage, expression of the *T* gene was mainly in the form of *T1170*, and decreased gradually with development, while the expression abundance of another transcript, *T1243*, showed the opposite trends. *T1243* was prevalent at the profuse flowering stage [13]. The *T* gene was proved to be absent from MF, which has the same genome background as the CMS line, except for the male sterility trait. This provides a tight correlation between the expression of the *T* gene and the phenotype of CMS. All of this information implied that the *T* gene is related to male sterility [13].

To provide direct evidence that the *T* gene can cause male sterility, over-expression constructs of *T1243* and *T1170* were made and used to transform Arabidopsis. We show here that expression of *T1243* caused male sterility in Arabidopsis whereas *T1170* over-expression does not show any phenotype. Further, when *T1243* transcripts were suppressed through RNAi, normal male fertile plants could be recovered. This clearly established the direct involvement of *T1243* in causing male sterility.

2. Materials and methods

2.1. Arabidopsis growth conditions

Arabidopsis thaliana (Columbia ecotype) plants were grown at 23 °C with 16 h light/8 h dark and were transformed with *Agrobacterium tumefaciens* GV3101 by vacuum infiltration transformation [14]. Harvested seeds were sterilized and sown on half-strength, hormone-free MS medium supplemented with 25 μg ml^{-1} hygromycin or 50 μg ml^{-1} kanamycin for resistance screening. All cultures were maintained at 23 °C for 2–3 weeks with 16 h light/8 h dark. Drug-resistant seedlings were transferred to soil in growth conditions as described above.

2.2. Over-expression constructs and Arabidopsis transformation

Two transcripts of the *T* gene, *T1170* and *T1243*, have been cloned into pGEM-T easy vector (Promega, USA) and named pGEM-T1170 and pGEM-T1243 as described [13]. The two alternative transcripts were derived by PCR amplification using primers 5′-GCTGGATTCAAATCGATGCCTCAACTGGATAAATTC-3′ ($P_{Bam}1$) and 5′-GCTGGTACCCCCAAGCTTCAGCGAAAGAGATCA AGGAT-3′ ($P_{Kpn}2$) with BamHI and KpnI restriction sites introduced. The PCR products were purified and digested with BamHI and KpnI, and then cloned in the vector pCAMBIA1301SN, derived from pCAMBIA1301 and adding a *35S* promoter and an *NOS* terminator, and named pCAMBIA1301-35S::T1243 and pCAMBIA301-35S::T1170, respectively. After the sequence was confirmed with BamHI and KpnI digestion and sequencing, they were mobilized into an *A. tumefaciens* strain GV3101. Wild type Arabidopsis plants were used as the recipients for Agrobacterium-mediated transformation. The seeds of transgenic plants were screened on selective medium as described above. The seedlings that survived in the selective medium were considered independent transgenic lines and were transferred to soil.

2.3. Making RNAi constructs and identifying stable RNAi lines

To prepare a plant RNAi vector, we first obtained a fragment including the *35S* promoter and the *NOS* terminator from pCAMBIA1301SN with HindI and EcoRI sites and ligated it to pCAMBIA2300 cut with the same restriction enzymes, and named it pCAMBIA-2301SN. The first intron of the potato *gibberellin 20 oxidase* gene used as the linker sequence was PCR amplified from potato DNA using the primers 5′-GGTACCCCTGCAGGCTCGAGAC-TAGTAGATCTGGTACGGACCGTACTAC-3′ ($P_{GA20}1$) and 5′-GTC-GACCCTGCAGGGTCGACTCTAGAGGATCCCCTATATAATTTAAGTAGG-A-3′ ($P_{GA20}2$). The fragment was digested with KpnI and SalI, and cloned into pCambia2300SN [15]. Subsequently, a 400 bp fragment of *T1243* cDNA was amplified from pGEM-T-T1243 by 5′ TACgtcga-cAAATCGATGCCTCAACTGGATAAATTC-3′($P_{Sal}1$) and 5′ GCTggattc-TATCGCCCACACGAATCAGGTTG-3′($P_{Bam}3$) or amplified by 5′ GGCggtaccAAATCGATGCCTCAACTGGATAAATTC-3′ ($P_{Kpn}1$) and 5′ GCGgagctc TATCGCCCACACGAATCAGGTTG-3′ ($P_{Sac}3$). These two fragments are referred to as *T1243F1* and *T1243F2*, respectively. The final RNAi vector was constructed by ligating the following DNA fragments in order: the KpnI–SalI spacer fragment; the SalI–BamHI fragment of *T1243F1*, the KpnI–SacI fragment of *T1243F2* and named pCAMBIA2300-T1243-RNAi. Then it was confirmed by restriction mapping (it is difficult to sequence the inverted repeat fragment). *T1243* was analyzed by BLAST search to confirm that it did not have significant sequence homology with any other Arabidopsis gene.

The T1 transgenic plants were screened on 1/2 MS medium containing 25 μg ml^{-1} kanamycin and confirmed by PCR amplification using primers 5′-AACAAGATGGATTGCACGCAGGTTC-3′ (Pnpt1) and 5′-TTCCATCCGAGTACGTGCTCGCTCGAT-3′ (Pnpt2). Positive independent plants with the typical wild type phenotype were self to obtain the T2 populations segregating for the T1243-RNAi homozygous mutants. The genotype of the analyzed plants was confirmed in T3 by kanamycin selection. The T3 seeds that did not segregate on MS plates with kanamycin were identified as homozygous RNAi lines (referred to as the T1243-RNAi background).

2.4. Reverse transcription PCR, Southern and Northern blot analysis of the transgenic plants

2.4.1. Detecting plants over-expressing the target genes

The hygromycin-resistant plants of the over-expression T1 generation were confirmed firstly by PCR using primers 5′-GCTCCTACAAATGCCATCA-3′ ($P_{35S}1$) and 5′-CCCAAGCTTCAGC-GAAAGAGATCAAGGAT-3′ ($P_{35S}2$). PCR positive plants were further analyzed by Southern blot. To prepare probes, full-length *T1243* or *T1170* genes were derived from pGEM-T-T1243 and pGEM-T-T1170 plasmids by PCR amplification using primers 5′-AAATC-GATGCCTCAACTGGATAAATTC-3′ (P1) and 5′-CCCAAGCTTCAGC-GAAAGAGATCAAGGAT-3′(P2) [13]. The PCR products were purified before being labeled. Genomic DNA (10 μg) extracted by CTAB was digested with the restriction enzyme EcoRI at 37 °C overnight. The Southern blot procedure was as described [16].

To detect the expression of *T1243* and *T1170* by RT-PCR, total RNA was prepared from 100 mg of 10 weeks old plant tissues using Trizol® Reagent (Invitrogen, USA), and 5 μg was used for reverse transcription using M-MLV reverse transcriptase as described by the manufacturer (Promega, USA). Products were diluted and used