

# RNA editing analysis of mitochondrial *nad3/rps12* genes in cytoplasmic male sterility and male-fertile cauliflower (*Brassica oleracea* var. *botrytis*) by cDNA-SSCP

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**ABSTRACT.** Characteristics of mitochondrial *nad3/rps12* locus have been shown to differ in cytoplasmic male sterility (CMS) and male-fertile cauliflower (maintainer line of CMS cauliflower). However, *nad3/rps12* can normally be co-transcribed in both lines. A specific fragment of about 800-bp has been detected by RT-PCR analysis. In order to further elucidate whether these two genes undergo different post-transcriptional modifications in the two lines, the RNA editing status of *nad3/rps12* was analyzed by cDNA-SSCP (single-strand conformation polymorphism). A total of 100 cDNA clones obtained from CMS and male-fertile cauliflower, respectively, were investigated. In CMS cauliflower, nine RNA editing patterns were identified while three were found in male-fertile cauliflower. To confirm the reliability of cDNA-SSCP analysis, four clones, randomly selected from each pattern, were sequenced. In total, 20 RNA editing sites were detected in the twelve different patterns, all within the coding region. In CMS cauliflower, except for the fact that one site was fully edited and one site was pre-edited, the editing of other sites (18) was incomplete. In contrast, in male-fertile cauliflower, 13 of the 20 sites were pre-edited; two sites were fully edited, and only five were partially edited. These results suggested significant differences in the RNA editing status of *nad3/rps12* between the two lines. Further phylogenetic tree analysis indicated that these genes belonged to different branches. Our data suggested that, given the same nuclear background, and excluding the effects of various growth conditions and developmental stages, the structure and origin of *nad3/rps12* may be the main factors affecting RNA editing status. Moreover, the relationship between the RNA editing status of *nad3/rps12* and the CMS trait in cauliflower is discussed.

**Keywords:** Cauliflower (*Brassica oleracea* var. *botrytis*); Cytoplasmic male sterility (CMS); RNA editing; SSCP (single-strand conformation polymorphism).

## INTRODUCTION

RNA editing is a phenomenon which occurs widely in very diverse groups of eukaryotes. It modifies the genetic information at the post-transcriptional level by exchanging, inserting or deleting standard nucleotides of the genetic alphabet (Benne, 1996; Smith et al., 1997; Maier et al., 1996; Steinhäuser et al., 1999). In higher-plant mitochondrial RNAs, this process is characterized by C-to-U, and more rarely U-to-C, exchanges. When editing occurs in coding regions, it is found to generate translational start codons by ACG to AUG conversions, as in *nad1* transcripts in wheat and *cox1* transcripts in tomato (Hoch et al., 1991; Kudla et al., 1992; Chapdelaine and Bonen, 1991; Kadowaki et al., 1995) or remove the translational stop codons such as in *atp6*, *atp9* and *rps10*

transcripts in several plant species (Wakasugi et al., 1996; Yoshinaga et al., 1996; Wintz and Hanson, 1991; Kempken et al., 1991; Zanolungo et al., 1995). However, the editing sites are mainly in the first or second positions, and the corresponding amino acids are usually altered, which improves the conservation of predicted protein as compared to other organisms, such as animals and fungi (Kurek et al., 1997; Bock, 2000). In rare cases, the editing events are observed at the third positions, and thus do not result in amino acid changes, a phenomenon that has been termed silent editing. In the mitochondria of *Arabidopsis*, 51 such sites have been identified, summing up to 11.6% of the total 441 sites identified in coding regions (Giege and Brennicke, 1999). In other cases, some RNA editing sites are also found outside of the protein-coding regions. The site, 32nt upstream of *psbF* in *Ginkgo biloba*, is edited (Kudla and Bock, 1999). Such RNA sites were also detected in the 5' untranslated regions of maize and rice *ndhG* mRNAs (Corneille et al., 2000). Moreover, the editing of "structural" RNAs has been described. In

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*Oenothera* mitochondria, two editing sites were found in a subpopulation of 26S rRNA molecules (Schuster et al., 1991), the first report that RNA editing takes place in ribosomal RNA. In *physarum* and *dictyostelium* mitochondria, the RNA editing of "structural" RNAs, the small subunit ribosomal RNAs (*SSU* rRNAs), was also observed (Mahendran et al., 1994; Barth et al., 1999). Although the function of RNA editing in these "structural" rRNAs is still unclear, some evidence has indicated that these editing events may be required for the formation of correct structure, suggesting their importance to efficient, accurate translation in the mitochondria (Barch et al., 1999).

To date, more than 1000 RNA editing sites have been found in all examined plant mitochondrial genes (Schuster and Brennicke, 1994), almost all undergoing specific post-transcriptional C-to-U conversion by RNA editing, with the rare exception of a few genes in specific plants. One of these is T-*urf13* in T-CMS maize (Ward and Levings, 1991), which is unique to the CMS-T cytoplasm of maize (Dewey et al., 1986). Another is *atp6* in radish (Krishnasamy et al., 1994), the unedited transcripts of which can produce proteins identical to those from edited transcripts in other plants, implying that editing of this gene in radish is unnecessary (Rankin et al., 1996).

Although RNA editing can be detected in a wide range of plant mitochondrial genes, in some cases the editing frequencies or editing patterns of certain genes are not identical in different plants, nor are they always identical in different tissues of the same plant, in plants of disparate growth conditions or developmental stages. In maize mitochondria, incompletely editing of *nad3* transcripts was detected in all tissues, while a temporal increase in the overall editing status from 50% at 3 days to about 75% at 7 days was also found (Grosskopf and Mulligan, 1996). In euplasmic and alloplasmic cytoplasmic male sterility wheat lines, the RNA editing of *atp6* occurred in twelve codons, all of which were fully edited in the euplasmic *Triticum timopheevi*, while in the CMS lines 17% of the clones were only partially edited. In *atp9* transcripts, eight codons were modified by RNA editing, and all eight were found fully edited in embryos, roots, shoots, and anthers in the euplasmic wheat lines, while in the transcripts obtained from the CMS lines, 19% of the clones were partially edited (Kurek et al., 1997). More interesting examples can be found in some cytoplasmic male sterility plants and their corresponding male-fertile materials, which usually have different nuclear backgrounds. The frequency of *atp6* RNA editing was specifically reduced in anthers of A3-CMS sorghum. However, it was increased in partially restored progeny (Tang et al., 1999; Howad et al., 1999; Pring et al., 1998). Similarly, the RNA editing of *orf107*, which is associated with CMS trait in A3 sorghum, has shown to be altered in leaf tissues of plants carrying *Rf3*, a fertility restoration gene of A3-CMS sorghum. While *orf107* is highly edited in CMS line A3Tx398, the lines carrying *Rf3Rf3* or *Rf3rf3* exhibit reduced editing in

the residual, non-processed transcripts (Pring et al., 1998). In CMS rice, two different N-*atp6* and B-*atp6* transcripts coexist, and the extent of RNA editing in the latter mRNAs was affected in the processing controlled by a nuclear gene involved in fertility restoration (Iwabuchi et al., 1993). In S-CMS maize, the sequence of *orf77* associated with the CMS trait, was similar to that of *atp9*. Although the *atp9* transcripts were fully edited, *orf77* nucleotides corresponding to edited nucleotides in *atp9* were either not edited or edited inefficiently (Gallagher et al., 2002). One explanation was that the editing sites in chimeric *orf77* could cause male sterility by compromising the expression of *atp9*. This evidence indicates that the RNA editing and cytoplasmic male sterility, two important phenomena associated with higher plant mitochondria, may have a relationship, although some ambiguous problems still require to be elucidated.

In previous study, we noticed that the characteristics of *nad3/rps12* locus were different in near-isogenic cytoplasmic male sterility and male-fertile cauliflower. To date, we have no further knowledge about these genes in either line. Here, we conduct the transcription and RNA editing analysis of *nad3/rps12*, which have been reported in several plant species (Rankin et al., 1996; Pesole et al., 1996; Itani and Handa, 1998; Wilson and Hanson, 1996). The cDNA-SSCP method, together with direct sequencing, was performed. The data indicated that this method was capable of analyzing the RNA editing of certain genes. Moreover, the origin of *nad3/rps12* in both lines was deduced by phylogenetic tree analysis. The possible relationship between RNA editing status of these genes and the CMS trait in cauliflower is also discussed.

## MATERIALS AND METHODS

### Plant materials

The CMS cauliflower, NKC-A and male-fertile cauliflower, NKC-B (the maintainer line of NKC-A) were kindly provided by Dr. Deling-Sun, Tianjin Vegetable Research Institute, Tianjin, China and grown under normal light conditions (day/light temperature of 25/19 °C, 16 h photoperiod). NKC-A had been backcrossed for twelve generations with the maintainer lines NKC-B, so theoretically, both lines possessed an identical nuclear background. The 10-day-old fresh leaves were used for total DNA and RNA extraction.

### DNA and RNA isolation

Total DNA was isolated using the CTAB method (Murray and Thompson, 1980) with some modifications. Fresh leaves (0.2 g) were ground in liquid nitrogen, and the frozen power was then directly added to 2 mL lysis buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 1.4 M NaCl, 0.2%  $\beta$ -mercaptoethanol, 2% PVP and 1 $\times$ CTAB). After incubation at 56°C for 30 min, 2 mL phenol : chloroform : isoamyl alcohol (25:24:1) was added. The supernatant was obtained by centrifugation at 10,000 g

for 10 min and extracted once with an equal volume of chloroform : isoamyl alcohol (24:1). After centrifuging at 11,000 g for 15 min, a two-thirds volume of cold isopropanol was added to the supernatant. The mixture was incubated for 30 min at -20°C. DNA was pelleted by centrifugation at 11,000 g for 30 min at 4°C and dissolved in 100 µL TE (pH 8.0) buffer. After the RNA was digested in the presence of 200 µg/µL RNase A for 1 h at 37°C, the DNA was detected by 1.0% agarose gel in 0.5×TBE and stored at -20°C.

Total RNA was isolated using TRIzol® (BBI, Canada), according to the manufacturer's instructions. After the contaminated DNA was digested with DNase I (TaKaRa, Japan) for 30 min at 37°C, total RNA was tested by 1.2% denaturing agarose gel and stored at -70°C.

### cDNA synthesis and PCR amplification

DNase I-treated total RNA (5 µg) were reverse transcribed to cDNA using 0.2 µg reverse primer of *nad3/rps12*, NR4, and 200 unit M-MLV reverse transcriptase under conditions specified by the enzyme supplier (Promega, USA) in a final volume of 10 µL. Following reverse transcription, PCR reactions were performed following the program for initial denaturation at 94°C for 2 min: 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min 15 s, and a final extension at 72°C for 8 min in a final volume of 25 µL containing 1 unit of *Taq* DNA polymerase (TaKaRa, Japan), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs, 0.2 µM of each forward and reverse primer, and about 50 ng of total DNA or 1 µL of the single-stranded reaction. After the amplification, the PCR products were separated by electrophoresis on 1.5% agarose gel. After being stained with ethidium bromide, gel was photographed under ultraviolet light using the GeneGenius (Syngene, USA). Oligonucleotide primers used for cDNA synthesis and PCR amplification are listed below. Primers ND1 and ND2 were designed to amplify long sequences containing *nad3/rps12* from total DNA. Primers ND3 and ND4 were used to amplify *nad3/rps12* from both total DNA and cDNA.

ND1: 5' AAGCGGGGTAGAGGAATTGGT 3'

ND2: 5' AGTTCAGAGGCATCTCCATTC 3'

ND3: 5' CAAAGTGGGCTGTAATGATGT 3'

ND4: 5' CATATCGATTTGGGTTTTTCTG 3'

### Cloning of *nad3/rps12* from DNA and cDNA

PCR products containing target sequences were extracted with the QIAquick Gel Extraction Kit (QIAGEN, USA), and were then directly cloned in pUCm-T easy vectors (BBI, Canada) according to the manufacturer's instructions and transformed into *Escherichia coli* DH5α. Positive clones were identified by the PCR method, and the PCR conditions were performed as described above. The positive clones with the insertion of *nad3/rps12* and flank sequence were directly sequenced

by the ABI3730 sequencer (Applied Biosystems, USA). Those positive clones with the insertion of *nad3/rps12* coming from both cDNA and total DNA were, prior to being sequenced, analyzed by the following cDNA-SSCP method to detect the RNA editing status in CMS and male-fertile cauliflower.

### cDNA-SSCP analysis and sequencing

1 µL samples of *nad3/rps12* amplified from positive clones were added to an equal volume of denaturing loading buffer (95% formamide, 0.05% bromo phenolblue, 0.05% xylene cyanol FF, and 20 mM EDTA), denatured at 97°C for 8 min, and immediately placed on ice for 2 min. After loading on a neutral 6% polyacrylamide gel (29:1, acryamide: bisacrylamide), the samples were electrophoresed in 1×TBE buffer at 20°C and run at 120v for 10-12 h. The gel then was stained with Silver Stain Kit (ATTO, Japan). Based on the results of SSCP, four clones randomly selected from each RNA editing pattern were sequenced by the ABI3730 sequencer (Applied Biosystems, USA). All the sequences were further analyzed by the Clustal W program (Thompson et al., 1994).

### Phylogenetic tree analysis

The sequences of *nad3/rps12* in other plant species, obtained from the DNA database of NCBI, and the two sequences in CMS and male-fertile cauliflower were aligned by the Clustal W program. After that, an unrooted neighbour-joining phylogenetic tree was constructed using the Phylip3.63 software (<http://evolution.genetics.washington.edu/phylip.html>) with the neighbor-joining (NJ) method (Saitou and Nei, 1987). The reliability of the tree was established by conducting 1000 neighbor-joining bootstrap sampling steps (Felsenstein, 1985). Phylogenetic trees were visualized using the Tree View, 1.6 program (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

## RESULTS

### The *nad3/rps12* locus structure is different between the two lines; however, the genes can normally be co-transcribed

Previous research identified a 21nt reverse repeat in the *nad3/rps12* locus of CMS cauliflower NKC-A that was not present in male-fertile cauliflower. In order to further clarify whether the *nad3/rps12* locus was different in CMS and fertile cauliflower, specific primers (ND1 and ND4) were designed to amplify the corresponding region of the *nad3/rps12* locus. A predicted fragment of approximately 2 kb was detected in CMS cauliflower NKC-A, but not in male-fertile cauliflower (Figure 1). Further sequence analysis revealed that this fragment contained *nad3/rps12* genes, found both in CMS and fertile cauliflower, as well as an unknown upstream region of *nad3/rps12* that was divergent in both lines (accession number: DQ219816, DQ219817). This suggested that the structure of the *nad3/*

*rps12* locus in the corresponding 2 kb region is different. Considering that structural differences may affect *nad3/rps12* expression, transcriptional analysis was performed. However, no significant differences were noted, as measured by RT-PCR. A specific cDNA fragment of *nad3/rps12* of about 800-bp was detected in both lines (Figure 2).

#### Different RNA editing patterns were detected by cDNA-SSCP in the two lines

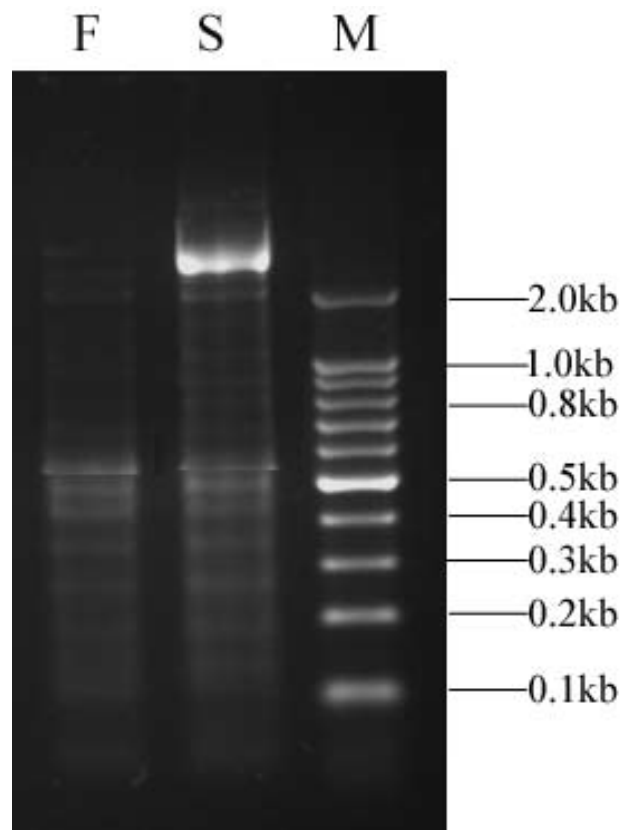
A total of 100 cDNA clones with *nad3/rps12* insertion obtained from CMS and male-fertile cauliflower, respectively, were investigated. In CMS cauliflower NKC-A, nine distinguishing RNA editing patterns were identified, while three were found in male-fertile cauliflower NKC-B (Figures 3 and 4). According to the number of cDNA clones representing each RNA editing pattern and the total number of cDNA clones (100) detected in each line, the frequency of each RNA editing pattern was calculated (Table 1). In CMS cauliflower NKC-A, the frequency was 2% to 36%. However, in male-fertile cauliflower NKC-B, the frequency was 28% to 42%. These results indicated that the RNA editing patterns in CMS cauliflower NKC-A were more complex than those in male-fertile cauliflower NKC-B.

#### RNA editing frequencies in corresponding sites are also different in the two lines

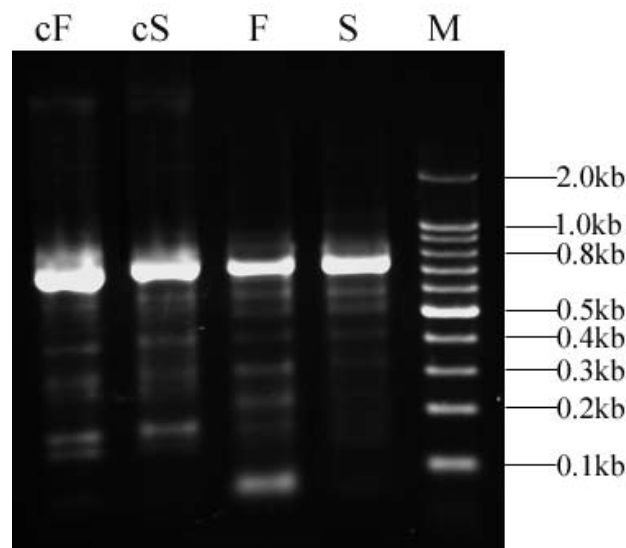
To confirm the reliability of cDNA-SSCP analysis and obtain further information regarding RNA editing sites, four clones, randomly selected from each pattern—except pattern 7, from which only two clones were selected—were sequenced. Analysis of these sequences indicated that the clones selected from the same RNA pattern exhibited identical sequences. A total of 20 RNA editing sites were detected within twelve different patterns (Figure 5), all within the coding region. In CMS cauliflower NKC-A, 19 sites were C-to-U edited. 18 of these were incompletely edited, and the one at position 5 was fully edited. Interestingly, the RNA editing site at position 5 could be detected in all nine RNA editing patterns, and another site at position 605 was pre-edited. The editing frequency was 6% to 98%. In male-fertile cauliflower NKC-B, 13 of the 20 sites were pre-edited, with two sites at positions 80 and

**Table 1.** The frequency of RNA editing pattern detected by cDNA-SSCP (Numbers 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 listed in the editing pattern column correspond with those in Figure 3).

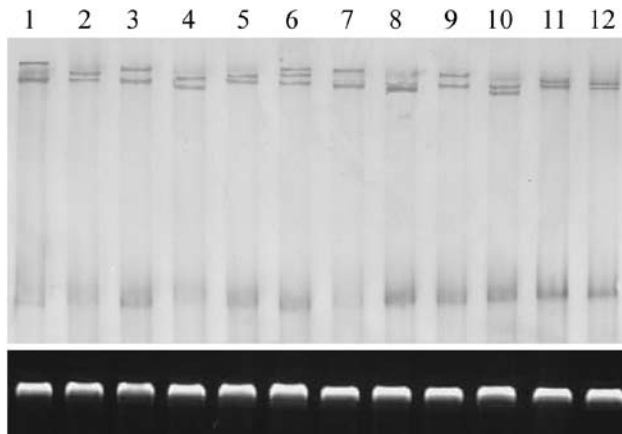
Editing pattern	Frequency (%)	Editing pattern	Frequency (%)	Editing pattern	Frequency (%)
1	20	5	4	9	6
2	36	6	8	10	42
3	10	7	2	11	30
4	6	8	8	12	28



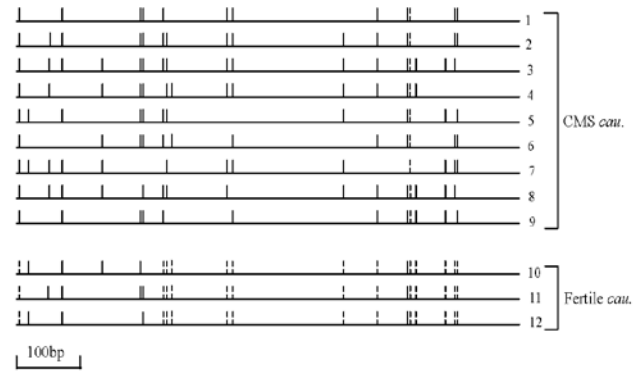
**Figure 1.** PCR amplified products of the *nad3/rps12* locus using primers ND1 and ND4 in CMS and male-fertile cauliflower, respectively. F: male-fertile cauliflower. S: cytoplasmic male sterility cauliflower. M: molecular weight marker (100-bp ladder).



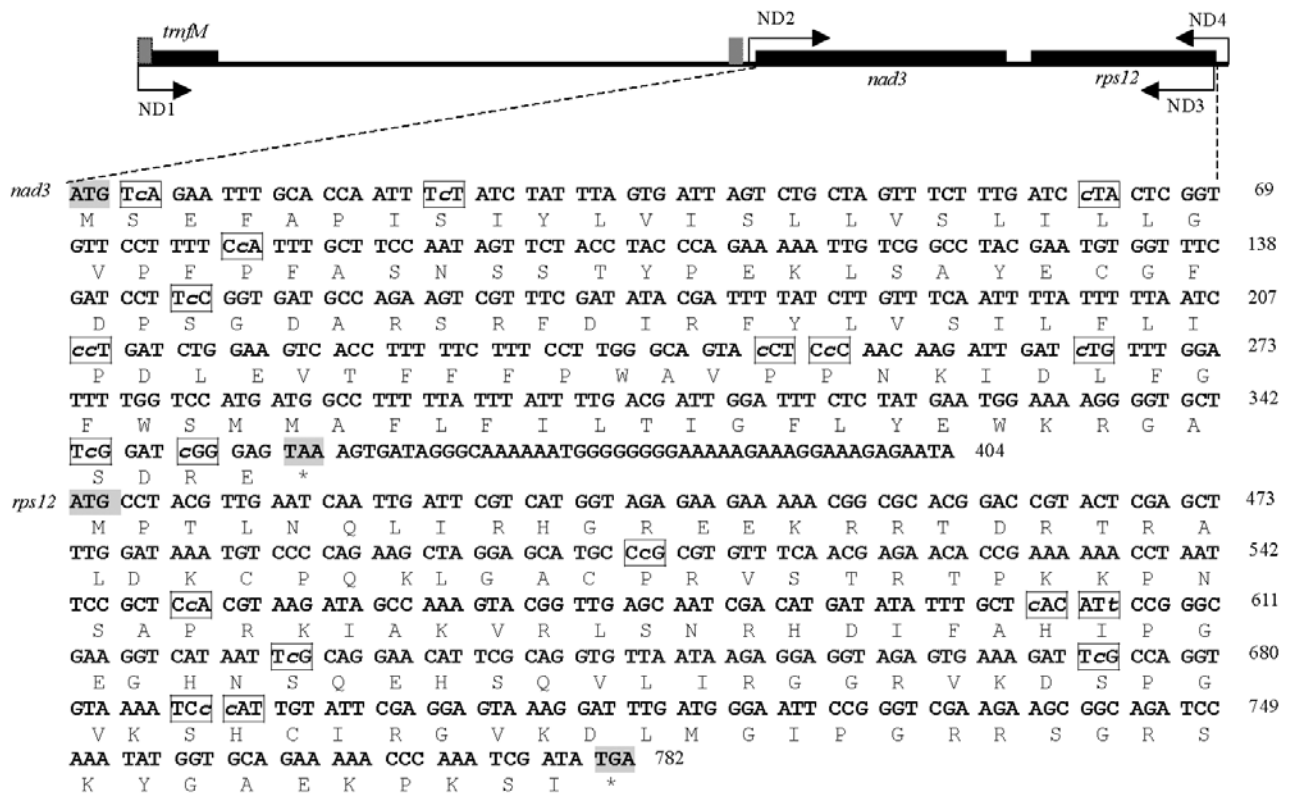
**Figure 2.** PCR and RT-PCR analysis of the *nad3/rps12* using primers ND2 and ND3 in CMS and male-fertile cauliflower, respectively. cF: RT-PCR amplified products of *nad3/rps12* in male-fertile cauliflower. cS: RT-PCR amplified products of *nad3/rps12* in cytoplasmic male sterility cauliflower. F: PCR amplified products of *nad3/rps12* in male-fertile cauliflower. S: PCR amplified products of *nad3/rps12* in CMS cauliflower. M: molecular weight marker (100-bp ladder).



**Figure 3.** Different RNA editing patterns of *nad3/rps12* in CMS and male-fertile cauliflower, respectively, detected by cDNA-SSCP. Lanes 1, 2, 3, 4, 5, 6, 7, 8 and 9 indicate different RNA editing patterns of *nad3/rps12* in CMS cauliflower. Lanes 10, 11 and 12 indicate different RNA editing patterns of *nad3/rps12* in male-fertile cauliflower. Prior to the analysis of cDNA-SSCP, the corresponding cDNA clones were firstly detected by agarose gel.



**Figure 4.** Distribution of RNA editing sites of *nad3/rps12* in each RNA editing pattern detected by cDNA-SSCP. Numbers 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 correspond with those in Figure 3. Drawing is approximately to scale, with C-to-U edits shown as vertical lines and those pre-edited sites shown as vertical broken lines (CMS cau.= CMS cauliflower; Fertile cau.= Fertile cauliflower).



**Figure 5.** Organization and sequence of the *nad3/rps12* locus in CMS cauliflower. The genes of *trnM*, *nad3* and *rps12* are indicated by black boxes. The repeat sequences found in this sequence were indicated by shaded boxes. Horizontal arrows showed the positions of primers used in reverse transcription (ND4) and PCR amplification experiments (ND1, ND2, ND3 and ND4). The sequence of *nad3/rps12* was shown in triplets. RNA editing sites (corresponding C-to-U changes at mRNA level) are boxed, and the altered nucleotides are italic and lowercase. The start and stop codons are marked by shadow following that. \* indicates the corresponding stop signal in amino acids level.

605 fully edited and five sites, at positions 23, 61, 146, 208 and 209, also incomplete edited. However, the editing frequency was from 30% to 72% (Table 2).

### The origin and structure of *nad3/rps12* may be associated with the RNA editing status in cauliflower

CMS and male-fertile cauliflower in this study possess an almost identical nuclear background while the RNA editing status of *nad3/rps12* in these two lines is significantly different. Excluding the effect of growth conditions and developmental stages, it may result from the genes themselves. The phylogenetic tree analysis was performed. Besides the two DNA sequences of *nad3/rps12* from CMS and male-fertile cauliflower, respectively, 21 other corresponding sequences from different species were available in the NCBI database (Table 3). The phylogenetic tree indicated that all these species could be distinguished by sequences of *nad3/rps12* (Figure 6). The *nad3/rps12* of CMS cauliflower NKC-A had a phylogenetic distance similar to that of *R. sativus* (accession numbers: U43506). The genes in male-fertile cauliflower NKC-B, on the other hand, obviously had a phylogenetic distance similar to that of *B. napus* (accession numbers: AP006444). This result implied that the origin of *nad3/rps12* in CMS and male-fertile cauliflower was different.

## DISCUSSION

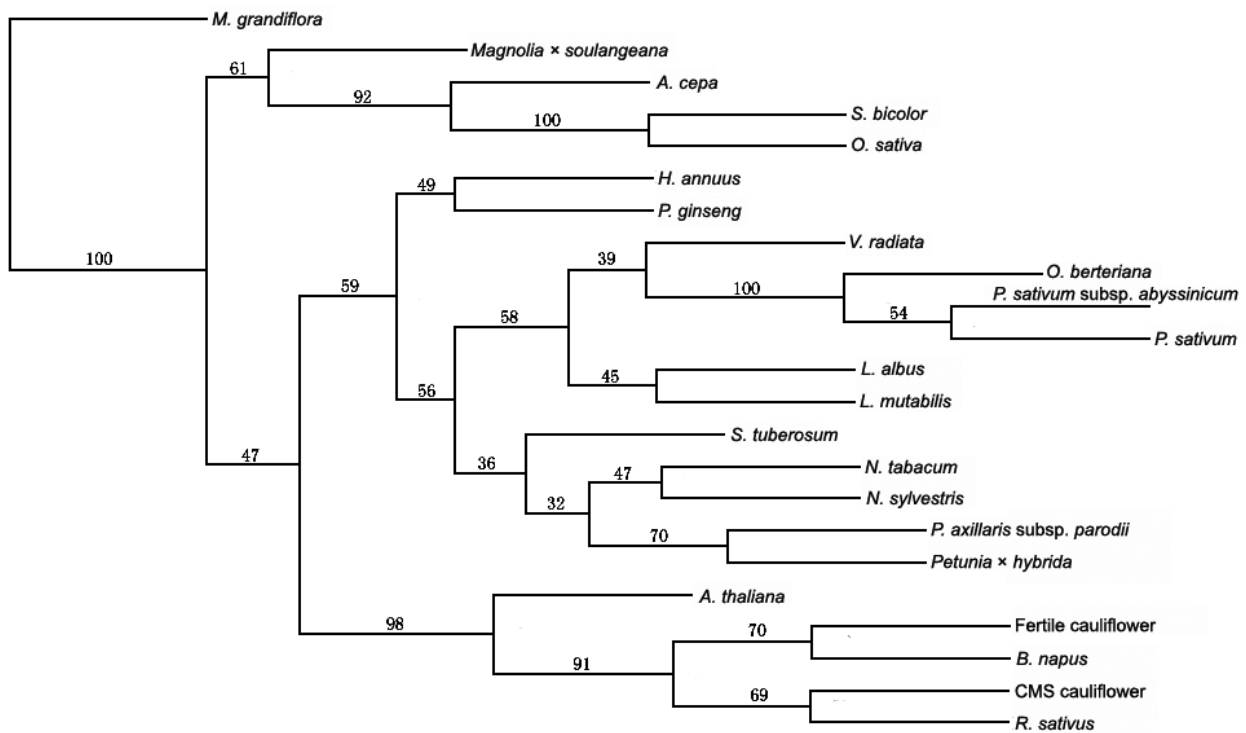
RNA editing, in higher plants, is essential to modifying mitochondrial genes at the post-transcriptional level. It affects not only the function genes, but also the structure genes. Direct sequencing of several cDNA clones is regarded as an ideal method of studying the RNA editing status of certain genes. However, in some cases, the balance between the sequencing cost and the accuracy must be considered. Here, we establish a method, cDNA-SSCP (single-strand conformation polymorphism), and combine it with direct sequencing to investigate the RNA editing status of *nad3/rps12* in cauliflower, dramatically reducing the cost without compromising accuracy. SSCP analysis depends on the conformation change of single-strand DNA having nucleotide substitutions (Orita et al., 1989; Nataraj et al., 1999). Because of the sensitive, inexpensive, and comparatively fast nature of this method, it has been widely used in detecting mutation sites, and single nucleotide polymorphisms (SNP) and in mapping and tracking candidate genes (Nataraj et al., 1999; Slabaugh et al., 1997; Sato and Nishio, 2002). C-to-U RNA editing in higher plant mitochondria can also be regarded as a mutation at the mRNA level and can, theoretically, be analyzed by SSCP. However, to date, little research has been reported in this field, with the exception of RNA editing of the *ndhA* gene in maize

**Table 2.** The RNA editing sites of *nad3/rps12* and the editing frequency of each site in CMS and male-fertile cauliflower, respectively. (*cau.*= cauliflower).

Editing position	Codon change	Residue change	Editing frequency	
			CMS <i>cau.</i> (%)	Fertile <i>cau.</i> (%)
5	TcA	S>L	100	Pre-edited
23	TcT	S>F	6	70
61	cTA	L (silent)	62	30
80	CcA	P>L	86	100
146	TcC	S>F	60	42
208	ccT	P>F	90	72
209	ccT	P>F	98	58
247	cCT	P>F	92	Pre-edited
251	CcC	P>F	66	Pre-edited
256	cTG	L (silent)	14	Pre-edited
344	TcG	S>L	82	Pre-edited
349	cGG	R>W	88	Pre-edited
508	CcG	P>L	66	Pre-edited
550	CcA	P>L	94	Pre-edited
600	cAC	H>Y	98	Pre-edited
605	ATr*	I>F	Pre-edited	100
625	TcG	S>L	30	Pre-edited
673	TcG	S>L	30	Pre-edited
689	TCc	S (silent)	84	Pre-edited
690	cAT	H>Y	76	Pre-edited

**Table 3.** Species and corresponding accession numbers of *nad3/rps12* obtained from the NCBI database.

Species	Accession numbers	Species	Accession numbers	Species	Accession numbers
<i>A. cepa</i>	Z49771	<i>M. grandiflora</i>	X84106	<i>P. ginseng</i>	M74169
<i>A. thaliana</i>	Y08501	<i>N. tabacum</i>	BA000042	<i>P. sativum</i>	AY043195
<i>B. napus</i>	AP006444	<i>N. sylvestris</i>	X96741	<i>P. sativum</i> subsp. <i>abyssinicum</i>	AY043192
<i>H. annuus</i>	X84008	<i>O. berteriana</i>	X52199	<i>R. sativus</i>	U43506
<i>L. albus</i>	AF035356	<i>O. sativa</i>	M57904	<i>S. tuberosum</i>	AF095279
<i>L. mutabilis</i>	AF035357	<i>P. axillaris</i> subsp. <i>parodii</i>	M16770	<i>S. bicolor</i>	Y13329
<i>Magnolia</i> × <i>soulangeana</i>	Z49796	<i>Petunia</i> × <i>hybrida</i>	U30458	<i>V. radiata</i>	AF071550

**Figure 6.** Phylogenetic analysis of *nad3/rps12* genes in CMS and male-fertile cauliflower and other species, which sequences of *nad3/rps12* now can be obtained from the databases of NCBI. The phylogenetic tree was constructed by neighbour-joining method, and the bootstrap values were below the branches. The *nad3/rps12* genes of CMS cauliflower were indicated as CMS cauliflower while that of male-fertile were indicated as male-fertile cauliflower. The other species and their accession numbers are listed in Table 3.

(Fuchs et al., 2001). This may be due to the complexity of the RNA editing status, which reduces the sensitivity of SSCP analysis. In the present study, *nad3/rps12* RT-PCR products from CMS and male-fertile cauliflower, respectively, were cloned. Each cDNA clone was then analyzed by SSCP, a process termed cDNA-SSCP. Because each cDNA clone represented only one status of RNA editing, the RNA editing pattern was dramatically simplified compared with the RT-PCR products directly used as samples. In 200 detected cDNA clones, each can produce distinguishable single-strand conformation. With strict control of the electrophoresis conditions, sample quantity and repeated experimentation ( $n=3/\text{cDNA clone}$ ), a total of 12 different RNA editing patterns were detected

(Figures 3 and 4). Random sequencing of four cDNA clones from each detected pattern confirmed this result. Our data demonstrated that SSCP analyzed the RNA editing of *nad3/rps12* of cauliflower nearly as efficiently as direct sequencing. However, as mentioned above, the complexity of the RNA editing in different genes, the condition of the electrophoresis, and the G+C content can all affect the accuracy of SSCP analysis (Nataraj et al., 1999), so further experiments will be conducted to confirm whether SSCP can analyze other genes as efficiently as it did *nad3/rps12* in cauliflower. Our results strongly suggest that cDNA-SSCP is a powerful tool that can be used to analyze the RNA editing status of target genes, especially the rudimental RNA editing status.

Based on SSCP analysis combined with direct sequencing, 12 different RNA editing patterns and 20 RNA editing sites (Table 2) have been found in cauliflower *nad3/rps12*, a number greater than found in Ogura and normal radish (Rankin et al., 1996), but smaller than in *Allium cepa*, *Triticum aestivum*, *Helianthus annuus*, *Oenothera berteriana*, *Petunia hybrida*, and *Magnolia soulangeana* (Pesole et al., 1996; Wilson and Hanson, 1996; Perrotta et al., 1996). In Ogura radish, two different RNA editing patterns and three *nad3/rps12* RNA editing sites have been described while no RNA editing has been found in normal radish genes. In the other six plants mentioned above, these genes contain from 25 to 35 editing sites, implying that although *nad3/rps2* genes are conserved in evolution, their RNA editing statuses may differ significantly. This may result from differences in the nuclear background or from the need to regulate gene expression. Interestingly, in both CMS and male-fertile cauliflower, the nuclear background is almost identical. However, the *nad3/rps12* RNA editing status is significantly different. In the 19 detected RNA editing sites of CMS cauliflower NKC-A, partial editing events are dominant (18/19), and each site can produce nine different RNA editing patterns (Figure 3 and 4). In contrast, in male-fertile cauliflower NKC-B more pre-edited and fully edited sites were detected (15/20), and only three different RNA editing patterns. Given that the differences in nuclear background, growth conditions, and developmental stages can be almost neglected, we speculate that the differences in *nad3/rps12* RNA editing status between the two lines may have resulted from the origin of these genes. Phylogenetic tree analysis data demonstrated this (Figure 6), indicating that *nad3/rps12* in CMS and fertile cauliflower belonged to different branches and suggesting these genes may have derived from different ancestors of the two lines. In addition, the *nad3/rps12* locus structure in both lines is different in the 5' untranslated regions. We can speculate then that given an identical nuclear background, the origin and the structure of the *nad3/rps12* locus may be the main factors affecting RNA editing status in cauliflower. Another unexpected result can also be obtained from the analysis of the phylogenetic tree: almost all the species can find their corresponding taxonomic positions in this tree reconstructed based on the *nad3/rps12*. This implies that the *nad3/rps12* genes may become very valuable candidates to evaluate the evolutionary relationships between higher plants. In previous studies, they have been used to analyze several angiosperms (Pesole et al., 1996).

Like RNA editing, cytoplasmic male sterility is an important phenomenon in higher plant mitochondria (Howad et al., 1999). Previous research has suggested that novel chimeric genes, resulting from the mitochondrial DNA rearrangements, were closely associated with the CMS trait, which are believed to interfere with normal pollen development (Schnable and Wise, 1998). Examples include T-*urf13* in T-maize (Dewey et al., 1986), *orf355/*

*orf77* in S-maize (Zabala et al., 1997), *orf79* in Bo-rice (Akagi et al., 1994, 1995), *pcf* in petunia (Young and Hanson, 1987), *orf522* in PET1 sunflower (Sabar et al., 2003), *pvs* in bean (He et al., 1995), *orf138* in Ogura radish (Bonhomme et al., 1992), *orf224* and *orf222* in pol and nap *Brassica napus*, respectively, (L'Homme and Brown, 1993; L'Homme et al., 1997), *orf107* in A3-sorghum (Tang et al., 1996), and *orf215* in sugar beet (Ivanov et al., 2004). However, the molecular mechanism of CMS in cauliflower is, to date, poorly understood. In our other study, a specific chimeric open reading frame similar to *orf138* in Ogura radish was confirmed to be closely associated with the CMS trait in cauliflower. It is of interest to note that all the progenies of CMS cauliflower NKC-A, used as the female parent, were 100% sterile, regardless of the status of the male parent. To our knowledge, over the past two decades, all attempts to create a restorer line of CMS cauliflower NKC-A have been unsatisfactory. These results suggest that the molecular mechanism of CMS in cauliflower NKC-A may be more complex than those in other CMS plants. We speculate that other factors, in addition to the novel chimeric genes found in CMS cauliflower NKC-A, may directly or indirectly affect this trait. Here, our study confirmed that the RNA editing status of *nad3/rps12* in CMS cauliflower NKC-A and male-fertile cauliflower NKC-B were significantly different, although no obvious differences were found at the transcriptional level. More RNA editing patterns and incomplete editing events were detected in CMS cauliflower NKC-A. The diversity of RNA editing patterns and insufficiency of certain RNA editing sites in some genes have been identified associated with the CMS trait in several plants such as *atp6* and *orf107* in CMS sorghum (Tang et al., 1999; Howad et al., 1999; Pring et al., 1998); *atp6* and *atp9* in CMS wheat (Kurek et al., 1997), and *orf77* in S-CMS maize (Gallagher et al., 2002). This implies that the RNA editing status of *nad3/rps12* in CMS cauliflower NKC-A may also be associated with the CMS trait. One possibility is that the chimeric genes similar to the *orf138* in Ogura radish, the functional mitochondrial genes *nad3/rps12*, and other unknown factors together affect the CMS trait in cauliflower NKC-A. However, no direct experiments have been done to confirm this hypothesis. To gain further insight into the different RNA editing status in CMS and male-fertile cauliflower and increase our knowledge of RNA editing and the CMS trait, further investigations are required. Our results offer significant clues toward their elucidation.

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## 細胞質雄性不育及可育花椰菜內線粒體基因 *nad3/rps12* 基於 cDNA-SSCP 的 RNA 編輯分析

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花椰菜細胞質雄性不育系及其保持系中線粒體 *nad3/rps12* 基因位點存在差異，但該基因在兩系中都能正常轉錄，RT-PCR 可檢測到一個 800-bp 大小的片段。為闡明 *nad3/rps12* 是否經受不同的轉錄後修飾，採用 cDNA-SSCP（單鏈構象多態性）的方法對該基因在兩系中的 RNA 編輯狀況進行了分析。各分析了 100 個 *nad3/rps12* cDNA 克隆，在細胞質雄性不育花椰菜中可檢測到九種不同的 RNA 編輯模式，而在保持系中只檢測到三種。為進一步驗證 cDNA-SSCP 分析的可靠性，在檢測到的每一種模式中隨機挑選四個克隆進行測序，共發現了二十個 RNA 編輯位點，這些位點都位於編碼區。在細胞質雄性不育花椰菜中，除一個位點發生完全編輯，一個位點提前被編輯，其他位點發生不充分編輯；而在保持系花椰菜中有十三個位點被提前編輯，二個被充分編輯，不充分編輯的位點僅有五個。以上結果顯示 *nad3/rps12* 在花椰菜細胞質雄性不育系及保持系中 RNA 編輯狀況有明顯不同。進一步的進化樹分析表明 *nad3/rps12* 在花椰菜兩系中分屬不同的進化分支，根據以上結果，排除生長條件及發育階段的影響，推測在相同的核背景下，花椰菜 *nad3/rps12* 的結構及進化來源可能是影響其 RNA 編輯狀況的主要原因，另外，文中對該基因的 RNA 編輯狀況與花椰菜細胞質雄性不育發生的關係進行了討論。

**關鍵詞：**花椰菜；細胞質雄性不育；RNA 編輯；單鏈構象多態性。