nad3 and *atp9* gene transcripts of safflower (*Carthamus tinctorius* L.) undergo extensive RNA editing

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Abstract
Transgenic male sterility has been induced in plants by targeting the nuclear encoded proteins of unedited-mitochondrial genes into mitochondria. With the aim of identifying candidate mitochondrial genes that could be used to induce transgenic male sterility in safflower (*Carthamus tinctorius L.*), we investigated the editing status in the transcripts of *atp9* and *nad3* genes, which encode subunits of ATPase and NADH-ubiquinone-oxidoreductase mitochondrial complexes. Here, we report, for the first time in safflower, the extensive editing seen in these transcripts. Comparison of the genomic and cDNA sequences revealed that the *nad3* transcripts had 19 edit sites including 12 complete and 7 partially edited sites leading to 14 amino acid changes in the protein while the *atp9* transcripts had a total of 12 edit sites, 10 completely edited and 2 partially edited, leading to 9 amino acid changes. Editing also led to creation of a stop codon that reduced the ATP9 polypeptide length by 12 amino acids. Comparison of the edit sites in safflower with those reported in other species clearly indicated that most of the edit sites are conserved and all the resultant aminoacid substitutions led to highly conserved proteins. Hydropathy analysis of the deduced proteins from genomic and cDNA sequences indicated that RNA editing led to increased hydrophobicity in both NAD3 and ATP9 proteins. Interestingly, most of the altered amino acids were in the transmembrane helices of the proteins. Hence, these results show that *u-atp9* and *u-nad3* genes could be potential candidate genes for induction of transgenic male sterility in safflower.

Key words: RNA editing - male sterility

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Introduction

RNA editing is a post-transcriptional process that modifies the sequence of RNA through nucleotide insertion, deletion or modification. In Plant mitochondria, RNA editing leads to C to U changes and very rarely U to C changes in some nucleotide positions of the transcripts. These alterations result in plant mitochondria result in production of proteins that are more conserved and this has suggested that RNA editing is important to produce functional proteins. This hypothesis was confirmed when male sterility was induced in transgenic tobacco plants by expression of unedited atp9 gene and targeting its protein (defective) into mitochondria (Hernould et al. 1993). Similar strategy has been adopted more recently by a private company to successfully obtain transgenic male sterile and restorer lines in rice (http://www.avesthagen.com/docs/newsletters/jan_feb_2005.pdf). We are working towards development of transgenic male sterile lines in safflower using the strategy adopted by Hernould *et al.* 1993. However, literature search revealed that mitochondrial genes of safflower have not been cloned so far. Since mitochondrial genes coding for proteins of the oxidative phosphorylation pathway are mostly implicated in cytoplasmic male sterility in different species, we intended to clone and study the editing patterns of two mitochondrial genes, *nad3* that codes for subunit 3 of the mitochondrial NADH ubiquinone oxidoreductase complex and *atp9* which codes for subunit 9 of the ATPase complex (a membrane-associated protein subunit involved in the formation of proton-translocating Fo portion of the ATPase in mitochondria) of safflower. Here, we report for the first time, RNA editing in the mitochondrial gene transcripts of safflower.

Materials and Methods

Primers were designed based on the *Helianthus annuus atp9* and *nad3* sequences. Mitochondrial DNA & RNA was isolated from young florets of safflower and used for PCR and RT-PCR respectively. Genomic and cDNA clones were developed, sequenced and compared to determine the edited sites. These edited sites were compared with those reported in other species. The protein sequences derived from the genomic and cDNA sequences were analyzed in-silico to study the effects of editing.

Results

Similar sizes of amplicons were obtained for *nad3* (357bp) and *atp9* genes (261bp), with both genomic as well as RT-PCR. A comparison of the genomic and cDNA clones revealed extensive editing of the transcripts of both the genes, all of which were C to U conversions. 21 C-U changes were seen in nad3, of which 19 were present in more than three clones and hence were considered for further analysis (to avoid considering errors that could have occurred during sequencing or PCR). Of these, 12 sites were edited consistently in all the clones (complete edit sites) whereas 7 were not edited in all the clones (partial edit sites) (Table 1). Editing led to 14 amino acid substitutions, the most common amino acid substitution being Proline to Leucine (P-L) (6 changes) (Table 1). Among the 12 cDNA clones of *nad3* analysed, only 4 clones had all the sites edited. Our results have shown that with 19 edit sites, safflower *nad3* transcript has the largest number of edited sites observed among the dicot plants analyzed so far.

Edit site no.	Partial/ complete editing (PE/CE)	Relative Editing Efficiency (REE)	Nucleotide position	Codon position	Amino acid change
1	CE	1.0	5*	2*	TCA(S)-TTA(L)
2	CE	1.0	39*	13*	ATC(I)-ATT(I)
3	CE	1.0	44*	15*	CCG(P)-CTG(L)
4	CE	1.0	61*	21*	CCA(P)-TTA(L
5	PE	0.9	62*		
6	CE	1.0	80*	27*	CCA(P)-CTA(L)
7	PE	0.3	147	49*	TTC(F)-TTT(F)
8	CE	1.0	208*	70*	CCT(P)-TTT(F)
9	CE	1.0	209*		
10	CE	1.0	215*	72*	CCG(P)-CTG(L)
11	PE	0.8	230*	77*	TCC(S)-TTT(F)
12	PE	0.7	231*		
13	PE	0.8	247*	83*	CCT(P)-TCT(S)
14	CE	1.0	251*	84*	CCC(P)-CTC(L)
15	CE	1.0	266*	89*	CCC(P)-CTC(L)
16	PE	0.9	275*	92*	TCT(S)-TTT(F)
17	CE	1.0	317*	106*	TCT(S)-TTT(F)
18	PE	0.9	344*	115*	TCG(S)-TTG(L)
19	CE	1.0	349*	117*	CGG(R)-TGG(W)

Table 1 RNA editing in *nad3* transcripts in safflower

* = edited nucleotide/edited amino acid conserved in the majority of the species
REE: number of clones showing editing /total number of clones,
CE: Complete edited site (edited in all clones)
PE: Partial edited site (not edited in all clones)

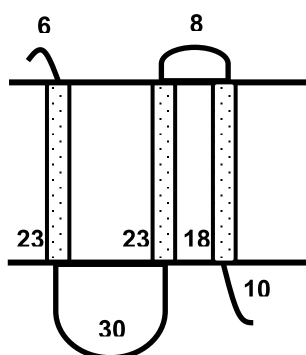
In the case of *atp9*, a total of 15 C-U changes were observed, of which 12 were seen in more than three clones and hence considered for further studies. Of these 12 edit sites, 10 were complete edit sites with 8 leading to amino acid substitutions. The most common amino acid substitution was S-L (observed for 5 edit sites) (Table 2). Editing at nucleotide position 223 modified an Arginine (A)(CGA) codon to a termination codon (TGA). Among the 11 clones studied, only four clones were edited at all the 12 edit sites. A comparison of the editing in these genes, with those reported in other angiosperms revealed that all the sites are edited in many species.

Table 2 RNA editing in *atp9* transcripts in safflower

Edit site no.	Partial/ complete editing P/C	Relative Editing Efficiency	Nucleotide position	Codon position	Amino acid change
1	CE	1.0	50*	17*	TCA(S)-TTA(L)
2	CE	1.0	81*	27*	GTC(V)-GTT(V)
3	CE	1.0	82*	28*	CTT(L)-TTT(F)
4	PE	0.5	90*	30*	TCC(S)-TCT(S)
5	CE	1.0	92*	31*	TCG(S)-TTG(L)
6	CE	1.0	134*	45*	TCA(S)-TTA(L)
7	CE	1.0	182*	61*	TCG(S)-TTG(L)
8	CE	1.0	191*	64*	CCA(P)-CTA(L)
9	PE	0.8	205*	69*	CTG(L)-TTG(L)
10	CE	1.0	212*	71*	TCA(S)-TTA(L)
11	CE	1.0	215*	72*	TCC(S)-TTC(F)
12	CE	1.0	223*	75*	CGA(R)-TGA(*)

[*] = edited nucleotide/edited amino acid conserved in the majority of the species
REE: number of clones showing editing /total number of clones,
CE: Complete edited site (edited in all clones)
PE: Partial edited site (not edited in all clones)

The derived protein sequences were subjected to in silico analysis to study the effect of editing on the hydrophobicity of the proteins as well as on their transmembrane structure. In case of both NAD3 and ATP9, editing led to increase in their hydrophobicity. In case of ATP9, editing resulted in shortening of the polypeptide due to a stop codon that was created at nucleotide position 223. Also it was found that amino acid changes brought about by editing were mainly located within the transmembrane helices for both the proteins (Figures 1 and 2).



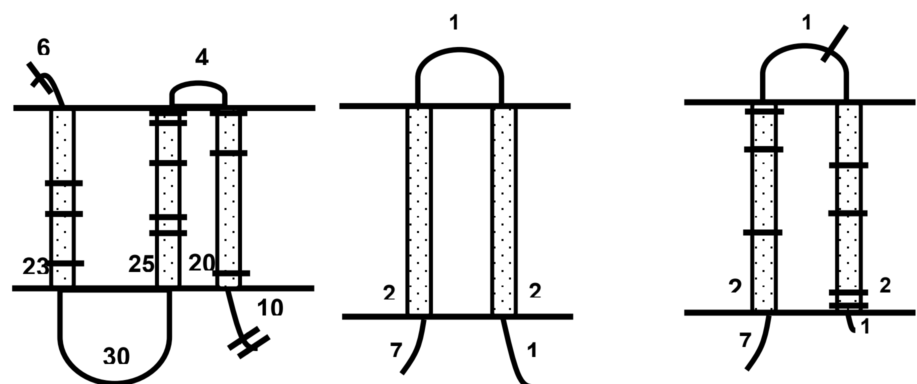


Fig. 1 Diagrammatic representation of the transmembrane helices predicted by HMMTOP for unedited NAD3(Left) and edited NAD3(Right). Figures indicate the number of amino acids in that region

Fig. 2 Diagrammatic representation of the transmembrane helices predicted by HMMTOP for unedited ATP9(Left) and edited ATP9(Right) Figures indicate the number of amino acids in that region

Discussion

The similar size of amplicons obtained for *atp9* and *nad3* genes, with both genomic as well as RT-PCR indicated that there were no introns. Both these gene transcripts in safflower were found to undergo extensive editing that increase the degree of conservation of their proteins with that of other species. The extensive and conserved patterns along with increased hydrophobicity of proteins observed in these two transcripts imply that these editings might be crucial for the functionality of NAD3 and ATP9 proteins. Though the *atp9* gene sequences of some species including safflower is longer, the creation of stop codon due to editing at nucleotide position 223, results in an equal polypeptide length (74 amino acids) for ATP9 across species. This high degree of conservation of the length and composition of both ATP9 and NAD3 across species, as a result of their transcript editing, indicates the functional importance of editing. It was interesting to note that the amino acid changes brought about by editing were mainly located within the transmembrane helices for both the proteins which corroborates well with the fact that editing leads to increased hydrophobicity of these proteins.

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