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Studies on the effects of nuclear background and tissue specificity on RNA editing of the mitochondrial ATP synthase subunits α, 6 and 9 in fertile and cytoplasmic male-sterile (CMS) wheat

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Abstract RNA editing occurs in transcripts encoded by the mitochondrial genome in all major groups of land plants and is characterised by C-to-U editing. When editing occurs, the amino acid is usually altered because the nucleotide modification is generally in the first or second position and the amino acid change improves the conservation of the predicted protein as compared with other organisms. In order to assess the effect of the nuclear background and tissue specificity of the RNA editing, we have analysed the RNA editing of the transcripts encoding for the mitochondrial ATPsynthase subunits *atpα*, *atp6*, and *atp9* in euplasmic and alloplasmic cytoplasmic male-sterile (CMS) wheat lines. The editing of *atpα* was detected in 6 codons. Although partially edited transcripts were found in all the cytoplasms, a larger number of partially edited clones were present in the CMS cytoplasm. The RNA editing of *atp6* occurred in 12 codons, and whereas in the euplasmic *Triticum timopheevi* all the clones were fully edited, in the CMS line 17% of the clones were only partially edited, suggesting an effect of the nuclear background on RNA editing efficiency. In *atp9* transcripts, 8 codons were modified by RNA editing. When the RNA editing of the *atp9* transcripts extracted from various tissues was compared, only fully edited clones were detected in embryos, roots, shoots, and anthers in the euplasmic wheat lines, whereas in the transcripts obtained from the CMS line, 19% of the clones were partially edited. This study reveals that RNA editing efficiency can be affected by the tissue and nuclear background. The mechanism by which incomplete and complete edited transcripts accumulate may indicate a link between the transcription kinetics and RNA editing which can be affected by nuclear background and tissue specificity.

Key words Wheat · CMS · *Triticum timopheevi* · Editing · ATP synthase

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Introduction

The term RNA editing is generally used to describe a large variety of biochemical processes which include the emergence of RNAs with nucleotide sequences differing from those encoded in the DNA genome. This process is not limited to messenger RNAs (mRNA) since alterations of non-informational RNA such as tRNAs and rRNAs have also been found (Scott 1995).

In plant mitochondria, C-to-U RNA editing was first described in angiosperms, but it occurs in all major groups of land plants (Malek et al. 1996). From every transcript so far analysed RNA editing has been found to affect predominantly the protein-coding regions (for recent reviews see Hanson et al. 1996; Araya et al. 1994). A rare exception to this rule is the *T-urf13* present in maize cytoplasmic male-sterile (CMS) mitochondria (Ward and Levings 1991). When editing occurs in mitochondria, the encoded amino acid is likely to be altered because the codon position of the edited C is usually in the first or second position (Gray and Covello 1993). Usually the amino acid change improves the conservation of the predicted protein as compared to other organisms. RNA editing may also shorten the predicted protein by creating stop codons, as in the *atp6*, *atp9* and *rps10* transcripts in several plant species (Wintz and Hanson 1991; Kempken et al. 1991, Begu et al. 1990; Zanlungo et al. 1995), or it may create a potential start codon, as in *nad1* transcripts in

wheat (Chapdelaine and Bonen 1991) and *cox1* transcripts in potato (Quinones et al. 1995).

The extent to which, different mitochondrial transcripts are RNA edited and the degree of edited codons vary among genes within the same species and the transcripts of the same gene between different species. Partially edited transcripts may represent a significant proportion of the mRNA population. Incompletely edited transcripts from plant mitochondria, evidenced as cDNAs, have been reported for several genes, such as *nad3* and *rps12* in wheat, Petunia, and *Oenothera* mitochondria (Schuster et al. 1990; Gualberto et al. 1991; Lu and Hanson 1992). Alternatively, almost fully edited cDNAs were detected for *cox3* in wheat (Gualberto et al. 1990), *atp9* in wheat (Begu et al. 1990) and Petunia (Wintz and Hanson 1991), and *nad4* from wheat (Lamattina and Grienenberger 1991). In Petunia mitochondria, it was shown that only the edited form of ATP6 accumulates although *atp6* partially edited transcripts were the most abundant (Lu and Hanson 1994). In contrast, RPS12 proteins translated from incompletely edited transcripts were found in the ribosomal pellet of Petunia and maize (Lu et al. 1996; Phreaner et al. 1996).

Although extensively studied, the molecular mechanism underlying CMS in many plant systems, including wheat, is not yet understood (for a review see Vedel et al. 1994). In CMS rice, where two different N-*atp6* and B-*atp6* transcripts coexist, the extent of the editing of the latter mRNA is affected by the processing controlled by a nuclear gene involved in fertility restoration (Iwabuchi et al. 1993). However, studies on RNA editing in other CMS plant models have not revealed any correlation between these two phenomena (Handa and Nakajumia 1992; Stahi et al. 1994; Lu et al. 1996).

The nuclear genome has been shown to affect the mitochondrial gene expression by altering the size, number, and/or abundance of transcripts (Moneger et al. 1994). In Petunia, a single nuclear locus determines transcript abundance and RNA editing of the *nad3* transcripts (Lu and Hanson 1994). In *Triticale*, the amplification of specific regions of the mitochondrial genome and the processing of the *atpα*/*atp9* transcripts are dependant on the rye nuclear genome (Kuck et al. 1993; Laser and Kuck 1995). In maize mitochondria, the developmental stage or growth conditions can affect RNA editing of the *nad3* transcripts (Grosskopf and Mulligan 1996).

In the study presented here, we have analysed the RNA editing of *atpα*, *atp6* and *atp9* transcripts encoding for subunits A, 6, and 9, respectively, of the ATP-synthase multiprotein complex from wheat mitochondria. In order to study the effect of the nuclear background on RNA editing, we compared the degree of RNA editing of these transcripts in alloplasmic and euplasmic wheat lines in various tissues. We present evidence that the nuclear background does affect the RNA editing rate since a higher level of partially edited transcripts were observed in an alloplasmic CMS line than in fertile lines.

Materials and methods

Nuclear and cytoplasm sources of wheat lines used

Plant material	Phenotype	Nuclear source	Cytoplasm source
T. aestivum (AABBDD) (cv Atir)	Fertile	*T. aestivum*	*T. aestivum*
T. timopheevi (AAGG) (01)	Fertile	*T. timopheevi*	*T. timopheevi*
Cytoplasmic male sterile (CMS) (17501)	Sterile	*T. aestivum*	*T. timopheevi*
Restored of fertility (RF) (17501/R122)	Fertile	*T. aestivum*	*T. timopheevi*

The *Triticum timopheevi* line used for mitochondria (mt)RNA extraction were provided by Prof. M. Feldman from the Department of Plant Genetics, Weizmann Institute of Science, Rehovot, Israel. The wheat CMS line 17501 and the restored to fertility lines hybrid line 17501/R122 were obtained from Pioneer Seed Company, Iowa, USA.

Isolation of nucleic acids

Mitochondria were isolated from embryos, roots, or coleoptiles as previously described (Schuster and Brennicke 1988), and mtRNA was treated with DNase RNase-free (Promega). The mtRNA for studies of RNA editing of *atpα* and *atp6* was extracted from 5-day-old etiolated coleoptiles. Anther total RNA was isolated by TRIzol reagent (BRL).

cDNA sequencing

RT-PCR (reverse transcriptase polymerase chain reaction) amplification of mtRNA was carried out for determination of the RNA sequence. Mitochondrial RNA was reverse-transcribed into a cDNA first strand with M-MLV reverse transcriptase (Promega) under standard conditions. The primers used for *atp6*, 5′ GCAGCTGTAGCCCCC 3′, located 27 nucleotides from the *atp6* genomic TGA stop codon (Bonen and Bird 1988); for *atpα*, 5′ GCGAGTAAGGGGCAACCTTTT 3′, 9 nucleotides from the *atpα* genomic TGA stop codon; and for *atp9*, 5′ GTAACTACATTCACACATTTC 3′, 10 nucleotides from the *atp9* genomic TGA stop codon (Begu et al. 1989). PCR amplification of the cDNA was carried out by *Taq* DNA polymerase (Promega) with the primer used in the first-strand cDNA synthesis and the following 5′ primers: for *atp6*, 5′ GGGCATATTAGAGGA 3′ located 34 nucleotides upstream of the *atp6* initiation codon (Bonen and Bird 1988); for *atpα*, 5′ GAATTCAAATCGAGATTGTGTG 3′ located 89 nucleotides upstream of the *atpα* initiation codon; and for *atp9*, 5′ AAAGTGTTTTCTCGACTCGA 3′ located 2 nucleotides upstream of the *atp9* initiation codon (Begu et al. 1989). After 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C the PCR products were analysed by electrophoresis on 1% agarose gel and extracted by the QIAEX extraction kit (QIAGEN). For cDNA sequencing the fragments were cloned into Bluescript KS^+ plasmid, and its nucleotide sequence determined by the dideoxy chain termination sequenase version 2.0 DNA polymerase (USB). In order to sequence *atpα* and

atp6 cDNAs we used the following oligonucleotides: for *atpα*, 5′ GAATGAGAATGTAGG 3′, located 207 bp from the start codon; 5′ TAATAGGGAACAG- AC 3′, located 506 bp from the start codon; 5′ TTATATATGATGATC 3′, located 806 bp from the start codon; and 5′ ACGTTGGCTTATCCG 3′, located 1106 bp from the start codon. The oligonucleotide 5′ CACTGTCGTTTTGGT 3′ located 138 bp from the start codon was used for the *atp6* sequence (Bonen and Bird 1988). The oligoucleotide that was used for sequencing *atp9* was 5′ AAAGTGTTTTCTCGACTCGA 3′, located 2 nucleotides upstream of the *atp9* initiation codon (Begu et al. 1989).

Results and discussion

atpα RNA editing

Fifty-one cDNA clones corresponding to the mitochondrial *atpα* mRNAs of *T. timopheevi* cytoplasm in three different nuclear backgrounds, *T. timopheevi*, *T. aestivum* – CMS, and *T. aestivum* restored to fertility line (RF), were sequenced (Table 1). Six C-to-U changes were observed when compared to the genomic sequence (Begu et al. 1989). Of the 6 modifications 4 created a leucine codon, 1 modification created a phenylalanine, and the remaining modification was silent (Table 1). In a previous communication, Laser and Kuck (1995) reported 2 additional editing sites (codon 393 and codon 1550) in the *T. timopheevi* cytoplasm. Although these codons were found to be pre-edited in our study this may be explained by a variation resulting from the plant mtDNA material used in the different studies. Our comparison with RNA editing of *atpα* transcripts in other plants revealed that the editing occurs at the same sites, except when the codons were pre-edited in the genomic sequence (Table 2) (Schuster and Brennicke 1991; Senda et al. 1993; Laser and Kuck 1995). The consequence of RNA editing is a change in the hydropathic profile of the putative protein (Fig. 1) and a better conservation between all the homologous subunits (Table 2).

Although the majority of the *atpα* cDNA clones (71% or 83%) were fully edited, 24% and 17% of the transcripts of the CMS and fertile cytoplasms, respectively, were partially edited, implying that *atpα* transcripts were present as an heterogeneous population in the fertile and cytoplasmic male-sterile wheat lines (Table 1). The number of partially edited clones in the RF and *T. timopheevi* fertile lines were 3 and 2, respectively. Five partially edited clones and 1 non-edited clone were found in the CMS line (Table 1). The degree of RNA editing was found to be identical between *T. aestivum* and *T. timopheevi* (data not shown).

As shown previously, in plant mitochondria, contrary to the situation in Trypanosomes the distribution of the edited sites was not polar. The codon at position 10 was preferentially not edited in all transcripts of the

Table 1 RNA editing rate (%) of *atpα* in *T. timopheevi*, CMS, and fertile wheat

Editing sites[a]						CMS		*T. timopheevi*		RF	
10 L>L	324 S>L	393 S>L	431 P>L	497 P>L	500 S>F	%	Number	%	Number	%	Number
+	+	+	+	+	+	71	(15)	83	(10)	83	(15)
−	+	+	+	+	+	9.5	(2)	8.5	(1)	5.6	(1)
−	+	+	+	+	−	5	(1)	0		0	
−	+	+	−	+	+	9.5	(2)	0		0	
−	+	+	−	−	−	0		0		5.6	(1)
−	+	−	−	−	−	0		0		5.6	(1)
+	+	−	+	−	−	0		8.5	(1)	0	
−	−	−	−	−	−	5	(1)	0		0	
Total clones						100	(21)	100	(12)	100	(18)
Total partial edited clones						24	(5)	17	(2)	17	(3)
Total unedited clones						5	(1)	0		0	

[a] Number indicate fully sequenced individual cDNAs clones (− , unedited; + , edited). They correspond to the amino acid positions at which editing occurred and are derived from Begu et al. (1989), they refer to the following nucleotide editing sites: +30, +971, +1178, +1292, +1490, +1499

Table 2 Comparison of codons edited in *atpα* in *T. timopheevi*, *T. aestivum*, *Triticale*, *Oenothera*, and sugar beet. The *Triticale* (Laser and Kuck 1995), *Oenothera* (Schuster and Brennicke 1991) and sugar beet (Senda et al. 1993) sequences were derived from cDNAs clones

Editing sites	10	13	324	393	431	472	497	500
T. timopheevi	L>L	L	S>L	S>L	P>L	L	P>L	S>F
T. aestivum	L>L	L	S>L	S>L	P>L	L	P>L	S>F
Triticale	L>L	L	S>L	S>L	P>L	L	P>L	S>F
Oenothera	L	L>L	S>L	L	L	P>L	P>L	F
Sugar beet	L	L	L	P>L	P>L	P>L	L	F

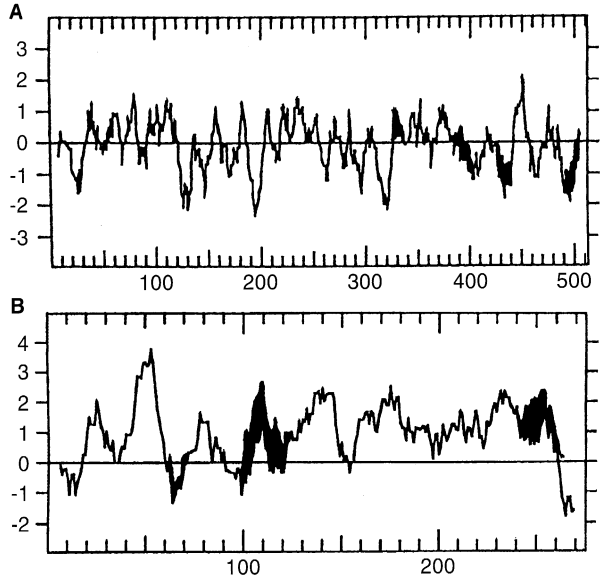

Fig. 1 Hydropathic profiles of *T. timopheevi atpα* (**A**) and *atp6* (**B**) proteins. Hydropathic profile of *atpα* and *atp6* protein according to Kyte and Doolittle (1982). The *middle line* indicates the limit between the hydrophobic (*upper side*) and hydrophilic domains (*lower side*). *Black areas* indicate the difference between edited (*upper side*) and unedited (*lower side*) proteins

CMS and fertile lines. Codon 324 was always edited, except in a completely non-edited clone isolated from the CMS cytoplasm (Table 1). The clustered sites 497 and were found to be both edited, except for 1 clone for which codon was not edited. No clear background effect on RNA editing was observed for the *atpα* transcripts, although a large number of partially edited cDNAs clones were present in the CMS cytoplasm.

atp6 RNA editing

In wheat mitochondria, RNA editing of *atp6* transcripts occurs by 12 C-to-U changes; this in contrast to 17 in sorghum (Kempken et al. 1991), 21 in *Oenothera* (Schuster and Brennicke 1991), 18 in maize (Kumar and Levings 1993), and 14 in Petunia (Lu and Hanson 1994). Comparison of the edited codons in different plants shows that the similarity of the primary structure of the putative ATP6 protein is improved by RNA editing between species (Table 3). Moreover, the hydrophobicity of these protein is also affected by the changes resulting from *atp6* mRNA editing (Fig. 1).

In wheat, 8 out of 12 changes occurred in the second nucleotide of the codon and 4 occurred in the first position, leading to 11 amino acid changes and the creation of a new stop codon similar to those of other plant *atp6* transcripts previously described. In *T. timopheevi*, the 12 clones sequenced were completely edited whereas 2 out of the 12 partially edited clones were detected in the CMS alloplasmic line (Table 4). The partially edited clones exhibited different non-edited codons except for codon 246, which was not edited in both clones. In addition, 2 clones among the fully edited transcripts in the CMS line carried an additional silent editing event at the third nucleotide of codons 103 and 116 (Fig. 2). The comparison of the degree of editing between *atp6* transcripts in the *T. timopheevi* euplasmic line and the CMS alloplasmic line suggests that the *T. aestivum* nuclear background may modulate the RNA editing efficiency.

atp9 RNA editing

The RNA editing process has been documented in several plants – wheat (Begu et al. 1990), sorghum (Salazar et al. 1991), barley (Yakar and Breiman 1992), maize (Grosskopf and Mulligan 1996). In *atp9* transcripts from wheat mitochondria, 8 codons are modified by RNA editing: 5 editing events lead to amino acids changes, 2 are silent, and 1 creates a new stop codon leading to a protein six amino acids shorter than the product predicted from the genomic sequence (Begu et al. 1990). In order to evaluate the effect of the nuclear background and tissue specificity on RNA editing, we have characterised the editing of *atp9* mRNAs extracted from embryos, shoots, roots, and anthers in *T. aestivum*, *T. timopheevi*, and the CMS line (Table 5). In *T. aestivum* and *T. timopheevi* 100% of the cDNA clones corresponding to *atp9* mRNAs extracted from embryos, roots, shoots, and anthers were found to be completely edited. In the CMS line, 100% of the cDNA clones of *atp9* mRNAs from roots, shoots, and anthers were fully edited, while only 81% of the cDNA clones isolated from wheat embryos were completely edited.

Other laboratories have reported that the editing level of the *atp9* mRNA analysed in undifferentiated maize callus cells, roots, and shoots were found to be always 100% (Grosskopf and Mulligan 1996). However, our extensive analysis of *atp9* editing in several tissues of maize and wheat (Table 5) indicated that a transcript that is usually found to be fully edited in a given tissue may become partially edited in a different nuclear background or tissue. The mechanism by which incompletely or completely edited transcripts accumulate may be related to an eventual link between the transcription kinetics and RNA editing: if the editing machinery is not capable of rapidly processing the newly synthesized transcripts, the accumulation of incompletely edited intermediates may occur. As the partially edited transcripts can produce altered proteins, a possible deleterious effect of such proteins in CMS lines cannot be ruled out.

RNA editing in chloroplasts revealed that although light-induced processes are not active determinants in eliciting the RNA editing process, the latter post-transcriptional step was down-regulated in seeds and roots,

Table 3 Comparison of codons edited in *atp6* in *T. timopheevi*, Sorghum, *Oenothera*, maize, and rice

Editing site	40[a]	43	49	66	83	85	101	103	105	111	114	116	
T. timopheevi	E	Y	L	S>L	L	L	F	R>C	S>L	S>L	R>C	P>L	
Sorghum	L	S>F	L>L	S>L	P>L	L	F	C	S>L	S>C	P>L	P>L	
Oenothera	P>S	F	L	S>L	P>L	P>L	F	R>C	S>L	S>L	R>C	P>L	
Petunia	F	P>S	L	S>L	L	L	S>F	R>C	S>L	S>L	C	P>L	
Maize	L	S>F	L>L	S>L	P>L	L	F	C	S>L	S>L	R>C	P>L	
Rice	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	
Editing site	161	181	182	189	203	239	246	249	251	254	255	263	267
T. timopheevi	L	S	Y	L	L	L	S>L	H>Y	S>F	S>L	I	T>I	Q>*[b]
Sorghum	L	S	H>Y	S>L	S>L	L	S>L	H>Y	S>F	S>L	I	T>I	Q>*
Oenothera	S>L	P>S	H>Y	S>L	S>L	P>L	S>L	Y	S>F	S>L	T>I	T>I	Q>*
Petunia	L	P>S	Y	S>L	S>L	L	S>L	H>Y	S>F	L	I	I	Q>*
Maize	L	S	H>Y	S>L	S>L	L	S>L	H>Y	S>F	S>L	T>I	T>I	Q>*
Rice	n.d.	n.d.	H>Y	S>L	S>L	L	L	H>Y	S>F	S>L	I	I	Q>*

The sorghum (Kempken et al. 1991), *Oenothera* (Schuster and Brennicke 1991), Petunia (Lu and Hanson 1994), maize (Kumar and Levings 1993) and rice (Iwabuchi et al. 1993) presented sequences are cDNAs clones (n.d. not detected)

[a] The numbers refer to the amino acid positions at which editing occurred, and the amino acid position is according to the *T. aestivum* genomic sequence (Bonen and Bird 1988)

[b] *, Stop codon

Table 4 RNA editing rate (%) of *atp6* in *T. timopheevi* and the wheat CMS line

Editing sites[a]												CMS		*T. timopheevi*	
66 S>L	103 R>C	105 S>L	111 S>L	114 R>C	116 P>L	246 S>L	249 H>Y	251 S>F	254 S>L	263 T>I	267 Q>*[b]	%	Number	%	Number
+	+	+	+	+	+	+	+	+	+	+	+	42	(5)	83	(10)
+	+	+	+	+	+ +	+	+	+	+	+	+	24	(3)	17	(2)
+	+ +	+	+	+	+ +	+	+	+	+	+	+	17	(2)	0	
+	+	+	+	−	+	−	+	+	+	+	+	8.5	(1)	0	
+	+	+	+	+	+	−	−	−	−	−	−	8.5	(1)	0	
Total clones												83	(10)	100	(12)
Total partial edited clones												17	(2)	0	(0)

[a] −, Unedited; +, edited; + +, additional silent editing site (as described in Fig. 2)

[b] *, Stop codon

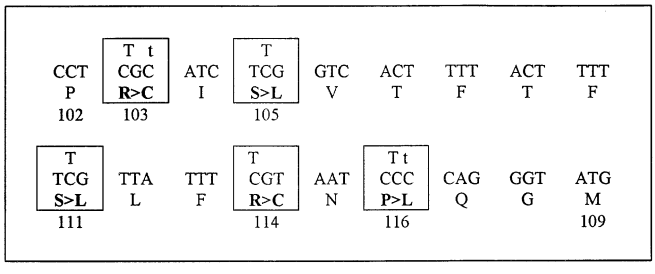

Fig. 2 Silent editing in *atp6* at position 103 and 116. Deduced amino acid sequence of the *atp6* cDNA clones at positions 102–119. Editing sites are *boxed* and amino acid changes are indicated by *boldface codons*. *Numbers* indicate amino acid position. The *top row of letters* indicate the nucleotide editing site (in *capitals*) and additional editing site (*lowercase*)

showing that RNA editing may be tissue and developmentally regulated (Bock et al. 1993; Hirose et al. 1996).

Since the *atp9* transcripts were fully edited in anthers, which is the target tissue of the CMS phenotype, results obtained by in vivo studies showed no direct correlation between partial editing of *atp9* and CMS in wheat. This conclusion is in agreement with studies showing full editing of the Brassica *orf224* and the sunflower *orf522*, which are associated with CMS, suggesting a lack of association between CMS and the RNA editing process.

In conclusion, our results strongly suggest that the nuclear background is correlated to the degree of editing, thus having a significant influence on the level of the partially or totally edited mitochondrial mRNAs of the *atpα*, *atp6*, and *atp9* transcripts isolated from a *T. timopheevi* cytoplasm wheat line. Even if the level of partially edited intermediates does not attain a value higher than 20–25%, we may assume that the nuclear effect is reflected by the abundance of the steady state level of partially edited mRNAs since their relative

Table 5 RNA editing rate (%) of *atp9* in *T. timopheevi*, *T. aestivum* , and the CMS line

Editing sites[a]								CMS				*T. tomopheevi*/*T. aestivum*[b]			
7 S>L	27 V>V	28 L>P	45 S>L	64 P>L	69 L>L	71 S>L	75 A>*[c]	Em %	R %	S %	An %	Em %	R %	S %	An %
+	+	+	+	+	+	+	+	81	100	100	100	100	100	100	100
−	−	+	+	+	+	+	+	4.7	0	0	0	0	0	0	0
+	−	+	+	+	+	+	+	14.3	0	0	0	0	0	0	0
Number of clones								21	16	14	19	16	13	15	13
Total partial edited clones								4	0	0	0	0	0	0	0

Em embryos · *R* roots · *S* shoots · *An* anthers
[a] −, Unedited; +, edited
[b] The editing rates and the number of clones analysed in *T. timopheevi* and *T. aestivum* tissues were identical
[c] *, Stop codon

amount may be enhanced by a tissue-specificity driven process whose mechanism remains elusive and which deserves to be studied in the future.

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