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Variability of Chloroplast DNA of Extranuclear Sunflower Mutants

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Abstract: A comparative analysis of full-genome sequences of chloroplast DNA (cpDNA) of the original inbred line 3629 and three extranuclear mutants, which were obtained by the method of mutagenesis induced with N-nitroso-N-methylurea (NMU) and characterized by different level of chlorophyll insufficiency (*en:chlorina*-7-yellow-green leaves; chlorophyll content (a + b)-67.8% with respect to the line 3629, *variegated*-10-leaves with white zones; chlorophyll content (a + b)-2.9% with respect to the line 3629 and *variegated*-13-leaves with yellow zones; chlorophyll content (a + b)-6.1% with respect to the line 3629), has been carried out. Single-parent maternal inheritance of chlorophyll defects was confirmed by analysis of progeny obtained from reciprocal crossbreedings between the original line 3629 and mutants. Chlorophyll mutants carried modified cpDNA unique for each mutant. We anticipate that chlorophyll defect of *en:chlorina*-7 may control the observed non-synonymous mutations (transitions) in the genes *rpoB*, *psaA* and *psbB*, which encode β-subunit of RNA-polymerase, the A1 apoprotein of chlorophyll *a* of the photosystem I, P700 and 47 kDa protein of the photosystem II respectively. In *variegated*-10, it may control mutations in the genes *rpoA* and *rpoC2*, which encode α and β" subunits of RNA-polymerase and in *variegated*-13-two mutations in the *ycf3* gene that encodes photosystem I assembly factor.

Keywords: Extranuclear Mutants, Reciprocal Crossbreeding, Chlorophyll Mutants, cpDNA, Sunflower

Introduction

Presently, a variety of DNA markers is used to assess plant gene polymorphisms (Wong *et al*., 2009; El-Awady *et al*., 2012; Usatov *et al*., 2014; Bhavsar *et al*., 2015). They are also effectively applicable in order to mark individual traits of plants (Poczai *et al*., 2013). However, analysis of full-genome sequences provides more accurate results.

It is common knowledge that mutants may be used as a suitable model for studying the "gene-trait" problem and extranuclear mutants are not excluded. However, their importance becomes even higher as biogenesis, functions of chloroplasts and mitochondria and their photosynthetic and respiratory activity are subjected to double nuclear-organelle regulation. Therefore, genetic analysis of these inheritable modifications allows us to reveal not only cytogene determined structural components of organelle, but also principles of nuclear-cytoplasmic relationships (Strand *et al*., 2003; Barajas-Lopez *et al*., 2013).

In the Southern Federal University, we obtained a series of chlorophyll mutations by mutagenesis of seeds of the inbred line 3629 induced with N-nitroso-N-methylurea (NMU) (Beletskii *et al*., 1969). The selected survivable mutants were referred to two phenotypic classes: Mutants with yellow-green leaves (*chlorina*) and poecilophyllous chimera with yellow and white zones on their leaves. Extranuclear nature of these mutations was confirmed by hybrid analysis. Extranuclear chlorophyll mutants are considered to be the appropriate model to study the role of chloroplast genome in chlorophyll biosynthesis and biogenesis of photosynthetic apparatus (Vezitskii *et al*., 1999;

Rassadina *et al*., 2001; Usatov *et al*., 2001; 2004; Rassadina *et al*., 2005).

Previously, it was attempted to map the mutations using the restriction analysis of cpDNA of the line 3629 and *en:chlorina* mutants (Triboush *et al*., 1999). In particular, it was shown that cpDNA of the *en:chlorina*-7 mutant contained an additional HindIII endonuclease restriction site as compared with the line 3629. However, this method appeared not to be sufficiently effective to locate the mutations. It became clear that more distinctive approaches are needed to locate mutations in cpDNA (Henry *et al*., 2014).

Threfore, the goal of our work was to study cpDNA variability in extranuclear chloroplast sunflower mutants with different level of chlorophyll defects, using the full-genome sequencing.

Materials and Methods

Plants

The objects of our study were plants of the original inbred line 3629 and three extranuclear mutant lines characterized by different level of chlorophyll insufficiency (*en:chlorina*-7-yellow-green leaves, *variegated*-10-leaves with white zones, *variegated*-13-leaves with yellow zones), which were obtained by mutagenesis induced with N-nitroso-N-methylurea (NMU).

Analysis of Hybrids

Analysis of hybrids was performed under the field conditions. The maternal plants were pollinated after sterilization and then, the plants were isolated. Plants were grown under the conditions of selection farm, in the 10 m plots of land with the area 40×60 cm.

The amounts of chlorophyll and carotinoids were assessed by the absorption spectra of 85% acetone extracts of leaves collected at the budding stage (Shlyk, 1971).

To obtain the full-genome sequences of cpDNA total DNA was isolated from green leaves of the original line 3629, yellow-green leaves of the *en:chlorina*-7 mutant and from white and yellow zones of leaves of the *variegated*-10 and *variegated*-13 mutants respectively. Total DNA was isolated by the modified CTAB method (Doyle and Doyle, 1990). Polyvinylpyrrolidone 40000 and sodium metabisulfite were added to the extraction buffer; for removing RNA, the DNA fractions obtained were incubated for 1 h at 37°C in the presence of RNase A.

Sequencing

Full-genome sequencing of cpDNA was performed in the HiSeq 2000 sequenator ("Illumina", USA) with the length of reading 100+100. The obtained sequences were mapped onto the chloroplast genome of sunflower, line HA383 [GenBank NC_007977 (Timme *et al*., 2007)]. The results were analyzed with the program CLC Genomics Workbenchv. 6.0.4. The obtained sequences were aligned with the program BioEdit 7.0.9.0. Synonymous and non-synonymous mutations were identified with the program ExPASy (http://web.expasy.org/translate/).

To perform the direct sequencing, the DNA amplicons were obtained and purified (Werle *et al*., 1994). Sequencing of amplicons was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) and ABI Prism 3130xl Genetic Analyser (Applied Biosystems, USA). In each line 11-15 plants were studied.

Results

The results of reciprocal crossbreedings of the chlorophyll mutants *variegated*-10, *variegated*-13 and *en:chlorina*-7 with plants of the original line 3629 are shown in Table 1 and 2. If mutant poecilophyllous plants were pollinated with pollen of green plants from the line 3629, their progeny was split in F1 into three types of seedlings: Green and variegated white and yellow (Table 1). If *en:chlorina*-7 mutant plants were pollinated, only mutant phenotype was inherited (Table 2). In further generations the self-pollinated poecilophyllous mutants split with nearly the same ration as in F1, while the *chlorina* phenotype remained unchanged. The reciprocal crossbreeding, i.e., when green plants of the line 3629 were used as maternal line and the mutant form was the paternal one, produced totally green progeny that did not split in further generations. Progeny of green plants produced by poecilophyllous mutants also remained unchanged.

Table 1. Reciprocal crossbreedings of the *variegated* -10 and *variegated* -13 mutants with green plants of the original line 3629

	F_1 plant phenotype			
Crossbreeding	Green	*Variegated*	White (yellow)	Number of mutants
Variegated -10×3629	688	373	188	44,9
3629×*variegated*-10	763	0	0	0
Variegated-13×3629	1329	598	345	41,5
3629×*variegated* -13	602	0	0	0

Table 2. Reciprocal crossbreedings of the *en:chlorina*-7 mutant with green plants of the original line 3629

	F_1 plant phenotype		F_2 plant phenotype	
Crossbreeding	Green	*Chlorina*	Green	*Chlorina*
en:chlorina-7×3629	0	44	0	412
3629×*en:chlorina*-7	41	0	786	0

Table 3. The level of chlorophylls (a + b) and carotinoids in the leaf tissue of sunflower mutants

		Chlorophyll (a+b)		Carotinoids	
Line	Tissue phenotype	mg/g of dry weight	% from the control	mg/g of dry weight	% from the control
3629	Green	8,53±0,50	100,0	2,17±0,70	100,0
en:chlorina-7	Yellow-green	5,78±0,16	67,8	1,81±0,50	82,9
variegated -10	White	0,25±0,11	2,9	0,17±0,03	7,8
variegated -13	Yellow	0,52±0,75	6,1	0,66±0,2	30,4

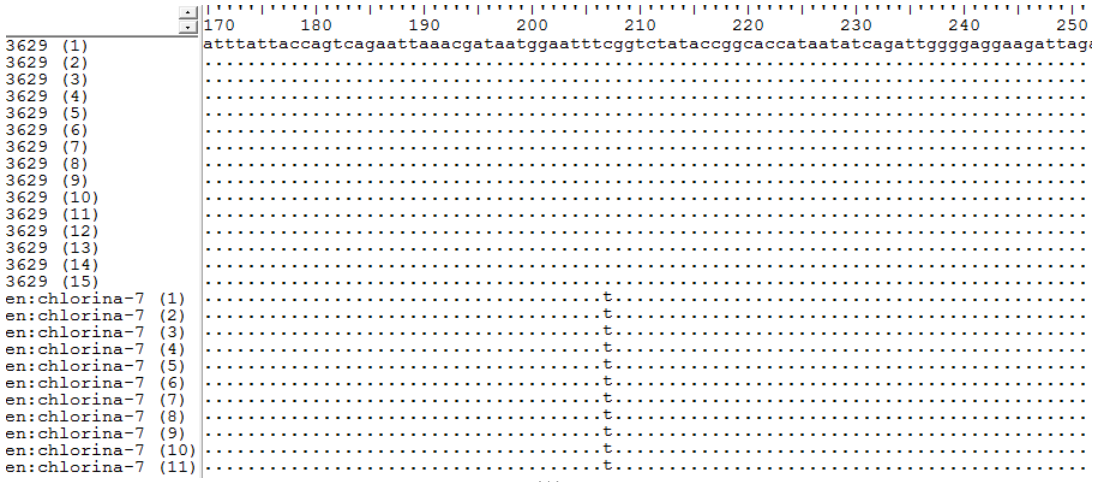
Table 4. Polymorphous sites of cpDNA of extranucleus sunflower mutants as compared with the original line 3629

Reference position	*en:chlorina*-7	*Var*-10	*Var*-13	Line 3629	Localization
5450	$(C)_9$	$(C)_{11}$	$(C)_{11}$	$(C)_9$	*rps16*, intron
10773	G	A	G	G	*ycf6-psbM*
13467	T	C	C	C	*rpoB* (Ser138Leu) (RNA polymerase β-subunit)
21493	C	T	C	C	*rpoC2* (Leu768Phe) (RNA polymerase β"-subunit)
28373	$(T)_{16}$	$(T)_{15}$	$(T)_{15}$	$(T)_{16}$	*atpF – atpA*
39945	A	G	G	G	*psaA* (Thr528Ile) (photosystem I P700 chlorophyll a apoprotein A1)
40855	G	A	G	G	*psaA* (photosystem I P700 chlorophyll a apoprotein A1)
43245	C	C	T	C	*ycf3* (Ala91Thr) (photosystem I assembly protein Ycf3)
43323	C	C	T	C	*ycf3* (Glu65Lys) (photosystem I assembly protein Ycf3)
63057-58	--	TA	TA	TA	*petA – psbJ*
69191	T	C	C	C	*rps12-clpP*
72247	T	C	C	C	*psbB* (His157Tyr) (photosystem II 47 kDa protein)
77386	A	G	G	G	*rpoA* (RNA polymerase α-subunit)
77663	G	A	G	G	*rpoA* (Thr203Ile) (RNA polymerase α-subunit)
78641	T	C	C	C	*rps11* (30S ribosomal protein S11)
81579	C	T	C	C	*rpl16-rps3*
110809	A	G	G	G	*ycf1* (hypotheticalchloroplast RF1)
117852	C	T	C	C	*ndhG* (NADH dehydrogenasesubunit 6)
122552	C	C	T	C	*trnL*-UAG-*rpl32*
123613	C	T	C	C	*rpl32-ndhF*

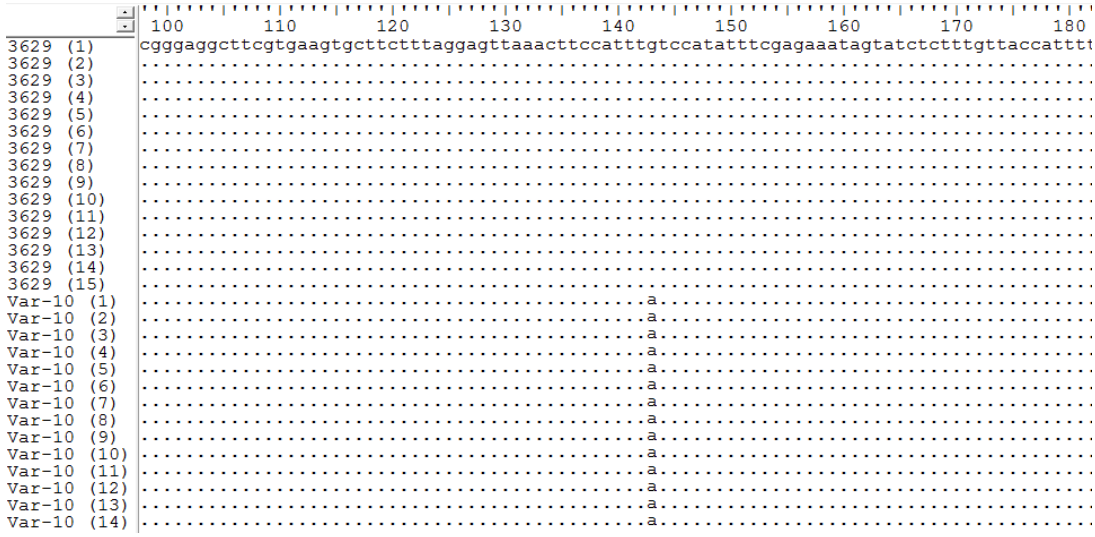
We performed a comparative analysis of chlorophyll and carotinoid level, because mutant plants were phenotypically different, especially by leaf color that differed from white to yellow green, from the original green plants (Table 3). It was shown that yellow-green leaves of the *en:chlorina*-7 mutant contained 5.78 mg g^{-1} of the dry weight of chlorophylls *a* and *b*. Total concentration of green pigments in the white leaf tissue of the *variegated*-10 mutant was 0.25 mg g^{-1} of dry weight. Yellow tissue of the *variegated*-3 mutant contained 0.52 mg chlorophyll per 1 g of dry weight. The total level of chlorophylls in the original green plants of the line 3629 was 8.53 mg g^{-1} of dry weight. The level of carotinoids in mutant plants was also lower than in the original green plants (Table 3).

The analysis of cpDNA structure of the line 3629 and the *en:chlorina*-7 revealed the following differences: One deletion (TA) in the intergene region (petA-psbJ) and seven single nucleotide polymorphisms, three of which were located in the genes *rpoA, rps11 u ycf1* (synonymous), one in the intergene region rps12-clpP (synonymous) and the other in the genes *rpoB*, *psaA*, *psbB* (non-synonymous). The latter were represented by the transition (C/T) in the gene, encoding β-subunit of RNA-polymerase (Ser138Leu), in which the reactive center located and G/A substitutions in the gene, encoding the A1 apoprotein of chlorophyll *a* (Thr528Ile), which is involved into the photosystem I and C/T substitution in the gene, encoding the 47 kDa protein (His157Tyr) involved into the photosystem II (Table 4).

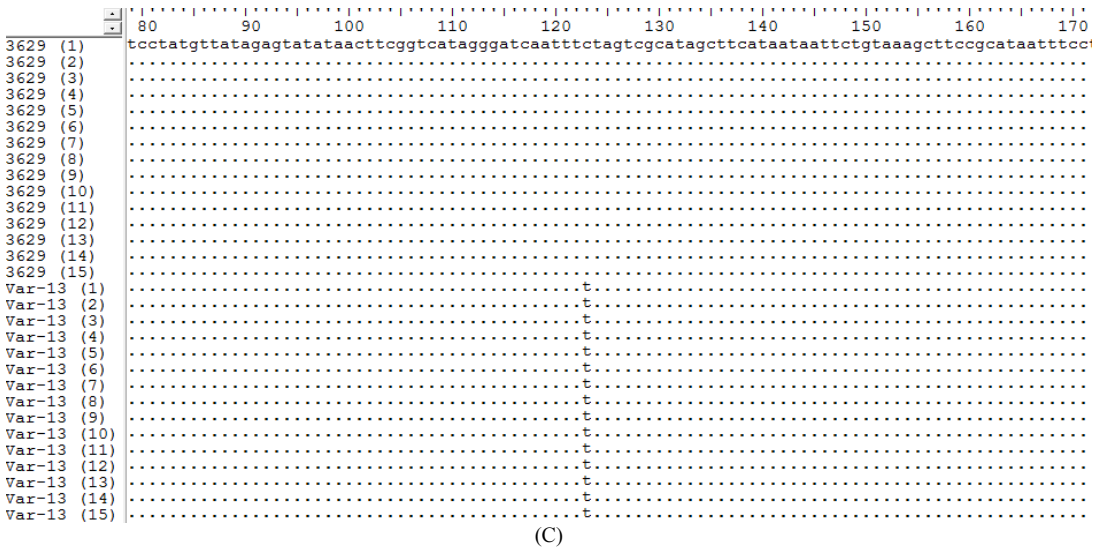
Structure of cpDNA of the *variegated*-10 mutant also differed from that of the line 3629 by two microsatellite loci, $(C)_{11}$ and $(T)_{15}$ and by seven SNPs. Two of the SNPs were represented by non-synonymous substitutions: G for A (*rpoA* (*Thr203Ile*)) and C for T (*rpoC2* (*Leu768Phe*)), which were located in the genes, encoding α and β" subunits of RNA-polymerase respectively (Table 4).



(A)



(B)



(C)

Fig. 1. Multiple alignment of sunflower chloroplast loci: A-*rpoB* fragment, B-*rpoA* fragment, C-*ycf3* fragment. Thus, Sanger sequencing data, confirm the results of NGS

These subunits represent a functionally important part of chloroplast RNA-polymerase, which participates in cpDNA transcription (Kremnev and Strand, 2014; Börner *et al*., 2015; Pfannschmidt *et al*., 2015). The other five SNPs, apparently, do not lead to modifications in the translation products, being either synonymous substitutions in the *psaA* and *ndhG* genes or located in the intergene regions of chloroplast genome (*ycf6-psbM*, *rpl16-rps3* and *rpl32-ndhF*).

We identified two polymorphous microsatellite loci, $(C)_{11}$ и $(T)_{15}$, in cpDNA of the *variegated*-13, which were similar to those of the *variegated*-10 mutant and three unique SNPs. One of them is located in the non-coding region of chloroplast genome (intergene region *trnL-UAG-rpl32*), while two non-synonymous SNPs-Ala91Thr and Glu65Lys are located in the *ycf3*, which encodes the photosystem I assembly factor. Ycf3 is known to work as a chaperone, which specifically interacts with PsaA and PsaD subunits during assembly of the photosystem I complex (Naver *et al*., 2001; Landau *et al*., 2009).

To confirm these results, we performed Sanger sequencing of chloroplast polymorphic loci, which had nonsynonymous substitutions (*rpoB, rpoC 2, psaA, ycf3, psbB, rpoA).* For Sanger sequencing we used 11-15 plants per line. As an example, Fig. 1 shows multiple alignments of *rpoB, rpoA, ycf3* sequences with variant sites in mutant lines-*en:chlorina*-7, *variegated*-10 and *variegated*-13, respectively.

Discussion

Former electron microscopic analysis of cell and organelle of mutant leaves revealed modifications of chloroplast ultrastructure that led to chlorophyll defects and, subsequently, to the decrease in photosynthetic activity (Usatov *et al*., 2004; Rassadina *et al*., 2005). It was shown that poecilophyllous mutants lacked thylakoid system and thus, suffered chlorophyll insufficiency. They often contained osmiophilic granule represented by non-structural aggregates of protein-lipid components of intracellular membranes. Lack of pigments well correlated with breaking of plastid structure of survivable mutants *en:chlorina*: Some of lamellae were vacuolated, general disorganization of the lamella structure was observed and thylakoid granae were poorly developed as compared with chloroplast of the line 3629.

It was also shown that the first traits of chlorophyll deficiency of the *variegated*-10 mutant are synchronic decrease in the synthesis of chlorophyll precursor, 5-aminolevulinic acid, lower ratio of *a* and *b* chlorophylls in comparison with the line 3629 and reduction in the photosystem II chlorophyll fluorescence (Usatov *et al*., 2004). The observed defects increased as mutants developed and eventually led to gradual destruction of the photosystem II and light-consuming complexes. The trait of chlorophyll deficiency of the *variegated*-10 was unstable and could either occur or disappear, depending on the plant growing conditions, such as temperature and light regimes. However, the dependence of the chlorophyll deficiency trait on light and temperature conditions was observed at early stages of pigment containing tissue development only. At this stage, low illumination not only did not induce chlorophyll insufficiency, but rather prevented destructive processes, when the mutant was transferred to the conditions, which induced most sever pigment abnormality. Such manifestation of the mutant trait may be due to defects of plastogene expression that control synthesis of pigment and protein components of the photosynthetic apparatus, nevertheless our study showed that mutations were located in structural genes of cpDNA.

Conclusion

A comparative analysis of the full-size sequences of cpDNA of the original inbred line 3629 and three extranuclear NMU-induced mutants characterized by different level of chlorophyll insufficiency (*en:chlorina*-7-yellow-green leaves, *variegated*-10-leaves with white zones and *variegated*-13-leaves with yellow zones) has been carried out. Each of the chlorophyll mutants carried uniquely modified cpDNA. We suggest that chlorophyll defect of *en:chlorina*-7 may control non-synonymous transitions in the *rpoB*, *psaA*, *psbB* genes, which encode β-subunit of RNA-polymerase, A1 apoprotein of chlorophyll *a* of the photosystem I P700 and the 47 kDa protein of the photosystem II respectively. In the *variegated*-10 mutant it may control mutations in the *rpoA* and *rpoC2* genes, which encode α and β" subunits of RNA-polymerase and in *variegated*-13, -two mutations in the *ycf3* gene, which encodes the photosystem I assembly factor.

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Author's Contributions

All authors equally contributed in this work.

N.V. Markin and A.V. Usatov: Designed and performed experiments and wrote the paper.

M.D. Logacheva, V.N. Vasilenko, A.I. Klimenko and N.S. Kolokolova: Designed and performed experiments.

M.Yu. Bibov and L.V. Getmantseva: Developed analytical tools and analysed data.

Ethics

This article is original and contains unpublished materials. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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