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The Investigation of Organelle Genomes of Extra Nuclear Sunflower Mutants with Variegated Phenotype

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Abstract: The comparative analysis of complete chloroplast and mitochondrial DNA sequences of the original inbred line 3629 and three extra nuclear mutant lines *variegated-1, variegated-15, variegated-17*, obtained by the N-nitroso-N-methylurea induced mutagenesis, was done. The studied mutant lines were presented two different phenotypes with diverse chlorophyll content: Pale/green mutant line *variegated-17* with 1.8% relative (to 3629 line) chlorophyll content in mutant leaf tissue and yellow/green lines *variegated-1, variegated-15* with 6.3% and 4.3% relative chlorophyll content, respectively. Each line had unique variation sites in chloroplast DNA. Among chloroplast SNP there were determined 7 nonsynonymous substitutions, which were located in *psaA* (*variegated-1), petD*, *rpl36*, *ccsA* (*variegated-15*), *rps4, rpoA, rpoC1* (*variegated-17*). The SNP of *variegated-17* line has resulted in the frameshift (*rpoC1*) and premature stop codon formations (*rpoA, rpoC1*). The whole genome sequencing of mitochondrial DNA mutant lines revealed no differences as compared with original line 3629. Summarizing current data and our previous research of variegated mutants we assume that pale/green variegated phenotype is associated with significant disturbance of RNA polymerase subunits genes, and the lesions in photosynthetic genes lead to yellow/green mutant phenotype.

Keywords: *Variegated* Mutants, Extra Nuclear Mutants, Chlorophyll Deficiency, Whole Genome Sequencing, Chloroplast DNA, Mitochondrial DNA, Sunflower, N-Nitroso-N-Methylurea

Introduction

Chloroplast mutants are a convenient model for studying the mechanisms of chloroplast biogenesis and photosynthetic apparatus functioning (Greiner, 2012). But the usage of mutant analysis may be quite limited, either because gene-of-interest mutations could be lethal (e.g., albinos) or because mutations lead to faintly discernible phenotype, as the defect may be buffered by a compensating activity (Yu *et al*., 2007). In its turn, plants with mosaic mutant phenotype, such as variegated, are more excellent models for research the function of the gene product by studying its mode of action in both tissue types – normal and mutant (Yu *et al*., 2007).

The variegated phenotype can be caused by mutations in nuclear, plastid and mitochondrial genes, as well as due to the transposition of mobile genetic elements, suppression of gene expression by RNA interference or incompatibility between nuclear and cytoplasmic genomes in inter specific hybrids (Yu *et al*., 2007). And the variegated mutants with extra nuclear genetic origin are of particular interest because the plastid or mitochondrial DNA mutations could not directly effect on chlorophyll biosynthesis and the reduction of pigments occurs by the involvement of retrograde signaling pathways (Makarenko *et al*., 2016a). Thus the variegated mutants with extra nuclear genetic origin are also the appropriate models for investigating such fundamental problems in biology as nuclear-cytoplasmic relationships. And the Whole Genome Sequencing (WGS) of chloroplast and mitochondrial DNA makes it possible the accurate identification of

mutational changes, which in turn helps to establish the contribution of nuclear, chloroplast and mitochondrial genome in one or another feature (Barajas-López *et al*., 2013).

In the years 1967-1977 in the Southern Federal University, there have been obtained a series of chlorophyll-deficient mutant lines of sunflower with extra nuclear genetic origin. The mutant plants were gained by N-nitroso-N-methylurea (NMU) induced mutagenesis of single inbred line (№ 3629) seeds (Beletskii *et al*., 1969; Beletsky and Razoriteleva, 1978). The most numerous mutant forms had a variegated phenotype. Variegated mutant lines were classified into two chimera types - pale/green and yellow/green (Fig. 1). Previously, using WGS sequencing, we have investigated the variability of chloroplast DNA (cpDNA) in extra nuclear chlorophyll mutants of sunflower with various types of chlorophyll deficiency (Markin *et al*., 2016). However, we studied complete chloroplast genome sequences of only two variegated mutant lines *(variegated-10* and *variegated-13)*. In this study, we investigated the complete chloroplast and mitochondrial DNA (mtDNA) sequence of three more variegated lines: *Variegated-1*, *variegated-15* and *variegated-17*. It allowed us to conduct more comprehensive comparative analysis of the extra nuclear DNA variability in mutants induced by NMU, as well as make assumptions about genetic nature of different types of variegation phenotype (pale/green, yellow/green).

Materials and Methods

The Objects of Study

The study was carried out on the sunflower (Helianthus annuus) inbred lines from the genetic collection of the Southern Federal University: Original line 3629 and three extra nuclear mutant lines *variegated-1*, *variegated-15*, *variegated-17*, which were obtained by NMU-induced mutagenesis from original line 3629. A technique of the inducible mutagenesis has been described earlier (Usatov *et al*., 2004). The variegated mutant lines were characterized with different color chimeric phenotypes: yellow/green - *variegated*-1*, variegated-15* and pale/green - *variegated-17* (Fig. 1).

Analysis of Progeny Chlorophyll Content Measurements

The hybridologic analysis was performed under the field conditions similar to past research (Markin *et al*., 2016). The chlorophyll content in the green and mutant tissues was evaluated by the absorption spectra of 85% acetone extracts of leaves collected at the budding phase (Shlyk, 1971).

Fig. 1: The examples of two different variegated phenotype of sunflower from Southern Federal University collection - pale/green (*variegated-17*) and yellow/green (*variegated*-1)

DNA Extraction WGS Sequencing and Data Analysis

DNA extraction was performed with PhytoSorb kit (Syntol, Russia) according to the manufacturer's instruction, from preliminarily isolated chloroplast fraction (Makarenko *et al*., 2016b) from leaf tissues - mutant (pale or yellow) and normal (green). NGS libraries preparations, their quantity and quality estimates were made as has been described earlier (Makarenko *et al*., 2016b), the exception is that in this study for libraries preparations we used the pool of DNA from 6 plants, mixed in equal concentrations. Libraries were sequenced with NextSeq 500 sequencer using High Output v2 kit (Illumina, USA). For each sample, the 1-2,1 mln of 150-bp paired reads were generated. The quality control and trimming of reads were performed with Fast QC and Trimmomatic software (Bolger *et al*., 2014). Using Bowtie2 tool (Langmead and Salzberg, 2012) reads were aligned to reference sequences - chloroplast and mitochondrial genomes of 3629 line, which were obtained earlier (Markin *et al*., 2015; Makarenko *et al*., 2016b). Variant calling was made by samtools/bcftools software (Li, 2011) and manually revised using IGV tool (Thorvaldsdóttir *et al*., 2013). Synonymous and non-synonymous mutations, as well as frameshifts, were identified with the program ExPASy (http://web.expasy.org/translate/).

Results

The results of cross-breeding events of the chlorophyll mutants *variegated-1*, *variegated-15*, *variegated-17* with plants of the original line 3629 are presented in Table 1. If mutant plants were pollinated with pollen from normal plants (line 3629), the F1 progeny splitted into three phenotype types: Green, variegated, totally pale or yellow. Totally pale (albino) or yellow plants are unviable and usually die to reach 1-2th leaf stage. In further generations of self-pollinated variegated plants, the progeny splitting pattern was almost the same as in F1. In the result of reciprocal cross-breeding tests, only green plants were produced in progeny, and normal phenotype remained unchanged in further generations. The results of chlorophyll content measurements are presented in Table 2. The concentration of green pigments (chlorophylls a+b) in pale leaf sectors was 0.15±0.05 mg/g of dry weight for *variegated-17* mutant line, while yellow-green mutants had chlorophyll content 2.5 fold (*variegated-15*) and 3.6 fold (*variegated-1*) higher in yellow leaf sectors (Table 2).

The obtained NGS data allowed us to get complete sequences of both chloroplast and mitochondrial genomes for sunflower mutant lines. The overall alignment rate for both genomes was diverse among samples (15-50% of total read number) and the average read coverage was within 50-300. These data were sufficient for a qualitative variant calling. The sequencing of green leaf sectors of variegated mutants revealed no significant variants as compared with original line 3629 (minor differences in some SSR locus), so the subsequent results concern only mutant phenotype (pale or yellow leaf sectors). Comparative analysis of mitochondrial genomes revealed no differences among all mutant lines and original line 3629, while the variations in chloroplast genomes were detected and presented in Table 3.

The comparison of variegated-1 mutant line with 3629 line revealed only single variant site. The SNP in psaA gene, coding core subunit of photosystem I reaction center, results in nonsynonymous substitution Gly734Glu (Table 3).

In chloroplast genome of *variegated-15* line, there were detected 4 SNP. Among them 1 SNP was located in Inter Genic Region (IGR) rps8-rpl14, the others 3 SNP affected coding sequences. The nonsynonymous substitutions were detected in subunit IV of cyt b6f (*Pro*72*Leu*), ribosomal protein L36 (*Arg*35*Lys*) and cytochrome c biogenesis protein (*Glu*259*Lys)* (Table 3).

The largest number of variant sites was detected in *variegated-17* cpDNA - 8 SNP. 2 out of 8 SNP were located in intergenic regions *rpl16-rps3* and *ndhI-ndhG*. 2 SNP localized in *psbI u rpoB* were synonymous mutations, and 2 nonsynonymous mutations were located in *rps4* gene. One more SNP was also nonsynonymous and resulted in the emergence of termination codon in 672 amino acid position (Trp672Ter) of RNA polymerase β'-subunit, which in turn shortens 18 amino acids on C-terminus of protein. However, the last most severe identified mutation was the last SNP - deletion in coding sequences of rpoA gene, leading to frameshift of RNA polymerase α-subunit at 265 amino acid position. The frameshift causes 266 aa polypeptide, instead of normal 335 aa.

Table 1: The results of cross-breeding events of the variegated mutant plants with green plants of the original line 3629

	F_1 plant phenotype			
Crosses	green	*variegated*	pale (yellow)	Number of mutants, %
Variegated-1 × 3629	688	283	278	44,9
3629 × *variegated*-1	763	0	0	0,0
Variegated-15 × 3629	327	118	127	42,8
3629 × *variegated-15*	264	0	0	0,0
Variegated-17 × 3629	1078	181	151	23,5
3629 × *variegated-17*	582	0	0	0,0

Table 2: The chlorophyll (a+b) content in the leaf tissue of sunflower mutants

		Chlorophyll (a+b)	
Line	Tissue phenotype	mg/g of dry weight	% from the control
3629	green	8,53±0,50	100,0
variegated -1	green/yellow	8,47±0,57/0,54±0,14	99,3/6,3
variegated -15	green/yellow	8,34±0,59/0,37±0,10	97,8/4,3
variegated -17	green/pale	8,22±0,65/0,15±0,05	96,4/1,8

Table 3: Polymorphic sites of chloroplast genomes of mutant lines *variegated-1, variegated-15, variegated-17* as compared with original line 3629

Reference position	Line 3629	*Var*-1	*Var*-15	*Var*-17	Localization
8396	C	C	C	T	*psbI* (photosystem II protein I)
13105	G	G	G	A	*rpoB* (RNA polymerase β subunit)
19031	G	G	G	A	*rpoC1 (Trp672Ter)* (RNA polymerase β'-subunit)
39327	C	T	C	C	*psaA* (Gly734Glu) (photosystem I P700 chlorophyll a apoprotein A1)
45975	C	C	C	T	*rps4* (*Gly29Arg*) (ribosomal protein S4)
45980	C	C	C	T	*rps4* (*Arg27Lys*) (ribosomal protein S4)
76799	C	C	T	C	*petD* (*Pro*72*Leu*) (cytochrome b6/f complex subunit IV)
77478	T	T	T	-	*rpoA* frameshift (RNA polymerase α-subunit)
78894	C	C	T	C	*rpl36* (*Arg*35*Lys*) (50S ribosomal protein L36)
80047	C	C	T	C	*rps8-rpl14*
81882	C	C	C	T	*rpl16-rps3*
117513	G	G	G	A	*ndhI-ndhG*
121402	C	C	T	C	*ccsA* (*Glu*259*Lys*) (cytochrome c biogenesis protein)

Discussion

The obtained data indicate that the NMU induces a diverse of mutations leading to variegated phenotype. Summarizing the data from this and previous studies (Markin *et al*., 2016) the interesting patterns could be observed. Considering variant sites of 2 pale/green variegated mutants (*variegated-10*, *variegated-17*) it can be noticed that the both have nonsynonymous mutation in genes coding the subunits of plastid polymerase *rpoC1* (*variegated-17*), rpoC2 (*variegated-10*) and *rpoA* (both mutant lines). At the same time, if we compare mutations in *rpoB* gene, then, on the one hand the *variegated-17* had more serious impact of mutation resulting in a frame shift with the significant reduction of polypeptide chain. From the other hand such frame shift influence only C-terminal domain, which is involved in interaction with transcriptional regulators and with upstream promoter elements, while the *variegated-10* mutation (*Thr203Ile*) affect an essential N-terminal domain, which is crucial for the plastid polymerase assembly and basal transcription function (Kimura *et al*., 1994; Kimura and Lshihama, 2000). In tobacco plants the targeted disruption of the plastid RNA polymerase genes *rpoA*, *rpoC1* has also lead to pale\green variegated phenotype (Santis-Maciossek *et al*., 1999). Noticeable that some non-photosynthetic higher plant parasites from Lathraea genius have impaired rpoA and rpoC2 genes due to multiple frameshifts and premature stop codon formations (Samigullin *et al*., 2016).

While comparing the nonsynonymous mutation of 3 yellow/green variegated mutants (*variegated-1*, *variegated-13, variegated-15*), the assumption could be made that such variegated phenotype results in disturbance of photosynthesis genes. However, the identified mutations affected different genes *psaA* (*variegated-1), petD (variegated-13)* and *ycf3 (variegated-15)* all these genes are involved in the photosynthetic activity and are essential for normal functioning photosystem I (*psaA, ycf3)* and cytochrome b6/f complex (*petD*) (Bock, 2007). Although there is a lack of extra nuclear mutant investigations, some data are confirming our assumptions about genetic causes of pale and yellow variegated phenotype. For instance, the yellowish phenotype in plastome mutants of different plant species (*Antirrhinum majus, Oenothera suaveolens*) with mutations in photosynthesis genes – *psaB*, *psbD*, *psbE*, as well as periodically pale phenotype of *Cryptomeria japonica* was associated with impairing of mRNA maturation function by premature stop codon in *matK* (Greiner, 2012).

It is also important to highlight, that we found no variant sites in mitochondrial genomes of *variegated-1, variegated-15, variegated-17* lines as compared with original line 3629. This fact shows the selective mutagenic effect of 0.01% NMU on chloroplast DNA of sunflower plastids (Beletskii *et al*., 1969; Usatov *et al*., 2004). At the same time, the absence of HMM-induced mutations in mtDNA point on conservatism of mtDNA, as among genes of all three plant genomes the mitochondrial ones have the slowest rate of evolution, and induced mutations in mtDNA are often lethal (Liu *et al*., 2011; Makarenko *et al*., 2016b).

Conclusion

The carried out comparative analysis of organelle genomes of sunflower variegated mutant lines with extra nuclear genetic origin - *variegated-1, variegated-15, variegated-17* with the original line 3629 revealed 13 variant sites in their chloroplast genomes, but no differences in their mitochondrial genomes. Each mutant line had unique variation sites in chloroplast DNA. Among chloroplast SNP there were determined 7 nonsynonymous substitutions, which were located in *psaA* (*variegated-1), petD*, *rpl36*, *ccsA* (*variegated-15*), *rps4, rpoA, rpoC1* (*variegated-17*). The SNP of *variegated-17* line has resulted in frameshift (rpoC1) and premature stop codon formations (*rpoA, rpoC1*). Summarizing the data from two studies, we assume that pale/green variegated phenotype is associated with significant disturbance of RNA polymerase subunits genes, and the lesions in photosynthetic genes lead to yellow/green mutant phenotype.

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Author's Contributions

All the eight authors participated in the laboratory study, data analysis and the entire process of the article preparation.

Ethics

The authors state that this article conforms to the ethical standards specified by the American Journal of Biochemistry and Biotechnology.

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