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Mitochondrial DNA amplification in albino plants of rye (*Secale cereale* L.) regenerated in vitro

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ABSTRACT

Somaclonal variation has been intensively studied by using various molecular markers, however, very little information has been published concerning the nature of the changes and the mutational events involved in the appearance of the marker pattern modifications. Albino plants appeared with a high frequency among the regenerated plants of rye. When RAPDs were performed using the OpF13 primer, all the albino plants presented a very conspicuous band, the ScF13c amplicon, which was very faint or imperceptible in the green plants. We investigated the reasons for the differential amplification of this sequence. IPCR and Southern data indicated that the ScF13c sequence is located in the mitochondrial DNA. The appearance of this band seemed to be related with the amplification of the mitochondrial genome in the albino plants, as the real time PCR data proved. We also observed an increase in the number of mitochondria per cell in these plants that could explain the rise in the copy number of the mitochondrial sequences studied. We observed a very high rate of mutation in a nuclear locus because the same recessive mutation involved in albinism appeared independently in three different cell lines, as the progeny study revealed. This very high rate of mutation in a nuclear locus has been rarely reported. The albino plants of these progenies also showed an increase in the mitochondrial DNA, and in the number of mitochondria.

1. Introduction

It is known that different stresses can exert a mutation pressure over the genome and induce changes through different mechanisms. Plant tissue culture has been indicated to be one of these stresses and the genetic variability generated is known as somaclonal variation. The rate of somaclonal variation can differ between species but in some the mutation frequency is quite high, as we have reported in rye [1]. Somaclonal variation, considered as a mutation inductive process, shows particular features which have been described as oddities [2]. For instance, in many cases, the mutation spectrum is not at random and high frequencies of specific alterations have been noticed. We have reported a high frequency of albinos which appeared among rye regenerated plants, this frequency being especially high in the case of the Ailes cultivar [3].

Somaclonal variation has been intensively studied by using various molecular markers. However, very little information has been published concerning the nature of the changes and the mutational events involved in the appearance of the marker patterns modifications. We have already performed several RAPD analyses to assess the variation among rye regenerated plants, and we observed several modifications of the amplification patterns. Some of these variable bands were modified in plants with different origins, so the same change occurred several times. We call these hypervariable bands, and we assume that they represent hot spot of mutation of the rye genome [4]. We studied some of these hypervariable amplicons as well as the flanking sequences in order to know which sequences were involved in the observed variation, and to have some indication of the mechanisms implicated in the appearance of the modified bands [5].

The aim of this work is the study of one of these amplicons, the so called ScF13c which appeared as a very patent band in all the albino regenerated plants. The analysis of the ScF13c flanking sequences indicates that this sequence is located in the mitochondrial DNA (mitDNA) which is amplified in the albino plants. This amplification is associated with an increase in the number of mitochondria.

2. Materials and methods

2.1. Plant material

Rye plants (cv. Ailes), were regenerated from embryogenic cell lines obtained by culturing immature embryos on a MS + 2 mg/l 2,4-D medium [6]. Each cell line was constituted for all the calli

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Abbreviations: TEM, transmission electron microscopy; *atpA*, ATP synthase subunit α; *cox*III, cytochrome C oxidase subunit III; *nadh4*, NADH dehydrogenase 4; RAPD, random amplification of polymorphic DNA; IPCR, inverse polymerase chain reaction; MS, Murashige and Skoog medium; 2,4 D, 2,4-dichlorophenoxyacetic acid; mitDNA, mitochondrial DNA; PCR, polymerase chain reaction.

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derived from the same embryo. Green regenerated plants were transferred to the in vivo conditions, grown in a greenhouse and reached maturity. Albino plants and green regenerated plantlets, in the same developmental stage as the albinos, were frozen to be studied subsequently. In vivo growing mature plants of the Ailes cultivar were also used. Moreover, seeds were germinated in darkness, or under a light regime of 16 h light at 25 ± 1 °C on wet filter paper and two weeks old green or etiolated plantlets were also studied.

2.2. Progeny analysis

Different crosses were performed with regenerated green plants obtained from different cell lines. The progeny of each cross was collected and germinated in order to verify the plantletś phenotype: green or albino. To quantify the data, plantlets obtained from crosses between plants regenerated from the same two cell lines were grouped. In those cases in which the two types of plantlets were observed, a statistical analysis was performed (a χ^2 test) in order to confirm the mendelian segregation of the trait.

2.3. RAPD analysis and Southern blot

The isolation and purification of total DNA from plant leaves was performed using the Plant Mini Kit from Quiagen Inc. RAPD reactions, using the primer OpF13 from OPERON Technologies, and Southern hybridization were performed as previously described [4]. DNA from the ScF13c variable amplicon was recovered from the gel (Geneclean Kit BIO 101 Inc.), and cloned into pCR II vector using the TOPO-cloning kit (Invitrogen).

2.4. Inverse polymerase chain reaction (IPCR)

Inverse PCR was used to clone the sequences flanking the Sc amplicon. DNA was digested with the appropriate endonuclease, determined by Southern hybridization, and self-ligated in the presence of the T4 DNA ligase during 16 h at 4 °C in a final volume of 200 μl. The sequences of the primers used were: L1 5′ATAGCTGCATTTCTGGTCAAGG3′; R1 5′GGAGGCCATTCAGGCATTGTGG 3′; L2 5′TGGTAGGGTCAAATCAGGCAAAGC 3′; R2 5′AAGAGTCAAGG AGAGCATTGCCA 3′. Two PCR reactions were carried out. In the first, the 25 μl reaction mixture contained 25 ng of self-ligated DNA as template, 0.20 μM of L1 and R1 primers, 200 μM of each dNTP and 1 U DNA polimerase DyNAzyme™ EXT (Finnzymes) with the corresponding buffer and 1.5 mM $MgCl_2$. Cycling conditions were as follows: one cycle 94 °C, 2 min; 32 cycles of 94 °C, 30 s; 68 °C, 30 s; 68 °C, 7 min and a last cycle of 70 °C, 10 min. The products of this first amplification were used in the second PCR which was performed under the same conditions, but using L2 and R2 primers.

Several fragments of the same size were sequenced.

2.5. DNA sequencing

DNA was sequenced using the ABI PRISM® 3730 (Applied Biosystems) at the Centro de Genómica y Proteómica, Parque Científico de Madrid. The sequences were compared with the ones present in the sequence databases using BLAST and FASTA programs.

2.6. Real-time PCR

Three sequences were used: ScF13c and the mitochondrial *atpA* and *coxIII*. For each sequence a pair of primers and a Taqman probe were designed with the program Primer Express Software v 2.0 (Applied Biosystem) (Table 1). The Taqman MGB (minor grove binder) was labelled at its 5′ end with the acceptor molecule 6-carboxyfluorescein (6-FAM).

The 25 μl reaction mixtures contained: 12.5 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystem), 300 nM of each forward and reverse primer, 250 nM of the TaqMan probe and 20 ng of DNA. The PCR was carried out in plates with 96 wells with optic covers in an ABI PRISM® 7700 *Sequence Detection System* (Applied Biosystems) Sistema ABI Prism 7700 (Applied Biosystems).

Cycling conditions were as follows: 2 min at 50 °C (uracil-*N*-glycosylase digest), 10 min at 95 °C (polymerase activation) and 40 cycles with 15 s at 95 °C and 1 min at 60 °C.

Standards for each target sequence (ScF13c, *atpA* y *coxIII*) were generated by making serial dilutions 1/10 from the cloned fragment, from 0.1 ng to 0.0001 ng DNA. The copy number of each sequence/dilution was calculated. Data were generated by running a single plate containing both the standard curve and the samples. The data were analysed using the *Sequence Detector Software* 2.1 (Applied Biosystems).

From two to five plants/plantlets of each kind were analysed. The differences between the mean values obtained in each case were statistically compared by applying a Student's *t*-test.

2.7. Transmission electron microscopy (TEM)

Leaf fragments were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 4 h at 4 °C. The samples were post-fixed in 1% osmium tetroxide during 1 h at room temperature, dehydrated in acetone series (increasing concentrations) and finally embedded in Spurr resin. Ultra-thin sections, 60 nm, were cut and stained with 2% uranyl acetate and Reynolds lead citrate. The sections were examined with a Jeol 1010 transmission electron microscope at 100Kv.

Electron micrographs of TEM sections taken at random from leaves of the studied plantlets were analysed. The number of mitochondria per cytoplasm area was scored measuring the cross-sectional area of the cells and counting the number of mitochondria present in this area (Adobe Photoshop Program).

3. Results

3.1. Regenerated plants

3.1.1. Study of the regenerated plants

Thirteen cell lines were studied (Table 2): some of them only regenerated green or albino plants, but in other lines both types of plants were obtained.

We performed RAPDs using the primer OpF13 and a very conspicuous band, which we shall refer to as the ScF13c amplicon, was present in the pattern obtained from all albino plants (Table 2), and was almost imperceptible or appeared to be absent in the one obtained from the green plants (Fig. 1). These results were

Table 1
Primers and Taqman probe used in the RT-PCR study.

	Direct primer	Probe	Reverse primer	bp
ScF13c	TGGTTCTGTCCTCATGCAACA	5′-FAM-AGACCAATTGCTTATTTC-MGB	CATTTCTGGGTCCAAGGGTTT	68
atpA	GCTAAACGATCGGACCAGACA	5′-FAM-CGACTGCGTTACCCG-MGB	CGTCTCCAGCTTGTGTTTCAATC	73
coxIII	GCGGGATGTTCTACGTGAATC	5′-FAM-CGTTGGAAGGGCATCA-MGB	CGAGGTCCTAATTGTACAGCTTTTG	66