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THE USE OF CYTOPLASMIC MARKERS IN ONION HYBRID BREEDING

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Abstract: We applied the RFLP approach to identify the cytoplasmic genotypes of selected onion breeding materials from Poland. For this purpose, mitochondrial DNA from cytoplasmic male-sterile (CMS) and male-fertile onions were hybridized with the probes for the following mitochondrial genes: *atpA*, *atp6*, *atp9*, *cob*, *cox1*, *nad3*, *nad4* and *nad6*. S-, T- or C-cytoplasm was represented in each analyzed sterile accession. Some new polymorphisms shared by S- and C-cytoplasmic onions were identified. We also used currently available PCR markers to test if cytoplasmic heterogenity occurs within onion inbreds. A fraction of the plants bearing S-cytoplasm were found within two male-fertile lines, but such plants were not detected in the open-pollinated cultivars *Sochaczewska*, *Wolska* and *Żytawska*. Both the RFLP and PCR approaches gave some proof of existing mitochondrial heteroplasmy in onions.

Key Words: Cytoplasmic Male Sterility, CMS, Mitochondrial DNA, mtDNA, Onion, *Allium cepa* L., Hybrid Seed

INTRODUCTION

In onion, hybrid seed production is based on cytoplasmic male sterility (CMS). In addition to normal male-fertile N-cytoplasm, three different male-sterile cytoplasms are used in onion breeding:

1. S – identified by Jones and Clarke [1] in the variety *Italian Red*,
2. C – identified by Banga and Petiet [2] in *Rijnsburger* onions,
3. T – identified by Berninger [3] in the variety *Jaune paille des vertus.*

All these CMS sources differ with respect to the genetic control and frequency of the maintainer genotypes. Due to this, a knowledge of the cytoplasm type is essential for hybrid breeding. At present, the identification of onion cytoplasms

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is significantly facilitated via the use of molecular techniques. Among them, RFLP profiling offers the best discrimination ability – it allows the distinction of most cytoplasm types [4 and references therein] – but due to the rather complex experimental procedure, it can hardly be used to screen large populations. As an alternative, PCR based markers have been proposed [5, 6]. The one reported by Havey [5] makes use of chloroplast DNA polymorphism, the one developed by Sato [6] takes advantage of rearrangements in the vicinity of the mitochondrial *cob* gene. Both markers differentiate S- from N-cytoplasmic plants.
A few seed companies in Poland reported ongoing efforts to develop hybrid onions. Our research was aimed at specifying which cytoplasm sources were exploited in their breeding programs, and at detecting cases of cytoplasmic heterogenity within their breeding accessions.

MATERIALS AND METHODS

Plant material

Mature bulbs and leaf tissue of the analyzed breeding accessions were provided by Polish seed companies. Different sets of accessions were used in RFLP and PCR analyses. As requested by the breeders, the exact origin of these materials remains confidential. The seed of open-pollinated (OP) cultivars originated from PlantiCo Zielonki (*Sochaczewska*) and Polan Kraków (*Wolska*, *Żytawska*).

DNA extraction

Mitochondrial DNA for RFLP analysis was isolated from bulbs using the procedure of Steinborn *et al.* [7] modified by Szklarczyk *et al.* [8]. Subsequently, mtDNA preparations (usually 100 µl) were supplemented with 5 volumes of 3 M guanidine thiocyanate, 10 mM TrisHCl pH 6.6, 5% ethanol. The sample was mixed, loaded onto a Qiagen DNeasy Mini Column and centrifuged at maximal speed for 1 minute. After flow-through disposal, the column was washed twice with 500 µl of 20 mM NaCl, 2 mM TrisHCl pH 7.5, 80% ethanol. DNA elution was performed with 50 µl of 10 mM TrisHCl pH 8.5.
The total cellular DNA for PCR was isolated from young leaves. After grinding in liquid nitrogen, 100 mg of the powdered tissue was suspended in 700 µl of extraction buffer: 200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS, 0.2% PVP. The resulting lysate was extracted with 600 µl of chloroform:isoamyl alcohol (24:1 v/v) for 5 minutes. After centrifugation at 13,000 rpm for 10 minutes, the upper aqueous phase was transferred to a new Eppendorf tube. DNA was precipitated with an equal volume of isopropanol, collected by 10 minute centrifugation at maximal speed, washed twice with 70% ethanol, dried and dissolved in 50 µl of water.

RFLP analysis

About 0.5 µg of mtDNA (from a single plant) was digested with 25 U of *Xba*I (MBI Fermentas) for 3 h. The subsequent procedure of gel blotting was

performed according to Szklarczyk *et al.* [8]. The molecular size of the DNA fragments corresponding to the hybridization signals was estimated using Gene Profiler 4.03 (Scanalytics).

DNA amplification

A 15 µl total volume of the reaction mixture contained: 10 mM TrisHCl pH 8.8, 50 mM KCl, 0.08% NP-40, 5 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 µM each primer, 5 ng template DNA and 1.1 U of Taq polymerase (MBI Fermentas). The amplifications were carried out in an Eppendorf Mastercycler gradient programmed for 1 cycle of 5 minutes at 94°C, followed by 36 cycles of 45 seconds at 92°C, 45 seconds at 57°C, 2 minutes at 72°C and 1 cycle of 10 minutes at 72°C. After PCR, each sample was separated in 1.5 % agarose.

RESULTS AND DISCUSSION

RFLP genotyping of sterility inducing cytoplasms

Southern hybridizations were carried using probes for the following mitochondrial genes: *atpA*, *atp6*, *atp9*, *cob*, *cox1*, *nad3* and *nad4* and *nad6*. The DNA fragments used as probes were PCR amplified from either carrot (*Daucus carota*) or beet (*Beta vulgaris*) total genomic DNA. A set of 13 accessions was included in the analysis: 8 male-sterile lines (S1-S8), 2 male-fertile lines (F1, F2) and 3 accessions of unraveled characteristics (U1-U3, phenotypes known only to the breeder). Table 1 shows the sizes of the DNA fragments exhibiting homology to the probes for all the studied accessions.

The *cob* and *nad3* probes clearly differentiated lines S1, S3 and U2 from the others (Fig. 1). No variation was observed between either S1, S3 and U2 or among the remaining accessions. According to the report of Havey [5], *cob*/*Xba*I polymorphism differentiated S- from N-cytoplasm. Taking into account that information and the male-sterile phenotype of S1 and S2, we may conclude that these three lines possess S-cytoplasm. Contrary to another report of Havey [4], we did not observe any fragment of 6 kb in *cob*/*Xba*I profiles. Another fragment of 8 kb probably corresponds to the one estimated here as 9 kb.

The probes *atpA*, *atp9* and *cox1* produced three distinct profiles which grouped the analyzed accessions in the same fashion. The first group, included lines S1, S3 and U2 – previously assigned to S-cytoplasm. Another type of profile was observed for lines S6, S8, U1 and U3. Probing with *cox1* yielded band patterns similar to those of *Hygro* F_1 [4] indicating that these lines contain the cytoplasm of *Rijnsburger* onions. The fertile accessions as well as the remaining sterile lines S2, S4, S5 and S7 showed the third type of profile. In the above context, it appears that these male-sterile lines possess T-cytoplasm.

These data concur with the results of Havey [4], who also used *cox1*/*Xba*I combination for genotyping purposes. The difference is that the largest fragment observed by Havey [4] for N-, T- and C-cytoplasms, in our experiments turned out to be a superposition of two co-segregating fragments of 6.4 and 5.9 kb. We

also observed that the profile of S2 contained a low intensity signal for a fragment of 3.9 kb. Because that signal was clearly visible only on overexposed blots, we assigned the cytoplasm of S2 to type T. However, it seems that at least with respect to *cox1* sequences, this accession represents a mitotype slightly intermediate between T- and *Rijnsburger* cytoplasms. Another low intensity signal was also observed for S2 when probe *atp9* was used – the respective restriction fragment of 4.2 kb appeared primarily in S-cytoplasm.

Tab. 1. *Xba*I restriction fragments [in kb] showing homology to the selected mitochondrial genes in a series of onion breeding accessions

Accession → Probe↓	S1	S2	S3	S4	S5	S6	S7	S8	F1	F2	U1	U2	U3
atpA [9]	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0	18.0
		12.0		12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0		12.0
		7.5		7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5		7.5
		1.4		1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4		1.4
		1.0		1.0	1.0		1.0		1.0	1.0			
atp6 [9]	4.0		4.0			4.0		4.0			4.0	4.0	4.0
	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
		1.0		1.0	1.0		1.0		1.0	1.0			
	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.6
atp9 [9]		9.4		9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4		9.4
		7.6		7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6		7.6
		6.0		6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0		6.0
	4.2	*4.2	4.2									4.2	
	3.9		3.9			3.9		3.9			3.9	3.9	3.9
		1.0		1.0	1.0		1.0		1.0	1.0			
cob [9]	9.8		9.8									9.8	
		9.0		9.0	9.0	9.0	9.0	9.0	9.0	9.0	9.0		9.0
cox1 [9]		6.4		6.4	6.4	6.4	6.4	6.4	6.4	6.4	6.4		6.4
		5.9		5.9	5.9	5.9	5.9	5.9	5.9	5.9	5.9		5.9
	3.9	*3.9	3.9			3.9		3.9			3.9	3.9	3.9
		3.1		3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1		3.1
nad3 [9]		11.5		11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.5		11.5
		9.4		9.4	9.4	9.4	9.4	9.4	9.4	9.4	9.4		9.4
	9.0		9.0									9.0	
nad4 [9]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
*nad6***	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0

*****clearly visible only after prolonged exposure, **5'-terminal part of the coding sequence (approx. 250 bp), isolated from carrot [8]

Two fragments of homology to the *atp6* probe were common among all investigated accessions. Each accession showed one additional fragment of either 4 or 1 kb. The 4 kb band was found in lines previously classified as S- or C-cytoplasmic – S1, S3, S6, S8, U1, U2 and U3. The 1 kb fragment appeared in

the remaining male-sterile lines – S2, S4, S5 and S7 as well as in both male-fertile accessions. Therefore, two alternative RFLP profiles were observed for this probe – of S/C and T/N type.

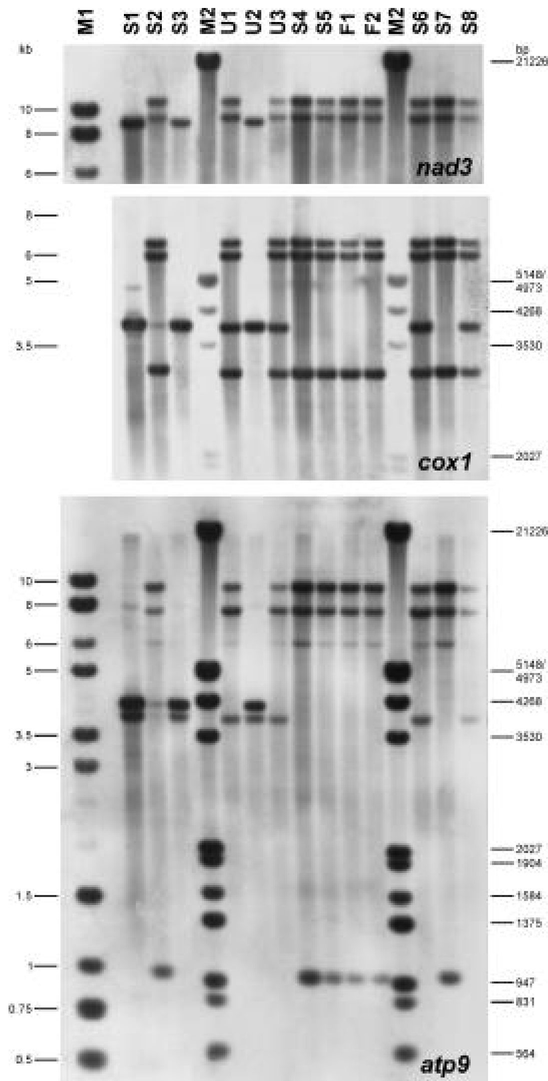

Fig. 1. Southern hybridizations of *Xba*I-digested onion mtDNA probed with *nad3*, *cox1* and *atp9* sequences. Each accession is indicated above the respective lane. M1, M2 – DNA size markers.

The hybridization patterns obtained for the *nad4* and *nad6* sequences did not reveal any polymorphism within the investigated material. Both probes detected a single restriction fragment in all the analyzed accessions.

PCR identification of plants with S cytoplasm

In this analysis, we applied the PCR markers of Havey [5] and Sato [6]. The former was reported to yield products of 1.1 and 1.2 kb [10, 11], the latter of 0.41 and 0.18 kb for S- and N-cytoplasm, respectively. In our study, some additional DNA fragments were amplified with both sets of primers. The chloroplast product of 1.2 kb was always accompanied by a weak band of 0.9 kb fragment (Fig. 2). For some accessions, the mitochondrial marker yielded a product of 0.4 kb (Fig. 3). It was never present in S-classified plants, and was always accompanied by strong band of 0.18 kb product. Since both unexpected DNA fragments were present either in fertile or non-S-cytoplasmic sterile onions and hence had no diagnostic value, they were ignored in subsequent descriptions. Using the assay of Sato [6], we also observed a faint band of 0.18 kb produced for plants classified as S-cytoplasmic, i.e. those showing 1.1 kb [5] and 0.41 kb [6] markers. This observation indicates that N-like variants of the *cob* sequence are present as sublimons in the mtDNA of S-cytoplasm [12]. For each analyzed plant, both markers showed accordant results.

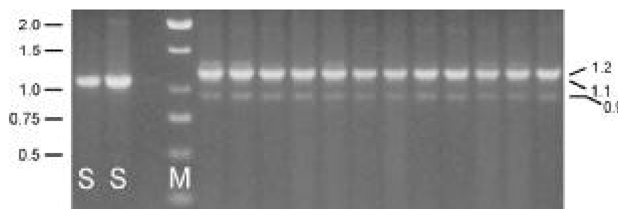

Fig. 2. PCR products generated with primers according to Havey [5] for line MS2. S – products obtained for S-cytoplasmic reference plants. M – DNA size marker [kb].

We analyzed 6 to 20 plants from each of the following accessions: 12 male-fertile lines (MF1-MF12), 1 male-fertile population (MFP), 4 male-sterile lines (MS1-MS4) and 2 hybrids (H1, H2). At this stage of our study, we also included open-pollinated (OP) cultivars: *Sochaczewska*, *Wolska* and *Żytawska*. Table 2 shows the PCR products obtained for all the above accessions using the primers recommended in both references.

The OP cultivars *Sochaczewska*, *Wolska* and *Żytawska* yielded PCR fragments identical to those expected for N-cytoplasm. Since *Wolska* onion is T-cytoplasmic [13], it appears that both markers do not distinguish between the N- and T-type of cytoplasm. We also observed that identical DNA fragments were produced for plants with *Rijnsburger* cytoplasm (data not shown).

Out of 13 male fertile accessions, 11 appeared to be monomorphic for the products not associated with the S-cytoplasm. PCR fragments specific for this

type of cytoplasm were found in a certain proportion of plants from the two remaining fertile lines. Such plants should be eliminated from accessions which are used as maintainers of CMS. Due to selection for fertility within maintaining lines these plants are likely to harbor restorer genes.

Plants of the two male-sterile lines exclusively exhibited DNA fragments specific to the S-cytoplasm. In another male-sterile line these amplification products we also predominant – only one plant showed the product of non-S-type. Such plants may represent either fertile N- or a different kind of sterile cytoplasm, and therefore they may result in a decreased stability of the male-sterile phenotype. For the male sterile line MS2, products of non-S-type were exclusively produced, therefore either T- or C-cytoplasm might be present in this material.

Tab. 2. Identified cytoplasmic markers for a series of onion accessions

	Cytoplasmic marker [kb]*	
Accession	Havey [5]	Sato [6]
MF1	1.2 (12 plants)	0.18 (13 plants)
MF2	1.2 (16 plants)	0.18 (16 plants)
MF3	1.2 (5 plants)	0.18 (8 plants)
	1.1 (2 plants)	0.41 (2 plants)
MF4	1.2 (12 plants)	0.18 (12 plants)
MF5	1.2 (15 plants)	0.18 (15 plants)
MF6	1.2 (6 plants)	0.18 (12 plants)
MF7	1.2 (7 plants)	0.18 (12 plants)
MF8	1.2 (12 plants)	0.18 (12 plants)
MF9	1.2 (13 plants)	0.18 (19 plants)
MF10	1.2 (10 plants)	0.18 (19 plants)
MF11	1.2 (19 plants)	0.18 (20 plants)
MF12	1.2 (12 plants)	0.18 (16 plants)
	1.1 (3 plants)	0.41 (3 plants)
MFP	1.2 (10 plants)	0.18 (12 plants)
MS1	1.1 (10 plants)	0.41 (11 plants)
		0.18 (1 plant)
MS2	1.2 (12 plants)	0.18 (12 plants)
MS3	1.1 (12 plants)	0.41 (12 plants)
MS4	1.1 (11 plants)	0.41 (12 plants)
H1	1.1 (9 plants)	0.41 (12 plants)
H2	1.2 (7 plants)	0.18 (12 plants)
Sochaczewska	1.2 (12 plants)	0.18 (19 plants)
Wolska	1.2 (14 plants)	0.18 (20 plants)
Żytawska	1.2 (14 plants)	0.18 (20 plants)

* - only high yield, diagnostic PCR products were allowed

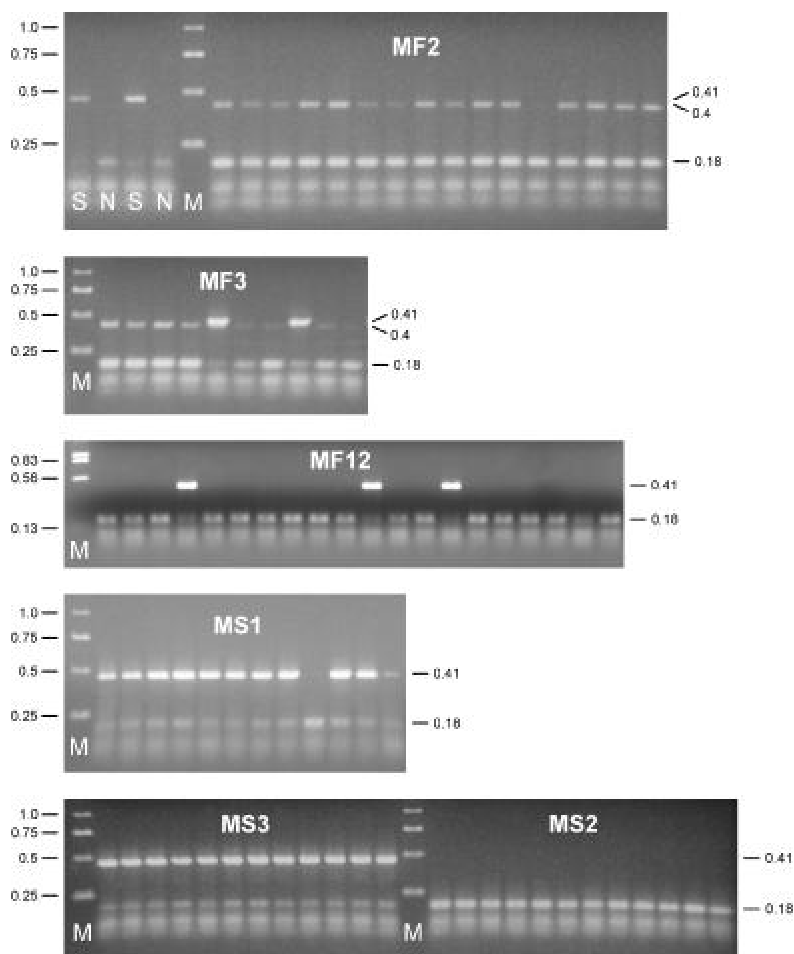

Fig. 3. PCR products generated with primers according to Sato [6] for lines MF2, MF3, MF12, MS1, MS3 and MS2. S, N – products obtained for S- and N-cytoplasmic reference plants. M – DNA size marker [kb].

Use of either marker shows that out of the two analyzed hybrids, H1 is based on S-cytoplasm, whereas for the production of H2, another sterile cytoplasm is used.

The data presented here indicate that all three identified sterile cytoplasms are used by the seed companies which work on hybrid onion in Poland. Hopefully, this fact will contribute to the genetic heterogenity of the resulting commercial hybrids. We also found that the probes *atpA* and *atp9*, used on *Xba*I-digested mtDNAs, differentiated onion sterile cytoplasms in a way identical to that previously reported for *cox1* sequence [4]. Such additional RFLPs may help to classify newly identified sources of sterility. Interestingly, some polimorphisms

generated with these probes, like that of the *atp6* gene, were shared between S- and C-cytoplasmic plants, indicating that the respective sequences may be associated with CMS in onion. Two of these probes produced low-intensity signals for S2. These signals have their highly-pronounced counterparts in the mtDNA, either from S- or S- and C-cytoplasm (Table 1). These data suggest that the corresponding genomic molecules are present in all cytoplasms but at different levels – undetectable in N/T, moderate in S2 and highly amplified in S/C. In the context of such intrinsic heteroplasmy, care should be taken when applying pooling strategies to estimate cytoplasm frequency within onion populations [11, 14]. Polymorphism of the *cob* sequence, used by Sato in his PCR assay [6], appears not to be exceptional with regard to the presence of substoichiometric DNA molecules. It explains why we were obtaining faint N-type products for onions with S-cytoplasm.

We also showed that, for some breeding accessions, genetic purity with respect to plasmatype might be a problem which can be resolved using the selection procedure based on cytoplasmic markers. There are reports indicating that some OP cultivars contain a significant proportion of S-cytoplasmic plants [14, 15]. In our work, we did not detect such plants within the tested Polish varieties. Nevertheless, we were able to show that S-cytoplasmic contaminants might even occur within male-fertile inbreds. Such events may result either from a mixture of seed or cytoplasmic heterogenity within the material the inbreds were derived from.

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