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Cytoplasmic male sterility in *Plantago lanceolata* L.: differences between male-sterile cytoplasms at the DNA- and RNA-level

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Summary. To establish the feasibility of a cytoplasm-type assay based on molecular hybridizations, mitochondrial DNAs from the two male-sterile cytoplasms of *Plantago lanceolata* (P and R) were compared by restriction endonuclease digestion. We cloned a 1.1 kbp *Eco* RI-HindIII fragment from P-mtDNA (pPl-311), which on hybridization to Southern blots of *Bam* HI digested mtDNA and total DNA from plants with P-cytoplasm, hybridized to a specific 1.1 kbp *Bam* HI fragment. Hybridizations of pPl-311 to Northern blots of total RNA resulted in the detection of 1,250 and 1,100 nucleotides transcripts specific to P- and R-cytoplasm, respectively, and of a common transcript of 930 nt. Evidence was obtained for additional cytoplasmic variation within *P. lanceolata* populations.

Key words: Cytoplasmic male sterility – Mitochondrial DNA – Cytoplasm-type assay – RNA – *Plantago lanceolata* L.

Introduction

Cytoplasmic male sterility (CMS) is a common phenomenon among plants and has received much attention due to its (potential) use in agriculture. Reports of male sterility in *Plantago lanceolata* (ribwort plantain) date back as far as the nineteenth century (Coleman 1876), but it was not until recently (Van Damme and Van Delden 1982) that two different male sterility types (P and R) were distinguished instead of the one identified until then. Inheritance of P- and R-type male sterility was shown to be complex and to involve both cytoplasmic and nuclear genes (Van Damme 1983).

Natural populations of *P. lanceolata* are usually gynodioecious, i.e. consisting of male-sterile and fertile plants (Van Damme and Van Delden 1982). The gynodioecious breeding system is of interest from an evolutionary point of view because of the selective disadvantage of male-sterile relative to fertile plants (Lewis 1941). In a study on the evolution and maintenance of gynodioecy in natural populations of *P. lanceolata,* there is the problem of estimating the frequencies of the two types of male-sterile cytoplasm. Both types are easily identified in male-sterile plants (or in plants with partially restored fertility) due to their characteristic differences in flower morphology. The combined frequencies of these plants within populations can range up to 29%, implying, however, that the cytoplasm of the majority of plants remains unidentified. Classification of the fertile plants into one of the two cytoplasm types can be done by the tedious method of crossing them to maintainers of each male sterility type and examining the flower morphology of the male-steriles (Van Damme 1986).

Several lines of evidence indicate that the cytoplasmic component of CMS generally resides in the mitochondria. In maize (*Zea mays*), sorghum (*Sorghum bicolore*), sugarbeet (*Beta vulgaris*) and faba bean (*Vicia faba*) the mitochondrial genomes of fertile and male-sterile plants can be distinguished by restriction enzyme analysis, while restriction patterns of their chloroplast DNAs show a lesser degree of variation (Pring and Levings 1978; Conde et al. 1982; Powling and Ellis 1983; Mikami et al. 1985; Boutry and Briquet 1982). In addition, the complement of low-molecular-weight DNA species in the mitochondria differ between maize cytoplasms and also between certain CMS and fertile lines of sorghum, sugarbeet and faba bean (Kemble and Bedbrook 1980; Pring et al. 1982; Powling 1981; Goblet

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et al. 1983). This variation at the DNA level is sometimes reflected at the protein level, i.e. isolated mitochondria from various CMS and fertile lines from a number of species synthesize unique polypeptides (Forde et al. 1978; Boutry and Briquet 1982; Dixon and Leaver 1982; Boutry et al. 1984).

If similar differences could be established between the two male-sterile cytoplasms within *P. lanceolata,* they could lead to a fast and relatively simple way of screening populations. As an initial step towards a cytoplasm-type assay we report here the cloning of a specific P-type mtDNA fragment, which enabled us to reliably identify P-cytoplasm on Southern blots of *Bam* HI-digested total DNA of plants from a number of populations. In addition to the detection of differences at the DNA level, this clone also differentiated between the cytoplasm types at the RNA level.

Materials and methods

Plant material

The *P. lanceolata* plants came from a crossing programme (Van Damme 1983 and unpublished data) or they were grown from seeds collected mainly from Dutch natural populations. Their cytoplasm types were known by the morphological characteristics of the flowers of the sampled plants or by ancestry in the case of fertile plants.

Seeds were germinated by incubating them for 2 days in Petri dishes on moist filter paper in a refrigerator at 6 °C and subsequently for 5 days in the dark at room temperature. After 2 days at room temperature a tip on the side of the radicle of non-germinating seeds was cut off and incubation continued. Germlings were grown in the greenhouse or in the garden.

Isolation of mtDNA

Leaves (40–50 g fresh weight) from 8- to 12-week-old greenhouse-grown plants were surface-sterilized in 0.5% sodium hypochlorite and rinsed with tap water. All subsequent operations up to lysis were conducted at 4 °C. The leaves were cut into small pieces and homogenized in 200 ml homogenization buffer (0.4 M sucrose, 50 mM Tris-HCl, 10 mM KH_2PO_4, pH 7.5, 5 mM β-mercaptoethanol, 1% PVP-25, 20 mM Na-ascorbate, 1 mM EGTA, 0.5% BSA) in a Waring blendor (twice for 2 seconds at low speed) followed by treatment with an Ultra Turrax (15–20 s). The homogenate was filtered through three layers of nylon gauze (mesh size 200 µm) and fluid squeezed from the residue. The filtrate was centrifuged for 10 min at 1,500 g and the supernatant centrifuged for 20 min at 12,000 g. The crude mitochondrial pellet was resuspended in 20 ml homogenization buffer with the aid of a loose-fitting Potter-Elvehjem homogenizer. Subsequent centrifugation at 1,500 g removed remaining chloroplasts. To digest contaminating nuclear or chloroplast DNA the supernatant was brought to 10 mM $MgCl_2$ and 50 µg · ml^{-1} DNAse 1 and incubated at 4 °C for 60 min. Digestion was terminated by addition of EDTA to 20 mM. The mitochondrial suspension (10 ml) was layered onto a 7.5 ml sucrose cushion (0.6 M sucrose, 10 mM EDTA) and the mitochondria centrifuged through the sucrose layer for 30 min at 12,000 g in a swing-out rotor. Mitochondrial DNA was isolated from the pellets by lysis in 1 ml 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 2% sarcosyl, 200 µg · ml^{-1} proteinase K for 15 min at 37 °C and extracted twice with phenol/chloroform (Maniatis et al. 1982). The aqueous phase was further purified by extractions with chloroform and ether followed by isopropanol precipitation of the DNA. The precipitate was washed once with 70% ethanol, dried under vacuum and rehydrated in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

Isolation of total DNA

Leaves of greenhouse or garden-grown plants were used for the isolation of total DNA, essentially according to Raeder and Broda (1985), except that *sometimes* two additional phenol/chloroform extractions were performed following the RNase treatment. Insoluble material remaining after rehydration of the final isopropanol precipitate was removed by centrifugation. 2 min at 10,000 *g*. The total DNAs from the plants, which did not come from the crossing programme, were also isolated by this procedure, but omitting the additional phenol/chloroform extractions, and selectively reprecipitating the DNA with spermine after rehydration of the final isopropanol precipitate (Hoopes and McClure 1981).

Isolation of total RNA

From 1 g of green leaves of greenhouse or garden-grown plants RNA was isolated as described by De Vries et al. (1982) and the yield assessed spectrophotometrically by measuring the A260. Intactness of the RNA was verified by electrophoresis in 1.5% agarose gels in 40 mM Tris-acetate, pH 7.8, 1 mM EDTA and 0.5 µg · ml^{-1} EtBr.

Restriction endonuclease digestions and ligations

Restriction enzymes and T4 DNA ligase from various manufacturers were used essentially as recommended except for the digestion of total DNA, where twice the recommended amount of enzyme was used during a 2 h incubation period.

Cloning of mtDNA fragments

DNA fragments were eluted from Ultra pure agarose gel according to Tautz and Renz (1983) and cloned in pUC8 with *E. coli* JM83 as a host (Vieira and Messing 1982). Subclones were also made in pUC8. Plasmid DNA was isolated as described by Birnboim and Doly (1979) and modified by Maniatis et al. (1982). This DNA was used for restriction enzyme digestions and nick translations with ^{32}P-α-dCTP without further purification.

Filter hybridizations

Southern blotting. After fractionation of restriction fragments on 0.8% agarose gels in 40 mM Tris-acetate, pH 7.8, 1 mM EDTA and 0.5 µg · ml^{-1} EtBr the DNA was transferred to GenescreenPlus membrane under alkaline conditions (Chomczinsky and Quasba 1984).

Northern blotting. Total RNA was denatured with glyoxal omitting DMSO (Thomas 1980) and fractionated on 1.5% agarose gels in 10 mM NaH_2PO_4, pH 7.0. The RNA was blotted onto GenescreenPlus membrane according to the manufacturer's protocol. Filters from both types of blottings were hybridized overnight at 65 °C in 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 1% SDS, 0.05% BSA and 100 µg · ml^{-1} denatured calfthymus DNA in the presence of nick translated probes (50 ng · ml^{-1}) and washed as recommended by the manufacturer. Autoradiography was done for various exposure times using Kodak XAR-5 film and intensifying screens (Ilford, Fast Tungstate).

Table 1. Classification of *P. lanceolata* progenies of crosses and field-sampled plants

Progeny	Origin	Cytoplasm type (from morphology)	1.1 kbp *Bam HI*[a] fragment	Length of transcripts[b] (nt)
63	Anlo	P	+	ND
637	Anlo	P	+	ND
78	Westduinen	P	+	ND
57	Westduinen	R	–	ND
739	Westduinen	R	–	ND
740	Westduinen	R	–	ND
826-X × An6	Anlo	P	+	1,250, 930
83	Westduinen	P	+	1,250, 930
791-X × 81-16	Westduinen	R	–	1,100, 930
An6 × An10	Anlo	An6[c]	+ /+/+	880 / 880 /880
An10 × An6	Anlo	An10[c]	– /–	840, 620/–
Field-sampled	Anlo	P	+ /+	1,250 /–
	Vlaardingen	P	+ /+	1,250 /1,250, 930
	Vlaardingen	R	– /–	1,100 /1,100
	Merrevliet	P	ND/ND	1,250, 930/1,250, 930
	Merrevliet	R	– /–	1,100 /–
	Haren	P	+ /–	1,250, 930/1,250, 930
	Haren	R	– /–	– /–
	Heteren	R	– /–	1,100 /1,100
	Westduinen	P	ND/ND	1,250 /1,250
	Westduinen	R	– /–	1,100 /1,100
	Durham (USA)	R	– /–/–	1,100 /1,100 /–

[a] + and –: presence or absence of the 1.1 kbp *Bam* HI fragment
[b] –: no signal was detected
[c] Plants with cytoplasms An6 and An10 never segregate male-steriles and consequently the cytoplasm is type unknown

Results

The green leaves used for the isolation of P- and R-type mtDNA were obtained by mixing leaves from the progenies of the crosses 63, 637 and 78 and of the crosses 57, 739 and 740, respectively (Table 1). None of these progenies were isogenic, and only progenies from the crosses 63 and 637 had the same female parent.

Bam HI digestion of mtDNA from plants with P- and R-cytoplasms followed by agarose gel electrophoresis resulted in complex restriction patterns in which one unique fragment in P-cytoplasm could be clearly recognized at 1.8 kbp (Fig. 1). Besides this obvious difference a number of other less conspicuous differences could be observed at approximately 20, 6 and 3.5 kbp. These four gel regions were cut from the gel and the DNA was isolated.

The putatively unique *Bam* HI fragments of approximately 20 and 1.8 kbp were cloned in pUC8 and used as probes in Southern blot hybridizations of *Bam* HI digested P- and R-mtDNA. Clones derived from the 6 and 3.5 kbp bands were not examined further. Hybridization with pPl-2, a clone which contains the 1.8 kbp P-type *Bam* HI fragment, demonstrated the fragment to be shared by both cytoplasms (data not shown). However, the intensities of the two signals differed at least two orders of magnitude. Similar hybridi-

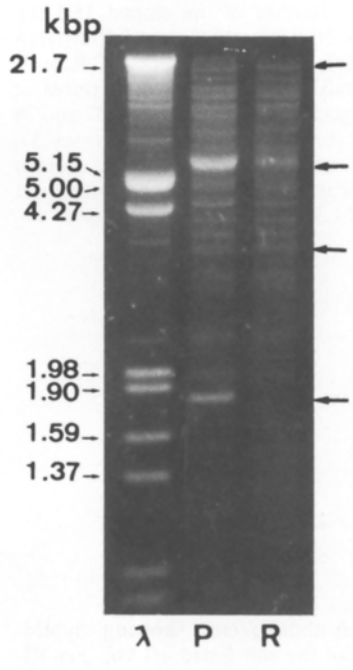

Fig. 1. Electrophoresis on 0.8% agarose of *Bam* HI digests of mtDNA from mixtures of progenies of the crosses 63, 637 and 78 (P-cytoplasm) and of the crosses 57, 739 and 740 (R-cytoplasm). Differences between restriction patterns are indicated by *arrows*. Molecular weight markers: *Eco* RI-*Hind*III fragments of lambda DNA (kbp)

zations with pPl-2 to Southern blots of *Bam* HI digested mtDNA from other plants of the same progenies showed the abundance of this fragment to be subject to variation. Hybridizations with pPl-3, a clone containing an 18.5 kbp *Bam* HI P-mtDNA fragment derived from the approximately 20 kbp region, resulted in the detection of a large number of P- and R-mtDNA fragments and between them some marked differences were apparent at 1.1 and 1.3 kbp (Fig. 2). In view of a possible application of (parts of) pPl-3 in a cytoplasm-type assay further research focussed on this clone.

Our aim was to find a subclone which would hybridize specifically to the two supposedly P-type specific *Bam* HI fragments of 1.1 and 1.3 kbp. To this end, pPl-3 was digested with *Hind*III and the fragments isolated after agarose gel electrophoresis. The largest *Hind*III fragment (6.6 kbp) hybridized strongly to the putatively P-specific, 1.1 and 1.3 kbp fragments but also to a large number of high-molecular-weight bands in both mtDNAs. A clone of the 6.6 kbp *Hind*III fragment contained in pUC8 (pPl-31) was digested with *Eco* RI. One of the four fragments (3.8 kbp) still hybridized to some high-molecular-weight bands in both mtDNAs but also to the 1.1 kbp *Bam* HI fragment of P-mtDNA (Fig. 3). This *Eco* RI fragment contained pUC8 and 1.1 kbp of P-mtDNA and it was cloned by selfligation and transformation to *E. coli* JM83 (pPl-311). Another *Eco* RI fragment of pPl-31 (1.8 kbp) hybridized only to the 1.3 kbp *Bam* HI fragment of P-mtDNA and was subcloned in pUC8 (pPl-313). However, it turned out that this fragment only occurred in the mtDNA of the plants from cross 78 (see below).

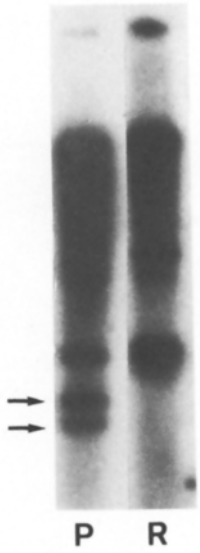

Fig. 2. Autoradiogram showing hybridization of the cloned 18.5 kbp *Bam* HI fragment from P-mtDNA (pPl-3) to Southern blots of *Bam* HI digests of mtDNA from mixtures of progenies of the crosses 63, 637 and 78 (P-cytoplasm) and of the crosses 57, 739 and 740 (R-cytoplasm). *Arrows* indicate the supposedly P-specific 1.1 and 1.3 kbp mtDNA fragments

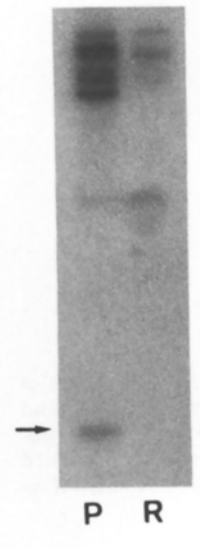

Fig. 3. Autoradiogram showing hybridization of the subcloned 1.1 kbp *Eco* RI-*Hind*III fragment from pPl-3 (pPl-311) to Southern blots of *Bam* HI-digested mtDNA from mixtures of progenies of the crosses 63, 637 and 78 (P-cytoplasm) and of the crosses 57, 739 and 740 (R-cytoplasm). The *arrow* indicates the 1.1 kbp P-specific mtDNA fragment

Suitability of pPl-311 and pPl-313 as probes in a cytoplasm-type assay

To screen large samples from natural populations, the number of manipulations should be kept to a minimum. Therefore differences in mtDNAs should be detectable in total DNA preparations. If the probe happened to be homologous to chloroplast or nuclear DNA sequences, this condition might not be met (Scott and Timmis 1984; Stern and Palmer 1984; Kemble et al. 1983).

Total DNA was isolated from one or two individuals of the progenies of every cross used for the isolation of mtDNA. This total DNA was *Bam* HI digested and electrophoresed on a 0.8% agarose gel and a Southern blot was made. Hybridization with pPl-31, containing both the inserts of pPl-311 and pPl-313, demonstrated the presence of the 1.1 kbp *Bam* HI fragment in both P-cytoplasms and its absence in all three R-cytoplasms (Fig. 4a). Surprisingly, the 1.3 kbp *Bam* HI fragment was present only in the DNA from the progeny of cross 78, indicating that pPl-313 could not be used in a cytoplasm-type assay. Consequently, hybridization of the same Southern blot with pPl-311, derived from pPl-31, again showed the 1.1 kbp *Bam* HI fragment exclusively present in P-cytoplasm besides a general lowering of the background (Fig. 4b). The large difference in total hybridization-signals between plants with P- and R-cytoplasms shown in Fig. 4b is usually less prominent. A further test of the suitability of probe pPl-311 was performed on other plants from the same and additional populations. The results of this screening are summarized in Table 1. In all cases the presence of a 1.1 kbp fragment hybridizable to pPl-311 correlated with the presence of P-cytoplasms as deduced from morphological examinations, except for one individual with P-cytoplasm from the Haren population which lacked the 1.1 kbp fragment. This fragment was absent in all R-cytoplasms.

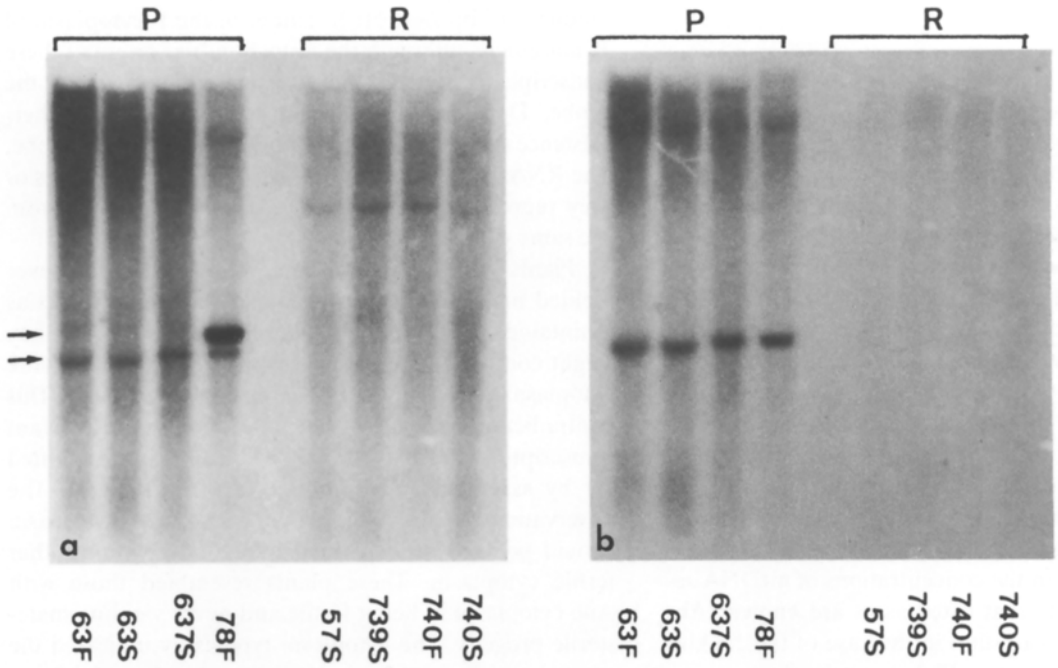

Fig. 4a, b. Autoradiograms of a Southern blot of *Bam* HI-digested total DNA from the progeny of the crosses 63, 637 and 78 (P-cytoplasm) and of the crosses 57, 739 and 740 (R-cytoplasm) and hybridized to pPl-31 (**a**) and pPl-311 (**b**). The P-specific 1.1 and 1.3 kbp fragments are indicated with *arrows*. pPl-31 contains a 6.6 kbp *Hind*III fragment from the insert of pPl-3 in pUC8. pPl-311 contains a 1.1 kbp *Eco* RI-*Hind*III fragment from the insert of pPl-31 in pUC8. *F*: fertile, *S*: male sterile

Northern blot hybridizations of total RNA with pPl-311 as a probe

Total RNA of *P. lanceolata* was electrophoresed, blotted and probed with pPl-311. Plants morphologically classified as having P-cytoplasm contained a unique 1,250 nucleotides transcript whereas plants with R-cytoplasm contained a unique 1,100 nt transcript hybridizable to pPl-311 (Fig. 5). In addition a common transcript of 930 nt was detected. As can be seen in Fig. 5 and in Table 1, progenies from the crosses An6×An10 and its reciprocal are exceptional in that they contain one or two transcripts hybridizing to pPl-311 which are absent in all the other plants. It should be noted that plants with An6 and An10 cytoplasms never yielded male-sterile progeny, in spite of an extensive crossing programme with maintainers of either male sterility type. Thus classical methods failed to classify An6 and An10 into male sterility types, while according to the DNA-test they have P- and R-cytoplasm, respectively.

A survey of the results of the northern blot analyses from all the plants used in the DNA-test shows that in a number of plants no pPl-311 homologous transcripts were detected (Table 1). The absence of these transcripts was not due to degradation of the RNA (data not shown). Despite the fact that the 930 nt transcript was not always detected and the 1,100 nt transcript was often faintly visible or absent, the results in general corroborate previous observations in that the 1,250 nt transcript is P-specific and the 1,100 nt transcript is R-specific. The deviant transcripts in An6 and An10 cytoplasms seem to be rare among *P. lanceolata* populations.

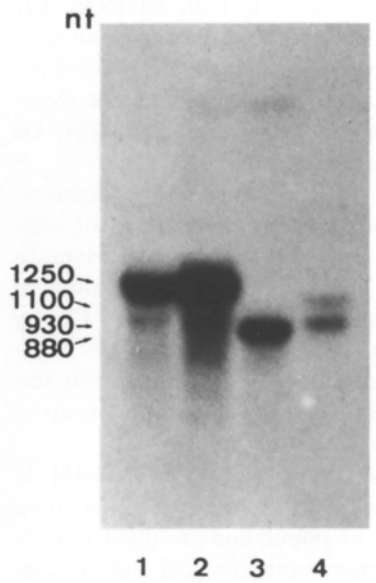

Fig. 5. Autoradiogram showing hybridization of the subcloned 1.1 kbp *Eco* RI-*Hind*III fragment from pP1-3 (pPl-311) to Northern blots of total RNA (25 µg/lane) from progeny of the crosses 83 (*lane 1*), 826-X×An6 (*lane 2*), An6×An10 (*lane 3*), 791-X×81-16 (*lane 4*)

Discussion

The feasibility of a cytoplasm-type assay based on molecular hybridizations for screening natural populations of *P. lanceolata* was indicated by the mtDNA differences regularly observed between fertile and CMS lines of other plants (for review see Hanson and Conde 1985). Restriction fragment analysis of mtDNA from representatives of both *P. lanceolata* male-sterile cytoplasms with *Bam* HI also suggested mitochondrial involvement with CMS. However, the number of different *Bam* HI fragments was rather small compared to maize in which up to 50% of fragments differ (Borck and Walbot 1982). Despite this overall similarity between restriction patterns some differences were apparent. Southern blot hybridizations with cloned DNA fragments as probes demonstrated that the most prominent difference was quantitative rather than qualitative. Although variations in the concentrations of mtDNA sequences between different cytoplasms are known (Abbott et al. 1985), the variation in the case of the 1.8 kbp *Bam* HI fragment cloned in pPl-2 was rather large.

Fortunately, similar hybridizations with pPl-3, a cloned 18.5 kbp *Bam* HI fragment from P-mtDNA, showed unambiguous differences between the mtDNAs from P- and R-plants, apart from a large number of hybridizing fragments common to both mtDNAs. The complex hybridization pattern of pPl-3 must result from the presence of repeated sequences in *P. lanceolata* mtDNA, possibly similar to those found in maize-*Oenothera*- or *Spirodela* mtDNA (Lonsdale et al. 1984; De Hey et al. 1985; Schuster and Brennicke 1986).

The 1.1 and 1.3 kbp P-mtDNA *Bam* HI fragments hybridizing to pPl-3 in particular seemed to be candidates for markers in a cytoplasm type assay. Hybridization of clone pPl-311, derived from pPl-3, to Southern blots of *Bam* HI digested mtDNA detected the 1.1 kbp P-mtDNA fragment, in addition to a number of high-molecular-weight bands in both cytoplasms. This implied that pPl-311 retained at least one of the repeats of pPl-3.

The patterns obtained by hybridizing pPl-311 to Southern blots of *Bam* HI digested total DNAs using plants from a number of populations resulted in only one inconsistency between morphological and molecular analyses. This single deviation could be due to restriction-fragment-length polymorphism or to contamination of the seed lot. However, as the deviant plant did contain the 1,250 nt transcript, the first explanation is preferred.

In the CMS-S cytoplasm of maize, the presence of a unique 6.6 kbp Xho I fragment in the mtDNA is associated with unique transcripts in the mtRNA (Dewey et al. 1986). This is reminiscent of the finding of P- and R-specific transcripts associated with the presence of a unique 1.1 kbp *Bam* HI fragment in the P-cytoplasm of *P. lanceolata,* although the mitochondrial origin of these transcripts is merely indicated by the origin of the probe. Despite the uncertainty of their origin, their existence reinforces the suitability of pPl-311 as a probe. The RNAs are either transcribed from different genes or they represent differentially processed transcripts from the same gene.

Plants with An6 cytoplasm were fertile and never yielded male-sterile progeny, but their ability to act as maintainers of P-type male sterility indicated that they might contain R-cytoplasm. However, according to the cytoplasm type assay they contained P-cytoplasm. This contradiction as well as the presence of their aberrant transcripts hybridizing to pPl-311 could be accounted for by assuming an additional, fertile cytoplasm. The observations concerning plants with An10 cytoplasm should perhaps be explained by assuming yet another fertile cytoplasm. These plants resembled those with An6 cytoplasm in being fertile and never yielding male-sterile progeny. The cytoplasm type assay indicated the presence of R-cytoplasm. However, the finding of two transcripts of 840 and 620 nt hybridizing to pPl-311, instead of the expected 1,100 and 930 nt RNAs, appears to contradict the presence of an R-cytoplasm.

In conclusion, pPl-311 seems to be a suitable probe for a cytoplasm type assay. If pPl-311 is to be used in such an assay, the significance of deviations like the P-plant from the Haren population will have to be evaluated by examining a larger number of plants, taking into account the occurrence of plants with An6 and An10 cytoplasms. The prospect of a more extensive cytoplasmic polymorphism in *P. lanceolata* than has been assumed hitherto opens an interesting perspective for further evolutionary molecular research.

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