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Linear molecules of tobacco ptDNA end at known replication origins and additional loci

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Abstract Higher plant plastid DNA (ptDNA) is generally described as a double-stranded circular molecule of the size of the monomer of the plastid genome. Also, the substrates and products of ptDNA replication are generally assumed to be circular molecules. Linear or partly linear ptDNA molecules were detected in our present study using pulsed-field gel electrophoresis and Southern blotting of ptDNA restricted with 'single cutter' restriction enzymes. These linear DNA molecules show discrete end points which were mapped using appropriate probes. One possible explanation of discrete ends would be that they represent origins of replication. Indeed, some of the mapped ends correlate well with the known origins of replication of tobacco plastids, i.e. both of the *oriA* sequences and—less pronouncedly—with the *oriB* elements. Other ends correspond to replication origins that were described for *Oenothera hookeri*, *Zea mays*, *Glycine max* and *Chlamydomonas reinhardtii*, respectively, while some of the mapped ends were not described previously and might therefore represent additional origins of replication.

Keywords Replication · Chloroplast · PFGE · Tobacco · Origin of replication · Plastome

Abbreviations

IR	Inverted repeat in the plastome
LSC	Large single copy region in the plastome
ORI	Origin of replication
PFGE	Pulsed-field gel electrophoresis
ptDNA	Plastid DNA
SSC	Small single copy region in the plastome

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Introduction

According to the present model plastid DNA (ptDNA) replication starts on circular molecules at two replication origins in θ mode (D-loop) and proceeds—after replication of a monomer—in σ mode (rolling circle) (Kolodner and Tewari 1975). Two replication origins (ORIs), which are able to initiate θ and σ replication, were identified in *Nicotiana tabacum* (tobacco) and termed *oriA* and *oriB* (Kunnimalaiyaan and Nielsen 1997). These ORIs are located in the *inverted repeats*. Therefore both ORIs are present twice in the *N. tabacum* plastome. In addition, in suspension cultures of *N. tabacum* a different replication origin—also in the *inverted repeats*—was found (Takeda et al. 1992). It was possible to delete both *oriA* sequences from all plastome copies and—in different lines—also one of the *oriB* sequences was completely removed (Mühlbauer et al. 2002). ptDNA replication in *N. tabacum* might therefore be possible from only one of the *oriB* elements. Alternatively, additional replication origins and/or replication modes different from θ and σ replication might be used in tobacco. In *Oryza sativa* (rice) different replication origins are used in suspension cultures, coleoptiles and mature leaves, respectively (Wang et al. 2003).

The template for the θ mode and σ mode of replication is a circular molecule. Most textbooks (e.g. Lodish et al. 1995; Buchanan et al. 2000) describe the

ptDNA as circular monomer. But ptDNA consists of a mixture of circular, linear und complex molecules. Already Kolodner and Tewari (1972) found, that only 37% of the ptDNA in *Pisum sativum* (pea) comprised circular molecules whereas the remaining fraction was predominantly linear. Using the FISH technique both circular and linear molecules were detected in *N. tabacum* (45% circular), *P. sativum* (25% circular) and *Arabidopsis* (42% circular) (Lilly et al. 2001). With fluorescence microscopy of ethidium bromide stained DNA molecules large, linear, branched complexes were found in *Zea mays* (maize) (Oldenburg and Bendich 2004), which accounted for 93% of the ptDNA mass, but only for 6% of the number of molecules. The branched complexes could correspond partially to the 17% of the ''unclassified molecules'', which Lilly et al. (2001) observed in *N. tabacum* using FISH.

Using pulsed field gel electrophoresis (PFGE) it was found, that most of the ptDNA of *N. tabacum* remains in the well, whereas a smaller portion could be separated as linear molecules of monomer, dimer, trimer and tetramer size and a submonomer size smear (Backert et al. 1995; Lilly et al. 2001; Swiatek et al. 2003). The part of the ptDNA, which remains in the well, could either be circular DNA or branched complexes, which cannot enter the gel. Though circular ptDNA of *Plasmodium falciparum* (malaria) was observed with PFGE (Williamson et al. 2002), in *Z. mays* the majority of the circular molecules remains in the well and supercoiled DNA was rarely observed. Circular molecules behave differently under different PFGE conditions compared to the linear marker molecules. Therefore they can be identified (Williamson et al. 2002). The circular molecules account for 93% of the number of well-bound molecules in *Z. mays*, but they only account for 7% of the mass of the well-bound DNA (Oldenburg and Bendich 2004).

After digestion of ptDNA of *Z. mays* with restriction enzymes, which cut only once per monomer, the ends of linear molecules were found to be located at sequences, which have homology to the *oriA* and *oriB* of *Oenothera hookeri* and the *oriA* of *N. tabacum*, but also to three additional positions. One of these was assumed to represent the known origin of replication in the *large single copy* region (Oldenburg and Bendich 2004). These data indicate that linear molecules and the linear smear, which probably represents replication intermediates, might represent products of replication and are not artefacts generated in the isolation process.

Taking the published data together it is still not clear, which ptDNA structure is the template for DNA replication in vivo and which role the branched complexes and linear ptDNA molecules might play. Here the ends of linear or partly linear ptDNA molecules in *N. tabacum* were mapped to test, if they correlate with known replication origins.

Materials and methods

Plant material

In vitro cultures of *N. tabacum* cv. Petit Havanna were grown on B_{5mod} medium (Dovzhenko et al. 1998) for 3–4 weeks. Only young, 10–50 mm long leaves and the shoot tips were used for analysis.

Chloroplast isolation

All work was carried out on ice or in the cold room. The plant material was homogenised in isolation medium (0.4 M sorbitol, 50 mM HEPES/KOH pH 8.0, 2 mM EDTA) in a Waring blender, filtered through Miracloth (Calbiochem, Darmstadt) and centrifuged for 3 min and 4000*g*. The pellet was resuspended in isolation medium and loaded on a 40%/80% Percoll gradient (0.4 M sorbitol, 50 mM Hepes/KOH pH 8.0, 2 mM EDTA, 40% or 80% Percoll) in Corex tubes, which was centrifuged for 25 min at 14,800*g*. The band with the intact chloroplasts between the 80% and the 40% phase was transferred to a fresh Corex tube, which was filled up to ca. 25 ml with isolation medium. After a centrifugation at 4000*g* for 5 min, the pellet was resuspended in isolation medium. The density of the chloroplast suspension was adjusted to 5 µg chlorophyll/µl.

PFGE

The chloroplast suspension was mixed with 3 volumes of LMP-Agarose (0.9% InCert-agarose (Cambrex, Verviers), 90 mM mercaptoethanol, 25 mM Na-citrate/HCl pH 7.0, 125 mM EDTA, 0.33 M sorbitol) at 42°C and cast to blocks. When the blocks were solidified, they were incubated for 15 min in lysis buffer (10 mM Tris/HCl pH 8.0, 100 mM EDTA, 1% SLS) at room temperature in the dark. Then the buffer was exchanged against fresh lysis buffer with 0.2 mg/ml proteinase K and incubated for 16 h at 50°C. The blocks were washed twice with TE and 1 mM PMSF and four times with TE at room temperature. The blocks were then ready for PFGE. For analysis of the fragment ends a pre-electrophoresis was necessary. The blocks were loaded on a 1% agarose gel in 1× TBE without ethidium bromide and run for 2 h with 3 V/cm. Then the blocks were cut out and soaked for 30 min in 100 µl of the

buffer, which is recommended for the restriction enzyme used. Then the buffer was exchanged against 100 µl fresh buffer and 20 units restriction enzyme. The digestion was carried out for 16 h at 37°C. The block was washed for 30 min in 0.5× TBE and loaded on a 1% agarose gel in 0.5× TBE without ethidium bromide. The PFGE program consisted of 7 s or 10 s impulses for 24 h at 10°C with 6 V/cm. Afterwards the gel was stained with ethidium bromide.

Southern analysis

The DNA was depurinated for 10 min in 0.125 HCl, than incubated in 0.4 M NaOH for 30 min. The alkaline transfer to a nylon membrane (Hybond-N+, Amersham, Freiburg) was executed according to standard protocols. The probes were labelled with (^{32}PdCTP using the ''prime-a-gene labelling system'' kit (Promega, Mannheim). The probes were hybridised in Church buffer (250 mM Na_2HPO_4/NaH_2PO_4 pH 7.5, 7% w/v SDS) at 65°C over night. The membrane was washed once with 0.5× SSC and 0.1% SDS at 55°C and twice with 0.1× SSC and 0.1% SDS at 60°C. The hybridisation signals were detected using a phosphoimager (Fujifilm FLA3000). The following probes were used: pTB22 (position 59314–64109 of the published *N. tabacum* plastome sequence, Shinozaki et al. 1986, NC_001879), pTB7 (position 18942–29830) (both Sugiura et al. 1986), *rpoA* (position 80455–81468), *ndhD* (position 117569–118713) and *ycf2* (positions 89565–89862 and 152764–153061) for detection of ptDNA and λ *Hin*dIII marker (Fermentas, St. Leon-Rot) for detection of the λ PFGE marker (NEB, Frankfurt/Main).

Results

Linear ends of ptDNA were analysed with PFGE and Southern analysis using the method, described by Oldenburg and Bendich (2004) for the analysis of ptDNA of *Z. mays*. After digestion with the restriction enzymes *Fsp*AI (Fermentas, St. Leon-Rot), *Sfi*I (Promega, Mannheim) or *Sgr*AI (NEB, Frankfurt/Main), which all cut only once in the monomer, the monomer size band was enriched, but no bands smaller than the monomer were visible. These bands were obscured by the linear smear (see Fig. 1A, lane A). Therefore this smear and the linear monomers, dimers, tetramers etc., which enter the PFGE gel, were removed by pre-electrophoresis (see Fig. 1A, lane D). After pre-electrophoresis and digestion with *Fsp*AI, *Sfi*I or *Sgr*AI the monomer size band was again enriched and the amount of the DNA remaining in the well was largely reduced. In addition, bands, which were smaller than the monomer were detected (see Fig. 1A, lane E, F, G).

The size of these fragments made it possible to map the position of the end of linear molecules. By Southern analysis using probes downstream or upstream of the restriction site it could be determined, where the end is relative to the restriction site. E.g. pTB22 was used as upstream probe and *rpoA* as downstream probe for fragments generated by *Sgr*AI digestion (see Fig. 1B, lane G for Southern analysis and Fig. 2 for positions of the restriction sites and probes according to the published sequence of the *N. tabacum* plastome, Shinozaki et al. 1986, NC_001879). To verify the mapping results achieved through the use of an initial probe, additional probes for the *SSC* (*ndhD*) and for the *inverted repeats* (*ycf2*) were used. Fragments of similar size found in independent experiments were combined in Table 1. The orientation of the end relative to the restriction site—upstream or downstream—could correspond to direction of replication, if the end is an origin of replication. In that case, the direction of replication would be in the opposite direction: starting at mapped end and progressing in the direction of the restriction site. E.g. an end detected downstream of the restriction site would mean an initiation of replication in the direction upstream of the mapped position (see Fig. 2 and Table 1).

Besides the bands, which were smaller than the monomer, after digestion with *Fsp*AI and *Sgr*AI also bands—fragments Fs3 and Sg12 (Table 1)—were found, which were bigger than the monomer, but smaller than the dimer (see Fig. 1A, lane F, G). These can be explained as parts of head-to-head or tail-to-tail dimers or multimers (see Fig. 3A, I) or as parts of head–tail dimers or multimers whose single copy regions are in opposite orientations, a configuration which was termed ''flipped'' by Palmer (1983) (see Fig. 3B, D). The expected sizes of linear or circular head–tail dimers or multimers with 'flipped' single copy regions generated by *Fsp*AI digestion are 109 kb and 203 kb, 154 kb and 157 kb in the case of *Sfi*I digestion and 86 kb and 226 kb in the case of *Sgr*AI digestion. Fragments Fs2 and Fs3 respectively Sg8 and Sg12 correspond with these expected sizes. Therefore these fragments are parts of linear or circular head–tail dimers or multimers with 'flipped' single copy regions, which was verified by Southern analysis. Fragments were only detected with those probes expected to hybridise to the molecules in question (e.g. all probes except of *rpoA* in the case of Sg12; see Table 1) There were no fragments bigger than the monomer

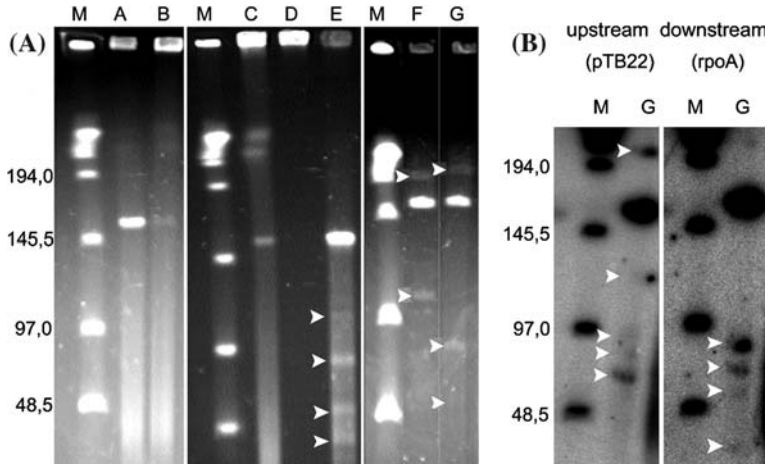

Fig. 1 (**A**) Pulsed-field gel electrophoresis (PFGE): M = λ PFGE marker (NEB), A = *Sgr*AI digestion without pre-electrophoresis, B/C = ptDNA without any treatment, D = with pre-electrophoresis, E = *Sfi*I digestion with pre-electrophoresis, F = *Fsp*AI digestion with pre-electrophoresis, G = *Sgr*AI digestion with pre-electrophoresis, arrowhead = analysed band. (**B**) Southern analysis: F = *Sgr*AI digestion with pre-electrophoresis, (pTB22) = probe to detect fragments upstream of the position restriction site of *Sgr*AI in the plastome sequence, (rpoA) = probe to detect fragments downstream of the position the restriction site of *Sgr*AI, arrowhead = analysed band

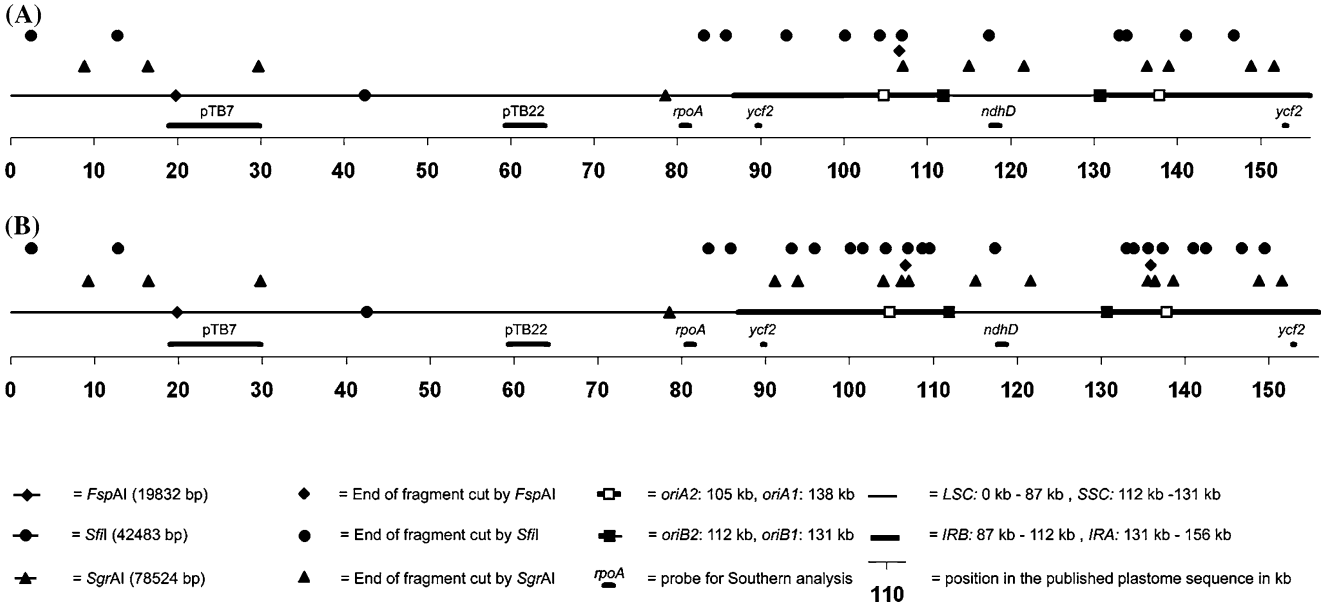

Fig. 2 Mapped ends of linear or partly linear ptDNA molecules. The positions of the fragment classes in Table 1 are shown excluding and including 'flipping'. The numbers indicate the position according to the published sequence of the *N. tabacum* plastome (NC_001879)

found after *Sfi*I digestion, because the fragments expected from 'flipped' dimers or oligomers have nearly the same size as the monomer. The submonomer sized fragments Fs2 and Sg8 (Table 1) were excluded from the mapping experiments since they respresent internal plastome sequences rather than ends of linear molecules (see discussion). The fragments derived from 'flipped' oligomers produced much stronger signals than other submonomer sized fragments, but clearly weaker signals than the monomer sized band (see Fig. 1 lane F, G).

The measured sizes of monomer bands were compared to the size found in the literature (Shinozaki et al. 1986, NC_001879). The standard deviation was 3.6 kb and the biggest absolute deviation was 9.7 kb. Therefore the ends of the detected linear molecules could be mapped with an accuracy of 3.6 kb. Under our conditions PFGE provided linearity between 30

Table 1 Ends of linear or partly linear molecules as detected by Southern blotting of PFGE gels

Enzyme	Position	Size	Standard deviation	Number of fragments found	Direction of replication	Probes				
						pTB22 (LSC)	pTB7 (LSC)	rpoA (LSC)	ndhD (SSC)	ycf2 (SSC)
*Fsp*AI										
Fs1	106,646	69,125	0.36	3	Downstream				Y	
Fs2		102,811	1.29	4			Y		Y	Y
Fs3		198,310	1.42	3			Y		Y	Y
*Sfi*I										
Sf1	12,804	29,680		1	Downstream		Y			
Sf2	2,428	40,055		1	Downstream		Y			
Sf3	85,830	41,793	2.92	2	Upstream	Y		Y		
Sf4	93,086	50,603	3.24	4	Upstream	Y				Y
Sf5	146,783	51,639	2.19	3	Downstream		Y			Y
Sf6	104,309	60,809	1.80	2	Upstream	Y		Y		
Sf7	133,080	65,342	3.64	2	Downstream		Y			
Sf8	117,396	81,026		1	Downstream		Y			
Sf9	133,933	90,996	0.94	3	Upstream	Y		Y	Y	Y
Sf10	107,014	91,302	0.48	4	Downstream		Y		Y	Y
Sf11	100,144	98,278	0.90	3	Downstream		Y			
Sf12	141,039	98,407	0.21	1	Upstream	Y		Y		
Sf13	83,176	115,246	1.51	3	Downstream		Y		Y	
Sf14	2,472	115,401	1.28	2	Upstream	Y		Y	Y	
*Sgr*AI										
Sg1	107,090	28,566	1.08	2	Upstream			Y		Y
Sg2	121,577	43,053		1	Upstream			Y		
Sg3	29,712	48,500	0.44	1	Downstream	Y	Y			
Sg4	136,385	57,861	2.19	5	Upstream			Y	Y	
Sg5	8,791	69,852	0.96	2	Downstream	Y	Y			
Sg6	148,836	70,312	0.68	2	Upstream			Y		
Sg7	151,575	83,033	1.95	2	Downstream	Y	Y			
Sg8		85,731	1.15	7				Y	Y	Y
Sg9	16,389	93,804		1	Upstream			Y		
Sg10	138,970	96,460	4.91	4	Downstream	Y	Y			
Sg11	115,022	119,441		1	Downstream	Y				
Sg12		212,401	6.38	5		Y	Y		Y	Y

75 individual fragments were grouped together to 29 classes. Members within a class are similar in size and were detected by the same probes. We assume that all fragments within a class represent the same DNA sequence. Fragment classes without mentioned position in the plastome sequence are internal parts of multimers. The direction of replication is based on the assumption, that the ends are oris and a rolling circle mechanism is used

and 190 kb. The results of a total of 16 independent experiments are presented with a total of 75 individual fragments different in size from mono– or multimers detected. These were grouped to a total of 29 classes in Table 1, according to sizes and the detection by probes. We assume that each class represents one individual sequence. The ends were mapped according to the published *N. tabacum* plastome sequence (Shinozaki et al. 1986, NC_001879). The positions of the combined fragments are shown in Fig. 2A. Because also molecules were found, whose single copy regions were 'flipped', i.e. the orientation of the *LSC* relative to the two *inverted repeats* is inverted, the mapped positions in one *inverted repeat* were transferred to the other *inverted repeat* (see Fig. 2B).

The majority of the ends of linear or partly linear molecules were mapped to the *inverted repeats*, the smaller part to the *single copy regions* (Fig. 2). For the distribution of the original 75 fragments see Table 2. Considering the standard deviation there is clear correlation of ends of these molecules with the two *oriA*, but a weaker correlation with the two *oriB* elements. In our analysis excluding 'flipping' of the LSC only a single fragment end was mapped to *oriB2*, but when 'flipping' was included the number of ends mapped to both *oriBs* is similar (see Table 2).

Additional ends were mapped to positions different from known *ori*s. There are ends of eight different fragment classes in the *LSC*—mostly near the border of the *inverted repeats*, of two classes in the *SSC*, of five

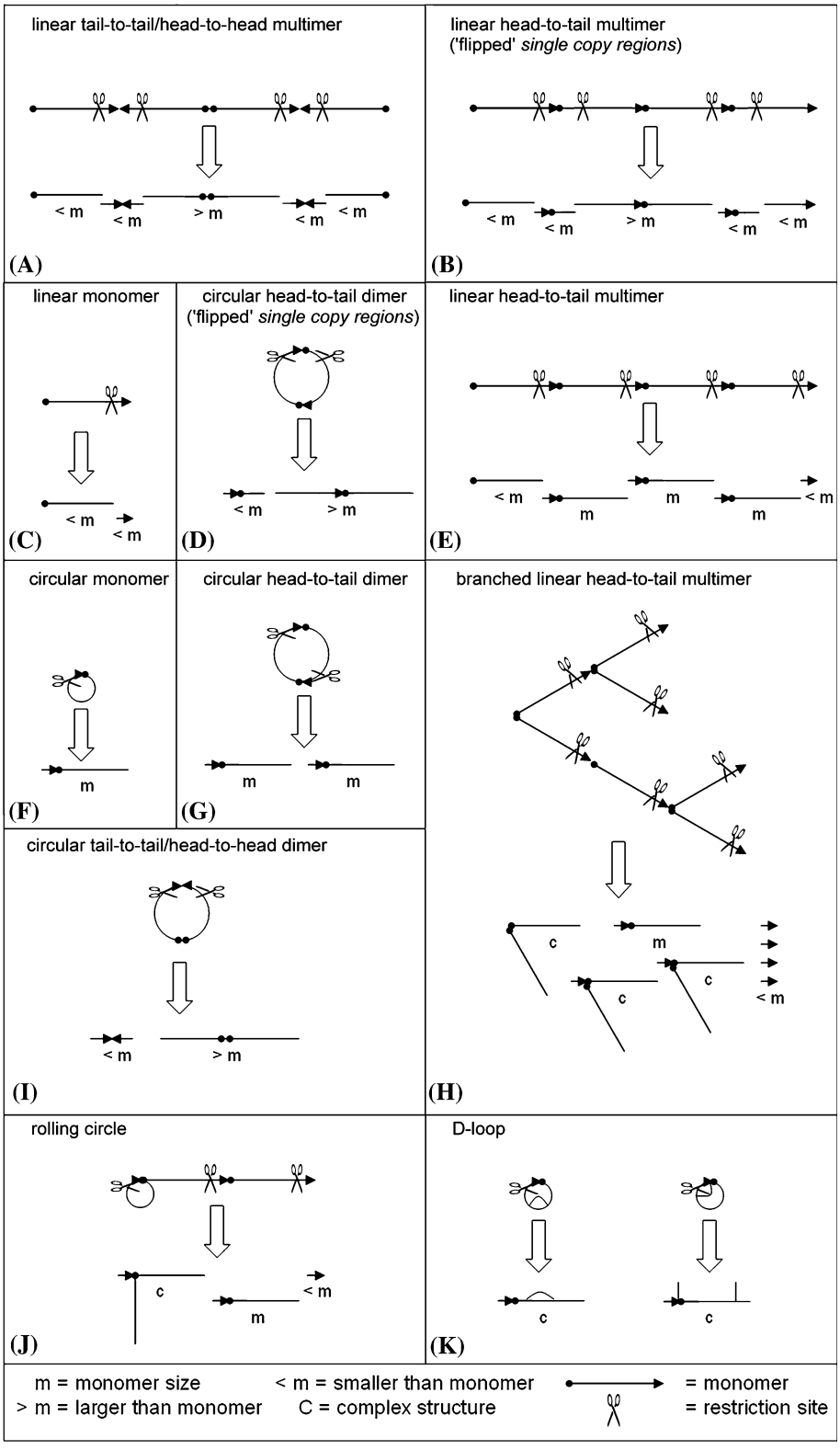

Fig. 3 Explanation for the detected ends. Note that configurations A, B, C and E are removed during pre-electrophoresis. Fragments derived from configuration I were not found. Therefore A is also improbable. Branched molecules (H) could also occur in 'flipped' orientation

in IR_B and of another five in IR_A (including flipping, see Fig. 2B). Even if the biggest absolute deviation is taken into consideration, the number of fragment ends mapped to different loci than known replication origins is only reduced to eight in the *LSC*, three in IR_B and three in IR_A (see Fig. 2A).

Table 2 Correlation of mapped ends of linear or partly linear molecules of ptDNA with known origins of replication and the inverted repeats and single copy regions, respectively

	Including flipping		Excluding flipping	
	Standard deviation	Absolute deviation	Standard deviation	Absolute deviation
oriA1 (IR_A)	9 (3)	17 (6)	21 (7)	32 (11)
oriA2 (IR_B)	12 (4)	15 (5)	21 (7)	32 (11)
oriB1 (IR_A)	5 (2)	12 (5)	5 (2)	23 (9)
oriB2 (IR_B)	1 (1)	13 (6)	6 (3)	24 (10)
Oenothera oriA (IR_A)	6 (2)	20 (7)	10 (3)	36 (13)
Oenothera oriA (*IRB*)	5 (2)	16 (6)	11 (4)	36 (13)
Suspension culture (IR_A)	10 (3)	15 (5)	20 (7)	29 (10)
Suspension culture (IR_B)	10 (3)	16 (7)	19 (6)	32 (12)
rpl16 (LSC)	5 (2)	7 (3)	5 (2)	13 (5)
LSC near IR_A	11 (5)	17 (8)	13 (8)	21 (16)
IR_A	26 (10)	31 (12)	45 (16)	51 (18)
IR_B	23 (9)	26 (11)	46 (17)	49 (19)
LSC	17 (8)	25 (12)	21 (8)	37 (16)
SSC	8 (5)	25 (11)	13 (7)	47 (19)

The total numbers of detected fragments are given, the number of fragment classes are given in brackets

Discussion

Identity of fragment ends

In contrast to what is generally assumed ptDNA molecules are not only circular, but there are also linear molecules (Kolodner and Tewari 1972; Lilly et al. 2001; Oldenburg and Bendich 2004). Here an analysis of the ends of linear or partly linear molecules of ptDNA of *N. tabacum* is presented. Linear ends can not only occur on simple linear molecules (see Fig. 3A, B, C, E), but also on linear branched molecules and rolling circle intermediates (see Fig. 3H, J). All these forms of molecules have been described to exist in plastids (Lilly et al. 2001; Oldenburg and Bendich 2004). After digestion with a restriction enzyme, which cuts once per monomer, a monomer-sized band is enriched (see Fig. 1A, B, lane A, E, F, G). This is consistent with digested linear and branched multimeric molecules (see Fig. 3A, B, C, E, H), but also with circular monomers and multimeric molecules, which can only enter the gel after linearisation (see Fig. 3D, F, G, I). Therefore digestion of the circular monomer-sized or head-tail multimeric molecules can only yield monomer-sized bands. Digested replication intermediates of θ replication—D-loops—result in complex molecules, which are larger than the monomer, but do not produce fragments smaller than the monomer (see Fig. 3K).

The digestion also yields submonomer-sized bands, whose size makes it possible to map the position of the ends in the sequence of the ptDNA. These bands are not part of the linear smear or the linear molecules observed with PFGE (see Fig. 1, lane B), because these molecules were removed before digestion. They are part of the ptDNA, which remains in the well and enters the gel only after digestion. The submonomer-sized fragments produced by digestion of the linear smear and the linear molecules or of the whole ptDNA of *Z. mays* were identical (Oldenburg and Bendich 2004). Therefore the fragments originating from digested linear molecules and digested DNA from the well are also expected to be identical in *N. tabacum*.

In addition to the monomer and submonomer-sized fragments, DNA molecules were found, which were between monomer and dimer size (see Fig. 1A, lane F, G, Table 1). These bands could originate from restriction of a multimeric molecule, whose monomers were not linked head-to-tail, but head-to-head or tail-to-tail (see Fig. 3A, I) or molecules, which were linked head-to-tail, but whose *single copy region* are 'flipped' (see Fig. 3B, D). Head-to-head or tail-to-tail multimers were observed by Kolodner and Tewari (1979) in *P. sativum* and Lilly et al. (2001) in *N. tabacum*, but not by Oldenburg and Bendich (2004) in *Z. mays*. But Oldenburg and Bendich (2004) and Palmer (1983, in *Phaseolus vulgaris*) found head-to-tail multimers with 'flipped' *single copy regions*. Our results show, that ptDNA of *N. tabacum* consists of head-to-tail multimers with both orientations of the *single copy region*s (see Table 1). The fragments, which were larger than the monomer (Fs3, Sg12, Table 1, Fig. 3B, D), and the corresponding submonomer sized fragments (Fs2, Sg8, Table 1, Fig. 3B, D) are internal parts of multimers. Therefore they cannot be used to map ends of linear molecules.

Alternative explanations for ends could be breakage points of the DNA. Because distinct bands were detected, these ends cannot be the result of random

breaks. Therefore the breaks would have to occur at defined positions possibly where the ptDNA is bound to membranes. In proplastids the PEND protein binds the ptDNA to the inner envelope membrane (Sato et al. 1993). PEND recognises a defined sequence. The *Brassica napus* BnPEND's binding site is found 24 times in the plastome of *N. tabacum* (Wycliffe et al. 2005), but there is no strong correlation with the linear ends described in this work. MFP1, another DNA binding protein, appears to bind the ptDNA to the thylakoids of suspension culture and leaf cell plastids in *Arabidopsis*. But MFP1 does not bind to a distinct sequence (Jeong et al. 2003). Topoisomerase II is covalently bound to the DNA (Wang 1996) and DNA isolation conditions can induce breakage of the DNA (Oldenburg and Bendich 2001). But it is unlikely that these would result in distinct bands, because for this topoisomerase II would have to be distributed non-randomly on the ptDNA. Another possible source of fragment ends would be secondary restriction sites of the restriction enzyme *Sgr*AI, which are known to occur, if the enzyme concentration is too high (Bitinaite and Schildkraut 2002). But there is no correlation between the observed ends and the secondary restriction sites of *Sgr*AI. Ends could also be produced at recombination branch points, e.g. on recombination hotspots. We cannot rule out, that some ends are caused by recombination, but there is connection of origins of replication and recombination hotspots in several species. In *N. tabacum* a recombination hotspot could be in the *trnI* intron (Johnson and Hattori 1996), but here also *oriA* is located (Kunnimalaiyaan and Nielsen 1997). In *Chlamydomonas reinhardtii* a recombination hotspot is near *psbA* (Newman et al. 1992), but here also a novobiocin-insensitive origin of replication was detected (Woelfle et al. 1993). Also in T4 phages origins of replication are recombination hotspots (Yap and Kreuzer 1991) too.

Therefore the observed linear submonomeric sized bands—except of the internal fragments of head-to-tail multimers with 'flipped' *single copy regions*—represent true ends of at least partly linear molecules, which most probably are products of DNA replication. If the rolling circle mechanism is used, the ends would be at the position of the origin of replication. If internal origins of replication as e.g. in T4 phages (review Kreuzer 2000) are used, the ends would be the termination points of replication.

Correlation with known origins of replication

In *N. tabacum* the two origins of replication—*oriA* and *oriB*—in the *inverted repeats* were characterised by 2D gel electrophoreses, electron microscopy, primer extension mapping and in vitro replication (Kunnimalaiyaan et al. 1997; Kunnimalaiyaan and Nielsen 1997; Lu et al. 1996). This made it possible to define the minimal origin of replication defined as sequence element that is able to initiate replication in vitro. Therefore the exact positions of the ORIs are known. In suspension culture cells Takeda et al. (1992) identified an additional origin of replication by mapping of the D-loop using electron microscopy. But the minimal origin was not defined in this study.

A considerable number of fragments were mapped to the region of both *oriA*s (see Fig. 2 and Table 2). Therefore there is a clear correlation between known replication origins and the ends of linear molecules described here. The correlation to the two *oriB*s is weaker—especially, if only the standard deviation is considered (see Fig. 2 and Table2), but still significant. If only the original data and not the extrapolation to include flipping are considered, then more ends are found at the position of *oriB1*. *oriB1* is in the coding region of the essential gene *ycf1*, whose function is still not known (Drescher et al. 2000). *oriB2* is in the reading frame *orf 350* and—unlike *oriB1*—it can be deleted from all plastome copies (Mühlbauer et al. 2002). It is not clear, if these differences between the *oriB*s reflect biological differences, e.g. if bordering SSC sequences or the *ycf1* gene have any influence.

The replication origin in suspension culture cells is 3′ of the 23S rRNA gene in *N. tabacum* (Takeda et al. 1992) and *O. sativa* (Wang et al. 2002). Because the minimal origin is not defined, the exact position of this replication origin is not known. The two copies of this ORI are located approximately at the positions 105 kb and 134 kb of the published sequence (Shinozaki et al. 1986, NC_001879) midpoint between *oriA* and *oriB* with a distance to each of them of ca. 3.5 kb. On these positions a considerable number of ends were mapped (see Table 2). But it is not possible to rule out, that these ends originated by activity of *oriA* or *oriB*, because the distance between these ORIs is too small for the resolution of mapping by PFGE.

Mapping of additional linear molecule end points

We detected 14 fragments (see Table 1 and Fig. 2), which do not correlate with any of the known replication origins—even when the biggest absolute deviation is considered. These ends are preferably found near the borders of the *inverted repeats* and the *large single copy* region. These ends might be additional sites, which are relevant for replication in *N. tabacum*.

In *Z. mays* ends were also found, which do not correlate with the described replication origin in the *large single copy* region (Carrillo and Bogorad 1988), but with sequences homologous to the *oriA* and *oriB* of *O. hookeri* and to the *oriA* of *N. tabacum* (Oldenburg and Bendich 2004). *oriB* of *Oenothera* and *oriA* of *N. tabacum* are mapped to the *trnI* gene. Chiu and Sears (1992) did not define the exact position of *oriB* in *Oenothera*. Hornung et al. (1996) assumed it would be in the spacer between *rrn16* and *trnI*, but did not present any data showing replication initiation there. In *N. tabacum oriA* is mapped to the intron of *trnI* (Kunnimalaiyaan and Nielsen 1997). Therefore *oriA* of *N. tabacum* and *oriB* of *Oenothera* could be homologous sequences at the same position. Both *oriA* in the *trnI* intron and the spacer between *rrn16* and *trnI* can be removed from all copies of the *N. tabacum* plastome (Mühlbauer et al. 2002). But ends can be mapped to this region (see Fig. 2 and Table 2). There could be an ORI in this region, which is homologous in *N. tabacum*, *Oenothera* and *Z. mays*.

Are there also ends at the position of *oriA* of *Oenothera* in *N. tabacum* as in *Z. mays*? *oriA* lies 1–2 kb upstream of *rrn16* in *Oenothera* (Chiu and Sears 1992), which would correspond to the position 101–102 kb of the published sequence of the *N. tabacum* plastome (Shinozaki et al. 1986, NC_001879) in the *inverted repeat B* respectively 141–142 kb in the *inverted repeat A*. Ends of at least partly linear molecules were mapped to those positions (see Fig. 2 and Table 2). These numbers are similar to the ends at the positions of known origins of replication of *N. tabacum*. Therefore there could be an origin of replication, which is a homologue to the *oriA* of *Oenothera*.

Ends were also found near the border of the two *inverted repeats* and the large single copy region. There is no report of an origin of replication in this area in the *inverted repeats*. But in *G. max* (soybean) two ORIs are reported to be in the *large single copy* region near one *inverted repeat* and the *rpl16* gene (Hedrick et al. 1993). Also in *Z. mays* (Carrillo and Bogorad 1988) and *C. reinhardtii* (Lou et al. 1987) a putative origin of replication is near *rpl16*. This gene is at the position 83.7–85 kb of the published sequence (Shinozaki et al. 1986, NC_001879) near the *inverted repeat B* in the *N. tabacum* plastome. Ends were found at this position (see Fig. 2 and Table 2). There is no counterpart in the literature, i.e. no description of an origin of replication in the *large single copy* region near the border to the *inverted repeat A*, but ends were found in this area (see Fig. 2 and Table 2).

There are also further hints in the literature to additional origins of replication. With hybridisation of enriched nascent ptDNA to a *Bam*HI liberary of ptDNA there are not only signals to the known replication origins *oriA* and *oriB*, but also to additional fragments (Lu et al. 1996). These signals were weak compared to the known ORIs, but Lu et al. (1996) used young leaves of 2 months old greenhouse plants, whereas in our work young, 10–50 mm long leaves and the shoot tips containing the leaf primordia of in vitro grown plants were used, which might explain the observed discrepancy. Also in the report, which shows σ mode replication starting at *oriA* and *oriB*, older, 75–100 mm long leaves of 5–7 week old plants were used (Lugo et al. 2004). In *Spinacia oleracea* (spinach) the highest replication activity is in the 5 mm of the leaf tip of 20 mm long leaves (Lawrence and Possingham 1986). Also in *Arabidopsis thaliana* the highest replication activity is found in the leaf primordia (Fujie 1994). The detected differences in the replication origin usage could hint to tissue and development specific differences in *N. tabacum* as they were observed in *O. sativa* (Wang et al. 2003).

An additional possible cause for observed differences could be that the minimal ORIs in *N. tabacum* were analysed using plasmids (Kunnimalaiyaan and Nielsen 1997; Lugo et al. 2004). In *E. coli* the binding site requirements for the ORIs on plasmids and the bacterial chromosome are different (Asai et al. 1998; Weigel et al. 2001). Therefore there could also be differences in the requirements for an origin of replication for the plastid chromosome and a plasmid.

The model of ptDNA replication

The development dependent differences and the differences between chromosomal and plasmid DNA could also explain why the observed correlation of the ends on linear or partly linear molecules with origins of replication is not compatible with the current model of plastid replication (Kolodner and Tewari 1975). θ mode replication cannot produce linear molecules or linear ends. σ replication should start 180° afar of *oriA* and *oriB*. Therefore the ends must be in the *large single copy* region approximately at the positions 26.8, 33.9, 52.8 and 59.8 kb of the published sequence (Shinozaki et al. 1986, NC_001879). But we found only a single end in this area (see Table 1 and Fig. 2), whereas the majority of the ends are at the positions of known replication origins or near the borders of the *inverted repeats* and the *large single copy* region. The observed θ mode replication could be employed only for maintenance of ptDNA in developed leaves, whereas some other replication mode is responsible for DNA synthesis in the early stages of leaf development.

In human mitochondria θ mode replication is only used for maintenance and coupled leading- and lagging strand synthesis for rapid increase of mtDNA (Holt et al. 2000). In contrast a rolling circle mechanism starting at the known origin of replication or at any of the mapped putative origins of replication can produce the described ends. But also other recombination dependent replication mechanisms could produce such ends (see Oldenburg and Bendich 2004).

Concluding remarks

The occurrence of defined fragment lengths in the plastome of *N. tabacum* confirms similar observation in *Z. mays* (Oldenburg and Bendich 2004). In the case that they indicate origins of replication, this would mean that besides the known origins of replication a considerable number of additional ORIs exist in the plastome of *N. tabacum*. Fine mapping of the detected ends and targeted inactivation of these elements might elucidate functionality of known and novel origins of replication.

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