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Mapping Major Replication Origins on the Rice Plastid DNA

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Abstract

To maintain and to differentiate into various plastid lineages, replication of the plastid DNA (ptDNA) and division of the plastid must take place. However, replication initiation of the ptDNA has been less understood. The present study describes identification of the initiation region (origin) of ptDNA replication in the rice cultured cells. RNA-primed newly replicated DNA strands pulse-labeled with bromodeoxyuridine were isolated and size-fractionated. Locations of these nascent strands on the ptDNA determined the two major origin regions around the 3' region of each 23S rDNA in the inverted repeats (IR_A and IR_B). Two-dimensional agarose gel electrophoresis of the replication intermediates suggested that replication from each origin proceeds bidirectionally. This contrasted to replication by the double D-loop mechanism.

Keywords: plastid DNA, replication, replication origin, rice, two-dimensional agarose gel electrophoresis.

Abbreviations

BND, benzoylated-naphthoylated DEAE; BrdU, 5-bromo-2-deoxyuridine; D-loop, displacement loop; EM, electron microscopy; EtBr, ethidium bromide; IR_A, inverted repeat A; IR_B, inverted repeat B; LSC, large single copy; SSC, small single copy; ptDNA, plastid DNA; rDNA, rRNA gene.

Introduction

Plastids are plant organelles derived from a progenitor proplastid, and give rise to various organelles including chloroplast, chromoplast, amyloplast and leucoplast under control of the host cells. To maintain and to differentiate into various plastid lineages, replication of the plastid DNA (ptDNA) and division of the plastids must take place. Recent reports (Osteryoung *et al.*, 1998; Colletti *et al.*, 2000) showed that division of the plastids occurs, in principle, like that of *E. coli*, in that plant homologues of bacterial cell division proteins FtsZ and MinD play important roles. Replication of ptDNA, however, has been less understood.

As to initiation of ptDNA, several experimental approaches have been made. Analysis of electron microscopic (EM) images of pea ptDNA revealed two regions with displacement loop (D-loop) locating on opposite strand, which was thought to be a replication intermediate (Kolodner and Tewari, 1975). These two D-loops extended toward each other until replication reaches the initiation sites of the D-loop on opposite strands, then Cairns-type (theta structure) replication starts (Kolodner and Tewari, 1975). Therefore, initiation sites of D-loops have been considered as initiation sites (origins) of unidirectional replication of ptDNA. Origins of this type of replication have been reported for several plant and algal species (Waddell *et al.*, 1984; Chiu and Sears, 1992; Kunnimalaiyaan and Nielsen, 1997b).

Different approaches have also been made for mapping origins of ptDNA, including *in vitro* replication using plastid or chloroplast extracts (Gold *et al.*, 1987; Carrillo and Bogorad, 1988; Hedrick *et al.*, 1993; Reddy *et al.*, 1994), two-dimensional agarose gel (2D gel) analysis of replication interme-

diates (Hedrick *et al.*, 1993; Nielsen *et al.*, 1993; Lu *et al.*, 1996; Kunnimalaiyaan *et al.*, 1997), and functional analysis of mutant ptDNA (Day and Ellis, 1984). However, different experimental approaches did not always result in the same location of the origins. For example, soybean origin regions determined by *in vitro* replication did not all correspond to the origins determined by 2D gel analysis (Hedrick *et al.*, 1993). Considering together with other examples (e.g., Takeda *et al.*, 1992), it seems necessary to employ several different experimental approaches for determination of the origin of ptDNA replication. In this respect, detailed analysis of *in vivo* replication intermediates might be of particular importance.

In the present study, we aimed to identify and characterize the replication origin of ptDNA of the rice suspension-cultured cells. For this purpose, we used two different experimental procedures that have not commonly been employed for plant studies. First, we characterized RNA-primed nascent DNA strands derived from the origin region. The bromodeoxyuridine (BrdU)-labeled nascent DNA chain was isolated using anti-BrdU antibody, digested with λ-exonuclease to remove nicked and degraded DNA, and then size-fractionated. These procedures could result in enrichment of intact RNA-primed replicating DNA strands. Second, the putative origin region was subjected to neutral (for the first dimension)/alkaline (second dimension) 2D agarose gel electrophoresis to see size distribution of the replication intermediates and direction of replication. These experiments revealed a major origin region at around 3' end of the 23S rDNA in each of IR_A and IR_B.

Materials and Methods

Maintenance of suspension-cultured cells of rice (Oryza sativa L.)

Suspension-cultured cells established from the rice *Oryza sativa* L. var. Nipponbare were maintained at 25 ℃ in AA medium (Müller and Grafe, 1978) containing 3% (w/v) sucrose. An aliquot of cultured cells was maintained by placing in the 90 ml fresh medium once a week. Lump cells were removed by filtration through a nylon net (200 μm pore size) every two weeks. Four-day-old cells after the passage were routinely used in this study.

Isolation of DNA

Cells were harvested at the 4th day after transfer into fresh medium. Total DNA was isolated as described by Lodhi *et al.* (1994). Plastid was prepared according to the procedure described by Heinhorst *et al.* (1990), and ptDNA was extracted according to the method described by Hirai *et al.* (1985). In some cases, ptDNA was further purified through cesium chloride density-gradient centrifugation (Zhao *et al.*, 1997).

Labeling with BrdU and immuno-detection of the BrdU-labeled DNA

Rice cells were cultured in the presence of 22 μM BrdU for the indicated time periods. Plastid DNA was then isolated as described above. Appropriate amounts of the BrdU-labeled ptDNA were blotted directly or after agarose gel electrophoresis onto a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech). The membrane was treated with 0.4 M NaOH containing 1.5 M NaCl for 10 min, washed two times with 2 × SSC, and baked at 80 ℃ for 10 min. Immuno-detection of the BrdU-containing DNA with anti-BrdU antibody (MBL Co. Ltd., Japan) was carried out by using ECL Western Blotting Detection System (Amersham Pharmacia Biotech).

Isolation of BrdU-labeled DNA using anti-BrdU antibody

BrdU-labeled ptDNA (50 μg) dissolved in 200 μl TBSE buffer (10 mM Tris-HCl, pH 9.5, containing 150 mM NaCl and 0.1 mM EDTA), was heat-denatured, and subjected to immuno-adsorption using anti-BrdU antibody and protein A+G agarose beads (Oncogene Research Products) as follows. BrdU-labeled ptDNA was mixed with 4.5 μg anti-BrdU antibody and incubated at 4 ℃ for 3 h with occasional mixing. Protein A+G agarose beads (50 μl), previously washed with 10 volume of TBSE buffer, was then added, and incubated for further 3 h. The beads were spun down, washed 3 times with 10 volume of TBSE buffer, and resuspended in 400 μl proteinase K buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM EDTA and 0.5% SDS). Proteinase K was then added to a final concentration of 0.5 mg ml^{-1}, and incubated overnight at 37 ℃. BrdU-DNA was recovered by centrifugation. The DNA in the supernatant was extracted twice with phenol/chloroform (1:1), once with chloroform/isoamyl alcohol (24:1), and ethanol precipitated. The BrdU-DNA preparation was further digested with λ-exonuclease to eliminate nicked and degraded DNA. Prior to this digestion, 5' end of DNA was phosphorylated using T4 polynucleotide kinase and ATP. After phenol/chloroform (1:1) extraction and ethanol precipitation, DNA was dissolved in 67 mM Glycine-KOH, pH 9.4, containing 2.5 mM $MgCl_2$ and 50 μg ml^{-1} BSA, and digested with 15 U λ-exonuclease (New England Biolab) at 37 ℃ over-

night. ^{32}P-labeled linearized plasmid DNA (pBS) was included in the reaction mixture as an internal control to monitor the digestion. RNA-primed BrdU-containing ptDNA thus obtained was further size-fractionated by 1% agarose gel electrophoresis.

Enrichment of replication intermediates by benzoylated-naphthoylated DEAE (BND)-cellulose chromatography

Enrichment of replication intermediates containing single-stranded DNA regions was performed by BND-cellulose column chromatography essentially as described by Huberman (1993). Total DNA from the rice cells was digested overnight with an appropriate restriction enzyme. After digestion, DNA was precipitated with ethanol and dissolved in NET buffer (10 mM Tris-HCl, pH 8.0, containing 800 mM NaCl and 1 mM EDTA). The digested DNA was adsorbed to BND-cellulose column (Sigma), which was pre-equilibrated with the same buffer. The column was washed with 10 volume of NET buffer and then DNA was eluted with NET buffer containing 1.8% (w/v) caffeine. Eluted DNA was ethanol-precipitated, and stored at −80 ℃.

Two dimensional (2D) agarose gel electrophoresis of replication intermediates

DNA purified by BND-cellulose column chromatography (25 to 30 μg) was loaded onto 0.4% agarose gel for the first dimension. Electrophoresis was performed in 40 mM Tris-acetate containing 1 mM EDTA and 0.1 $\mu g\,ml^{-1}$ EtBr at 0.72 V cm^{-1} for about 30 h, as described by Little *et al.* (1993) and Huberman (1993). For electrophoresis in the second dimension, the gel strip of the first dimension was placed on top of 1% agarose gel. The agarose gel was placed in circulating alkaline electrophoresis buffer (40 mM NaOH containing 2 mM EDTA) and incubated at room temperature for 1 h. Then electrophoresis was started at 0.56 V cm^{-1} for 37 h. After electrophoresis, DNA was transferred onto a nylon membrane and hybridized with various probes. Probe labeling, hybridization, and detection were performed using Gene Images Labeling and Detection Kit (Amersham Pharmacia Biotech).

Results

Incorporation of BrdU into replicating ptDNA

One way to determine origin of DNA replication is to identify newly replicated short DNA fragment. Incorporation of BrdU into DNA during replication is a useful method for marking newly replicated DNA. To our knowledge, however, studies showing incorporation of BrdU into ptDNA have not been reported, except for a unicellular chrysophyte (Nerozzi and Coleman, 1997). Therefore, we examined incorporation of BrdU into ptDNA in the rice cultured cells.

ptDNA (ng)	1000	1000	1000	1000	1000
BrdU-labeled ptDNA(ng)	0	5	25	50	100

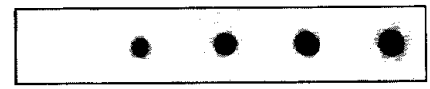

Fig. 1 Incorporation of BrdU into rice ptDNA. Rice cells were labeled with BrdU for 24 h, and the ptDNA was prepared. Indicated amounts of the BrdU-labeled ptDNA were mixed with 1000 ng of non-labeled ptDNA, spotted onto a nylon filter, and incubated with anti-BrdU antibody. BrdU-bound antibodies were detected using HRP-linked anti-IgG antibody and ECL Western Blotting Detection System (Amersham Phamacia Biotech).

Rice cells were cultured for 24 h in the presence of 22 μM BrdU, and ptDNA was prepared. Incorporation of BrdU into ptDNA was examined by using anti-BrdU antibody. Various amounts of BrdU-labeled and non-labeled ptDNA were spotted onto a nylon filter and incubated with the antibody. BrdU-DNA-bound antibody on the filter was detected by using HRP-linked anti-IgG antibody. As shown in **Fig. 1**, anti-BrdU antibody bound to BrdU-labeled ptDNA with increasing intensities in proportion to the amount of DNA spotted, while no binding to the nonlabeled DNA was detected. Thus, BrdU was incorporated into ptDNA in the rice cultured cells. We next performed similar immunodetection using ptDNA labeled with BrdU for 30 min, 60 min and 24 h. Equal amounts of the labeled ptDNAs were digested with *Sal*I, separated on an agarose gel and blotted onto a nylon filter. BrdU-containing DNA fragments were detected by binding of anti-BrdU antibody as described above. As shown in **Fig. 2**, incorporation of BrdU into ptDNA increased with the period of labeling.

Mapping initiation region of ptDNA replication by labeling with BrdU

To determine origin region of replication, rice cells were pulse-labeled for 30 min with BrdU. The BrdU-labeled nascent strands were purified by binding to anti-BrdU antibody. As described in Materials and Methods, λ-exonuclease was used to eliminate nicked and degraded DNA molecules

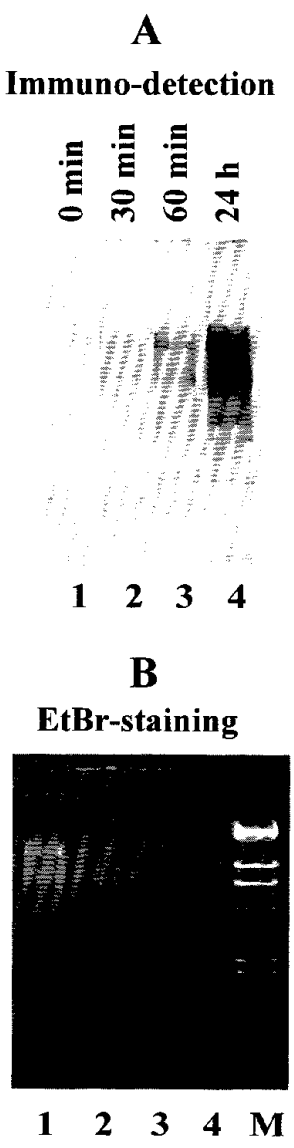

Fig. 2 Immuno-detection of BrdU-labeled ptDNA fragments. (A) ptDNAs were prepared from rice cells labeled with BrdU for 0 min (lane 1), 30 min (lane 2), 60 min (lane 3) and 24 h (lane 4). The BrdU-labeled ptDNA (3 μg each) was digested with *Sal*I, separated on a 0.8% agarose gel, blotted, and detected using anti-BrdU antibody as described in **Fig. 1**. (B) shows EtBr-staining of the gel. Lane M, *Hind*III-digested λ-DNA as a size marker.

possibly generated during DNA preparation. Complete digestion was monitored by including ^{32}P-labeled plasmid DNA in the reaction mixture (data not shown). The nuclease does not attack RNA-primed DNA molecules (Abdurashidova *et al.*, 2000). Thus, RNA-primed nascent DNA strand remains undigested.

RNA-primed BrdU-containing DNA strands were further size-fractionated by agarose gel electrophoresis, and the BrdU-DNA ranging from 0.7 to 0.9 kb was extracted. Because this size was expected to be larger than unligated Okazaki fragment of the lagging strand, although it was uncertain if it existed. In addition, for determination of replication origin, the nascent DNA should be short and origin-proximal.

To delimit the ptDNA region from which the nascent DNA derived, the 0.7-0.9 kb nascent BrdU-DNA and total BrdU-DNA without size-fractionation were hybridized with ptDNA digested with *Bgl*II, *Sac*I or *Sal*I. As shown in **Fig. 3A**, the 0.7-0.9 kb nascent DNA probe preferentially hybridized with 5.3 kb fragment in the *Bgl*II digest, with 8.7 and 11.4 kb fragments in the *Sac*I digest, and with 7.5 and 14.7 kb fragments in the *Sal*I digest. In contrast, the total BrdU-DNA probe hybridized with much more fragments, indicating that BrdU was incorporated randomly into replicating DNA over the entire ptDNA region. Since the size-fractionated 0.7-0.9 kb probe represents RNA-primed short nascent strands synthesized from origin, the fragments that the probe hybridized must contain origin of replication.

However, it should be noted that the 0.7-0.9 kb probe hybridized, although to a lesser extent, with several fragments in addition to those described above (marked by asterisks in **Fig. 3A** and **B**). They distributed randomly over the ptDNA genome and did not overlap. It is not clear at present whether these signals represent multiple replication origins or contamination of nuclear or mitochondrial DNAs.

Considering together, these results indicated that within or in the very vicinity of the overlapping region of the DNA fragments that the 0.7-0.9 kb DNA probe preferentially hybridized, the preferred or major origin of ptDNA replication locates (**Fig. 3B** and **C**). According to the registered sequence of the rice ptDNA (GenBank/EMBL/DDBJ Accession no. NC_001320), the overlapped region corresponded to 2.3 kb DNA region including 3' end of the 23S rDNA in each of IR_A and IR_B.

In tobacco, a pair of D-loop type replication origins has been mapped in each IR; *ori*A between the 16S rDNA and the 23S rDNA, and *ori*B downstream of the 23S rDNA (Kunnimalaiyaan and Nielsen, 1997b). By contrast, there seems to be only one major origin region in each IR in the rice ptDNA, the position of which corresponds to that between *ori*A and *ori*B in tobacco. Therefore, we next intended to know how replication begins and proceeds by 2D agarose gel electrophoresis.

2D agarose gel electrophoresis of replication intermediates

Fig. 4 shows schematic representation of neu-

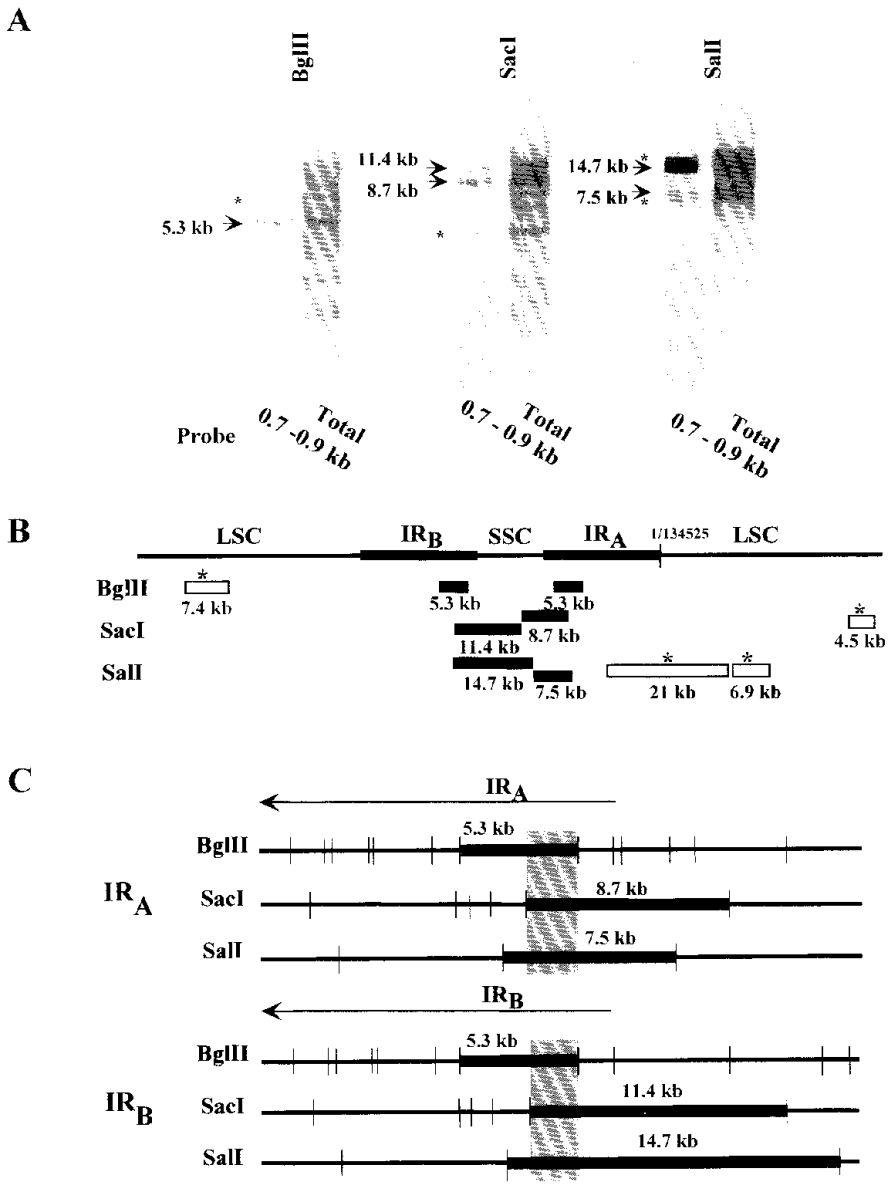

Fig. 3 Location of the BrdU-labeled newly replicated nascent DNA strands on the ptDNA. (A) BrdU-labeled RNA-primed nascent strands (see text) were size-fractionated, and those with sizes of 0.7-0.9 kb and total BrdU-labeled nascent strands (total) were used as probes for hybridization with ptDNA (5 μg each) digested with *Bgl*II, *Sac*I and *Sal*I. Probe labeling and detection were carried out using Gene Images Labeling and Detection Kit (Amersham Pharmacia). (B) Summary of hybridization of the BrdU-labeled nascent strands. Positions of the preferentially hybridized fragments are depicted by filled boxes. The fragments that the probe hybridized to a lesser extent (marked by asterisks in A) are also depicted by open boxes with asterisks. (C) Restriction map around the IR_A and IR_B regions and the fragments to which the 0.7-0.9 kb probe preferentially hybridized. Filled boxes with their sizes indicate the hybridized fragments. Overlapping region (about 2.3 kb) of the hybridized fragments is indicated by gray zone.

tral/alkaline (N/A) 2D gel electrophoresis. N/A 2D gel method detects the direction of replication fork movement through a particular restriction fragment (Huberman *et al.*, 1987; Huberman, 1993). In this approach, restriction enzyme-digested DNA is separated on a first dimension gel according to their molecular weight, that is, the extent of replication. The second dimension gel is run under alkaline condition to denature DNA. Because template DNA strands have a constant size regardless of the extent of replication, they generate horizontal signal after hybridization with a specific probe. However, replicating nascent strands vary in their length, and thus generate a diagonal signal. If DNAs from different positions of a restriction fragment are used as probes (e.g., probes 1, 2 and 3 in **Fig. 4**), the

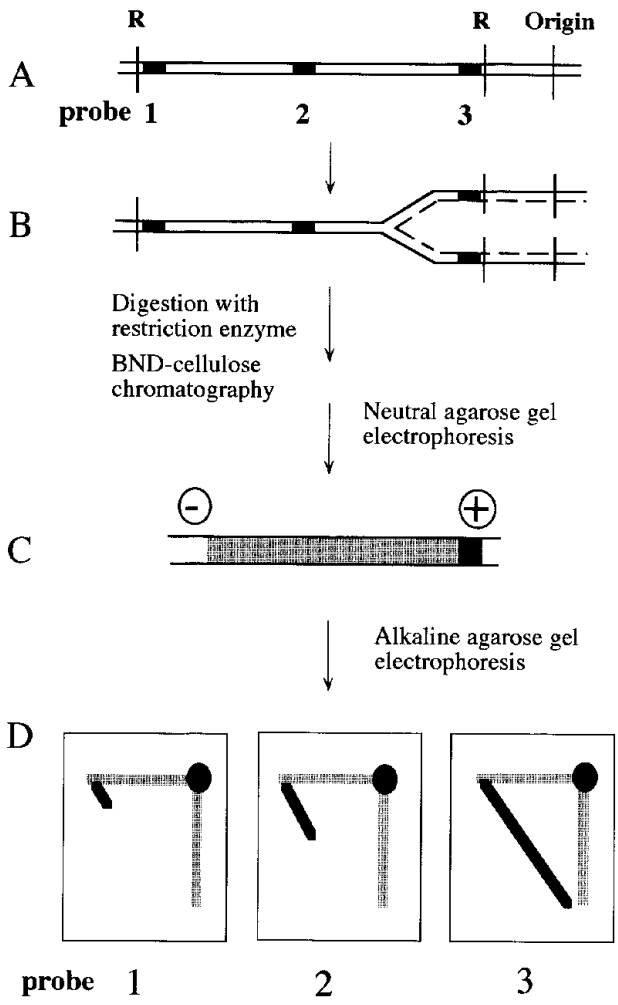

Fig. 4 Schematic representation of N/A 2D agarose gel electrophoresis for determination of replication direction. At the top (A), the positions of restriction sites (R), probes 1, 2 and 3, and origin are shown. Replication intermediates (B), are digested with the restriction enzyme, enriched by BND-cellulose chromatography and subjected to neutral (C, for the first dimension) and alkaline (D, for the second dimension) agarose gel electrophoresis. Different hybridization patterns will be detected with different probes 1, 2, and 3. Origin-distal probe 1 detects only the long nascent DNA strands, whereas origin-proximal probe 3 detects the full range of nascent strands.

direction of replication can be deduced. The origin-distal probe (probe 1) detects only the long nascent strands, whereas the origin-proximal probe (probe 3) detects the full range of nascent strands.

Replication intermediates around the origin regions were analyzed by N/A 2D gel electrophoresis. Direction of replication was examined for the 5.3 kb *Bgl*II fragment, 4.8 kb, 4.1 kb and 4.4 kb *Dra*I fragments as shown in **Fig. 5**. In the 5.3 kb *Bgl*II fragment, only long nascent strands were detected with the 593 bp probe, suggesting the probe region to be origin-distal. This shows that replication fork moves in the opposite direction of 23S rDNA transcription. However, nascent strands extending in both directions were detected in the *Dra*I 4.8 kb and *Dra*I 4.4 kb regions, because full range (from short to long) of nascent strands was detected using probes corresponding to either end of each fragment. This means that in these regions replication proceeds from both ends. These results, together with the data obtained by nascent strand analysis (**Fig. 3**) suggested the replication fork movement shown in **Fig. 6**. Two replication origins locate around the 3' end of the 23S rDNA in the two IRs, and replication from each origin proceeds in both directions.

Discussion

In the present study, two major replication origins of the rice ptDNA were mapped around the 3' region of each 23S rDNA in IRs, based on localization of RNA-primed, BrdU-labeled nascent DNA strands. It seems that ptDNA replication origins may have a general characteristic of proximity to the rDNAs but a flexible positioning relative to them. In ptDNA from tobacco seedlings (**Fig. 6**), two D-loop-started origins, *ori*A and *ori*B, were mapped around 16S and 23S rDNA (Kunnimalaiyaan and Nielsen, 1997b). Nucleotide sequence of the *ori*A region is highly conserved among plant species (Hiratsuka *et al.*, 1989; Kunnimalaiyaan and Nielsen, 1997a), and *ori*A was mapped in the same region in pea and tobacco. However, no replication origin was found in the corresponding region in soybean (Hedrick *et al.*, 1993) and rice (this study). Initiation of ptDNA replication does, therefore, not primarily depend on nucleotide sequence itself.

Analysis by N/A 2D agarose gel electrophoresis suggested that replication extends bidirectionally from each of the two origins (see **Fig. 6** for summary). However, this does not simply mean that both parental strands are simultaneously replicated. An alternative is also possible that two opposite strands, which are switched at the origin, are used as templates. In any case, the mode of ptDNA replication in the rice cultured cells apparently differs from the double D-loop mechanism (Kolodoner and Tewari, 1975), in which the two D-loops expand toward each other and only one parental strand serves as a template. However, replication of the ptDNA so far reported is not restricted to the D-loop mechanism. EM studies of *Euglena gracilis* ptDNA indicated that replication from a single origin near the 5'-end of the supplementary 16S rDNA proceeds bidirectionally and that both paren-

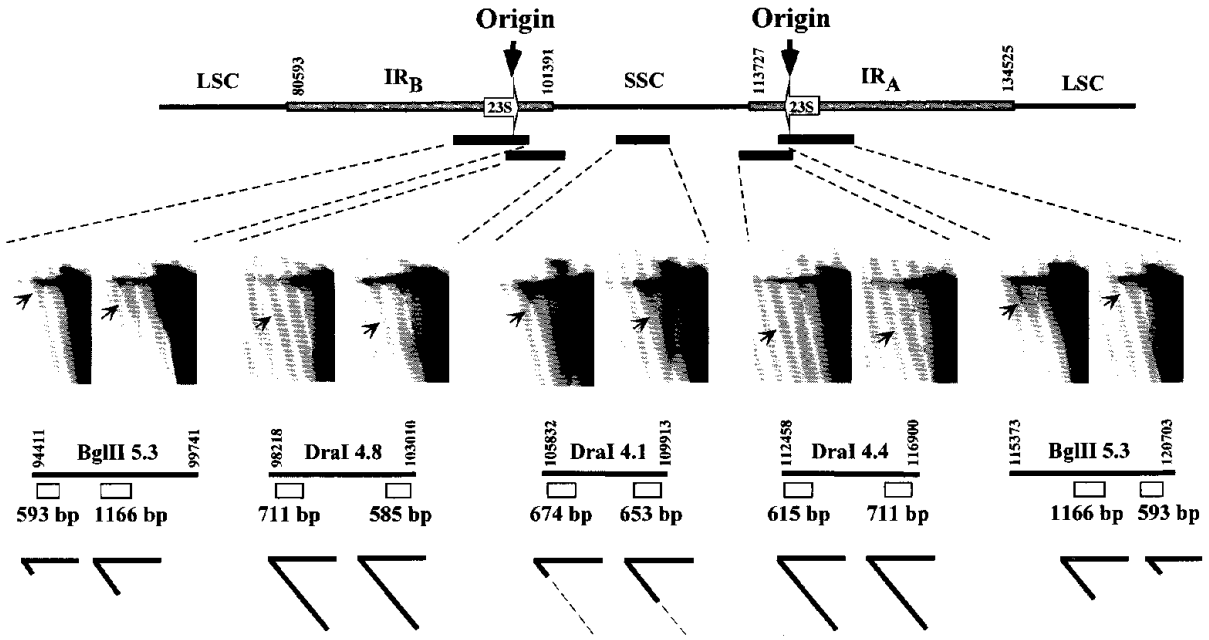

Fig. 5 N/A 2D agarose gel electrophoresis of the replication intermediates around the origin regions. Structure of the rice ptDNA is shown at the top. Large single copy (LSC), small single copy (SSC), inverted repeat regions (IR_A and IR_B, filled boxes) and the replication origins are indicated. The 23S rRNA gene is depicted by open arrows. 2D agarose gel patterns for the 5.3 kb *Bgl*II, 4.8 kb *Dra*I, 4.1 kb *Dra*I and 4.4 kb DraI fragments are shown in the middle panel. In each 2D gel, 25 to 30 μg of the BND-cellulose-enriched replication intermediates was subjected to the electrophoresis. Arrows indicate replicating nascent strands. Cartoon forms of each result and the position of the probes (open box) are shown at the bottom.

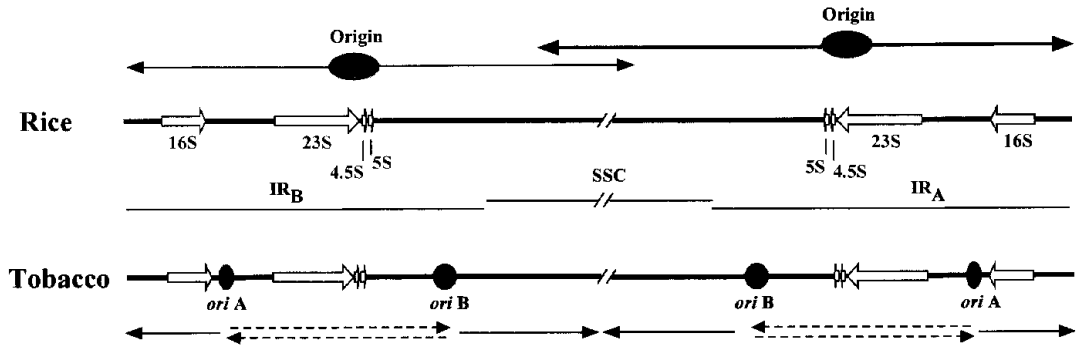

Fig. 6 Replication origins and the direction of replication of ptDNA in the rice cultured cells and the tobacco plant. Origins are indicated by filled circles, and the direction of replication by arrows. SSC and IRs, and rRNA genes are also shown. Dotted arrows indicate unidirectional replication according to the double D-loop mechanism (see text).

tal strands are simultaneously replicated (Ravel-Chapuis *et al.*, 1982). *In vitro* replication experiments using maize ptDNA also showed that both DNA strands were simultaneously used for templates (Carrillo and Bogorad, 1988). Moreover, an interesting observation has recently been reported for human mitochondrial DNA (mtDNA) that replication occurs by coupled leading- and lagging-strand synthesis in addition to D-loop-started unidirectional replication (Holt *et al.*, 2000). It is considered that D-loop-started replication is employed for maintenance of mtDNA copy number, while coupled leading- and lagging-strand replication for rapid increase of mtDNA copies. Replication of ptDNA might be fundamentally similar to that of mtDNA. However, we do not know at present whether coupled leading- and lagging-strand synthesis occurs.

Whether the two origins in IR_A and IR_B are both fired on the same ptDNA molecule is not clear at present. A possibility still remains to be elucidated that replication of one ptDNA molecule starts from the origin in one of the IRs while the other molecule from the other origin.

Finally, it should be noted that immuno-detection of BrdU-labeled DNA shown in **Fig. 2** resulted in the discrete bands of the labeled DNA fragments that contain replication origins, i.e., 14.7 and 7.5 kb *Sal*I fragments (not indicated in **Fig. 2**). BrdU-labeling was performed using randomly growing rice cells, so that incorporation of BrdU should occur randomly and uniformly into the ptDNA. Therefore, the results may indicate paused replication intermediates. Indeed, replication pause has been found in tobacco ptDNA (Kunnimalaiyaan and Nielsen, 1997a). In tobacco, paused nascent strands were reported to be 0.8 to 2.5 kb-long.

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