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Physical Localization of the 18S-5·8S-26S rDNA and Sequence Analysis of ITS Regions in *Thinopyrum ponticum* (Poaceae: Triticeae): Implications for Concerted Evolution

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Fluorescence *in situ* hybridization was used in *Thinopyrum ponticum*, a decaploid species, and its related diploid species, to investigate the distribution of the 18S-5·8S-26S rDNA. The distribution of rDNA was similar in all three diploid species (*Th. bessarabicum*, *Th. elongatum* and *Pseudoroegneria stipifolia*). Two pairs of loci were observed in each somatic cell at metaphase and interphase. One pair was located near the terminal end and the other in the interstitial regions of the short arms of one pair of chromosomes. However, all of the major loci in *Th. ponticum* were located on the terminal end of the short arms of chromosomes, and one chromosome had only one major locus. The maximum number of major loci detected on metaphase spreads was 20, which was the sum of that of its progenitors. The interstitial loci that exist in the possible diploid genome donor species were probably 'lost' during the evolutionary process of the decaploid species. A number of minor loci were also detected on whole regions of two pairs of homologous chromosomes. These results suggested that the position of rDNA loci in the Triticeae might be changeable rather than fixed. Positional changes of 18S-5·8S-26S rDNA loci between *Th. ponticum* and its candidate genome donors indicate that it is almost impossible to find a genome in the polyploid species that is completely identical to that of its diploid donors. The possible evolutionary significance of the distribution of the rDNA is also discussed. Internal transcribed spacer (ITS) regions of nuclear DNA in *Th. ponticum* were investigated by PCR amplification and sequencing. The sequence data from five positive clones selected at random, together with restriction site analysis, indicated that the ITS repeated units are nearly homogeneous in this autoallodecapolypoid species. Combined with *in situ* hybridization results, the data led to the conclusion that the ITS region has experienced interlocus as well as intralocus concerted evolution. Phylogenetic analyses showed that the sequences from *Th. ponticum* have concerted to the **E** genome repeat type.

Key words: *Thinopyrum ponticum*, *Thinopyrum elongatum*, *Thinopyrum bessarabicum*, *Pseudoroegneria,* 18S-5·8S-26S rDNA, fluorescence *in situ* hybridization (FISH), internal transcribed spacer (ITS), concerted evolution.

INTRODUCTION

Thinopyrum ponticum (Podp.) Liu & Wang is a perennial decaploid species ($2n = 10x = 70$) in the tribe Triticeae. It is known to possess a number of elite genes for wheat improvement, such as those for rust resistance, wheat curl mite resistance, wheat streak mosaic virus (WSMV) resistance, barley yellow dwarf virus (BYDV) resistance, and tolerance to abiotic stresses, such as salinity and drought (Zhang *et al*., 1996*a*, *b*; Chen *et al*., 1998). All of these characters make this species useful as a source of genes for improving the genetic diversity of cultivated wheat. Because it hybridizes easily with common wheat, a number of useful genes have been transferred from this species to wheat, which has led to the development of many important wheat germplasms and cultivars (Zhang *et al*., 1996*a*, *b*; Fedak *et al*., 2000).

Th. ponticum is a complex decaploid species that has been used to improve wheat for more than half a century. However, our knowledge of this species is still very limited. The origin and genome composition of *Th. ponticum* is an interesting and puzzling subject (Dewey, 1984). Genomic relationships among the genomes of *Th. ponticum* and its related species have been studied several times, and different genome formulae have been proposed. Muramatsu (1990) and Wang *et al*. (1991) concluded that *Th. ponticum* was an autodecaploid, and proposed $\mathbf{J_1J_2J_3J_4J_5}$ and **JJJJJ**, respectively, as its genome formula based on chromosome pairing. The **J** genome was from *Th. bessarabicum* (Savul and Rayss) A Löve ($2n = 2x = 14$, currently designated as $\mathbf{E^b}$), and was almost identical to the **E** genome of *Th. elongatum* (Host) D. R. Dewey ($2n = 2x = 14$, $\mathbf{E^e}$). Recently, this species was shown to be an autoallodecaploid. Its genome formula was designated by Zhang *et al*. (1996*a*, *b*) as $\mathbf{StStE^eE^bE^x}$, based on genomic *in situ* hybridization (GISH) and genome-specific markers. The **St** genome was homologous to the **St** genome of *Pseudoroegneria*. However, Chen *et al*. (1998) proposed $\mathbf{JJJJ^sJ^s}$ for this decaploid based on the same GISH results. In their paper, the $\mathbf{J^s}$ genome referred to modified **J**- or **E**-genome chromosomes conveying **St** segments by translocation between the **St** and **E** (or **J**) genomes (Chen *et al*.,

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1998). They proposed that *Th. ponticum* conveyed only segments of the **St** genome rather than any intact **St** genome or chromosomes. The major disagreement between Zhang *et al*. (1996*a*, *b*) and Chen *et al*. (1998) centred on the explanation of GISH results of *Th. ponticum* probed by **St** genomic DNA and blocked by **E** genomic DNA. The **St** genomic probe, even with a very high rate of **E**-genome blocking DNA, hybridized all 70 chromosomes, but 28 chromosomes hybridized more strongly at their centromeres and nearby regions. In the reverse GISH analysis, these 28 chromosomes were also hybridized by the **E** genomic probe except for their centromeric and nearby regions which were completely blocked by the **St** genomic DNA (Zhang *et al*., 1996*a*). Zhang *et al*. believed that the unexpected signals appearing beyond the probe genome chromosomes were mainly caused by cross-hybridization between **St** and **E** genomes because of their too close relationship in *Th. ponticum*. Therefore, Zhang *et al*. (1996*a*) proposed that the centromeres and nearby regions might be critical in the discrimination of **St** and **E** genomes. Similar GISH phenomena have also been reported in allotetraploid *Triticum dicoccoides* (Belyayev *et al*., 2000) and in the genus *Hordeum* (Heslop-Harrison, 1996). Inter-genomic translocations may not be appropriate explanations of this phenomenon. Therefore, we accepted the opinion of Zhang *et al*. (1996*a*) and used $\mathbf{StStE^eE^bE^x}$ as the genome formula for *Th. ponticum*. Nowadays, the candidate donor species of *Th. ponticum* have been narrowed down to a few species, including two diploid species of the genus *Thinopyrum* (*Th. bessarabicum* and *Th. elongatum*) and possibly several species of *Pseudoroegneria,* a unique genome (**St**) genus (Dewey, 1984).

Fluorescence *in situ* hybridization (FISH) is a powerful technique used to localize nucleic acid sequences on chromosomes. It provides a tool to construct physical maps, analyse chromosome structure and aberrations, and investigate structure, function and evolution of chromosomes and genomes (Maluszynsky and Heslop-Harrison, 1993; Leitch *et al*., 1994; Sang and Liang, 2000). The most frequently mapped gene is that for 18S-5·8S-26S rDNA (Zhang and Sang, 1999). In recent years, hybridization of rDNA probes using the FISH technique has been applied to assist karyological and genome analysis in a large number of plant species (Schmidt and Heslop-Harrison, 1998; Snowdon *et al*., 2000). Physical mapping of rDNAs can also provide information to help understand the relationships and evolution of polyploid species, for example, better understanding of concerted evolution of rDNA (Zhang and Sang, 1999).

That individual members of a gene family evolve as a unit and not independently of other members of the family is frequently referred to as concerted evolution (Elder and Turner, 1994; Waters and Schaal, 1996). Concerted evolution among repeated sequences has been observed in many organisms, from fungi through higher plants and animals (Waters and Schaal, 1996). Generally, the basic unit of repeated sequences homogenizes within individuals and among individuals within a species by the force of concerted evolution, but varies greatly between species.

In the evolution of polyploids, concerted evolution plays an essential role in the maintenance of sequence homogeneity of multigene families (Zhang and Sang, 1999). Studies on concerted evolution have mainly focused on rRNA multigene families (Wang and Zhang, 2000). However, previous studies have shown that the mode and rate of concerted evolution of rDNA differ among various plant groups (Wang *et al*., 2000). To reveal the evolutionary patterns of rDNA in the formation of allopolyploids, more data are needed. *Th. ponticum* has been shown to be an autoallodecaploid and has a relatively well-established phylogenetic framework (Zhang *et al*., 1996*a*; Chen *et al*., 1998). According to the criteria of Wendel *et al*. (1995), it is an ideal plant material for studying the concerted evolution of rDNA following allopolyploid speciation.

In this study, we employed FISH to locate 18S-5·8S-26S rDNA loci on chromosomes of *Th. ponticum* and its possible diploid progenitors (*Th. elongatum*, *Th. bessarabicum* and *Ps. stipifolia*) and investigated the DNA sequence of internal transcribed spacer (ITS) regions of *Th. ponticum* by PCR amplification and clone sequencing. Our primary goals were to: (1) determine the number and location of 18S-5·8S-26S rDNA loci in these species; (2) infer evolutionary changes of the rDNA loci; (3) investigate whether ITS sequences have been homogeneous; and (4) determine the direction of concerted evolution of rDNA in *Th. ponticum* if it has indeed occurred.

MATERIALS AND METHODS

Plant material

The plants used in this study were *Thinopyrum ponticum* (Podp.) Liu & Wang ($2n = 10x = 70$, accessions PI 578683, PI 578684 and PI 578686), *Th. elongatum* (Host) D. Dewey ($2n = 2x = 14$, $\mathbf{E^e}$ genome, accession Z1371), *Th. bessarabicum* (Save ex Rayss) A Löve ($2n = 2x = 14$, $\mathbf{E^b}$ genome, accession PI 531712) and *Ps. stipifolia* (Czern ex Nevski) A Löve ($2n = 2x = 14$, **St** genome, accession PI 313960). In this paper, we follow the standardized genome symbols designated by Wang *et al*. (1995).

DNA extraction

Seeds were sown and maintained in a glasshouse. Total genomic DNA was extracted from young fresh leaves collected from ten individuals, following a modified DNA extraction procedure of Sharp *et al*. (1989).

Chromosome preparation

Seeds were germinated on filter paper wetted by distilled water at room temperature (20–25 °C). When root-tips were 1–2 cm long, they were excised and pre-treated in ice water for 36 h before fixation in 3 : 1 (v/v) ethanol : acetic acid fixing solution. The single root-tip was squashed in a drop of 45 % acetic acid. Cover slips were removed after freezing in liquefied nitrogen, and slides were air-dried.

Probe DNA labelling

The probe, p*Ta*71, a highly tandem repetitive sequence isolated from bread wheat (*Triticum aestivum* L.) contains a 9 kb *Eco*RI fragment of the coding sequences for 18S, 5·8S and 26S rDNAs and the non-coding spacer sequences (Gerlach and Bedbrook, 1979). It was labelled with digoxigenin using the DIG-Nick Translation Mix (Boehringer Mannheim, GmbH, Germany).

Fluorescence in situ *hybridization*

Slides were incubated in 100 μg ml^{-1} RNase A at 37 °C for 1 h followed by three washings in 2 × SSC for 5 min. They were then dehydrated in a graded ethanol series and air-dried. The hybridization mixture solution containing 100 ng labelled probe (for one slide), 0·125 mg ml^{-1} salmon sperm DNA, 50 % formamide, 10 % dextran sulfate, 2 × SSC and 0·1 % SDS was denatured at 80 °C for 5 min and chilled on ice for 3–5 min. The solution was added to the slide and covered with a plastic membrane. Slides were put into the hybridization chamber (PTC™ Programmable Thermal Controller; M J Research, Inc., Watertown, MA, USA). The temperature regime was as follows: 75 °C (5 min), 60 °C (2 min), 55 °C (2 min), 50 °C (30 s), 45 °C (1 min), 42 °C (2 min), 40 °C (5 min), 38 °C (5 min), then 37 °C overnight.

Detection of hybridization

Plastic membranes were carefully removed after hybridization. Slides were then washed once at 42 °C in 2 × SSC for 5 min, twice for 5 min each in 0·1 × SSC containing 20 % formamide, three times for 5 min each in 2 × SSC, and then three times for 5 min each in 2 × SSC at 20–25 °C. Slides were washed a final time for 5 min in 4 × SSC containing 0·2 % Tween-20 at 20–25 °C. The slide was then blocked for 5 min at 37 °C by 5 % BSA in 4 × SSC containing 0·2 % Tween-20. Hybridization sites were detected with anti-digoxigenin conjugated by fluorescence isothiocyanate (FITC). The antibody binding reaction was carried out at 37 °C in 5 % BSA in 4 × SSC containing 0·2 % Tween-20 for about 1 h. The slide was washed three times in 5 % BSA in 4 × SSC containing 0·2 % Tween-20 for 8 min each at 37 °C. Chromosomes were counter-stained with propidium iodide (PI), mounted with anti-fade solution (Vector Laboratories Inc., Burlingame, CA, USA). Details of FISH can be found in Leitch *et al*. (1994).

The hybridization signal was observed using a fluorescence microscope (Olympus BX60, Japan). Images were captured by a charge-coupled device system (SPOT™; Diagnostic Instruments, Inc., USA) and brought together to make the plate using Adobe Photoshop 6·0 software. During this process, no modification was made to the individual images.

PCR amplification and sequencing

Total genomic DNA from *Th. ponticum* was used directly in PCR amplifications. PCR amplification of ITS regions generally followed Hsiao *et al*. (1994, 1995) using primers ITS-4 and ITS-L. PCR was performed in a PTC–100 thermal cycler (MJ-Research, Inc., Watertown, MA, USA) and consisted of 35 cycles (93 °C for 35 s, 49 °C for 35 s, 72 °C for 2 min), followed by a final extension of 7 min at 72 °C. Six independent reactions were carried out in this study. Their amplification products were mixed and purified using the Wizard PCR Pres DNA purification system (Promega, Madison, WI, USA). PCR products were cloned using pGEM-T Easy Vector Systems (Promega). Five positive clones were selected at random and identified by PCR using the same primers (ITS-4 and ITS-L). Sequencing was carried out by the BioAsia Biotechnology Co. Ltd (Shanghai, China) using an ABI 377 DNA Sequencer (Perkin Elmer, Foster City, CA, USA). The boundaries of the ITS regions were determined by comparison with the sequence of *Pseudoroegneria spicata* (GenBank accession no. L36502) (Hsiao *et al*., 1995)

Restriction site analysis (PCR-RFLP)

To investigate whether sequences of the ITS region have been homogenous, restriction site analysis was applied following the procedure of Booy *et al*. (2000). The mixed PCR products of ITS regions were purified and concentrated. Several inner-restriction enzymes (*Eco*RV, *Mse*I, *Mlu*I, *Msp*I, *Hpa*II, *Sau*3AI) were used to digest the PCR product. Digested fragments were separated on a 4 % agarose gel to determine the polymorphic sites.

The PCR products of ITS regions from the three diploid species (*Th. bessarabicum*, *Th. elongatum* and *Ps. stipifolia*) were also analysed to investigate their homogeneity following the same procedures.

Sequence alignment and phylogenetic analysis

To construct phylogenetic trees and determine the direction of concerted evolution of rDNA, we compared the ITS sequences from *Th. ponticum* with those of its candidate diploid donor species. Two diploid species containing the **St** genome, *Pseudoroegneria spicata* and *Pseudoroegneria libantica* (Hackel) D. R. Dewey, and two species containing the **E** genome, *Th. bessarabicum* ($\mathbf{E^b}$) and *Th. elongatum* ($\mathbf{E^e}$) were used. The ITS sequences of these species were sequenced by Hsiao *et al*. (1994). The data were downloaded from the website (http://www.ncbi.nlm.nih.gov), and their GenBank accession numbers were L36501 (*Ps. libantica*), L36502 (*Ps. spicata*), L36506 (*Th. bessarabicum*) and L36495 (*Th. elongatum*). Based on the phylogenetic trees of Hsiao *et al*. (1994), *Hordeum vulgare* L. (GenBank accession no.: Z68921) was selected as outgroup. All of these sequences were aligned using Clustal X program (Thompson *et al*., 1997).

Phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei, 1987) using programs of PHYLIP version 3·572c (Felsenstein, 1985). Bootstrap analysis (Felsenstein, 1997) was carried out with 1000 replicates. Phylogenetic trees were

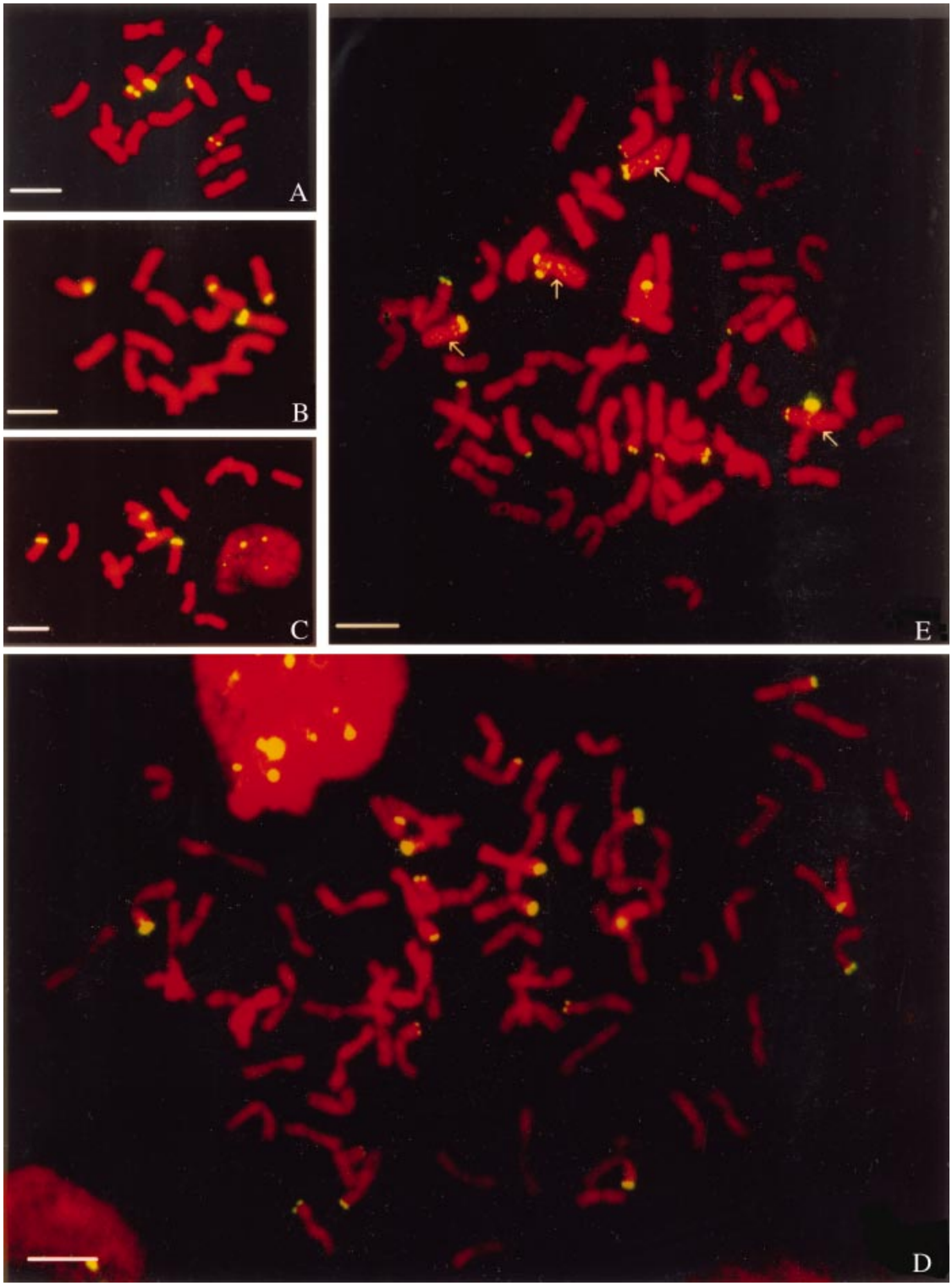

FIG. 1. Fluorescence *in situ* hybridization of metaphase chromosomes of *Th. bessarabicum* (A), *Th. elongatum* (B), *Ps. stipifolia* (C), and *Th. ponticum* (D and E) with the 18S-5·8S-26S rDNA probe, p*Ta*71 (yellow-green). Orange-red fluorescence shows chromosomes counterstained with propidium iodide. Bars = 10 μm.

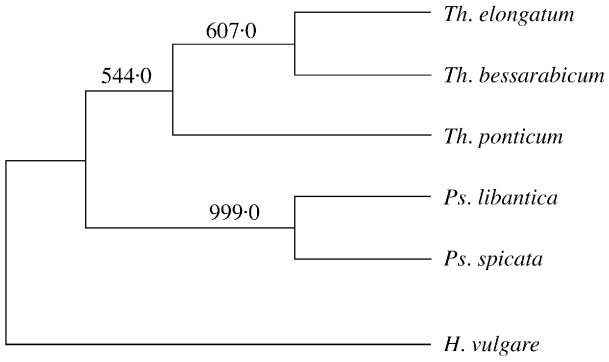

FIG. 2. The phylogenetic tree inferred from ITS sequences of *Th. ponticum* and its related diploid species generated by the neighbour-joining method, using *Hordeum vulgare* as outgroup. Numbers above branches represent bootstrap support in 1000 replicates.

constructed with Kimura two-parameter distances (Kimura, 1980).

RESULTS

Fluorescence in situ *hybridization*

Yellow-green fluorescence signals were indicative of 18S-5·8S-26S rDNA loci (Fig. 1). The pattern of distribution of 18S-5·8S-26S rDNA was similar in all three diploid species. Two pairs of 16S-5·8S-26S rDNA loci were observed on each of the metaphase and interphase spreads of their somatic cell. The number of these loci corresponded to that of satellite chromosomes, and they were located in the region of the secondary constrictions. One pair was located near the terminal end of the short arms, and another pair was located on the interstitial regions of the short arms. Fluorescence signals of all detected loci were strong, indicating that many copies of the 16S-5·8S-26S rDNA repetitive unit existed at these loci (Fig. 1A–C). Kosina and Heslop-Harrison (1996) have described the chromosomal location of rDNA in *Th. elongatum* (syn. *Lophopyrum elongatum*). Our result was in accordance with theirs. It was interesting to note that the two loci on homologous chromosomes were unequal in size: one was obviously bigger than the other in all three diploid species.

For each accession of *Th. ponticum*, four individuals were evaluated in our study. Hybridization patterns did not differ among accessions or individuals. Eleven integrated and several incomplete metaphase chromosome spreads were observed. All of the major 18S-5·8S-26S rDNA loci were located on the terminal end of the short arms of chromosomes. One chromosome only conveyed one major locus. The maximum number detected on metaphase spreads of root-tips in the present study was 20 (Fig. 1D). The strength of fluorescence signals differed among these loci, indicating that they had different copy numbers of the rDNA repetitive unit. A great number of minor loci were detected on two or more pairs of homologous chromosomes (Fig. 1E).

ITS sequences in Thinopyrum ponticum

The sequences of the five positive clones selected at random among over 1000 positive clones were very similar. Three were sequenced completely. The entire ITS region in *Th. ponticum,* including both non-coding spacers (ITS1 and ITS2) and the 5·8S rDNA, was 605 base pairs (bp). The ITS1 region was 221 bp, and the ITS2 region was 220 bp. The 5·8S subunit was 164 bp. The other two segments that were successfully sequenced were 530 bp, including the entire ITS1 and 5·8S regions and partial ITS2. These sequences were aligned by the Clustal X program to compare their homogeneity. Results showed that they were almost identical. Only one base 'g' was substituted by 'c' at position 80 in one of these sequences. The phenomenon could be explained as a point mutation, sequencing or amplification error. These sequences have been nearly homogeneous, suggesting that the ITS repeats have undergone concerted evolution. The sequence reported in this paper has been deposited in the GenBank database (Accession no.: AY090768).

Restriction site analysis

If PCR products contain different sequences, restriction enzymes will cut at heterogeneous sites, and the sum of the sizes of the restriction fragments will be greater than the entire length of the undigested ITS fragment. In this study, restriction site analysis of ITS PCR fragments showed that the sum of the sizes of restriction fragments was equal to the size of the initial amplified fragment itself in each enzyme-cutting reaction. Thus, the ITS repeats in *Th. ponticum* and its three related diploid species have been homogeneous, at least at the restriction sites detected in this study.

Phylogenetic analysis

The phylogenetic tree inferred from ITS sequences of *Th. ponticum* and its related diploid species is shown in Fig. 2. Given the controversy concerning the potential distortion induced by species of hybrid-origin allopolyploids in cladistic analyses (McDade, 1995), the tree was compared with the monogenomic species trees of Hsiao *et al*. (1995). Figure 2 shows that the topological relationships among the diploid species were not changed after the introduction of the polyploid species, *Th. ponticum*. The two diploid species conveying the **E** genome, *Th. bessarabicum* ($\mathbf{E^b}$) and *Th. elongatum* ($\mathbf{E^e}$), formed a clade. The other two diploid species that conveyed the **St** genome, *Ps. spicata* and *Ps. libantica*, formed another clade. *Th. ponticum* was grouped with *Th. bessarabicum* ($\mathbf{E^b}$) and *Th. elongatum* ($\mathbf{E^e}$), suggesting the ITS region of the rDNA in *Th. ponticum* has been homogenized or concerted to the **E** genome type.

DISCUSSION

Most studies have shown that much variability exists for 18S-5·8S-26S rDNA loci among species, subspecies, populations and even individuals (Sánchez-Gea *et al*., 2000). In

most alloploployploid plants, the number of 18S-5·8S-26S rDNA loci equals the sum of that of their progenitors (Wang and Zhang, 2000). However, loss of some loci has been observed in several alloploployploid species (Vaughan *et al*., 1993; Leggett and Markand, 1995; Snowdon *et al*., 1997). *Th. ponticum* is a decaploid, containing ten genomes in its somatic cells. The related diploid species (*Th. bessarabicum, Th. elongatum* and *Ps. stipifolia*) have the same number of 18S-5·8S-26S rDNA loci, with two loci per genome. The expectation is that the decaploid species would have 20 loci if the number of major 18S-5·8S-26S rDNA loci has remained stable during the evolutionary process. The maximum number of major loci detected in *Th. ponticum* using the present method was 20, which is the sum of that of its progenitors. However, all of the rDNA loci of *Th. ponticum* were located on the terminal regions of short arms (Fig. 1D and E). The interstitial loci have apparently been 'lost'. These results suggest that there has been distinct differentiation between *Th. ponticum* and its diploid relatives during the evolutionary process. It is impossible to determine directly the ancient ancestor(s) of *Th. ponticum*. The interstitial position is probably an ancestral trait whereas the terminal position is probably derived (Dubcovsky and Dvoøák, 1995). Therefore, the distribution pattern of rDNA loci in the ancient ancestor may be similar to that of the modern diploid species, and it may possess a similar type of interstitial locus. During polyploidization of *Th. ponticum*, all of the interstitial loci have been deleted or migrated, and 'novel' loci have been positioned on terminal regions of the chromosomes. A similar phenomenon has been observed in other species (Gill and Apples, 1988; Dubcovsky and Dvoøák, 1995), though the exact mechanism for this is still unknown. According to studies of comparative linkage maps, Dubcovsky and Dvoøák (1995) suggested that the loci might change position via dispersion of minor loci without structural rearrangements of chromosomes. In addition to the 20 major loci, many minor loci were detected in our study (Fig. 1D), supporting the suggestion of Dubcovsky and Dvoøák (1995). The positional changes of 18S-5·8S-26S rDNA loci between *Th. ponticum* and its candidate donors indicated that it is almost impossible to find a genome in the polyploid species that is completely identical to that of its diploid donor.

Hybridization between different species with subsequent polyploidization is a prominent process in the evolution of higher plants (Masterson, 1994). In the evolution of polyploid species, concerted evolution plays an essential role in the maintenance of sequence homogeneity in multigene families through inter-chromosomal interactions (Zhang and Sang, 1999). A remarkable example of concerted evolution in plants is cotton (*Gossypium*). Sequence data from the ITS regions have indicated that rDNA arrays are homogeneous or nearly homogeneous in all five allotetraploids (**AD** genome) and their diploid progenitors (**A** genome and **D** genome). In four tetraploids, the 18S-5·8S-26S rDNA was homogenized to the **D** genome repeat type, but in the other tetraploid it was concerted to the **A** genome repeat type, although both **A** and **D** conveyed the major rDNA loci in the tetraploid (Wendel *et al*., 1995; Hanson *et al*., 1996; Wendel, 2000). Inter-locus homogenization of alternative rDNA repeat types (concerted evolution) has also been reported in other polyploid species such as *Microseris*, *Paeonia* and *Saxifraga* (Wendel, 2000). This phenomenon may be common during polyploidization (Wendel, 2000). Unequal crossing over (unequal exchange) and gene conversion are possible mechanisms for concerted evolution (Zhang and Sang, 1999; Booy *et al*., 2000; Wendel, 2000).

Physical mapping of rDNA can increase understanding of concerted evolution (Zhang and Sang, 1999). The 5S rDNAs of cotton were located on the interstitial regions of chromosomes. Their sequence homogeneity was very low, indicating a high rate of polymorphism among species and even individuals. Inter-chromosomal exchanges may be facilitated in taxa such as *Gossypium* and *Paeonia* by the terminal or near-terminal location of the rDNA loci, which may permit unequal crossing-over without deleterious recombination among non-homologous chromosomes (Zhang and Sang, 1999; Wendel, 2000). The terminal positions of the 18S-5·8S-26S rDNA and the loss of its interstitial loci found in our study suggest that concerted evolution of 18S-5·8S-26S rDNA probably occurred, and the sequences may have become homologous or nearly homogeneous.

Generally, rDNA repeated units are considered to be homogenous as a result of concerted evolution (Dover, 1982, 1989; Dvoøák, 1990; Booy *et al*., 2000). However, heterogeneity of ITS regions within individuals of some species has been reported (Booy *et al*., 2000). Such heterogeneity may occur if concerted evolution does not occur quickly enough or if it fails to homogenize rDNA repeated units as a result of recent hybridization, the development of pseudogenes, the large number of rDNA loci, the occurrence of asexual reproduction, and so on (Dover, 1982; Wendel *et al*., 1995; Zhang and Sang, 1999: Booy *et al*., 2000; Wendel, 2000).

Although the distribution of major rDNA loci in *Th. ponticum* implied that concerted evolution may have occurred, the possibility of heterogeneity also exists. The sequence data of the five ITS region clones and the results of restriction site analysis showed high levels of homogeneity, with most of the ITS sequences in *Th. ponticum* being identical. An alternative explanation for sequence homogeneity is that rDNA loci of one or more genomes have been lost following allopolyploid speciation (Wendel *et al*., 1995). We can ignore this possibility because the FISH results in our study showed that the number of major rDNA loci in *Th. ponticum* was the sum of that of its progenitors. Thus, we conclude that the homogeneity of the ITS within *Th. ponticum* genomes is mainly the result of concerted evolution. The phylogenetic tree suggests that the ITS region of the rDNA in *Th. ponticum* has been homogenized or concerted to the **E** genome type. The sequences of the **St** genome type have possibly been changed into or 'overwritten' by those of the **E** genome type through concerted evolution.

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