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Cytological and Molecular Analyses of the Ns Genome in Russian Wildrye, *Psathyrostachys juncea* (Fisch.)

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CYTOLOGICAL AND MOLECULAR ANALYSES OF THE Ns GENOME IN RUSSIAN WILDRYE, *PSATHYROSTACHYS JUNCEA* (FISCH.)

by

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ABSTRACT

Cytological and Molecular Analyses of the Ns Genome in

Russian Wildrye, *Psathyrostachys juncea* (Fisch.)

by

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Russian wildrye (*Psathyrostachys juncea* Fisch. 2n=2x=14, NsNs) is an important forage grass and a potentially useful germplasm in wheat improvement. A standard C-banding karyotype of *Ps. juncea* has been developed based on the C-bands of chromosomes in geographically diverse materials. Although there are C-banding polymorphisms, the seven pairs of chromosomes can be distinguished from each other according to their basic banding patterns. Based on C-banded karyotype, one deletion-translocation heterozygote, four primary trisomics, one double-deletion trisomic, and two tertiary trisomics were identified. These cytogenetic stocks will be useful in genetic studies of Russian wildrye.

Genetic variations in Russian wildrye were analyzed at chromosomal, protein,

and DNA levels using C-banding, isozymes, and randomly amplified polymorphic DNA (RAPD) techniques, respectively. Due to the self-incompatibility in Russia wildrye, a high level of genetic diversity existed both within and among accessions. In general, accessions originated from the same or neighboring geographical areas showed closer genetic relationships. The results of various approaches for genetic variation analysis suggest that there are tremendous genetic variations in the Russian wildrye germplasm for the effective improvement of this forage grass.

Some molecular markers were isolated and characterized in Russian wildrye using RAPD and cloning techniques. These markers may be useful in gene mapping, species identification, studies of evolutionary relationships, and transferring useful genes into cereal crops.

(178 pages)

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CHAPTER 1

INTRODUCTION

Taxonomy

Russian wildrye belongs to *the genus Psathyrostachys* (Nevski) of the tribe Triticeae. *Psathyrostachys* is a small genus with no more than 10 species. This genus was erected by Nevski (1934) and was almost unanimously recognized by agrostrologists (Dewey 1984). Although species in this genus had been previously placed in the traditional *genus Elymus* or *Hordeum*, they are now placed in the genus *Psathyrostachys* with almost no controversy around the world. Plants in this genus are characterized by caespitose, long-anthered, cross-pollinating, long-lived perennials with multiple spikelets per node, one or more florets per spikelet, subulate glumes, and a fragile rachis (Dewey 1984). The genome of this genus was designated with the letter N (Löve 1984), which was recently changed to Ns, according to the new genome symbol system (Wang et al. in press). The well known species in this genus are *Ps. fragilis* (Boiss.) Nevski, *Ps. juncea* (Fisch.) Nevski, *Ps. lanuginosa* (Trin.) Nevski, *Ps. stoloniformis, Ps. huashanica* Keng, and *Ps. kronenburgii* (Havk) Nevski. Baden (1991) described the morphologies of eight species of *Psathyrostachys* in detail.

Russian wildrye was transferred from *Elymus* to *Psathyrostachys* based on its cytology (Dewey and Hsiao 1983). For a long time, it was thought that diploid

plants (2n=2x=14) occur in *Psathyrostachys*. Although diploid is the predominant form in all *Psathyrostachys* species, some tetraploid plants have been recently reported in *Ps. fragilis, Ps. lanuginosa* (Linde-Laursen and Baden 1994), and in some accessions of *Ps. juncea* (Asay et al. 1994). It was assumed that all *Psathyrostachys* species contain one basic Ns genome, even though the interspecific hybrids were almost completely sterile due to chromosome modifications in each species (Dewey and Hsiao 1983). The Ns genome is also one of the genome donors of the genera *Leymus* (NsX) and *Pascopyrum* (StHNsX).

Forage utilization

Psathyrostachys grasses grow on rocky open slopes and steppes from the Middle East and European Russia across central Asia to northern China. Only one species, Ps. juncea (Russian wildrye), has gained importance as a forage grass.

Russian wildrye is a cross-pollinated, long-lived, cool-season perennial bunchgrass. It is native to the steppe and desert regions from Iran northward to the lower Volga River and lower Don River regions of Russia in the north, eastward into western Siberia, and across Asia to Outer Mongolia and northern China (Dewey 1984). It is exceptionally cold-, drought- and alkalinity-tolerant, and it has dense basal leaves that are high in nutritive value and palatable to grazing animals. Although noted for its productivity during the early spring, its nutritive value is

retained better during the late summer and fall than in many other grasses. Therefore, Russian wildrye provides excellent grazing for livestock and wildlife on semiarid rangelands (Asay 1992).

Russian wildrye was first introduced into Canada in 1926 and into the United State in 1927, and it is now widely used to revegetate arid rangelands in North America. It was estimated in 1980 that about 100,000 ha had been seeded to Russian wildrye in the Prairie Provinces of Canada and more that 300,000 ha had been seeded in the Great Plains and Intermountain regions of the U. S. (Smoliak and Johnson 1980). Forage yield and quality (White 1986), selection (Berdahl and Barker 1984), and germination and emergence (Lawrence et al. 1991) of Russian wildrye have been studied. Many cultivars, such as 'Vinall', 'Sawki', 'Mayak" (Hanson 1972), 'Cabree' (Smoliak 1976), 'Swift' (Lawrence 1979), 'Bozoisky' (Asay et al. 1985), and 'Mankota' (Berdahl and Barker 1992) have been released. Seedling vigor and seed retention have been improved (Asay 1992). Autotetraploid Russian wildrye produced with nitrous oxide (Berdahl and Barker 1991) and with colchicine (Lawrence et al. 1990) is reported to have larger seeds and greater seedling vigor than its diploid counterparts. Natural tetraploids (2n=4x=28) were also recently identified in accessions collected from Kazakhstan. In comparison with standard diploid cultivars, the tetraploids were taller and more productive, had wider and longer leaves, and had higher water-use efficiency (Asay et al. 1994). It is a desirable ploidy level for improving the agronomic performance of

Russian wildrye.

Genome relationship

Hybridization between species of the same genome or of different genomes is one approach to study genome relationships and in the meantime new hybrids can widen the germplasm base. Hybridizations of *Ps. juncea* with *Elymus scribneri* (StStHH) (Dewey 1967), *Leymus* (XNs) (Dewey 1970, 1972), *Hordeum* (H) (Baden et al. 1989), other *Psathyrostachys* species (Dewey and Hsiao 1983; Wang 1987; Lu et al. 1989), *Critesion. violaceum* (H^v) and *Ps. juncea* (Wang 1984, 1986), *Pseudoroegneria* (St) (Wang 1987, 1991), *and Thinopyrum*(E) (Wang 1989) have enriched the germplasm of perennial Triticeae. According to the levels and patterns of chromosome pairing in these hybrids, species of *Psathyrostachys* have very close relationships and the Ns genome is distinctively distant from all other basic genomes in Triticeae (Wang 1989, 1992).

Karyotype analyses of *Ps. fragilis, Ps. juncea, Ps. kronenburigil, Ps. lanuginosa,* and *Ps. huashanica* show that the morphology and size of the chromosomes in this species, except *Ps. huashanica*, are very similar (Hsiao et al. 1986). The chromosomes of *Ps. fragilis, Ps. juncea,* and *Ps. huashanica* were compared using C- and Ag-staining (Linde-Laursen and Bothmer 1984b, 1986). The karyotypes of the diploid and tetraploid *Ps. lanuginosa* are analyzed by C- and N-banding as well as $AgNO_3$ staining (Linde-Laursen and Baden 1994). The C-banding analysis

indicated that *Ps. fragile, Ps. juncea, Ps. stoloniformis,* and *Ps. lanuginosa* have similar karyotypes and close relationships.

The relationships among species in Ns genome and other genomes in Triticeae have also been studied with isozymes and some DNA sequences (McIntyre 1988, McIntyre et al. 1988, Scoles et al. 1988; West et al. 1988; Hsiao et al. in press).

Crop improvement

In addition to tolerance to salinity and drought, Russian wildrye is also reported to possess resistance to barley yellow dwarf virus (BYDV) (Comeau and Plourde 1987), and to powdery mildew (*Erysiphe graminis*) (Dong et al. 1992) and immunity to leaf rust (*Puccinia rubigovera*) and stem rust (*Puccinia graminis*) (Li et al. 1992). Russian wildrye is considered to be an important germplasm in cereal improvement (Mujeeb-Kazi and Asiedu 1990). Hybrids between barley and *Ps. fragilis* (Linde-Laursen and Bothmer 1984a; Baden et al. 1989), between common wheat and *Ps. juncea* (Chen et al. 1988; Plourde et al. 1990; Zhou et al. 1992), and between durum (*T. turgidum*) and *Ps. juncea* (Mujeeb-Kazi et al. 1995) have been obtained, although the crosses were very difficult to make. The hybrids were sterile and showed some necrosis and chlorosis. Backcrossing the hybrid with common wheat also made it difficult to obtain seed. Only backcross progeny was obtained when using amphiploid *Triticum aestivum* x *Triticum tauschii* as pollen donor. The backcross offspring was vigorous and showed a considerable reduction in leaf

chlorosis (Zhou et al. 1992).

Previous studies and further studies needed

The karyotype of Russian wildrye was analyzed with acetoorcein staining (Hsiao et al. 1986). The meioses in diploid, triploid and tetraploid plants of *Ps. juncea* have been reported (Wang and Berdahl 1990).

The C-banding in *Ps. juncea* has been studied by many groups of researchers (Gill 1981; Endo and Gill 1984; Linde-Laursen and Bothmer 1986; William and Mujeeb-Kazi 1992). The C-banded karyotypes of this species varied from one study to another. The results showed distinct differences from the karyotypes of Hsiao et al. (1986). No systematic research based on diverse plant materials has been made, and further genetic studies, such as chromosome identification and gene location, are limited. The C-band polymorphism, which has been noticed by several researchers (Endo and Gill 1984; Linde-Laursen and Bothmer 1986), has not been completely studied.

Several isozymes of *Ps. juncea* have been studied, and some of them can be used as genomic markers for tracking *Ps. juncea* chromosomes in a wheat background (William and Mujeeb-Kazi 1992).

Significant genetic variations have been found in Russian wildrye breeding populations in both laboratory and field trials for characters relating to stand establishment (Asay and Johnson 1980). Morphological polymorphisms are evident

due to the self-incompatibility in Russian wildrye (Jensen et al. 1990). Characterization of genetic variations is required prior to improve such traits as seedling vigor, forage yield, and quality through breeding. The genetic variation in this species has not been studied systematically.

There is still a long way to go to transfer useful genes from Russian wildrye to wheat. The number, location, and heredity of these genes should be studied. Then the useful genes can be incorporated into wheat through chromosome engineering procedures. Alternatively, these genes may be isolated and introduced into crops with molecular transformation techniques. For either approach, knowledge of the genome of Russian wildrye is the prerequisite of successful gene transfer.

About this research

The objectives of this study were to establish a standard karyotype for Russian wildrye with the aid of chromosome banding to identify trisomics of Russian wildrye, to evaluate the polymorphisms in this species using chromosome banding, isozyme and random amplified polymorphic DNA (RAPD), and to develop some DNA markers for the Ns genome, for *Ps. juncea*, or for specific chromosomes.

C-banding has been the most commonly used banding technique to identify chromosomes. C-banding, which stains constitutive heterochromatin (Pardue and Gall 1970; Hsu 1973), has been successfully used in the establishment of standard

karyotypes (Gill et al. 1991), analysis of chromosome structural variation (Zhu et al. 1993), identification of alien chromosomes and chromosome segments (Friebe et al. 1990a, 1992), and identification of trisomics (Friebe et al. 1990b).

Aneuploids are very useful in cytogenetic analysis. In particular, primary trisomics have been used frequently to locate genes to specific chromosomes and construct linkage groups (Tanksley and Rick 1980; Tsuchiya 1983; Khush et al. 1984; Kaiser and Friedts 1989). A complete set of trisomics has not been reported in this species.

Isozymes are enzymes that share a common substrate but differ in electrophoretic mobilities. They are detected when tissue extracts are subjected to electrophoresis in a type of gel and subsequently submersed in solutions containing enzyme-specific stains. Isozyme analysis has become particularly prominent in systematic and evolutionary biology as well as agronomy (Tanksley and Orantan 1983). Isozyme markers are widely used in gene mapping of crop plants (Wendel and Weeden 1989). Isozyme variations among accessions of Russian wildrye have not been reported.

The RAPD technique, developed by Welsh et al. (1990) and Williams et al. (1990) provides a simpler and cheaper method for DNA analysis. Since its development, this method has been widely used in the measurement of genetic variations to establish genetic and evolutionary relationships within and/or among species, subspecies, and populations (Wilkie et al. 1993; Jain et al. 1994).

Combined with cloning techniques, RAPD products can be used to establish molecular markers for genome, species or chromosomes. No RAPD work on Russian wildrye has been carried out.

In this study, we tried different methods, including C-banding, isozyme electrophoresis, and RAPD, to analyze genetic variations in Russian wildrye, and tried to evaluate the usefulness and to compare the sensitivity of these methods.

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CHAPTER 2

STANDARD C-BANDING KARYOTYPE OF RUSSIAN WILDRYE (*PSATHYROSTACHYS JUNCEAE* FISCH.) AND ITS USE IN IDENTIFICATION OF A DELETION-TRANSLOCATION

Abstract

Russian wildrye, *Psathyrostachys juncea* (Fisch.) Nevski (2n=2x=14; **NsNs**), is an important forage grass and a potentially useful germplasm resource for wheat improvement. Ten accessions of this grass collected from different geographical regions were analyzed using the C-banding technique. C-banding polymorphisms were observed at all levels, i.e., within some homologous chromosome pairs, among different individuals in the same accession, between different accessions of the same geographic area, and among accessions of different origins. The seven homologous groups varied in the level of C-banding polymorphism. Chromosomes A, B, E, and F were more variable than chromosomes C, D, and G. The polymorphisms did not prevent chromosome identification in Ps. juncea because each chromosome had a different C-banding pattern and karyotypic characters. A standard C-banding karyotype of *Ps. juncea* was proposed based on the overall karyotypes and C-bands in the 10 accessions. The C-bands on the **Ns**-genome chromosomes were designated according to nomenclatural rules. A deletion-translocation heterozygote of Russian wildrye was identified using the

established karyotype and C-banding patterns. The chromosome F pair consists of a deletion having the distal segment in the long arm deleted and a translocation having the distal segment of long arm replaced by the distal segment of chromosome E. The plant has a normal chromosome E and a translocation having the proximal segment of the long arm of E and the distal segment of long arm F.

Introduction

Russian wildrye, *Psathyrostachys juncea* (Fisch.) Nevski (2n =2x=14; **NsNs**), is a cool-season perennial bunchgrass native to rocky open slopes, steppes, and desert regions from the Middle East and European Russia across central Asia to northern China (Dewey 1984). The species is relatively tolerant to alkalinity and drought, has dense basal leaves that are high in nutritive value, and is palatable to grazing animals. It provides excellent grazing for livestock and wildlife on semiarid rangelands (Asay 1992). Russian wildrye was first introduced to the United States in 1927 and is now widely used to revegetate arid rangelands in North America.

In addition to tolerance for salinity and drought, Russian wildrye possesses resistance to barley yellow dwarf virus (BYDV), which can reduce yields of wheat and barley (Comeau and Plourde 1987). It is also resistant to powdery mildew (*Erysiphe graminis*) (Dong et al. 1992), yellow rust (*Puccinia striformis*), and leaf rust (*Puccinia recondita*) (Li et al. 1992). Russian wildrye, therefore, is an important gene reservoir for wheat improvement (Mujeeb-Kazi and Asiedu 1990).

Hybridization between Russian wildrye and other diploid Triticeae (Wang 1992), and between Russian wildrye and common wheat (Chen et al. 1988; Plourde et al. 1990) has been reported.

The somatic karyotype (Hsiao et al. 1986) and meiotic behavior (Wang and Berdahl 1990) of Russian wildrye have been described. C-banding, which stains constitutive heterochromatin (Pardue and Gall 1970; Hsu 1973), is the banding technique most often used to identify plant chromosomes. Because Russian wildrye is an outcrossing species, its high heterogeneity is reflected by polymorphisms of chromosome banding patterns (Gill 1981; Endo and Gill 1984; Linde-Laursen and Bothmer 1986; William and Mujeeb-Kazi 1992). However, diverse materials have not been systematically studied to establish a standard C-banded karyotype for this species.

Therefore, we analyzed C-banded chromosomes in 10 different accessions of this species and established a standard C-banding karyotype. Utility of this C-banding karyotype was then tested on a male-sterile Russian wildrye.

Materials and methods

Ten accessions, with four to six plants in each accession, from different geographic regions were analyzed (Table 2-1).

The procedures of Endo and Gill (1984) for C-banding analyses were followed with some modifications. Fresh root tips were collected and pretreated with ice

Table 2-1. Accessions and geographical origins of *Ps. juncea* used in this study.

Number	Accession	Geographical origin	No. Plants
1	PI314668	USSR	5
2	PI314521	USSR	5
3	DJ 3955	USSR	5
4	"Bozoisky"	USSR	6
5	PI499558	China	5
6	PI206684	Turkey	4
7	"Cabree"	via Canada	5
8	KJ 130	Iran	4
9	KJ 237	Mongolia	5
10	KJ 240	Mongolia	5

water for 24 h, and fixed in 95% ethanol and acetic acid (3:1) for 1-3 days. Fixed root tips were stained in 1% acetocarmine for 2 h, followed by squashing in 45% acetic acid. Cells with well spread chromosomes were photographed at this step. After cover slips were removed following rapid freezing in liquid nitrogen, slides were treated at 60°C in 45% acetic acid for 15 min and were air dried overnight. The slides were incubated in 5% $Ba(OH)_2$ for 15 min at 60°C and in 2x SSC (0.3 M NaCl and 0.03 M sodium citrate) for 20 min 60°C, with thorough washing after each step. The slides were air-dried and then stained in 2% Giemsa for 1-2 h. C-

banded chromosomes were photographed.

The images of metaphase cells before and after C-banding were compared. The karyotype and banding pattern were analyzed by computer programs CHROMPAC III (Green et al. 1984) and PCAS (Plant Chromosome Analysis System; Lu Y-Q., R. R-C. Wang, and N. J. Chatterton, unpublished). Chromosomes were arranged from the longest to the shortest and were designated with the letters A to G. C-band nomenclature employed was that used for human (Rowley 1974) and wheat (Gill et al. 1991) chromosomes.

A male-sterile *Ps. juncea* plant, HD-33-6, was grown in a greenhouse of USDA-ARS-FRRL, Logan, Utah. HD-33-6 was derived from a seed harvested from a *Ps. juncea* plant. Ten root-tip cells with good chromosome morphology and C-bands were analyzed. Spikes of the plant were fixed in Farmer's solution (3:1 95% ethanol:acetic acid) for 24 h before transfer into 70% ethanol for storage. Pollen mother cells (PMCs) were stained and squashed in 1% acetocarmine. The C-banding method for meiotic chromosomes was the same as that used for mitotic chromosomes.

Results and discussion

Homoeology between the chromosomes of the genome and the chromosomes in other genomes of Triticeae is unknown. Therefore, chromosomes of *Ps. juncea* were arranged from the longest to the shortest and labelled with letters A to G,

respectively. The somatic metaphase chromosome length, arm ratio, and centromere index in the 10 accessions were averaged (Table 2-2). The ratio of longest to the shortest chromosome was 1.37.

There are three different chromosome arrangements and designations of *Ps. juncea* in the literature (Table 2-3). Gill (1981) and Endo and Gill (1984) tentatively designated the seven chromosomes by letters A to G, but did not arrange chromosomes according to chromosome length as did Hsiao et al. (1986). Linde-Laursen and Bothmer (1986) used the designations of Endo and Gill (1984), but arranged the chromosome from the longest to the shortest. In the present study, we use the same arrangement as that of Hsiao et al. (1986) but use letters A to G for the designation of chromosomes. The only other difference is the allocation of the fifth and sixth chromosomes. These two chromosomes are very similar in length and the difference in measurements may be caused by different chromosome preparation methods. Hsiao et al. (1986) treated the root tips with colchicine and 8-hydroxyquinoline and then stained the cell with aceto-orcein, while we treated root tips with ice water and stained with acetocarmine.

All of the 14 chromosomes in this species showed terminal and/or interstitial bands (Fig. 2-1). The 10 accessions were collected from different geographical areas in the world. All of the accessions showed similar karyotype and banding patterns, although significant banding variations were observed (Fig. 2-2). Variations in band number, size, and position occurred at different levels. Firstly,

Table 2-2. Length*, arm ratios and centromere index of *Ps. juncea* chromosomes.

Chromosome designation	Average length(μm)	Relative length(%)	Arm ratio L/S	Centromere index
A	9.77(0.65)	16.66(0.27)	1.09(0.03)	0.48
B	9.38(0.60)	15.99(0.28)	1.29(0.04)	0.44
C	8.66(0.59)	14.77(0.22)	1.51(0.07)	0.40
D	8.17(0.60)	13.93(0.18)	2.18(0.07)	0.31
E	7.89(0.54)	13.45(0.27)	1.11(0.03)	0.47
F	7.68(0.56)	13.09(0.25)	1.74(0.09)	0.36
G	7.11(0.51)	12.11(0.24)	1.43(0.06)	0.41
Total	58.65(0.45)			

* The length of satellite is not included.

the banding patterns of the homologues were often nonidentical (Fig. 2-2), especially, the interstitial bands in the long arms of chromosomes A and E. Secondly, different plants in the same accession showed different banding patterns, although the banding patterns in different cells of a plant were very stable. For example, the interstitial band in the short arm of chromosome A was a different size in two plants of the accession PI314668 (1-1 and 1-2 in Fig. 2-2). Thirdly, different accessions from the various geographical regions had different C-banding patterns (Fig. 2-2). Accessions originated from close geographical regions shared more similar C-banding patterns than accessions from distant geographical regions.

Table 2-3. Chromosome arrangement comparisons

Authors	Chromosome arrangements						
This study	A	B	C	D	E	F	G
Gill (1981)	D	E	F	G	A	B	C
Endo & Gill (1984)	A	G	F	D	E	B	C
Hsiao et al. (1986)	1	2	3	4	6	5	7

Additionally, the degree of polymorphism varied among chromosomes. The bands in chromosomes A, B, E, and F were more variable than those in chromosomes C, D, and G. Banding polymorphisms increased from a low level among plants of an accession, to a higher level within accessions from the same geographical region, and then to the highest amount of polymorphism among accessions from different geographical regions.

B-chromosomes, varying from one to three, were observed in plants of the accession DJ-3955 (No. 3 of Fig. 2-2). The uniform staining after Giemsa treatment indicates the totally heterochromatinized nature of B-chromosomes.

Although banding polymorphisms were present, each of the seven chromosomes had a unique banding pattern that allowed identification of each chromosome. A generalized C-banding karyotype was established based on the C-bands and chromosome karyotype data of the 10 accessions (Fig. 2-3). The

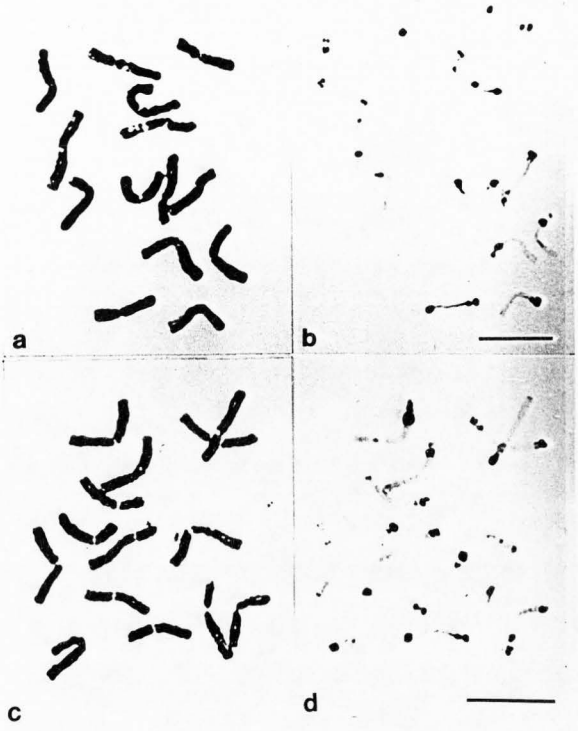

Fig. 2-1. Acetocarmine stained and C-banded metaphase chromosomes of *Ps. juncea* in accession PI314668 (a and b) and accession Bozoisky (c and d). a and c were stained with acetocarmine, and b and d were C-banded. Bar=10 µm.

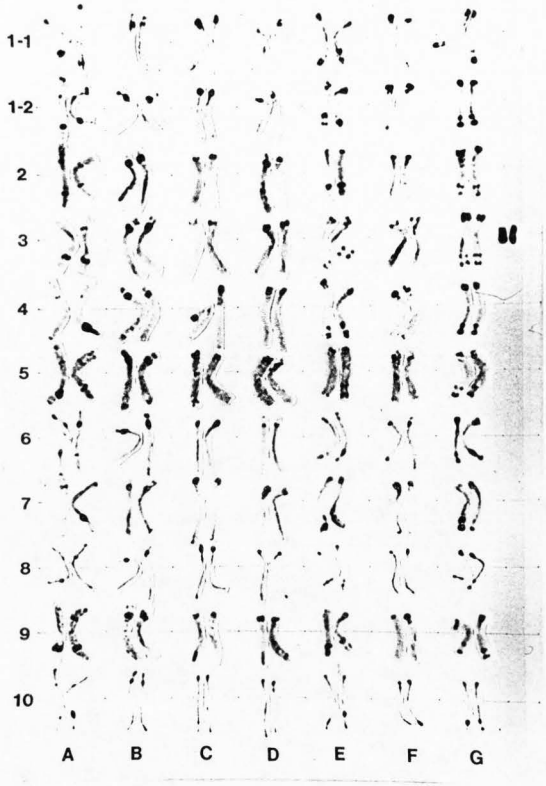

Fig. 2-2. C-banding karyotypes in 10 accessions of *Ps. juncea*. 1-1 and 1-2 were two plants of accession PI314668; 2, PI314552; 3, DJ3955; 4, Bozoisky; 5, PI499558; 6, PI206684; 7, Cabree; 8, KJ130; 9, KJ237, and 10, KJ240.

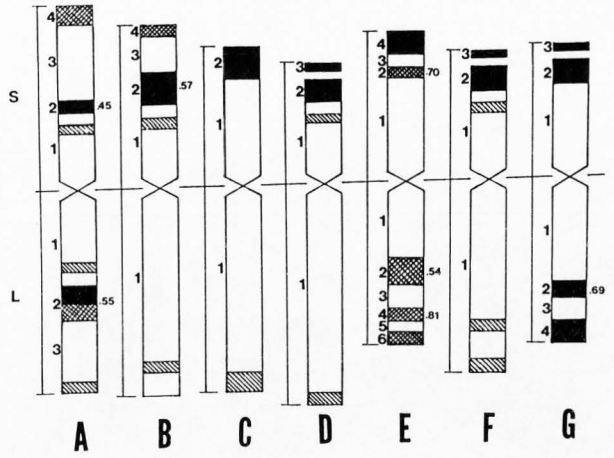

Fig. 2-3. Generalized C-banding idiogram of *Ps. juncea*. Band numbers are indicated on the left and FL positions on the right of the band. Bands present more than 95% of the time (solid dark bands) and more than 80% (crosshatched bands) were numbered. The band with slant lines were variable and not numbered.

solid dark bands were observed with a frequency higher than 95%, the crosshatched bands were observed with a frequency of 80% to 94%, and the slant-lined bands were rarely observed. The designation of the chromosome C-bands is based on the standardized nomenclature for human cytogenetics (Rowley 1974) and for common wheat (Gill et al. 1991). The short arm is designated by the letter S and the long arm by the letter L. Each chromosome consists of a continuous series of light and dark bands. A band is defined as "a part of a chromosome clearly distinguishable from adjacent parts by virtue of its lighter or darker staining ability" (Gill et al. 1991, p. 833). Only the bands observed frequently (higher than 80% frequency) were used as chromosome landmarks in this outcrossing species. The fraction length (FL) of the diagnostic bands from the centromere is indicated on the right of the band. Bands were numbered consecutively from the centromere outward along each chromosome.

To designate a particular band, the chromosome number, the arm symbol, and the band number, separated by a full stop between items, should be indicated. For example, A.S.2 is the interstitial band in the short arm of chromosome A. Because of the simple band pattern, subdivision, as in human and wheat chromosomes, is not necessary in this species.

The characters of each chromosome are described as follows:

Chromosome A (L/S=1.09, RL=16.66%): It is the longest chromosome with the smallest arm ratio in the genome. It has an interstitial and a terminal band in the

short arm, and a major interstitial band with variable band size in the long arm.

Chromosome B (L/S=1.29, RL=15.99%): This chromosome has a major interstitial band in the distal region of the short arm. The short arm frequently had a terminal band, whereas either the long arm or short arm occasionally had an interstitial band.

Chromosome C (L/S=1.51, RL=14.77%): This chromosome has a major terminal band in the short arm and sometimes a minor terminal band in the long arm.

Chromosome D (L/S=2.18, RL=13.93%): This chromosome, with the largest arm ratio, is the only subcentromeric chromosome in the genome. It has a satellite band and a terminal band in the short arm. A terminal band was rarely observed in the long arm.

Chromosome E (L/S=1.11, RL=13.45%): This chromosome has almost the same arm ratio as chromosome A, but was much shorter than A. There is a terminal band in the short arm. An interstitial band near the terminal band in the short arm and one to two interstitial bands with or without a terminal band in the long arm were frequently observed. The band number, band size, and band position in the long arm of this chromosome were the most variable.

Chromosome F (L/S=1.74, RL=13.09%): This is a satellited chromosome with the second largest arm ratio and the second shortest length. This chromosome has a satellite band and a terminal band, and sometimes an interstitial band in the short

arm and a terminal and/or an interstitial band in the long arm.

Chromosome G (L/S=1.43, RL=12.11%): The shortest chromosome also has a microsatellite. It has a satellite band, a terminal band in the short arm, and an interstitial and a terminal band in the long arm. Chromosome G has the most stable banding pattern of all.

Each D, F, and G chromosomes had a microsatellite in the short arm. The size of the microsatellite was less than a tenth of the short arm. Due to their separation from the chromosome or tight association with the end of a chromosome, the satellites were not always observed in all cells. That might be caused by squashing during slide preparation or the low activity of the nucleolar organizer. Only 31.26%, 19.40%, and 9.03% of the chromosomes D, F, and G were observed with a microsatellite.

The Russian wildrye plant HD-33-6 originated from a plant of the cultivar "Bozoisky", which was selected from the accession PI406468 (Asay et al. 1985). By comparing its mitotic karyotype and C-banding pattern with "Bozoisky", HD-33-6 was evidently a Russian wildrye, not a hybrid between *Ps. juncea* and *Agropyron cristatum* (L.) Gaertner. However, some chromosome structural aberrations occurred in this plant. Before banding treatment, a unique small metacentric chromosome was observed. After C-banded karyotype analysis, additional aberrations were identified in detail. In addition to the small metacentric chromosome, two other chromosomes were abnormal. The small chromosome was

identified as chromosome F with a deletion in the long arm. Both arms of the small chromosome were almost equal in length, but they can be distinguished by the terminal band in the short arm (Fig. 2-4a).

In the chromosome E pair, one was normal with terminal bands in both arms and an interstitial band in the long arm. However, the other homologue of chromosome E had no band in the long arm (Figs.2- 4b and 2-4c). Even though the bands in the long arm of this chromosome were the most variable, chromosome E without a C-band in the long arm was never observed in other plants of "Bozoisky" (Fig. 2-2) or in other studied accessions.

In the chromosome F pair, one homologue was the small chromosome mentioned above. The other chromosome F had two major bands in the long arm, one interstitial and a terminal band (Fig. 2-4b), which are similar to those of chromosome E. It was, therefore, considered a reciprocal translocation. The distal part of chromosome E (having two bands) and the distal segment of chromosome F (having no band) exchanged their positions. So this plant had a deletion and a reciprocal translocation. The chromosome F with a deletion was designated F^-, and the two translocation chromosomes were E^f and F^e, respectively.

To confirm this deletion-translocation, the chromosome behavior in meiotic PMCs was examined. Nearly all (98%) of the PMCs in diakinesis showed five bivalents and a quadrivalent (Fig. 2-5a). After C-banding, all of the seven chromosomes were easily identified and chromosomes E and F were involved in the

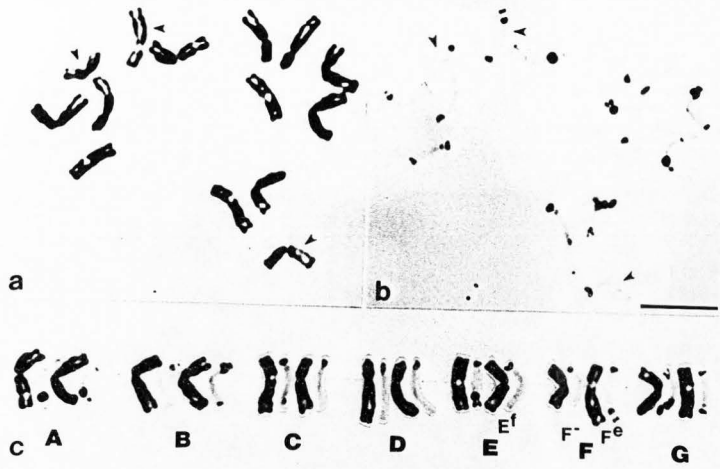

Fig. 2-4. The karyotype and C-banding pattern of HD-33-6. (a) stained by acetocarmine, (b) C-banding, and (c) karyotype. Bar = 10 μm.

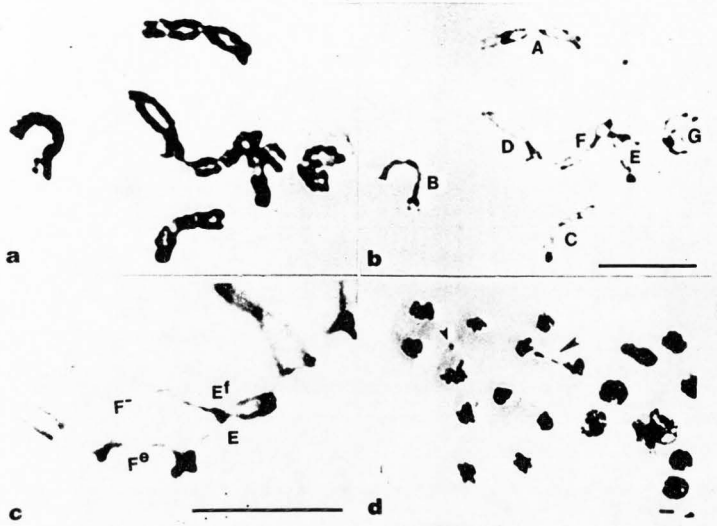

Fig. 2-5. Meiosis of HD-33-6. (a) aceto-carmine stained, (b) C-banded, (c) the quadrivalent, and (d) micronucleus and chromosome bridge. Bar = 10 μm.

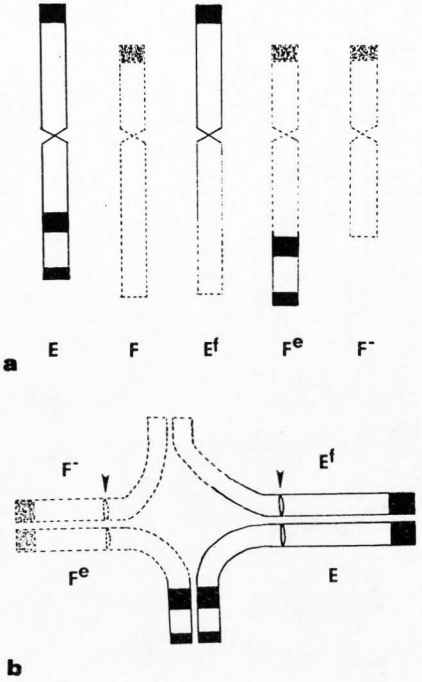

Fig. 2-6. Idiograms of the aberrated chromosomes in mitotic and meiotic cells. (a) normal and aberrated chromosome E and F, and (b) the quadrivalent. The solid line represents chromosome E and the broken line represents chromosome F.

formation of the quadrivalent (Fig. 2-5b). Unexpectly, chromosome F^- paired with E^f in the distal part of their long arm (Fig. 2-5c). It could be interpreted that the deletion involved not the terminal segment but the interstitial segment of the long arm of chromosome F. The translocation occurred between a segment proximal to the interstitial band in the long arm of chromosome E and the distal segment of the long arm of chromosome F. The ideograms of the normal and aberrant chromosomes E and F in mitosis and meiosis are shown in Figs. 2-6a and b. A chromosome bridge and micronucleus were also observed in telophase and anaphase of PMCs (Fig. 2-5d). Only 2% of the pollen can be stained by IKI_2; but anthers of this plant were non-dehiscent. This plant can be maintained vegetatively.

Some within genome translocations and their tranlocation sites have been reported in barley and rye (Xu and Kasha 1992; Marthe and Kunzel 1994; Alvarez et al. 1994). Reciprocal translocation can be used for gene mapping (Benito et al. 1991; Brewster et al. 1992; Nonomura et al. 1993). Therefore, the deletion-translocation plant of Russian wildrye may be useful for gene mapping of some important traits in this species.

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CHAPTER 3

CYTOLOGICAL IDENTIFICATION OF SOME TRISOMICS OF RUSSIAN WILDRYE (*PSATHYROSTACHYS JUNCEA*)

Abstract

Russian wildrye, *Psathyrostachys juncea* (Fisch.) Nevski (2n=2x=14; **NsNs**), is an important forage grass and a potential source of germplasm for cereal crop improvement. Because of genetic heterogeneity as a result of its self-incompatibility, it is difficult to identify trisomics of this diploid species based on morphological characters alone. Putative trisomics (2n=15), derived from open-pollination of a triploid plant by pollen grains of diploid plants, were characterized by Giemsa C-banding. Based on both karyotypic criteria and C-banding patterns, four of the seven possible primary trisomics, a double-deletion trisomic and two tertiary trisomics, were identified.

Introduction

Russian wildrye is an important species in the genus *Psathyrostachys*, one of the most easily circumscribed and least controversial genera in the Triticeae. *Psathyrostachys* was erected by Nevski (1934) and is recognized by most agrostologists (Dewey 1984). Most *Psathyrostachys* species are diploid, although some tetraploid accessions were recently found in *Ps. juncea* (Asay et al. 1994).

The genome of *Psathyrostachys* species was originally designated as **N**, but was changed to **Ns** according to the newly proposed system (Wang et al. in press).

Although it is native to the steppe and desert regions of Russia and China, Russian wildrye is now widely used in North America as a forage grass. Its nutritive value and resistance to alkalinity and drought provide excellent grazing for livestock and wildlife on semiarid range lands (Asay 1992).

Russian wildrye is resistant to many cereal diseases, such as barley yellow dwarf virus (Comeau and Plourde 1987), powdery mildew (Dong et al. 1992), yellow rust, and leaf rust (Li et al. 1992). Hybrids between Russian wildrye and common wheat have been obtained (Chen et al. 1988; Plourde et al. 1990). Therefore, Russian wildrye is considered an important potential source of disease resistance for cereal improvement. The cytology of Russian wildrye, including meiotic behavior (Wang and Berdahl 1990), karyotyping (Hsiao et al. 1986), and chromosome banding (Gill 1981; Endo and Gill 1984; Linde-Laursen and Bothmer 1986; William and Mujeeb-Kazi 1992; J-Z. Wei, W.F. Campbell, and R.R-C.Wang, unpublished) have been studied.

Trisomics can be used to construct linkage groups, to assign genes to specific chromosomes, and to study phenotypic effects of individual chromosomes (Khush 1973). Generally, trisomics are produced by firstly crossing an autotetraploid to the diploid, subsequently selfing the triploid or backcrossing to the diploid parent, and finally selecting among the progeny for trisomics. Tetraploids of Russian wildrye

have been artificially induced by nitrous oxide (Berdahl and Barker 1991) and by colchicine (Lawrence et al. 1990). Triploid plants were obtained through natural pollination of the tetraploid by diploid plants (Wang and Berdahl 1990; A.E. Slinkard and G.J. Scoles, unpublished). Triploids can then be used to create trisomics. Because of polymorphism resulting from its self-incompatibility (Jensen et al. 1990), trisomic plants in this species cannot be classified by morphological characteristics alone.

C-banding is the most useful banding technique for plant chromosomes and has been successfully used to identify trisomics in some plant species (Linde-Laursen 1978; Friebe et al. 1990; Imanywoha et al. 1994). A standard C-banding karyotype of Russian wildrye developed by J-Z. Wei, W.F. Campbell, and R.R-C.Wang (unpublished) was used to identify the available trisomics of Russian wildrye.

Materials and methods

Two triploid plants were obtained by open-pollination of the tetraploid with pollen grains from diploids (A.E. Slinkard and G.J. Scoles, unpublished). The triploids and their progenies were grown in a field nursery in Saskatoon, Saskatchewan, Canada. Young inflorescences of these plants were placed on callus induction medium (Wang et al. 1991) and brought back to Logan, Utah. Regenerated plants were potted and maintained in a greenhouse. Root tips were sampled from all regenerants for chromosome counting. Plants with 15 chromosomes were

subjected to detailed karyotype and C-banding analyses using procedures described previously (J-Z. Wei, W.F. Campbell, and R.R-C.Wang, unpublished). Somatic cells with good chromosome spread were photographed before and after C-banding. Chromosomes were analyzed manually, or automatically by the "Plant Chromosome Analyses System" (PCAS) computer program (Y. Lu, R. R-C. Wang, and N. J. Chatterton, unpublished). Available trisomics were tentatively identified through comparison with established karyotype and C-bands in Russian wildrye (J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished).

Results and discussion

A total of 60 regenerants was obtained from 33 inflorescences, of which 29 plants from 13 (39.5%) infloresences had 15 chromosomes in the somatic cells (Table 3-1). Two plants from two inflorescences were triploid and 29 regenerants obtained from 18 inflorescences (54.5%) was diploid.

The C-banding karyotypes of the 29 plants with 15 chromosomes were analyzed in detail. Seven different trisomics, including four kinds of primary trisomics, one double deletion trisomic and two tertiary trisomics, were tentatively identified (Table 3-2).

In most diploid plant species, such as *Spinacea oleracea* L. (Janick et al. 1959) and *Hordeum spontaneum* K. Koch (Tsuchiya 1960), all or most of the different trisomics can be distinguished morphologically. Only two of the seven primary

Table 3-1. Chromosome numbers of regenerated plants from 33 inflorescences of *Ps. juncea*.

Chromosome number	No. of inflorescences	No. of regenerants
2n=14	18	29
2n=15	13	29
2n=21	2	2
Total	33	60

trisomics in *Triticum monococcum* L. produced distinct morphological features (Friebe et al. 1990). Because of its self-incompatibility, Russian wildrye is highly polymorphic even among normal diploid plants. Few distinct morphological features were found to differentiate the trisomics (2n=15) from the diploids (2n=14). One plant, which was cytologically identified as a double deletion-trisomic, induced from an inflorescence, was less vigorous, slower growing, and produced fewer tillers. In addition, four plants induced from two inflorescences had wider leaves and stronger stems than the diploids. These four plants were trisomics for chromosome G and they are described below.

Some diploid plants analyzed by the C-banding procedure showed a banding pattern very similar to the established standard C-banding karyotype. The only exception was a major terminal band on one chromosome C in some plants, which was switched from the short arm to the long arm and the arm ratio of this chromo-

Table 3-2. Classification of identified trisomics and number of plants regenerated from 13 source inflorescences of trisomic *Ps. juncea*.

Trisomic	No. of source inflorescences	No. of plants
Trisomic C	1	3
Trisomic D	4	7
Trisomic F	3	7
Trisomic G	2	4
Trisomic E with double deletions	1	1
Tertiary trisomic DS/AL	1	4
Tertiary trisomic DS/FS	1	3
Total	13	29

some was also changed (Fig. 3-1). It is highly probable that a paracentric inversion occurred to this chromosome. The cytological identification of the seven different trisomics are described as follows:

Primary trisomic for chromosome C: Three regenerants from one original plant (inflorescence) were identified as trisomics for chromosome C. All of the three C chromosomes were normal instead of the inverted one as shown in Fig. 3-1. Chromosome C, the third longest chromosome, had a major terminal band and sometimes an interstitial band in the short arm (Fig.3-2). It was distinct from

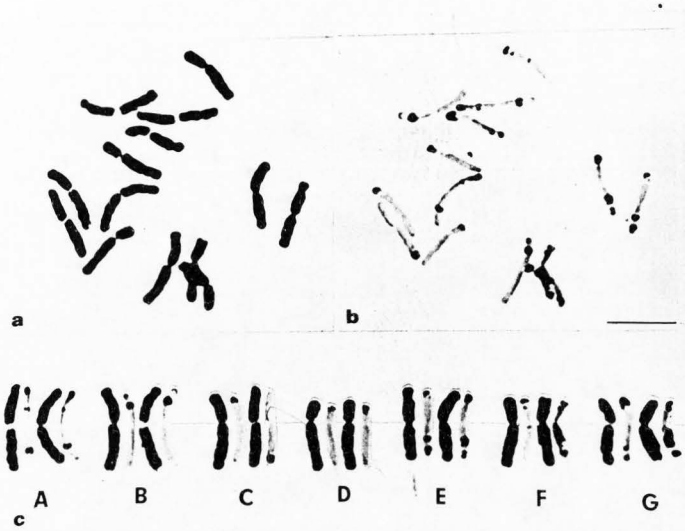

Fig. 3-1. Karyotype and C-banding pattern of diploid *Ps. juncea*. (a) Stained by acetocarmine, (b) C-banded chromosomes, and (c) karyotype. Bar = 10 μm.

chromosomes A, B, E, and G. Chromosome D had a much larger long arm/short arm ratio. Chromosome F was shorter than chromosome C, and sometimes had an interstitial band or a terminal band in the long arm.

Primary trisomic for chromosome D: Chromosome D had the largest arm ratio and was the only submetacentric chromosome in this species. It had a terminal band and sometimes an interstitial band in the short arm. A small satellite was frequently observed (Fig. 3-3). These features made this chromosome distinct from the others. Seven regenerants from four original inflorescences were identified as trisomics for chromosome D.

Primary trisomic for chromosome F: Seven plants from 3 inflorescences were identified as trisomic F (Fig. 3-4). Because of their similar banding patterns, chromosome F could be easily confused with chromosome C or D. Chromosome length and arm ratio were carefully measured. Arm ratio distinguished chromosome F from D and overall chromosome length distinguished chromosome F from C.

Primary trisomic for chromosome G: Chromosome G was the shortest chromosome in the genome. It exhibited a terminal band, and a interstitial band in the long arm, a major terminal band, and sometimes a small satellite in the short arm (Fig. 3-5). Its banding pattern made chromosome G distinct from other chromosomes except chromosome E. The arm ratio and a slightly different banding pattern in the long arm helped distinguish chromosome G from E. Four plants regenerated from two inflorescences were identified as trisomic G. These trisomics

Fig. 3-2. Karyotype and C-banding pattern of trisomic for chromosome C in *Ps. juncea*. (a) Stained by acetocarmine, (b) C-banding, and (c) karyotype. Bar = 10 μm.

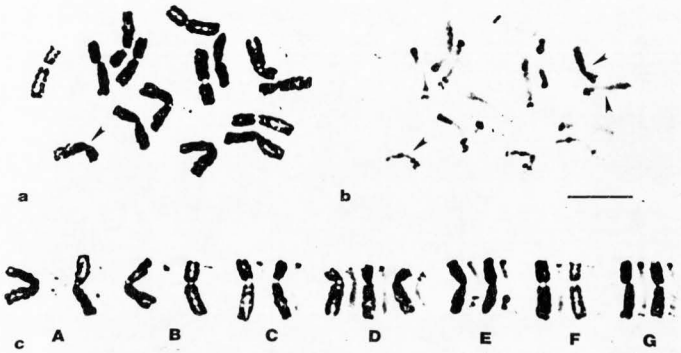

Fig. 3-3. Karyotype and C-banding pattern of trisomic for chromosome D in *Ps. juncea*. (a) Stained by acetocarmine, (b) C-banding, and (c) karyotype. Bar = 10 μm.

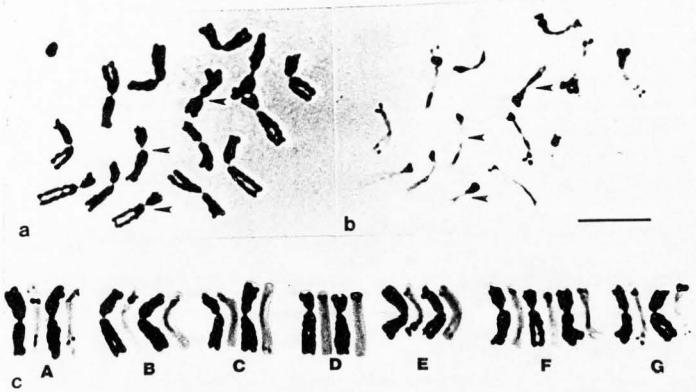

Fig. 3-4. Karyotype and C-banding pattern of trisomic for chromosome F in *Ps. juncea*. (a) Stained by acetocarmine, (b) C-banding, and (c) karyotype. Bar = 10 μm.

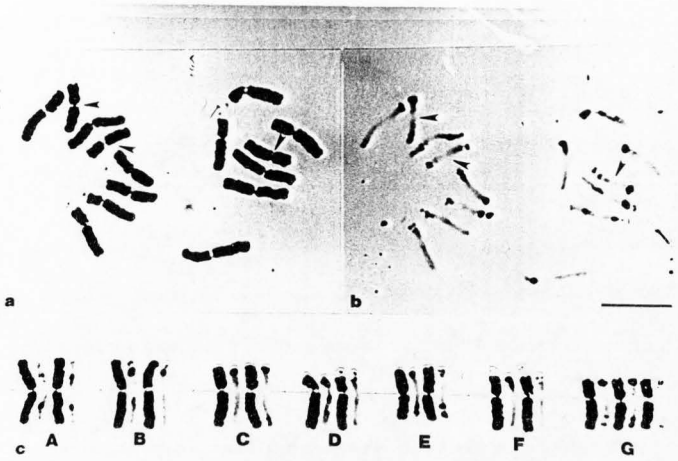

Fig. 3-5. Karyotype and C-banding pattern of trisomic for chromosome G in *Ps. juncea*. (a) Stained by acetocarmine, (b) C-banding, and (c) karyotype. Bar = 10 μm.

had slightly wider leaves and stronger culms than the diploids and other trisomics.

Double deletion-trisomic for chromosome E: One trisomic plant was observed to have two small acrocentric chromosomes (Fig. 3-6). C-banding and karyotype analysis identified it as a trisomic for chromosome E with two deletions. One deletion was the distal segment in the short arm of one chromosome D and the other was the distal segment in the short arm of one of the three E chromosomes. This trisomic exhibited less vigor, was slower growing, and had fewer tillers than the diploids and other trisomics.

Tertiary trisomic DS/AL: The tertiary trisomic contained an extra chromosome consisted of arms from two different chromosomes. The extra chromosome in four regenerants from one original plant had a large arm ratio, a terminal band in the short arm, and an interstitial band in the long arm (Fig. 3-7). Arm length (the short arm of chromosome D was the shortest arm in this species) and the banding patterns were used to show that the extra chromosome was composed of the short arm of chromosome D and the long arm of chromosome A.

Tertiary trisomic DS/FS: One very short extra chromosome (about half the length of the longest chromosome in the **Ns** genome) was observed in three plants regenerated from one inflorescence. A small satellite was frequently present in the longer arm of the extra chromosome, and both arms had terminal and minor interstitial bands (Fig. 3-8). This extra chromosome was composed of the short arm of chromosome D and the short arm of chromosome F.

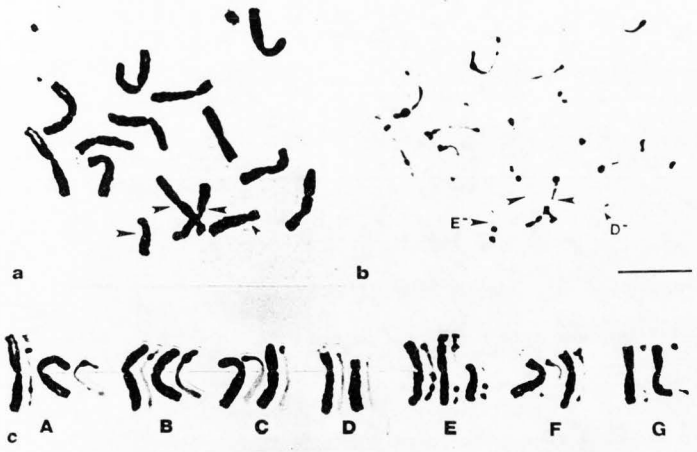

Fig. 3-6. Karyotype and C-banding pattern of a double-deletion trisomic for chromosome E in *Ps. juncea*. (a) Stained by acetocarmine, (b) C-banding, and (c) karyotype. Bar = 10 μm.

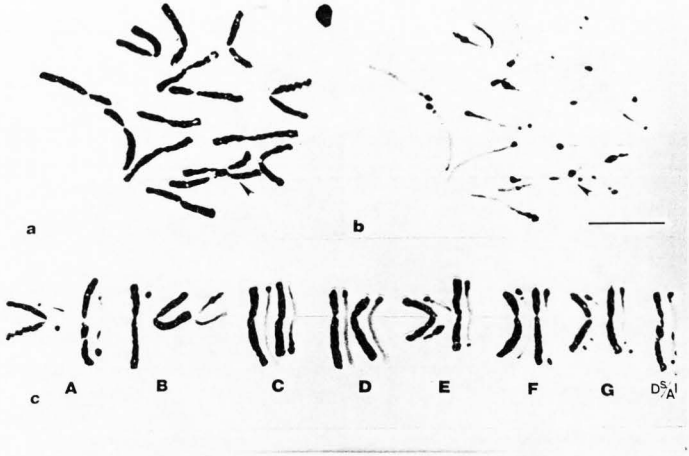

Fig. 3-7. Karyotype and C-banding pattern of a tertiary trisomic for DS/AL in *Ps. juncea*. (a) Stained by acetocarmine, (b) C-banding, and (c) karyotype. Bar = 10 μm.

No primary trisomic for chromosomes A, B, or E was found in this study, although the long arm of chromosome A and most of chromosome E were involved in the tertiary trisomic DS/AL and the double deletion-trisomic E, respectively. Some primary trisomics may be difficult to obtain. Examples include the trisomic for chromosome 3A in *T. monococcum* (Friebe et al. 1990) and the trisomic for chromosome F in *Agropyron cristatum* (Imanywoha et al. 1994).

Telosomics are usually obtained during the production of trisomics in some species (Friebe et al. 1990; Kim and Kuspira 1992; Imanywoha et al. 1994). In this study of Russian wildrye no telosomics were observed. However, the occurrence of some tertiary trisomics suggests that telocentric chromosomes may have fused into the tertiary trisomic. It appears that satellite chromosomes frequently become trisomic. Of the 13 original trisomic plants 9 were trisomics for satellited chromosomes. Three of the four arms of the tertiary trisomics contained arms with a satellite. Our previous study indicated the frequency of satellites on chromosomes D, F, and G were about 31%, 19%, and 9%, respectively (J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished). In this study, although the sample size was small, a similar trend in trisomics for chromosomes D, F, and G was found. Four, three, and two trisomic plants for chromosomes D, F, and G were found. In *A. cristatum*, more trisomics for satellited chromosomes D and E were produced (Imanywoha et al. 1994). There appears to be some relationship between the satellited chromosomes, or the nucleolar organizer, and the viability of gametes.

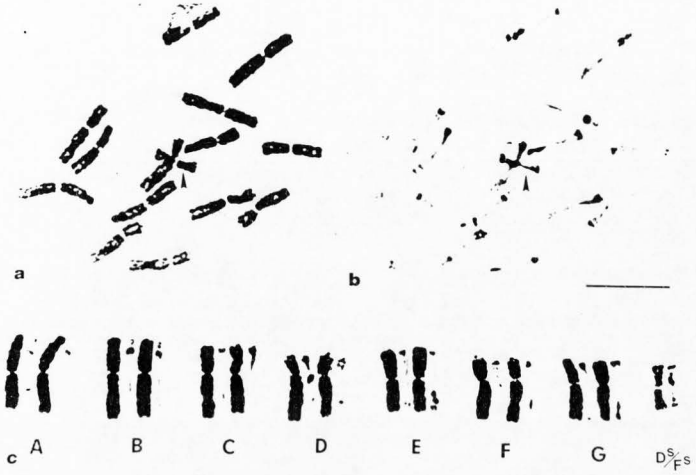

Fig. 3-8. Karyotype and C-banding pattern of a tertiary trisomic for DS/FS in *Ps. juncea*. (a) Stained by acetocarmine, (b) C-banding, and (c) karyotype. Bar = 10 μm.

Numerical and structural chromosome variation can be induced in tissue culture of rye, and the cytological variation is genotype-dependent (Puolimatka and Karp 1993). In this study, the constant C-banding karyotype observed in all plants regenerated from the same inflorescence excluded the possibility that the tertiary trisomics and double-deletion trisomic resulted from the inflorescence culture procedure.

In summary, four primary trisomics, one double-deletion trisomic, and two tertiary trisomics were tentatively identified by chromosome C-banding techniques. The trisomics need to be confirmed by meiotic and molecular evidence. It is also desirable to obtain the primary trisomics for chromosomes A, B, and F to make the trisomic series complete. When the trisomic series is complete, they will be useful in genetic studies.

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CHAPTER 4

ALLOZYME VARIATION IN ACCESSIONS OF RUSSIAN WILDRYE (*PSATHYROSTACHYS JUNCEA*)

Abstract

One hundred and sixty individuals representing 11 accessions of Russian wildrye, *Psathyrostachys juncea* (Fisch.) Nevski (2n=2x=14; **NsNs**), collected from five countries, were analyzed by starch gel electrophoresis to measure genetic variation. Ten enzymes revealed 14 putative and scorable loci, of which 11 showed polymorphisms (on average, 66.2% loci polymorphisms and 2.6 alleles per locus). High levels of isozyme diversity were found mainly within accessions. Fixation of locus *Idh* occurred in accessions from Russia, Turkey, and China, whereas polymorphisms at that locus existed in accessions from Mongolia and Iran. Allelic variation at locus *Ak*-1 was only detected in accessions of Russian origin. A large amount of allelic diversity occurred at most polymorphic loci. Approximately 90% of the allelic diversity existed within accessions. Most of the accessions, except one from Turkey, are genetically similar. Accessions collected from Russia and Mongolia are closely related, except for accession JA251, which was collected at a location distant from other Russian accessions' habitats. The isozyme data may be used in conjunction with other data by gene banks to select accessions for the formation of core collections.

Introduction

Psathyrostachys (Nevski) is a small genus of the tribe Triticeae with no more than 10 species. These grasses are characterized by caespitose, long-anthered, cross-pollinating, long-lived perennials with multiple spikelets per node, one or more florets per spikelets, subulate glumes, and a fragile rachis (Dewey 1984). The only species within this genus to gain importance as a forage grass is *Ps. juncea* (Russia wildrye).

Russian wildrye (2n = 14, **NsNs**), is tolerant to alkalinity and drought, and is resistant to barley yellow dwarf virus (BYDV) (Comeau and Plourde 1987) and some other wheat diseases. Hybrids between common wheat and *Ps. juncea* have been obtained (Chen et al. 1988; Plourde et al. 1990).

The study of genetic variation in accessions of Russian wildrye is important to 1) evaluate genetic composition and structure for further utilization of the germplasm, and 2) provide genetic information for breeding of this forage grass. Russian wildrye is self-incompatible, and genetic diversity widely exists within populations according to chromosomal banding studies (Endo and Gill 1984; Linde-Laursen and Bothmer 1984; J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished).

Allozyme data have been widely used to measure genetic variation within and among populations (Tanksley and Orton 1983; Chung and Hamrick 1991; Novak et al. 1991; Ahmad et al. 1992; Spooner et al. 1992). McIntyre (1988) studied

isozyme variation in Triticeae, including *Ps. juncea*, and the genetic distances in 16 species are estimated. William and Mujeeb-Kazi (1992) reported some isozyme markers of *Ps. juncea*. These markers could be used in tracking of *Ps. juncea* chromosomes in a wheat background.

Isozyme variation within and among accessions of *Ps. juncea* has not been reported previously. In the present study, isozyme variation in 11 different accessions was analyzed.

Materials and methods

Materials and sampling

One hundred and sixty plants representing 11 accessions were examined (Table 4-1). Accessions of Russian wildrye were collected from Russia, China, Mongolia, Iran, and Turkey by scientists of the Forage and Range Research Laboratory (FRRL), USDA-ARS, Logan, Utah. Ten to 20 spikes were randomly collected from each location and mixed together. Seeds of each accession were chosen randomly from the mixture, germinated, and seedlings were transplanted to the field (K. Jensen, personal communication). Ten to 20 individuals in each accession were analyzed.

Isozyme electrophoresis

Young leaves were collected from each plant and stored at -70° C. The leaf tissue was ground in an extraction buffer (0.1 M Tris-HCl, pH 7.0, 0.01 M EDTA, 0.5

Table 4-1. Accessions of *Ps. juncea* used and their geographical origins.

Number	Accession	Geographical origin	No. of plants
1	JA 239	Russia	15
2	JA 246	Russia	20
3	JA 251	Russia	20
4	JA 285	Russia	20
5	JA 305	Russia	20
6	KJ 130	Iran	10
7	KJ 237	Mongolia	15
8	KJ 238	Mongolia	11
9	KJ 240	Mongolia	10
10	PI206684	Turkey	10
11	PI499558	China	10

g/L NAD, 0.2 g/L NADP, 10 ml 2-mercaptoethanol; Wendel and Weeden, 1989). Starch gel electrophoresis procedures followed those of Wendel and Weeden (1989) and Murphy et al. (1990). Ten enzymes were examined in three buffer systems (Table 4-2).

Data analysis

Electrophoretic data were analyzed using BIOSYS-1. The data were entered

Table 4-2. Enzymes, buffer systems, locus, and numbers of alleles detected in 11 accessions of *Ps. juncea*.

Enzyme	E. C. No.	Buffer system[1]	Locus	No. of allele detected
Adenylate kinase (AK)	2.7.4.3	M-C	*Ak*-1	2
			Ak-2	1
Aldolase (ALD)	4.1.2.13	M-C	*Ald*	1
Glucose Dehydrogenase (GDH)	1.1.1.118	7	*Gdh*	2
Glucose-6-phosphate dehydrogenase (G6PDH)	1.1.1.49	7	*G6pdh*	1
Glucose phosphate isomerase (GPI)	5.3.1.9	7	*Gpi*-1	5
			Gpi-2	3
Isocitrate dehydrogenase (IDH)	1.1.1.42	M-C	*Idh*	2
Malate dehydrogenase (MDH)	1.1.1.37	T-C	*Mdh*-1	5
			Mdh-2	5
Phosphoglucomutase (PGM)	2.7.5.1	7	*Pgm*	3
6-Phosphogluconate dehydrogenase (PGDH)	1.1.1.44	T-C	*Pgdh*-1	6
			Pgdh-2	4
Shikimic dehydrogenase (SDH)	1.1.1.25	M-C	*Sdh*	4

1. See electrophoretic buffer system in Appendix.

as genotype of each individual for each accession. Allelic frequencies of each locus were calculated in each accession. Genetic variability was assessed using the number of alleles per locus, the percent polymorphic loci per population, and the observed and expected mean heterozygosity across an accession. A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95. A chi-square test for deviation from Hardy-Weinberg equilibrium was carried out for each locus in each accession. The gene diversity statistics of Nei (1973) were used to partition the total allelic diversity within these accessions. At each polymorphic locus the total allelic diversity is represented by H_T, which is partitioned into the mean allelic diversity within accessions (H_S), and the allelic diversity among accessions (D_{ST}). The proportion of the total allelic diversity found among accessions (G_{ST}), is calculated as the ratio D_{ST}/H_T. The distribution of genetic variation among populations was estimated using Nei's genetic diversity statistics (Nei 1973). The unbiased genetic identity coefficient of Nei (1978) was calculated for all possible pairwise comparisons among the 11 accessions. Cluster analysis was carried out and a phenogram was prepared using the unweighted pairgroup method and arithmetic average (UPGMA) with SHAN (Sequential, Hierarchical, Agglomerative, and Nested Clustering) routine in NTSYS (Numerical Taxonomy System) programs.

Results

All 160 individual plants were analyzed for electrophoretic variability and the enzyme bands that migrated anodally from the origin were scored. The 10 enzymes were encoded by 14 putative loci (Table 4-2). Four enzymes, *MDH, AK, PGDH,* and *GPI*, have two loci; all other enzymes have one locus. Among the 14 loci, only three loci, *Ald*, *G6pdh*, and *Ak*-2, were monomorphic; the other 11 loci (78.57%) were polymorphic. The allele number at each locus ranged from 1 to 6 (Table 4-2). *Pgdh*-1 was the most polymorphic locus. Table 4-3 shows allelic frequencies in the 11 accessions. Among the polymorphic loci, most of the alleles were distributed across all 11 accessions. Some alleles were fixed in several accessions and other alleles were present or absent in just one accession. The accessions from Russia (Nos. 1 - 5), Turkey (No. 10), and China (No. 11) were fixed for allele *Idh*-a at the locus *Idh*. With the exception of accession JA285 (No. 4), allele *Ak*-1 was fixed in all accessions other than those from Russia. The loci *Pgdh*-1 and *Pgdh*-2 are fixed in accession KJ130 (No. 6) and PI206684 (No. 11), respectively. *Mdh*-2d and *Mdh*-2e were detected only in accession KJ130 (No.6), one that lacks the allele *Mdh*-2c. Accession KJ130 has only the allele d at the locus *Pgdh*-1; and only allele *Pgdh*-2a was detected in accession PI206684. Various accessions showed different allele polymorphisms. Accession PI206684 showed the least allele polymorphisms.

The mean number of allele per locus, percentage of loci polymorphism, and

Table 4-3. Allelic frequencies in the 11 accessions of *Ps. juncea* (numbers of individuals in parentheses).

Locus		Accessions 1 (15)	2 (20)	3 (20)	4 (20)	5 (19)	6 (10)	7 (15)	8 (11)	9 (10)	10 (10)	11 (10)
Pgm	a	.100	.000	.325	.100	.053	.056	.000	.091	.000	.000	.000
	b	.867	.825	.625	.825	.789	.944	.967	.818	.778	.750	.950
	c	.033	.175	.050	.075	.158	.000	.033	.091	.222	.250	.050
Idh	a	1.00	1.00	1.00	1.00	1.00	.950	.900	.955	.944	1.00	1.00
	b	.000	.000	.000	.000	.000	.050	.100	.045	.056	.000	.000
Sdh	a	.200	.150	.100	.325	.263	.050	.200	.273	.500	.150	.000
	b	.467	.550	.450	.375	.342	.700	.500	.273	.700	.250	.250
	c	.333	.300	.450	.300	.395	.150	.300	.409	.200	.600	.750
	d	.000	.000	.000	.000	.000	.100	.000	.045	.050	.000	.000
Mdh-1	a	.100	.000	.100	.175	.079	.000	.333	.227	.050	.650	.100
	b	.400	.000	.325	.225	.474	.450	.200	.091	.250	.150	.500
	c	.200	.425	.175	.275	.237	.500	.200.	.182 .	400	.000	.100
	d	.267	.250	.350	.300	.079	.050	.033	.182	.200	.000	.000
	e	.033	.325	.050	.025	.132	.000	.233	.318	.100	.000	.000
Mdh-2	a	.267	.200	.525	.450	.553	.450	.500	.091	.350	.450	.600
	b	.667	.600	.400	.425	.342	.450	.433	.455	.450	.550	.300
	c	.067	.200	.075	.125	.105	.000	.067	.455	.200	.000	.100
	d	.000	.000	.000	.000	.000	.050	.000	.000	.000	.000	.000
	e	.000	.000	.000	.000	.000	.050	.000	.000	.000	.000	.000
Ald	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Gdh	a	.533	.825	.250	.600	.694	.650	.733	.682	.750	.900	.750
	b	.467	.175	.750	.400	.306	.350	.267	.318	.250	.100	.250
G6pdh	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ak-1	a	.067	.125	.025	.000	.053	.000	.000	.000	.000	.000	.000
	b	.933	.875	.975	1.00	.947	1.00	1.00	1.00	1.00	1.00	1.00
Ak-2	a	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Pgdh-1	a	.300	.100	.250	.175	.053	.000	.067	.136	.200	.100	.050
	b	.367	.125	.350	.275	.026	.000	.167	.182	.050	.650	.700
	c	.067	.050	.125	.050	.184	.000	.067	.273	.000	.000	.050
	d	.033	.450	.050	.375	.658	1.00	.500	.182	.450	.000	.100
	e	.133	.125	.125	.125	.000	.000	.133	.091	.300	.000	.100
	f	.100	.150	.100	.000	.079	.000	.067	.136	.000	.250	.000
Pgdh-2	a	.000	.275	.550	.400	.605	.100	.533	.455	.000	1.00	.800
	b	.300	.300	.400	.325	.368	.650	.400	.500	.350	.000	.100
	c	.500	.350	.050	.250	.026	.250	.067	.045	.500	.000	.100
	d	.200	.075	.000	.025	.000	.000	.000	.000	.150	.000	.000
Gpi-1	a	.300	.350	.475	.375	.289	.350	.300	.455	.250	.000	.600
	b	.267	.175	.225	.225	.211	.150	.167	.273	.050	.200	.100
	c	.200	.150	.150	.125	.158	.400	.433	.182	.000	.800	.250
	d	.067	.200	.025	.200	.026	.100	.000	.045	.450	.000	.000
	e	.167	.125	.125	.075	.316	.000	.100	.045	.250	.000	.050
Gpi-2	a	.333	.350	.400	.525	.395	.250	.167	.136	.150	.000	.150
	b	.400	.300	.250	.125	.289	.450	.267	.182	.200	.400	.150
	c	.267	.350	.350	.350	.316	.300	.567	.682	.650	.600	.700

the observed and expected mean heterozygosity are given in Table 4-4. Across the 11 accessions, the mean number of allele per locus ranged from 1.9 to 2.8 with a mean of 2.6. All accessions had high polymorphism, with 57.1 - 71.4 percentage (66% on average) of loci polymorphic. In general, accessions from Russia (Nos. 1 - 5) and Mongolia (Nos. 7 -9) had the highest polymorphism. The direct-counted mean heterozygosities were close to the Hardy-Weinberg expected mean heterozygosities in all the accessions except PI499558.

The chi-square test for Hardy-Weinberg equilibrium (Table 4-5) indicated that no locus was in equilibrium in all of the 11 populations and no population was in equilibrium at all 11 polymorphic loci. Most of the accessions reached H-W equilibrium at 4 to 9 loci. The more alleles a locus has, the less chance can all the populations reach equilibrium. The fixation index across all loci in the 11 accessions ranged from -0.695 to +1.

The total allelic diversity and partitions of the 11 loci are shown in Table 4-6. Locus *Mdh*-1 had the highest H_T value, 0.781, while locus *Idh* had the lowest, 0.045. The mean value for polymorphic loci was 0.517. The distribution of the total allelic diversity within and among accessions (Table 4-7) indicated that 91% of the diversity (ranged from 79 - 96%) was found within accessions. A very small part (9%) was attributable to that among accessions. On average, the allelic diversity within accessions was about 10 times that of among accessions. Diversity within countries was about two times that of among countries. Few allelic diversities were

Table 4-4. Genetic variability at 14 loci in 11 accessions of *Ps. juncea* (standard errors in parentheses).

Accession	Mean sample size per locus	Mean no. of allele per locus	Polymorphic loci %	Mean heterozygosity	
				Direct-count	H - W expected*
1. JA 239	15.0	2.8	71.4	.338	.405
	(.0)	(.4)		(.083)	(.087)
2. JA 246	20.0	2.6	71.4	.368	.400
	(.0)	(.4)		(.085)	(.084)
3. JA 251	20.0	2.8	64.3	.361	.399
	(.0)	(.4)		(.091)	(.085)
4. JA 285	20.0	2.7	64.3	.321	.406
	(.0)	(.4)		(.079)	(.090)
5. JA 305	18.9	2.7	71.4	.328	.383
	(.1)	(.4)		(.102)	(.079)
6. KJ 130	9.9	2.3	64.3	.365	.308
	(.1)	(.3)		(.102)	(.079)
7. KJ 237	15.0	2.6	64.3	.295	.375
	(.0)	(.4)		(.091)	(.084)
8. KJ 238	11.0	2.9	64.3	.331	.403
	(.0)	(.4)		(.091)	(.088)
9. KJ 240	9.9	2.6	71.4	.347	.384
	(.1)	(.4)		(.081)	(.081)
10. PI206684	10.0	.9	57.1	.293	.259
	(.0)	(.3)		(.082)	(.068)
11. PI499558	10.0	2.4	64.3	.193	.293
	(.0)	(.4)		(.065)	(.070)
Mean	14.5	2.6	66.2	.322	.365

* Unbiased estimate (see Nei 1978)

detected within countries for *Idh* and *Pgi*-2, and among countries for *Pgm, Mdh*-2, and *Gdh*. The unbiased genetic identity coefficient of Nei (1978) ranged from 0.0 to 1.0, where a genetic similarity value of 1.0 indicates that a population pair is

Table 4-5. P values of chi-square test for deviation from Hardy-Weinberg equilibrium for isozyme loci in 11 accessions of *Ps. juncea*.

Locus	Accession										
	1	2	3	4	5	6	7	8	9	10	11
Pgm	.025	.032	.000	.036	.717	.860	.894	.909	.391	.292	.868
Idh	--	--	--	--	--	.865	.667	.875	.860	--	--
Sdh	.333	.096	.631	.683	.509	.094	.031	.125	.577	.708	.292
Mdh-1	.066	.002	.000	.000	.015	.770	.000	.081	.005	.030	.001
Mdh-2	.552	.135	.000	.141	.038	.000	.001	.010	.016	.975	.006
Gdh	.072	.343	.766	.264	.010	.754	.930	.875	.292	.725	.292
AK	.782	.523	.909	--	.809	--	--	--	--	--	--
Pgdh-1	.027	.001	.000	.001	.028	--	.000	.006	.077	.407	.000
Pgdh-2	.001	.010	.000	.144	.601	.407	.000	.011	.063	--	.000
Pgi-1	.455	.093	.099	.440	.266	.059	.016	.587	.355	.429	.311
Pgi-2	.913	.491	.229	.372	.068	.455	.032	.109	.407	.035	.607

genetically identical. The cluster test of Nei's genetic identity values showed that the 11 accessions were genetically similar (Table 4-8). The phenogram (Fig. 4-1) measured the overall similarity among the 11 accessions. All of the accessions collected from Russia and Mongolia, except accession JA251, showed more similarity than those with other accessions. Accession PI206684, which originated from Turkey, had the least similarity with other accessions.

Table 4-6. Nei's allelic diversity statistics summarized for the 11 accessions of *Ps. juncea*.

Locus	H_T	H_S	D_{ST}	G_{ST}
Pgm	0.295	0.281	0.014	0.048
Idh	0.045	0.043	0.002	0.036
Sdh	0.634	0.558	0.043	0.068
Mdh-1	0.781	0.694	0.087	0.091
Mdh-1	0.609	0.581	0.028	0.046
Gdh	0.442	0.403	0.039	0.089
Ak	0.048	0.046	0.002	0.039
Pgdh-1	0.772	0.628	0.144	0.187
Pgdh-2	0.664	0.522	0.142	0.215
Pgi-1	0.759	0.689	0.070	0.092
Pgi-2	0.640	0.594	0.046	0.072
Mean	0.517	0.471	0.046	0.089

Discussion

A study of isozyme variations in 17 species of Triticeae (McIntyre 1988) that used four accessions of *Ps. juncea* showed the presence of 8 polymorphic loci of 16 loci examined. William and Mujeeb-Kazi (1992) also observed isozyme variation

Table 4-7. Distribution of the total gene diversity within and among accessions and countries of origin for 11 accessions of *Ps. juncea* at all polymorphic loci.

Locus	H_T	Within accessions	Among accessions	Among countries
Pgm	0.295	0.952	0.056	-0.008
Idh	0.045	0.964	0.000	0.036
Sdh	0.634	0.932	0.027	0.041
Mdh-1	0.781	0.909	0.057	0.034
Mdh-2	0.609	0.939	0.063	-0.002
Gdh	0.442	0.911	0.109	-0.020
Ak-1	0.048	0.961	0.037	0.002
Pgdh-1	0.772	0.823	0.104	0.083
Pgdh-2	0.664	0.785	0.168	0.047
Pgi-1	0.759	0.908	0.052	0.040
Pgi-2	0.640	0.928	0.001	0.071
Mean	0.517	0.911	0.060	0.029

in their attempt to establish isozyme markers for tracking Russian wildrye chromosomes in a wheat background. In the present study, 11 out of 14 loci were polymorphic in 11 accessions of *Ps. juncea*. Similar to C-banding polymorphisms (J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished), isozyme polymorphisms

Table 4-8. Nei's unbiased genetic identity between 11 accessions of *Ps. juncea*.

Accession	1	2	3	4	5	6	7	8	9	10	11
1 JA239	****										
2 JA246	.865	****									
3 JA251	.867	.815	****								
4 JA285	.881	.880	.879	****							
5 JA305	.844	.862	.863	.889	****						
6 KJ130	.827	.827	.783	.836	.845	****					
7 KJ237	.829	.861	.837	.878	.886	.858	****				
8 KJ238	.830	.850	.837	.872	.854	.794	.880	****			
9 KJ240	.840	.873	.782	.852	.825	.840	.850	.832	****		
10 PI206684	.754	.760	.758	.770	.777	.715	.816	.784	.739	****	
11PI499558	.799	.785	.825	.832	.840	.775	.849	.831	.792	.829	****

were common both within and among the accessions. More within than among accession polymorphisms were present. The high frequency of polymorphisms and marked heterozygosity of this species may be attributed to its self-incompatibility. The close fit between the direct-counted and H-W expected mean heterozygosity showed that most populations of Russian wildrye were nearly randomly mated in their habitats. The self-incompatibility, which prevents selfing, enhances gene flows within a population and increases both heterozygosity and polymorphism.

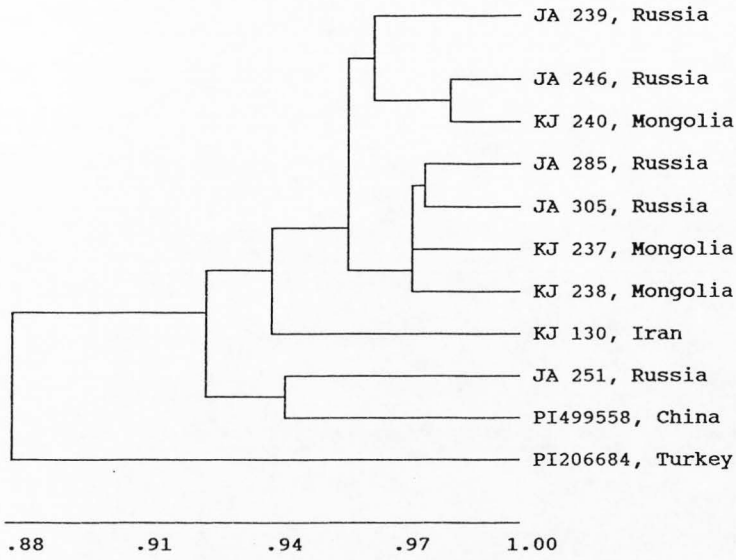

Fig. 4-1. Cluster phenogram of genetic identity values for the 11 accessions of *Ps. juncea*. This phenogram was generated using the UPGMA algorithm.

If the population is small, fixation can be reached in a short time. When populations are not separated by long geographic distance, gene flow among populations can also occur. Therefore, accessions that originated in the same or proximal geographic areas showed high genetic similarity. In the 11 accessions studied, accessions from Russia and Mongolia were most similar. While the Russian and Mongolian accessions had only one locus fixed, other accessions from Iran, Turkey, and China had two or three loci fixed (Table 4-3). Accession JA251, which was collected from the Novosibirsk region, was distinct from the four Russian accessions collected from Kazakhstan. Accession PI206684, collected from Turkey, shared the least similarity (<0.90) with all other accessions. It may be from a small and geographically isolated population. It did have 3 of the 11 examined polymorphic loci fixed. However, the deviation from the Hardy-Weinberg equilibrium might be due to the small sample size because disequilibrium frequently occurred at higher polymorphic loci in these 11 accessions.

The major allelic diversity was partitioned into the within-population component. That contrasts with selfing populations, in which among-population diversity contributed a large part of the total diversity (Novak et al. 1991). Because Russian wildrye is self-incompatible, its within-population allelic diversity was higher than other outcrossers (Hamrick and Godt 1990). Two thirds of the variability among accessions were within countries and about one third (3% in 9%) was among countries. Thus, the allelic diversity among countries contributed a very small

portion to the total diversity, because geographic location, rather than the national boundary, is the real factor that affects gene flow among populations. In the 11 accessions studied here, the accessions originated in Russia and Mongolia may be geographically closer to each other than either is with the other accessions. However, the overall diversity among accessions accounted for about 9% of the total allelic diversity.

In conclusion, isozyme analyses indicate that most accessions show high genetic similarity; however, polymorphisms were widely observed at isozyme loci in *Ps. juncea*. Most of the polymorphic loci showed a high allelic diversity primarily within accessions.

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CHAPTER 5

GENETIC VARIABILITY IN RUSSIAN WILDRYE, (*PSATHYROSTACHYS JUNCEA*) ASSESSED BY RAPD

Abstract

To assess the genetic variation within and among accessions of Russian wildrye (*Psathyrostachys juncea*), 88 individuals of 11 accessions originated from geographically diverse locations were analyzed using randomly amplified polymorphic DNA (RAPD). Under the optimized condition, 56% of the 200 tested primers produced polymorphic RAPDs among accessions. High level RAPD marker variations existed both within and among accessions. 55.7% and 47.8% of the amplified fragments are polymorphic within and among accessions, respectively. Accessions from neighboring geographical regions are more similar than those from distant regions. These results corroborate those obtained from studies on chromosome C-banding and isozyme electrophoresis. RAPD allows a rapid and efficient detection of genetic variations within and among populations of this outcrossing species although it is not suitable for species identification or classification. Some of the amplification products may be further used for genetic mapping.

Introduction

The random amplified polymorphic DNA (RAPD) technique (Welsh and McClelland 1990; Williams et al. 1990) allows working with anonymous genetic markers, requires only a small amount of DNA, and is simpler, less costly, and less labor intensive than other DNA marker methodologies (Caetano-Anolles et al. 1991). RAPDs have been extensively used in many research areas, including 1) construction of genetic maps (William et al. 1990; Welsh et al. 1991; Klein-Lankhorst et al. 1991; Carlson et al. 1991; Giovannoni et al. 1991; Faure et al.1994; Nelson et al. 1993); 2) the identification of hybrids, cultivars, and species (Welsh et al. 1991; Hu and Quiros 1991; Koller et al. 1993; Wu and Lin 1994; Stiles et al. 1993; Yang and Quiros, 1993; Transue et al. 1994); 3) identification and localization of molecular markers linked to important genes, such as disease resistant genes (Penner et al. 1993; Schachermayr et al. 1994; Byrum et al. 1994); 4) the development of chromosome and genome-specific markers (Quiros et al. 1991) and 5) the measurement of genetic variation to establish genetic and evolutionary relationship within and/or among species, subspecies or populations (Wilkie et al. 1993; Tinker et al. 1993; Vierling and Nguyen 1992; He et al. 1992; Kresovich et al 1992; Jain et al. 1994).

Most of the research on RAPD marker variations in populations is carried out using inbred species or cultivars (Joshi and Nguyen 1993; Heun et al. 1994) while less has been done with outcrossing species (Huff et al. 1993). Russian wildrye

(*Psathyrostachys juncea*) is an outcrossing diploid species (2n =14, **NsNs**). It is an important forage crop for revegetating rangeland in North America (Dewey 1984). This species is also considered to be an important germplasm in crop improvement because it possesses resistance to barley yellow dwarf virus (BYDV) (Comeau and Plourde 1987). Barley yellow dwarf causes a serious decrease in the production of wheat, barley, and oats. Hybrids between common wheat and *Ps. juncea* have been obtained in attempts to transfer resistance into wheat (Chen et al. 1988; Plourde et al. 1990).

Russian wildrye is highly polymorphic in morphology due to self-incompatibility (Jensen et al. 1990). Its extensive genetic diversity has been demonstrated by chromosome banding (Endo and Gill 1984; Linde-Laursen and Bothmer 1986; J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished) and isozyme analysis (J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished). In this research, we analyzed RAPD marker variations within and among accessions of Russian wildrye to evaluate the usefulness of RAPD markers in this germplasm and to compare the results with those from chromosome banding and isozyme analysis.

Materials and Methods

Plant materials

Eighty-eight individual Russian wildrye plants representing eleven accessions were analyzed and 4 to 13 plants of each accession were examined in this study

(Table 5-1).

Template DNA preparation

Individual plant genomic DNA was extracted from young leaves following protocols of Williams et al. (1992) and Lassner et al. (1989). About 100 mg of young leaf tissue was ground in liquid nitrogen and mixed with 1.0 ml of CTAB buffer (0.14 M sorbitol, 0.22 M Tris-HCl, 0.022 M EDTA, 0.8 M NaCl, 0.8% CTAB and 1% N-laurlysarcosine) and 0.4 ml of chloroform. After a 10 min incubation at 55° C, the sample was centrifuged at 12,000 g for 10 min. DNA in the supernatant was recovered following addition of 1.2 vol of isopropanol, centrifugation at 12,000 g, washing twice with chilled 70% ethanol and drying in a SpeedVac. The dried pellet was resuspended in 80 µl TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA). The DNA concentration was determined by a TKO100 DNA fluorometer and by comparing band intensities with known standards of lambda DNA on an agarose gel. The individual genomic DNA was diluted in sterile water to a concentration of 20 ng/µl. Equal amounts of DNA from each individual of each accession were mixed to form the pooled template DNA for that accession.

RAPD assay

Decamer oligonucleotides from Operon Technologies were used in this study. The Stoffel fragment, 10x buffer and 25 mM $MgCl_2$ were obtained from Perkin Elmer. All reagents except template DNA were premixed and aliquoted. One

Table 5-1. Accessions and their geographic origin

Number	Accession	Geographic location	No. of plants
1	PI206684	Turkey	5
2	PI499558	China	10
3	x93174	China	11
4	x93257	China	13
5	PI314668	Russia	9
6	JA239	Russia	8
7	JA305	Russia	9
8	JA240	Mongolia	4
9	JA237	Mongolia	5
10	JA130	Iran	5
11	Cabree	Canada	9

optimized amplification reaction (25 µl) contained 1x buffer, 3 mM $MgCl_2$, 0.24 mM dNTP, 100 ng primer, 40 ng template DNA, and 2 Units of the Stoffel fragment. About 30 µl of mineral oil was overlaid on each reaction mixture. DNA amplification was preformed using a Gene Amp PCR System 9600 programmed for 40 cycles of 1 min at 94°C, 1 min at 36 C and 2 min at 72 C using the fastest available transitions followed by storage at 4°C. The RAPD products were separated by agarose gel electrophoresis in 1x TBE buffer on 2% agarose (Perkin Elmer)

containing 0.5 µg/ml ethidine bromide. DNA fragments were visualized and photographed under UV light.

Data analysis

Photographs were used to score the RAPD data. DNA fragment sizes were determined by comparing with DNA size markers. Reproducible DNA bands were scored for their presence (1) or absence (0) in each individual or accession. These data matrices were entered into NTSYS-PC program (Rohlf 1993). Data were analyzed using SIMQUAL (Similarity for Qualitative Data) routine to generate Jaccard's similarity coefficients. Similarity coefficients were used to construct dendrograms using the unweighted pairgroup method and arithmetic average (UPGMA) with SHAN (Sequential, Hierarchical, Agglomerative, and Nested Clustering) routine in NTSYS programs.

Results

Optimization of RAPD reaction

The concentrations of all regents in RAPD reactions were optimized. The effectiveness of Taq DNA polymerase and the Stoffel fragment were compared (Fig. 5-1). They produced entirely different products with many primers. In some instances, the Stoffel fragment yielded products while Taq DNA polymerase did not. In general, more products (either band number or band intensity) were obtained with the less expensive Stoffel fragment than with Taq DNA polymerase. A test of

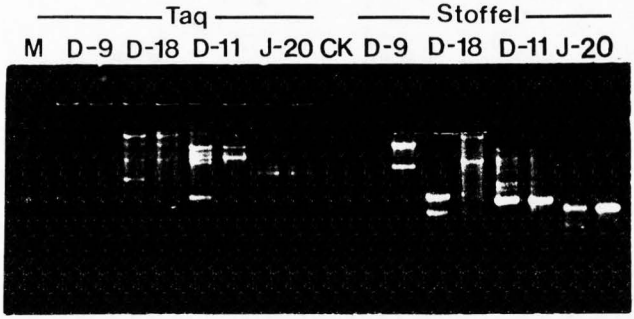

Fig. 5-1. Comparison of RAPD profiles produced with Taq DNA polymerase and the Stoffel fragment with DNA of *Ps. juncea*. Two accessions (JA130 and JA305) were used as templates with each primer. RAPD was carried out with a 25 µl of reaction mix that contained 1x buffer, 3 mM $MgCl_2$, 0.24 mM dNTP, 40 ng template DNA, and either 2 units of the Stoffel fragment or 1 unit of Taq DNA polymerase. Molecular size markers (M, from top to bottom) are 2000, 1500, 1000, 700, 500, 400, 300, 200, 100, and 50 bp. The control (CK) contained everything except the template DNA.

primer concentrations (Fig. 5-2) showed that low primer concentrations produced large size DNA fragments. Increased primer concentration resulted in more small size fragments and less large size fragments. $MgCl_2$ concentrations from 0 to 5 mM were tested (Fig. 5-3). $MgCl_2$ below 1 mM did not give any product, and lower $MgCl_2$ concentrations resulted in only small size fragments. In contrast, when there was too much $MgCl_2$ fragment numbers decreased and the bands were diffuse. The best results were obtained using 3 mM $MgCl_2$. Template DNA concentrations of 0 to 640 ng per 25 µl reaction were tested (Fig. 5-4). Both low and high amounts of template DNA gave poor results. The optimal template DNA concentration was determined to be 40 ng. Finally, dNTP greater than 120 µM and at least 1 unit of the Stoffel fragment per reaction were required (data not shown). These optimized conditions were suitable also for other perennial Triticeae grasses.

Primer Selection

A total of 200 primers were tested to select those that produced polymorphic DNA bands. A polymorphic band was defined as an amplified DNA fragment that was present in at least one individual plant or accession and was absent in at least one individual plant or accession. Of the 200 primers tested, 55 (27.5%) had no detectable product or produced unambiguous products, 34 (17%) produced uniform fragments among accessions, and 111 (55.5%) produced some polymorphic fragments. In most cases, the major bands were uniform, while the minor bands were polymorphic and often hard to score. Various primers produced different

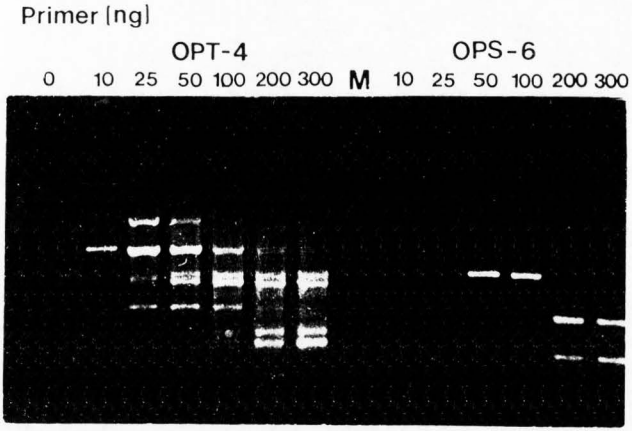

Fig. 5-2. Effects of primer concentration on RAPD profiles of *Ps. juncea* accession JA130. Numbers above the wells are primer amount (ng) per 25 µl of reaction mix, which also contains 1x buffer, 3 mM $MgCl_2$, 0.24 mM dNTP, 40 ng template DNA, and 2 units of the Stoffel fragment. Lane 1 to 7: primer OPT-4; Lane 9 to 14: primer OPS-6.

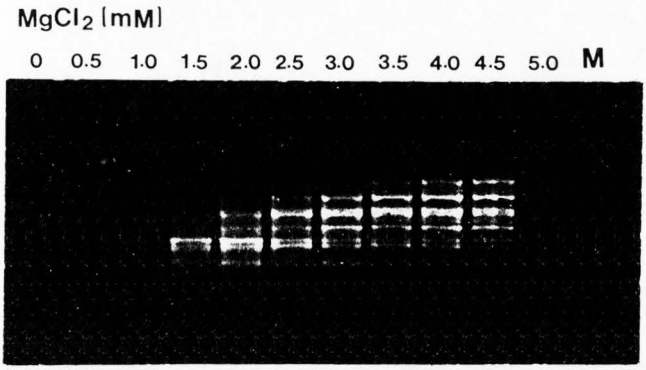

Fig. 5-3. Effects of $MgCl_2$ concentrations on RAPD profiles of *Ps. juncea* accession JA130. Each 25 µl of reaction mix contained 1x buffer, 0.24 mM dNTP, 100 ng OPT-4, 40 ng template DNA, 2 units of the Stoffel fragment, and varying amounts of $MgCl_2$.

fragments from the same template DNA. Figure 5-5 showed the RAPD products of a set of Operon primers on the same template DNA. Use of a combination of primers increased the amplified fragments, but did not increase polymorphic products (Fig. 5-6). Seventeen primers were selected for RAPD assay (Table 5-2). Each of these primers produced about seven bands ranging from 205 bp to 1050 bp. Thirty-nine polymorphic bands out of a total of 70 bands produced by the 10 of the 17 primers were analyzed within each accession. Fifty six polymorphic bands out of 117 bands (47.8%) produced by 17 primers were analyzed.

RAPD marker variation within accessions

Only 12 fragments of the 70 fragments amplified by the 10 different primers (17.2%) were fixed in all 88 individuals. The 19 (27.1%) bands that were ambiguous in some individuals were not considered. Thirty-nine unambiguous RAPD markers (55.7%) were used to analyze genetic variations within each accession and all 88 individuals. There was considerable variation within each of the 11 accessions (Fig. 5-7). The Jaccard's genetic similarity coefficients ranged from 0.442 to 0.897 (Table 5-3). Twenty-three RAPD bands (59%) were fixed in all of the individuals of at least one accession, and 20 markers (51%) were absent in all of the individuals of at least one accession. No bands were present in all of the individuals of one accession and absent in all other individuals. Relationships among the accessions were not revealed because individuals were mixed by the UPGMA cluster analysis. Fourteen of the 39 RAPD markers used to analyze

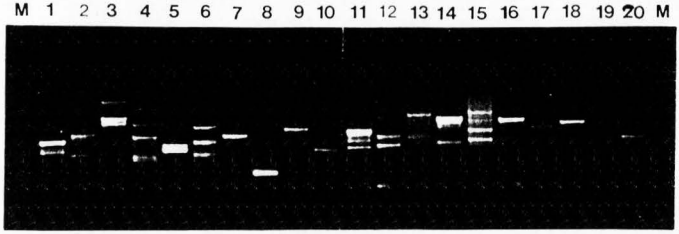

Fig. 5-5. RAPD results of a set of Operon primers (OPP-1 to OPP-20) on the same DNA template of *Ps. juncea*. RAPD was carried out with a 25 µl of reaction mix that contained 1x buffer, 3 mM $MgCl_2$, 0.24 mM dNTP, 40 ng template DNA of accession KJ-130, and 2 units of the Stoffel fragment.

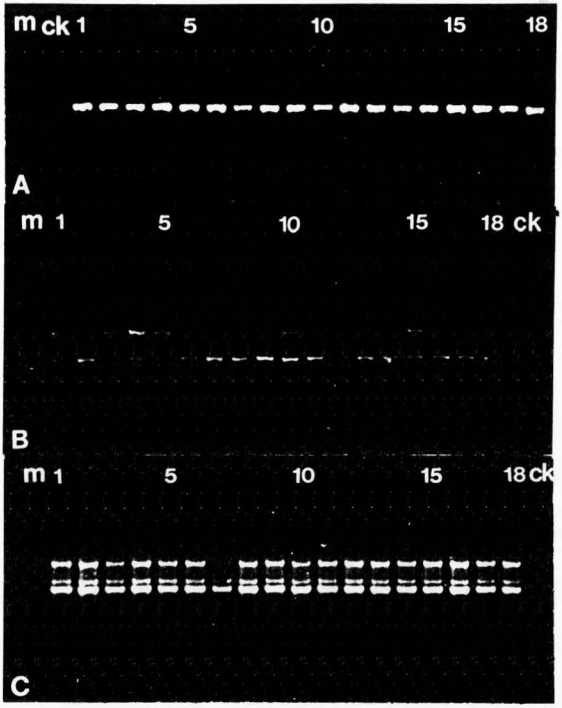

Fig. 5-6. Comparison of polymorphism with single and combined primers. (A) OPD-16, (B) OPC-3, and (C) OPD-16 and OPC-3. Individuals in lane 1-5, 6-10, 11-14, and 15-18, are from accessions DJ-3955, KJ-130, KJ-240, and PI314668, respectively.

Table 5-2. List of primers, their sequences, and amplification results.

Primer	Sequence	Total bands	Scorable polymorphic bands
OPD-01	5'ACCGCGAAGG3'	4	D1-740
OPD-09	5'CTCTGGAGAC3'	8	D9-275, D9-360
OPD-14	5'CTTCCCCAAG3'	7	D14-340, D14-430, D14-760
OPD-19	5'CTGGGGACTT3'	9	D19-1050, D19-640, D19-280
OPM-08	5'TCTGTTCCCC3'	6	M8-420, M8-510, M8-560 M8-600, M8-730
OPM-11	5'GTCCACTGTG3'	8	M11-300, M11-620, M11-770 M11-950, M11-1050
OPM-17	5'TCAGTCCGGG3'	5	M17-305, M17-560, M17-590
OPP-09	5'GTGGTCCGCA3'	6	P9-230, P9-290, P9-430
OPQ-05	5'CCGCGTCTTG3'	6	Q5-250, Q5-540
OPQ-06	5'GAGCGCCTTG3'	5	Q6-740
OPS-02	5'CCTCTGACTG3'	7	S2-395, S2-620, S2-800
OPS-04	5'CACCCCCTTG3'	7	S4-265, S4-460, S4-535, S4-490, S4-620
OPS-06	5'GATACCTAGG3'	6	S6-290, S6-355, S6-395, S6-590, S6-900
OPT-02	5'GGAGGAACTC3'	7	T2-205, T2-240, T2-330, T2-535, T2-780, T2-970
OPT-06	5'CAAGGGCAGA3'	9	T6-210, T6-280
OPW-17	5'GTCCTGGGTT3'	6	W17-250, W17-355, W17-585
OPW-19	5'CAAAGCGCTC3'	11	W19-275, W19-340, W19-440, W19-560
Total		117	56

variation within accessions were present in all 11 accessions when individual plants' data within each accession were pooled.

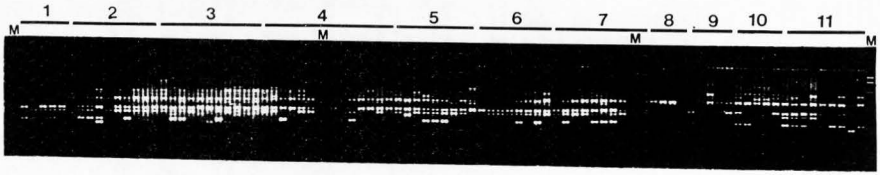

Fig. 5-7. RAPD results in 88 individuals from 11 accessions (Table 1) of *Ps. juncea* with primer OPD-14. RAPD was carried out with a 25 µl of reaction mix that contained 1x buffer, 3 mM $MgCl_2$, 0.24 mM dNTP, 40 ng template DNA, and 2 units of the Stoffel fragment.

Table 5-3. Jaccard's genetic similarity coefficients within accessions

Accessions	Similarity Coefficients
PI206684	0.611 - 0.795
PI499558	0.631 - 0.846
X93173	0.542 - 0.821
X93257	0.573 - 0.821
PI314668	0.650 - 0.897
JA239	0.612 - 0.872
JA305	0.442 - 0.821
JA240	0.615 - 0.769
JA237	0.615 - 0.769
JA130	0.673 - 0.872
Cabree	0.583 - 0.821

RAPD marker variation among accessions

Genomic DNA of individuals was bulked to form template DNA for each accession. The 17 selected primers (Table 5-2) amplified 117 fragments of which 39 fragments (33.4%) were fixed in the 11 accessions. Twenty-two (18.8%) bands were not considered due to their ambiguity. Fifty-six unambiguous polymorphic markers (47.8%) were used to analyze the genetic variation among the accessions. Considerable genetic variation occurred among accessions (Fig.5-8). The

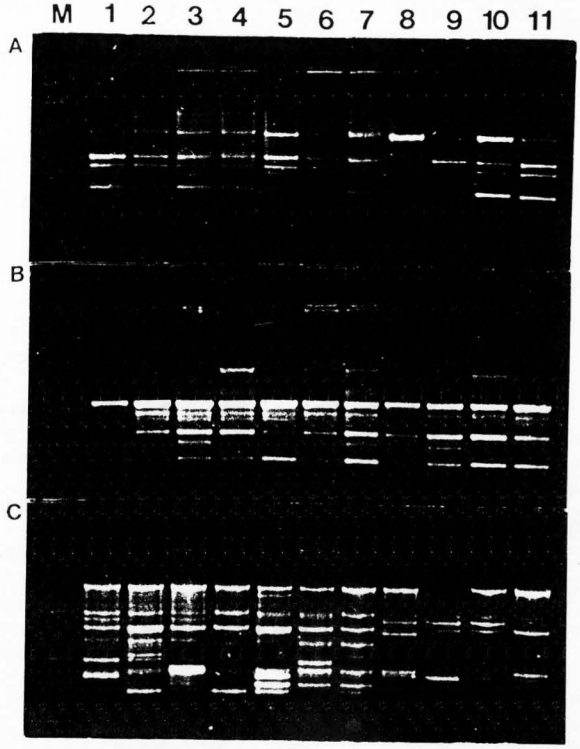

Fig. 5-8. RAPD products from 11 different accessions (Table 5-1) of *Ps. juncea* with OPD-14 (A), OPS-06 (B), and OPD-19 (C) as the primer. RAPD was carried out with a 25 µl of reaction mix that contained 1x buffer, 3 mM $MgCl_2$, 0.24 mM dNTP, 40 ng template DNA, and 2 units of the Stoffel fragment.

Jaccard's genetic similarity coefficients ranged from 0.702 to 0.860. Accessions from China, Russia, and Mongolia showed close genetic relationships (Fig. 5-9). The accession PI206884 from Turkey and Cabree from Canada was less closely related. Accessions originating from China and Russia had closer genetic relationships than some accessions from close geographic location. No accession-specific RAPD markers were found in *Ps. juncea*.

Discussion

Reproducibility of RAPD

Reproducibility of RAPD is often regarded as the most critical factor in its use (Devos and Gale 1992; Chou et al 1992; Joshi and Nguyen 1993). In this study, we found that DNA polymerase (Taq DNA polymerase or the Stoffel fragment), primer/template ratios, annealing temperature and Mg^{++} concentration can affect the reproducibility of RAPDs. When each condition was optimized, RAPD results could be reproduced very well. As reported in sugarcane (Sobral and Honeycutt 1993), we found the Stoffel fragment to produce more RAPD products and more polymorphisms than Taq DNA polymerase in Russian wildrye. With optimized reaction condition, our RAPD products ranged from 150 to 1500 bp in size. Both individual and accession DNA samples yielded reproducible RAPDs under optimized reaction conditions. These conditions also may be suitable for many other grasses in Triticeae.

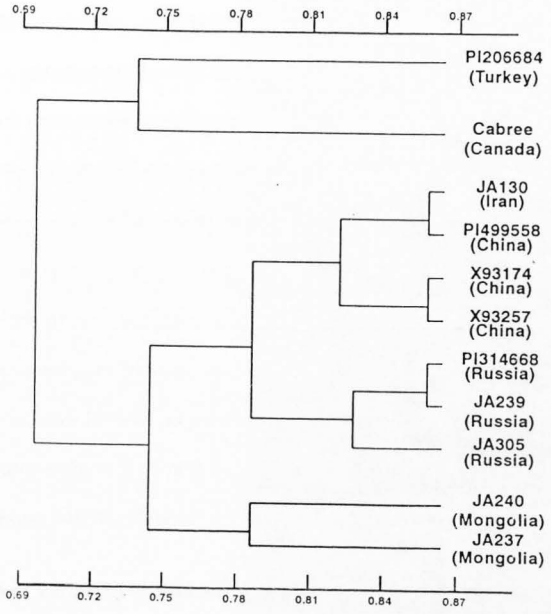

Fig. 5-9. A dendrogram generated from RAPD markers for 11 Russian wildrye (*Ps. juncea*) accessions. Scale is Jaccard's coefficients of similarity.

Level of RAPD polymorphism

Various levels of polymorphism are generated from RAPD in different organisms and with different primers. In tetraploid wheat, 52.2% of the primers tested yielded polymorphic patterns, while 40% did not amplify detectable products or produce unambiguous data (Joshi and Nguyen 1993). Polymorphic fragments were found with 68% of tested primers in diploid wheat (Vierling and Nguyen, 1992), 57.5% in spring barley (Tinker et al. 1993), 94% in Indian mustard (Jain et al. 1994), and 41% in cranberry(Novy et al. 1994). We found 55.5% of tested primers showed polymorphisms among accessions, and 55.7% and 47.8% of RAPD bands were polymorphic among individuals and accessions, respectively. A combination of primers generated additional polymorphism in Indian mustard (Jain et al. 1994). We found that combinations of primers increased the number of amplified fragment but did not increase polymorphism.

RAPD marker variation in Russian wildrye

This is the first report on genetic variation in Russian wildrye using RAPD markers. Our major objectives was to assess the suitability of the RAPD method for detecting the genetic variation within and among accessions of Russian wildrye. Results from this study and previous chromosome banding (J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished) and isozyme techniques (J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished) are in considerable agreement. High genetic diversity exists within and among accessions although most of the

accessions are genetically similar. Accessions collected from neighboring geographical areas have close genetic relationships. This study demonstrates that RAPDs can be successfully used in an outcrossing species like Russian wildrye to detect the genetic variation within and among accessions. One major difference among procedures was that RAPDs showed accessions from China and Russia to be genetically close, whereas data from isozyme analysis indicated that accessions from Mongolia and Russia were genetically close. Because RAPDs are based on DNA sequence differences, they may be more accurate for determinating genetic relationships than C-banding and isozyme analysis (Heun et al. 1994, M'Ribu and Hilu 1994).

RAPD markers are extensively divergent among, but exhibit little variation within, inbred species or cultivars (Wilkie et al. 1993). In outcrossing species, individual plants exhibit considerable genetic heterogeneity that gives rise to RAPD variations (Huff et al. 1993). RAPD marker variation exists extensively both within and among accessions of Russian wildrye. The variation within accessions occurs at about the same level as among accessions, and constitutes a major part of the genetic variation within this species. The large amount of genetic variation within accessions and the outcrossing nature of Russian wildrye apparently slows down genetic drift but maximizes population diversity.

Although the number of RAPD markers was small, the results provide genetic variation patterns within and among accessions. This information is useful in

genetic research, germplasm evaluation and breeding of Russian wildrye and other outcrossing species in Triticeae. We conclude that RAPDs are additional characters for assessing genetic variation among and within accessions and the genetic relationships among accessions from various regions.

Limitations of RAPD

Although RAPDs are undoubtedly a powerful tool in quantifying genetic variation, the RAPD technique has some limitations. In addition to problems of reproducibility, artifacts on agarose gel have also been reported. Unstable bands may result from the formation of artificial heteroduplexes between multiple amplified fragments (Wenger and Nielson 1991) or from nonspecific amplification, i.e. amplification even when primer/template homology is not perfect (He et al. 1992). In this study, some variable bands were difficult to score in all of the samples tested. Therefore, only the consistent and unambiguous bands were considered in the assay. Furthermore, in our consideration of genetic variation using RAPD, only amplified fragment size and not the copy number (band intensity) was considered. Changes in the copy number as well as in the sequence of a specific DNA fragment are important in evolutionary processes. In most data analysis programs, all bands of a given size are excuted as homologs even if they differ in band intensity. Thus, considerable genetic information is not used.

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CHAPTER 6

IDENTIFICATION, ISOLATION, AND CHARACTERIZATION OF SOME RAPD MARKERS IN RUSSIAN WILDRYE (*PSATHYROSTACHYS JUNCEA*)

Abstract

Sixty RAPD markers from Russian wildrye (*Psathyrostachy juncea*) (2n=2x=14, NsNs) and related species were identified based on analysis of individual plant RAPD products or by comparing the RAPD products of different genomes or species. Some of those markers were genome- or species-specific. Forty-four of the markers were isolated and 16 RAPD markers were cloned with the RAPD-cloning technique. Some of the cloned markers were characterized with southern hybridization and DNA sequencing. The results indicated that some of RAPD markers were amplified from repetitive sequences and some from low copy sequences. Specific RAPD markers revealed the nucleotide length between specific priming sites in the template. The internal sequences of specific markers were not necessary to be genome- or species-specific sequences as RAPD markers *per se*.

Introduction

Russian wildrye (*Psathyrostachys juncea*, 2n=2x=14, NsNs) is an important cool-

season forage grass. It is tolerant to alkalinity and drought, and its leaves are high in nutritive value and palatable to grazing animals (Asay 1992). Russian wildrye is also a potential germplasm resource for wheat improvement because it is resistant to barley yellow dwarf virus (BYDV) (Comeau and Plourde 1987), powdery mildew (*Erysiphe graminis*) (Dong et al. 1992), leaf rust (*Puccinia rubigovera*), and stem rust (*Puccinia graminis*) (Li et al. 1992). Hybrids between *Ps. juncea* and common wheat J have been obtained (Chen et al. 1988; Plourde et al. 1990).

A standard karyotype of *Ps. juncea* has been established and cytological markers, chromosome bands, make the chromosomes easily identified (J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished). Molecular markers will be useful in identification of chromosomes or chromosome segments and in gene location.

The random amplified polymorphic DNA (RAPD) technique (Welsh and McClelland 1990; Williams et al. 1990) provides an easier and simpler approach than the traditional RFLP (restriction fragment length polymorphism) technique for identifying DNA markers. Some genome-, species- and chromosome-specific RAPD markers (Quiros et al. 1991; Kleig-Lankhorst et al. 1991; King et al. 1993; Wang et al. 1995) have been reported.

In a previous study, we have analyzed the genetic variation of Russian wildrye using RAPD (J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished). High levels of RAPD variation existed both within and among accessions. Some RAPD profiles are uniform in all of the tested accessions, even in all of the tested plants.

In this study, we identified, isolated, and characterized some RAPD markers in Russian wildrye.

Materials and methods

Plant Materials

Eighty-eight plants from 11 accessions in *Ps. juncea* were used in this study. In developing genome- or species-specific RAPD markers, some other species having the Ns genome and other genomes were used (Table 6-1). Except *Ps. juncea*, one or two accessions in each species and 5 to 10 plants in each accession were used. All of these species are diploids.

DNA preparation

Plant genomic DNA was extracted from young leaves as described previously (J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished). The genomic DNA of each accession was extracted from the bulked leaves of 5 to 10 individual plants in that accession. The accession genomic DNA was diluted in sterile water to a concentration of 20 ng/µl. Equal amounts of diluted accession DNA from different accessions of one species were pooled to constitute the species genomic DNA, and then an equal amount of species DNA from different species having the same genome was pooled to form the genome genomic DNA. In *Ps. juncea*, genomic DNA of individual plants was also extracted and kept separately.

Table 6-1. Genomes and species used in this study.

Genome	Species	2n
Ns	*Psathyrostachys juncea*	14
	Psathyrostachys fragili	14
	Psathyrostachys huashanica	14
	Psathyrostachys stolonformis	14
P	*Agropyron cristatum*	14
	Agropyron mongolicum	14
	Agropyron puberulum	14
St	*Pseudoroegneria spicata*	14
	Pseudoroegneria libanotica	14
	Pseudoroegneria inermis	14
	Pseudoroegneria stipifolia	14
H	*Hordeum bogdanii*	14
	Hordeum chilense	14
	Hordeum violaceum	14
	Hordeum marinum	14
I	*Hordeum bulbosum*	14
	Hordeum vulgare	14
R	*Secale montanum*	14
	Secale cereale	14
E^e	*Thinopyrum elongatum*	14
E^b	*Thinopyrum bessarabicum*	14
W	*Australopyrum rectrofractum*	14

RAPD assay

RAPD was carried out as described previously (J-Z. Wei, W.F. Campbell, and R.R-C. Wang, unpublished). The RAPD products were separated by agarose gel electrophoresis in 1x TBE buffer with a 2% agarose (PE XPRESS) containing 0.5 µg/ml ethidium bromide. DNA fragments were visualized and photographed under UV light. RAPD marker sizes were determined by comparisons with the 50 - 2000bp DNA ladder markers from BIO-RAD.

DNA recovery from agarose

RAPD markers were precipitated from agarose according to procedures of Qian and Wilkinson (1991) with some modifications. RAPD products were separated on a 1.5% low-melting agarose gel with 1x TAE buffer in a cold room (4°C). The band of interest was cut with a sharp scalpel under UV light and put in a 1.5-ml microcentrifuge tube. Three volumes of TE (pH 8.0) were added and the tube was incubated at 70°C for 5 min. After being frozen at -70° C for 1 h, the sample was thawed at room temperature and spun at 14K for 15 min. The supernatant was saved and the DNA was precipitated with cold 100% ethanol.

RAPD cloning

The cloning of RAPD markers was carried out with the Prime PCR Cloner™ Cloning System according to the protocol provided by the manufacturer (5 Primer→ 3 Primer, Inc.). After modification, DNA fragments were ligated to the pNoTA

vector, which then was used to transform the host cell JM109.

Plasmid extraction

Plasmid DNA was prepared with the CTAB-DNA precipitation method. Overnight culture was centrifuged at 14 K for 5 min. The cell pellet was resuspended in STET buffer with 1 mg/ml lysozyme. After boiling for 45 seconds and spinning for 10 min, the soft pellet was discarded. RNase A, with a final concentration of 200 µg/ml, was added and incubated at 68° C for 10 min, followed by adding CTAB to a final concentration of 0.5% and leaving at room temperature for 3 min. The pellet was saved after centrifugation and dissolved in 300 µl 1.2 M NaCl and then precipitated with 750 µl 100% ethanol. Following centrifugation, the DNA pellet was rinsed twice in 70% ethanol, dried, and resuspended in TE.

Probe labeling

Probes were labeled and detected with the DIG DNA Labeling and Detection Kit (Boehringer Mannheim). Before labeling, the template DNA was boiled for 10 min and quickly chilled on ice/ethanol.

Southern hybridization

The plasmid was digested with *BamHI*, and the plant genomic DNA was digested with *HindIII*. Digested DNA samples were separated on a 1.5% regular agarose gel. After electrophoresis, the gel was treated in 0.25 N HCl for 15 min, 0.5 M NaOH/1.5 M NaCl for 2x15 min, and 1.0 M Tris (pH 8.0)/1.5 M NaCl for 2x15 min.

DNA was transferred to a nylon membrane (GeneScreen) with 10x SSC overnight. DNA was bonded to the membrane with a UV crosslinker. After a prehybridization treatment for 2 h in hybridization buffer (5x SSC, 1% Block reagent, 0.1% N-lauroylsarcosine, 0.02% SDS), the membrane was hybridized with a particular probe in the hybridization buffer overnight. The hybridization signal was detected by color reaction using the Detection Kit of Boehringer Mannheim.

DNA sequencing

The inserts in the transformed plasmids were sequenced in both directions by the ABI 373A DNA sequencer using fluorescent dye with M 13 forward and reverse primers.

Results

Identification of RAPD markers

RAPD markers of Russian wildrye were identified from two sources. One was the RAPD products of individuals of different accessions. If a band was present in all the of tested individuals of each tested accession, it was considered a potential marker. Figure 6-1 shows some of the uniform bands in all individuals of the different accessions. The other source was RAPD products of different genomes. RAPD products generated from the Ns genome and four different species having different versions of the Ns genome were compared with other eight different genomes. According to the RAPD data, the four species of the Ns genome had a

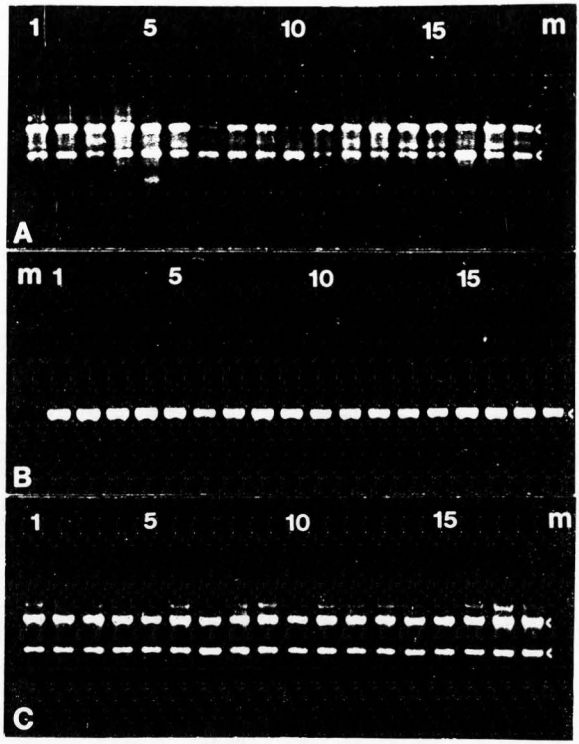

Fig. 6-1. Uniform RAPD markers among individuals from different accessions of *Ps. juncea*. (A) with primer OPD-20; (B) with primer OPG-6; (C) with primer OPG-10. Individuals in lane 1-5, 6-10, 11-14, 15-18 are from accession DJ-3955, KJ-130, KJ-240, and PI314668, respectively. Arrows point the uniform markers. M is DNA molecular size marker (Bio-Rad) (from top to bottom: 2000, 1500, 1000, 700, 500, 400, 300, 200, 100, and 50 bp).

very close relationship (data not shown) and some Ns genome-specific and species-specific RAPD markers were identified (Table 6-2, Fig. 6-2). Sixty RAPD markers, ranging from 200bp to 1210bp, were identified.

Isolation and cloning of RAPD markers

Among the two kinds of RAPD markers, only those bands that were well separated from other bands and were intensely stained by ethidium bromide in the gel were selected, because they were easier to isolate from the gel and were consistent RAPD markers. Forty-four different markers were isolated and 16 of them were cloned (Table 6-3, Fig. 6-3).

Characterization of RAPD markers

To confirm that the cloned sequence was the exact RAPD marker, the plasmid with insert was labeled and hybridized with the original RAPD products. In most cases, only the band of interest showed a hybridization signal (Fig. 6-4), although some weak signals were occasionally observed in the same lane or other lanes.

When using the genomic DNA as a probe to hybridize with the digested plasmid, some inserts showed very strong hybridization bands, while some were too weak to be visible (data not shown). Some Ns genome-specific RAPD markers were Southern hybridized with different genomic DNAs. Although some weak signals were observed in some other genomes, the probes were mainly hybridized with species having the Ns genome with uniform hybridization patterns for all these

Table 6-2. Genomes and genome-specific markers developed by RAPD.

Genome	RAPD Markers
Ns	M7-750, C9-530, K7-300, Q5-440, X11-380, W5-330
Ns^j	T7-1110, C8-470, D19-1000, B3-400
Ns^f	K7-650
Ns^h	W5-220, F3-350
Ns^s	J7-490
Ns^j & Ns^s	K7-450, R5-650
Ns^j & Ns^h	D19-1210
Ns^j, Ns^s & Ns^h	C9-320
Ns^f, Ns^h & Ns^s	B3-200, R5-600, C9-450

species (Fig. 6-5). Twelve cloned markers have been sequenced. Figure 6-6 shows the nucleotide sequences of three cloned markers. The first 10 base pairs in each end of the sequence are the primer sites.

Discussion

In this study, the species having various versions of the Ns genome showed a close relationship based on the RAPD analysis. This result corroborates those obtained from the analyses of karyotype, chromosome banding, and chromosome

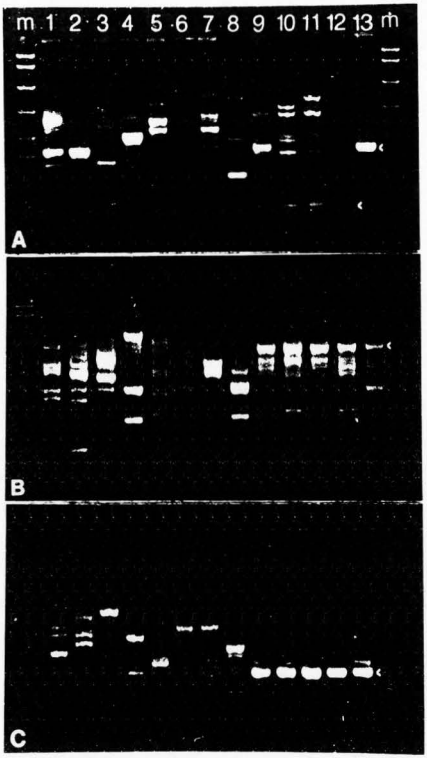

Fig. 6-2. RAPD results in different genomes with primer OPB-3 (A), OPM-7 (B), and OPW-5 (C). Templates from lane 1 to 13 are E^e, E^b, H, I, P, R, St, W, Ns, Ns^f, Ns^h, Ns^s, and Ns^j, respectively (see Table 1) N^f, N^h, N^s, and N^j are *Ps. fragilis, Ps. huashanica, Ps. stolonformis , and Ps. juncea,* respectively. Ns is the pooled DNA of the four species. Arrowheads point to the specific bands.

Table 6-3. Cloned RAPD markers and their primers.

Number	Name	Primer	Primer Sequence	Marker Size
1	C9N3	OPC-09	5'-CTCACCGTCC-3'	326*
2	C9N5	OPC-09	5'-CTCACCGTCC-3'	500
3	C9N6	OPC-09	5'-CTCACCGTCC-3'	548*
4	C11N3	OPC-11	5'-AAAGCTGCGG-3'	300
5	C14N3	OPC-14	5'-TGCGTGCTTG-3'	356*
6	D1N5	OPD-01	5'-ACCGCGAAGG-3'	508*
7	D20N4	OPD-20	5'-ACCCGGTCAC-3'	462*
8	G6N4	OPG-06	5'-GTGCCTAACC-3'	375*
9	G11N3	OPG-10	5'-TGCCCGTCGT-3'	343*
10	G11N5	OPG-10	5'-TGCCCGTCGT-3'	520
11	J18N3	OPJ-18	5'-TGGTCGCAGA-3'	270*
12	J18N4	OPJ-18	5'-TGGTCGCAGA-3'	455*
13	M7N4	OPM-07	5'-CCGTGACTCA-3'	443*
14	T4N3	OPT-04	5'-CACAGAGGGA-3'	289*
15	W5N3	OPW-05	5'-GGCGGATAAG-3'	338*
16	X11N4	OPX-11	5'-GGAGCCTCAG-3'	420

* These markers have been sequenced.

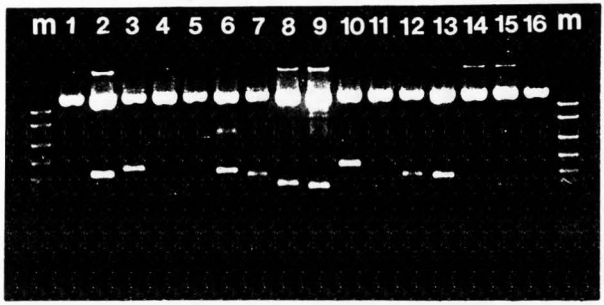

Fig. 6-3. *BamH I* digested pNoTA plasmid DNA . Lanes 1 to 16 are C9N3, C9N5, C9N6, C11N3, C14N3, D1N5, D20N4, G6N4, G10N3, G10N5, J18N3, J18N4, M7N4, T4N3, W5N3, and X11N4, respectively (see Table 6-3).

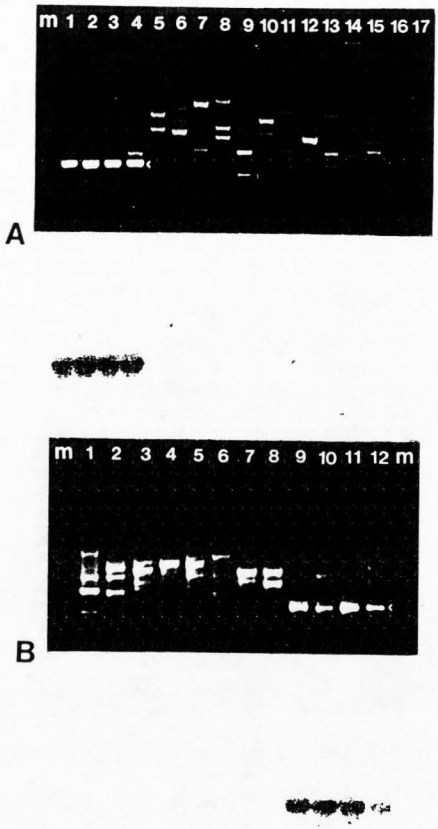

Fig. 6-4. RAPD products and southern hybridization. (A) RAPD was preformed with primer OPW-5 and the products were hybridized with labelled plasmid W5N3. The templates of lane 1 to 17 are Ns^{i}, Ns^{f}, Ns^{h}, Ns^{s}, C (*Ae. caudata*), J^{e}, H, I, P, R, St, W, A (*T. monococcum*), B (*T. speltodise*), D (*T. tauchii*), AB (*T. durum*), and ABD (*T. aestivum*), respectively. (B) RAPD was preformed with primer OPX-11 and the products were hybridized with labelled plasmid X11N4. The templates in lane 1 to 12 are E^{e}, E^{b}, H, I, P, R, St, W^{f}, Ns^{h}, Ns^{s}, Ns, and Ns, respectively. Arrowheads point to the specific bands.

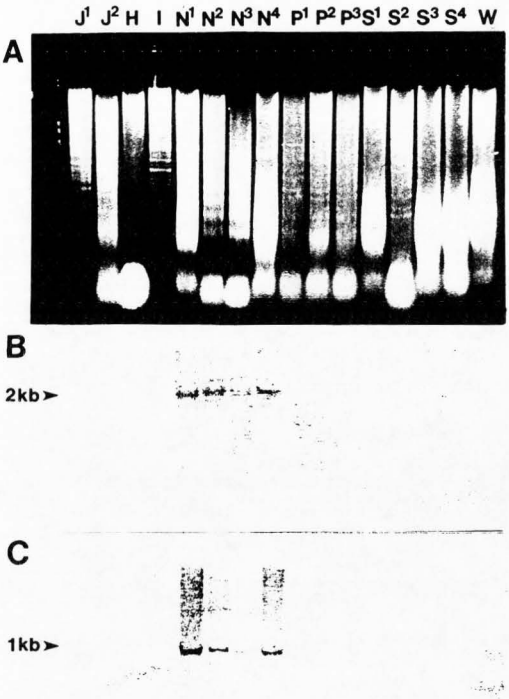

Fig. 6-5. *Hind III* digested genomic DNA and southern hybridization. (A) *Hind III* digested species genomic DNA. J^1 -- *Th. elongatum*, J^2 -- *Th. bessarabicum*, H -- *H. bogdanii*, I -- *H. vulgare*, N^1 -- *Ps. juncea*. N^2 -- *Ps. fragilis*, N^3 -- *Ps. huashanica*, N^4 -- *Ps. stolonformis*, P^1 -- *A. cristatum*, P^2 -- *A. mongolicum*, P^3 -- *A. puberulum*, S^1 -- *P. spicata*, S^2 -- *P. libanotica*, S^3 -- *P. inermis*, S^4 -- *P. stipifolia*, and W -- *Au. rectrofractum*. (B) Southern hybridization with labeled plasmid C14N3. (C) Southern hybridization with labeled plasmid W5N3.

A
```
001  TGCGTGCTTG GTCCTAGGTT GCTCCAATAC AGCTGTTAAC ACAAGAATCC
051  AAGGGAAAGT AGTGAAAACC AAATATACAC ACACGTGTGG AATGGTCACA
101  ATGAGGAGGA TAAAAACCGG AAATCATGCG TGGTGGACAT ATATTTGGGT
151  CATTGGGGCA TAATATATGA AGAGAACATG CAACAGATGA GCTCTAAAAA
201  TGCATAAAAT ATAGAGACAT CAGCCTCCCC ACGCTTAAAC CTTGGTTGTC
251  CTCGAGTACG GAGGTTGACT ACCAAAAGCT CAATGTTACC AACTCTAAAG
301  CTTACATTGA ACCAAGGCCC AAAAGTGATA TCCATGTGCC ATCATGCAAG
351  CACGCA
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B
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001  ACCGCGAAGG GAACAAGAAT GGANTTTCCA GTGGAGATTT CCGCACNNTG
051  AAGCCACCAT GATGACATAN AAGAAGATGG ACGAAATATA CAAGATGGCC
101  TTTCATAAAT TTCGTCCATA GCTTATTATT GGTNNTGCGN CACCTTATNT
151  TTGGGCCAGG CCNATGTANT TTCNAAATAC TANTTAATAG GNTGTTTTTA
201  GANTCCGTAT TGTAGAGAAA ATGACTNAGG AGGGATTTTA GTCCNACCNT
251  GCCAAGGGTG GGANNAAATT CCCTCTCTTT TNCCCTATAA ATACAGCCCT
301  TAGGGCGTCN TTTAGACTTG GGTTTTAATT AGTTAAAAGT TAGCCATNGC
351  TGCAACTTNG TGTACTTNGT TTGTGTCCAA NGACCAGNCC AAGACNGCTT
401  TTGGAACCCC ACNTTTATCA ANACTTCATC TATATTCGCA ATATTCAGAT
451  TGCTNTTATC ATATTCTTNC TNGTTCTTCG ATTGCTTGNA GGAATAGACC
501  TTCGCGGT
```

C
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001  GGCGGATAAG AAGAAGAACG GTTCTTCCGG TAAAGGTATA AAAGTTATAC
051  AAGTCAATGT TATTTGTATT CTACTTGCTG GAAATACCTG TAAGTCATGG
101  GTATTTGATA CCGGATCTGT TGCTCACATT TGCAACACGA TGCAGGGACT
151  ACAGAAAGTT CGCAAGCTGG AAAGGAATGA AGTGATGATG CGCGTCGGGA
201  ACGGTGCCGG GATCTCCGCT CAAGCCGTTG GCATCATGTC TTTAAGTCTC
251  CCTTCAGGAT TTATCTTAGA ACTGAATAAT TGTTATTACA TTCCGAAGTT
301  GTGTAAAAAC ATTATCTCTG GATCATGTCT TATCCGCC
```

Fig. 6-6. Nucleotide sequences of RAPD markers. (A) Insert sequence of C14N3. (B) Insert sequence of D1N5. (C) Insert sequence of W5N3. The first ten nucleotides in each ends are priming sites.

pairing (Hsiao et al. 1986; Linde-Laursen and Bothmer 1986; Linde-Laursen and Baden 1994; Wang 1992).

The majority of RAPD products are believed to be generated from repetitive DNA sequences (Devos and Gale 1992). More than 70% of the DNA in higher eukaryotes is repetitive DNA, and the repetitive sequences change rapidly during evolution and account for speciation, while much of the single- and low-copy number sequences is highly conserved in related species (Flavell et al. 1981; Anamthawat-Jonsson and Heslop-Harrison 1992, 1993). Therefore, RAPD provides a simple, fast, and easy method for isolating molecular markers, which could be genome-, species-, population-, or chromosome-specific. Genome- and species-specific RAPD markers have been developed in *Brassica*, and some of the markers have been located on specific chromosomes with additional lines (Quiros et al. 1991). Some chromosome-specific RAPD markers have also been identified in some other species (Kleig-Lankhorst et al. 1991; King et al. 1993; Wang et al. 1995).

According to the RAPD principle, RAPD markers (or bands) reveal the presence and the distribution of the specific primer sequence in the template DNA, and specific RAPD markers indicate the specific priming sites in that template. Little information about the internal sequences can be directly deducted from RAPD products without DNA sequencing. In this study, we cloned and characterized some Ns genome-specific RAPD markers. The results suggested that the internal

sequences of a few markers were genome-specific, whereas a majority of them were not genome-specific because they can hybridize with certain digested fragments of other genomes. If the internal sequence is also specific, the isolated RAPD marker can be used as a specific sequence to identify the existence of a specific genome, or chromosomes. If the internal sequence is not specific, the RAPD marker is useful only by its presence in the RAPD profiles. In this case, the RAPD marker reveals amplified fragment length polymorphism (AFLP). In a comparison with restriction fragment length polymorphism (RFLP), AFLP reveals the differences in priming sites, while RFLP reveals the differences in restriction enzyme recognition sites. In the latter case, the internal sequence may have other variations among different genomes or species. These kinds of sequences can be used to study DNA variation in the evolutionary and phylogenetic relationships.

The hybridization of genomic DNAs (as the probe) to different inserts indicated that there are great differences in copy numbers of the insert sequences in the native genomic DNAs. It is hard to explain why some of the inserts (the isolated markers) do not show a hybridization signal even if the original RAPD bands are very intense, when most of the RAPD products arise from repetitive DNA. The results may suggest that not only repetitive sequences, but also some low copy sequences are amplified in the RAPD reactions. That may be the reason why there were no visible signals when some of the isolated markers were used as probes for *in situ* hybridization (data not shown).

In conclusion, 16 RAPD markers were isolated and some of them were preliminarily characterized. This research provided a new approach in developing molecular markers and isolating specific DNA sequences. These isolated RAPD markers may be useful in genetic studies and gene mapping of Russian wildrye, marker-assisted crop improvement (i.e., transfer useful genes from Russian wildrye or other species having the Ns genome to cereal crops), and phylogenetic studies in Triticeae.

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CHAPTER 7

CONCLUSIONS

Russian wildrye, *Psathyrostachys juncea* (Fisch.) Nevski (2n=2x=14, NsNs), is an important forage grass in Triticeae tribe. It is native to steppe and desert regions in the Middle East, European Russian, and Central Asia. Russian wildrye was first introduced into the United States in 1927, and it is now widely used in North America. Russian wildrye possesses many disease-resistant genes and it is a potential germplasm for cereal crop improvement. The lack of genetic studies in this species has limited further research, exploration, and utilization. This dissertation research was conducted to analyze variations in Russian wildrye from three different approaches, i.e., cytological, biochemical, and molecular techniques.

Ten accessions originating from different geographical regions were analyzed with the C-banding technique. C-banding polymorphisms were observed within homologous chromosome pairs, among individuals, accessions, and geographical regions. The bands on chromosomes A, B, E, and F were more variable than those on chromosomes C, D, and G. These polymorphisms did not prevent chromosome identification. A standard C-banding karyotype was developed based on the overall karyotypes and common C-bands in the 10 accessions. The bands on the chromosomes were designated and the frequencies of their occurrences indicated. The chromosome arrangements in this study and those in the literature were compared and analyzed. Because it was based on diverse materials, this result will

provide a basic karyotype for further cytogenetic studies and linkage analysis.

Due to its self-incompatibility, Russian wildrye exhibits morphological polymorphisms. It is difficult to distinguish and identify trisomics from diploid plants. Based on the established karyotype, four out of seven possible primary trisomics, a double-deletion trisomic and two tertiary trisomics, were identified from 29 plants that contained 15 chromosomes. The four primary trisomics were primary trisomics for chromosomes C, D, F, and G. The double deletion-trisomic for chromosome E had a distal segment in the short arm of one chromosome D and one chromosome E deleted. This plant was less vigorous, was slower growing, and had fewer tillers than the diploid and other trisomics. The two tertiary trisomics were DS/AL and DS/FS.

A deletion-translocation heterozygote was also identified. A distal intercalary segment in the long arm of chromosome F was deleted and the distal regions in the long arms of chromosomes E and F were reciprocally translocated. This result was confirmed by C-banding and chromosome behavior in meiosis. These trisomic and deletion-translocation materials may be useful in gene mapping.

One to three B chromosomes (supernumerary chromosome) were observed in some plants of the accession DJ-3955. The uniform staining after C-banding indicated the heterochromatinized nature of the B chromosome.

One hundred and sixty individuals in 11 accessions of Russian wildrye were analyzed by starch gel electrophoresis. Among the 14 scorable loci of 10

enzymes, 11 loci showed polymorphisms. High genetic diversity existed at most of the polymorphic loci. The genetic diversity was partitioned into within-accessions, within-countries, and among-countries. The result indicated that 91.1% of the allelic diversity existed within accessions.

The reaction conditions in randomly amplified polymorphic DNA (RAPD) were optimized for Russian wildrye and 88 individuals from 11 different accessions were analyzed with this technique. Thirty-nine and 56 unambiguous RAPD markers were used to analyze the genetic variation within and among accessions, respectively. A high polymorphic rate (55.7% and 47.8%) of the amplified fragments existed in both within- and among-accessions. Accessions from neighboring geographical regions were more similar than those from distant regions.

Generally, the results from chromosome banding, isozymes, and RAPD were highly corroborated. They indicated that genetic diversity widely existed in Russian wildrye, both within and among accessions. Although more genetic similarity existed in neighboring accessions, most genetic diversity came from within accessions. The results provided useful information for genetic research, germplasm management, and breeding programs of Russian wildrye. This study also suggested that existing collections of Russian wildrye may be adequately variable for improvement of this forage grass.

Some specific RAPD markers were identified, isolated, and characterized with

southern hybridization and DNA sequencing for *Ps. juncea* and related species. The RAPD products were amplified from both repetitive and low copy sequences. The specific RAPD markers revealed the specific priming sites in the DNA templates, whereas the internal sequences could be specific or nonspecific. These molecular markers may be useful in gene mapping, species identification, and phylogenetic study.

APPENDIX

Protocols used in this research

Protocol - 1

Chromosome Number Checking

Procedure

1. Incubate fresh root tips, collected from geminated seeds or from plants in green house in 0.05% colchicine for 2-4 h.

2. Rinse root tips in water and put them in 1% aceto-orcein for 2 h.

3. Put a stained root tip on a clean slide, remove tissue rather than meristem from the tip, drop a small drop of 45% acetic acid and heat the slide on an alcohol light for 3 seconds.

4. Gently squash the meristem tissue with a needle or a tweezers, put on a coverslip and squash the coverslip gently with a thumb.

5. Check the slide under a microscope.

Notes:

1.Root tips also can be pretreated in ice water for 24-40 h in stead of in colchicine. In pretreatment of root tips with ice water, root tips are usually put in vials filled with water and the vials are put in a container with ice. The container is then put in a refrigerator or cold room.

2. Root tips in 1% aceta-orcein can be stored in a refrigerator for several months. Alternatively, pretreated root tips with ice water can be fixed with 3:1 (95% ethanol : acetic acid) for one day and stored in 70% ethanol. Before using, root tips are transferred to 1% aceta-orcein.

Solutions

1. 0.05% Colchicine: dissolve 50 mg colchicine and 25 mg 8-hydroxyquinoline in 100 ml dH_2O (distilled water), then add 25 drops dimethylsulfoxide (DMFO), store in a refrigerator protecting from light.

2. 1% Aceta-orcein: 1 g orcein dissolved in 100 ml boiled 45% acetic acid, filtration after cool down.

3. 45% acetic acid: 45 ml glacial acetic acid + 65 ml dH_2O.

4. Fixative: 30 ml 95% ethanol + 10 ml glacial acetic acid.

References

Hsiao, C., Wang, R.R-C., and Dewey, D.R. 1986. Karyotype analysis and genome relationships of 22 diploid species in the tribe Triticeae. Can. J. Genet. Cytol. **28**:109-120.

Protocol - 2

Giemsa C-banding

Procedure

1. Collect and pretreat root tips as in protocol 1.

2. Fix root tips in fresh fixative of 95% ethanol acetic acid (3:1) for at least one day. Root tips can be stored in fixative for one month at 4°C or longer time at -20°C.

3. Stain root tips in 1% acetocarmine for 2 h in room temperature or at 4°C for days, squash preparations in 45% acetic acid as described in protocol 1.

4. Check slides and take pictures of cells with well-spread chromosomes under microscope.

5. Remove coverslips by freezing in liquid nitrogen.

6. Put slides in 60°C 45% acetic acid for 10 min, then in 95% ethanol at room temperature for 10 min, air dry overnight.

7. Incubate the slides in saturated $Ba(OH)_2$ at 60°C for 15 min, then completely rinse with water.

8. Incubate the slides in 2xSSC at 60°C for 30 min, rinse with water, then air dry.

9. Stain the slides in Giemsa working solution for 15 to 30 min, rinse in water and air dry. The slides without mounting can be stored in room temperature for years.

10. Observe the bands under microscope and take pictures with high contrast film.

11. Develop the film with D-19 solution for 4 min and fix in fixer for 5 min.

Solutions

1. 1% acetocarmine: Slowly put 1 g acetocarmine in 100 ml boiling 45% acetic acid and boil 30 seconds, filter the solution after cool down.

2. $Ba(OH)_2$: dissolve 5 grams of $Ba(OH)_2$.8H2O in 100 ml boiling distilled water,

stir for 5 min and filter the solution. The solution has to be prepared freshly every time.

3. 2xSSC: 8.84 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$, MW 294.1) and 17.56 g NaCl (MW 58.44) in 1000 ml distilled water, pH 7.0.

4. Phosphate buffers: 1/15 M Na_2HPO_4 -- 9.465 g Na_2HPO_4 in 1000 ml dH_2O
1/15 M KH_2PO_4 -- 9.078 g KH_2PO_4 in 1000 ml dH_2O

5. Giemsa stock solution: Completely ground 1 g Giemsa in 66 ml glycerol, incubate in oven at 60°C for 2 h, then add 66 ml methanol and mix completely. This solution needs to maturate for one week and it can be stored at room temperature for many years.

6. Giemsa working solution: 55 ml 1/15 M Na_2HPO_4 + 45 ml 1/15 M KH_2PO_4 + 2 ml Giemsa stock solution.

References

Endo, T.R., and Gill B.S. 1984. The heterochromatin distribution and genome evolution in diploid species of *Elymus* and *Agropyron*. Can. J. Genet. Cytol. **26**:669-678.

Protocol - 3

Meiotic Chromosome

Procedure

1. Collect young spikes from plants, remove leaves and sheath.

2. Fix young spikes in farmer's solution (3:1) for one day, transfer the spikes to 70% ethanol and store in a refrigerator.

3. Stain spike in 1% acetocamine for 2 h.

4. Pick an anther on a clean slides and drop a small drop of 45% acetic acid.

5. Cut the anther into two to three parts, and squash the anther gently with a dissect knife or tweezers, let pollens out of the anther and into the solution.

6. Remove the debris of the anther and put on a coverslip, then squash the coverslip gently with a thumb.

7. Check under a microscope.

8. For C-banding of meiotic chromosomes, as same as protocol-2 from step 5 to step 11.

Reference

Wang, R. R.-C., and Berdahl, J. D. 1990. Meiotic association at metaphase I in diploid, triploid and tetraploid Russian wildrye [*Psathyrostachys juncea* (Fisch) Neviski]. Cytologia, **55**:639-643.

Protocol - 4

Starch Gel Electrophoresis

Procedure

1. Starch gel preparation

1). Put 50 g potato starch into a dry 1000 ml armed flask.

2). Heat 325 ml gel buffer to boiling in the microwave.

3). Add another 125 ml cold gel buffer to the starch in the flask, agitate the flask to get all of the starch into suspension quickly.

4). Pour the hot 325 ml gel buffer slowly into the flask with agitation.

5). Heat the flask in the microwave to boiling , then carefully vacuum the air bubbles from the gel.

6). Quickly pour the gel into the mold, the sides of the mold are sealed with tapes before pouring.

7). After an hour, cover the gel with wrap to prevent drying out.

8). Put the gel in a refrigerator for 2 h before using.

2. Sample preparation

1). Collect plant leaves in the field or greenhouse and store in -70°C till use.

2). Grind about 0.01 g leaf tissue in extraction buffer on a chilled spot plates.

3). Place wicks into wells to saturated with solution.

3. Load and run the gel

1). Cut the gel at about 1 cm from the top with a sharp spatula, and push the gel gently away from the cut.

2). Put saturated wicks into the cut orderly and evenly, the first and last wicks are saturated with blue dye to indicate the positions of the samples.

3). Put the spacers in the mold and put the wrap over the gel.

4). Put the gel into a electrophoretic tank which is filled with electrode buffer.

5). Turn on the power supply and run the gel at 4°C with 60 volts overnight.

4. Gel staining

1). Slice the gel with a bow slicer into four slices.

2). Put slice in separate tray.

3). Prepare different staining solutions, pour staining solution on the gel and stain at 37°C.

4). Record the data.

Notes

1. Extraction buffer

	For 100 ml buffer
Tris-HCl 0.1 M pH 7.0	1.21 g
EDTA 0.01 M	0.37 g
NAD 0.5 g/l	0.05 g
NADP 0.2 g/l	0.02 g
2-mercaptoethanol	10 ml

2. Electrophoretic buffer system

1). M-C Buffer

Stock buffer: dissolve 8.41 g citrate acid in 990 ml dH_2O, add 11.5 ml M-C Buffer, pH 6.1

Stock for electrode buffer, 1:19 diluted for gel buffer.

2). T-C Buffer

Stock: 0.223 M Tris, 0.069 M citrate acid--- 27.8 g Tris and 13.33 g citrate acid in 1000 ml dH_2O.

Stock for electrode buffer, 1:29 diluted for gel buffer.

3). Buffer 7

Electrode buffer: 0.038 M LiOH, 0.188 M boric acid--- dissolve 1.60 g $LiOH.H_2O$, 11.60 g boric acid to 900 ml dH_2O, adjust pH to 8.3, bring the volume to 1000 ml with dH_2O.

Gel buffer--- 0.045 M Tris, 0.007 M citrate acid, 0.004 M LiOH, 0.019 M boric acid: dissolve 5.45 g Tris and 1.28 g citric acid in 850 ml dH_2O, add 100 ml electrode buffer, adjust pH to 8.3 with 1.0 m NaOH, bring volume to 1000 ml with dH_2O.

3. Staining solutions

1). Adenylate kinase (AK) (EC 2.7.4.3)

1.0 M Tris-HCl	10 ml
dH_2O	40 ml
0.1 M $MgCl_2.6H_2O$	6 ml
adenosine 5'-diphosphate	0.03 g
D(+)-glucose	0.1 g
hexakinase	20 units
G6PDH	40 units
NAD (5mg/ml)	4 ml
MTT (5mg/ml)	1 ml
PMS (1mg/ml)	1 ml

2). Aldolase (ALD) (EC 4.1.2.13)

1 M Tris-HCl Ph 8.5	5 ml
dH_2O	45 ml
fructose, trisodium salt	100 mg
1 M arsenic acid, sodium salt	0.5 ml
glyceraldehyde 3-phosphate dehydrogenase	100 units
NAD (5mg/ml)	2 ml
MTT (5mg/ml)	2 ml
PMS (1mg/ml)	2.5 ml

3).Glucose dehydrogenase (GDH) (EC 1.1.1.118)

1 M Tris-HCl Ph 8.0	5 ml

dH_2O	35 ml
1 M L-glutamic acid Ph 8.0	10 ml
NAD (5mg/ml)	2 ml
MTT (5mg/ml)	1 ml
PMS (1mg/ml)	1 ml

4). Glucose-6- phosphate dehydrogenase (G6PDH) (EC 1.1.1.49)

1.0 M Tris-HCl Ph 8.0	5 ml
dH_2O	45 ml
$MgCl_2.6H_2O$	50 mg
glucose-6-phosphate	50 mg
NAD (5mg/ml)	1 ml
MTT (5mg/ml)	2 ml
PMS (1mg/ml)	1 ml

5). Glucose phosphate isomerase (GPI) (EC 5.3.1.9)

1 M Tris-HCl Ph 8.0	5 ml
dH_2O	45 ml
1.0 M $MgCl_2.6H_2O$	0.5 ml
fructose-6-phosphate, disodium salt	15 mg
glucose-6-phosphate dehydrogenase	20 units
NADP (5mg/ml)	1 ml
MTT (5mg/ml)	2 ml
PMS (1mg/ml)	1 ml

6). Isocitrate dehydrogenase (IDH) (EC 1.1.1.42)

1 M Tris-HCl Ph 8.0	5 ml
dH_2O	45 ml
isocitric acid, trisodium salt	50 mg
1.0 M $MgCl_2.6H_2O$	2.5 ml
NADP (5mg/ml)	1 ml
MTT (5mg/ml)	1.5 ml
PMS (1mg/ml)	1 ml

7). Malate dehydrogenase (MDH) (EC 1.1.1.37)

1.0 M Tris-HCl Ph 8.0	5 ml
dH_2O	45 ml
D.L-malic acid Ph 8.0	1.34 g

NAD (5mg/ml)	1 ml
MTT (5mg/ml)	1 ml
PMS (1 ml)	1 ml

8). Phosphoglucomutase (PGM) (EC 5.4.2.2)

1 M Tris-HCl Ph 8.0	10 ml
dH_2O	40 ml
$MgCl_2.6H_2O$	0.102 g
a-D-glucose-1-phosphate	0.1 g
G6PDH	40 units
NAD (5mg/ml)	4 ml
MTT (5mg/ml)	1 ml
PMS (1mg.ml)	2.5 ml

9). 6-Phosphogluconate dehydrogenase (PGDH) (EC 1.1.1.44)

1 M Tris-HCl Ph 8.0	10 ml
dH_2O	40 ml
$MgCl_2.6H_2O$	0.102 g
6-phosphogluconic acid	0.01 g
NADP (5 mg/ml)	2 ml
MTT (5 mg/ml)	1 ml
PMS (1 mg/ml)	5 ml

10). Shikimic dehydrogenase (SDH) (EC 1.1.1.25)

1 M Tris-HCl Ph 8.0	10 ml
dH_2O	40 ml
Shikimic acid	0.05 g
NADP (5 mg/ml)	2 ml
MTT (5 mg/ml)	1 ml
PMS (1 mg/ml)	5 ml

References

Murphy, R.W., Sites, J.W., Jr., Buth, D.G., and Haufler C.H. 1990. Protein I: isozyme electrophoresis. *In* Molecular systematics. *Edited by* D. M. Hillis and C. Morits Sinaur Associates, Sunderland, Mass. pp. 45-126.

Wendel, J.F., and Weeden, N.F. 1989. Visualization and interpretation of plant

isozymes. *In* Isozymes in plant biology. *Edited by* D. E. Scotis and P.E. Scotis Discorides Press, portland, Ore. pp. 5-45.

Protocol - 5

Extraction of Plant DNA

Procedure

1. Cut 0.1 - 0.5 g plant leaves into 0.5 cm long pieces and put the leave pieces and 3 ball bearings into a 2 ml microcentrifuge tube.

2. After leaving in liquid nitrogen about one minute, vortex till the leaves to fine powder.

3. Add 1 ml preheated extraction buffer and briefly vortex to suspend the leave tissue.

4. Add 0.4 ml chloroform and vortex .

5. Heat the sample for 10 min at 55°C.

6. Centrifuge the sample at 12 k for 10 min and transfer the supernatant to a new 1.5 ml tube.

7. Mix the supernatant with 1.2 volumes of isopropanol, reverse the tube several times till DNA precipitated.

8. Centrifuge at 12 k for 10 min, wash the DNA pellet twice with 1 ml of 70% ethanol.

9. Vacuum dry for 10 min or air dry for 20 min at 37°C, redissolve DNA in 100 µl TE.

Notes:

Different plant materials vary in the temperature and length of incubation in extraction buffer.

Solutions

1. Extraction Buffer: 0.14 M sorbitol, 0.22 M Tris-Hcl, 0.022 M EDTA, 0.8 M NaCl 0.8% CTAB(Hexadecyltrimethylamomonium bromide) and 1% N-laurlysarcosine.

For 200 ml extraction buffer: dissolve 5.1 g sorbitol, 5.32 g Trizma base, 1.64 g

EDTA, 9.34 g NaCl, 1.6 g CTAB and 2 g N-larylsarcosine in 150 distilled water, Ph 7.8-8.0, add water to 200 ml, autoclave.

2. TE: 10 Mm Tris-Hcl (7.5) and 0.1 Mm EDTA.

References

Lassner, M.W., Peterson P., and Yoder J.I. 1989. Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. Plant Mol. Biol. Rep. **7**:116-128.

Williams, J.G.K., M.K. Hanafey, J.A. Rafalski, and S.V. Tingey. 1992. Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymol. **218**:704-740.

Protocol - 6

Electrophoresis of DNA Samples

Procedure

1. Prepare 1x TAE or 1x TBE buffer by diluting the 10x stock buffer.

2. Completely dissolve 0.8 - 1.5 g regular agarose in per 100 ml 1x buffer by heating in microwave.

3. Add 3 µl 10 mg/ml ethidium bromide for per 100 ml gel when cool down to 50°C.

4. Pour the gel to the electrophoretic mode with comb(s) set and place in room temperature for at least 30 min or in cold room for 15 min.

5. Carefully pull the comb(s) straight upward, fill the tank with 1x buffer till the gel is just immersed in the buffer.

6. Prepare DNA samples by adding appreciate DNA, dH_2O and loading dye.

7. Loading the samples in the wells of the gel.

8. Close the lid, connect and turn on the power supply.

Notes:

1. For PCR or RAPD products, 2 - 3% agarose specific for PCR can give better resolution. For DNA precipitate for the gel, 1.5% of Low melting agarose in 1 x TAE is better.

2. The buffer used to make the gel and running buffer (in the tank) should be the same.

3. Voltage of the power is about 4.5 vol/cm.

Solutions

1. 10x TAE: 0.4 M Tris-acetate, 0.01 M EDTA.
Per liter: Tris base 48.4 g, glacial acetic acid 11.42 ml, 0.5 M EDTA (pH 8.0) 20 ml.

2. 10x TBE: 0.89 M Tris-borate, 0.89 M boric acid, 0.02 M EDTA.
Per liter: Tris base 108 g, boric acid 55 g, 0.5 M EDTA (pH 8.0) 40 ml.

3. Loading dye: 0.25% bromophenol blue and 40% sucrose in water.
10 ml: 0.025 g bromophenol blue and 4 g sucrose, storage at 4°C.
(Loading dye : loading sample = 1 : 4).

4. Ethidium bromide
Stock solution: 10 µg/µl, storage at 4°C in dark.
Working solution: 0.5 µg/µl.
In agarose gel: 0.5 µg/ml. For example, for 100 ml agarose gel, add 100 µl working solution.

Protocol - 7

DNA Qualification

DNA concentration can be decided by either comparing the band with a concentration known DNA sample or reading with a TKO 100 mini-fluorometer as the follows.

Procedure

1. Turn on the TKO 100 mini-fluorometer at least 15 min before use.

2. Prepare 1x TNE buffer by diluting 10x TNE with distilled water.

3. Make working solution by adding 10 µl dye stock to per 100 ml 1x TNE.

4. Turn scale control to fully counter clockwise position.

5. Turn scale control five clockwise position.

6. Put the cuvette into the well with "G" symbol facing forward. Pipet 2 ml of working solution into the cuvette and close the lid.

7. Zero the instrument by turning zero control knob until the display reads "000".

8. Pipet 2 µl of standard DNA into the cuvette, mix with 1 ml pipette, close the lid.

9. Adjust the scale control to display reads "100".

10. Repeat step 6 to 9 once or twice until display reproducible reads "100±3".

11. Measure DNA samples as step 6 to 9, do not adjust the scale control and zero control during the measurement.

Solutions

1. 10x TNE: dissolve 3.03 g Tris base (MW 121.2),0.903 g Na_2-EDTA (MW) and 29.2 g NaCl in 200 ml distilled water, adjust pH with concentrated HCl to 7.4, add distilled water to 250 ml, store the solution in refrigerator up to 12 months.

2. Dye stock: 10 mg Hoechst 33258 in 10 ml dH_2O, filter and protect from light, store in refrigerator up to 6 months.

3. Standard DNA: dilute 1 mg/ml calf thymus DNA with dH_2O ten times to 100 ng/µl.

Protocol - 8

Randomly Amplified Polymorphic DNA (RAPD)

Procedure

1. Design the experiment on a RAPD sheet.

2. Move buffer, dNTP, $MgCl_2$, primers, template DNA from -20°C onto ice to defrozen.

3. Prepare and label 0.2 ml PCR tubes.

4. Prepare RAPD reactions. For per 25 ul RAPD reaction, add 14.3 µl ddH_2O, 2.5 µl of 10X buffer ($MgCl_2$ free), 3 µl of 8 Mm dNTP, 3 µl of 10 µM primer, 3 µl of 25 mM $MgCl_2$, 2 µl of 20 ng/µl template DNA, 0.2 µl of 10 U/µl Stoffel Fragment and 30 µl mineral oil, then briefly centrifuge.

For more than one reactions, master mix can be prepared accordingly and then divide into reaction tubes.

5. Load the reaction tubes into sample block of a PCR system (GeneAmp PCR System 9600), make sure that the tube are tightly contacted with the well.

6. Close the block cover and turn on the power. Select a suitable stored program and run the RAPD. For RAPD, 40 cycles of 93°C 1 min, 35°C 1 min and 71°C 2 min.

7. When finished, stop the program and turn off the machine. Put reaction tube in 4°C till using.

Notes:

1. Template DNA

Dilute genomic DNA to 20 ng/µl with ddH_2O and store in refrigerator. If plasmid or specific DNA fragment is used as template, the concentration of template should be much lower.

2. Primers

Primers used for RAPD are diluted to 10 µM, the random primers purchased from Operon Company are dissolved in 500 µl ddH_2O.

3. dNTP

For 0.5 ml 8 mM dNTP:
10 µl of 100 mM dATP
10 µl of 100 mM dCTP
10 µl of 100 mM dGTP
10 µl of 100 mM dTTP
5 µl of 1M Tris-HCl (pH 7.9)
455 µl ddH_2O
mix well, aliquot and store in -20°C.

4.Concentrations in reaction.

	original concentration	amount for 25 µl reaction	amount for50 µl reaction	final concentration
Buffer	10x	2.5	5.0	1x
dNTP	8 mM	3	6	0.96 mM
Primer	10 uM	2	2	0.8 µM
Primer	10 uM	3	6	1.2 µM
MgCl2	25 mM	2.5 3	5 6	2.5 mM 3 mM
Enzyme	10 U/µl	0.2	0.4	2 U/25 µl
Template	20 ng/µl	2	4	1.6 ng/µl

5. Buffer

Buffer can be made as followings or purchased from the company alone with the amplification enzyme.

10x Taq buffer: 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$ and 0.01% (w/v) gelatin

10x Stoffel buffer: 100 mM KCl and 100 mM Tris-HCl (pH 8.3)

6. Enzyme

For PCR, Taq DNA polymerase produces more specific products; for RAPD, Stoffel fragment can produce more bands. Enzymes from different companies show different activity.

Reference

Williams, J.G.K., Hanafey, M.K., Rafalski, J.A., and Tingey, S.V. 1992. Genetic analysis using random amplified polymorphic DNA markers. Methods Enzymol. **218**: 704-740.

Protocol - 9

DNA precipitate from agarose

Procedure

1. Prepare 1.5% low melting point agarose gel in 1x TAE containing 0.5 µg/ml ethidium bromide.

2. Load 10 µl RAPD products per lane and run at 50 V for about three hours in cold room.

3. Locate bands with short (weak) wave UV.

4. With a sharp scalpel cut out the bands of interest, removing as much of the agarose as possible. Place the agarose with DNA into a 1.5 ml microcentrifuge tube, add 400 µl of TE pH 8.0 (about 3 volume), then incubate at 70°C for 2-5 min.

5. Tap the tube and quick freeze the sample in a -70°C freezer for at least one hour. Let sample freeze completely.

6. Thaw the sample at room temperature, vigorously tap the tube and centrifuge the sample in a microcentrifuge at full speed for at least 15 min. Carefully save the supernatant that contains the agarose-free DNA fragment.

7. Add 1/10 volume (40 µl) of 3M NaOAc (pH 5.2) and 2-2.5 volumes (800-1000 µl) 100% ETOH. Precipitate for overnight, at 4°C.

8. Next morning centrifuge for 20 min. Wash the pellet with 70% ETOH, dry the pellet and resuspend in TE or sterile water.

Note:

If desired, any residual agarose can be removed by phenol/chloroform extraction, followed by ETOH precipitation.

Reference

Qian, L., and M. Wilkinson. 1991. DNA fragment purification: removal of agarose 10 minutes after electrophoresis. BioTechniques **10** (6): 736-738.

Protocol - 10

Plasmid Extraction

Procedure

1. Pick up single colony from LB (Amp+) plate and culture it in 5 ml to 25 ml liquid LB (Amp+) medium overnight at 37°C with 200 rpm agitation.

2. Next morning, transfer the culture into 2 ml microcentrifuge tubes (for small amount of culture) or 25 ml centrifuge tubes (for large amount of culture), centrifuge at 14K for 10 min (microcentrifuge tube) or at 8K for 10 min (large tube) and save the pellet.

3. Suspend the pellet in STET buffer by vortex. (200 µl STET for microcentrifuge tube, 1.25 ml for large tube and then transfer into microcentrifuge tubes.)

4. Add 0.1 mg/µl lysozyme to the sample (The final concentration of lysozyme is 1mg/ml), vortex for 3 seconds and leave at room temperature for 5 min.

5. Boil the sample for 50 second and centrifuge at 14k for 10 min.

6. Pick away the soft pellet with sterile toothpicks and centrifuge again at 14k for 10 min to avoid the debris.

7. Add 10 mg/ml RNase A (The final concentration of RNase A is 100 µg/ml.), then incubate at 68°C for 10 min.

8. Add 5% CTAB to the sample (The final concentration of CTAB is 0.5%.), mix by vortex and leave at room temperature for 3 min and centrifuge at 14 k for 10 min.

9. Save the CTAB-DNA pellet and suspend the pellet in 0.3 ml of 1.2 M NaCl, add 0.75 ml 100% ethanol and mix.

10. Centrifuge at 14 k for 10 min, wash the pellet with 70% ethanol and dry the DNA pellet and dissolve DNA in 30 µl TE.

Solutions

1. 1 M Tris: 12.11 g Tris base (MW=121.1) in 100 ml H_2O, pH 8.0.

2. 0.5 M EDTA:

3. 1.2 M NaCl:

4. STET Buffer (50 ml): sucrose 4 g
1 m Tris 2.5 ml
0.5 M EDTA 5 ml
Triton x-100 5 µl
dH_2O 42.5 ml.

6. 0.5% CTAB; 5mg CTAB (hexadecyl trimethylammonium bromide, FM=364.5) in 100 ml dH_2O.

7. Lysozyme: make 0.1 mg/µl of lysozyme freshly in STET buffer.

8. RNase: dilute stock RNase A solution with water to 10 mg/ml or dissolve RNase A powder in 2xSSC to 10 mg/ml.

Protocol- 11

RAPD Cloning With Prime PCR Cloner Cloning System

Procedure

1. DNA preparation

Elute the band of RAPD product from agarose gel with protocol-9. Read the DNA concentration with a fluorometer as protocol-7.

2. Modification

1) Set up 16°C and 75°C water baths.

2) In a 0.65 ml chilled microcentrifuge tube, add the followings:

MBG H_2O	µl
10 x Prime PCR cloning regent	2 µl
Prime PCR cloner nucleotide stock	1 µl
0.1 M DTT solution	1 µl
Eluted RAPD product	µl
Total	18.5 µl

Note: The amount of PCR product is determined according to the product length, effective molar ratio and PCR product concentration. For 5:1 effective molar ratio,

(y) ng of PCR product to use = (n) bp x 0.555
( ) µl of PCR product = (y) ng/concentration (ng/µl)

If DNA concentration is too low, concentrate the DNA before use.

3) Mix and short spin the above reaction.

4) Add 1.5 µl of Prime PCR modification regent to the tube, mix the mixture (cloning reaction) by gentle vortex, spin briefly at 4°C, incubate at 16°C for 25 min, at 75°C for 15 min, then place at room temperature.

3. Ligation

1) Set up 25°C and 65°C water baths.

2) In a 0.65 ml tube, preparing ligation mix by adding the followings:

MBG H_2O	5 µl
0.1 M DTT	1 µl
10 x Prime efficiency ligation buffer	2 µl
cloning reaction	10 µl
pNoTA	1 µl

Note: the other 10 µl of cloning reaction can be stored in -20°C.

3) Mix briefly and add 1 µl T4 DNA ligase, mix well by gentle vortex and short spin at room temperature.

4) Incubate at 25°C for 30 min, then place at room temperature.

4. Transformation

1) Streak single colony of JM 109 on a 2x YT plate to grow 14-16 hours at 37°C in the previous day.

2) During ligation, add competency buffer to a 2 ml tube, put the tube on ice.

3) Take about 30 single colonies for per 200 µl competency buffer, suspend the cells by vertex and put the tube back on ice for 40 - 60 min.

4) Aliquot 200 µl competent cells into the ligation mix, mix gently by tapping the tube.

5) Leave the tube on ice for 20 min, heat shock at 42°C for 90 seconds then put back on ice.

6) Add 0.8 ml 2x YT liquid medium to each tube and shake the tube at 37°C with 200 rpm for 45 min.

7) Spin 10 seconds at full speed, remove 800 µl of the supernatant, suspend the cells in remaining supernatant.

8) Spread the cells onto LB/AMP/X-gal/IPTG plates, culture overnight at 37°C.

9) Pick up white colony and streak on LB/AMP plates, culture overnight at 37°C.

10) Pick single colony and culture in 10 ml liquid LB/AMP medium at 37°C with 200 rpm overnight.

Medium

1. LB medium

Dissolve 10 g NaCl, 10 g trypton, 5 g yeast extract and 15 g agar in 1000 ml dH_2O, autoclave for 30 min, pour about 25 ml medium into each plate in an aseptic hood, place the plates in hood till solid, then store at 4°C.

LB/AMP

After autoclave,keep the medium in 55°C, add 1 ml 100 mg/ml ampicillin (final concentration of ampicillin is 100 mg/l). Without agar, the medium is LB liquid medium.

2. 2x YT medium

Dissolve 5 g NaCl, 16 g trypton, 10 g yeast extraction and 15 g agar to 1000 dH_2O, autoclave for 30 minute, pour into plates.

Solutions

1. X-gal stock (10%): dissolve 100 mg X-gal (MW 408.64) in 1 ml filter sterile DMF, aliquot to 200 µl and store at -20°C.

2. IPTG stock (100 mM): dissolve 0.024 g IPTG in i ml ddH_2O, aliquot to 200 µl and store at -20°C.

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Protocol -12

Probe Labeling

I. Random primer labeling

1. Thaw frozen hexanucleotide mixture and dNTP labeling mixture on ice.

2. Add 2 µg DNA in 15 µl ddH_2O into a 1.5 ml microcentrifuge tube. Denature the DNA by heating in boiling water for 10 min and chilling quickly on ice/alcohol for 5 min.

3. Pipette the following components into the tube:

hexanucleotide mixture	2 µl
dNTP mixture	2 µl
Klenow enzyme	1 µl

4. Centrifuge briefly and incubate at 37°C for 2 h to overnight.

5. Stop the reaction by adding 2 µl 0.5 M EDTA (pH 8.0).

6. Add 1/10 volume of 3 M sodium citrate and 2 volumes of 100% ethanol, mix well.

7. Leave at -70°C for 30 min or -20°C for 2 h.

8. Centrifuge at 14 K for 20 min, wash with 70% ethanol and vacuum dry.

9. Dissolve in 50 µl TE buffer and store at -20°C.

Notes:

dNTP labeling mixture contains DIG-dUTP or biotin-dUTP.

II. Nicktranslation labeling

1. Thaw frozen dNTP labeling mixture on ice.

2. Pipette the following components into a microcentrifuge tube on ice:

10x dNTP mix	5 µl
DNA (1 µg)	___
ddH_2O	___
10x enzyme mix	5 µl
total	50 µl.

3. Mix well and briefly centrifuge.

4. Incubate at 16°C for 2 h.

5. Stop by adding 2 µl 0.5 M EDTA (pH 8.0).

6. Purify the probe as same as described for the random primer labeling.

Protocol - 13

Restriction Digestion

Digestion of plasmid

1. Add 100 - 200 ng plasmid with insert in 17.8 µl ddH_2O in a 1.5 ml microcentrifuge tube.

2. Warm the tube at 65°C for 5 min.

3. Add the following components to the tube:

10x high salt buffer (USB)	2 µl
enzyme BamH1 (10U/µl) (UBS)	0.2 µl

4. Briefly centrifuge and incubate at 37°C for 2 h.

5. Heat at 65°C for 5 min.

6. Add 5 µl loading dye and run a regular agarose gel.

Digestion of plant genomic DNA

1. Add the following components into a 1.5 ml microcentrifuge tube:

plant genomic DNA (2 µg)	____
ddH_2O	____
10x medium salt buffer	2 µl
Hind III (10 U/µl)	0.5 µl
total	20 µl

2. Incubate at 37°C for 4 h.

3. Add 5 µl loading dye and run a regular agarose gel.

Protocol - 14

Southern Hybridization

Procedures

1. Run agarose gel (1.5% regular agarose in 1x TAE or 1x TBE buffer).

2. Take a picture under UV light.

3. Cut the edges of the gel.

4. Soak the gel in 0.25 N HCl at room temperature for 15 min with gentle agitation, rinse in dH_2O.

5. Denature the gel in 0.5 M NaOH/1.5 M NaCl at room temperature 2x15 min, rinse in dH_2O.

6. Neuturelize the gel in 1.0 M Tris-HCl (pH 8.0)/1.5M NaCl at room temperature 2x15 min, rinse in dH_2O then in 2x SSC.

7. Wet the same size membrane in dH_2O, then soak in 2x SSC for 15 min.

8. Prepare 10x SSC, tray, 3 mm paper and tower paper.

9. Set up the transfer in order: tray, 10x SSC, plate, 3 wicks, gel, membrane, 3x3 mm paper, tower paper and weight. make sure there is no air bubbles between the gel and the membrane.

10. After overnight transferring, rinse the membrane in 0.4 N NaOH 1 min, 0.2 M Tris-HCl(pH 7.5)/1x SSC 1 min, and 2x SSC 1 min.

11. UV crosslink for 1 min and store the membrane between 3 mm papers or use it immediately.

12. Prehybridization with hybridization buffer at 65°C for 2 h.

13. Replace the hybridization buffer with hybridization buffer + probe, and hybridize at 65°C overnight.

14. Wash the membrane with 2x SSC at room temperature for 5 min.

15. Wash the membrane in Wash I 2x5 min, Wash II 2x5 min respectively at room temperature.

16. Wash the Membrane in Wash III at 65°C 2x15 min.

17. Rinse membrane in Buffer I for 1 min.

18. Incubate membrane in Buffer II at room temprature for 30 min.

19. Incubate membrane in fresh antibody solution (1:10000) for 30 min.

20. Wash in Buffer I 2x15 min, then Buffer III 5 min.

21. Seal membrane in color reaction in dark for 20 min to several hours.

22. Wash membrane in dH_2O and air dry.

Solutions

1. 10xSSC: 21.94 g NaCl (MW 58.44), 11.03 g Na-citrate (FW 294.1) in 200 ml. ddH_2O, adjust pH to 7.0, bring the volume to 250 ml with ddH_2O, autoclave 15 min.

2. 20xSSC: 43.87 g NaCl (MW 58.44), 22.06 g Na-citrate (FW 294.1) in 200 ml. ddH_2O, adjust pH to 7.0, bring the volume to 250 ml with ddH_2O, autoclave 15 min.

3. 5% SDS: 10 g SDS in 200 ml ddH_2O, stir to dissolve, do not autoclave.

4. Wash I (500 ml): 100 ml 10xSSC, 8 ml 5% SDS in 392 ml ddH_2O.

5. Wash II (500 ml): 10 ml 10xSSC, 8 ml 5% SDS in 482 ml ddH_2O.

6. Wash III (500 ml): 8 ml 10xSSC, 8 ml 5% SDS in 486 ml ddH_2O.

7. Buffer I: 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl

12.12 g Trizma base(FW 121.1) in 1000 ml ddH_2O, adjust pH to 7.5 with HCl, add 8.76 g NaCl (MW 58.44), filter through 0.45µm filter paper, autoclave.

8. Buffer II: 3% BSA

0.6 g BSA (Fraction V) dissolve in 20 ml Buffer I, do not autoclave. Or 1% block

regent in Buffer I.

9. Buffer III: 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$

3.03 g Trizma base (FW 121.1) in 250 ml ddH_2O, adjust pH to 9.5 with HCl, add 1.46 g NaCl(MW 58.44), 2.54 g $MgCl_2$ (FW 203.3), filter with 0.45 µm filter paper, autoclave.

10. SA-AP solution (7ml)

Add 7µl 1.0mg/ml SA-AP stock to 7ml Buffer I and mix, use freshly.

11. Dye solution (7.5ml)

Add 45 µl 75 mg/ml NBT and 35 µl 50 mg/ml BCIP to 10 ml Buffer III and mix, use freshly.

12. Hybridization buffer

5x SSC, 1% (w/v) blocking reagent, 0.1% (w/v) N-lauroysarcosine and 0.02% SDS. For 500 ml hybridization buffer: add 125 ml 20x SSC, 50 ml 10% blocking stock reageat, 0.5 g N-lauroylsarcosine and 2 ml 5% SDS to 325 ml dH_2O.

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Publications

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