Theor Appl Genet (2001) 102:782–786

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A *Thinopyrum ponticum* and *Triticum aestivum* hybrid. Electrophoretic patterns of their endogenous phosphorylation, Diphosphonucleoside kinases, DNases and RNases

Received: 29 June 2000 / Accepted: 28 July 2000

Abstract Endogenous protein phosphorylation, DNase and RNase electrophoretic patterns, and the detection of NDP-kinases by TLC (Thin Layer Chromatography) were performed in *Thinopyrum ponticum* (2n=10*x*=70), *Triticum aestivum* (2n=6*x*=42), and their hybrid seedlings in order to accomplish intergeneric hybridization. Octoploid intergeneric hybrids (2n=8*x*=56) were synthesized in less than 50% of the hybrids. The F_1 hybrid plants resembled *Th. ponticum* with regard to morphological features and were sterile. Hybrid seedlings revealed very low endogenous phosphorylation and very low NDP-kinase activity in comparison to their parents. In addition hybrid seedlings expressed a new nuclease.

Keywords *Thinopyrum ponticum* · *Triticum aestivum* · Hybridization · Endogenous phosphorylation · Diphosphonucleoside kinase · DNase · RNase

Introduction

Thinopyrum ponticum (Podp.) Barkworth and D.R. Dewey (2n=10*x*=70) which belongs to the *Thinopyrum elongatum* polyploid complex of the section *Lophopyron* (Dewey 1984) is a self-fertilizing wheat grass. Its unique properties of salt tolerance and disease resistance provide a potential means for the transfer of these important and complex traits to cultivated wheat through intergeneric hybridization (Zhong et al. 1994). It is well-known that taxa of the polyploid complex possess agronomically desirable traits, such as the perennial habit, disease resistance, drought and salt tolerance, that are of interest to wheat, rye and barley breeders (Dewey 1984). Several *Agropyron* species have been successfully hybridized to *Triticum aestivum* L. (Alonso and Kimber 1980; Sharma and Gill 1983a, b; William and Mujeeb-Kazi 1993; Jauhar 1995) and, as a result, their desirable traits have been transferred into wheat. For example *Th. ponticum* is the source of genes for resistance in wheat to leaf and stem rust (McIntosh 1991) and salt tolerance (Shannon 1978; Dvorak et al. 1985). However, these intergeneric hybrids were sterile and low chromosome pairing was observed (Jauhar 1995).

Enzymes as biochemical markers (William and Mujeeb-Kazi 1993) and gliadin electrophoretic patterns (Jauhar 1995) formed the basis necessary to study the biochemical characteristic of hybrids and their parents. Similar studies could be based on electrophoretic patterns of their endogenous protein phosphorylation. As is well known, protein phosphorylation represents a ubiquitous post-translational modification mechanism for regulating the function of a number of enzymes as well as structural and modulator proteins (Trewavas 1976; Ranjena and Boudet 1987).

Nucleoside diphosphate kinase (NDP-kinase, E.C. 2.7.4.6) is a ubiquitous enzyme which catalyzes the transfer of the terminal phosphate of 5' triphosphate nucleotides (NTPs) to 5' diphosphate nucleotides (NDPs) through a "ping-pong" mechanism according to the following scheme:

$$E+NTP \rightleftarrows E–P+NDP,$$
$$E–P+N' DP \rightleftarrows E+N' TP.$$

This transfer is associated with the regulation of intracellular di and tri-phosphonucleotide levels and recent evidence suggest that it may also participate in the regulation of growth, development and signal-transduction processes (Sommer and Song 1994; Zhang et al. 1995).

Nucleolytic enzymes [DNases, RNases and type-I nucleases (E.C. 3.1.30.2)] may be expected to participate in a variety of intracellular processes involving the hydrolysis, recombination, replication, transcription or repair of nucleic acids (Wilson 1975; Yupsanis and Pantazaki 1988).

In the present work, *Th. ponticum* has been hybridized with *T. aestivum*. Then, hybrid seedlings (confirmed by cytological observation) and seedlings of their parents were evaluated for electrophoretic analysis of endogenous protein phosphorylation, DNases and RNases, as well as

Communicated by H.F. Liskens

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for the detection of nucleoside diphosphate kinases in order to identify biochemical characteristics among parental species and their hybrids. In addition, the gross morphological and cytological features are presented.

Materials and methods

Plant material

The wheat cultivar '4 FAWWON' *T. aestivum* L. (2n=6*x*=42) was crossed with the wheatgrass *Th. ponticum* (2n=10*x*=70) using wheat as the female parent. Both were generously provided by the Cereal Institute of Thessaloniki. The wheat spikes were emasculated and pollinated with wheatgrass pollen. Hybrid caryopses were allowed to ripen on the wheat parent. Hybrid seeds, as well as seeds from the two parents, were treated and germinated for 5 days in the dark at 21.5±1°C as described previously (Yupsanis et al. 1996). The hybrid nature of seeds was confirmed by cytological observation of the somatic cells prepared from root tips of the seedlings. The standard Feulgen technique of staining and squashing was applied for cytological observations (Coucoli and Symeonidis 1980). Cytologically confirmed hybrids were grown in the greenhouse and some were transferred to the field of the Cereal Institute of Thessaloniki. Five-day old hybrid seedlings, as well as seedlings of its parents, were utilized for the comparison of enzyme-activity patterns.

Extraction of enzymes

The five-day frozen seedlings were ground in a pestle and mortar with 10 ml/g of ice-cold buffer consisting of 0.1 M Tris-acetate, pH 7.0, containing 10 µM of β-mercaptoethanol and 0.5 mM of PMSF. The homogenate was centrifuged at 13000 g for 15 min and the supernatant was used as the source of the enzyme activities.

Protein determination

Protein was measured colorometrically by the method of Bradford, as modified by Bearden (1978).

Activity detection of DNases/RNases in native polyacrylamide-gel electrophoresis (Native-PAGE)

DNases and RNases isozymes (30 µg of seedling-protein extraction) were separated on a 9.5% (w/v) polyacrylamide slab gel (6.5 cm×1 mm) polymerized in the presence of 80 µg/ml of ssDNA or RNA. Procedures for electrophoresis and enzyme detection are given by Matousek and Tupý (1985), as modified by Kefalas and Yupsanis (1995).

Endogenous protein phosphorylation

SDS-PAGE protein kinase activity was assayed by incubating the enzyme preparation (30 µg) at 37°C for 5 min in the presence of 33 mM of Tris-HCl, pH 7.6, 1 mM of $MgCl_2$, 10 mM of K-Pi pH 7.6, and 3/8 µC of [γ-^{32}P] ATP (specific activity 4500 Ci/mmol). Then, the phosphorylated endogenous proteins were analyzed by SDS-polyacrylamide gel electrophoresis (12% w/v; 5 cm×1 mm; mini vertical-slab cell of the Biorad Laboratory, Richmond, USA) according to the Laemmli (1970) system. The gels were dried under vacuum and autoradiographied using Kodak-O-Mat-X-ray film (Yupsanis et al. 1989).

Detection of nucleoside diphosphate kinase (NDK) activity

After SDS-electrophoresis and visualization of the phosphorylated peptides the main labelled bands were excised. To remove SDS every excised band was washed twice at 4°C for 1 h each with 50 mM of Tris-HCl, pH 7.5, containing 20% (v/v) isopropanol and once, after removing the Whatman paper, with 50 mM of Tris-HCl, pH 7.5, containing 5 mM of β-mercaptoethanol. The ability of kinase to transfer [^{32}P]-phosphate from [γ-^{32}P]-ATP to nucleoside diphosphate was assayed by incubating the above-washed piece of gel for every labelled band in 50 mM of Tris-HCl, pH 7.5, (60 µl) containing 1 mM of $MgCl_2$, 10 mM of K-Pi, pH 7.6, 0.1 mM of GDP and 4/8 µCi of [γ-^{32}P]-ATP (specific activity 4500 Ci/mmol). After incubation at 30°C for 1 h, 50 µl of each reaction mixture was withdrawn and applied to a TLC-cellulose plate. The TLC plate was developed with 0.1 M of phosphate pH 6.8/ammonium sulphate/propan-1-ol (100:60:2). The resulting spots were visualized by autoradiography (see above).

Cytology

Root tips were pre-treated with a saturated solution of bromonaphalene at room temperature for up to 5 h, fixed in glacial acetic acid and hydrolysed in 1 N HCl for 10 min at 60°C. Then, they were stained with leucobasic-fuchsin and the meristematic tip was squashed in a drop of 1% acetocarmine. Metaphase cells with a complete chromosome complement were photographed.

Results

Morphology and cytology

Regarding the morphological features, the F_1 hybrids were sterile perennials and generally resembled the male *Th. ponticum* parent. Hybrid spikes (Fig. 1B) had spikelets without awns, larger than those of either parents and the spikelet density was intermediate between the two parents (Fig. 1A, C). Less than 50% of the hybrids had 56 somatic chromosomes with six characteristic (marker) chromosomes, four satellited chromosomes (one metacentric, two submetacentric and one acrocentric), and two acrocentric chromosomes (Fig. 1b).

Protein phosphorylation – detection of nucleoside diphosphate kinase (NDK) activity

Figure 2 displays the SDS-PAGE autoradiograms of the in vitro phosphorylated soluble proteins from the hybrid (Fig. 2, tracks 1, 3, 5), wheat (Fig. 2, track 2) and *Th. ponticum* (Fig. 2, track 4) after different exposure times for autoradiography.

The autoradiograms of wheat and *Th. ponticum* revealed mainly two major groups of phosphorylated endogenous protein bands: a strong phosphorylated lowmolecular-group (Mrs approximately 10000–20000; LMG) and a low phosphorylated medium-molecular-group (Mrs approximately 35000–50000; MMG) which appeared after the gel had been exposed for over 12 h (Fig. 2, tracks 2 and 4). In autoradiograms of the hybrids the MMG was absent and the phosphorylation of LMG was very low and appeared after it had been exposed to autoradiography for over 12 h (Fig. 2, tracks 1, 3 and 5).

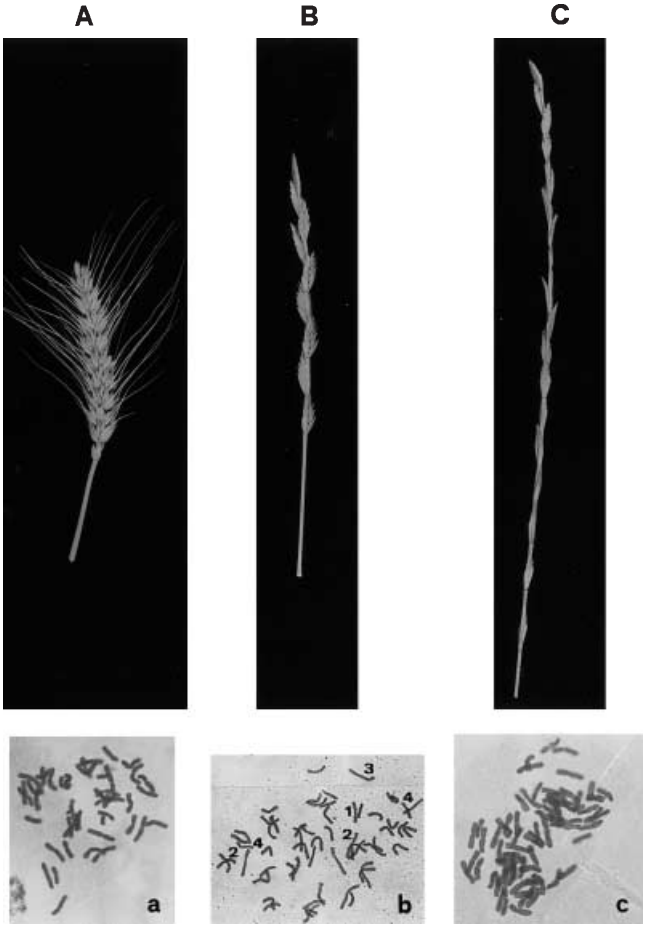

Fig. 1 Spike morphology of female *T. aestivum* (**A**), the intergeneric hybrid (**B**) and male *Th. ponticum* (**C**). Somatic chromosomes of *T. aestivum* (**a**), octaploid hybrid with six marker chromosomes, four satellited (one metacentric[1], two submetacentric[2], and one acrocentric[3]) and two acrocentric[4] (**b**), and *Th. ponticum* (**c**)

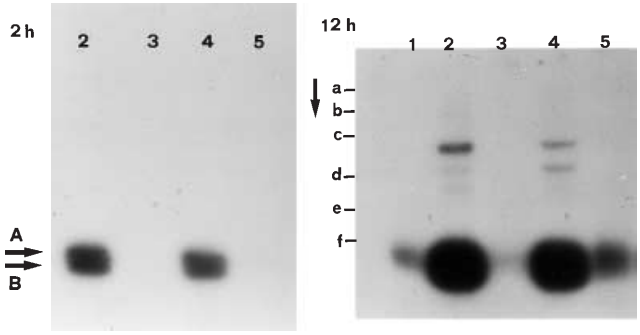

Fig. 2 Analysis by SDS-PAGE and autoradiography of the endogenous phosphorylated proteins of the extractions prepared from *Th. ponticum* (*track 4*) wheat (*track 2*) and hybrid (*tracks 1, 3, 5*) seeds. Exposure time 2 h (left) and 12 h (right). Pre-stained molecular standards: *a* phosphorylase b (Mr approximately 130000); *b* bovine serum albumin (Mr approximately 75000; *c* ovalbumin (Mr approximately 50000); *d* carbonic anhydrase (Mr approximately 39000); *e* soybean trypsin inhibitor (Mr approximately 27000); *f* lysozyme (Mr approximately 17000). The *horizontal arrows* indicate the position of the catalytic subunits of the NDP kinases (*A and B*)

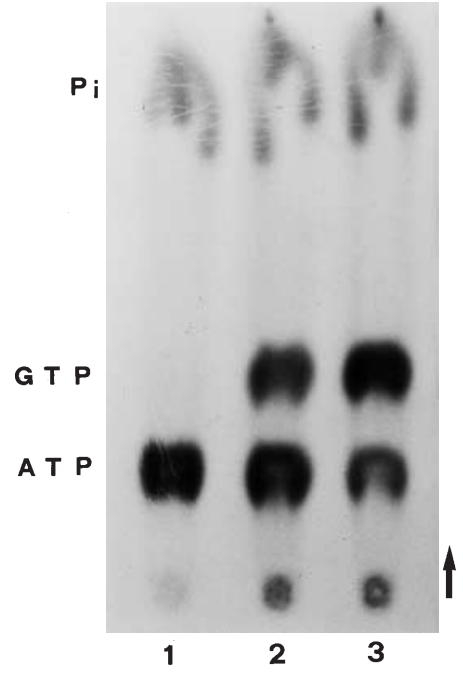

Fig. 3 Autoradiography of a TLC plate showing the reaction products of the gel-extracted catalytic subunits of NDP-kinases (*A* and *B*, see Fig. 2) with nucleoside diphosphate (GDP). Extracted catalytic subunits of NDP-kinases were incubated at pH 7.5 in the presence of 1 mM of $MgCl_2$ with (γ-^{32}P)-ATP and -GDP. *Track 1* control (GDP but no enzyme); *track 2* as *1* but with catalytic subunits of NDP-kinase (*A*); *track 3* as *2* but with catalytic subunits of NDP-kinase (*B*) (see Fig. 2). Exposure time 24 h

The LMG consisted of two protein bands: A and B (indicated by arrows in Fig. 2) with molecular weights (Mrs) of about 14 and 16 kDa. These protein bands were extracted and their nucleoside diphosphate kinase (NDP) activity was examined using nucleoside diphosphates as substrates and [γ-^{32}P]-ATP as a phosphate donor. Autoradiography of a TLC plate (Fig. 3) showing the reaction products of gel-extracted peptides A and B (Fig. 2) with nucleoside diphosphate proved that the above peptides were catalytic subunits of NDP-kinases.

Detection of DNase and RNase isozymes

The DNase pattern in the active gel polymerized in the presence of ssDNA is shown in Fig. 4A. Wheat and *Th. ponticum* showed similar DNase isozymes (Fig. 4A, tracks 2 and 4 respectively) while hybrids showed an additional strong, DNase band (Fig. 4A, tracks 1 and 3 indicated by an arrow).

Wheat and *Th. ponticum* exhibited very faint RNase isoforms in gels polymerized in the presence of RNA (Fig. 4B, tracks 2 and 4 respectively). In contrast, hybrids showed a strong RNase activity (Fig. 4B, tracks 1 and 3, indicated by an arrow) mainly in the same region as its DNase activity (see above).

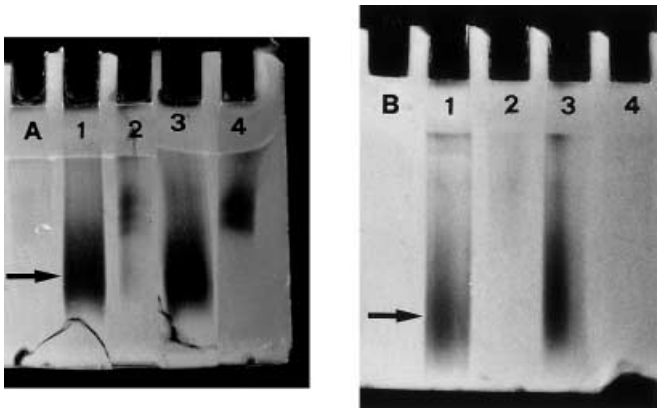

Fig. 4 Analysis of PAGE on a 9.5% gel polymerized in the presence of ssDNA (*A*) and RNA (*B*) of the DNases and RNases extracted from *Th. ponticum* (*track 4*), wheat (*track 2*) and hybrid seeds (*tracks 1, 3*). The gels were incubated at 37°C for the detection of nuclease activities (*dark bands*). The bands appeared after staining the gels with ethidium bromide. *A* 1-h incubation *B* 3-h incubation. The *arrows* indicate the position of the new nuclease

Discussion

The F_1 hybrid plants were highly sterile. This must have been the result of poor chromosomal pairing (Bai and Knott 1993) and the irregular distribution of chromosomes at anaphase-I, since it is believed that this causes sterility (Jauhar 1995). Features of the wild parent which dominated in the hybrids, such as awnlessness, plant morphology and perennial habit in growth, may be attributed to the much-higher chromosomal contribution (five genomes) from the male wheatgrass parent compared to only three genomes from the wheat parent.

There have been no data available concerning endogenous protein phosphorylation and crucial enzymes (DNases, RNases and NDP-kinases) of nuclei-acid metabolism, as biochemical markers for detecting the presence of *Th. ponticum* chromosomes in a wheat background during intergeneric hybridization between them. In similar experiments, a number of enzymes, malate dehydrogenase, grain esterase, b-amylase, a-amylase and superoxide dismutase (William and Mujeeb-Kazi 1993), as well as the electrophoretic pattern of storage proteins, gliadins (Jauhar 1995) and high-molecular-weight glutenin (William and Mujeeb-Kazi 1993) were studied.

Similarity to the endogenous strongly phosphorylated LMG protein pattern of wheat and *Th. ponticum* (Fig. 2, tracks 2 and 4 respectively) was observed in barley chromatin (Van Loon et al. 1975; Yupsanis and Vatzaki 1991) and in alfalfa seedlings (Yupsanis et al. 1994); in other words, in both monocotyledons and dicotyledons. A similar group of proteins (LMG) was also labelled by in vitro phosphorylation of soluble proteins from germinating barley (Yupsanis et al. 1989), and one of them was identified as a NDP-kinase (Georgatsos and Fisentzides 1996). As in barley, the gel-extracted LMG peptides of parental plants (wheat and *Th. ponticum*) were identified as catalytic subunits of the NDP-kinases (Fig. 3), while their hybrids revealed very low endogenous protein phosphorylation (Fig. 2, tracks 1, 3 and 5) with very low NDP-kinase activity (data not shown). The formation of a phosphorylated enzyme intermediate in NDP-kinase catalysis reaction has been known for over two decades (Parks and Agarwal 1973).

Regarding nucleolytic activity, it is well-known that an increase of nucleolytic activity was observed in germinating barley (Yupsanis and Pantazaki 1988), lentil (Kefalas and Yupsanis 1995) and alfalfa (Yupsanis et al. 1996) seeds. In all cases, nucleolytic activity is absent in the ungerminated seeds of the investigated wheat, *Th. ponticum*, and seeds of the hybrids.

Active gel-analysis revealed the appearance of a new fast-migrated nuclease (an enzyme that hydrolyzed both DNA and RNA substrates) in intergeneric hybrid seedlings (Fig. 4, indicated by arrow). On the whole, the nucleolytic profile of the hybrids was different from those of either parent, that were both similar.

According to our results, hybrid seeds gave sterile plants. Consequently, it would seem that the very low endogenous protein phosphorylation, the very low NDP-kinase activity, and the appearance of the new nuclease in hybrid seeds indicated sterile hybrids. In other words, sterile hybrids are unable to express the parental enzymes that were involved mainly in the metabolism of nucleic acids.

Acknowledgements We thank the Greek General Secretariat of Research and Technology (PENED 91 ED 891)

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