Theor Appl Genet (1983) 66:1–7

Study on nuclear and cytoplasmic genome expression in wheat by two-dimensional gel electrophoresis

1. First results on 18 alloplasmic lines

M. Zivy, H. Thiellement, D. de Vienne and J.-P. Hofmann

Laboratoire d'Amélioration des Plantes-G.I.S. Moulon, Bat. 360, Université de Paris-Sud, F-91405 Orsay, France

Received January 3, 1983
Communicated by K. Tsunewaki

Summary. In this first analysis the protein patterns obtained by two-dimensional gel electrophoresis of 8 day-old leaves from 18 alloplasmic wheat lines are compared. From 440 spots retained on the basis of their reproducibility, 36 proteins were observed to vary in different cytoplasms, allowing us to distinguish the *T. aestivum* cytoplasm from 5 *Aegilops* cytoplasms. Twenty-four of the 36 variable proteins could be structurally related to the large subunit of RuBPCase. Nuclear variation between 3 wheat varieties was observed for 14 proteins.

Key words: *Triticum aestivum* – *Aegilops* species – Alloplasmic lines – 2D gel electrophoresis – Cytoplasmic inheritance

Introduction

The ability to produce alloplasmic lines by repeated backcrosses of common wheat (*Triticum aestivum* L.) to wild and related species in the genera *Triticum* and *Aegilops* has been extensively used for phylogenetical and agronomic purposes. The most important and best studied effect is male sterility (Kihara 1940, 1951; Fukasawa 1955). Many other manifestations at the level of the phenotype of whole plants have been reported – pistilloidy (Kihara 1951), delayed heading (Fukasawa 1953), leaf variegation (Fukasawa 1953; Hori and Tsunewaki 1967), production of haploids and twins (Kihara and Tsunewaki 1962; Tsunewaki et al. 1974) and many other agronomic characters (Maan and Lucken 1970; Kofoid and Maan 1982). All these experiments have been recently reviewed (Maan 1979; Tsunewaki 1980).

Joint experiments have been performed between different laboratories and led to the classification of 75 cytoplasms from 33 *Triticum* and *Aegilops* species using alloplasmic lines of common wheat varieties (Mukai et al. 1978; Tsunewaki 1980). Phylogenetic relationships were deduced from these experiments. These were also confirmed at the molecular level using cytoplasmic (mitochondrial and chloroplastic) DNAs (Vedel et al. 1978, 1981; Ogihara and Tsunewaki 1982).

The purpose of our work is to investigate nuclear and cytoplasmic genome expression by two-dimensional (2D) gel electrophoresis of denaturated soluble proteins (O'Farrell 1975). This technique enables us to observe several hundreds of protein markers as spots on the 2D gels, throughout the life of the plant. By comparing the protein patterns obtained at different stages on different organs and by taking into account nuclear and cytoplasmic genetic variability, developmental genetic studies can be initiated. We may be able to demonstrate variation at the level and timing of protein synthesis and thus have access to regulatory genes. The same kind of approach has been very recently developed by Klose (1982) on the mouse.

In this paper, we report the results obtained by comparing 18 alloplasmic lines at one developmental stage: the 8 day-old leaf. The lines studied are 3 varieties of spring wheat ('Chinese Spring', 'Selkirk' and 'Penjamo 62') in combination with 6 cytoplasms (*Triticum aestivum, Aegilops kostchyi, Ae. squarrosa, Ae. ventricosa, Ae. cylindrica,* and *Ae. juvenalis*).

Materials and methods

Plant material

The alloplasmic lines were furnished by 3 different laboratories: the 'Selkirk' lines (Sk) by Dr. S. S. Maan of North Dakota State University, Fargo, ND, USA, the 'Chinese Spring' lines (CS) by Dr. K. Tsunewaki of Kyoto University,

Kyoto, Japan and the 'Penjamo 62' lines (Pj) by Dr. I. Panayotov of the Wheat and Sunflower Institute near General Toshevo, Bulgaria. These lines were multiplied by G. Doussinault at the Station d'Amélioration des Plantes de l'I.N.R.A., Rennes, France.

According to the cytoplasm classification commonly used *Triticum aestivum* is type B; *Ae. kostchyi* is type S^v; *Ae. ventricosa, Ae. cylindrica* and *Ae. squarrosa* are type D and *Ae. juvenalis* is type D^2 (Mukai et al. 1978; Tsunewaki 1980; Ogihara and Tsunewaki 1982). None of these cytoplasms induce drastic phenotypic change compared to *T. aestivum*. Each one is considered to be homogeneous whatever its geographical origin. The 6 cytoplasms are considered to be very similar to each other compared with other taxa in the same complex (Tsunewaki 1980). Each of the 3 lines were crossed onto each of the 6 sources of cytoplasm. Each hybrid was backcrossed at least 8 times with pollen taken from the original line, except for Pj with *Ae. squarrosa* and CS with *Ae. cylindrica*, which were backcrossed 6 and 7 times, respectively.

Seeds were allowed to germinate in Petri dishes on water imbibed filter paper and after 4 days were planted out in small pots. The first 8 day-old leaves from 3 different plantlets of the same genotype were cut in pieces and the 3 were mixed to obtain one sample. This was done at least twice for each combination providing the 36 samples compared in this paper. No delay of development was noticed at this stage between the euplasmic and alloplasmic lines.

Extraction

Proteins were extracted as follows: 100 mg of leaf pieces were dry crushed in a mortar cooled by liquid nitrogen. The powder was resuspended at 0 °C in 2 ml of 30 mM Tris-Cl^- buffer at pH 8.7 with 1 mM dithiothreitol, 1 mM ascorbic acid, 1 mM EDTA Na_2, 5 mM $MgCl_2$ and 10 mg of insoluble polyvinylpyrrolidone. This sample was centrifugated twice at 33,000 g, for 15 min and for 10 min, with the pellet drawn off after each centrifugation. Cold acetone (8 ml) with 10 mM 2-mercaptoethanol was then added to the supernatant, allowing the proteins to precipitate for 1 h at −30 °C. After 10 min of centrifugation at 33,000 g, the pellet was air-dried and resuspended in 200 µl of O'Farrell's lysis buffer (O'Farrell 1975). The samples were stored at −80 °C. Before isoelectrofocusing (IEF) they were recentrifugated at 2,500 g for 2 min. Twenty microliters of supernatant were used for the IEF.

Electrophoresis

The IEF was done according to the procedures described by O'Farrell (1975) except for the urea concentration which was 9.5 M and the Ampholine mixture (4% with 4 volumes of pH 3.5–10 and 1 volume of pH 5–7 Ampholine). The IEF gels were in the form of columns 120 mm long and 2 mm in diameter. The IEF was performed at 400 V for 16 h, then at 800 V for 1 h. Gels were equilibrated in O'Farrell's equilibration buffer (1975) for 45 min. After drying, the first dimension gels were stored at −80 °C. The second dimension electrophoresis was performed according to the method of O'Farrell (1975) in a 10–15% acrylamide exponential gradient gel.

The 2D gels were silver stained as described by Oakley et al. (1980).

Results

As shown in Fig. 1 a good resolution can be obtained by this method on leaf proteins since about 500 spots can be distinguished. The main spot, noted LS in Fig. 2, corresponds to the large subunit of ribulose bisphosphate carboxylase (RuBPCase), which is the most abundant enzyme in green tissues. The molecular weight, estimated using 3 standard proteins (bovine serum albumin, egg albumin and chymotrypsinogen from Serva), is in the range of 55,000 daltons.

From this analysis, 440 spots, schematized in Fig. 2, were retained for further study. The non-reproducible spots were eliminated from further consideration. When nuclear variation was detected between varieties it was retained only if it occurred in every cytoplasm and, in the same manner, cytoplasmic variation was retained only if it occurred in every variety. In this way, we eliminated the spots resulting from possible residual heterozygosity, especially in the case of the 2 lines with only 6 or 7 backcrosses. However, this method also ruled out any variation dependent upon specific combinations of one nuclear genotype with one cytoplasm.

Cytoplasmic variation

On the whole, 36 proteins behaved as expected for cytoplasmic gene products. The 36 proteins can be divided in 2 groups. In the first group are the large subunit of RuBPCase, which is known to be cytoplasmically coded (Kawashima and Wildman 1972; Chan and Wildman 1972; Coen et al. 1977), and 23 other proteins. In this group no differences were observed between the 5 *Aegilops* cytoplasms but all these spots shifted in the same basic direction in the *T. aestivum* cytoplasm as indicated in Fig. 2 by the solid lines. They can be found at different isoelectric points and various molecular weights. Four of them (No. 1, 2, 3 and 4) are located at higher molecular weights than the LS. Apart from their common basic shift, we can also notice a slight variation of molecular weight between corresponding spots, polypeptides from the *T. aestivum* cytoplasm being heavier than those from the 5 other cytoplasms. This is indicated in Fig. 2 and can be observed on a coelectrophoresis (Fig. 3) where the deposit was a 1/1 mixture of extracts from two 'Selkirk' lines with *T. aestivum* and *Ae. juvenalis* cytoplasm. At this stage of development, the large amount of RuBPCase precludes precise comparison of molecular weights for LS. However, at the germination stage, where RuBPCase was in a smaller amount (3 day-old etiolated shoots), molecular weight variation can also be observed for LS and for spots No. 22 and No. 23 (Zivy et al., in preparation).

The second group is constituted by 12 proteins showing another kind of variation. This variation corresponds to the presence or absence of spots and to spots varying in relative intensity depending upon the cytoplasm.

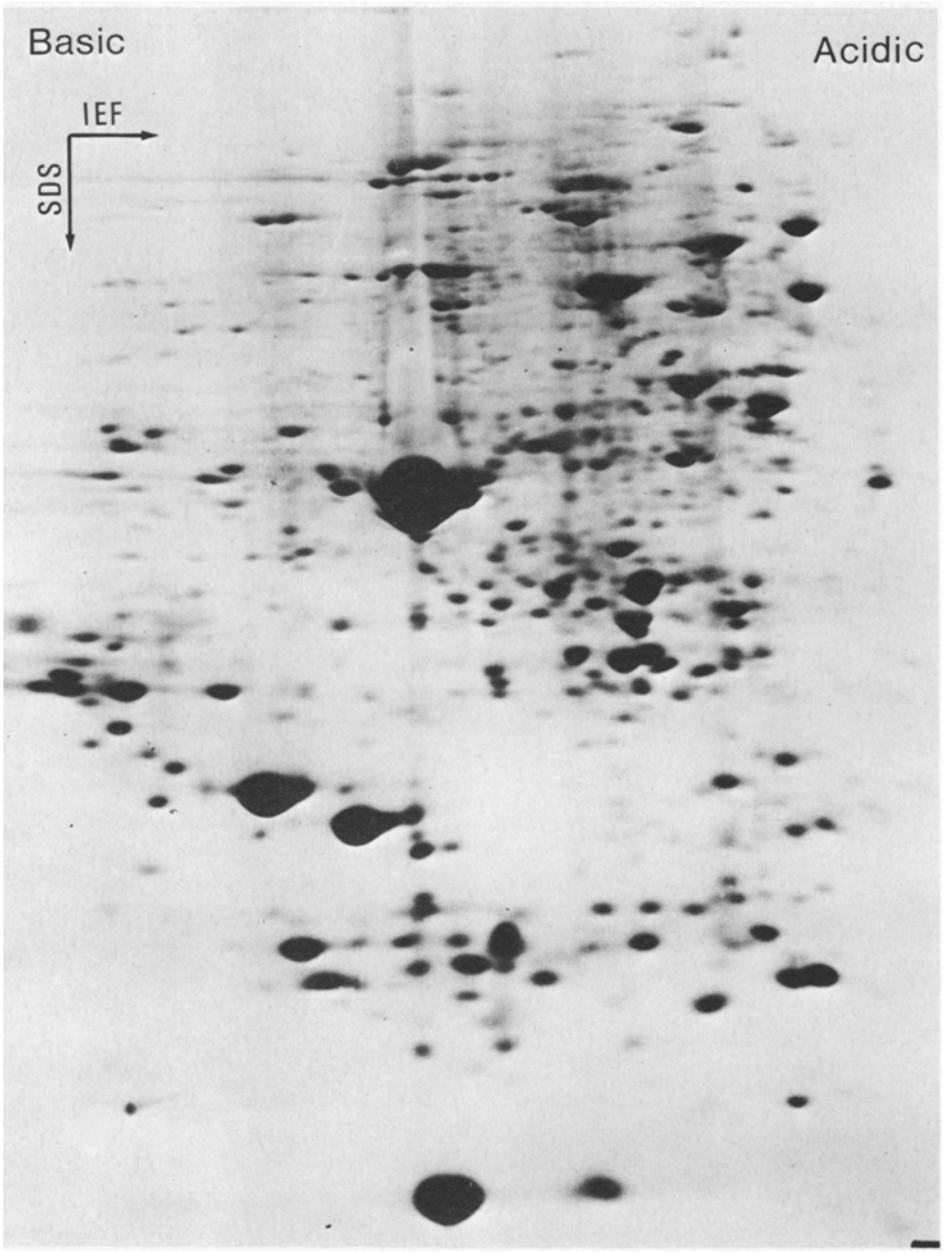

Fig. 1. Two-dimensional (2D) pattern of 8 day-old leaf proteins of 'Chinese Spring' with *Ae. ventricosca* cytoplasm. *IEF* isoelectric focusing, *SDS* electrophoresis in gel containing sodium dodecyl sulfate

In this group also only two patterns are found: one for the *T. aestivum* cytoplasm and the other for the 5 *Aegilops* cytoplasms. Nine of these 12 spots are more intense (spots No. 6, 15 and 16) or present only (spots No. 8, 9, 10, 14, 17 and 29) in *T. aestivum* cytoplasm. For the other 3, spots No. 5 and No. 7 are more intense and spot No. 30 is present only in the *Aegilops* cytoplasms.

Nuclear variation

From the 440 proteins retained for this analysis, 14 are different according to nuclear genotype. They are indicated by letters in Fig. 2. The 3 varieties can be differentiated.

For proteins C, E, G, H and I there was a slight shift between 2 varieties in the IEF horizontal dimension. This was to be expected for 2 alleles of one gene, noted 1 and 2, which differed by their charge.

The other proteins (A, B, D, F, J, K, L, M and N) are present in some varieties, and missing in others without any corresponding allelic spot. Two explanations are suggested: either the allelic form of the protein is hidden in a cluster of spots in the vicinity (this could be the case for all of them except spot B), or

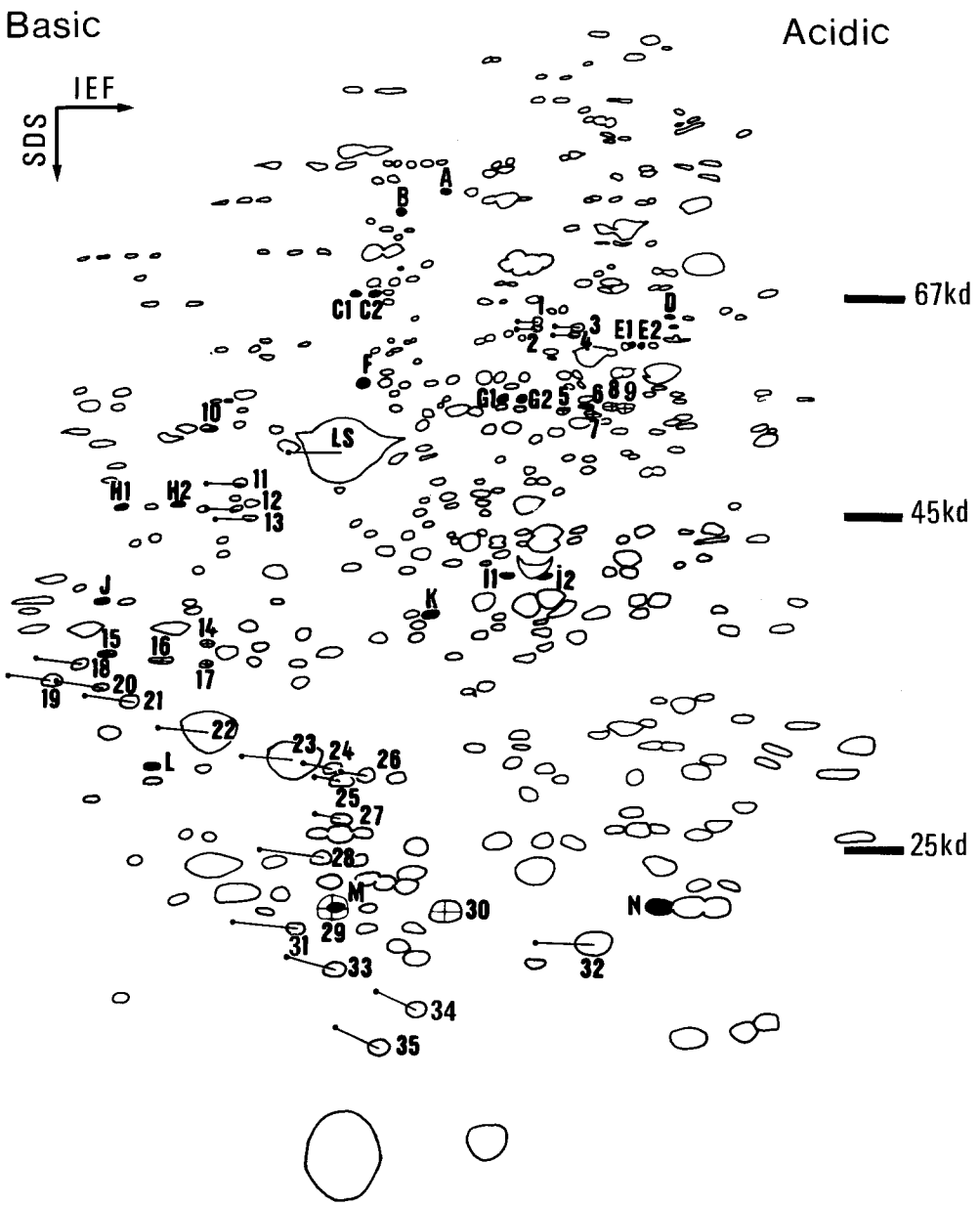

Fig. 2. Schematic representation (2D pattern) of wheat leaf proteins. *LS* large subunit of RuBPCase. Other letters are for spots varying according to the nucleus (*black spots*). Numbers are for spots varying for different cytoplasms in relative intensity (*white spots* marked by a *cross*) or in position (the displacement of the *LS* and of 23 other spots between the *Aegilops* pattern represented here and the *T. aestivum* pattern is indicated by a *solid line*). Molecular weights of 3 standard proteins are indicated of the *right*. *kd* kilodaltons

the genetic difference concerns the amount of protein synthesized (this could be the case for spot B).

The differences noticed between the 3 varieties are listed in Table 1 and illustrated by two examples in Fig. 4.

Discussion

The present analysis has led to the detection of 36 proteins as cytoplasmic markers differing by their relative intensity or by their isoelectric point. Only 2 patterns are found: one for the *T. aestivum* cytoplasm and the other common to the 5 *Aegilops* cytoplasms studied. Every cytoplasmically controlled spot behaves according to this rule. This was previously shown using IEF analysis by Hirai and Tsunewaki (1981) for the large subunit of RuBPCase. Many more markers can be revealed by 2D analysis but no distinction can yet be made between the 5 *Aegilops* cytoplasms. Two kinds of explanations can be suggested. The first is the genetical proximity of these cytoplasms as demon-

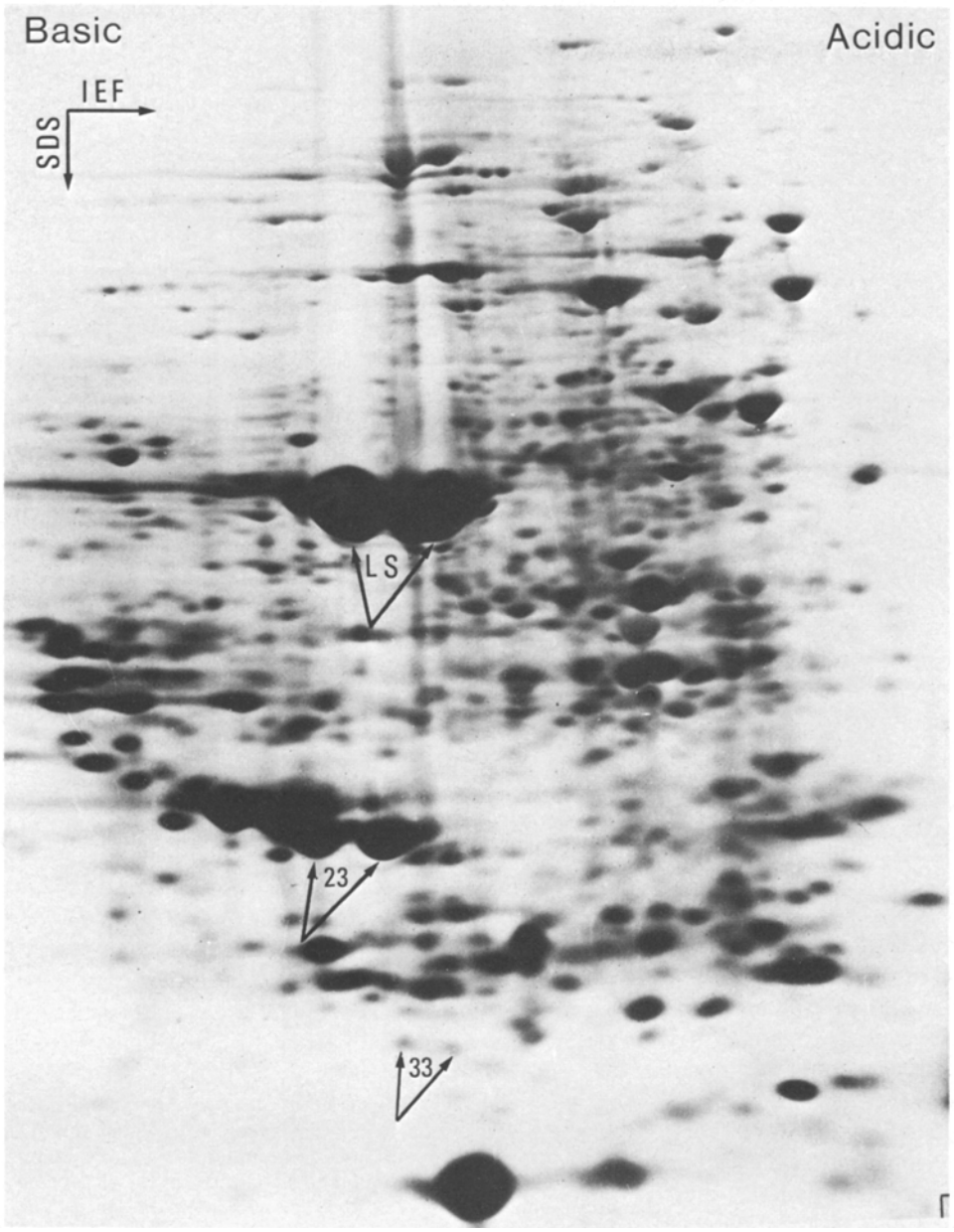

Fig. 3. Coelectrophoresis of a 1/1 mixture of 'Selkirk' lines with *T. aestivum* and *Ae. juvenalis* cytoplasms. *Arrows* some examples of double spots

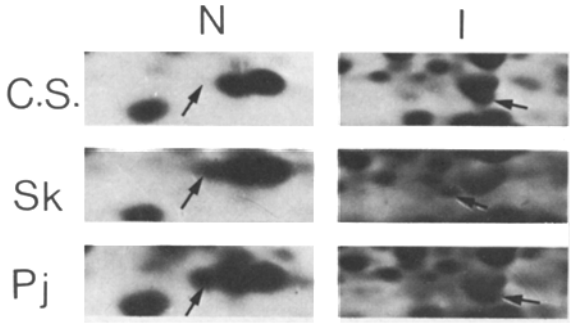

Fig. 4. Two examples of nuclear variation found in the 3 varieties. The spot N is present in 'Selkirk' (*Sk*) and 'Penjamo 62' (*Pj*) but not in 'Chinese Spring' (*CS*). Protein I shows allelic variation: spot I2 is found in *CS* and *Pj*, spot I1 in *Sk*

strated for phenotypic and agronomic characters (Tsunewaki 1980). The second is methodological: up to now we have limited our analysis to qualitative distinctions (presence/absence or displacement of spots) and we have only used quantitative distinction when very clear relative intensity differences were observed. It is thus plausible that quantitative variation exists for some spots between the *Aegilops* cytoplasms, as can be the case for specific combination of one variety with one cytoplasm, but requires further and more precise techniques to be detected.

The 24 proteins of the first group (see above) that show an identical shift between the electrophoregrams

Table 1. List of the spots differing between the 3 varieties. Spots are labelled as in Fig. 2.

Spot	'Selkirk'	'Chinese Spring'	'Penjamo 62'
A	+	–	–
B	+	+	–
C1	+	–	–
C2	–	+	+
D	+	–	–
E1	+	–	–
E2	–	+	+
F	–	+	–
G1	–	+	–
G2	+	–	+
H1	–	+	–
H2	+	–	+
I1	+	–	–
I2	–	+	+
J	+	–	–
K	+	–	+
L	+	–	–
M	+	–	+
N	+	–	+

+ and –: spot present and missing, respectively

of *T. aestivum* and the 5 *Aegilops* cytoplasms raise a few questions. If they are products of different cytoplasmic genes why should they all show an isoelectric modification in the same direction? Moreover, why should they all differ from those of *T. aestivum* without any diversity among those of the various *Aegilops* species? The simplest explanation is that all 24 proteins are different products of the same LS cytoplasmic gene, being degradation or non-terminated translation intermediaries (Coen et al. 1977). In that case a difference between *Aegilops* and *T. aestivum* in the LS gene or in the processing of the polypeptide encoded by this gene would affect the other products. The parallel change between their isoelectric point shift and their slight variation in molecular weight leads to the interpretation that the *Aegilops* polypeptides differ from the corresponding *T. aestivum* ones by their length. The addition or deletion of charged amino acids could explain the simultaneous variation in isoelectric point and in molecular weight.

If these 23 proteins are degradation products of the LS, are they due to in vivo turnover or to our experimental procedures? Biochemical investigations are presently in progress in our laboratory in order to clarify this point. We can already note that the spots No. 1, 2, 3 and 4 have higher molecular weights than the LS and thus cannot be explained in that way. They are probably not precursors of the LS since, according to Langridge (1981), precursors are 1,000 to 2,000 daltons heavier than the LS.

Among the 440 spots retained in this analysis, 14 present a variation between nuclear genotypes and allow us to recognize each variety without ambiguity. The percentage of variation is then $14/440 = 3.2\%$. This value may not be compared to the proportions of polymorphic loci obtained using 2D electrophoresis in *Drosophila melanogaster* (11%, Leigh Brown and Langley 1979; Ohnishi et al. 1982), in *Mus musculus* (4%, Racine and Langley 1979) or in man (1.6%, Comings 1979) for the following reasons: (1) we do not work on natural populations, (2) only 3 varieties were studied and (3) the proportion of cytoplasmically encoded proteins is probably higher in plants than in animals. In any case, such percentages are always approximations. First, when many spots are clustered together or when one major spot occupies a large area, we are no longer able to say how many spots there are and whether or not they show any variation. Second, several spots may be the products of one single gene. This may be the case for the 23 proteins correlated with LS. It must be noticed that RuBPCase is in a very large concentration in green tissues and even small quantities of its metabolites are likely to be stained. This would not occur for proteins which are present in lesser quantities. Anderson and Anderson (1979) and Klose (1982) have reported that in certain cases of nuclear variation, spots that are very close to each other correspond to products of single genes. This is not true for the nuclear variation observed here, since the spots are spread out all over the gel.

This study will be extended to other developmental stages and to different organs. For instance, on 2D gels obtained from 3 day-old etiolated shoots we noticed many differences from the 8 day-old leaf studied here (Zivy et al., in preparation). The way we read, analyse and compare our gels by looking at the spots one by one on the 36 gels is a long and eye-tiring method. Moreover it only allows a very rough quantitative approach. We are presently developing a reading system of the gel image based on a video camera and a microcomputer. The comparative analysis will be performed with the use of programs developed by Anderson's laboratory at the Argonne National Laboratory (Anderson et al. 1981). It is very important to obtain reliable quantitative estimations of spots since, according to Klose (1982), many genetical differences can be noticed at the level of protein concentration, i.e. at the regulation level.

Acknowledgements. We wish to acknowledge the skilful technical assistance of Germinal Delbos and Marie-Pierre Digard. We are grateful to Dr. C. Mouches and Dr. Ph. Tarroux for helpful discussions on the technique. We thank Dr. K. Tsunewaki, Dr. S. S. Maan and Dr. I. Panayotov for providing the seeds. This work was supported in part by an A.T.P. I.N.R.A. grant No. 4309: "Expression du génôme et contexte cytoplasmique."

References

Anderson NL, Anderson NJ (1979) Microheterogeneity of serum transferrin, haptoglobin and 2HS glycoprotein examined by high resolution two-dimensional electrophoresis. Biochem Biophys Res Commun 88:258–265

Anderson NL, Taylor J, Scandora AE, Coulter BP, Anderson NG (1981) The TYCHO system for computer analysis of two-dimensional gel electrophoresis patterns. Clin Chem 27:1807–1820

Chan PH, Wildman SG (1972) Chloroplast DNA codes for the primary structure of the large subunit of Fraction 1 protein. Biochim Biophys Acta 277:677–680

Coen DM, Bedbrook JR, Bogorad L, Rich A (1977) Maize chloroplast DNA fragment encoding the large subunit of ribulosebisphosphate carboxylase. Proc Natl Acad Sci USA 74:5487–5491

Comings DE (1979) Pcl Duarte, a common polymorphism of a human brain protein, and its relationship to depressive desease and multiple sclerosis. Nature 277:28–32

Fukasawa H (1953) Studies on restoration and substitution of nucleus in *Aegilotriticum*. 1. Appearance of male-sterile *durum* in substitution crosses. Cytologia 18:167–175

Fukasawa H (1955) Studies on restoration and substitution of nucleus in *Aegilotriticum*. 2. The interrelationships between *ovata* cytoplasm and fertility restoring factors. Cytologia 20:211–217

Hirai A, Tsunewaki K (1981) Genetic diversity of the cytoplasm in *Triticum* and *Aegilops*. 8. Fraction I protein of 39 cytoplasms. Genetics 99:487–493

Hori T, Tsunewaki K (1967) Study on substitution lines of several Emmer wheats having the cytoplasm of *Triticum boeoticum*. Seiken Ziho 19:55–59

Kawashima N, Wildman SG (1972) Studies on Fraction 1 protein. 4. Mode of inheritance of primary structure in relation to whether chloroplast or nuclear DNA contains the code for a chloroplast protein. Biochim Biophys Acta 262:42–49

Kihara H (1940) Anwendung der Genomanalyse für die Systematik von *Triticum* und *Aegilops*. Jpn J Genet 16: 309–320

Kihara H (1951) Substitution of nucleus and its effect on genome manifestations. Cytologia 19:177–193

Kihara H, Tsunewaki K (1962) Use of an alien cytoplasm as a new method of producing haploids. Jpn J Genet 37: 310–313

Klose J (1982) Genetic variability of soluble proteins studied by two-dimensional electrophoresis on different inbred mouse strains and on different mouse organs. J Mol Evol 18:315–328

Kofoid KD, Maan SS (1982) Agronomic and bread-making performance of fertile alloplasmic wheats. Crop Sci 22: 725–729

Langridge P (1981) Synthesis of the large subunit of spinach ribulose bisphosphate carboxylase may involve a precursor polypeptide. FEBS Lett 123:85–89

Leigh Brown AJ, Langley CH (1979) Reevaluation of genic heterozygosity in natural populations of *Drosophila melanogaster* by two-dimensional electrophoresis. Proc Natl Acad Sci USA 76:2381–2384

Maan SS (1979) Specificity of nucleo-cytoplasmic interactions in *Triticum* and *Aegilops* species (a review). Wheat Inf Serv 50:71–79

Maan SS, Lucken KA (1970) Interaction of *Triticum boeoticum* cytoplasm and genomes of *T. aestivum* and *T. durum:* restoration of male fertility and plant vigor. Euphytica 19:498–508

Mukai Y, Maan SS, Panayotov I, Tsunewaki K (1978) Comparative studies of the nucleus-cytoplasm hybrids of wheat produced by three research groups. Proc 5th Int Wheat Genet Symp 1:282–292

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021

Oakley BR, Kirsch DR, Morris R (1980) A simplified silver stain for detecting proteins in polyacrylamide gels. Anal Biochem 105:361–363

Ogihara Y, Tsunewaki K (1982) Molecular basis of the genetic diversity of the cytoplasm in *Triticum* and *Aegilops*. 1. Diversity of the chloroplast genome and its lineage revealed by the restriction pattern of ct-DNAs. Jpn J Genet 57:371–396

Ohnishi S, Leigh Brown AJ, Voelker RA, Langley CH (1982) Estimation of genetic variability in natural populations of *Drosophila simulans* by two-dimensional and starch gel electrophoresis. Genetics 100:127–136

Racine RR, Langley CH (1980) Genetic heterozygosity in a natural population of *Mus musculus* assesed using two-dimensional electrophoresis. Nature 283:855–857

Tsunewaki K (1980) Genetic diversity of the cytoplasm in *Triticum* and *Aegilops*. Jpn Soc Promotion Sci, Tokyo

Tsunewaki K, Endo TR, Mukai Y (1974) Further discovery of alien cytoplasms inducing haploids and twins in common wheat. Theor Appl Genet 45:104–109

Vedel F, Quetier F, Dosba F, Doussinault G (1978) Study of wheat phylogeny by *Eco* RI analysis of chloroplastic and mitochondrial DNAs. Plant Sci Lett 13:97–102

Vedel F, Quetier F, Cauderon Y, Dosba F, Doussinault G (1981) Studies on maternal inheritance in polyploid wheats with cytoplasmic DNAs as genetic markers. Theor Appl Genet 59:239–245