STUDY ON CHANGES OF PROTEINS, ENZYMES AND CHROMOSOME NUMBER IN REGENERATED PLANTS OF WHEAT (*TRITICUM AESTIVUM* L.)

Kh. Kiarostami and H. Ebrahimzadeh*

Department of Biology, faculty of Science, Tehran University, Tehran, Islamic Republic of Iran

Abstract

Somaclonal variation is one of the possible sources of variation in plant breeding. To evaluate the usefulness of somaclonal variation for creating variation in Iranian cultivars of wheat, proteins, enzymes and chromosome number of regenerated and seed-produced plants of wheat (Alamout, Hyrmand and Maroon) were studied. Plantlets obtained from the immature embryos and seed culture were collected and used for studying proteins, peroxidases, polyphenoloxidases and superoxidedismutases by using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and PAGE. The root tips of plantlets were used for chromosome counting. In the SDS-PAGES system, regenerated plants had 5 additional protein bands. The number of protein bands in PAGE system were less than the SDS-PAGE system but the later system represented a greater difference between seed-produced plants and regenerated plants. Peroxidase had 6 isozymes, 3 of which were similar among all plants. Nine isozymes of superoxidedismutase were found, 5 of which were common in all. Polyphenoloxidase showed no changes in regenerated plants. Cytological study of the three regenerated cultivars indicated the Maroon cultivar to possess a greater chromosome number (2n=54 or 60) than the other two (2n=6x=42). Somaclonal variation was observed in cultivars, but the nature of variation was different.

Introduction

Wheat is one of the most important products, and many investigators have attempted to produce its desirable genotypes. But their efforts are reaching a plateau, especially with respect to yield. For such crops, breeding needs alternative techniques. The recombinant DNA technology is considered as a new path for this field [8].

Keywords: Chromosomes; Embryo culture; Embryogenesis; Enzymes; Isozymes; Micropropagation

This technology requires regeneration of cells or tissue cultured *in vitro*. By using this method, variation caused by tissue culture is significant.

In spite of low variation in regenerated plants from embryogenic culture in Graminae, there is also no assurance for genetic uniformity in the regenerated

* *E-mail: ebrahimzadeh@khayam.ut.ac.ir*

somatic embryo.

Variation and unstableness of regenerated plants have been shown. The usefulness of these changes depends on selected desirable mutants, but unstableness of this variation is one of the most important problems. Scowcroft and Larkin (1981) studied the somaclonal variation induced by tissue culture for the first time. Many studies have been performed on different species [8], indicating the ability of somaclonal variation to cause genetic variation in wheat, resulting in variation of plant height, protein content, amylase amount, tolerance to aluminum and freezing conditions, change of flowering time, variation in ploidy level, decline in chromosome number and activation of transposable elements. Changes in isozyme patterns represented a wide variation. Ryan and Scowcroft analyzed β-amylase isozymes and showed five additional isozymes in regenerated plants [9].

Bapat and Rawal studied peroxidase isozymes in different developmental stages of somatic and germinated embryos and reported three peroxidase isozymes in germinated embryo of wheat, one of which was exclusively formed in the germinated embryo while the other two were found in the callus as well [2].

Phenotypic and genetical methods are common for evaluating somaclonal variation [7]. We selected electrophoresis of proteins and enzymes and chromosome number from the two above-mentioned methods and investigated changes in proteins, peroxidase, superoxidedismutase and polyphenol-oxidase isozymes and chromosome number in regenerated wheat plants and compared them with the results obtained from intact plants.

Materials and Methods

Culture of Immature Embryos and Their Regeneration

Seeds of three cultivars of bread wheat (Alamout, Maroon and Hyrmand) were grown in the field of Seed and Plant Improvement Institute. Caryopses were dissected out of the spikes 10-14 days after anthesis and then slightly washed out with water and soft soap. They were later sterilized with 3% NaOCl for 15 min, and rinsed with sterile distilled water 4 times. Under sterile conditions, embryos were excised and scutellum located on MS medium with 1 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.8% agar. The obtained callus was subcultured on MS medium supplemented with 2 mg L^{-1} 2,4-D, 500 mg L^{-1} glutamine and 100 mg L^{-1} casein hydrolysate, for one month. The embryogenic calli then were transferred to hormone free MS medium under a 16 h photoperiod and 2000 Lux (light intensity) for developing embryos to whole plants.

Culture of Seeds

Seeds of the same cultivars were slightly washed out with water and soft soap, then soaked in 0.2% benomile for 5 h. After washing with water, they were sterilized with 3% NaOCl for 15 min and 0.1% $HgCl_2$ for 15 min, then rinsed 4 times with sterile distilled water, and cultured on hormone-free MS medium and incubated for a 16 h photoperiod with 2000 Lux intensity light and collected after 10 days.

Proteins and Enzymes Electrophoresis

Protein extraction. Tris-glycine buffer was used for protein extraction. The ratio of buffer to dry material was 3 v/1w. SDS-PAGE and PAGE electrophoresis techniques utilized 10% acrylamide gel and electrode buffer in PAGE system consisted of 25 mM tris, 192 mM glycine, but buffer in SDS-PAGE system was supplemented with 1% SDS [5,9].

The reaction mixtures used for visualization of enzyme activities in the gel were as follows:

Peroxidases. 0.2 M benzidine in 50° methanol, 14.7% H_2O_2 in 0.2 M acetate buffer (pH=5). 72, 90 and 36 ml were used, respectively [10].

Polyphenoloxidases. 0.2 M phosphate buffer (pH=6.8) 100 ml, 73.5% $CaCl_2$. $2H_2O$ 104 ml, 0.5% L-dopa 20 ml, gel was kept in this solution for 1 h [10].

Superoxidedismutases. The gel was first immersed in a solution that consisted of 50 mM potassium phosphate buffer (pH=7.8), 0.03 mM riboflavin, 1.25 mM NBT, 326% v/w (TEMED) for 30 min, and then illuminated on light box for 20 min [4].

For protein determination, the Bradford method was utilized [3] and the gel was loaded with an equivalent protein of each extract.

Proteins. SDS-PAGE and PAGE gels stained with coomassi blue R-250 [5,9].

Determination of chromosome number. The root tips of the regenerated plants were cut and soaked in 0.1% v/w colchicine solution for 2 h at room temperature. They were then fixed in acetic acid: ethanol (1:3) for overnight and stored in 70% ethanol. For staining, the tips were put in 0.5% aceto-carmin solution for several days and then hydrolyzed with 1 N HCl at 60°C for 3-5 min [1]. Finally, the tips were squashed by using acetic acid-glycerol (10:1). Chromosomes were counted under light microscope.

Results

Study of Protein and Enzyme Changes in SDS-PAGE and PAGE System

In SDS-PAGE system investigation on proteins in

leaves of the regenerated and seed plants showed presence of 54 protein bands (Fig. 1) most of which were similar in all plants. Bands 1,2 (MW* more than 66000) were absent in leaves of regenerated plants. Bands 15,16 and 18 (MW about 34000, 33000 and 24000, respectively) were found in leaves of regenerated plants, bands 31,32 (MW about 34000, 33000 and 24000, respectively) were observed in leaves of plants of Alamout cultivar.

In the PAGE system, determination of proteins in leaves of regenerated and seed plants showed 31 protein bands (Fig. 2) some of which were different in both plants. Band 11 (MW about 46000) was absent in regenerated and seed plants of Maroon cv., bands 12,13 (MW about 45000 and 40000, respectively) were absent in seed plants of Maroon cv. Band 14 (MW about 36000) was absent in seed plants of Alamout cv. Band 15 (MW about 33000) was absent in seed plants of Hyrmand cv., and band 19 (MW about 24000) was absent in regenerated and seed plants of Maroon cv.

Peroxidases. Six isozymes of peroxidase have been shown in regenerated and seed plants (Fig. 3). Isozymes 1,2,3 were observed in all plants, isozyme 4 found in regenerated plants of Hyrmand cultivar and isozymes 5 and 6 found in regenerated plant of Alamout and Hyrmand cultivars and seed plants of Maroon and Hyrmand cultivars.

Polyphenoloxidases. Nine isozymes of polyphenol-oxidase were found in the plants studied (Fig. 5) showing no difference among them.

Superoxidedismutases. Nine isozymes of superoxide-dismutase existed in the leaves of regenerated and seed plants of wheat (Fig. 5). Isozymes 1,2,5,7 and 9 were found in the leaves of all plants. Isozyme 3 was absent in regenerated plants of Maroon cv., and isozymes 4 and 6 were absent in regenerated plants of Alamout cv. Isozyme 8 was observed only in regenerated plants of Alamout cv.

Determination of chromosome number. Investigation on chromosome number of regenerated plants of Alamout and Hyrmand showed normal chromosome number (2n=6x=42), while in regenerated plants of Maroon cv., the chromosome number was increased to 54 (85% of cells) or 60 (10% of cells).

Conclusion

The results obtained by utilizing the SDS-PAGE system showed that leaves of regenerated and seed plants, both had 54 protein bands, most of which were similar; regenerated plants had 5 additional bands.

In the PAGE system, although the protein bands

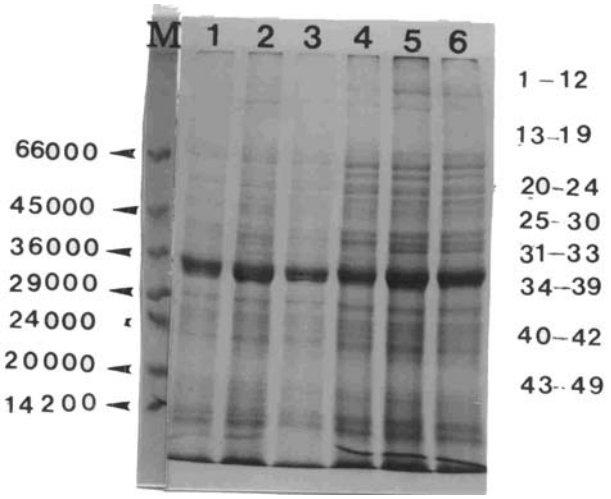

Figure 1. Electrophoretic pattern of leaf proteins of regenerated and seed plants of wheat in SDS-PAGE system. Lanes 1,2,3: Leaves of regenerated plants of Alamout, Maroon and Hyrmand cv. Lanes 4,5,6: Leaves of seed plants of Alamout, Maroon and Hyrmand cultivars, respectively (M: Marker; B: Bands number).

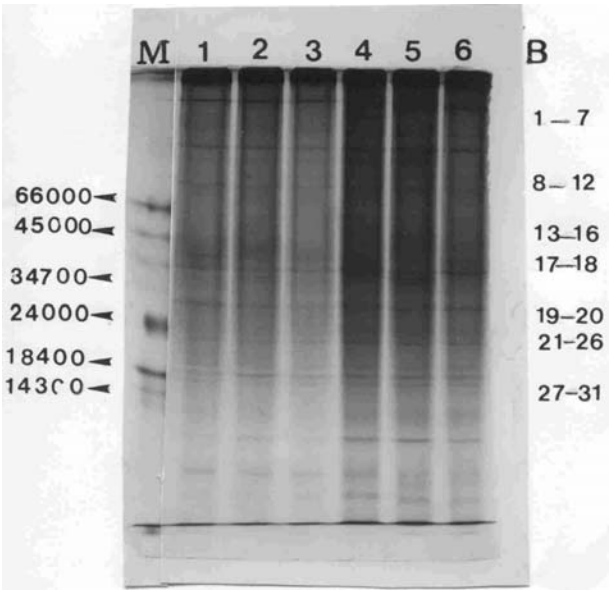

Figure 2. Electrophoretic pattern of leaf proteins of regenerated and seed plants of wheat in the PAGE system. Lanes 1,2,3: Leaves of regenerated plants of Alamout, Maroon and Hyrmand cv. Lanes 4,5,6: Leaves of seed plants of Alamout, Maroon and Hyrmand cultivars, respectively (M: Marker; B: Bands number).

* Molecular Weight

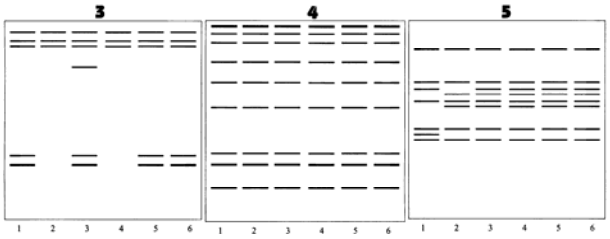

Figure 3, 4 and 5. Electrophoretic patterns of peroxides, polyphenoloxidase and superoxidedismutase isozymes, respectively. Lanes 1,2,3: Leaves of regenerated plants of Alamout, Maroon and Hyrmand cultivars and Lanes 4,5,6: Leaves of seed plants of Alamout, Maroon and Hyrmand cultivars, respectively.

obtained were less than the SDS-PAGE, a greater difference was observed between the leaves of regenerated and those of seed plants.

There were 6 isozymes of peroxidases in the leaves of regenerated and seed plants of wheat, three of which were similar between regenerated and seed plants of the three cultivars. In regenerated plants of Hyrmand cv. one additional isozymes was observed. Two isozymes were absent in the regenerated plants of Maroon cultivar.

Nine isozymes of polyphenoloxidases were observed in all the materials under study. Therefore, polyphenoloxidase did not indicate any variation.

Nine isozymes of superoxidedismutase were observed, 5 of which were common in all. In regenerated plants of Alamout and Maroon cultivars, the number of isozymes was decreased. Isozyme 8 was only observed in regenerated plants of Alamout cv. In Hyrmand cv. no difference was observed between seed plants and regenerated plants. Superoxidedismutase had no variation between seed plants of these cultivars but in regenerated plants of Alamout and Maroon cultivars somaclonal variation was observed.

There was no report of any isozyme changes in regenerated plants of wheat, but 5 additional β-amylases have been reported in plants obtained from tissue culture [8]. In germinated embryos of wheat, 3 isozymes of peroxidase were reported, one of which specifically belonged to germinated embryos [2].

In regenerated plants of Alamout and Hyrmand cultivars, the chromosome number did not differ but in Maroon, it increased to 54 or 60. There was a report of declined chromosome number in suspension cultures of wheat [8].

2,4-D is usually used for induction of somatic embryogenesis. When used in high concentration, it was able to affect chromosomes [6]. So the changes of chromosome number in regenerated plants of Maroon cv. was expected. We can also attribute the changes of proteins and isozymes patterns to somaclonal variation, especially in Maroon cultivar. The difference observed between isozyme patterns in the three cultivars are attributed to genotype. Species and their genotypes may show completely different variations after regeneration. For example, variation of regenerated plants of the Mexican wheat belong to yaqui 50 E line are more than that of British cultivar; some maize lines on the medium are more unstable from others [7]. These variations have previously been reported and not unexpected. They can be referred to somaclonal variation.

References

1. Ahmed, K. and Sagi, F. Culture of fertile plant regeneration from regenerable embryogenic suspension cell–derived protoplasts of wheat (*Triticum aestivum* L.), *Plant Cell Reports*, **12**, 175-179, (1993).
2. Bapat, S. A., Rawal, S. K. and Mascarenhas, A. F. Isozyme profiles during ontogeny of somatic embryos in wheat (*Triticum aestivum* L.), *Plant Science*, **82**, 235-242, (1992).
3. Bradford, M. A rapid and sensitive method for the quantification of microgram quantities in utilizing the principle of protein-dye binding, *Analytical Biochemistry*, **72**, 254-284, (1976).
4. Constantine, N. and Stanley, K. Superoxidedismutase occurrence in higher plants, *Plant Physiol.*, **59**, 309-314, (1977).
5. Hames, B. D. and Rickwood, D. *Gel electrophoresis of proteins, A practical approach*, 2nd Ed., 383 pp., Oxford University Press, (1990).
6. Karp, A. *Chromosome variation in regenerated plants, in genetic manipulation in plant breeding*, Walter de Gruyter and Co., pp. 547-556, (1986).
7. Lindsey, K. and Jones, M. G. K. *Plant biotechnology in agriculture*, John Wiley and Sons, England, Open University Press, pp. 78-94, (1989).
8. Maheshwari, N., Rajyalakshmi, K., Bawejak, K., Dhris, K., Chowdhry, N. and Maheshwari S. C. In vitro culture of wheat and genetic transformation, retrospect and prospect, (Critical Review), *Plant Sciences*, **14**, 149-178, (1995).
9. Smith, B. J. SDS-Polyacrylamide gel electrophoresis of proteins in method biology, *Hamana Press*, **1**, 41-56, (1984).
10. van Loon, L. C. Tobacco polyphenoloxidase, A specific staining method indicating non-identity with peroxidase, *Phytochemistry*, **10**, 503-597, (1971).