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Heritable somaclonal variation in gliadin proteins of wheat plants derived from immature embryo callus culture**

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Summary. Fertile R_0 plants of the winter wheat line ND7532 (*Triticum aestivum L.*) were regenerated from callus tissue after 60–190 days in culture. Seeds produced from these self-pollinated plants were planted in the field. Of the 5586 R_1 plants, 32 differed for one or more agronomic traits from plants not passed through tissue culture process. Gliadin electrophoregrams were prepared from bulk samples of R_2 seed from these 32 plants. Four of the 32 produced gliadin patterns different from controls, so 12 seeds of each of these four lines were examined individually. Three of the four mutant lines were fixed for the presence of a mutant protein of 50 relative mobility units (RMU) and the corresponding loss of a parental protein of 26 RMU. The remaining line segregated for the presence/absence of band 50 and the corresponding loss/retention of band 26. The mutant protein of 50 RMU was never seen in control plants. This indicated that either band 50 was coded for by a mutant gene allelic to the gene that coded for band 26 or that bands 26 and 50 were coded for by two different structural alleles under the control of a common regulatory locus. Each of the 12 seeds from the four mutant lines contained a prominent protein band at 30 (RMU), which was only observed as a faint band in one control seed. The types of variation in gliadin patterns observed in somaclones of ND7532 were similar to those reported for the line 'Yaqui 50E', except that, gliadin changes occurred less frequently in ND7532.

Key words: Tissue culture – Electrophoresis – Storage proteins – Mutation – *Triticum aestivum*

Introduction

Callus cultures of wheat (*Triticum aestivum* L.) can be established by explanting various plant parts onto artificial media. Immature embryos of wheat are the most efficient sources of tissue for regenerating whole plants in large numbers (Shimada 1978; Shimada and Yamada 1979; Gosh-Wakerle et al. 1979; Sears and Deckert 1982).

Many whole plants can be regenerated from a single callus using this technique. The majority of these regenerated plants are indistinguishable from sexually derived plants of the same genotype. However, some regenerated plants show somaclonal variation for one or more morphological and/or biochemical traits. The variant trait(s) are frequently inherited by the progeny of the primary regenerates. This has been termed heritable somaclonal variation (Larkin and Scowcroft 1981; Sears and Deckert 1982; Larkin et al. 1984). These variations are apparently the result of genetic changes that occur during the time the calli are in culture. Somaclonal variation has been observed in many cereals: barley (Orton 1980); maize (Edallo et al. 1981); rice (Shaeffer 1982); oats (McCoy et al. 1982); triticale (Nakamura and Keller 1982) and wheat (Larkin et al. 1984). Some traits of agronomic importance frequently demonstrate heritable somaclonal variation. Among these are height, maturity, and tiller number (which may effect yield), seed color (which will affect marketing classes), variation in enzymes (which may effect physiological processes), and variation in storage proteins (which may effect end-use properties).

Gliadins are wheat storage proteins (prolamins), which are insoluble in water and dilute salt solutions but are soluble in 70% ethanol (Osborn 1907). Chemically, gliadins are charac-

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terized by high contents of glutamine and proline and low contents of basic amino acids, especially lysine (Kasarda et al. 1976). The genes that code for gliadin proteins are located in tight linkage groups on the shortarms of homoeologous chromosomes 1 and 6 (Shepard 1968; Wrigley and Shepard 1973). Gliadins are excellent biochemical markers for assessing the genetic constitutions of wheat plants for the following reasons: 1) they are insensitive to environmental variation; 2) they show codominant inheritance, so mutant genes are expressed in heterozygotes; 3) they are selectively neutral, so mutant types generally are not eliminated; 4) most wheats produce 25–30 distinct gliadin bands with electrophoresis, so many genotypes can be discriminated easily. Aspects of the biochemistry and genetics of gliadins are reviewed by Mecham et al. (1978); Garcia-Olmedo et al. (1982) and Porceddu et al. (1983).

Heritable variations in gliadin proteins of plants derived from somaclones of the line 'Yaqui 50E' were reported by Larkin et al. (1984). Among the changes were deletions of specific proteins, appearances of new proteins, and different quantities of specific proteins relative to the parent line. A mean of 3.0 changes per individual R_2 plant was observed.

There is considerable diversity in the response of different genotypes to tissue culture. Sears and Deckert (1982) identified a highly inbred line (ND7532) that was very amenable to having its immature embryos cultured. We have successfully regenerated numerous plants of this line and have evaluated their progeny for several agronomic traits over several sexual cycles.

The objectives of this study were to determine whether heritable variation of gliadins occurred in plants derived from somaclones of the line ND7532 and, if so, to compare the types and frequencies of such changes with those found in 'Yaqui 50E'.

Materials and methods

Plant materials

Self pollinated inflorescences of the hexaploid wheat line, ND7532, were used as the source of immature embryos for callus initiation for tissue-culture-derived plants, and as pure seed for control plants. ND7532 is a highly inbred line, derived from the cross 'Froid'/'Centurk'. The inbred condition and varietal purity of the embryo donor plants and the pure seed source for controls was confirmed by both bulk and single seed gliadin electrophoretic patterns. Gliadin patterns from bulk extracts of 33 somaclone lines (Table 1) were determined. Four somaclone lines of ND7532 were examined in greater detail with single-seed analysis. Three of the four lines were derived from a single R_0 plant (GH80-1131) and the remaining line was derived from another R_0 plant (GH80-1130). The four lines were designated as 1083-1, 1083-3, 1083-7, and 1082-9, respectively. The mutant gliadin phenotypes, in at least three (and probably all four) of the lines are thus apparently the result of the same mutational event in a common callus progenitor.

Nomenclature

The nomenclature system proposed by Chaleff (1981) is used to describe the various generations of tissue-culture derived plants and seeds in this study:

R_0 plants – Primary regenerate plants from tissue culture.
R_1 seed – Seed produced by a selfed pollinated R_0 plant.
R_1 plants – Plants grown from R_1 seed.
R_2 seed – Seed produced by a self pollinated R_1 plant.
Controls – Plants and seed derived from the same source of ND7532 used for immature embryo explants, but having passed through the tissue culture process.

Culture and regeneration

Immature embryos of ND7532 wheat were explanted onto a modified MS medium (Murashige and Skoog 1962) to induce callus formation according to the method of Sears and Deckert (1982). Fertile R_0 plants were regenerated from calli after 60, 90, 120, 150, and 190 days in culture, and grown to maturity in the greenhouse during the spring of 1981. Control seeds of ND7532 and R_1 seeds from ND7532 somaclones were planted on 30 cm centers in the field in the fall of 1981. The R_1 plants were compared to control plants for height, heading date, grain yield, and other agronomic traits. Of the 5586 R_1 plants grown, 32 were markedly different from control plants for one or more of the traits evaluated (Sears et al. 1982). Table 1 lists the duration of the culture period and the agronomic distinctions of these 32 ND7532 somaclones. The R_2 seed produced by each R_1 plant was harvested and bulked on an individual-plant basis prior to gliadin extraction.

Gliadin extraction and electrophoresis

Bulk analysis. Gliadins were extracted with 70% ethanol from 1 g bulk samples of ground R_2 seed from each of the 32 plants previously identified as distinct from the parent line ND7532. The gliadins were separated by polyacrylamide gel electrophoresis (PAGE) using the methods of Lookhart et al. (1982), except that electrophoresis was performed in an EC-490 vertical gel apparatus at 10 °C for 7 h using a constant voltage of 300 vdc. Gliadins extracted from ground ND7532 control seed were electrophoresed in four of the 16 sample slots of each gel as controls. Following electrophoresis, the gels were fixed overnight in 12% trichloroacetic acid. The fixed gels were stained with Coomassie Blue-R 250, destained, and photographed using the methods of Lookhart et al. (1982). Photographs were scanned with a Schoeffel densitometer and the data were analyzed with the computer program described by Lookhart et al. (1983). Gliadins of the cultivar 'Marquis' were used as an internal standard for determining the relative mobilities of the protein bands in the bulk samples (Zillman and Bushuk 1979). Four of the 32 lines examined in the bulk analysis had a densely staining protein band with the same mobility as the reference band of 'Marquis', which has arbitrarily been assigned a value of 50 relative mobility units (RMU). This coincidence permitted us to delete the 'Marquis' internal standard from subsequent gels, as we could determine the mobilities of other protein bands relative to the 50 RMU reference bands of the four ND7532 somaclones.

Single-seed analysis. Four of the 32 bulked samples gave altered electrophoregrams. Twelve individual seeds from each of these four lines were electrophoresed and analyzed, along with a total of 16 individual seeds from ND7532 controls. The embryos were needed for further study, so the seeds were cut in half and only the distal (brush) half of each seed was ground with a mortar and pestle and extracted with 200 µl of 70% ethanol (1 h at 24 °C with sonification). The resulting suspension was centrifuged at 4,000 × g for 10 min. The supernate was lyophilized, then redissolved in 200 µl of 4.2 mM aluminium-lactate buffer, pH 3.1. After centrifuging as before, a 20 µl aliquot was removed and the protein concentration was determined with a BioRadTM[1] protein assay (Bradford 1976).

[1] Mention of firm names or trade products does not constitute endorsement by the U.S. Department of Agriculture over others not mentioned

Table 1. Length of culture period, heading date, height and grain yield for 32 selected R_2 somaclones of ND7532

Days in culture	R_0 plant no.	R_1 plant no.	Heading[a] date	Height (cm)	Yield (g/plant)
60	GH80-1026	2156-bulk	–	–	–
	GH80-1060	1024-6	25	119	29.2
	GH80-1100	1053-bulk	–	–	–
90	GH80-1110	1062-1	28	114	25.7
	GH80-1110	1062-3	26	115	23.7
	GH80-1110	2090-3	26	108	25.2
	GH80-1130	1082-9	23	104	22.0[b]
	GH80-1131	1083-1	31	114	25.3[b]
	GH80-1131	1083-3	28	118	29.9[b]
	GH80-1131	1083-7	24	112	23.5[b]
	GH80-1137	2027-3	24	111	40.6
	GH80-1164	3083-8	28	99	5.6
	GH80-1169	1115-6	28	108	12.2
	GH80-1169	2055-10	26	112	27.3
	GH80-1174	2092-8	26	109	22.7
	GH80-1175	2133-1	27	102	23.7
	GH80-1175	2133-2	27	111	29.6
	GH80-1175	3133-6	26	103	22.0
120	GH80-1182	3063-5	27	66	23.2
	GH80-1198	1142-2	27	115	30.2
	GH80-1198	1142-7	27	112	22.6
	GH80-1198	2147-4	26	110	23.1
	GH80-1200	1144-2	35	56	31.1
	GH80-1200	1144-6	27	110	25.6
	GH80-1200	1144-8	28	103	27.9
	GH80-1222	1153-6	23	108	24.0
	GH80-1222	2182-9	25	113	26.8
	GH80-1234	3160-2	39	109	6.4
	GH80-1234	3160-5	27	105	6.7
	GH80-1280	1202-6	29	106	22.6
	GH80-1280	3084-3	27	107	24.7
	GH80-1280	3084-4	26	44	24.7

[a] Days to anthesis after 1 May 1982
[b] Lines with altered gliadin electrophoregrams

A 150 µl aliquot was relyophilized and then dissolved in 10% sucrose plus methyl green tracking dye to provide a final protein concentration of 100 µg/20 µl, for PAGE. A half kernel of wheat yielded enough gliadins for two to five electrophoretic separations of 100 µg each.

Results

Electrophoresis of bulked samples showed that four of the 32 lines selected on the basis of altered agronomic traits contained mutant gliadins. Single-seed analyses revealed the frequencies and distributions of the mutations within the four lines, as compared to ND7532 controls. Figure 1 is a photograph of the typical electrophoretic separation of gliadin extracts from single R_2 seeds and controls. The electrophoretic patterns of all of the single seeds examined from each of the four R_2 plants are summarized in Fig. 2. This figure was generated with a computer program, which compared the densitometer scans for each single seed examined, according to Lookhart et al. (1983). For clarity, only 5 controls are shown in Fig. 2. The electrophoretic patterns of 15 of the 16 ND7532 control seeds were identical, the remaining seed (Fig. 1, lane 1) had a faint band at 30 RMU in addition to the typical gliadin band pattern for ND7532. The faintest traces of this band were also observed in bulk extracts of ND7532 pure seed grown under a higher nitrogen regime in the greenhouse (a condition which enhances the relative amounts of higher molecular weight gliadins). All of the single seeds examined from the four somaclone lines had a prominent band at 30 RMU. All of the single seeds examined from three of the four lines (1082-9, 1083-3 and 1083-7) contained a second mutant protein band of 50 RMU and the corresponding loss of a 'parental' protein band of 26 RMU. The remaining line (1083-1) segregated 5 : 7 for the presence : absence

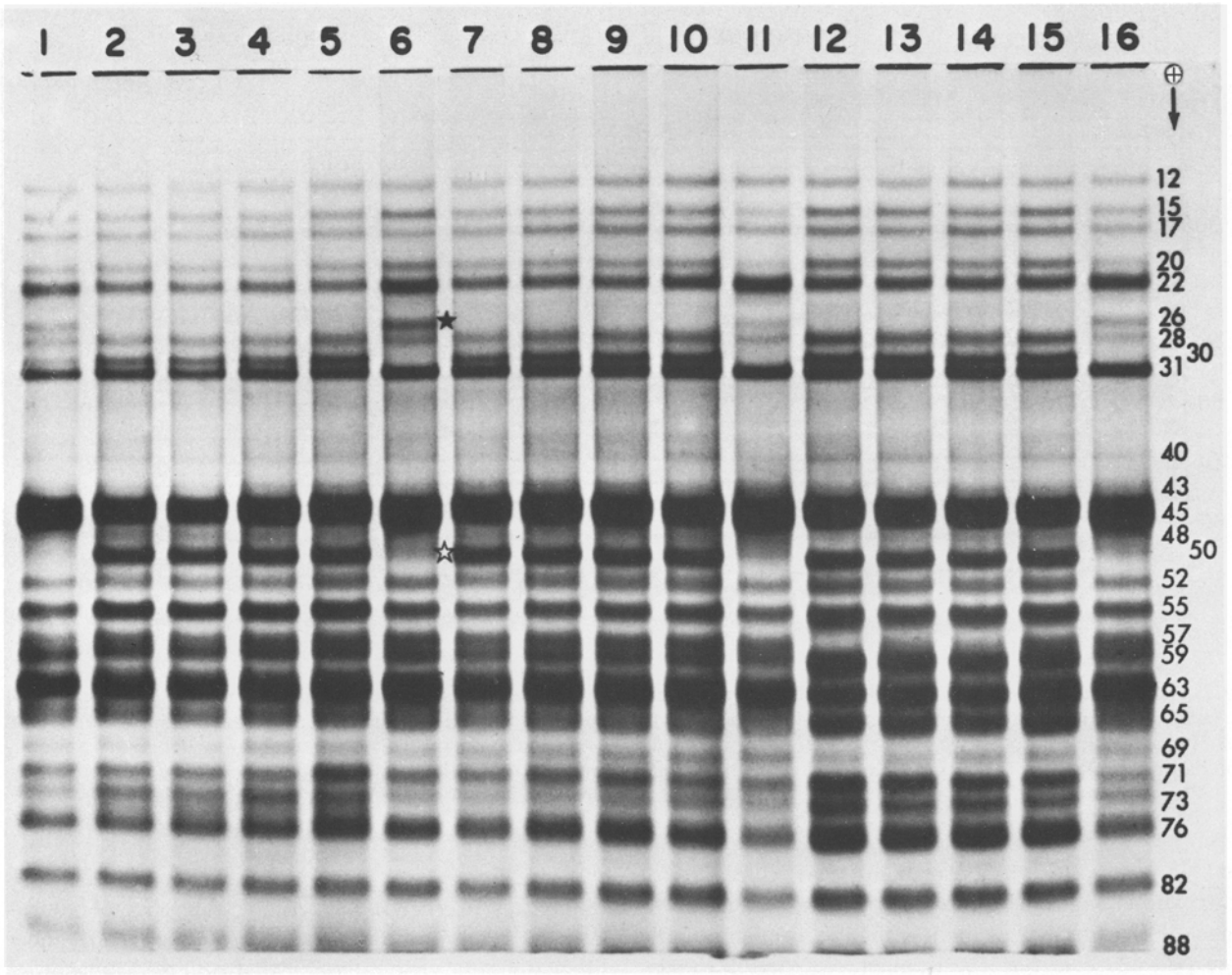

Fig. 1. Typical gliadin band patterns from single seed extracts. Relative mobilities (RMU) of the protein bands are on the right. The *arrow* shows the direction of migration. ND7532 control plants are in *lanes 1, 6, 11,* and *16. Lanes 2–5* are from somaclone 1083-3. *Lanes 7–10* are from somaclone 1083-7. *Lanes 12–15* are from somaclone 1082-9. Note the relationship between protein bands 26 (*closed star*) and 50 (*open star*). All of the somaclones contained a prominent band of 30 RMU, which was seen faintly in only one control seed (*lane 1*)

of the mutant protein of 50 RMU and the corresponding loss: retention of the parental protein of 26 RMU. The protein band of 50 RMU was never seen in single seed or bulk extracts of ND7532 controls. Some individuals within two of the four lines (1082-9 and 1083-1) either have a reduced amount of, or are completely missing, a protein of 57 RMU, but this does not appear to be correlated with the presence or absence of any other protein bands. Some of the single seeds were missing one or both of the proteins of 43 and 48 RMU. Those differences may not be significant in that both bands 43 and 48 are low density bands adjacent to the very dense band of 45 RMU, making their detection difficult.

The frequencies of changes in the gliadin bands of each individual averaged for each of the four lines are shown in Table 2. Changes involving the presence or

Table 2. Mean change[a] in gliadin pattern per individual R_2 somaclone seed

R_1 somaclone line no.	No. of band changes	
	Presence or absence only	Density changes included
1082-9	3.08	4.58
1083-1	2.50	3.00
1083-3	2.50	2.83
1083-7	2.27	2.27
Average for single seed analyses	2.59	3.17
Average including bulk analyses	0.32	0.40

[a] Mean of 12 individual seed per somaclone line. The presence of band 30 was not considered as a change

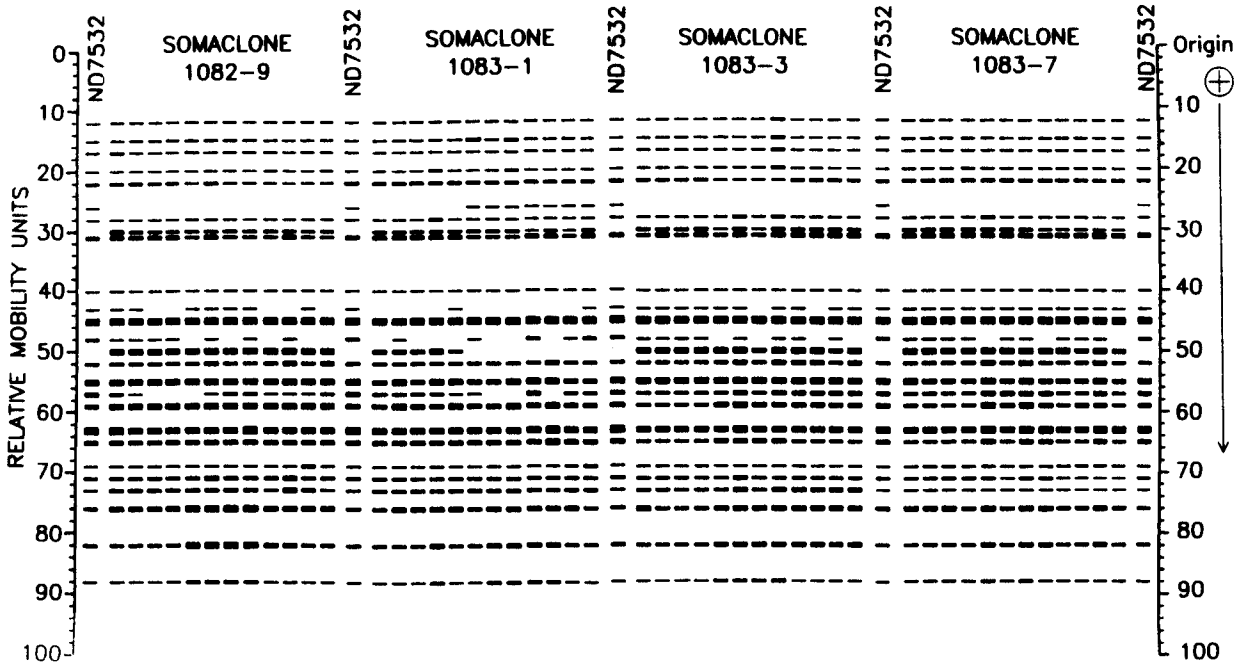

Fig. 2. A computer generated summary of the four somaclone lines examined with single seed analyses. The relative mobilities of the bands are indicated by the displacement of bars on the vertical axis, and their relative densities are indicated by the bars thickness. Typical patterns for ND7352 controls are shown between the somaclone patterns. All of the somaclones contained a prominent band of 30 RMU, which was seen faintly in only one of sixteen control seeds analyzed (not shown)

total absence of a particular band were considered, along with changes in band density outside the normal range of densities associated with a particular band from ND7532 controls. The frequency of gliadin band changes per individual averaged over the four ND7532 somaclones examined with single seed analysis was 3.17. When the results of the bulk analysis are considered, in which only 4 out of the 32 lines examined showed any appreciable change in gliadin pattern, a lower mean frequency of change per individual (0.40) seems more reasonable for the line ND7532 as a whole.

Discussion

Gliadin patterns of ND7532 controls were invariant, except for the rare occurrence of a faint band at 30 RMU, which is readily explained by the fact that ND7532 was never selected for gliadin uniformity during the course of its development. Band 30 is apparently a natural variant, which occurs at a low frequency within the population of ND7532. All of the single seeds examined from the four somaclone lines had a prominent band at 30 RMU, therefore, they are apparently homozygous selections derived from a naturally occuring variant for band 30 within the population of ND7532.

With regard to protein bands 26 and 50, the differences in gliadin patterns between tissue-culture-derived plants and ND7532 control plants are most likely due to a gene mutation that occurred during the tissue culture process rather than a selection of a pre-existing variant within the population of ND7532. A simultaneous occurrence of mutations at two loci is unlikely. This suggests single locus control of protein bands 26 and 50. Several observations confirm that these mutations occurred in culture.

The parental line is highly inbred and the electrophoretic patterns of bulk and single seed extracts of ND7532 controls (with the exception of a variant of 30 RMU) were always identical within the limits of detectability and of the minor variations associated with PAGE. The mutant protein of 50 RMU was found in 41 of the 48 R_2 seeds examined. The mutant protein of 50 RMU was never seen in single-seed or bulk extracts of ND7532 controls. If the 50 RMU protein band was an existing variant within the population of ND7532, the large amount of protein 50 present in some of the somaclones would have been detected in bulk extracts of ND7532 controls (as was band 30) even if it was present in only a low frequency within the population.

Gliadin genes show codominant inheritance (Mecham et al. 1978), so any outcrosses to other cultivars

would be readily detected by the complex additive gliadin patterns. The electrophoretic patterns of the tissue-culture-derived plants are similar enough to the controls to rule out any outcrosses with pollen from other sources.

Root-tip chromosome counts of R_3 seedlings indicated that the somatic number of all four tissue-culture-derived lines was 42, so aneuploidy was not the source of the somaclonal variation observed.

The segregation of line 1083-1 for proteins 26 and 50 cannot be accounted for by the simple clonal propagation of a pure line, unless a mutation (somaclonal variation) occurred during the culture period. The frequency of variation observed was much too great to be attributed to whole plant mutations that might have occurred after the callus culture period. The variation among the four lines for mean changes per individual can not be attributed to batch effects as noted by Larkin et al. (1984), because the four were all cultured simultaneously.

Finally, the differences in gliadins between somaclones and ND7532 control plants were not due to environment, because both were grown in the same environment and gliadins are relatively stable to environmental variation. Because the gliadin patterns of the four lines were generally similar, and the lines were derived from a common source, it is reasonable to infer that the mutational event(s) that produced the mutant phenotypes occurred sometime early in the culture period, prior to the derivation of the lines.

The allelic nature of the proteins at 26 and 50 RMU is of particular interest. In the gliadin patterns of all of the single seeds from the four somaclones, as well as the ND7532 controls (64 seeds total), either band 26 or 50 was present but both were never present in the same individual. There are at least two possible explanations for these results. First, the protein at 50 RMU may be the result of a mutation within the structural gene that codes for protein 26, producing an altered transcript that now codes for protein 50. The difference in electrophoretic mobility between bands 26 and 50 could be due to either a shortened transcript, or changes in the primary amino acid sequence that results in a protein with greater net charge, and/or a more compactly folded secondary structure. Second, there could be a mutation in a regulatory gene (perhaps on chromosome 2A as described by Brown and Flavell 1981) which represses the synthesis of protein band 26 and promotes synthesis of protein band 50. Compared to band 26, band 50 is present in greater quantity. This is consistent with both of the above hypotheses. If the mutation was in the structural gene for protein 26, the resulting m-RNA transcript could be produced in greater quantity, have a faster turn-over rate during translation, or have a reduced sensitivity to enzymatic degradation than the normal transcripts. Because gliadin m-RNA's have relatively long lifetimes, and a direct relationship existed between gliadin m-RNA levels and gliadin synthesis, Greene (1983) concluded that control of gliadin gene expression was primarily at the level of m-RNA transcription. If the mutation was in a regulatory gene that controls the production of proteins 26 and 50, the normally latent structural gene responsible for protein 50 could have been present in a higher dosage in the triploid endosperm than the structural gene for protein 26, or the regulation itself could have been altered.

Peptide mapping of purified proteins 26 and 50 with HPLC or two-dimensional electrophoresis would show whether there is any homology between the two proteins. Considerable homology would favor the hypothesis that a mutation occurred in the structural gene for protein 26, and a lack of homology would favor the regulatory gene theory.

In summary, the types of variation that we observed in the electrophoretic patterns of gliadins from somaclones of ND7532 confirm the results reported by Larkin et al. (1984) using the line 'Yaqui 50E'. The frequency of variation for gliadins in the four selected somaclones of ND7532 was similar to that reported for 'Yaqui 50E'. However, the frequency of somaclonal variation for gliadin proteins in ND7532, as a whole, was less than that reported for the line 'Yaqui 50E'. Based on the phenotypic distributions of electrophoretic variants in the R_1 progeny plants, three distinct classes of variation were apparently present in the R_0 plants: 1) a homozygous mutation for a protein band of 30 RMU, which is most likley a selection of an existing variant within the population of ND7532, and not due to somaclonal variation; 2) a heterozygous mutation for the simultaneous loss of a parental protein band of 26 RMU and the addition of a new protein band of 50 RMU, which apparently occurred during the tissue culture process; and 3) heterozygous mutations for the loss and/or significant reduction in protein band 57 and possibly in bands 43 and 48.

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