Control of Wheat Protein Biosynthesis\textsuperscript{1}

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ABSTRACT

Gliadins and glutenins, the most abundant proteins of the wheat seed, are important (as storage proteins) to the nutrition of young wheat seedlings, and (as gluten proteins) to the quality of flour-based foods. The rate of synthesis and deposition of these proteins changes dramatically during a transition period early in the development of the wheat seed, from a minor fraction of total proteins synthesized in very young seeds, to a major fraction in seeds during middle and later stages of development. The rates of storage protein synthesis in developing wheat seeds change in direct proportion to the amounts and rate of accumulation of seed messenger RNA, and control of the gene expression may be primarily transcriptional. Synthesis of gliadins can be observed as early as six days after flowering, and the expression of these genes appears to be coordinately controlled. Analysis of populations of moderate-to-abundant messenger RNA indicate that species present in seeds at 16 days after flowering were also present at seven days after flowering, although not in the same relative proportions. This indicates that the corresponding gene population is activated by seven days after flowering, and that subsequent differences observed in the protein population may result from quantitative variations in control of the expression of the genes. Gliadin genes are present in multiple copies in the wheat genome, and genes coding for proteins of the A-gliadin complex are present in different numbers in the cultivars Cheyenne (hard red winter) and Yamhill (soft white winter). The nucleotide sequence of an A-gliadin gene isolated from a genomic clone library has been determined, and the coding region shown to be free of introns. This gliadin gene contains all the consensus regulatory sequences of eukaryotic genes and is probably functional in the endosperm cell.

Key words: Gene expression, Gene structure, Protein biosynthesis, Recombinant DNA

Traditional approaches to the genetic improvement of wheat generally utilize a series of crosses and backcrosses to transfer desirable genes, either between different wheat cultivars or to wheat from its grass relatives (Smith and Heyne 1967). Such approaches are responsible for the high-yielding, disease-resistant wheats available today. Because the economic and nutritional importance of wheat is due primarily to proteins, especially gluten proteins that give wheat flour the unique ability to form doughs, the maintenance or enhancement of dough-forming characteristics is an integral goal of these improvement efforts. With the development of recombinant DNA technologies, there exists at least the potential for making specific genetic improvements in wheat through direct molecular manipulations or alterations of its DNA. Possible goals of such manipulations might be, for example, increases in proportions of selected grain protein components or of total grain protein. These molecular approaches currently have potential rather than actual impact on the genetic improvement of wheat, however, because wheat is monocotyledonous, and gene transfer systems have not yet been developed for this class of plants. The possibility now exists, however, that the Agrobacterium transfer system being developed for dicotyledonous plants may also be useful for monocotyledonous ones (Hooykaas-van Slogteren et al 1984, Hernalsteens et al 1984). Tissue culture

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research (e.g., Shaeffer et al. 1979, Nabors et al. 1983) will also be a necessary element of successful gene manipulation in wheat.

In addition to work on the development of gene transfer systems, the molecular genetics and biology of wheat are now being studied in detail not possible in the recent past. These studies provide insight into mechanisms that control gene activity and help to identify targets for genetic manipulation. This report describes elements of one such study. Information on developmental aspects of grain protein biosynthesis, characteristics of the protein synthetic apparatus, and gliadin gene structure is presented and discussed in relation to findings of other investigators in examination of the control of gene expression in the developing wheat seed.

MATERIALS AND METHODS

Wheat Seeds

Triticum aestivum L. cv. Cheyenne (Cl 8885) and INIA 66R (Cl 13528) were grown in a greenhouse (Greene 1981). Heads were tagged at flowering (anthesis) and harvested at defined times afterward. They were either used immediately or frozen in liquid nitrogen and stored at −35°C until needed. Yamhill (Cl 14563) seeds were obtained from T.M. Ching, Department of Agronomic Crop Science, Oregon State University, Corvallis.

Reagents

Radiochemicals were purchased from New England Nuclear Company. Wheat germ was the generous gift of Herbert Sanguinetti, General Mills, Inc., Vallejo, CA. X-ray film (XAR) and developer (GBX) were purchased from Eastman Kodak. Phenol was redistilled and stored at −35°C until needed. Other reagents and enzymes were the highest quality commercially available. Solutions, glassware, and plastic ware were sterile when received from manufacturers or autoclaved prior to use.

Methods

Seed poly A(+) RNA was isolated as described previously (Greene 1981, Okita and Greene 1982). Single-stranded (ss) cDNA probes were prepared according to Land et al. (1983) with [γ³²P]-dCTP label. The Cheyenne genomic library was screened with the ss-cDNA probes by plaque hybridization, essentially according to Benton and Davis (1977). Bacteriophage plaques containing genes of interest were rescreened and isolated. The gliadin structural gene probe was subcloned from an A-gliadin genomic clone (Anderson et al. 1984) and labeled by Nick-translation (Maniatis et al. 1982). DNA sequencing was carried out by the dideoxy method (Sanger et al. 1977), using the M13 primer system (Messing et al. 1981). Other methods are described in Maniatis et al. (1982) or are summarized and cited in figure legends of this report.

RESULTS AND DISCUSSION

Developmental Aspects

The growth curve of a developing wheat seed, measured as dry weight increase per unit time from fertilization to senescence, consists of a period of approximately constant weight gain at a rate of 4-6% of the mature dry weight per day, preceded and followed by periods of slower growth (Gleadow et al. 1982). The period preceding that of maximal growth is characterized by a high rate of cell division and a low rate of protein accumulation. The proteins synthesized during this period are predominantly albumins and globulins in the classification of Osborne (1907) and are relatively lysine rich (Kasarda et al. 1976). In the period following that of maximal growth, the number of endosperm cell nuclei declines (Briarty et al. 1979), the rate of protein accumulation decreases, and the tissue becomes senescent. Endosperm proteins synthesized during and following the phase of maximal seed growth are predominantly prolams (gliadins) and glutelins (glutenins), by Osborne's criteria, and make up 60-70% of the total endosperm protein in the dry seed.

The time course of the change in endosperm protein composition, and its relation to the regulation of the synthesis of the proteins, have been the focus of a number of studies. Graham et al. (1962) observed that storage proteins accumulated in deposits (protein bodies) in the endosperm of developing wheat grain. Butrose (1963), Campbell et al. (1981), and Bechtel et al. (1982) reported electron micrographic observations of protein bodies in endosperm cells as early as six days after flowering (DAF), whereas studies based on electrophoresis of accumulated seed protein have generally detected the gliadin class of storage proteins 10-12 DAF (Meckham et al. 1981, Bollini et al. 1981, Greene 1983). Khan and Bushuk (1976) have described the subunit composition of gliadin in developing wheat. To examine the question of the time of appearance of storage proteins with more sensitivity, immature INIA 66R heads were labeled with [³⁵S]-leucine for 4-hr periods to reveal proteins being synthesized at specific times of seed development (Greene 1983). Analysis of total labeled seed proteins (Fig. 1) shows significant differences in the population of proteins being synthesized at 9 DAF (and earlier) and that being synthesized at 15 DAF (and later). There is a change in the pattern of gene expression at about 12 days after anthesis, with synthesis of storage proteins predominant after this time. This analysis does not clearly fix the earliest time of storage protein synthesis in the developing seed, however; nor does it reveal whether the observed changes in protein synthesis result from qualitatively or quantitatively different gene expression. That is, it does not determine whether the genes in question are turned off and on, or regulated up and down. In vitro translation of messenger RNA (mRNA) from seeds of these same ages indicated that gliadin genes were being expressed at a low level as early as 9 DAF. Examination of the gliadin fraction of [³⁵S]-leucine-labeled Cheyenne seed proteins (Fig. 2) shows clearly that they are synthesized as early as 6 DAF. The initial

Fig. 1. Protein synthesis in vivo in developing wheat kernels. INIA 66R wheat heads were harvested at different times after flowering and labeled for 4-hr periods with [³⁵S]-leucine. Embryos were removed from seeds and endosperm/testa pericarp tissues extracted with 20 mM tris-HCl (pH 6.8)/1.9% (w/v) SDS/5% (w/v) β-mercaptoethanol/5% sucrose, and extracts analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The dots identify polypeptides whose relative synthetic rates change during seed development. Arrowheads indicate major gliadin bands. From Greene (1983).
The development of wheat endosperm involves only divisions of nuclei, with cell wall formation beginning about 2 DAF (Morrison and O'Brien 1976). The evidence of Figure 2 thus indicates that gliadin genes are active shortly after cellularization of the endosperm and well in advance of the cessation of cell division in this tissue. This is consistent with the possibility that endosperm cells become competent to synthesize storage proteins individually and that the endosperm does not require a whole-tissue activation signal such as a diffusible chemical messenger.

It is thought that, after the initial cellularization of the multinucleate endosperm at about 2-3 DAF, additional endosperm cells arise from division of cells in the peripheral meristematic layer (Morrison and O'Brien 1976). Differentiated aleurome, subaleurome, and starchy endosperm cells are apparent by 6 DAF (Bechtel et al. 1982). These new endosperm cells may become committed to storage protein synthesis as a result of signals generated during the process of cell division. Another such possibility is that the commitment to storage protein synthesis is an integral part of the initial endosperm cellularization, and that newly generated cells receive commitment signals from the original cluster of endosperm cells. They might, in turn, progressively pass signals to still newer cells generated on their periphery.

The data of Figure 2 are also relevant to the question of coordinate expression of gliadin genes. All major classes of gliadins (α, β, γ, ω; see Kasarda et al. 1976) are present in the proteins of the youngest seeds examined, indicating that all the corresponding families of gliadin genes are active. This is consistent with the simultaneous activation of all gliadin genes, although still younger tissue will have to be examined to establish if this, in fact, does occur. The differences in intensities of individual protein bands in seeds of different maturities (see 15 vs. 27 DAF α/β-ratio, for example) are suggestive of time-dependent changes in relative rates of protein synthesis. The cause is not apparent but could be related to differences in individual rates of gene transcription or to differences in mRNA stability. Both mechanisms could lead to changes in individual gliadin mRNA levels and yield differences in synthesis rates of individual proteins.

In another approach to determining the basis of observed differences in protein synthesis between early and middle periods of seed development, the moderate-to-abundant mRNA populations of seeds 7 and 16 DAF were studied. Labeled single-stranded (ss)-cDNA was prepared from mRNA populations of each of the two seed ages. These were separately blot-hybridized to replica

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**Fig. 2.** Gliadin synthesis in vivo in developing wheat kernels. Cheyenne wheat heads were harvested at different times after flowering and labeled for 4-hr periods with [14C]-leucine. Endosperm/testa pericarp tissues were extracted with 1.5 M dimethylformamide and extracts analyzed by aluminum lactate polyacrylamide gel electrophoresis. Greek letters indicate gliadin mobility classes.

**Fig. 3.** Comparative analysis of gene expression in developing Cheyenne wheat seeds. Genomic clones that exhibited strong hybridization signals with single-stranded (ss) cDNA from 7 and/or 16 DAF messenger RNA (mRNA) populations were selected and screened a second time with 7 DAF ss-cDNA, 16 DAF ss-cDNA, and A-gliadin structural gene probes. E10, genomic clone corresponding to an mRNA present at a higher level at 7 DAF than at 16 DAF; B16, genomic clone corresponding to a non-A-gliadin mRNA present at both 7 and 16 DAF; B43, genomic clone corresponding to a gliadin mRNA present at both 7 and 16 DAF.
platings of a Cheyenne genomic library prepared (O. D. Anderson et al., unpublished) in bacteriophage lambda (Maniatis et al. 1982). Three potential classes of positive hybridization signals were sought:

1) Those that indicated the expression of a specific gene only in the population of 7 DAF mRNAs, 2) those that indicated the expression of a gene only in the population of 16 DAF mRNAs, and 3) those that indicated the expression of a gene in both mRNA populations. A total of 68 clones corresponding to strong hybridization signals were selected for a repeat hybridization screening. Probes used in this second screening were 7 and 16 DAF (ss)-cDNA and a genomic subclone, Nco I, containing the protein-coding region of an α-glutelin gene (Anderson et al. 1984). The latter probe allowed cross-screening for α/β-glutelin gene expression in the 7 and 16 DAF messenger populations. Useful signals were obtained from 49 clones in this second screening, and examples are shown in Figure 3. Of the 49 useful signals, 44 identified genes expressed at both 7 and 16 DAF, and a maximum of five identified genes expressed at 7 DAF but possibly not at 16 DAF. In all cases where gene activity was detected at 16 DAF, it was also present at 7 DAF. One possibility of qualitative differences in gene expression between the 7 and 16 DAF seeds is in the genes that correspond to clones recognized by 7 DAF ss-cDNA, but not 16 DAF ss-cDNA. Low levels of expression of these genes in 16 DAF seed cannot be ruled out by this experiment, however. When 18 of the clones corresponding to genes expressed at both 7 and 16 DAF were screened with a cloned α-glutelin gene, 12 gave positive signals, verifying that glutelin genes are expressed as early as 7 DAF. Expression of glutelin genes as early as 5–6 DAF has been reported by Reeves et al. (1984). These overall results indicate that the differences in gene expression observed in Figure 1 result from primarily quantitative regulation of genes activated before 7 DAF and indicate that the general characteristics of gene expression in developing wheat seeds do not change qualitatively between 7 and 16 DAF. It may be necessary to analyze mRNA populations of very young (<3 DAF) seeds or to examine transcription in isolated endosperm cell nuclei to detect gene expression qualitatively different from that occurring in the 7–16 DAF period.

The Protein Synthetic Apparatus

Biogenesis of proteins by higher organisms usually occurs in one of two characteristic ways: in the cell cytosol, in a process directed by “free” mRNA; or in association with internal membranes of the cell, directed by “membrane-bound” mRNA. Two types of observations in wheat endosperm indicate that
synthesis of the gliadins, and probably of other storage proteins, occurs in association with membranes: 1) Electron microscopic observations by Graham et al. (1962), Campbell et al. (1981), and Bechtel et al. (1982) showed that much of the protein of endosperm cells accumulated in membrane-bound organelles, termed protein bodies, which were subsequently shown to contain mostly proteins with aluminum lactate electrophoretic mobilities similar to those of the prolamin class. 2) When free and membrane-bound mRNA populations were isolated from immature wheat endosperm tissue and used to direct protein synthesis in vitro, the prolamin products from the membrane-bound mRNA were much more highly enriched in prolamins than those from free mRNA (Greene 1981, Donovan et al. 1982; Fig. 4). In fact, prolamins are the major protein product from in vitro translation of membrane-bound mRNAs. Their RNAs constitute more than 60% of the messenger population in the endosperm of wheat grains during the period of maximal growth (Okita and Greene 1982, Pernollet and Vaillant 1984) and are discernible as ethidium bromide-stained bands in electrophoresis of poly A(+) enriched membrane-bound RNA (Fig. 5). Membrane-bound endosperm RNAs have also been shown to direct the synthesis of high molecular weight glutenin subunits of wheat (Forde and Miflin 1983). The proteins from in vitro translation of free endosperm mRNAs have not been very well characterized but are probably rich in messages for cellular enzymes.

It is thought that, in vivo, mRNAs such as those for gliadins become bound, in a complex with ribosomes, to membranes of the rough endoplasmic reticulum (RER) and that the protein translation products are transported through the membrane into the lumen of the RER (Blobel and Dobberstein 1975). There is some uncertainty concerning the relative contributions of RER and golgi apparatus to the formation of protein bodies (Campbell et al. 1981, Bechtel et al. 1982. Parker 1982), but the role of the RER in facilitating synthesis of wheat storage proteins is acknowledged. In this context, observations of Briarty et al. (1979) on the rate of proliferation of RER in developing wheat endosperm may be important clues to the regulation of storage protein synthesis. They noted that the RER surface area per endosperm cell increased by a factor of 4.7 during the period between 12 and 16 days after anthesis. This is a much greater actual and relative increase than occurs in similar time periods either preceding 12 DAF or following 16 DAF. This rapid RER proliferation coincides with the period during which an approximately threefold increase occurs in the level of seed mRNA, as measured by incorporation of [3H]-uridine into poly A(+) (Fig. 6). The synthesis of storage protein related to other seed proteins also increases during this period (Fig. 1). In maize, the endosperm RER is apparently adapted for more efficient processing of nascent zeins than is RER from other tissues (Burr and Burr 1981). In addition, the synthesis of zeins must occur in association with the RER for efficient processing and transport to take place. The association of gliadin polysomes with endosperm RER may also be required for efficient processing and deposition of wheat storage proteins. It is worthwhile to consider the possibility that the two instances of gene expression represented by increases in endosperm mRNA levels and in proliferation of RER are not simply coincidental but mechanistically linked.

With respect to mechanisms of control of storage protein biosynthesis, the coincidence, in time, of increased levels of translatable mRNA (Fig. 6) and increased protein synthesis in vivo (Fig. 1) indicate that the rate of storage protein synthesis in the developing kernel may also be limited by the level of mRNA present. The persistence of measurable RNA levels after decline in the rates of mRNA accumulation facilitates continued storage protein synthesis over an extended time period. The factors that control the level of translatable mRNA have not been directly determined, but available evidence is consistent with control primarily at the level of RNA synthesis (transcription/processing) during the period of rapid mRNA accumulation, and by mRNA stabilization during the later period of seed development (Greene 1983).

**STRUCTURAL CHARACTERISTICS OF GLIADIN GENES**

The storage proteins of wheat, especially the gliadins, are very heterogenous (Mecham et al. 1978; Fig. 7) and reflect the heterogeneity of the genes that code for them. This heterogeneity is thought to result from a series of divergences and duplications, over evolutionary time, of ancestral genes (Kasarda et al. 1976). Different combinations of these evolutionary changes, enhanced by the manipulations of ancient and modern-day breeders, have produced the genotypes of modern wheat varieties and determine their different baking qualities. In order to analyze more precisely the mechanism(s) that induce high levels of storage protein synthesis specifically in endosperm tissue of developing wheat seeds, some knowledge of the structural characteristics of their genes is required. Since the expression of gliadin genes is apparently coordinately controlled, and the amino acid sequences of members of each subclass are similar, some general conclusions can be based on detailed examinations of relatively few genes, complemented by less detailed examinations of others.

The high abundance of gliadin mRNA in the developing wheat endosperm has made the purification of their mRNAs relatively uncomplicated (Okita and Greene 1982), and cDNAs to several gliadin mRNAs have been prepared, cloned, and sequenced (Kasarda et al. 1984, Bartels and Thompson 1983, Okita 1984). All sequenced gliadins include some elements of repeated sequence, as suggested by Kasarda (1980) on the basis of amino acid sequence data. In the protein coding sequence of α-type gliadins from Cheyenne wheat, this repeat unit is based on the 36 nucleotide/12 amino acid sequence:

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CAAG - CAG - CGCG - CAAA - CCC-TTT - CCCA - CCAA - CAAA - CCAA - CAAA - CAAA - CAAA - CAAA
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Fig. 6. Developmental profiles of poly A(+) RNA accumulation and in vitro protein synthesis capacity in INIA 66R wheat. Wheat heads were labeled for 24 hr with [3H]-uridine, and poly A(+) accumulation was determined by binding to agarose poly (U), oxidation to tritiated water, and scintillation counting. Total RNA was extracted from endosperm tissue and translated in vitro using a wheat germ extract in the presence of [3H]-leucine. Labeled polypeptides were precipitated in 1% trichloroacetic acid and counted in a scintillation counter. From Greene (1983).
Fig. 7. Gliadin proteins in Cheyenne and Yamhill wheat cultivars. Seeds were extracted with 1.5M dimethylformamide and proteins separated by two-dimensional electrophoresis (pH 3.2 aluminum lactate and pH 9.2 tris-glycine) after Mecham et al (1978). The 6A coded A-gliadin proteins and two comigrating 6D coded gliadins are shown in the insert.

Fig. 8. Structural features of an A-gliadin gene. A gliadin-containing clone was isolated from a Yamhill genomic library, and 2.35 kb of its nucleotide sequence was determined. The thickened lines indicate the protein coding region. Structural domains and approximate positions of sequences discussed in the text are indicated in the lower part of the figure. Modified from Anderson et al (1984).
The total protein coding sequence of mature α-type gliadins can be represented as consisting of five regions, or structural domains: 1) an N-terminal region composed essentially of repeats of the 12 amino acid sequence; 2) a polypeptide region; 3) a central unique amino acid sequence region; 4) a second polypeptide region; and 5) a C-terminal region of unique sequence (Fig. 8). These may have evolved independently of the repeated sequence region. Region “S” is not contained in the mature molecule. It is the signal peptide, which is cleaved as it facilitates processing and accumulation of the protein in protein bodies.

Cloned gliadin cDNA molecules contain all the information of the corresponding mRNAs, and this information is useful in understanding the structural features of the protein and the control of its synthesis at the level of translation. Information controlling the time, place, and rate of gliadin mRNA synthesis resides in the structure of the gene itself; however, and can only be obtained by studying the structure of the genomic DNA. This is facilitated by isolation and study of cloned copies of the genes; studies of this kind are underway in our laboratory and others (Anderson et al. 1984, Rafalski et al. 1984). Using a cloned A-gliadin cDNA as a hybridization probe, we have identified and isolated cloned copies of over 30 gliadin genes from two genomic libraries. One of these libraries was prepared from total DNA of the variety Yamhill by Murray et al. (1984) and generously provided to us. The other library was prepared in our laboratory (Anderson et al., unpublished) from DNA of the variety Cheyenne. Preliminary characterization of gliadin genes isolated from these two libraries revealed structural similarities, so two-dimensional gel electrophoretic behavior of gliadin proteins from Yamhill and Cheyenne was compared to assess the genetic relatedness of these cultivars. Results indicated that the two wheats share the ancestry that has yielded the proteins of the A-gliadin complex and others (Fig. 7), so some of the cloned genes from the two libraries were expected to have protein coding sequences essentially identical to each other and to that of the probe used to identify them. The expected identities have been confirmed by nucleotide sequence analysis (Anderson et al., unpublished) in at least two cases.

The cloned genes isolated from these libraries were assigned to classes based on the degree of their similarity to the gliadin cDNA probe, and several were selected for sequencing. The characteristic structural features of one such gliadin gene are presented in Figure 8. About 25 similar genes are located on the 6A chromosome of Cheyenne wheats, about 10 are found in the variety Yamhill, and about two in the variety Chinese Spring (Anderson et al. 1984). Restriction analysis and nucleotide sequence indicate that these genes are at least 10 kb apart in the wheat genome. The protein coding sequence of the characterized gene is continuous from the ATG translation start signal to the TGA translation stop signal, and codes for an N-terminal signal peptide, S, followed by the five structural domains similar to those described in the cDNA by Kasarda et al. (1984). The mature protein coded by this gene has 97% sequence homology with the directly determined sequence of an A-gliadin molecule (Kasarda et al. 1984). The protein coding region is preceded on its 5’ flank by the consensus nucleotide sequences (“CAAT” and “TATA” boxes) associated with initiation of RNA synthesis (transcription) and followed on its 3’ flank by sequences (AATAAA and AATAAA) associated with attachment of a polyadenylate sequence (at “poly A” in Fig. 8) during conversion of the initial RNA transcript to gliadin mRNA. A similar gene sequence has been reported by Rafalski et al. (1984), and this general structure is representative of (α/β) gliadin genes. The noncoding features described above are common to most nuclear genes, and their presence in the cloned wheat gene indicates that it is a copy of a functional gene. The specific sequence and location of the gene regions that interact with incoming signals to activate gliadin genes is not yet known, but information obtained from the study of other nuclear genes suggests that they will be located in the DNA sequences in the 5’ flanking regions. The cloned gliadin gene described here will be a valuable substrate for studies of the relation of the fine structure of such regions to control of gene activity, for gene modification and transfer studies in model systems, and eventually in wheat.

CONCLUSION

Protein biosynthesis in the developing wheat seed is subject to a variety of control mechanisms from gene activation through post-translational protein processing. Recent progress has extended our knowledge of some of these mechanisms to the molecular level and has allowed reasonable conclusions to be drawn concerning their importance. Among these, the processes of gene activation and transcription are of particular interest. The coordinate nature of gene expression for storage proteins indicates the possibility that manipulation of a relatively small number of regulatory factors could affect the synthesis of a relatively large number of proteins. This would involve the difficult task of identifying and isolating the genes that control the levels of regulatory factors. Alternatively, the availability of cloned, characterized gliadin genes makes manipulation of the expression of single genes potentially feasible by modification of regulatory nucleotide sequences, or by construction of hybrids containing regulatory sequences of one gene and protein coding sequences of another. Effective methods for carrying out this type of molecular genetic manipulation in vivo in wheat and other monocotyledsen crop plants are not available, although some exciting observations have been made. Practical application of the rapidly accumulating knowledge of control of wheat protein biosynthesis should swiftly follow the development of appropriate gene transfer and regeneration methods.

LITERATURE CITED


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