Tissue-Specific Expression of a Wheat High Molecular Weight Glutenin Gene in Transgenic Tobacco

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The expression of a wheat genomic clone containing the entire coding sequence of the high molecular weight glutenin subunit 12 gene flanked by 2.6 kilobases of 5' and 1.5 kilobases of 3' sequences has been studied after introduction into tobacco. Seeds of different tobacco plants containing the full-length wheat genomic clone accumulated different amounts of intact high molecular weight glutenin subunit mRNA and of a polypeptide displaying the solubility, molecular weight, and antigenic properties of the high molecular weight glutenin subunit 12. The wheat protein accumulated without obvious degradation products and constituted up to approximately 0.1% of the total tobacco endosperm protein. Restriction fragments corresponding to 2.6 kilobases, 1.4 kilobases, and 433 base pairs of high molecular weight glutenin 5' upstream sequence were fused to the coding sequence of the chloramphenicol acetyltransferase (CAT) gene in the vector polyCATter and transferred into tobacco. Chloramphenicol acetyltransferase enzyme activity was detected only in the seed endosperm tissue of the transformed plants. It was detected in tobacco seeds 8 days after anthesis and persisted until seed maturity. It is concluded that 433 base pairs of high molecular weight glutenin upstream sequence are sufficient to confer endosperm-specific expression of this monocot gene in the dicot tobacco.

INTRODUCTION

The high molecular weight (HMW) glutenin subunits found in wheat (Triticum aestivum) constitute approximately 10% of the total seed storage proteins deposited in the endosperm of the developing grain (Payne et al., 1984). A wheat cultivar contains between 3 and 5 of the possible 20 different HMW glutenin subunits that have been identified (Payne, Holt, and Law, 1981). Each subunit is believed to be coded by a single gene located on the long arm of one of the homoeologous group 1 chromosomes (Harberd, Bartels, and Thompson, 1986). The HMW glutenin subunits together with the low molecular weight (LMW) glutenin subunits form a large disulfide-linked protein aggregate which is responsible for the viscoelasticity of wheat dough (Kasarda, Bernadin, and Nimmo, 1976). The bread-making quality of a wheat cultivar is directly influenced by the particular combination of HMW glutenin subunits present in its flour (Payne et al., 1984).

The complete nucleotide sequences of several genes coding for HMW glutenin subunits have now been published. These sequences have revealed that these subunits contain three distinct domains, a nonrepetitive NH₂-terminal domain, a large central domain of repeated sequences, and a nonrepetitive COOH-terminal domain (Forde et al., 1985b; Sugiyama et al., 1985; Thompson, Bartels, and Harberd, 1985; Halford et al., 1987). The sequences of the HMW glutenin genes which regulate expression of the genes in the cereal endosperm are less well understood (Forde et al., 1985a). In the absence of an efficient transformation-regeneration system for cereals, it is not currently possible to study the effect of promoter mutations on gene expression after reintroduction of an isolated gene into wheat. However, the Agrobacterium tumefaciens-mediated gene transfer system is available for tobacco and other dicots to study the expression of wheat genes inserted into these plants. In this paper we describe the expression, transcription, and translation of a wheat HMW glutenin gene in tobacco and initial experiments to define and analyze the promoter region responsible for the observed tissue-specific expression.
RESULTS

Detection of HMW Glutenin DNA and mRNA in Transformed Tobacco Plants

A 6-kb EcoRI DNA fragment corresponding to the HMW glutenin genomic clone λC11 (Figure 1A), which consists of the complete intronless coding sequence for HMW glutenin subunit 12 flanked by 2.6 kb of 5' upstream sequence and 1.5 kb of 3' sequence (Thompson, Bartels, and Harberd, 1985), was inserted into the binary vector Bin 19 (Bevan, 1984) and used to transform tobacco. DNA gel blot analysis of the DNA isolated from independent tobacco transformants using pTag 1290, an HMW glutenin cDNA clone previously described (Thompson et al., 1983), revealed the presence of 1 to 5 copies of the 6-kb HMW glutenin insert within the tobacco genome (Figure 2). All DNA samples, including that of untransformed tobacco, displayed minor unidentified bands which hybridized to pTag 1290. Twelve individual transformants were examined and four contained glutenin genes that were structurally rearranged (results not shown). Further analyses were performed only on tobacco plants containing unmodified HMW glutenin inserts.

To investigate whether the HMW glutenin gene was actively transcribed in tobacco, polyadenylated RNA was isolated from developing seeds [10 days to 12 days after anthesis (daa)] of three independent tobacco transformants and equal amounts were hybridized on RNA gel blots with the HMW glutenin cDNA clone pTag 1290. As shown in Figure 3, all three transformants possess an mRNA of the same size which hybridized with the HMW glutenin cDNA clone (lanes 3 to 5). This mRNA is 2200 nucleotides in length, the size previously determined for the mRNA...
Expression of a Wheat HMW Glutenin Gene in Tobacco

Figure 3. Detection of the Wheat HMW Glutenin Subunit 12 mRNA in Transgenic Tobacco Plants.

The polyadenylated RNA analyzed in lanes 3 and 5 of Figure 3 was isolated from the same tobacco transformants whose DNA was used in lanes 4 and 6, respectively, of Figure 2. Whereas one of the transformants displays a relatively high copy number (Figure 2, lane 4) and a low level of HMW glutenin mRNA (Figure 3, lane 3), the other transformant shows a lower number of inserts (Figure 2, lane 6) but a higher level of mRNA (Figure 3, lane 5). Hence, a high number of inserts within the tobacco genome does not necessarily imply an elevated level of mRNA synthesized.

Detection of the HMW Glutenin Polypeptide in Tobacco Seeds

Alcohol-soluble proteins extracted under reducing conditions from mature seeds of untransformed and transformed tobacco plants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels. Two tobacco transformants contained sufficiently high quantities of an additional protein band to allow its detection by Coomassie Blue staining (Figure 4, lanes 2 and 3). The new polypeptide found in tobacco corresponded exactly in size to the HMW glutenin subunit 12 found in wheat (Figure 4, lane 4, arrow) and was absent in untransformed tobacco seeds (lane 1).

Protein gel blot analysis using antibodies raised specifically against purified HMW glutenin subunits was performed to confirm the identity of the additional polypeptide present in the two tobacco transformants and possibly to uncover altered forms of this protein. The novel polypeptide did cross-react with the HMW glutenin subunit antibodies (Figure 5, lanes 5 and 6). The increased sensitivity of the protein gel blot analysis revealed the presence of this polypeptide in other transformants (Figure 5, lanes 2–4), and in each case it appeared within the resolution of the gels that the signal peptide had been cleaved from the native polypeptide. Lower molecular weight forms were not detected. The presence of the HMW glutenin subunit was not detected in some transformants (Figure 5, lanes 7 and 8) which showed unmodified wheat DNA insertions (Figure 2, lanes 7 and 8). The tobacco transformants analyzed in lanes 2 to 8 of the DNA gel blot analysis (Figure 2) are the same plants as those used in lanes 2 to 8 for the protein gel blot analysis. Assuming that the wheat glutenin subunit was fully extracted, by the isopropyl alcohol:2-mercaptoethanol solution, the highest level of wheat protein observed in tobacco seeds was approximately 0.1% of the total endosperm protein, on the basis that nonembryonic tissues account for 70% of the mature tobacco seed (Sengupta-Gopalan et al., 1985).

It was shown that, in the case of two particular transformants, the number of copies of wheat DNA inserted into the tobacco genome did not necessarily correlate with the level of mRNA produced. The protein gel blot analysis coding for the HMW glutenin subunit 12 (Thompson et al., 1983; Thompson, Bartels, and Harberd, 1985). Untransformed tobacco seeds or leaves of transformed plants did not contain the HMW glutenin mRNA (Figure 3, lanes 1 and 6, respectively). The HMW glutenin mRNA was also absent from the seed poly A+ fraction (results not shown).
displayed the highest number of inserts (Figure 2, lane 5). Therefore, there is no consistent correlation between the levels of mRNA or protein accumulated in transgenic plants and the number of DNA inserts integrated in the genome.

Construction of HMW Glutenin/Chloramphenicol Acetyltransferase (CAT) Gene Fusions

To investigate the approximate location of the regulatory sequences responsible for the seed-specific expression of the HMW glutenin gene in tobacco and their developmental activation, 5′ upstream DNA fragments of this gene were fused to the coding sequence of the chloramphenicol acetyltransferase enzyme, whose activity can be assayed with great sensitivity. The wheat promoter region used in the chimeric constructs corresponded to a 2.6-kb upstream deletion fragment obtained from the genomic clone λC11 (Figure 1B). Separate restriction fragments of 2.6 kb, 1.4 kb, and 433 bp representing decreasing amounts of wheat 5′ upstream sequence were isolated and fused

Figure 4. Detection of the HMW Glutenin Subunit 12 Polypeptide in Mature Seeds of Tobacco Plants Transformed with the HMW Glutenin Genomic Clone λC11.

SDS-PAGE of alcohol-soluble proteins (200 µg) extracted from seeds of tobacco plants. Lane 1, proteins extracted from the seeds of an untransformed tobacco plant; lanes 2 and 3, proteins extracted from the seeds of two independent tobacco transformants; lane 4, wheat (cv Chinese Spring) glutenin fraction; lane 5, molecular weight markers. The arrow indicates the position of the HMW glutenin subunit 12 polypeptide. Kd, kilodaltons.

Figure 5. Immunological Detection of the HMW Glutenin Subunit 12 Polypeptide in Tobacco Plants Transformed with the HMW Glutenin Genomic Clone λC11.

Alcohol-soluble proteins (200 µg) were separated by SDS-PAGE, transferred to a nitrocellulose filter, and reacted with anti-HMW glutenin serum and 125I-labeled protein A. Lane 1, proteins extracted from seeds of an untransformed tobacco plant; lanes 2 to 8, proteins extracted from mature seeds of independent tobacco transformants; lane 9, wheat (cv Chinese Spring) glutenin fraction. The arrow indicates the position of the HMW glutenin subunit 12 polypeptide.
downstream of the transcription start to the CAT gene of the Bin 19-derived vector polyCATter previously described (Colot et al., 1987). Each wheat upstream fragment was inserted in both orientations relative to the CAT gene producing transcriptional fusion constructs pCAT12-1 to pCAT12-6 (Figure 1C). The six HMW glutenin/CAT fusion constructs were separately introduced into the nuclear genome of *Nicotiana tabacum* (cv Samsun) using kanamycin for the selection of stable transformants (Bevan, 1984; Horsch et al., 1985). Promoter activity in the transformed tissues was then determined by assaying for CAT enzyme activity (Herrera-Estrella et al., 1983; Colot et al., 1987).

**Wheat HMW Glutenin Promoter Activity in Transformed Tobacco Plants**

The integrity of the HMW glutenin/CAT fusion DNA inserted into the tobacco genome was verified by DNA gel blot hybridization (results not shown).

The majority (≈70%) of the transformed plants examined which contained CAT fusions with the smaller upstream fragments (pCAT12-3 to pCAT12-6) did not undergo any structural rearrangements in tobacco, and the expected promoter or CAT restriction fragments were observed. However, most (≈80%) of the fusions involving the 2.6-kb upstream fragment exhibited some rearrangement. Although some transformants were found with an unmodiﬁed pCAT12-1 insertion, all six independent transformants analyzed which contained pCAT12-2 [the 2.6-kb upstream fragment in the reverse orientation (3' to 5') relative to the CAT gene] displayed structurally altered fusion DNA.

Restriction fragments defining the sites of integration of the HMW glutenin/CAT fusion DNA within the tobacco genome varied among the different transformants. The number of copies inserted also differed signiﬁcantly, and in some cases there is evidence to indicate that the foreign DNA is found in multiple (possibly tandem) copies.

Seeds of three tobacco transformants containing single-copy inserts of pCAT12-1, pCAT12-3, or pCAT12-5 were germinated on a medium containing kanamycin (500 μg/ml), and in all three cases the 3:1 segregation ratio of resistant to sensitive phenotype expected of a single dominant Mendelian trait was observed (results not shown).

To investigate the promoter activity of the wheat upstream sequences in tobacco, CAT enzyme assays were performed on transformants which displayed structurally unmodiﬁed HMW glutenin/CAT DNA insertions. When extracts from different tissues were examined, CAT activity was detected only in the seeds of transgenic plants transformed with pCAT12-1, pCAT12-3, and pCAT12-5 and in no other organ (Figure 6A). The three different hybrid HMW glutenin/CAT genes were able to function in tobacco and...
confer organ specificity in all the transgenic plants tested. Interestingly, tobacco plants containing pCAT12-4 or pCAT12-6, which possess, respectively, the 433-bp and 1.4-kb wheat upstream fragments in the opposite orientation relative to the CAT gene, also consistently showed some, albeit reduced, seed-specific CAT activity (Figure 6A). No CAT activity was observed in untransformed tobacco tissues (Figure 6A). Following hand dissection of individual tobacco seeds, CAT enzyme activity was found to be further restricted to the endosperm tissue of seeds from plants transformed with pCAT12-1, pCAT12-3, and pCAT12-5 (Figure 6B). No CAT activity was detected in the seed embryo tissue obtained from these transgenic plants or in the component tissues of untransformed tobacco seeds. Hence, sequences sufficient to determine endosperm-specific expression of the HMW glutenin gene must be localized within the 433-bp 5' upstream fragment of this gene.

It is difficult to correlate the length of the HMW glutenin upstream fragment used in a chimeric construct with the level of CAT enzyme activity observed in a tobacco transformant because of variations in, for example, sites of insertion and copy number. However, detection of any effect of the different HMW glutenin upstream fragments on the time of appearance of CAT activity during tobacco seed development should not be biased by these difficulties. Seeds from transgenic plants containing pCAT12-1, pCAT12-3, and pCAT12-5 were therefore harvested every 4 days after anthesis (daa) and assayed for CAT activity. The pattern of appearance of CAT activity during tobacco seed development was essentially identical for all three constructs examined, and is exemplified in Figure 6C. CAT activity was not detected before 8 daa and persisted until seed maturity. On a per-seed basis, the level of CAT activity generally increased from 8 to 28 daa, but it remained a similar proportion of the amount of protein extracted for the assays. There were no major fluctuations observed in the appearance or disappearance of CAT activity during seed development.

**DISCUSSION**

We have presented evidence demonstrating that a wheat HMW glutenin genomic clone containing the complete coding sequence for the HMW glutenin subunit 12 with 2.6 kb of 5' and 1.5 kb of 3' flanking DNA possesses the necessary cis-acting elements for the correct tissue-specific transcription and translation of this gene in tobacco. Furthermore, only 433 bp of the HMW glutenin upstream DNA sequence was required to direct endosperm-specific CAT expression, and an additional 2.2 kb of 5' flanking sequence was not essential for the correct tissue-specific or developmental accumulation of CAT activity in transformed tobacco seeds.

The presence of an mRNA or protein produced by a cereal storage protein gene driven by its own promoter has not been demonstrated previously in the seeds of a transgenic dicot plant. However, zein mRNA was shown to be correctly transcribed at low levels in undifferentiated sunflower tissue (Matzke et al., 1984; Goldsborough, Gelvin, and Larkin, 1986), and, more recently, zein has been detected in the seeds of tobacco plants transformed with a chimeric construct having the 15-kD zein coding sequence inserted between β-phaseolin flanking sequences (Hoffman et al., 1987).

Our results clearly indicate that the HMW glutenin genomic clone λC11 can specify the transcription of a 2200 nucleotide mRNA in transformed tobacco plants. This mRNA accumulates in the seeds but not the leaves of these plants and no other mRNA transcript was found to hybridize with an HMW glutenin cDNA probe. There was no apparent correlation between the level of HMW glutenin mRNA observed and the number of wheat genes inserted in the tobacco genome. An analogous lack of association between the number of inserted copies and the amount of mRNA produced in transgenic plants has been reported previously (Jones, Dunsdmuir, and Bedbrook, 1985).

The HMW glutenin subunit was not found to be proteolytically cleaved in any of the tobacco transformants where it was detected. The apparent stability of the HMW glutenin subunit in tobacco seeds is particularly interesting in view of its relatively large size and numerous repeats, and contrasts with the post-translational degradation observed when β-phaseolin (Sengupta-Gopalan et al., 1985) or β-conglycinin (Beachy et al., 1985) are accumulated in tobacco and petunia seeds, respectively. However, zein, which is also a hydrophobic protein likely to aggregate, was also found to be especially stable in tobacco cells.

The highest level of HMW glutenin protein observed in tobacco seeds corresponded to approximately 0.1% of the total endosperm protein. Assuming that, in the wheat cultivar Chinese Spring, a single copy of the subunit 12 gene produces about 2.5% of the total endosperm protein, it is probable that the synthesis of HMW glutenin observed in the dicot seed was not as efficient as that found in the homologous system. The quantity of HMW glutenin protein detected in tobacco seeds varied enormously among different transformants and no correlation with the number of copies of the wheat gene present in the tobacco genome was evident. Such quantitative fluctuations among transformants are common and can be attributed to a variety of factors ranging from influences associated with the different sites of integration of the foreign DNA to physiological and epigenetic effects (Czernilofsky et al., 1986). Similar results have been described for zein in tobacco (Hoffman et al., 1987). The quantity of a foreign protein synthesized in a transformed plant may thus be estimated better by the level of mRNA produced rather than the number of foreign genes inserted.

Only two other monocot gene sequences have been
shown to function in a dicot plant and regulate correctly their own expression: the wheat chlorophyll $a/b$ binding protein gene (Lamppa, Nagy, and Chua, 1985) and the maize heat-shock gene (Rochester, Winer, and Shah, 1986). Like the HMW glutenin gene, the wheat chlorophyll $a/b$ binding protein gene possesses no introns, whereas the maize heat-shock gene contains one intron. The ribulose-1,5-bisphosphate carboxylase small subunit gene, which possesses introns, was not expressed in transformed tobacco plants, and this was believed to result from differences in the pre-mRNA processing machinery of monocot and dicot cells (Keith and Chua, 1986). Maize Adh-1 upstream sequences do not function in tobacco without being coupled to promoter elements from the octopine synthase gene or the cauliflower mosaic virus 35S promoter (Ellis et al., 1987). In this case, additional factors are required to express the monocot gene in a dicot tissue.

The sequences that determine the tissue-specific expression of the HMW glutenin gene in tobacco lie within the DNA 433 bp upstream from the start of translation. It has recently been demonstrated that the sequences −326 to −160 are similarly capable of directing tissue-specific expression of a wheat LMW glutenin gene in tobacco (Colot et al., 1987), and 5′ upstream sequences of a B hordein gene also confer endosperm specificity of expression (Marnis, Gallois, and Kreis, 1988). Regulation of gene expression is probably achieved by the binding of trans-acting factors, probably proteins, to this region of DNA. The precise region of DNA which binds to a protein is currently under investigation. An endosperm nuclear protein was shown to bind to the promoter region of zein genes which are expressed specifically in the endosperm of maize (Maier et al., 1987).

Transgenic tobacco plants with constructs having 1.4 kb (pCAT12-4) or 433 bp (pCAT12-6) of HMW glutenin upstream sequence in the opposite (3′ to 5′) orientation with respect to the CAT gene consistently displayed some seed-specific CAT activity. This phenomenon was also observed with a 938-bp LMW glutenin upstream fragment, which had similarly been inverted and fused to CAT (Colot et al., 1987). We assume that the fragments in an inverted orientation supplied TATA and/or CAAT signals to initiate transcription. Further experiments are required to ascertain which sequences can act as promoter- and/or enhancer-like elements in both orientations to give rise to the observed results.

The levels of CAT activity directed by the HMW glutenin upstream fragments which were observed during tobacco seed development essentially paralleled that of the total tobacco seed protein (Sehgal and Gifford, 1979; Erdelska, 1985; Colot et al., 1987). However, CAT activity is detected relatively early in tobacco seed development contrasting with the later onset of accumulation reported for proteins coded by genes regulated by conglycinin or phaselin promoters (Beachy et al., 1985; Sengupta-Gopalan et al., 1985; Hoffman et al., 1987). This early appearance of CAT activity in tobacco seeds agrees with the observed accumulation of glutenins in wheat grains (Bartels and Thompson, 1986) and the relative sequence of development and tobacco storage protein accumulation of the endosperm and embryo tissues in tobacco (Sehgal and Gifford, 1979; Erdelska, 1985).

METHODS

Plasmid Constructions

The 6.0-kb EcoRI DNA fragment from the HMW glutenin clone λC11 (Thompson, Bartels, and Harberd, 1985) consisting of the complete coding sequence for the HMW subunit 12 gene flanked by 2.6 kb and 1.5 kb of 5′ and 3′ sequences, respectively (Figure 1A), was inserted into the EcoRI site of the Ti-derived disarmed binary vector Bin 19 (Bevan, 1984).

A deletion fragment of DNA consisting of the 5′-untranslated region and the beginning of the coding sequence of the HMW glutenin gene was obtained from the genomic clone λC11 and inserted into pUC 9. To remove the coding sequence, a series of Bal-31 deletions were generated from the 3′ end of this insert, subcloned, and sequenced in M13 vectors (Sanger et al., 1980; Poncz et al., 1982). A deletion fragment containing 2.6 kb of 5′ upstream DNA sequence beginning at −5 bp relative to the start codon of the HMW glutenin gene was selected and subcloned in pUC 9 (Figure 1B). The full length 2.6-kb upstream fragment was excised by digestion with restriction endonucleases BamHI and EcoRI, made blunt-ended using the Klenow fragment of DNA polymerase I (Maniatis, Fritsch, and Sambrook, 1982), and fused transcriptionally to the CAT gene by insertion into the Smal site of the vector polyCATter described previously (Colot et al., 1987). This fragment was inserted separately in the same or the reverse orientation relative to the direction of transcription of the CAT gene generating constructs pCAT12-1 and pCAT12-2, respectively (Figure 1C). The partial HindIII fragment of 5′-flanking DNA extending to −1.4 kb was also introduced in the same (pCAT12-3) or reverse (pCAT12-4) orientation into the HindIII site of polyCATter. Similarly, the HindIII fragment extending from −5 to −438 bp was inserted in both orientations into polyCATter to form pCAT12-5 and pCAT12-6.

Tobacco Transformation

The Bin 19 vector containing the HMW glutenin subunit 12 genomic clone λC11 and the polyCATter plasmids harboring the chimeric HMW glutenin/CAT constructs were separately introduced into Agrobacterium tumefaciens LBA4404 via triparental mating as previously described (Bevan, 1984). The Agrobacterium cells were then used to inoculate sterile leaf discs of Nicotiana tabacum (cv Samsun), and transformed tobacco cells were selected on a shoot-inducing medium containing 100 μg/ml kanamycin and 200 μg/ml carbenicillin (Horsch et al., 1985). Following the transfer and rooting of young shoots on root-inducing medium containing 100 μg/ml kanamycin and 200 μg/ml carbenicillin, cuttings were made of each regenerated plant and the duplicate transformants grown to maturity and self-pollinated in the green-
house. From 10 to 20 different pairs of duplicate tobacco plants were grown from each transformation experiment with constructs containing one of the HMW glutenin upstream DNA fragments inserted in the 5' to 3' orientation relative to the CAT gene or with the full-length HMW glutenin genomic clone in Bin 19. From 5 to 12 pairs of transformants were grown per construct containing an HMW glutenin upstream fragment in the opposite orientation.

**Nucleic Acid Analysis**

Total DNA was isolated from frozen tobacco leaf tissue kept at −80°C. Approximately 1 g of tissue was ground to a fine powder in liquid nitrogen. The frozen powder was mixed with 5 ml of DNA extraction buffer (100 mM Tris-Cl, pH 8.5, 100 mM NaCl, 50 mM EDTA, 2.0% SDS, 10 mM dithiothreitol, 0.1 mg/ml proteinase K) and incubated with occasional swirling at 37°C for 1 hr. The mixture was then extracted twice with an equal volume of 50:50 (v/v) phenol/chloroform solution previously equilibrated with extraction buffer. The aqueous phase was adjusted to 0.3 M sodium acetate and the nucleic acids were precipitated with 2 volumes of ethanol. Following resuspension, RNA was removed by a treatment with 100 μg/ml boiled RNase A for 1 hr at 37°C, followed by the phenol chloroform extraction and DNA precipitation steps described above. DNA samples (10 μg) were restricted and electrophoresed in 1.25% agarose gels prior to transfer to nitrocellulose (Southern, 1975) and hybridized (Maniatis, Fritsch, and Sambrook, 1982) with purified DNA fragments labeled with 32P (Feinberg and Vogelstein, 1983).

RNA was isolated from tobacco leaves and developing seeds by a modification of the method described by Apel and Kloppstech (1978). Developing seeds were harvested 2 weeks postanthesis and frozen immediately in liquid nitrogen. Leaf and seed samples were kept at −80°C until use. One gram of tissue was ground to a fine powder in liquid nitrogen and dispersed in 10 ml of RNA extraction buffer (50 mM Tris-Cl, pH 9.0, 100 mM NaCl, 10 mM EDTA, 2.0% SDS, 10 mM β-mercaptoethanol, 0.1 mg/ml proteinase K) for 30 sec using a Polytron. Following repeated phenol chloroform extraction and DNA precipitation steps described above, RNA samples (10 μg) were restricted and electrophoresed in 1.25% agarose gels prior to transfer to nitrocellulose (Southern, 1975) and hybridized (Maniatis, Fritsch, and Sambrook, 1982) with purified DNA fragments labeled with 32P (Feinberg and Vogelstein, 1983).

**Protein Analysis**

Proteins were extracted from dry mature tobacco seeds by grinding in 55% isopropyl alcohol and 2.0% β-mercaptoethanol (30 mg of seeds/400 μl of extraction buffer) in 1.5-ml Eppendorf tubes. The homogenate was centrifuged at 12,000 rpm for 15 min, the supernatant recovered, and the centrifugation step repeated. A small aliquot of the extract was removed for protein determination by the dye-binding method of Bradford (1976) using the Bio-Rad Laboratories Kit. Quantities of extract containing equal amounts of protein were vacuum-dried and redissolved in gel-loading buffer prior to boiling and electrophoresis in 10% polyacrylamide gels (Laemmli, 1970). Protein gels were stained for 3 hr with 0.2% Coomassie Brilliant Blue R-250 in acetic acid/methanol/water (10:25:65, v/v/v) and destained in acetic acid/methanol/water (10:25:65, v/v/v). Molecular weight markers (Bethesda Research Laboratories) were: myosin (H-chain), 200 kD; phosphorylase b, 97.4 kD; bovine serum albumin, 68 kD; ovalbumin, 43 kD; α-chymotrypsinogen, 25.7 kD; β-lactoglobulin, 18.4 kD; and lysozyme, 14.3 kD. The wheat (cv Chinese Spring) glutenin fraction was obtained by incubating in gel-loading buffer wheat flour from which the salt-soluble and alcohol-soluble proteins had been sequentially extracted with 50 mM Tris-Cl, pH 8.5, 1.0 M NaCl, and with 70% ethanol, respectively.

Protein gel blot analysis was performed according to a modified procedure of Towbin, Staehelin, and Gordon (1979). Immediately following gel electrophoresis, proteins were transferred electrophoretically onto a nitrocellulose filter for 24 hr at 135 mA in 25 mM Tris, 200 mM glycine, and 0.1% SDS. The filter was prehybridized in 10 mM Tris-Cl, pH 7.5, 0.35 M NaCl, and 2.0% defatted milk powder (Marvel) for 2 hr at room temperature. The filter was rinsed with 50 mM Tris-Cl, pH 7.5, 0.75 M NaCl, 50% (w/v) Triton X-100, 0.5% deoxycholate, 0.5% SDS, and hybridized for 16 hr at 4°C with the same buffer containing 25 μl of rabbit immune serum with antibodies raised specifically against purified HMW glutenin (Bartels, Thompson, and Rothstein, 1985). The filter was washed extensively with hybridization buffer, incubated for 2 hr at room temperature in the same buffer containing 0.5 μCi of 125I-protein A, washed, and exposed to Kodak X-Omat AT film at −80°C.

**CAT Assays**

CAT activity was assayed following the method of Herrera-Estrella et al. (1983) as modified by Colot et al. (1987). Root, stem, or leaf tissues were harvested, frozen immediately in liquid nitrogen, and assayed the same day. Developing seeds were kept at −80°C until use. Assays were carried out on 15 μg of protein extracted from the different tissues. CAT activity was also measured from the total supernatant obtained from homogenates of 100-seed batches.

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