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Genetics

Wheat tetrameric inhibitors of insect α-amylases: Alloploid heterosis at the molecular level

(*Triticum aestivum/Triticum monococcum/Triticum tauschii*/inhibitor reconstitution/genome interaction)

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ABSTRACT **Tetrameric inhibitors of heterologous α-amylases have been characterized in allohexaploid wheat, *Triticum aestivum* (genomes AABBDD), as well as in *Triticum turgidum* (AABB) and *Triticum tauschii* (DD). Their subunits have been identified as the previously described CM proteins. Single oligomeric species were observed in *T. Turgidum* (subunits CM2, CM3A, and CM16) and in *T. tauschii* (CM1, CM3D, and CM17) by a two-dimensional electrophoretic method that does not dissociate the inhibitors in the first dimension. Multiple tetrameric species, resulting from different combinations of the subunits contributed by the two ancestral species, are observed by the same procedure in *T. aestivum*. The three types of subunits were required for significant activity when the inhibitor of *T. turgidum* was reconstituted from the purified subunits, whereas, in the case of *T. tauschii*, binary mixtures involving subunit CM1 also had some activity. Additional combinations of the subunits present in these two species, which occur in the allohexaploid *T. aestivum*, were also reconstituted, and their inhibitory activities ranged from 144% to 33% the activity of the reconstituted inhibitor from *T. tauschii*. The activity of these inhibitors toward the α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) of the insect *Tenebrio molitor* is much greater than that against the salivary enzyme. These observations, together with the previously established chromosomal locations of genes encoding CM proteins, fit a model of alloploid heterosis at the molecular level.**

Allopolyploidy has played a major role in the evolution of higher plants, as possibly over one-third of the present species, including many important crops, have an obvious alloploid origin (1). At least two features inherent to an alloploid genetic structure can be considered as relevant to its apparent success: the long-term diversification or loss (diploidization) of redundant genetic information and the immediate fixation of possible intergenomic heterotic interactions. Diploidization, which can be envisaged as a mechanism of elimination of deleterious interactions, has been extensively studied in fish (refs. 2 and 3) and, to a lesser extent, in plants (4–9), but evidence of alloploid heterosis at the molecular level is lacking.

Plant proteins that inhibit heterologous proteinases and α-amylases are receiving considerable attention because of recent evidence concerning their possible role in plant protection and the possibility of interspecies genetic transfer by recombinant DNA techniques (10). Subunits of tetrameric inhibitors of heterologous α-amylases are encoded in wheat and barley by multigene families that also include genes for dimeric and monomeric inhibitors of α-amylases and for trypsin inhibitors and that are dispersed over several chromosomes (11–15). Although the wheat monomeric and dimeric inhibitors have been extensively studied (16), less information is available concerning the tetrameric inhibitors (17–19). In particular, their subunits have not been purified and characterized, and there is no direct evidence of their homology to the subunits of dimeric and monomeric inhibitors. This type of evidence has been recently reported for the barley tetrameric inhibitor (20). We report here the characterization of native and reconstituted tetrameric inhibitors, whose subunit associations and inhibitory properties imply intergenomic interactions that fit a heterotic model.

MATERIALS AND METHODS

Plant Material. Ground endosperms from *Triticum aestivum* cv. Chinese Spring and *Triticum turgidum* cv. Senatore Capelli and from Spanish accessions of *Triticum tauschii* (UP-2) and *Triticum monococcum* (UP-1) were used in this study.

Preparation of Tetrameric Inhibitors. Ground endosperms were extracted with 150 mM NaCl (5 vol/wt, 1 hr). After centrifugation for 30 min at 12,000 rpm (23,300 × *g*), the supernatant was precipitated with 50% (saturated) $(NH_4)_2SO_4$, centrifuged for 30 min at 15,000 rpm, suspended in 100 mM ammonium acetate, dialyzed against water, and lyophilized.

The crude inhibitor preparations were fractionated by gel filtration on Sephadex G-100 (90- × 1.6-cm column; 60 mg of protein; 25 ml/hr; 3.3-ml fractions) with 100 mM ammonium acetate (pH 6.8) as elution buffer. Appropriate volumes (≈10 μl) of the eluted fractions were assayed for their inhibitory activities against α-amylases (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) from the larvae of the insect *Tenebrio molitor* and from human saliva. Inactivation of potential amylase activity in all inhibitor fractions was carried out by heating for 40 min at 60°C.

Inhibition Tests. Inhibitory activity against α-amylase was tested by the method of Benfeld (21) with 20 mM sodium acetate/100 mM NaCl/0.1 mM $CaCl_2$, pH 5.4, for *Tenebrio molitor* α-amylase and 20 mM potassium phosphate/67 mM NaCl/0.1 mM $CaCl_2$, pH 6.9, for human salivary α-amylase.

All tests were carried out against 1 unit of α-amylase, defined as the amount of enzyme required to produce the reducing equivalents of 1 μmol of maltose in our experimental conditions.

Protein concentration in gel filtration fractions or purified protein solutions was quantitated by the methods of Lowry *et al.* (22) and Smith *et al.* (23).

Electrophoretic Procedures. $NaDodSO_4$/PAGE was performed according to Laemmli (24). Two-dimensional electrophoresis was carried out as follows: Isoelectrofocusing on a pH 4–9 ampholine gradient (140- × 2-mm column; 35 V/cm; 7 hr; sample insertion at acid end) was performed in the first dimension. Nondissociating conditions in this separation were achieved by omitting the 6 M urea and including the

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protein sample in the polymerization mixture, to avoid the extreme pH of the electrode buffers. Starch gel electrophoresis was performed in 0.1 M aluminum lactate, pH 3.2/3 M urea (28 × 18 × 0.1 cm slabs; 20 V/cm; 3.5 hr).

Chromatography. Purification and quantitation of α-amylase inhibitor subunits from *T. turgidum* and *T. tauschii* were performed by analytical (30–100 μg of protein) or preparative (≈5 mg of protein) HPLC on a Nucleosil 300-5 C4 column (250 × 4.5 mm or 250 × 8 mm; particle size, 5 μm). *T. turgidum* subunits were eluted with a linear gradient of 25–50% isopropanol in the presence of 0.1% trifluoroacetic acid at a flow rate of 0.5 ml/min (total run, 125 min). *T. tauschii* subunits were separated with a 25–32% acetonitrile gradient (curve profile 5, Waters automated gradient controller 680; total run, 70 min).

RESULTS

Crude inhibitor preparations were obtained by salt extraction and $(NH_4)_2SO_4$ precipitation from the endosperms of *T. aestivum* (genomes AABBDD), *T. turgidum* (AABB), *T. tauschii* (DD), and *T. monococcum* (AA). The crude inhibitors were subjected to gel filtration as indicated in Fig. 1, and the inhibitory activities against salivary and insect α-amylases were monitored to identify the fractions containing tetrameric, dimeric, and monomeric inhibitors. In agreement with previous reports (17, 19), three types of inhibitors, with apparent molecular masses of 60, 24, and 12 kDa, respectively, were thus detected in the *T. aestivum* extract: the tetrameric and monomeric fractions were more active against the insect α-amylase, and the dimeric fraction was highly active against both enzymes (Fig. 1*A*). The elution patterns of the *T. turgidum* (AABB) and *T. tauschii* (DD) inhibitors differed from that of *T. aestivum* in that their crude monomeric fractions were less abundant and about 15 times less active (Fig. 1 *B* and *C*). In contrast, no inhibitors of the tested α-amylases were found in *T. monococcum* (AA), even at concentrations of protein 20 times greater than those used to assay the inhibitors in the other species (Fig. 1*D*).

Proteins from the four fractions indicated for each elution profile in Fig. 1 were separated by $NaDodSO_4$/PAGE, as shown in Fig. 2. Except in the case of *T. monococcum*, fraction 2 from each of the extracts (Fig. 1) was shown to include prominent components with apparent molecular masses in the range of 12–15 kDa, as expected for the subunits of the tetrameric inhibitors, in addition to nondissociating components of higher molecular mass. The fractions containing the tetrameric inhibitors were subjected to combined isoelectric focusing (pH 4–9) and starch gel electrophoresis (pH 3.2), using dissociating conditions in both dimensions, and compared with the corresponding crude inhibitor preparations fractionated by the same procedure (Fig. 3 *A–F*). The conditions of the two-dimensional separation excluded most of the high molecular mass components. Fraction 2 from *T. aestivum* (AABBDD) presented several components (numbered 1, 2, 3, 16, and 17 in Fig. 3 *A* and *D*) that corresponded to previously described CM proteins (hydrophobic endosperm globulins; refs. 25–29) as demonstrated by coelectrophoresis with purified components (data not shown). *T. turgidum* (AABB) presented CM proteins 2, 3, and 16 (Fig. 3 *B* and *E*), and *T. tauschii* (DD) presented components 1, 3, and 17 (Fig. 3 *C* and *F*). None of the known α-amylase inhibitors was detected by two-dimensional electrophoresis of either the crude extract or fraction 2 from *T. monococcum* (data not shown). Fraction 2 from each of the three species was then subjected to the same two-dimensional procedure, except that dissociating conditions (6 M urea) were omitted in the first dimension, to investigate association among the components (Fig. 3 *G–I*). Although a single three-component association was detected in *T. turgidum* (components 2, 3, and 16) and in *T. tauschii* (components 1, 3, and 17), a complex pattern of hybrid associations was detected in *T. aestivum*.

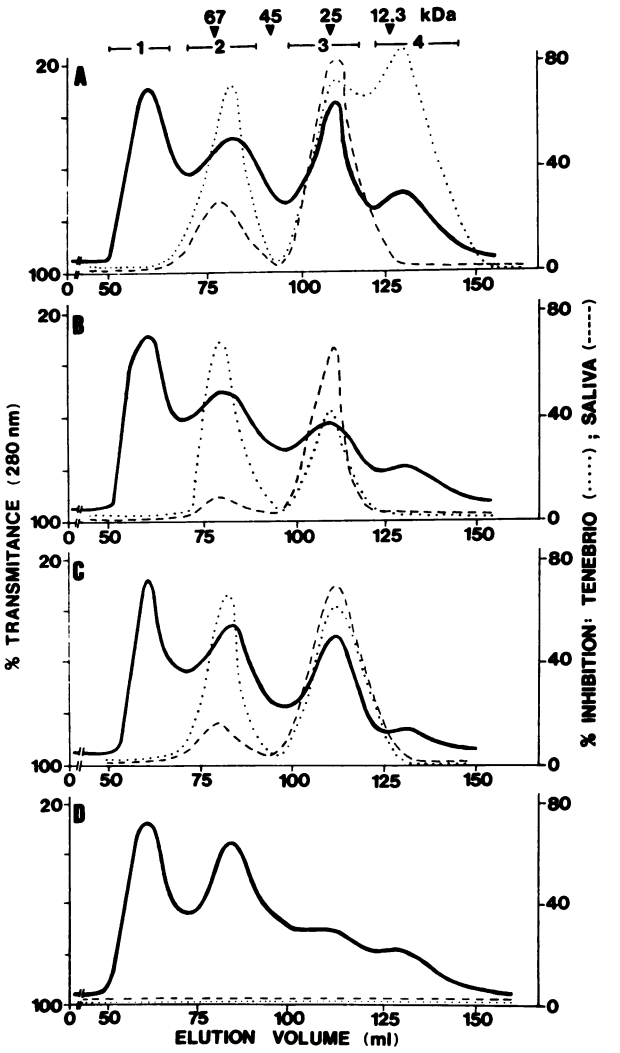

FIG. 1. Gel filtration on Sephadex G-100 of crude inhibitor preparations from mature endosperms of *T. aestivum* cv. Chinese Spring (*A*), *T. turgidum* cv. Senatore Capelli (*B*), *T. tauschii* (*C*), and *T. monococcum* (*D*). Bovine serum albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), and cytochrome *c* (12.3 kDa) were used to calibrate the column as indicated. Appropriate samples of the eluted fractions were assayed for their inhibitory activities against the α-amylases from the larvae of *Tenebrio molitor* and from human saliva. Four pools, based on the inhibitory activity profiles, were collected as indicated in the top of the figure (fractions 1–4). In *T. turgidum* and *T. tauschii*, no activity was detected in the fraction 4 region in the assay conditions used, but it could be detected at higher protein concentrations (see text).

Components of the tetrameric inhibitors from *T. turgidum* and *T. tauschii* were purified by previously described methods (30). Stoichiometry of the components was determined by analytical HPLC, using the purified components to ascertain that the ratio of peak area to molar amount injected was not significantly different for any of them. Thus, the CM1/CM3/CM17 and CM2/CM3/CM16 ratios were, respectively, 1:2.08 ± 0.35:1.16 ± 0.18 and 1:1.34 ± 0.19:0.94 ± 0.22 (mean ± SD), suggesting that two copies of CM3 were present in each tetramer.

Reconstitution of the tetrameric inhibitors from *T. turgidum* and *T. tauschii* was carried out by mixing the purified components under nondissociating conditions, and the results are summarized in Fig. 4. In the case of *T. turgidum*,

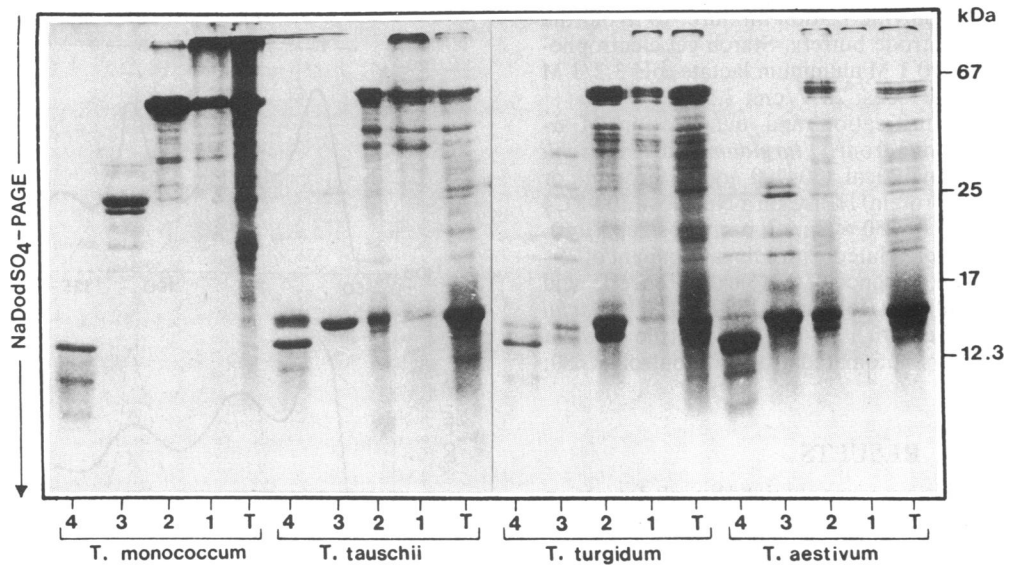

FIG. 2. $NaDodSO_4$/PAGE of crude inhibitor preparations (T) and gel filtration fractions (1–4), pooled as indicated in Fig. 1, from *T. aestivum* cv. Chinese Spring, *T. turgidum* cv. Senatore Capelli, *T. tauschii*, and *T. monococcum*.

only the mixture of the three components showed inhibitory activity at the protein concentrations tested (Fig. 4*A*), whereas, although the components of the *T. tauschii* inhibitor were maximally active in the ternary mixture, binary mixtures involving CM1 were also active (Fig. 4*B*). No significant inhibition of salivary α-amylase was detected at the inhibitor concentrations used in these tests.

A number of heterologous combinations, in which each of the three subunits from the *T. tauschii* inhibitor were exchanged for their equivalents from *T. turgidum*, were also

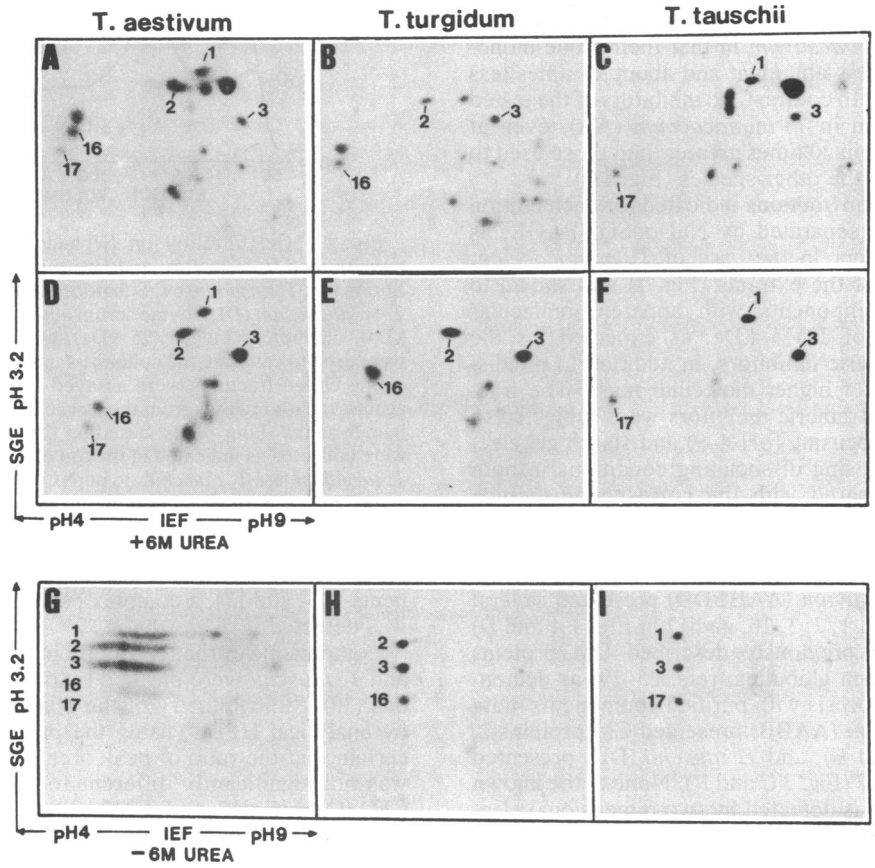

FIG. 3. Two-dimensional electrophoresis (first dimension: isoelectric focusing, pH 4–9; second dimension: starch gel electrophoresis, pH 3.2) of crude inhibitor preparations (*A–C*) and gel filtration fractions (fraction 2) containing the tetrameric inhibitors (*D–I*) from *T. aestivum* cv. Chinese Spring (*A*, *D*, and *G*), *T. turgidum* cv. Senatore Capelli (*B*, *E*, and *H*), and *T. tauschii* (*C*, *F*, and *I*). (*A–F*) Dissociating conditions (+ 6 M urea) in the first dimension. (*G–I*) Nondissociating conditions (− 6 M urea) in the first dimension. Inhibitor subunits (CM proteins) are identified by numbers in the different two-dimensional maps. IEF, isoelectric focusing; SGE, starch gel electrophoresis.

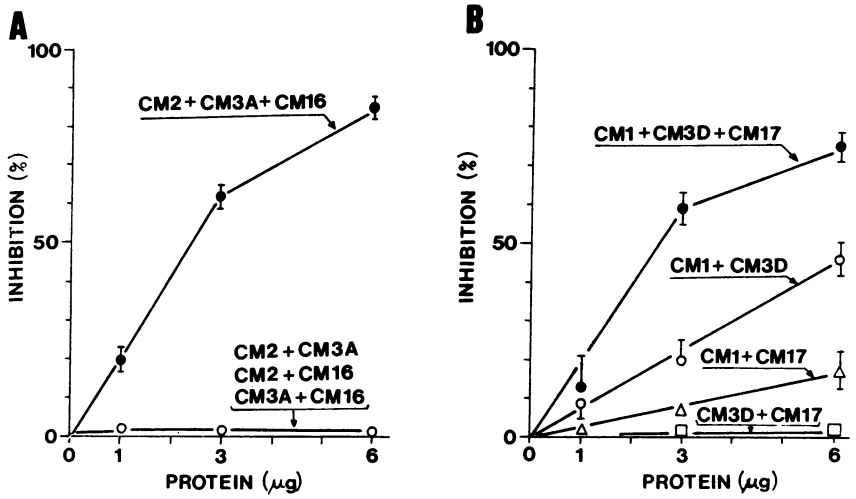

FIG. 4. Inhibitory activity against the α-amylase from the insect *Tenebrio molitor* of the indicated mixtures of purified subunits from the tetrameric inhibitors from *T. turgidum* (*A*) and *T. tauschii* (*B*). None of the purified subunits by themselves showed inhibitory activity at up to 12 μg per assay. Mean values and SD were obtained from at least three independent experiments carried out with different preparations of the purified subunits.

tested, and the results are summarized in Table 1. The novel subunit combinations showed either enhanced or decreased inhibitory activities with respect to the controls.

DISCUSSION

We have previously studied a group of endosperm globulins, designated CM proteins, that are soluble in 70% ethanol and in chloroform/methanol mixtures and whose homology relationships to the monomeric and dimeric α-amylase inhibitors have been established based on their N-terminal amino acid sequences (25–30, 32). We have now shown that the previously purified CM proteins (CM1, CM2, CM3, CM16, and CM17), whose correspondence with spots of the two-dimensional globulin pattern have also been previously determined (26, 27, 32), are the subunits of the wheat tetrameric inhibitors of heterologous α-amylases. This identification is in line with our previous observations concerning the barley tetrameric inhibitor (20). Three types of subunits, which have little or no inhibitory activity by themselves or in binary combinations, are required to obtain fully active inhibitors (Fig. 4). Previous data concerning amino acid sequences and gene locations of CM proteins in wheat and barley (11–14, 25–30, 32) indicate the existence of three types of loci (*CM1/CM2/CMa* in group 7 chromosomes; *CM3A/CM3D/CMd* and *CM16/CM17/CMb* in group 4 chromosomes), which correspond to the three types of subunits that integrate the tetrameric inhibitors.

Table 1. Inhibitory activity toward the α-amylase from the insect *Tenebrio molitor* of tetrameric inhibitors reconstituted with exchanged subunits

Inhibitor subunits*			% inhibition†	Activity, % of *T. tauschii*
CM1	CM3D	CM17	59 ± 9	100
CM2	CM3D	CM17	20 ± 4	33
CM1	CM3A	CM17	81 ± 6	137
CM1	CM3D	CM16	59 ± 7	100
CM1	CM3A	CM16	85 ± 8	144
CM2	CM3A	CM16	72 ± 5	122

*Genetic and homology relationships of subunits CM1/CM2, CM3A/CM3D, and CM16/CM17 have been previously reported (11, 12, 14, 29, 31). CM3 is designated A when isolated from *T. turgidum* and D when isolated from *T. tauschii*. Three micrograms of inhibitor was used per assay. The *T. turgidum* subunits are underlined.

†Mean values and sample SD were from at least two different experiments.

The apparent molecular mass of the tetrameric inhibitor deduced from Sephadex G-100 chromatography was corrected to 47–48 kDa by Buonocore *et al.* (19) by using more accurate procedures, a figure that is in close agreement with that expected from the molecular masses and stoichiometry of the subunits reported here.

The CM2/CM3A/CM16 subunits of the *T. turgidum* inhibitor are encoded in *T. aestivum* by genes located in B-genome chromosomes (7B and 4A, whose designation has been changed officially to 4B at the 1988 International Wheat Genetics Symposium, Cambridge, UK), whereas the CM1/CM3D/CM17 subunits of the *T. tauschii* inhibitor are associated with the D genome. Both the electrophoretic analysis of native inhibitors and the reconstitution experiments with purified subunits have demonstrated the formation of new tetrameric structures with specific activities outside the range of those of the inhibitors present in the ancestral species of the hexaploid wheat (Fig. 3 and Table 1). It is accepted that, in terms of measurement of specific characters, heterosis implies phenotypic values outside the parental range. An alloploid can be considered as a permanent heterozygote, in which both positive and negative intergenomic heterotic interactions are effectively fixed in the short term and then either stabilized by selection or eroded through the slow process of diploidization. The tetrameric inhibitors of insect α-amylase would fit this model of alloploid heterosis.

The new official classification of chromosome 4A as 4B, as had been suggested by several authors (33, 34), is in agreement with the absence of any of the subunits of the tetrameric inhibitor in the *T. monococcum* accession analyzed in this study (Figs. 1 and 2) and the lack of anti-α-amylase activity in 14 accessions of the same species (35).

Finally, it should be pointed out that, before the present structural relationships among the CM proteins were even suspected, an interdependence of the net amounts of the different subunits accumulated in endosperm per gene dose was demonstrated: in the allohexaploid, the amount of a given subunit was increased when analyzed in a nullitetrasomic line in which the chromosome carrying the gene for an equivalent subunit was missing (31).

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