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Molecular phylogenetic analysis of amylase trypsin inhibitors (ATIs) from a selection of ancient and modern wheat

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28 **Abstract**

29 Wheat amylase-trypsin inhibitors (ATIs) are a family of wheat proteins, which play an important
30 role in plant defence against pest attacks. Recently, ATIs have been identified as major stimulators
31 of human innate immune cells leading to cause Non-coeliac Wheat Sensitivity. Information about
32 ATI sequence differences among wheat species is scarce, especially considering ancient wheat
33 genotypes. In this study, ten selected wheat accessions with different ploidy level and year of
34 release were used for gene sequencing of four representative alpha-amylase/trypsin inhibitor genes
35 (WMAI, WDAI, WTAI-CM3 and CMx genes). The phylogenetic analysis and the PCA analysis
36 performed on the deduced amino acid sequences of ATI genes evidenced that the ten wheat
37 genotypes can be differentiated on the basis of their ploidy level, but not with respect to ancient or
38 recently developed wheat genotypes. The haplotype analysis based on Nei's genetic distances,
39 beside confirming these results, also allowed the separation of the hulled from the naked wheat
40 genotypes. The genetic sequence differences highlighted in this study among the ten genotypes can
41 be the basis for further studies aimed at identifying proinflammatory sequences in ATI genes. This
42 is the first study analyzing the ATI genetic sequences of a set of ancient and modern wheat
43 genotypes with a different ploidy level.

44 **1. Introduction**

45 Wheat amylase-trypsin inhibitors (ATIs) represent ~2–4% of total wheat protein and are an
46 important family of low molecular weight, water-soluble proteins located in the endosperm of the
47 grain kernel where they play a crucial role in plant defence against pest attacks (Carbonero and
48 García-Olmedo, 1999). ATIs are able to inhibit enzymes of common parasites such as mealworms
49 and mealy bugs from digesting wheat starch and protein. Besides having a protective role, they act
50 as storage proteins (Altenbach et al., 2011).

51 ATIs are classified into four groups: three groups of alpha-amylase inhibitors differentiated
52 according to their degree of aggregation (monomeric, homodimeric and heterotetrameric forms) and
53 one group of trypsin inhibitors (Carbonero and García-Olmedo, 1999). The three groups of alpha-
54 amylase inhibitors include the wheat monomeric amylase inhibitors (WMAI), often called 0.28
55 proteins; proteins that form the wheat homodimeric amylase inhibitors group (WDAI), sometimes
56 called 0.19 and 0.53 proteins and the proteins of the wheat heterotetrameric amylase inhibitors
57 (WTAI). The WMAI inhibitors are encoded by genes on the short arm of chromosome 6 of *T.*
58 *aestivum*, while the genes for WDAI are located in the short arm of chromosome 3 (Carbonero and
59 García-Olmedo, 1999), in particular on 3BS and 3DS chromosomes, though not much is known
60 about homologous common wheat chromosome 3AS (Pandey et al., 2016). The tetrameric
61 inhibitors are often called CM proteins because of their solubility in chloroform/methanol and in
62 modern hexaploid wheat five sub-units have been identified: CM1, CM2, CM3, CM16 and CM17.
63 CM proteins are generally composed by one copy of either CM1 or CM2 sub-unit, encoded by
64 genes on chromosomes 7D or 7B, plus one copy of either CM16 or CM17 sub-unit, encoded by
65 genes on chromosomes 4B and 4D, plus two copies of CM3 sub-unit, also encoded on
66 chromosomes 4B and 4D. WMAI, WDAI and CM proteins have a molecular weight ranging from
67 13.0 to 13.5 kDa at the subunit level, with the exception of CM3 which shows a mass of about 15.5
68 kDa (Geisslitz, 2021). All these alpha-amylase inhibitors are active with different specificity against
69 insect, mite and mammalian alpha-amylases, but are not active against cereal enzymes. The WTAI
70 inhibitors are also able to inhibit papain, bovine trypsin and subtilisin. The putative wheat trypsin
71 inhibitors are referred to as CMx proteins, are encoded by genes on the group 4 chromosomes and
72 are monomeric (Altenbach, 2011; Carbonero and García-Olmedo, 1999).

73 Altenbach et al. (2011) identified 19 different alpha-amylase/protease inhibitors of ~120- to 150-
74 amino acids expressed in the modern wheat genome. The main ATI species in wheat are 0.19
75 (WDAI) and CM3 (WTAI).

76 All ATIs are characterized by high homology in their amino acid sequence and by an analogous
77 compact secondary structure showing five or less (often four) intrachain disulphide bridges and a
78 main body of the molecule built around four α -helices arranged in an “up and down” pattern linked
79 together by loop segments (Carbonero and García-Olmedo, 1999).

80 ATIs are of high interest because they have an important impact on human health. Some ATI
81 proteins are involved in wheat allergies (Tatham and Shewry, 2008) and, recently, ATIs have been
82 identified to play a role in the onset of celiac disease (Huebener et al., 2015) and Non-celiac Wheat
83 Sensitivity (Junker et al., 2012). Several *in vitro* and *in vivo* studies identified ATIs (especially
84 CM3 and 0.19), but not gluten, as a major nutritional trigger of human and murine innate immunity
85 on wheat by the activation of the toll-like receptor 4 (TLR4) complex (Geisslitz, 2021; Junker et al.,
86 2012; Zevallos, 2017).

87 Thus far, there is little information in literature about the diversity of ATIs between different types
88 of wheat, especially between so-called “ancient” and modern wheat genotypes. Recently, Zevallos
89 et al. (2017) found that modern hexaploid wheat showed higher ATI inflammatory activity than
90 some ancient variants like diploid (einkorn) and tetraploid wheat (emmer, KAMUT® khorasan
91 wheat) or older hexaploid variants like spelt. Another recent paper (Gélinas and Gagnon, 2018)
92 determined the alpha-amylase inhibitory activity against human α -amylase in several different
93 wheat cultivars and showed that the inhibitory potential did not vary with respect to ancient or
94 recently developed wheat cultivars. To the same conclusion came Call et al. (2020) who analysed
95 the ATI concentrations and the trypsin inhibitor activity in a set of different *Triticum* species and
96 EL Hassouni et al. (2021) who determined the content of eight ATI proteins as well as of the total
97 ATI of 149 European old and modern bread wheat cultivars.

98 However, information about ATI genetic sequences is available only for a few types of wheat,
99 especially for common wheat (*T. aestivum* L. subsp. *aestivum*) and for wild emmer wheat (*T.*
100 *turgidum* L. subsp. *dicoccoides*) (Pandey et al., 2016; Wang et al., 2010, 2008, 2007), while ATI
101 genetic sequences of some other types of wheat like spelt (*T. aestivum* L. subsp. *spelta*), emmer (*T.*
102 *turgidum* L. subsp. *dicoccum*) and khorasan wheat (*T. turgidum* L. subsp. *turanicum*) have never
103 been studied. Moreover, gene sequences of some ATIs like WTAI and CMx are very scarce (Liu
104 and Wang, 2012). Lastly, little is known of the genetic diversity of ATIs between genotypes, in
105 particular between ancient and modern wheat genotypes (Geisslitz et al., 2021).

106 In the present study, ten wheat accessions were selected to cover different *Triticum* species with a
107 different genome composition (diploid, tetraploid, hexaploid) and with a different year of release to
108 determine the genetic sequences of four representative alpha-amylase/trypsin inhibitor genes

109 (WMAI, WDAI, WTAI-CM3 and CMx genes). The genetic sequence differences among the wheat
110 genotypes were investigated and discussed.

111 2. Material and methods

112 2.1 Plant material

113 In the present study, ten selected accessions belonging to different *Triticum* species i.e. *T.*
114 *monococcum* L. subsp. *monococcum* (2n = 2x = 14 chromosomes, AA genome), *T. turgidum* L.
115 subsp. *dicoccum*, *T. turgidum* L. subsp. *turanicum*, *T. turgidum* L. subsp. *durum* (all 2n = 4x = 28
116 chromosomes, AABB genomes), *T. aestivum* L. subsp. *aestivum*, *T. aestivum* L. subsp. *spelta* (all
117 2n = 6x = 42 chromosomes, AABBDD genomes) were used for the characterization of ATI gene
118 sequences (Table 1). These genotypes were selected to cover different wheat species with a
119 different genome composition (diploid, tetraploid, hexaploid) and with a different year of release.
120 The pedigrees of heritage and modern wheat varieties were retrieved in GRIS – Genetic Resources
121 Information System for Wheat and Triticale (<http://wheatpedigree.net>) and are shown in Table 1.

122 2.2 DNA isolation and PCR amplification

123 Ten seeds for each wheat genotype were grown in sprouters at room temperature in daylight for
124 seven days. The leaf tissues were sampled at the four-leaf stage from ten different plants per
125 genotype, immediately frozen in liquid nitrogen and ground in a mortar with a pestle. Total DNA
126 extraction was performed with the Nucleospin® Plant II kit (Macherey Nagel, Düren, Germany)
127 following manufacturer's instructions. DNA quality and quantity was measured by
128 NanoPhotometer® P-Class (Implen GmbH, München, Germany).

129 The primers used to amplify WMAI, WDAI, WTAI-CM3 and CMx genes are listed in Table 2.
130 Primers F1, F2, F3, F4, R1 and R2 were previously used by Wang et al. (2008), primers WDAIfor
131 and WDAIrev were previously used by Wang et al. (2007), the other primers were designed *in*
132 *house* with the software Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) on the conserved
133 sequences of the coding regions of genes obtained from the GenBank database.

134 WMAI 0.28 genes were amplified in common wheat samples using F2 and R1 primers and in
135 einkorn, spelt and Peliss genotypes using Mfor3 and Mrev2. Emmer, turanicum and Alzada samples
136 were also amplified using the forward primers F1, F3, F4, Mfor1, Mfor2, Mfor4, Mfor5 and the
137 reverse primers R2, Mrev1, Mrev3, Mrev4, Mrev5, Mrev6. The different combinations of primer
138 sets used for the amplification of WMAI genes are listed in Supplementary Table 1 with the
139 corresponding annealing temperatures used and the expected amplicon sizes.

140 WDAI 0.53 and 0.19 genes were amplified using WDAIfor and WDAIrev primers with an
141 annealing temperature of 58 °C. The expected amplicon size was 446 bp.

142 WTAI-CM3 genes were amplified using CM3for and CM3rev primers and an annealing
143 temperature of 55 °C. The expected amplicon size was 647 bp. In order to check for the possible
144 presence of WTAI-CM3 gene sequences also in einkorn, the internal forward primers CM3intfor
145 and CM3intfor2 and the internal reverse primers CM3intrev and CM3intrev2 were designed and
146 used in combinations at the annealing temperature of 50 °C.

147 CMx1/CMx3 and CMx2 genes were amplified using CMxfor and CMxrev primers and an
148 annealing temperature of 60 °C. The expected amplicon size was 498 bp.

149 All the PCR amplifications were performed with Biometra[®] ThermoCycler (Biosense srl, Milan,
150 Italy) in 50 µl volume, consisting of 200-400 ng genomic DNA, 1 µM each primer, 1x HotStar
151 HiFidelity PCR Buffer (containing 1.5 mM MgSO₄ and 0.3 mM dNTPs), 2.5 U of HotStar
152 HiFidelity DNA Polymerase (QIAGEN, Hilden, Germany). Due to the lack of amplification of
153 WMAI genes in emmer, turanicum and Alzada genotypes and WTAI-CM3 genes in einkorn,
154 MgSO₄ was also tested at final concentration of 2 and 3 mM to enhance the stability of primer-
155 template complexes in order to further check the presence of these genes in the mentioned
156 genotypes.

157 The cycling parameters were 95 °C for 5 min to pre-denature, followed by 45 cycles of 94 °C for 15
158 sec, annealing temperature (specific for each primer set) for 1 min, and 72 °C for 90 sec, and a final
159 extension at 72 °C for 10 min.

160 The desired DNA fragment was recovered from 1.5 % agarose gel using QIAquick gel extraction
161 kit (QIAGEN, Hilden, Germany), quantified by NanoPhotometer[®] P-Class (Implen GmbH,
162 München, Germany), ligated to the pDrive Cloning Vector and used to transform *E. coli* competent
163 cells by using QIAGEN[®] PCR Cloning Plus Kit (QIAGEN, Hilden, Germany). For each amplified
164 gene (WMAI, WDAI, WTAI-CM3, CMx), five positive clones obtained from each wheat genotype
165 were screened and sequenced with the Sanger method using the Brilliant Dye Terminator 1.1 kit
166 (NimaGen BV, The Netherlands) and with ABI3730 automated sequencer. Sequences were
167 obtained using the service provided by BMR genomics (Padova, Italy).

168 **2.3 Bioinformatics analysis**

169 **2.3.1 Haplotypes identification**

170 Open reading frames were obtained by using ORF Finder
171 (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>) and EMBL-EBI server
172 (https://www.ebi.ac.uk/Tools/st/emboss_transeq/). A search for similarity was executed with the
173 BLASTN and BLASTP programs available at the National Centre for Biotechnology Information

174 (NCBI). Haplotypes identification was performed by MEGA software Version 7.0.26 (Kumar,
175 2016). Multiple sequence alignments were performed using Clustal Omega program
176 (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), MEGA software Version 7.0.26 and Jalview version
177 2.11.0 (Waterhouse, 2009). Sequence identity analysis was carried out with SIAS server
178 (<http://imed.med.ucm.es/Tools/sias.html>). Amino acid sequences of reference proteins were
179 retrieved from the Universal Protein Resource database (Uniprot) (<https://www.uniprot.org/>).

180 The signal peptides and the location of their cleavage sites in proteins were determined with
181 SignalP 5.0 (<http://www.cbs.dtu.dk/services/SignalP/>).

182 Obtained sequence data have been deposited in EMBL/GenBank Data Libraries under accession
183 nos. MT739524-38 (WMAI genes); MT861996-2029 (WDAI genes); MT887935-58 (WTAI-CM3
184 genes) and MT897967-88 (CMx genes).

185 **2.3.2 Phylogenetic analysis**

186 The phylogenetic relationships between haplotypes were performed using MEGA software Version
187 7.0.26. The FASTA multiple sequence alignment was used to infer the Neighbor-Joining (NJ)
188 phylogenetic tree. The tree is drawn to scale, with branch lengths in the same units as those of the
189 evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were
190 computed using the Poisson correction method and are in the units of the number of amino acid
191 substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary
192 analyses were conducted with 1000 bootstrap replicates (Kumar, 2016).

193 **2.3.3 Principal component analysis (PCA)**

194 PCA was used to transform a number of correlated variables into a smaller number of uncorrelated
195 variables called principal components. With this method the original space for variable
196 measurements was projected down onto two low dimensional subspaces. One of these was case-
197 related (ten wheat genotypes), the other was variable-related. The variables were the amino acid
198 substitutions at a specific position. The variable-related subspace was analysed (factor loading) to
199 understand the correlation between the variables and wheat genotypes (principal component).

200 First, an interim PCA was run with all the variables included and the quality of representation
201 (squared cosine, \cos^2) of each variable was checked. Second, since a high \cos^2 indicates a good
202 representation of the variable on the principal component, a definitive PCA was run with those
203 variables with a quality of representation higher than 0.5. The data were not pretreated before
204 submission to PCA since a homogeneous set of variables was used (i.e. amino acid substitutions at

205 a specific position). The PCA analysis was based on correlation matrix and was performed using
206 STATISTICA Software v. 7.1 (StatSoft, Tulsa, Oklahoma, USA).

207 **2.3.4 Haplotype analysis**

208 The program POPGENE 1.31 was used to carry out the haplotype analysis. The genetic distances
209 were calculated to compare the ten wheat genotypes (Nei, 1972) based on the cluster analysis of a
210 0-1 matrix, where the presence or absence of the haplotypes of all the four genes analysed in this
211 study was recorded as 1 and 0, respectively. The dendrogram was constructed based on the genetic
212 distances using UPGMA (unweighted pair group with arithmetic average) method modified from
213 Neighbor procedure of PHYLIP Version 3.5.

214 3. Results and discussion

215 3.1 Sequence analysis of monomeric alpha-amylase inhibitors (WMAI)

216 One desirable PCR product of the expected size was obtained in common wheat samples Judee,
217 Turkey Red, Marquis, Vida (~529 bp using F2 + R1) and in einkorn, spelt and Peliss genotypes
218 (~502 bp using Mfor3 + Mrev2).

219 Emmer, turanicum and Alzada samples did not produce any PCR product of the expected size with
220 the two sets of primers cited above and other primers were used (see Supplementary Table 1). The
221 primers were designed to cover the most part of the WMAI ORF region. Supplementary Figure 1
222 shows the gene sequence of 0.28 WMAI and the sequences of all the primers used in this study to
223 amplify the corresponding ORF. All the combinations of primers listed in Supplementary Table 1
224 were used, but it was not possible to obtain any PCR product corresponding to the ORF of WMAI
225 genes in the tetraploid emmer, turanicum and Alzada wheat samples.

226 A previous study (Wang et al., 2010) determined the monomeric α -amylase inhibitor gene
227 sequences of 14 populations of wild emmer wheat (*T. dicoccoides*), which is the wild progenitor of
228 modern tetraploid and hexaploid wheat, however, it was not possible to retrieve any monomeric α -
229 amylase inhibitor gene sequence from *T. durum*, *T. turanicum* and *T. dicoccum* in the GenBank
230 non-redundant DNA database (access performed on May 11st, 2021).

231 Regarding the available studies determining the amount of monomeric α -amylase inhibitors in
232 tetraploid wheat, the results were mixed. Rogniaux et al. (2015) using a targeted MS/MS approach
233 were able to detect and quantify WMAI protein in *T. aestivum*, *T. durum* and *T. monococcum*, but
234 not in KAMUT[®] khorasan wheat sample (*T. turgidum* ssp. *turanicum*). Geisslitz et al. (2020) used a
235 new targeted LC-MS/MS method and found that emmer had similar contents of ATI 0.28 as
236 common wheat and spelt but obtained mixed results for durum wheat with six out of eight cultivars
237 showing a content near or even below LOD.

238 The lack of WMAI sequences in *T. durum*, *T. turanicum* and *T. dicoccum* from available databases
239 together with the amplification of WMAI genes in *T. durum* cv. Peliss, but not in other tetraploid
240 wheat in this study, and the mixed results regarding the presence of WMAI protein in tetraploid
241 wheat confirmed that further studies are needed to deepen knowledge about WMAI in tetraploid
242 wheat.

243 Regarding the other seven wheat genotypes (einkorn, Peliss, spelt, Turkey Red, Judee, Marquis and
244 Vida) the DNA sequences from five clones per each wheat genotype were determined and used to

245 deduce the full amino acid sequence of the proteins. As described before (Wang et al., 2008), there
246 was no intron in the monomeric α -amylase inhibitor sequences. So, it was possible to isolate the
247 complete coding sequences of monomeric α -amylase inhibitor gene by direct PCR amplification. In
248 this study, no ins/del in the coding region of WMAI were found in any of the wheat samples.

249 From a total of 35 deduced amino acid sequences, 16 haplotypes were identified (HM1-HM16).
250 Haplotype HM7 was shared by all the hexaploid wheat samples (it occurred in 13 out of 25
251 hexaploid wheat sequences) and it turned out to be the most abundant WMAI haplotype. No
252 pseudogenes were found (Table 3).

253 All the putatively functional genes encoded for a 151 amino acid protein (30 amino acid signal
254 peptide and a 121 amino acid mature protein).

255 In this study, after aligning the 16 monomeric α -amylase inhibitor haplotypes from 7 wheat
256 genotypes and WMAI 0.28 (GenBank: AJ223492), 18 nsSNPs were identified. The frequency of
257 nsSNPs was 1 out of 25.2 bases.

258 The alignment of the 16 monomeric α -amylase inhibitor haplotypes and the position of the nsSNPs
259 were shown in Supplementary Figure 2.

260 Sequence alignment showed that all the monomeric α -amylase inhibitors were highly homologous
261 and are part of a monomeric α -amylase inhibitor family including WMAI 0.28 and suggested that
262 these inhibitors might have derived from a very limited number of ancestral genes (Wang et al.,
263 2008). Haplotype HM7, which is the most abundant WMAI haplotype, had the same sequence as
264 WMAI 0.28; the other haplotypes showed a percentage of identity with WMAI 0.28 ranging from
265 96.7% to 99.3%.

266 All the deduced proteins of monomeric α -amylase inhibitors had 10 Cys residues which form five
267 disulphide bonds. The disulphide bonds are essential for the inhibitory activity (Carbonero and
268 García-Olmedo, 1999) and in fact the Cys residues were at conserved positions.

269 Monomeric α -amylase inhibitors are highly active against α -amylase of *Tenebrio molitor* and show
270 a low inhibitory activity against mammalian and some avian α -amylases. The 3-D structure of the
271 complex between α -amylase from *T. molitor* and 0.28 α -amylase inhibitor has been determined
272 (Payan, 2004) and three regions of contact have been identified in the mature protein: the N-
273 terminal segment (residues 1-10); residue 53 (inside the second loop segment) and sequence
274 including residues 103-119, which are part of the fourth loop (shown as residues 31-40, 83 and 133-
275 149 respectively in Supplementary Figure 2). In particular, the last region, which corresponds to the

276 C-terminal segment of 0.28, plays an important role in filling the central substrate-binding subsites
277 of *T. molitor* α -amylase and in targeting its catalytic residues. Moreover, this region plays an
278 important role in the specificity of 0.28 inhibitor. Most of the haplotypes shared the same sequence
279 as WMAI 0.28 in these regions, with the following exceptions: HM1, HM9, HM10 and HM13.

280 Other two regions critical for the inhibitory activity have been identified in the mature protein: the
281 N-terminal sequence (positions 1-6) and the sequence after the CRC motif (positions 57-59) (Liu
282 and Wang, 2012). Only haplotype HM1 (found in einkorn) displayed a change at position 6 (shown
283 as residue 36 in Supplementary Figure 2).

284 The current literature lacks information on the peptide sequences and epitopes responsible for the
285 allergies triggered by wheat albumin and globulin proteins (Tatham and Shewry, 2008). Regarding
286 ATIs, there is only one study which identified a high IgE-binding region at the position 9 to 26 in
287 the mature WMAI protein (Walsh and Howden, 1989). As shown as residues 39-56 in
288 Supplementary Figure 2, only two haplotypes had an amino acid change at this level: HM5 and
289 HM14.

290 Whether the sequence changes found in this study and cited above are able to reduce or even delete
291 the inhibitory activity and/or the immunogenic properties is not possible to say from these data.

292 **3.2 Sequence analysis of dimeric alpha-amylase inhibitors (WDAI)**

293 The primers WDAIfor and WDAIrev were used to amplify the ORF of the dimeric alpha-amylase
294 genes (WDAI). All the ten wheat genotypes gave the PCR product of the expected size (~509 bp).

295 From a total of 50 deduced amino acid sequences, 35 haplotypes were identified (HD1-HD35),
296 among which 6 haplotypes represented pseudogenes (indicated with a p after the number), due to
297 the presence of one or more in-frame stop codons because of a single nucleotide polymorphism
298 (substitution, insertion or deletion), although we cannot predict from the genomic data whether
299 these sequences are being expressed (Table 3). All the sequences obtained from the einkorn sample
300 showed an insertion of C at position 160 (from the start codon ATG) which resulted in a premature
301 stop codon and the impossibility to synthesize the correct mature protein. The same insertion of C
302 was observed in the DNA sequences from all the *T. monococcum* accessions and from above all *T.*
303 *boeoticum* accessions in a previous study (Wang, 2007). The absence of gene sequences coding for
304 functional WDAI genes in *T. monococcum* is in agreement with functional studies using targeted
305 MS/MS approach to detect WDAI proteins (Geisslitz et al., 2020, 2018; Rogniaux et al., 2015).

306 All the putatively functional genes were 426 bp long, encoding for a 141 amino acids protein (17
307 amino acids signal peptide and 124 amino acids mature protein). Haplotype HD18 was found in 6
308 clones from 5 different wheat genotypes and it was the most abundant.

309 In this study, after aligning the 35 dimeric α -amylase inhibitor haplotypes from ten wheat
310 genotypes, 69 nsSNPs were identified (Supplementary Figure 3).

311 The frequency of nsSNPs was 1 out of 6.2 bases. The WDAI gene sequences had more divergence
312 than the WMAI gene sequences of this study, which is in agreement with previous studies (Liu and
313 Wang, 2012).

314 All the deduced proteins of WDAI but HD1 and ATI 0.53 had 10 Cys residues which formed five
315 disulphide bonds and were at conserved positions.

316 Studying the modelled complex of human salivary α -amylase (HSA) with 0.19 inhibitor, three
317 spots important for the inhibitory activity of WDAI have been highlighted (Payan, 2004): His47;
318 Ser49 and the sequence Val104 – Val105 – Asp106 – Ala107. Amino acids changes at the level of
319 these three spots can result in a different ability to inhibit human α -amylases. As shown as residues
320 64, 66 and 121-124 respectively in Supplementary Figure 3, the only haplotypes displaying all the
321 above-mentioned amino acids residues at the three inhibitor spots were HD5, HD9, HD11, HD15
322 and HD17. Interestingly, all these haplotypes were found only in hexaploid wheat genotypes, which
323 showed in fact the highest α -amylase inhibitory activity *in vitro* (data not published yet).

324 Considering the percentage of identity with the two amino acid sequences of the well-studied
325 dimeric α -amylase inhibitors 0.19 and 0.53, only the haplotype HD9 had a 100% of identity with
326 0.19. However, the level of homology was high with both 0.19 and 0.53 (ranged from 87.1% to
327 100% for 0.19 and from 84.7% to 99.2% for 0.53), with the exception of the pseudogene HD29p.

328 **3.3 Sequence analysis of tetrameric alpha-amylase inhibitors CM3 (WTAI-CM3)**

329 WTAI-CM3 was selected as representative of WTAI genes in this study because of its extended
330 interaction with α -amylases compared to the other CM subunits (Capocchi et al., 2013) and because
331 it was the most abundant expressed WTAI proteins in cv. Butte 86 (Altenbach, 2011).

332 The sequence of CM3 encoding gene revealed that there is only one ORF encoding the entire
333 protein (Singh et al., 2012).

334 The primers CM3for and CM3rev were designed *in house* and were used to amplify the ORFs of
335 the tetrameric α -amylase gene CM3 and all the samples but einkorn gave the PCR product of the

336 expected size (~ 647 bp). Two sets of internal primers were designed *in house* and were used in all
337 the possible combinations with the einkorn sample, but no band of the expected size was amplified.
338 Moreover, using the sequence of inhibitor WTAI-CM3 (NCBI: X17574) a blast search against the
339 GenBank non-redundant DNA database was performed (access performed on May 11th, 2021), but
340 it was not possible to retrieve any WTAI-CM3 sequence from *T. monococcum*.

341 Some studies were previously performed to quantify allergens abundance in different wheat
342 genotypes and raised doubts on the presence of WTAI-CM3 proteins in diploid wheats. Rogniaux et
343 al. (2015) detected WTAI-CM3 proteins only at very low levels in two *T. monococcum* (cv.
344 Engrain, cv. DV92). Geisslitz et al. (2020, 2018) detected WTAI-CM3 in only four out of eight
345 einkorn cultivars, but in very low concentrations near the LOQ or even below.

346 From a total of 45 deduced amino acid sequences derived from 9 wheat genotypes, 25 haplotypes
347 were obtained (HC1-HC25), among which one haplotype was identified as a pseudogene (HC20p)
348 due to the presence of one in-frame stop codon (table 3). As described before (Singh et al, 2012)
349 there was no intron in the WTAI-CM3 sequences. In this study, no ins/del in the coding region of
350 WTAI-CM3 was found in any of the wheat samples.

351 All the putatively functional genes encoded for a 168 amino acid protein (25 amino acid signal
352 peptide and 143 amino acid mature protein).

353 In this study, after aligning the 25 WTAI-CM3 haplotypes from 9 wheat genotypes, 34 nsSNPs
354 were identified. The frequency of nsSNPs was 1 out of 14.8 bases. Previously, Liu et al. (2012)
355 stated that WTAI genes (CM2, CM3, CM16) are more conserved than WMAI and WDAI genes
356 probably because these subunits are combined to form the tetrameric inhibitor and an amino acid
357 change in one of them can affect the structure and consequently the resulting inhibitory activity.
358 However, in this study results showed that WTAI-CM3 was more conserved than WDAI genes
359 where it was found a snSNPs frequency of 1 out of 6.2 bases, but less conserved than WMAI genes
360 where the snSNP frequency was 1 out of 25.2 bases.

361 The alignment of the 25 WTAI-CM3 haplotypes and the position of the nsSNPs were shown in
362 Supplementary Figure 4.

363 Haplotype HC6, which was the most abundant WTAI-CM3 haplotype, had the same sequence as
364 WTAI-CM3 (X17574) studied by García-Maroto et al. (1990), the other haplotypes showed a high
365 percentage of identity with WTAI-CM3 (X17574) ranging from 92.9% to 99.4%.

366 The most part of the deduced proteins of WTAI-CM3 had 10 Cys residues which form five
367 disulphide bonds and are at conserved positions. The exceptions were HC3 (Tyr instead of the

368 second Cys of the CRC motif); HC9 (Tyr at position 148); HC11 (Arg at position 29); HC18 (Tyr at
369 position 52).

370 The sequence DLPGCPRE (amino acid positions 101-108 of the mature protein, shown as residues
371 126-133 in Supplementary Fig. 4) is the most conserved in CM proteins and may be the
372 characteristic sequence of alpha amylase/ trypsin - bifunctional inhibitors. Most of the haplotypes
373 shared this conserved sequence, with the following exceptions: HC14, HC3, HC4, HC7, HC8,
374 HC21 and HC25 (Supplementary Fig. 4).

375 Capocchi et al. (2013) proposed a structural model for the emmer tetrameric α -amylase inhibitor
376 and showed that two sequences Phe30-Lys55 and Lys116-Gln122 of the mature protein (shown as
377 residues 55-80 and 141-148 respectively in Supplementary Fig. 4) are important for the inhibitory
378 activity against *T. molitor* α -amylase. In this study four amino acid changes occurred in some
379 haplotypes at level of the first sequence: Thr instead of Met at amino acid position 65 occurred in
380 HC16; Val instead of Ala at amino acid position 68 occurred in HC1; Phe instead of Tyr at amino
381 acid position 71 and Met instead of Gly at position 74 both occurred in HC3, HC7, HC8, HC21,
382 HC25 (Supplementary Fig. 4). Interestingly, the haplotypes which had the last two amino acid
383 changes also had changes at the conserved sequence DLPGCPRE and all these were found only in
384 hexaploid wheat genotypes.

385 ATI proteins from wheat have been demonstrated to trigger inflammation by eliciting strong innate
386 immune effects *in vitro* and *in vivo* with the activation of the TLR4–MD2–CD14 complex (Junker
387 et al., 2012). Cuccioloni et al. (2016) investigated the interaction between WTAI-CM3 and human
388 TLR4 and predicted the ATI-TLR4 binding interface regions. These sequences are residues 33-44
389 and 90-100 in the mature protein sequence (shown as residues 58-69 and 115-125 respectively in
390 Supplementary Fig. 4). The haplotypes which showed one or two amino acid changes within these
391 regions were: HC1, HC5, HC8, HC15 and HC16 (Supplementary Figure 4). From the existing
392 evidence it is not possible to say if these changes are able to decrease or delete the ability of CM3-
393 WTAI to bind human TLR4.

394 Several studies suggested that the apparent absence of alpha-amylase inhibitory activity of *T.*
395 *monococcum* against mammalian enzymes could be explained by the fact that the corresponding
396 coding genes might be expressed at very low level or even silenced, perhaps because of gene
397 mutations that prevent the translation into the mature protein (García-Maroto et al., 1990). This
398 hypothesis is in agreement with the gene sequencing results of this study. In fact, einkorn did not
399 show any WTAI-CM3 gene sequence and so it should not be able to produce any tetrameric alpha-
400 amylase inhibitor proteins. Moreover, all the WDAI gene sequences showed an insertion of C at

401 position 160 which resulted in a premature stop codon and the impossibility to synthesize the
402 correct WDAI mature protein. Lastly, einkorn showed WMAI sequences, but it is known that
403 WMAI proteins are highly active against alpha-amylase of *T. molitor* and only weakly inhibits the
404 alpha-amylases from human saliva and pancreas (Payan, 2004). Notwithstanding low levels of ATI
405 proteins and a low alpha-amylase inhibitory activity, Call et al. (2020) found that einkorn samples
406 have high trypsin inhibitory activities and they hypothesized that it could be due to the fact that
407 besides ATIs also lipid transfer proteins (LTPs), grain softness proteins and beta-amylases show
408 high affinity to trypsin.

409 **3.4 Sequence analysis of trypsin inhibitors CMx**

410 The primers CMxfor and CMxrev were designed *in house* and were used to amplify the ORFs of
411 the CMx genes and all the ten wheat genotypes gave the PCR product of the expected size (~ 498
412 bp). Since CMxfor was designed 9 nucleotides downstream the A of the start codon, these first 9
413 nucleotides were taken from the reference sequence (GenBank: X75608) and added to each DNA
414 sequence in order to deduce complete protein sequences for subsequent analyses.

415 From a total of 50 deduced amino acid sequences, 22 haplotypes were obtained (HX1-HX22),
416 among which two haplotypes were identified as pseudogenes due to the presence of one in-frame
417 stop codon (Table 3).

418 HX20 occurred in 15 out of 50 CMx sequences and it turned out to be the most abundant CMx
419 haplotype.

420 The deduced proteins from haplotypes HX1-HX10 were 145 amino acids long and were found only
421 in hexaploid wheat genotypes and the deduced proteins from haplotypes HX13-HX20 were 146
422 amino acids long. Differently, two nucleotide sequences showed a premature stop codon after
423 nucleotide 363 as observed by Sanchez de la Hoz et al. (1994), which resulted in a deduced protein
424 of 121 amino acids (haplotypes HX11 and HX12). Interestingly, emmer wheat showed only the two
425 haplotypes with the deduced protein of 121 amino acids. The homology between the deduced HX11
426 and HX12 amino acid sequences and those of the other CMx haplotypes was maintained beyond the
427 premature stop codon, up to the second stop codon which appeared in the same position as the other
428 haplotypes. All the deduced proteins showed a 25 amino acid signal peptide.

429 In this study, after aligning the 22 CMx haplotypes from 10 wheat genotypes, 39 nsSNPs were
430 identified (Supplementary Figure 5). The frequency of nsSNPs was 1 out of 11.2 bases, similar to
431 what was found for CM3 genes in this study.

432 The most part of the CMx deduced proteins had 10 Cys residues which form up to five disulphide

433 bonds and were at conserved positions. The exceptions were HX3, HX4, HX9, HX11 HX12 and
434 HX17. Interestingly, all the haplotypes found in emmer in this study didn't show all the 10 Cys
435 residues.

436 **3.5 Phylogenetic analysis**

437 The Neighbor-Joining method was used to calculate the phylogenetic distances and to construct the
438 phylogenetic tree among all the haplotypes for each gene (Figure 1).

439 For the 16 WMAI haplotypes the optimal tree with the sum of branch length 0.12061355 was
440 shown (Figure 1A). Cluster III, which displayed all the haplotypes of the tetraploid Peliss and the
441 diploid einkorn, diverged from cluster I, cluster II and HM8, the latter displaying only hexaploid
442 wheat genotypes (in particular cluster II and HM8 displayed only haplotypes from spelt). So, the
443 clusters clearly separated the wheat genotypes according to their ploidy.

444 For the 35 WDAI haplotypes the optimal tree with the sum of branch length 0.96668030 was shown
445 (Figure 1B). HD29p was the most divergent and was highly separated from the other haplotypes.
446 The other pseudogenes from einkorn (HD28p, HD34p, HD35p) diverged together in a separated
447 group within cluster III. Each cluster showed haplotypes from both tetraploid and hexaploid wheat
448 genotypes and from both modern and ancient wheat genotypes, so it was not possible to make any
449 differentiation based on WDAI gene sequences.

450 For the 25 WTAI-CM3 haplotypes the optimal tree with the sum of branch length 0.20342301 was
451 shown (Figure 1C). Two clusters were identified with cluster II displaying only haplotypes from
452 hexaploid wheat genotypes (both modern and ancient genotypes).

453 For the 22 CMx haplotypes the optimal tree with the sum of branch length 0.27715519 was shown
454 (Figure 1D). A first subdivision was between haplotypes HX1-HX10 (which were all 145 amino
455 acids long and found only in hexaploid wheat genotypes) and HX11-HX22p. Then a further
456 subdivision distinguished HX9 and HX10 (cluster IV) from the other haplotypes of the first group
457 (cluster V), while in the second group three clusters were identified: cluster I with haplotypes found
458 in both tetraploid and hexaploid genotypes, cluster II showing the two 121 amino acids long CMx
459 proteins, cluster III showing only haplotypes found in einkorn.

460 According to the phylogenetic analysis of the haplotypes of all the four genes analysed, it was not
461 possible to differentiate the wheat genotypes with respect to ancient or recently developed wheat
462 genotypes.

463 **3.6 Principal Component Analysis (PCA) of WMAI, WDAI, WTAI-CM3 and CMx sequences**

464 A PCA analysis was performed based on deduced amino acid sequences of all the four genes
465 sequenced in this study (WMAI, WDAI, WTAI-CM3 and CMx).

466 To visualise relationships between the tested variables, principal component analysis was run for
467 each species in two steps. First, an interim PCA was run including all the variables (160 nsSNPs),
468 and the quality of representation (Cos2) of each variable was checked. Second, a definitive PCA
469 was run with those variables with a quality of representation higher than 0.5 (103 nsSNPs).

470 The scatter plot reported the projection of cases (10 wheat genotypes) on the first two components
471 PC1 and PC2 and explained the 63.6% of total variance with the first PC accounting for 40.9% and
472 the second PC for 22.7% (Figure 2). Einkorn diverged from all the other genotypes according to the
473 projection of the first component (PC1 axis). The tetraploid wheat genotypes can be divided from
474 the hexaploid wheat genotypes according to the second projection (PC2 axis) with the tetraploid
475 Peliss which was closer to the hexaploid group. This fact can be explained in part by the fact that
476 Peliss was the only tetraploid wheat which was amplified with primers for WMAI genes. It was not
477 possible to differentiate the wheat genotypes with respect to ancient or recently developed wheat
478 genotypes.

479 **3.7 Haplotype analysis**

480 The Nei's genetic distances were calculated for paired comparisons of the ten wheat genotypes
481 (Supplementary Table 2). The values of genetic distances varied from 0.0085 to 0.0321 with an
482 average of 0.022. The genetic distance between turanicum and Alzada was the lowest (0.0085)
483 whereas those between Peliss and both spelt and Vida were the largest.

484 Figure 3 showed the dendrogram based on the Nei's genetic distances. First, einkorn diverged from
485 the other genotypes and this confirmed the results of the PCA analysis and this is in line with the
486 literature which affirmed that *T. monococcum* did not contribute to the evolution of both tetraploid
487 species of *Triticum* and *T. aestivum* through polyploidization (Zhao et al., 2021).

488 Then, the group of the naked tetraploid wheat genotypes diverged from the others (in this group
489 turanicum and Alzada are closer to each other than Peliss probably because they lacked WMAI
490 sequences, unlike Peliss). The hulled tetra/hexaploid wheat genotypes diverged from the naked
491 hexaploid wheat genotypes. In this last group it is interesting to note that the heritage genotypes
492 (Turkey Red and Marquis) were closer than the modern genotypes (Judee and Vida). It is also
493 interesting to highlight that emmer was close to spelt and this is in line with recent studies based on

494 HMW glutenin analysis which showed that the European spelt is a result of hybridization between
495 free-threshing hexaploid wheat and domesticated emmer (Blatter et al., 2004).

496 **4. Conclusions**

497 In this study, the sequences of four representative alpha-amylase/trypsin inhibitor genes have been
498 determined in ten wheat genotypes with different ploidy level and year of release, and some of
499 these, for example *T. monococcum* and *T. turgidum* spp. *turanicum*, have never been sequenced for
500 some or all ATI genes before. So, this study has expanded the available information about ATI
501 sequences.

502 Considering the deduced amino acid sequences of all the four ATI genes studied, both the
503 phylogenetic analysis and the PCA analysis evidenced that the ten wheat genotypes can be
504 differentiated on the basis of their ploidy level. This is in agreement with the fact that ATI genes are
505 present in different chromosomal sets. Overall, einkorn was the most divergent genotype.

506 Based on both the phylogenetic analysis and the PCA analysis of the haplotypes of the four genes
507 analysed, it was not possible to differentiate the wheat genotypes with respect to ancient or recently
508 developed wheat genotypes. Further studies with a higher number of genotypes per each ploidy
509 level are needed to confirm these results. The haplotype analysis, beside confirming these results,
510 also allowed the separation of the hulled from the naked wheat genotypes.

511 The literature lacks information on which sequences of ATIs have immunogenic and inflammatory
512 potential (Geisslitz et al., 2021), so it was not possible to make this kind of consideration on the
513 basis of the deduced amino acid sequences. However, the genetic sequence differences highlighted
514 in this study among the ten genotypes can be the basis for further studies aimed at identifying those
515 sequences in ATI genes able to trigger innate and adaptive immune response. Moreover, the
516 sequence variants of ATIs can be useful information for future breeding programs aimed at
517 selecting wheat varieties with potential pest resistance and minimum inflammatory effect on human
518 health.

519 Finally, it is desirable to measure the inhibitory activities against both alpha-amylase and trypsin
520 enzymes to find possible differences among the ten wheat genotypes and to eventually find a
521 correlation with their genetic sequences.

522 To the best of our knowledge, this is the first study analyzing the ATI sequences of a set of ancient
523 and modern wheat genotypes with a different ploidy level.

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Tables

Table 1

List of wheat genotypes used in this study.

<i>Triticum</i> species	Genotypes	Genome formula	Year of release	Typology	Pedigree	Provenance	Name used in this study
<i>T. monococcum</i> L. subsp. <i>monococcum</i>	Local landrace	AA	/	Hulled, ancient wheat	N.D.	Montana State University (Montana, USA)	Einkorn
<i>T. turgidum</i> L. subsp. <i>dicoccum</i>	Local landrace	AABB	/	Hulled, ancient wheat	N.D.	Montana State University (Montana, USA)	Emmer
<i>T. turgidum</i> L. subsp. <i>turanicum</i> *	QK-77	AABB	/	Naked, ancient wheat	N.D.	Kamut International (Montana, USA)	Turanicum
<i>T. turgidum</i> L. subsp. <i>durum</i>	Peliss	AABB	1900	Naked, heritage durum wheat	LV-Oran	University of Saskatchewan (Canada)	Peliss
<i>T. turgidum</i> L. subsp. <i>durum</i>	Alzada	AABB	2004	Naked, modern durum wheat	Mohawk/Kofa	Montana State University (Montana, USA)	Alzada
<i>T. aestivum</i> L. subsp. <i>spelta</i>	Local landrace	AABBDD	/	Hulled, ancient wheat	N.D.	Montana State University (Montana, USA)	Spelt
<i>T. aestivum</i> L. subsp. <i>aestivum</i>	Turkey Red	AABBDD	1873	Naked, heritage hard red common wheat	(S)Crimean	Hartland Mills (Kansas, USA)	Turkey Red
<i>T. aestivum</i> L. subsp. <i>aestivum</i>	Judee	AABBDD	2011	Naked, modern hard red common wheat	93-X-312-E-14/NuHorizon	Montana State University (Montana, USA)	Judee
<i>T. aestivum</i> L. subsp. <i>aestivum</i>	Marquis	AABBDD	1913	Naked, heritage hard red common wheat	Hard-Red-Calcutta/Red-Fife	Montana State University (Montana, USA)	Marquis
<i>T. aestivum</i> L. subsp. <i>aestivum</i>	Vida	AABBDD	2006	Naked, modern hard red common wheat	Scholar/Reeder	Montana State University (Montana, USA)	Vida

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Table 2

List of primers used in this study.

Gene	Name	Sequence
WMAI (NCBI GenBank: AJ223492)	Mfor1	5' – CACCACTTATATCCAAGGACCA – 3'
	Mfor2	5' – AGCTTGCTTTGATTCTGCTGAT – 3'
	Mfor3	5' – ACTAAATTGAAACAATGTGGAT – 3'
	F2	5' – ATGTGGATGAAGACCGKGT – 3'
	F4	5' – ATGCTCGTGCGACAACAAT – 3'
	F3	5' – ACAACAATGGCGGTTCGAGTA – 3'
	F1	5' – CATAACAGTGGTCCTTGGAGT – 3'
	Mfor4	5' – GCAATGGTGAAGCTCCAGT – 3'
	Mfor5	5' – AGCTGGCCGACATCAACA – 3'
	Mrev1	5' – TCTCTGAGAGGACACATACACCA – 3'
	R1	5' – CACGCACCGCACCATTACTT – 3'
	Mrev2	5' – CACCGCACCAATTAAGAT – 3'
	Mrev3	5' – CGCACCAATTAAGATGCAGA – 3'
	Mrev4	5' – TTAAGATGCAGATTCGCTTGAC – 3'
	R2	5' – GACTAGRYGTCCGKATACGC – 3'
	Mrev5	5' – GATGGGCACCTTGCAGAC – 3'
Mrev6	5' – GCACCTCCTTCCCCTCAC – 3'	
WDAI (NCBI GenBank: AK330823)	WDAIfor	5' – CTATGTATGCTCGTGCGAC – 3'
	WDAIrev	5' – ACTCATTYGCTTGACTAGGC – 3'
WTAI-CM3 (NCBI GenBank: X17574)	CM3for	5' – CGAACCAGACTTGGCTAGAATA – 3'
	CM3intfor2	5' – ACAACAAACTTGTGGCACCT – 3'
	CM3intfor	5' – GCGCTGCGCTACTTCATA – 3'
	CM3rev	5' – ATTCATAGCAGATAGCCCACAC – 3'
	CM3intrev2	5' – TGTGAATGGTCGCCAAGT – 3'
	CM3intrev	5' – GCCGCTCTCACCAACAT – 3'
CMx (NCBI GenBank: X75608.1)	CMxfor	5' – AAGCACCAGCTCATCCTCTC – 3'
	CMxrev	5' – ATACACATATGCGATTTCGTCCA – 3'

Table 3

Distribution of WMAI, WDAI, WTAI-CM3 and CMx haplotypes in the ten wheat genotypes. The numbers under parenthesis indicate how many times the haplotype occurred in the 5 sequences analysed per each wheat genotype.

GENOTYPES	WMAI HAPLOTYPES	WDAI HAPLOTYPES	WTAI-CM3 HAPLOTYPES	CMx HAPLOTYPES
Einkorn	HM1 (1), HM2 (1), HM3 (3)	HD28p (2), HD29p (1), HD34p (1), HD35p (1)	/	HX13 (3), HX14 (1), HX21p (1)
Emmer	/	HD8 (1), HD18 (1), HD26 (2), HD27 (1)	HC6 (3), HC16 (1), HC17 (1)	HX11 (4), HX12 (1)
Turanicum	/	HD18 (2), HD20 (1), HD21 (1), HD22 (1)	HC6 (1), HC9 (1), HC10 (1), HC11 (1), HC12 (1)	HX17 (1), HX18 (1), HX20 (3)
Peliss	HM3 (3), HM4 (1), HM5 (1)	HD1 (1), HD2 (1), HD4 (1), HD18 (1), HD32 (1)	HC6 (4), HC18 (1)	HX20 (5)
Alzada	/	HD8 (1), HD18 (1), HD23 (1), HD24 (1), HD25 (1)	HC6 (2), HC13 (1), HC14 (1), HC15 (1)	HX19 (1), HX20 (4)
Spelt	HM6 (1), HM7 (1), HM8 (1), HM9 (1), HM10 (1)	HD15 (2), HD31 (1), HD30p (2)	HC6 (2), HC7 (1), HC22 (1), HC23 (1)	HX3 (1), HX8 (1), HX11 (3)
Turkey Red	HM7 (3), HM11 (1), HM12 (1)	HD5 (1), HD6 (1), HD7 (1), HD8 (1), HD9 (1)	HC6 (2), HC19 (1), HC25 (1), HC20p (1)	HX1 (1), HX5 (1), HX8 (2), HX9 (1)
Judee	HM7 (3), HM13 (1), HM14 (1)	HD9 (1), HD10 (1), HD11 (1), HD12 (1), HD33 (1)	HC1 (1), HC2 (1), HC3 (1), HC4 (1), HC5 (1)	HX6 (1), HX15 (1), HX16 (1), HX20 (2)
Marquis	HM7 (4), HM15 (1)	HD13 (1), HD14 (1), HD15 (2), HD16 (1)	HC6 (2), HC7 (1), HC21 (1), HC24 (1)	HX7 (1), HX8 (1), HX10 (1), HX20 (1), HX22p (1)
Vida	HM7 (2), HM15 (2), HM16 (1)	HD3 (1), HD9 (1), HD17 (1), HD18 (1), HD19p (1)	HC6 (2), HC7 (1), HC8 (2)	HX2 (1), HX4 (1), HX8 (3)

Supplementary Table 1

Combinations of primer sets used to amplify WMAI genes. For each primer set the expected amplicon size (bp) and annealing temperature used (°C) are listed.

Reverse Forward	R1	R2	Mrev2	Mrev3	Mrev5	Mrev6	Mrev4	Mrev1
F1	445 bp 50°C	374 bp 62°C	404 bp 50°C	401 bp 50°C				
F2	529 bp 58°C	458 bp 50°C	488 bp 50°C	485 bp 56°C				
F3	478 bp 50°C	407 bp 56°C	437 bp 52°C	434 bp 55°C				
F4	490 bp 50°C	419 bp 50°C	449 bp 50°C	446 bp 55°C				
Mfor3	543 bp 54°C	472 bp 50°C	502 bp 50°C	502 bp 50°C				
Mfor4				329 bp 54°C	240 bp 54°C	169 bp 54°C		
Mfor5				262 bp 58°C	173 bp 54°C	102 bp 54°C		
Mfor1							550 bp 50°C	
Mfor2								623 bp 50°C

Supplementary Table 2

Nei's genetic distance matrix of WMAI, WDAI, WTAI-CM3 and CMx haplotypes in the ten wheat genotypes.

Wheat genotype	Einkorn	Emmer	Turanicum	Peliss	Alzada	Spelt	Turkey Red	Judee	Marquis
Emmer	0.0274								
Turanicum	0.0224	0.0206							
Peliss	0.0296	0.0287	0.0167						
Alzada	0.0249	0.0205	0.0085	0.0124					
Spelt	0.0264	0.0133	0.0221	0.0321	0.0229				
Turkey Red	0.0256	0.0220	0.0212	0.0312	0.0212	0.0192			
Judee	0.0247	0.0272	0.0169	0.0286	0.0177	0.0237	0.0166		
Marquis	0.0281	0.0254	0.0212	0.0294	0.0211	0.0174	0.0130	0.0157	
Vida	0.0273	0.0237	0.0212	0.0321	0.0229	0.0201	0.0130	0.0210	0.0148

Figure captions

Figure 1.

Phylogenetic analysis of WMAI (A), WDAI (B), CMx (C) and WTAI-CM3 (D) haplotypes. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. In red are indicated in which genotypes the haplotypes occurred. *T. turgidum* L. subsp. *durum* and *T. aestivum* L. subsp. *aestivum* genotypes are indicated with the abbreviations DU and AE respectively.

Figure 2.

Principal component analysis based on deduced WMAI, WDAI, WTAI-CM3 and CMx amino acid sequences. The scatter plot reports the projection of cases (10 wheat genotypes) on the first two components PC1 and PC2 (accounting for 63.6% of total variability): the diameter of each balloon is proportional to the variability observed for the wheat genotype. Each colour of the balloons corresponds to a different wheat ploidy: green for diploid wheat; red for tetraploid wheat; blue for hexaploid wheat.

Figure 3.

The dendrogram of the ten wheat genotypes based on Nei's genetic distance matrix.

Supplementary Figure 1.

The gene sequence of monomeric α -amylase inhibitor 0.28 (AJ223492) and the primers used to amplify WMAI genes. The sequences of signal peptide and mature protein are highlighted in red and blue respectively. The stop codon is marked with an asterisk.

Supplementary Figure 2.

Multiple sequence alignment of 16 WMAI haplotypes obtained from seven wheat genotypes and WMAI 0.28 (GenBank: AJ223492). The sequences related to the signal peptide have been included (residues 1 – 30 highlighted with a light blue box). The dots indicate conserved residues and the letters correspond to the substituted amino acid residues for each alignment gap. Blue arrows highlight the positions of the 10 Cys residues. The amino acids at the three inhibitor spots (Payan et al., 2004) are highlighted with yellow boxes. The two regions critical for the inhibitory activity (Garcia-Maroto et al., 1991) are marked with red rectangles.

Supplementary Figure 3.

Multiple sequence alignment of 35 WDAI haplotypes obtained from ten wheat genotypes with WDAI 0.19 (Uniprot: P01085) and 0.53 (Uniprot: P01084). The sequences related to the signal peptide have been included (residues 1 – 17 highlighted with a light blue box). The dots indicate conserved residues and the letters correspond to the substituted amino acid residues for each alignment gap. The pseudogenes have been included: in-frame stop codons are indicated with asterisks *; insertions and deletions are indicated with blue and red triangles respectively and the sequences after these changes until the end of the ORF of the corresponding functional gene is shown. Blue arrows highlight the positions of the 10 Cys residues. The amino acids at the three inhibitor spots are highlighted with yellow boxes.

Supplementary Figure 4.

Multiple sequence alignment of 25 WTAI-CM3 haplotypes obtained from nine wheat genotypes and WTAI-CM3 (GenBank: X17574). The sequences related to the signal peptide have been included (residues 1 – 25 highlighted with a light blue box). The dots indicate conserved residues and the letters correspond to the substituted amino acid residues for each alignment gap. The asterisk * shows in-frame stop codons of pseudogene. Blue arrows highlight the positions of the 10 Cys residues.

The conserved sequence DLPGCPRE is marked with a red rectangle, the amino acid residues of contact with TMA with yellow boxes and the ATI-TLR4 binding interface regions with blue rectangles.

Supplementary Figure 5.

Multiple sequence alignment of 22 CMx haplotypes obtained from different wheat genotypes with CMx1/CMx3 (deduced from the sequence GenBank: X75608) and with CMx2 (deduced from the sequence GenBank: X75609). The sequences related to the signal peptide have been included (residues 1 – 24 highlighted with a light blue box).

The asterisks * show in-frame stop codons. The red triangle shows the positions of the deletion found in the nucleotide sequence used to deduce the amino acid sequence. The dots indicate conserved residues and the letters correspond to the substituted amino acid residues for each alignment gap. Blue arrows highlight the positions of the 10 Cys residues.











