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Molecular Evolution of Exogenous Alpha-amylase Inhibitors in Triticeae - An Update

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ABSTRACT

Exogenous α-amylase inhibitors in seeds and vegetative organs are attractive candidates for the control of seed weevils as these insects are highly dependent on starch as an energy source. In cereal seeds, α-amylase inhibitors proteins were known as one great family on the basis of the homology of their sequences. The overall information on the monomeric, dimeric and tetrameric α-amylase inhibitors strongly support the view that these inhibitors have evolved from a common ancestor gene through duplication and mutation. All α-amylase inhibitors from Triticeae (21 different genomes) have 10 conserved cysteine (5 disulfide bonds) sharing common cysteine skeleton. In the past years the α-amylase inhibitors have been well studied, including large amount of attention that directed towards their evolutionary relationship. Adaptive evolution of monomeric and dimeric α-amylase inhibitor genes and its ecological association has been investigated in wild emmer wheat. They were contributed by both natural selection and co-evolution, which ensures the conserved function as well as the inhibition of a variety of insect amylases. The known aspects of α-amylase inhibitors and their molecular evolution in Triticeae have been discussed in this review.

Keywords: adaptive evolution; alpha-amylase inhibitor; cysteine skeleton; Triticeae
Abbreviations: 3D, three-dimensional; **AMY**, alpha-amylase; **cSNP**, coding sequences single nucleotide polymorphism; **FAB-MS**, fast atom bombardment mass spectrometry; **MS**, mass spectrometry; **RDAI**, rye dimeric alpha-amylase inhibitor; **SNP**, single nucleotide polymorphism; **WDAI**, wheat dimeric alpha-amylase inhibitor; **WMAI**, wheat monomeric alpha-amylase inhibitor; **WTAI**, wheat tetrameric alpha-amylase inhibitor

CONTENTS

INTRODUCTION

Starch is the main carbon-storage molecule of plants and is the main source of energy for animals, including humans. The kernel of wheat and related Triticeae species contains a number of protein components capable of inhibiting many α-amylase [α- (1, 4)-glucan-4-glucanohydrolases], which are a family of enzymes that hydrolyze α-D-(1,4)-glucan linkages in starch, and play an important role in the carbohydrate metabolism of many autotrophic and heterotrophic organisms (MacGregor *et al*. 2001; Kumar *et al.* 2009). The endogenous α-amylase (AMY1, AMY2 and AMY3) in cereals are very important in initiating starch degradation in cereal grains, which are expressed during seed maturation and germination. Heterotrophic organisms use exogenous α-amylase primarily to digest starch in their food sources (Silva *et al*. 2000; Gorjanović 2009). Chitinase and β-1, 3-glucanase enzymes, lectins, arcelins, vicilins, systemins and enzyme inhibitors are the proteins, which could protect plants against the attack of insect pests and pathogens (Ryan 1990; Ryan and Pearce 1998; Sales *et al*. 2000; Nieuwenhuizen *et al*. 2007; Gorjanović 2009; Gbaye *et al.* 2011). Several α-amylase and proteinase inhibitors present in seeds and vegetative organs act to resist phytophagous insects (Konarev 1996; Chrispeels *et al*. 1998; Gatehouse and Gatehouse 1998; Wisessing *et al.* 2010). α-Amylase inhibitors are attractive candidates for the control of seed weevils as these insects are highly dependent on starch as an energy source (Franco *et al*. 2000; Nagy-Gasztonyi *et al*. 2010). For weevil control, the members from α-amylase inhibitor family could be used by plant genetic engineering such as transgenic (Prescott *et al*. 2005). Many insects have several α-amylases that differ in specificity, and successful utilization of a food source is dependent on the presence of α-amylase for which there is no specific inhibitor (Silva *et al*. 2000; Bonavides *et al*. 2007).

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Invited Review

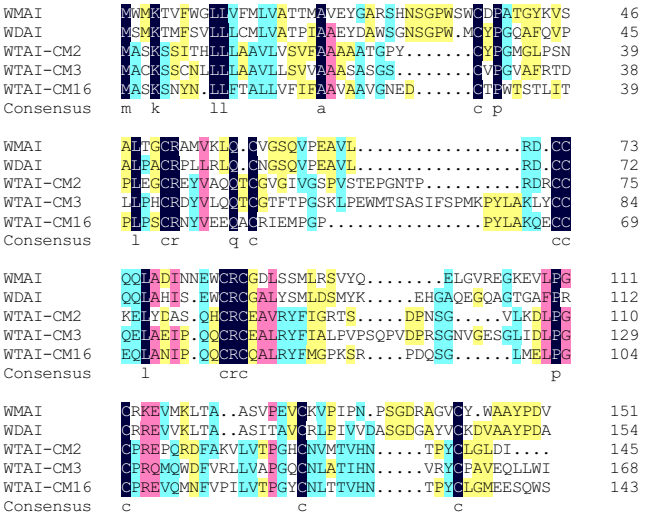

Fig. 1 The sequences alignment of WMAI, WDAI, WTAI-CM2, WTAI-CM3 and WTAI-CM16 in wheat. The protein sequences were obtained from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/): WMAI (AJ223492), WDAI (AY856085), WTAI-CM2 (X55454), WTAI-CM3 (X17574), and WTAI-CM16 (X17573). *Highlight homology level: 100% (dark blue), ≥ 75% (pink), ≥ 50% (sky blue), ≥ 33% (yellow).

It is known that the bulk of seed albumins consist of a few amylase isoinhibitor families which are likely phylogenetically related and which are coded by a small number of parental genes (Buonocore *et al*. 1977; Nagy-Gasztonyi *et al*. 2010; Dupont *et al.* 2011). In cereal seeds, α-amylase inhibitors proteins with 120-130 amino acids, which include trypsin inhibitors as well as α-amylase inhibitors, are known as one great family on the basis of the homology of their amino acid sequence (Strobl *et al*. 1995; Wang *et al*. 2005, 2010a). The monomeric, homo-dimeric and hetero-tetrameric α-amylase inhibitor (WMAI, WDAI and WTAI) are the main members that are active on exogenous α-amylases from various origins in the wheat kernel (**Fig. 1**). In the absence of dissociating agents, wheat α-amylases inhibitors which are active to exogenous α-amylases could be fractionated by gel filtration into three isoinhibitor families with apparent molecular weight close to 60 kDa, 24 kDa and 12 kDa (Deponte *et al*. 1976; Pelegrini *et al*. 2008). WMAI is an inhibitor with molecular weight 12 kDa; WDAI is combined with two 12 kDa units, that is 24 kDa; WTAI is a mixture (about 60 kDa) of WTAI-CM2 plus 2 WTAI-CM3 plus WTAI-CM16, where none of the subunits is active on its own (Gomez *et al*. 1989; Alfonso *et al*. 1997). Although WTAI-CM3 and WTAI-CM16, have no *in vitro* α-amylase inhibitory activity individually, they do show increased *in vitro* inhibitory activity to insect α-amylases when they are combined with WTAI-CM2 to form a tetrameric protein (Buonocore *et al*. 1985; Sánchez-Monge *et al*. 1986; Garcia-Olmedo *et al*. 1987; Gomez *et al*. 1989). It is well established that each family is closely related by having largely identical amino acid sequences and conformational structure and it was suggest that the α-amylase inhibitors are derived from common ancestral genes (Silano *et al*. 1977; Wang *et al*. 2010a).

MOLECULAR CHARACTERIZATION OF ALPHA-AMYLASE IN CEREALS

Nucleotide sequences encoding alpha-amylase in cereals

Recently, many genes encoding mono-, di- and tetrameric α-amylase inhibitors were identified from members of the Triticeae family (**Table 1**). Among them, inhibitors from wild emmer wheat (*Triticum dicoccoides*), common wheat (*T. aestivum*), rye (*Secale cereale*) and barley (*Hordeum vulgare*) were well studied.

The WMAI genes in cereals were obtained with similar nucleotide sequences without any insertion or deletion in the coding region. The monomeric α-amylase inhibitors share very high homology (99.54%). Little evidence could show that there were WMAI genes in the A genome of hexaploid wheat, whereas the A genome of diploid wheat had WMAI genes (Wang *et al*. 2008b). The monomeric α-amylase inhibitors from wheat and *Aegilops* strongly support the view that these inhibitors have evolved from a common ancestor gene through duplication and mutation.

Sequence alignment indicated that all WDAI sequences from the Triticeae were also highly homologous (Wang *et al*. 2010a). Most of the sequences had 375 nucleotides and only few had 3/6/9 bp insertions or deletions, which could encode dimeric α-amylase inhibitors with 121 to 125 amino acid residues. Compared with WDAI from common wheat,

Table 1 The gene sequences of monomeric, dimeric and tetrameric α-amylase inhibitor identified from Triticeae.

Origin (genome)/Gene	WMAI	WDAI	T-CM2**	T-CM3**	T-CM16**
Triticum urartu (Au)	4	42	0	0	0
Triticum boeoticum (Am)	0	18	0	0	0
Triticum monococcum (Am)	16	49	0	0	0
Triticum dicoccoides (AB)	353	244	115	131	100
Triticum aestivum (ABD)	246	551	324	479	417
Hordeum vulgare (I)	605	9	373	475	238
Triticum araraticum (AG)	0	20	0	0	0
Aegilops tauschii (D)	8	34	0	0	0
Aegilops bicornis (Sb)	3	10	0	0	0
Aegilops speltoides (S)	0	28	0	0	0
Aegilops sharonensis (Ssh)	0	27	0	0	0
Aegilops searsii (Ss)	0	24	0	0	0
Aegilops longissima (Sl)	3	23	0	0	0
Aegilops uniaristata (N)	0	6	0	0	0
Eremopyrum bonaepartis (F)	0	10	0	0	0
Heteranthelium piliferum (Q)	0	5	0	0	0
Secale cereale (R)	0	9	0	0	0
Elytrigia bessarabicum (Eb)	0	6	0	0	0
Australopyrum retrofractum (W)	0	4	0	0	0
Aegilops comosa (M)	0	8	0	0	0
Aegilops umbellutata (U)	0	5	0	0	0
Henrardia persica (O)	0	7	0	0	0
Agropyron desertorum (P)	0	4	0	0	0

The data were obtained from NCBI (National Center for Biotechnology Information) on 4th Feb 2012.
** Tetrameric alpha-amylase inhibitors WTAI-CM2, WTAI-CM3, and WTAI-CM16 in common wheat were similar to BTAI-CMa, BTAI-CMd, and BTAI-CMb in barley.

the sequences from Q, W and O genomes had 3/6/9 bp deletions respectively, while the sequences from R and E genomes had a 3-bp insertion (Wang *et al*. 2010a). All the WDAI gene sequences from *T*. *urartu* and only few sequences from *T*. *boeoticum* were 375 bp in length, and the others were 376 bp. One insertion was observed in the sequences from 72% *T*. *boeoticum* and all *T*. *monococcum* accessions since these sequences could not encode the correct mature proteins. Genes from the A^m genome were more conserved than those of the A^u genome. The frequency of SNPs (Single Nucleotide Polymorphisms) in the α-amylase inhibitor genes from the A^m genome was 3.45% (Wang *et al*. 2007a). The frequency of SNPs in the B (a.k.a. S) genomes encoding WDAI genes was 17% (Wang *et al*. 2007b). As expected, most of the SNPs in dimeric α-amylase inhibitor genes were transitions and the rest were transversions. The A-T transversions were not detected in these dimeric α-amylase inhibitor genes. A total of 59 haplotypes were defined in the B (a.k.a. S) genomes coding for WDAI genes, among which 4 main haplotypes occurring in more than 10 genes and 36 haplotypes with a single gene were found (Wang *et al*. 2007b). Dimeric α-amylase inhibitors might be derived from a very limited number of ancestral genes.

Hundreds of tetrameric α-amylase inhibitor coding gene sequences (114, 124 and 96 of WTAI-CM2, WTAI-CM3 and WTAI-CM16, respectively) were characterized form emmer wheat (unpublished data). The frequency of SNPs in them was 1 out of 87.6, 101.4, and 108.0 bases, where 5, 5 and 4 cSNPs were detected in the coding sequence, which were lower than the SNPs observed form WDAI and WMAI genes in wild emmer wheat (Wang *et al*. 2008a, 2010b). It was found that the coding sequences of mono-, di- and tetrameric α-amylase inhibitors had very high polymorphisms. However, WTAI was much more conserved than WMAI and WDAI.

Recently, coding sequences for mono-, di- and tetrameric α-amylase inhibitors were unequivocally associated with specific proteins identified by tandem mass spectrometry (MS/MS) in proteomic analysis of milled white flour from US wheat cultivar Butte 86 (Altenbach *et al.* 2011). And proteins corresponding to WDAI, WMAI and WTAI subunits CM2, CM3 and CM16 were accumulated to the highest levels in flour (Altenbach *et al.* 2011). Characterization of inhibitors and their coding genes in a certain cultivar will help us to understand their expression pattern, their relationship, and the role of individual inhibitors in cereals.

Characterization of protein sequences of inhibitor

In molecular genetics, there is a growing interest in the relationship between genetic variation and individual differences in biological function. Different amino acid residues determined by polymorphic sites significantly affected the structure, charge and function between inhibitors (Maeda *et al*. 1985; Wang *et al*. 2005). The charge difference might result in the relative mobility of gel electrophoresis and the differential inhibitory activities of α-amylase inhibitors (Maeda *et al*. 1985; Lin *et al*. 2007; Fontanini *et al*. 2007). In the former investigations, more than 5 inhibitor units of the monomeric α-amylase inhibitor family were found viz. inhibitor 0.28, 0.32, 0.35, 0.39 and 0.48. They had similar molecular weight but different inhibitory activity (Buonocore *et al*. 1997). Moreover, the different structures of inhibitors affect the specificity and activity against different mammalian and insect α-amylases (Feng *et al*. 1996; Franco *et al*. 2002; Murayama *et al*. 2009). The data concerning a new α-amylase inhibitor named Inhibitor II is more confusing because, although the structural information clearly indicates that this protein is either identical with or very closely related to WMAI 0.28, the inhibition specificity differs from that reported for inhibitor 0.28. WMAI 0.28 was supposed to inhibit only the insect enzyme, whereas Inhibitor II inhibits both mammalian and insect enzymes (Sánchez-Monge *et al*. 1986; Murayama *et al*. 2009). Moreover, in this case of Inhibitor II, the only significant discrepancy of WMAI 0.28 seems to be the possible presence in its protein sequence of 1 His and 1 Phe (Sánchez-Monge *et al*. 1986). It suggested that there are at least two members with different functions of the WMAI family. However, it was found that the amino acid sequences of WMAI members had high homology (99.24%), which had less divergence than WDAI.

The presence of CC and $CX_{1-4}C$ motifs is usually observed in this kind of seed protein. It was clear that the members of α-amylase inhibitor super family had a common conserved cysteine skeleton C-Xn-C-Xn-C-Xn-CC-Xn-C-X-C-Xn-C-Xn-C-Xn-C (**Table 2**). This cysteine skeleton pattern also appeared in many other cereal protein allergens namely, Acyl-CoA oxidase, Thioredoxin, Fructose bisphosphate aldolase, and Peroxidase (Wang *et al*. 2010c; Sander *et al*. 2011). Both WMAI 0.28 and 0.39, similar to WDAI 0.19, were readily inactivated by treatments that break disulphide bonds, thus indicating that their stability is mainly dependent on the integrity of their disulphide bridges (Petrucci *et al*. 1978; Om and Nivedita 2010). The assignment of the five disulfide bridges in WMAI 0.28 from wheat kernel was achieved by combining fast-atom-bombardment mass spectrometry (FAB-MS) and automatic sequencing based on Edman degradation (Poerio *et al*. 1991). By combining FAB-MS and automatic sequencing, it was possible to assign the five disulfide bonds of WMAI 0.28 from wheat kernel as follows: Cys7-Cys54, Cys21 - Cys42, Cys29-Cys82, Cys43-Cys98 and Cys56-Cysl13 (Poerio *et al*. 1991). The deduced proteins of the monomeric α-amylase inhibitors had 10 Cys, and their positions were conserved, which indicated that the Cys were important for these inhibitors' three dimensional structure. Additionally the substantial difference present in the C-terminal of the loop region including residues 103-119 played an important role in the specificities of WMAI 0.28 and WDAI 0.19 (Franco *et al*. 2000; Payan *et al*. 2004). Overall, the 3D structure of WMAI 0.28 was similar to that of WDAI 0.19 (Oda *et al*. 1997; Om and Nivedita 2010).

Most polymorphic sites in WMAI alleles did not occur at a conservative site, which ensures that the inhibitors maintain their activity structure to combine with α-amylase. Different amino acid residues could also affect the charge, structural and inhibitory activities between WMAI 0.28 and 0.39. The two inhibitors undergo significant structural changes with only one different amino acid residue (Silano *et al*. 1977). The mutations made a difference in charge that might result in the relative mobility of gel electrophoresis and the differential inhibitory activities of monomeric inhibitors 0.28, 0.32, 0.35, 0.39 and 0.48 with similar mole-

Table 2 Conserved cysteine skeleton of some seed proteins (from Wang *et al*. 2010c).

Seed proteins	Cys Pattern and specific Cys motifs
Monomeric alpha-amylase inhibitor	C-X_n-C-X_n-C-X_n-CC-X_n-C-X-C-X_n-C-X_n-C-X_n-C
Dimeric alpha-amylase inhibitor	C-X_n-C-X_n-C-X_n-CC-X_n-C-X-C-X_n-C-X_n-C-X_n-C
Tetrameric alpha-amylase inhibitor	C-X_n-C-X_n-C-X_n-CC-X_n-C-X-C-X_n-C-X_n-C-X_n-C
Acyl-CoA oxidase	C-X_n-C-X_n-C-X_n-C-X_3-C-X_n-C-X_n-C-X-C-X_n-C-X_n-C-X_n-C
Fructose bisphosphate aldolase	C-X_n-C-X_n-C-X_n-C-X_n-C-X_n-C-X_n-C
Thioredoxin	C-X_2-C
Peroxidase	C-X_n-C-X_4-C-X_n-C-X_n-C-X_n-C-X_n-C-X_n-C
Lipid transfer protein	C-X_n-C-X_n-CC-X_n-C-X-C-X_n-C-X_n-C
Triosephosphate isomerase	C-X_n-C-X_n-C-X_n-C

cular weight (Buonocore *et al*. 1977).

Earlier site-directed insertion mutagenesis of WMAI 0.28 from wheat surprisingly showed structural changes at amino acid position 1 or 4 to reduce, but not completely destroy the ability to inhibit α-amylase (García-Maroto *et al*. 1991). A synthetic gene encoding WMAI 0.28 was introduced into a vector for expression in *Escherichia coli* and 13 mutants were obtained at six different sites. Expression studies of these genes would broaden our knowledge on the functional behavior of WMAI. Synthetic WMAI had the correct N-terminal sequence, the same electrophoretic mobility and specific activity towards the α-amylase from the insect *Tenebrio molitor* as the native WMAI isolated from wheat. Two regions of WMAI amino acid sequences are critical for the inhibition mechanisms: 1) N-terminal sequence before the 1st Cys, and 2) sequence after the 7th Cys that was right after a CRC motif (positions 54-56). They are conserved throughout the WMAI family (García-Maroto *et al*. 1991). The roles of these regions seemed to be different, since the mutations in the first region affected the kinetics formation of the enzyme-inhibitor complex and tended to have a moderate effect on inhibitory activity, whereas all the mutations at the second region, even a single amino acid insertion, rendered the inhibitor completely inactive (García-Maroto *et al*. 1991). Thus, the first region plays a role in the postulated conformational change and is less critical to the stability of the enzyme-inhibitor complex, for which the second region would be rather stringently critical. According to the amino acid sequences alignment, only the 4th (Trp-Gly), 5th (Ser-Asn), 109th (Arg-Gly), 111st (Gly-Arg-Ser) and 120th (Asp-Gly) amino acids of WMAI were changed by nucleotide mutations. Most of the amino acids in central domain were conserved that ensured the stability of WMAI. However the change of the 4th and 5th amino acid might result in the different conformational change, these mutations were in low frequency.

The crystal structure of WDAI-0.19 was determined by the multiple-isomorphous replacement method coupled with density modification and noncrystallographic symmetry averaging and then refined by simulated annealing using diffraction data to 2.06 Å resolution (Oda *et al*. 1997). From the structure of WDAI-0.19, it was known that not only the 10 Cys residues were of importance, but Asp110, Lys116, Asn29, Glu35, Ser94, Leu90, Trp51, His47 and Gln13 were also important to form the structure of inhibitors (Oda *et al*. 1997). Three inhibitor spots of interest were proposed using the modeled complex of human salivary α-amylase with α-amylase inhibitor 0.19. The first was residue His47, the second concerned Ser49, and the third region of interest was the sequence Val104-Val105-Asp106-Ala107 (Franco *et al*. 2000). Additionally the substantial difference present in the C-terminal of the loop region including residues 103-119 played an important role in the specificities of WDAI (Payan 2004). The first 9 amino acid residues were one of the most conserved regions of the inhibitor from Triticeae, but the Q genome inhibitors had 3 amino acid mutations and 1 insertion, which occurred only in *Heteranthelium piliferum* α-amylase inhibitors. The Lys116, Asn29, Gln13, Ser49, and Val104-Val105-Asp106-Ala107 motifs were probably mutated in the Q genome α-amylase inhibitors. This might be the reason why the sequences from the Q genome samples did not appear like the other inhibitors.

Compared with WDAI 0.19 and WDAI 0.53 from common wheat, the sequences from Q, W, O genomes had 3, 2, 1 amino acid residues deletion respectively, while the sequences from R and E genome had an amino acid residues insertion. Most mutations did not occur at the conserved sites, which ensured the stable structure and activity to combine with the α-amylase. All the dimeric α-amylase inhibitors (except the Q genome sequences) had the Ser49, which was closely packed by Lys352 and Asp356 of human salivary α-amylase and by Trp51 and Cys52 of its own (Franco *et al*. 2000). This meant that this position was conserved. It was noteworthy that only few α-amylase inhibitors closely related to WDAI 0.19 (D genome sequence from *Ae. tauschii* and common wheat) from D, S, N, R, I, U and E^b genomes had the His47 which was replaced by the Asp, Lys or Asn amino acids in most of the other inhibitors. It was proposed that His47 was situated near to Glu349 of human salivary α-amylase (Franco *et al*. 2000). Furthermore, each inhibitor of the 24 kDa α-amylase inhibitor family consisted of four similar subunits. Therefore, one amino acid change in one subunit would have resulted in four times change in the inhibitor. This could explain that all the inhibitors in this family shared very high sequence coherence but showed dramatically different abilities to inhibit human salivary α-amylase activity.

Chromosome location

The chromosome locations of the main α-amylase inhibitor genes are list in **Table 3**. Compensating nulli-tetrasomic and ditelosomic lines of 'Chinese Spring' had been analyzed by two-dimensional electrophoresis, under conditions in which there was no overlap of the inhibitors with other proteins, and the chromosome locations of the genes encoding these inhibitors have been established: genes for WDAI were in the short arm of chromosome 3, and that for WMAI in the short arm of chromosome 6 (Sánchez-Monge *et al*. 1986). Major components of the monomeric inhibitors from Chinese Spring had been purified and characterized. Their molecular size and amino acid composition are quite similar, but their different inhibitory activities indicate that two different pairs of closely related components can be distinguished (Gomez *et al*. 1991). The sequence homology between these inhibitors and the equivalent positions of their respective genes in the short arms of chromosomes 6D and 6B clearly show that the genes were homoeologous (Gomez *et al*. 1991). A cDNA probe corresponding to WMAI did hybridize with DNA in the 6BS and 6DS, but did not recognize any DNA fragment from the A genome (Gomez *et al*. 1991). In the former investigations, no monomeric inhibitor was found in association with the A genome of hexaploid wheat, as was the case for other inhibitor classes. No WDAI activity has been detected in diploid wheat *T. monococcum* and *T. boeoticum* also (Bedetti *et al*. 1974; Vittozzi *et al*. 1976). Moreover, it was absent from the A genome in the tetraploid and hexaploid wheat according to the analysis of ditelosomic stocks by two-dimensional electrophoresis and isoelectric focusing of wheat pro-

Table 3 The chromosome locations of the main α-amylase inhibitor genes in cereals.

Species	Genes	Protein aggregation	Chromosome location	References
Triticum aestivum	WMAI	monomeric	6BS/6DS	Sánchez-Monge *et al*. 1986; Gomez *et al*. 1991
Triticum aestivum	WDAI	dimeric	3BS/3DS	Sánchez-Monge *et al*. 1986; Singh *et al*. 2001; Wang *et al*. 2006a
Triticum aestivum	WTAI-CM2	tetrameric	7BS	Framon *et al*. 1984
Triticum aestivum	WTAI-CM3	tetrameric	4AS	Framon *et al*. 1984
Triticum aestivum	WTAI-CM16	tetrameric	4AS	Framon *et al*., 1984
Hordeum vulgare	BMAI	monomeirc	2H	Witzel *et al*. 2010
Hordeum vulgare	BDAI	dimeric	6H	Lázaro *et al*. 1988; Witzel *et al*. 2010
Hordeum vulgare	BTAI-CMa	tetrameric	7HS	Salcedo *et al*. 1984
Hordeum vulgare	BTAI-CMb	tetrameric	4HS	Salcedo *et al*. 1984
Hordeum vulgare	BTAI-CMd	tetrameric	4HS	Salcedo *et al*. 1984
Secale cereale	RDAI	dimeric	3RS	Lyons *et al*. 1987; García-Casado *et al*. 1994

teins on ultrathin gels (Sánchez-Monge *et al*. 1986, 1989; Masojć *et al*. 1993; Singh *et al*. 2001). In particular, genes for inhibitors WDAI-0.53 and WDAI-0.19 have been assigned to the chromosomes 3BS and 3DS, whereas there was no evidence of a homoeologous locus or loci on chromosome 3AS (Sánchez-Monge *et al*. 1986, 1989; Masojć *et al*. 1993; Singh *et al*. 2001; Wang *et al*. 2006a). However, WDAI encoding genes could be amplified from *T. monococcum* and *T. urartu*, suggesting that this gene was present in the A-genome of diploid species (Wang *et al*. 2005, 2007a). Furthermore, a new inhibitor from rye (*Secale cereale* L.) named RDAI (dimeric α-amylase inhibitor from rye), which was homologous to wheat homodimeric inhibitors, was also located on chromosome 3RS (Lyons *et al*. 1987; García-Casado *et al*. 1994).

α-Amylase inhibitors are located on the short arm of chromosome 3 in Triticeae (barley, wheat, rye). However, coding gene sequences of WMAI and WDAI could be found from A genome of diploid wheat, it is still not known the reason about the absent of α-amylase inhibitors from A genome of polyploid wheat accessions (Wang *et al.* 2007a, 2008b). Recently, next generation sequencing technology is used to obtain large quantity of sequences form certain species. It could assay the variance of WMAI and WDAI between diploid and polyploid wheat in future.

EVOLUTION

Different genomes

The important food crops for human nutrition and animal feed production such as barley, rye and wheat belong to the Triticeae tribe that comprises more than 500 taxa in a system of 37 genomically defined genera (Löve 1984). The origin of *T. aestivum* (2n=6x=42, AABBDD) has been the subject of much research in recent years and involved the related genus *Aegilops*. It is accepted that the diploid donors of the A and D genomes were *T. urartu* (2n=2x=14, A^uA^u), *T. monococcum* (2n=2x=14, A^mA^m) and *Ae. tauschii* (2n=2x=14, DD) respectively, whereas the identity of the donor(s) of the B genomes remains a contentious issue. *T. urartu* has been recognized as the A^u genome ancestor of emmer wheat, durum wheat and common wheat, whereas *T. monococcum* is the donor of the A^m genome of *T. Zhukovskyi* (Dvorak *et al*. 1993; Jiang and Gill 1994; Feldman 2000; Baum and Bailey 2004). In several cases the B genome of common wheat appears closer to the S genome of *Ae. speltoides* than with those of the other species (Talbert *et al*. 1995; Sasanuma *et al*. 1996; Blake *et al*. 1999).

Three kinds of exogenous α-amylase inhibitors have different evolutionary rate in Triticeae. The sequence polymorphisms of their genes were not similar. WMAI is more conserved than WDAI. The tetrameric inhibitor is combined by three subunits. Therefore the mutation of nucleotide in each one of them might affect the structure and then the inhibitory ability. Thus, all the three subunits of WTAI (CM2, CM3, and CM16) were much more conserved than WDAI and WMAI. In the former investigation, it was found that WMAI genes had high homology according to sequence alignment. One hundred and fifty one WMAI genes were divided into two subgroups, and the genes from diploid wheat were clustered into both of the 2 subgroups, while the genes from *Ae. tauschii*, *Ae. bicornis*, and *Ae. longissima* were in either of subgroup (Wang *et al*. 2008b). The presence of high homology among monomeric α-amylase inhibitors indicated that the inhibitors might derive from a very limited number of ancestral genes coding for peptide units and there was a duplication of these ancestral genes followed by divergence of the duplicated genes through mutation, which had the similar evolution model to WDAI genes (Buonocore *et al*. 1977, 1985).

WDAI was well studied for its evolutionary event in Triticeae. The sequence variation and molecular phylogenetic relationship of WDAI from different genomes in Triticeae were characterized by sequence analysis. 630 sequences of WDAI representatives from diploid, tetraploid and hexaploid wheat (containing the A^m, A^u, A, B, D and G genomes), and from 17 wheat wild related species with the D, E^b, F, I, M, N, O, P, Q, R, S, S^b, S^l, S^s, S^{sh}, U, and W genomes were used for phylogenetic analysis (Wang *et al*. 2010a). The phylogenetic tree indicated that the dimeric α-amylase inhibitors encoded by genes from different genomes form several separate clusters. The inhibitors encoded by genes from of A^u, A^m, Q, P, W, F, I, N, M, O, G and D genomes were clustered together, respectively (Wang *et al*. 2010a). Among the genomes, the sequences from the Q genome were most distant from sequences from other genomes (Wang *et al*. 2010a). It was clear that the A^u genome was far from the A^m genome, and that the P, F, and W genomes were also separate and distant from the other genomes. The rest of the genomes could be clustered into three groups, 1) D, N, I, U; 2) M, A^u, S^b, S, S^s, S^{sh}, S^l; and 3) R, A^m, O, E^b. All the sequences obtained from wheat and its related species in Triticeae are very similar to each other, indicating a remarkable conservation of these protein genes (Wang *et al*. 2010a).

Isoelectric focusing, two-dimensional gel electrophoresis, and both direct and clone sequencing also revealed multiple copies of the dimeric α-amylase inhibitor genes in polyploid wheat (Barber *et al*. 1986; Sánchez-Monge *et al*. 1986, 1989; Masojć *et al*. 1993; Wang *et al*. 2005). However, there was no dimeric α-amylase inhibitor activity detected in diploid wheat, but there were WDAI genes in the diploid wheats, and some of them could encode mature protein sequences (Bedetti *et al*. 1974; Vittozzi *et al*. 1976; Wang *et al*. 2007a). The A^m genome sequences were close to a few sequences of the R, S^{sh}, S, S^b, and O genomes from *Secale cereale*, *Ae. sharonensis*, *Ae. speltoides*, *Ae. bicornis*, *Henrardia persica* respectively; while the sequences from A^u genome were clustered together to the M genome sequences from *Ae. comosa*. The genes originally present in the progenitor of diploid wheat were modified or deleted from all species after the formation of common wheat, or the genes were mutated in the diverging species but remained similar in a lineage that gave rise to the donor of the A genome of common wheat.

There was a large group containing the sequences present in hexaploid wheat, *Ae. tauschii*, *H. vulgare*, *Ae. Uniaristata*, *S. cereale* and *Ae. umbellutata*. Since *Aegilops* is a large and diverse group in Triticeae, it was clear that two distinguishable subgroups could be found in *Aegilops* species based on the dimeric α-amylase inhibitor gene sequences, one similar to *Ae. speltoides* and other similar to *Ae. tauschii*. The dimeric α-amylase inhibitor genes evolutionary relationships among B, S and G genomes was also interpreted and the results indicated an uncertain relationship of these three genomes, which was also complex with sequences from O, E^b, and R genomes. The α-amylase inhibitor gene sequences from the B, S and G genomes fell into two groups, which were separated by sequences from the A^u genome, and a larger group with 4 subgroups. Subgroup I had the sequences from the S, S^l, S^b, S^{sh}, S^s and B genomes; subgroup II had many sequence from the B genome and a few sequences from O, E^b, R, S^s and S^b genomes; subgroup III had sequences from S, B, G and E^b genomes; and the last subgroup IV contained the B genome sequences and only one sequence from S^{sh} genome (Wang *et al*. 2010a).

The tetraploid and hexaploid species exhibited B, S genome sequences of higher complexity than in the diploid species, suggesting that different diploid species could have contributed inhibitor-coding genes to polyploid wheats (Vittozzi *et al*. 1976). It was shown that the sequences from hexaploid wheat have divergence less than those from *T. dicoccoides*. While, the dimeric α-amylase inhibitors coding by the B genome from common wheat were divided into two groups, which indicated that the dimeric α-amylase inhibitors originated from at least two separate *T. dicoccoides* populations. Possibly species from *Aegilops* Section Sitopsis might have undergone homologous recombination in the

highly identical dimeric α-amylase inhibitor gene domains because the *Aegilops* Section Sitopsis are heterogeneous and out-crossing species (Wang *et al*. 2007b). This suggests that different diploid species of *Aegilops* contributed the B genome dimeric α-amylase inhibitor genes to the polyploid wheats by gene introgression via inter-specific hybridizations (Vittozzi *et al*. 1976; Wang *et al*. 2007b).

Recent years, the seed proteins such as high molecular weight glutenin subunits, grain softness protein, gliadin were used to do the evolutionary studies. α-Amylase inhibitors are main albumin in cereal seed. Like other seed proteins, their evolutionary relationship among species in Triticeae could also give some important evidence for plant evolution on the functional protein aspect.

Adaptive evolution

Wild emmer wheat is tetraploid and predominantly self-pollinated wheat which is distributed over the Near East Fertile Crescent (Israel, Jordan, Lebanon, Syria, east Turkey, north Iraq, and west Iran) (Harlan and Zohary 1966). The center of distribution and diversity of emmer wheat was found in the catchment area of the upper Jordan Valley (Golan Heights, eastern Upper Galilee Mountains, etc.) in Israel and its vicinity (Nevo and Beiles 1989). Wild emmer wheat covers wide ranges of eco-geographical conditions and ranges over wide altitudinal amplitude in Israel. However, towards their marginal and peripheral areas, both in Israel and Turkey, wild emmer wheat became semi-isolated or isolated, and smaller in size. This distributional pattern has a dramatic effect on their population genetic structure and differentiation (Nevo and Beiles 1989). Individual plants of emmer wheat were collected at random, at least 1 m apart, from populations differing in major ecological properties. These collection sites and populations have been described in detail elsewhere (Nevo and Beiles 1989; Nevo *et al*. 1982).

The adaptive evolution of α-amylase inhibitor genes in emmer wheat was assayed. It was found that the WMAI, WDAI and WTAI genes were associated with environments, including temperature, water, geographic factors (Wang *et al*. 2008a, 2010b). Great diversity at these loci, both between and within populations, was detected in the populations of Israeli wild emmer wheat. The sequences of WMAI and WDAI were contributed by both natural selection and co-evolution, which ensures the conserved function as well as the inhibition of a variety of insect amylases. Ecological factors, singly or in combination, explained a significant proportion of the variations in SNPs and the SNPs could be classified into several categories as ecogeographical predictors (Wang *et al*. 2008a, 2010b). The conflict between genetic divergence and geographic distances also suggested that the SNPs in WMAI and WDAI were subjected to natural selection, and ecological factors had an important evolutionary role in gene differentiation at these loci. It was clear that the nucleotide sequence of tetrameric α-amylase inhibitors were much more conserved than that of dimeric and monomeric α-amylase inhibitors. Two dimeric α-amylase inhibitors could combine with 2 α-amylase and 2 monomeric α-amylase inhibitors could combine 1 α-amylase. However, the four units of terameric α-amylase inhibitors (1 CM2, 1 CM 16 and 2 CM3) should combine first and then inhibit the α-amylase. It might be the reason that the gene/protein sequences of WTAI-CM2, CM3 and CM16 were more conserved than WDAI or WMAI, to remain the inhibitory activity. The whole sequences $\omega<1$ suggested that the inhibitors were under strong purifying selection pressure (indicating there might be a structural requirement). Most positions of WTAI were conserved, position analysis of the ratio of synonymous and non-synonymous substitutions provided strong evidence for natural selection acting on WTAI (unpublished data). In other words, amino acid-altering substitutions in either unit of WTAI might affect the compound's structure, then the ability to combine amylase.

It is well known that all cereal-type α-amylase inhibitors had 10 Cys (5 disulfide bonds). The WMAI, WDAI, WTAI-CM2, CM3 and CM16 also had 10 Cys, and the positions of the 10 Cys were conserved. Most of the SNPs did not occur at highly conserved positions, which ensured that the α-amylase inhibitors would keep their correct 3D structure to combine with α-amylase, even under different environments.

FUTURE PROSPECTS

The insect-resistance genes transferred into plants to date mainly target the insect digestive system. Several kinds of α-amylase and proteinase inhibitors in seeds and vegetative organs act to regulate the numbers of phytophagous insects, which could be used through plant genetic engineering. Many insects have several α-amylases that differ in specificity and successful utilization of a food source is dependent on the presence of α-amylase for which there is no specific inhibitor. Screening inhibitors in cereals without taxonomic constraint and the identification of their evolutionary relationship can help identify novel insecticidal determinants. Hundreds of monomeric, dimeric and tetrameric α-amylase inhibitor genes were obtained from Triticeae. The continuing discovery of new α-amylase inhibitors suggests that the list of α-amylase inhibitors is far from complete. Even though lots of genes encoding exogenous α-amylase inhibitors were characterized, basic information pertaining to gene's expression and regulation, protein's sequence and 3D structure studies are lacking, as well as the molecular correlation between enzyme and its inhibitor. Thus, the search for new genes is ongoing and aims to expand the range of insects affected, to combat the development of resistance in the target insects by identifying genes to improve potency.

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REFERENCES

Alfonso J, Ortego F, Sánchez-Monge R, García-Casado G, Pujol M, Castañera P, Salcedo G (1997) Wheat and barley inhibitors active towards α-amylase and trypsin-like activities from *Spodoptera frugiperda*. *Journal of Chemical Ecology* **23**, 1729-1741

Altenbach SB, Vensel WH, Dupont FM (2011) The spectrum of low molecular weight alphaamylase/protease inhibitor genes expressed in the US bread wheat cultivar Butte 86. *BMC Research Notes* **4**, 242

Barber D, Sánchez-Monge R, Mendez E, Lazaro A, García-Olmedo F, Salcedo G (1986) New α-amylase and trypsin inhibitors among the CM-proteins of barley (*Hordeum vulgare*). *Biochimica et Biophysica Acta* **869**, 115-118

Baum BR, Bailey LG (2004) The origin of the A genome donor of wheats (*Triticum:* Poaceae): A perspective based on the sequence variation of the 5S DNA gene units. *Genetic Resources and Crop Evolution* **51**, 183-196

Bedetti C, Bozzini A, Silano V, Luciano V (1974) Amylase protein inhibitors and the role of *Aegilops* species in polyploid wheat speciation. *Biochimica et Biophysica Acta* **362**, 299-307

Blake NK, Lehfeldt BR, Lavin M, Talbert LE (1999) Phylogenetic reconstruction based on low copy DNA sequence data in an allopolyploid: The B genome of wheat. *Genome* **42**, 351-360

Bonavides KB, Pelegrini PB, Laumann RA, Grossi-de-Sá MF, Bloch C, Melo JAT, Quirino BF, Noronha EF, Franco OL (2007) Molecular identification of four different α-amylase inhibitors from baru (*Dipteryx alata*) seeds with activity toward insect enzymes. *Journal of Biochemistry and Molecular Biology*, **40**, 494-500

Buonocore V, Biasi MGD, Giardina P, Poreio E, Silano V (1985) Purification and properties of an α-amylase tetrameric inhibitor from wheat kernel. *Biochimica et Biophysica Acta* **831**, 40-48

Buonocore V, Petrucci T, Silano V (1977) Wheat protein inhibitors of α-amylase. *Phytochemistry* **16**, 811-820

Chrispeels MJ, Grossi-de-Sá MF, Higgins TJV (1998) Genetic engineering with α-amylase inhibitors seeds resistant to bruchids. *Seed Science Research*

8, 257-263
da Silva MC, de sá MF, Chrispeels MJ, Togawa RC, Neshich G (2000) Analysis of structural and physico-chemical parameters involved in the specificity of binding between α-amylases and their inhibitors. *Protein Engineering* **13**, 167-177
Deponte R, Parlamenti R, Petrucci V, Silano V, Tomasi M (1976) Albumin α-amylase inhibitor families from wheat flour. *Cereal Chemistry* **53**, 805-820
Dupont FM, Vensel WH, Tanaka CK, Hurkman WJ, Altenbach SB (2011) Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases and tandem mass spectrometry. *Proteome Science* **9**, 10
Dvorak J, Tetizi P, Zhang HB, Resta P (1993) The evolution of polyploid wheats: identification of the A genome donor species. *Genome* **36**, 21-31
Feldman M (2001) Origin of cultivated wheat. In: Bonjean AP, Angus WJ (Eds) *The World Wheat Book: A History of Wheat Breeding*, Lavoisier Publishers, Lavoisier, France, pp 3-58
Feng GH, Richardson M, Chen MS, Kramer KJ, Morgan TD, Reeck GR (1996) α-Amylase inhibitors from wheat: A sequences and patterns of inhibition of insect and human α-amylases. *Insect Biochemistry and Molecular Biology* **26**, 419-426
Fontanini D, Capocchi A, Muccilli V, Saviozzi F, Cunsolo V, Saletti R, Foti S, Galleschi L (2007) Dimeric inhibitors of human salivary α-amylase from emmer (*Triticum dicoccon* Schrank) seeds. *Journal of Agricultural and Food Chemistry* **55**, 10452-10460
Fra-Mon P, Salcedo G, Aragoncillo C, García-Olmedo F (1984) Chromosomal assignment of genes controlling salt-soluble proteins (albumins and globulins) in wheat and related species. *Theoretical and Applied Genetics* **69**, 167-172
Franco OL, Ridgen DJ, Melo FR, Bloch C Jr., Silva CP, Grossi de Sá MF (2000) Activity of wheat α-amylase inhibitors toward bruchid α-amylases and structural explanation of observed specificities. *European Journal of Biochemistry* **267**, 2166-2173
Franco OL, Rigden DJ, Melo FR, Grossi de Sá MF (2002) Plant α-amylase inhibitors and their interaction with insect α-amylases structure, function and potential for crop protection. *European Journal of Biochemistry* **269**, 397-412
García-Casado F, Cabonero P, García-Olmedo F (1991) Site-directed mutagenesis and expression in *Escherichia coli* of WMAI-1, a wheat monomeric inhibitor of insect α-amylase. *Plant Molecular Biology* **17**, 1005-1011
García-Olmedo F, Salcedo G, Sánchez-Monge R, Gomez L, Royo J, Carbonero P (1987) Plant proteinaceous inhibitors of proteinases and α-amylases. *Oxford Surveys of Plant Molecular and Cell Biology* **4**, 275-334
García-Casado G, Sánchez-Monge R, López-Otín C, Salcedo G (1994) Rye chromosome arm 3RS encodes a homodimeric inhibitor of insect α-amylase. *Theoretical and Applied Genetics* **89**, 60-63
Gatehouse AMR, Gatehouse JA (1998) Identifying proteins with insecticidal activity: Use of encoding genes to produce insect-resistant transgenic crops. *Pest Science* **52**, 165-175
Gbaye OA, Millard JC, Holloway GJ (2011) Legume type and temperature effects on the toxicity of insecticide to the genus Callosobruchus (Coleoptera: Bruchidae). *Journal of Stored Products Research* **47**, 8-12
Gomez L, Sánchez-Monge R, Lopez-Otín C, Salcedo G (1991) Wheat inhibitors of heterologous α-amylases. *Plant Physiology* **96**, 768-774
Gomez L, Sánchez-Monge R, García-Olmedo F, Salcedo G (1989) Wheat tetrameric inhibitors of insect s-amylases: Alloploid heterosis at the molecular level. *Proceedings of the National Academy of Sciences USA* **86**, 3242-3246
Gorjanović S (2009) Biological and technological functions of barley seed pathogenesis-related proteins (PRs). *Journal of the Institute of Brewing* **115**, 334-360
Harlan JR, Zohary D (1966) Distribution of wild wheat and barley. *Science* **153**, 1074-1080
Jiang J, Gill BS (1994) Different species-specific chromosome translocations in *Triticum timopheevi* and *Triticum turgidum* support the diphyletic origin of polyploid wheats. *Chromosome Research* **2**, 59-64
Konarev AV (1996) Interaction of insect digestive enzymes with plant protein inhibitors and host-parasite co-evolution. *Euphytica* **92**, 89-94
Kumar RSS, Singh SA, Rao AGA (2009) Conformational stability of α-amylase from malted sorghum (*Sorghum bicolor*): Reversible unfolding by denaturants. *Biochimie* **91**, 548-557
Lazaro A, Sánchez-Monge R, Salcedo G, Paz-Ares J, Carbonero P, García-Olmedo F (1988) A dimeric inhibitor or insect alpha-amylase from barley. *European Journal of Biochemistry* **172**, 129-134
Lin KF, Lee TR, Tsai PH, Hsu MP, Chen CS, Lyu PC (2007) Structure-based protein engineering for α-amylase inhibitory activity of plant defensin. *Proteins: Structure, Function, and Bioinformatics* **68**, 530-540
Löve A (1984) Conspectus of Triticeae. *Feddes Repertorium* **95**, 425-521
Lyons A, Richardson M, Tatham AS, Shewry PR (1987) Characterization of homologous inhibitors of trypsin and the α-amylase. *Biochimica et Biophysica Acta* **915**, 305-313
MacGregor EA, Janecek S, Svensson B (2001) Relationship of sequence and structure to specificity in the α-amylase family of enzymes. *Biochimica et Biophysica Acta* **1546**, 1-20
Maeda K, Kakabayashi S, Matubara H (1985) Complete amino acid sequence of an α-amylase inhibitor in wheat kernel (0.19-inhibitor). *Biochimica et Biophysica Acta* **828**, 213-221
Masojć P, Zawitowski J, Howes NK (1993) Polymorphim and chromosomal location of endogenous α-amylase inhibitor genes in common wheat. *Theoretical and Applied Genetics* **85**, 1043-1048
Murayama Y, Mochizuki K, Shimada M, Fujimoto S, Nukui K, Shibata K, Goda T (2009) Dietary supplementation with α-amylase inhibitor wheat albumin to high-fat diet-induced insulin-resistant rats is associated with increased expression of genes related to fatty acid synthesis in adipose tissue. *Journal of Agricultural and Food Chemistry* **57**, 9332-9338
Nagy-Gasztonyi M, Nagy A, Nemeth-Szerdahelyi E, Pauk J, Gelencser E (2010) The activities of amylases and α-amylase inhibitor in wide-range herbicide resistant wheat lines. *Czech Journal of Food Sciences* **28**, 217-224
Nevo E, Beiles A (1989) Genetic diversity of wild emmer wheat in Israel and Turkey: Structure, evolution and application in breeding. *Theoretical and Applied Genetics* **77**, 421-455
Nevo E, Golenberg E, Beiles A (1982) Genetic diversity and environmental associations of wild wheat, *Triticum dicoccoides*, in Israel. *Theoretical and Applied Genetics* **62**, 241-254
Nieuwenhuizen NJ, Beuning LL, Sutherland PW, Sharma NN, Cooney JM, Bieleski LRF, Schröder R, MacRae EA, Atkinson RG (2007) Identification and characterisation of acidic and novel basic forms of actinidin, the highly abundant cysteine protease from kiwifruit. *Functional Plant Biology* **34**, 946-961
Oda Y, Matsunaga T, Fukuyama K, Miyazaki T, Morimoto T (1997) Tertiary and quaternary structures of 0.19 α-amylase inhibitor from wheat kernel determined by X-ray analysis at 2.06 Å resolution. *Biochemistry* **36**, 13503-13511
Om P, Nivedita J (2010) α-Amylase inhibition: Some practical considerations. *Current Enzyme Inhibition* **6**, 158-163
Payan F (2004) Structural basis for the inhibition of mammalian and insect α-amylase by plant inhibitors. *Biochimica et Biophysica Acta* **1696**, 171-180
Pelegrini PB, Lay FT, Murad AM, Anderson MA, Franco OL (2008) Novel insights on the mechanism of action of α-amylase inhibitors from the plant defensin family. *Proteins: Structure, Function, and Bioinformatics* **73**, 719-729
Petrucci T, Sannia G, Parlamenti R, Silano V (1978) Structural studies of wheat monomeric and dimeric protein inhibitors of alpha-amylase. *Biochemical Journal* **173**, 229-235
Poerio E, Caporale C, Carrano L, Pucci P, Buonocore V (1991) Assignment of the five disulphide bridges in an alpha-amylase inhibitor from wheat kernel by FAB/MS and Edman degradation. *European Journal of Biochemistry* **199**, 595-600
Prescott VE, Campbell PM, Moore A, Mattes J, Rothenberg ME, Foster PS, Higgins TJV, Hogan SP (2005) Transgenic expression of bean α-amylase inhibitor in peas results in altered structure and immunogenicity. *Journal of Agricultural and Food Chemistry* **53**, 9023-9030
Ryan CA, Pearce G (1998) Systemin: A polypeptide signal for plant defensive genes. *Annual Review of Cell and Developmental Biology* **14**, 1-17
Ryan CA (1990) Protease inhibitors in plants: Genes for improving defenses against insect and pathogens. *Annual Review of Phytopathology* **28**, 425-449
Salcedo G, Fra-Mon P, Molina-Cano JL, Aragoncillo C, García-Olmedo F (1984) Genetics of CM-proteins (A-hordeins) in barley. *Theoretical and Applied Genetics* **68**, 53-59
Sales MP, Gerhardt IR, Grossi-de-Sá MF, Xavier-Filho J (2000) Do legumes storage proteins play a role in defending seeds against bruchids? *Plant Physiology* **124**, 515-522
Sánchez-Monge R, Barber D, Mendez E, García-Olmedo F, Salcedo G (1986) Gene encoding alpha-amylase inhibitors are located in the short arms of chromosomes 3B, 3D and 6D of wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics* **72**, 108-113
Sánchez-Monge R, Gomez L, García-Olmedo F, Salcedo G (1989) New dimeric inhibitor of heterologous alpha-amylase encoded by a duplicated gene in the short arm of chromosome 3B of wheat (*Triticum aestivum* L.). *European Journal of Biochemistry* **183**, 37-40
Sander I, Rozynek P, Rihs H-P, van Kampen V, Chew FT, Lee WS, Kotschy-Lang N, Merget R, Brüning T, Raulf-Heimsoth M (2011) Multiple wheat flour allergens and cross-reactive carbohydrate determinants bind IgE in baker's asthma. *Allergy* **66**, 1208-1215
Sasanuma T, Miyashita NT, Tsunewaki K (1996) Wheat phylogeny determined by RFLP analysis of nuclear DNA. 3. Intra- and interspecific variations of five *Aegilops sitopsis* species. *Theoretical and Applied Genetics* **92**, 928-934
Silano V, Poerio E, Buonocore V (1977) A model for the interaction of wheat monomeric and dimeric protein inhibitors with α-amylase. *Molecular and Cellular Biochemistry* **18**, 87-91
Singh J, Skerritt JH (2001) Chromosomal control of albumins and globulins in wheat grain assessed using different fractionation procedures. *Journal of Cereal Science* **33**, 163-181
Strobl S, Muhlhahn P, Bernstein R, Wiltscheck R, Maskos K, Wunderlich M, Huber R, Glockshuber R, Holak TA (1995) Determination of the three-dimensional structure of the bifunctional alphα-amylase/trypsin inhibitor from ragi seeds by NMR spectroscopy. *Biochemistry* **34**, 8281-8293

Talbert LE, Blake TK, Storlie EW, Levin M (1995) Variability in wheat based on low-copy DNA sequence comparisons, *Genome* **38**, 951-957

Vittozzi L, Morisi G, Silano V (1976) Continuous automated assay of alpha-amylase inhibitors. *Journal of the Science of Food and Agriculture* **27**, 449-453

Wang JR, Pu ZN, Lan XJ, Baum BR, Yan ZH, Zheng YL, Wei YM (2010a) Phylogenetic analysis of the dimeric alpha-amylase inhibitor sequences from an orthologous region in 21 different genomes of the tribe Triticeae (Poaceae). *Biochemical Systematics and Ecology* **38**, 708-714

Wang JR, Wei YM, Deng M, Nevo E, Yan ZH, Zheng YL (2010b) The impact of single nucleotide polymorphism in monomeric alpha-amylase inhibitor genes from wild emmer wheat, primarily from Israel and Golan. *BMC Evolutionary Biology* **10**, 170

Wang JR, Wei YM, Fedak G, Liu ZG, Jiang QT, Pu ZE, Zheng YL (2010c) Molecular characterization of major allergens associated with baker's asthma in wheat flour. *Food Science and Technology Research* **16**, 341-346

Wang JR, Wei YM, Long XY, Yan ZH, Nevo E, Baum BR, Zheng YL (2008a) Molecular evolution of dimeric α-amylase inhibitor genes in wild emmer wheat and its ecological association. *BMC Evolutionary Biology* **8**, 91

Wang JR, Wei YM, Yan ZH, Nevo E, Baum BR, Zheng YL (2006) Molecular characterization of dimeric α-amylase inhibitor genes in wheat and development of genome allele-specific primers for the genes located on chromosome 3BS and 3DS. *Journal of Cereal Science* **43**, 360-368

Wang JR, Wei YM, Yan ZH, Zheng YL (2005) Detection of single nucleotide polymorphisms in the 24 kDa dimeric α-amylase inhibitors from cultivated wheat and its diploid putative progenitors. *Biochimica et Biophysica Acta* **1723**, 309-320

Wang JR, Wei YM, Yan ZH, Zheng YL (2008b) SNP analysis and haplotype identification of the genes encoding monomeric α-amylase inhibitors. *Genetica* **134**, 277-285

Wang JR, Yan ZH, Wei YM, Baum BR, Zheng YL (2007a) Sequence variation and haplotype identification of wheat dimeric α-amylase inhibitor genes in einkorn wheats. *Biochemical Genetics* **45**, 803-814

Wang JR, Zhang L, Wei YM, Yan ZH, Baum BR, Nevo E, Zheng YL (2007b) Sequence polymorphisms and relationships of dimeric α-amylase inhibitor genes in the B genomes of *Triticum* and S genomes of *Aegilops*. *Plant Science* **173**, 1-11

Wisessing A, Engkagul A, Wongpiyasatid A, Choowongkomon K (2010) Biochemical characterization of the α-amylase inhibitor in mungbeans and its application in inhibiting the growth of *Callosobruchus maculatus*. *Journal of Agricultural and Food Chemistry* **58**, 2131-2137

Witzel K, Pietsch C, Strickert M, Matros A, Röder MS, Weschke W, Wobus U, Mock HP (2010) Mapping of quantitative trait loci associated with protein expression variation in barley grains. *Molecular Breeding* **27**, 301-314