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CHITINASE ACTIVITIES IN WHEAT AND ITS RELATIVE SPECIES

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Defense components such as chitinases (EC 3.2.1.14) are crucial for plants to cope diseases. Despite of that the pattern and activities of these enzymes in agronomically important Triticale is unexplored. This work is aimed to study chitinase activities in the leaves of plants of early developmental stages in two diploids (*Aegilops tauschii* Coss., *Triticum monococcum* L.), four tetraploids (*Ae. cylindrical* Host, *Ae. triuncialis* L., *T. araraticum* Jakubyz, *T. dicoccum* Schrank) and two hexaploids (*T. aestivum* L., *T. spelta* L.). The leaves were subjected to quantitative and qualitative activity assays using synthetic 4-methylumbelliferyl-β-D-*N*,*N*′,*N*′′-triacetylchitotrioside and glycolchitin as substrates, respectively. Our results showed that the activities of chitinases with specificity towards short oligomers were variable and genotype dependent. The enzyme activities in the tetra- and hexaploid genotypes were significantly higher than in diplod counterparts. In the gel detection assays were revealed up to four fractions (~20, 30, 42 and 95 kDa) of proteins with the chitinase activity towards long chain polymers. The isoform of ~30 kDa was identified in all analyzed genotypes. Among the seven acidic and three basic chitinase fractions identified, three acidic (ChiA, ChiB, ChiC) and two (ChiH, ChiI) fractions were present in all genotypes. None of the isoforms can be assigned as specific with respect to ploidy.

Key words: defense proteins, hydrolases, glycolchitin, chitinases, ploidy, PR protein, wheat

Chitinases are widely distributed enzymes in plants. Plant chitinases (EC 3.2.1.14) hydrolyze β-1,4-N-acetyl-D-glucosamine (GlcNAc) linkages of chitin. Their true substrate in plants is unknown since they lack chitin, but chitinases are suggested to cleave arabinogalactan proteins (AGPs) and N-acetylglucosamine-containing glycoproteins in the plant cell walls (van Hengel *et al.* 2001; van

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Hengel *et al.* 2002). Afterwards, the released substances can act as an elicitor of plant defence response (Fessel & Zuccaro 2016). Chitinases that are induced in response to pathogen attack are also referred as "Pathogenesis related proteins" (PR3, PR4, PR8 and PR11) (Kasprzewska 2003). They were shown to inhibit fungal growth *in vitro* (Sela-Buurlage *et al.* 1993), while their over-expression in transgenic plants enhanced fungal resistance (Moravcikova *et al.* 2004).

According to the CAZy database chitinases form two glycoside hydrolase families GH18 and GH19 which show different structures and catalytic mechanisms. The family GH19 chitinases are mainly found in plants. Based on their amino acid composition, plant chitinases are mainly grouped into five classes (Neuhaus 1999). The classes I, II and IV belong to the family GH19 while III and V to the family GH18. These enzymes can be expressed in plants constitutively (Colligne *et al.* 1993) or they can be induced upon pathogen infection (Žur *et al.* 2013), low temperature (Yeh *et al.* 2000), ethylene (Zhong *et al.* 2002), drought (Gregova *et al.* 2015) or heavy metals (Meszaros *et al.* 2014). Besides, it was suggested that they might take part in programmed cell death (Kim *et al.* 2015). Chitinases could also play a role in developmental processes such us pollination, senescence, seed germination and somatic embryogenesis (Kasprzewska 2003; Grover 2012).

Bread wheat is one of the most important grain crops worldwide. Its hexaploid genome is a result of the evolutionary hybridization, domestication and/or selection steps. However, genetic improvement for human purposes caused genetic erosion and increased susceptibility to environmental and biotic stresses. Not surprisingly that some wild ancestors and inter-crossable wheat relatives are more resistant to some disease than bread wheat (Peng *et al.* 2011).

In this work we studied the activities of chitinases in two wheat (*T. aestivum* L., *T. spelta* L.) and crop relative species (*Aegilops tauschii* Coss., *Ae. cylindrical* Host, *Ae. triuncialis* L., *T. monococcum* L., *T. araraticum* Jakubyz, *T. dicoccum* Schrank). Qualitative and quantitative chitinase activity assays were used to reveal in detail the profile and activities of individual isoforms in wheat and wild relative species at early developmental stage.

MATERIAL AND METHODS

Plant material

Seeds of eight wheat genotypes (Table 1) were obtained from the Gene Bank of the Slovak Republic (National Agricultural and Food Centre, Slovak Republic). The seeds were germinated on the watered sterile filter paper in dark at room temperature for 3 days. Then, germinated seeds were transferred to the pots with the commercial substrate BORA and cultivated at 22°C and 16 h/8 h light/dark photoperiod under 50 μE/m^2/s light intensity for 3 weeks. The leaves of wheat plants (10 plants/genotype) at two leaf stage (Zadoks stage 12) were collected and used for analyses.

Protein extraction

Crude protein extracts were isolated from the leaves of plants using an extraction buffer that contained 0.1 mol/dm^3 sodium acetate (pH 5.0) and 0.02% (v/v) β–mercaptoethanol according to the protocol described previously (Žur *et al.* 2013). Protein concentration was determined according to Bradford (1976).

Gel electrophoresis and chitinase activity staining

Protein extracts (30 μg) were separated on 12.5% (w/v) SDS-containing polyacrylamide slab gels (Laemmli 1970) with 0.01% (w/v) glycol chitin as an enzyme substrate. Glycol chitin was obtained by acetylation of glycol chitosan (Sigma G-7753) as described by Trudel and Asselin (1989). The gels were run at 8°C at a constant voltage of 120 V for 2 h. After electrophoresis, proteins were re-naturated by shaking the gel in 50 mmol/dm^3 sodium acetate buffer (pH 5.0), 1% (v/v) Triton X-100 for 1 hour.

Separation of proteins under native conditions (for acidic/neutral or basic/neutral proteins) was performed according to Konotop *et al.* (2012) using 11% (w/v) acrylamide gels with 0.01% (w/v) glycol chitin.

The chitinase activity was detected by staining with 0.01% (w/v) Fluorescent Brightener 28 (Pan *et al.* 1991).

Chitinase quantitative assays

The chitinase activity was assayed fluorimetrically using the synthetic substrate 4-methylumbelliferyl-β-D-N,N′,N′′-triacetylchitotrioside [4-MU-

$(GlcNAc)_3$](Fluka 69615) as was described previously (Libantova *et al.* 2009). The fluorescence was measured using excitation/emission filters 360 nm/450 nm (Synergy H1,Bioetek). The chitinase activity was expressed in picomoles of methylumbelliferone (4-MU) generated per hour per milligram of soluble proteins.

The shown data represent the means of three replications. Statistical significance of the experimental results was evaluated by ANOVA/MANOVA and Duncan's tests, with help of STATISTICA® version 7.1.

RESULTS AND DISCUSSION

Chitinases from different plant species have been studied mainly for their inducibility upon biotic stresses. Their role in plant growth and development has also been proven. However, there are still limited information about chitinases in wheat and its relative species. So far, the Uniprot and NCBI databases contain up to 18 characterized sequences concerning chitinases in the hexaploid *T. aestivum* L. (Table 2). In literature, wheat chitinases were studied mainly for their antifungal potential (Liao *et al.* 1994; Caruso *et al.* 1999; Li *et al.* 2001; Kong *et al.* 2005), drought stress (Gregorova *et al.* 2015), heavy metal accumulation (Lyubka *et al.* 2008) or different concentration of nitrogen as a nutritional supply (Maglovski *et al.* 2017).

In this work we studied the activities of chitinases in wheat and relative species (Table 1). The plants were grown under controlled conditions up to two leaf stage. Afterwards, the leaves were collected and subjected to the chitinase activity assays.

The enzyme activities were evaluated quantitatively based on the ability of plant chitinases to release $(GlcNAc)_3$ from the tetramer 4MU-$(GlcNAc)_3$, a fluorogenic substrate for short oligomer-specific endochitinases. Our results showed that chitinases with hydrolytic activity towards oligomers were active in all analysed genotypes. Data are summarized in Figure 1a. The effect of genotype was found to be significant (at $p \leq 0.001$). The highest chitinase activity was detected for *T. diccocum* Schrank (59.05 nmol MU/h/mg) and the lowest ones for *T. monococcum* L. (5.89 nmol MU/h/mg) and *Ae. tauschii* Coss. (7.96

T a b l e 1

Wheat and crop relatives species used in the experiments and their resistance to important wheat diseases

Genotype	Disease resistance*					
	Gene bank**	Ploidy/ Genome	Powdery mildew Leaf/spike	Wheat rust leaf	Take-all of wheat	Blotch of wheat
Aegilops cylindrical Host	ARME N06-02	Tetraploid CCDD	9/9	1	9	9
Aegilops tauschii Coss.	ARME N06-40	Diploid DD	9/9	1	9	9
Aegilops triuncialis L.	ARME N06-06	Tetraploid UUCC	9/9	9	9	9
Triticum aestivum L.	Astella	Hexaploid $BBAA^uDD$	5/5	7	8	8
Triticum araraticum Jakubyz	AZESVK 2009-47	Tetraploid $GGAA^u$	7/6	5	8	8
Triticum dicoccum Schrank	AZESVK 2009-78	Tetraploid $BBAA^u$	8/7	6	6	6
Triticum monococcum L.	AZESVK 2 009-84	Diploid AA^m	9/9	8	9	9
Triticum spelta L.	Brun 5/9	Hexaploid $BBAA^uDD$	4/6	5	9	9

*Disease severity rating based on the data obtained from the Gene Bank of the Slovak Republic; 9 – very high resistance, 1 – very low resistance

**Accession number in the Gene bank of the Slovak Republic

nmol MU/h/mg). The activities in the tetraploid and hexaploid species were significantly higher than in the diploid species (at $p \leq 0.01$) (Figure 1b). It might coincide with successive polyploidization of wheat genome since such genomic can reprogram gene expression patterns (Chen *et al.* 2007).

As is shown in Table 1 the analysed wheat relatives were evaluated as more resistant to the selected fungal diseases. However, no correlation between chitinase activities and disease severity rating was observed. Chitinases are multifunctional enzymes that take part not only in plant pathogenesis but

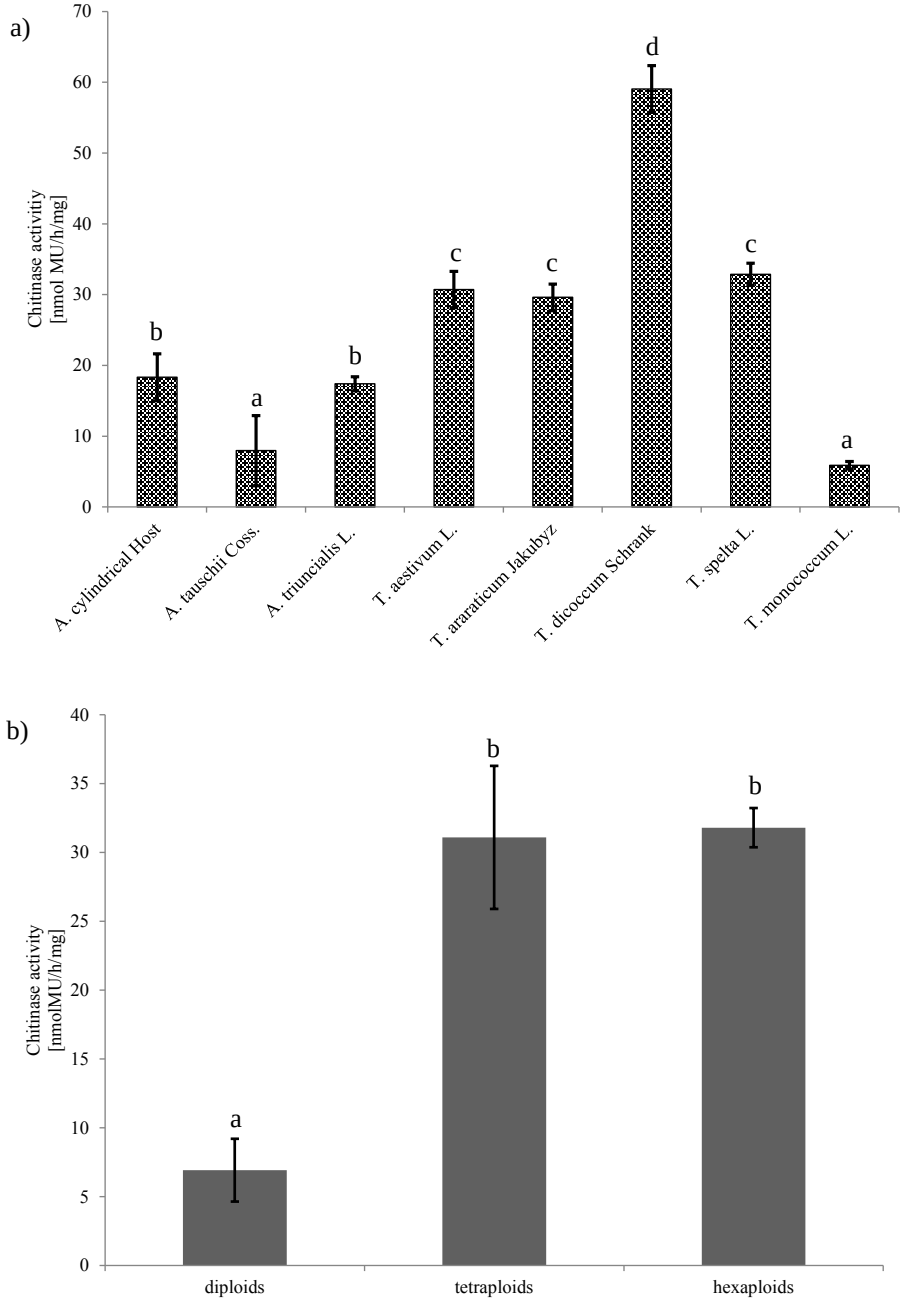

Figure 1. Chitinase activities in the studied wheat and its (wild) relatives (a) and in dependence on ploidy level (b). The enzyme activities were assayed fluorimetrically using [4-MU-$(GlcNAc)_3$] as a substrate. The activity was expressed in nmol of methylumberylferone (MU) released per hour per mg of soluble proteins. Bars represent means ± standard deviations of three replications. Distinct letters denote statistically significant differences with Duncan's test at $p \leq 0.001$ (a) and at $p \leq 0.01$ (b).

T a b l e 2

Chitinases in *T. aestivum* L. and their characterization based on the data in the NCBI and UNIPROT databases (September 2016)

UNIPROT/ NCBI	Name	AA	MW [kDa]	DNA [bp]	Description/function	Literature
Q8W427/ AB029936	Chitinase 3, Chi3 cht1	319	33.5	960	Biotic stress *Puccinia striiformis West. f. sp. tritici*	–
Q8W428/ AB029935	Chitinase 2, Chi2 Chitinase 2	323	34.2	1,163	–	–
A0A0H4TIG5/ KR049249	Chitinase Chitinase, cht3	317	33.4	954	Biotic stress *P. striiformis West. f. sp. tritici*	–
Q6T484/ AY437443	Class I, chitinase Class I, chitinase	319	33.5	1,121	Botic stress *Fusarium graminearum*	Kong *et al*. (2005)
Q8W429/ AB029934	Chitinase 1, Chi 1 Chitinase 1	256	27.1	979	–	–
Q4Z8L7/ AY973230	Class II, chitinase Class II, chitinase	266	28.3	811	Seed development	–
Q4Z8L8/ AY973229	class II, chitinase class II,chitinase	266	28.2	892	Seed development	–
A0A0H4TM98/ KR049250	Chitinase cht4, chitinase	320	33.6	963	Biotic stress *P. striiformis West. f. sp. tritici*	–
A0A077RF77/ KR049248	Chitinasecht2, chitinase	320	33.6	963	Biotic stress *P. striiformis West. f. sp. tritic*i	–
Q9XEN3/ AF112963	Chitinase II, Cht2 Chitinase II, Cht2	230	24.7	956	Biotic stress *F. graminearum*	Li *et al*. (2001)
Q41539/ X76041	Endochitinase CHI Endochitinase CHI	320	33.6	1,985	Biotic stress *P. graminis*	Liao *et al*. (1994)
A0A023W638/ KJ507390	Endochitinase Endochitinase	320	33.6	963	–	–
A0A023W4F1/ KJ507387	Endochitinase Endochitinase	318	33.5	957	–	–
A0A023W636/ KJ507385	Endochitinase Endochitinase	319	33.5	960	–	–
A0A023W5U7/ KJ507389	Endochitinase Endochitinase	320	33.6	963	–	–
A0A023W4N4/ KJ507388	Endochitinase Endochitinase	318	35.3	957	–	–
A0A023W594/ KJ507386	Endochitinase Endochitinase	317	33.5	954	–	–

AA – amino acids; MW – molecular weight

also they regulate processes of plant growth and development. It has been reported that upon (a)biotic stresses the activities of some constitutively synthesized chitinases can be increased (Žur *et al.* 2013).

The activities measured comprise several individual isoforms with different activities and functions. Therefore the substrate glycol chitin was used to detect chitinases with hydrolytic activities towards long polymers after their separation in gels. Our analyses identified up to four enzyme fractions (~20, 30, 42 and 95 kDa) with the chitinolytic activities (Figure 2b, Table 3). Only the ~30 kDa fraction was identified in all analysed genotypes. For comparison, the purified wheat chitinases in the sequence databases are of molecular mass in a range between 27 kDa to 36 kDa (Table 2). The molecular mass of plant chitinases commonly ranges from 25 kDa to 35 kDa, but plant isoforms of 20 kDa or ≥40 kDa were also described (Chang *et al.* 2014). Most plant chitinases of 30–46 kDa are referred as PR proteins (Ferreira *et al.* 2007) thus the identified chitinase isoforms of ~30 kDa and ~42 kDa might be significantly induced upon (a)biotic stresses.

Plant chitinases have been found to exist in acidic and basic forms according to their isoelectric points (Kasprzewska 2003). Based on the classification by Stintzi *et al.* (1993) the acidic isoforms are considered as extracellular while basic as vacuolar. We identified up to seven acidic (ChiA-ChiG) (Figure 2c) and three basic (Figure 2d) fractions with hydrolytic activities towards the long polymer glycolchitin (Table 3). The three acidic (ChiA, ChiB, ChiC) and two (ChiH, ChiI) isoforms were detected in all genotypes. In most of analysed genotypes, combination of four acidic fractions was found. However, none of the acidic isoforms can be associated with ploidy level.

Our results (Figure 1, Figure 2) showed that chitinases with specificities towards short oligomers and long chitin polymers are accumulated in the leaves of all analysed genotypes. The genotype had significant effect on short oligomer-specific chitinase ac-

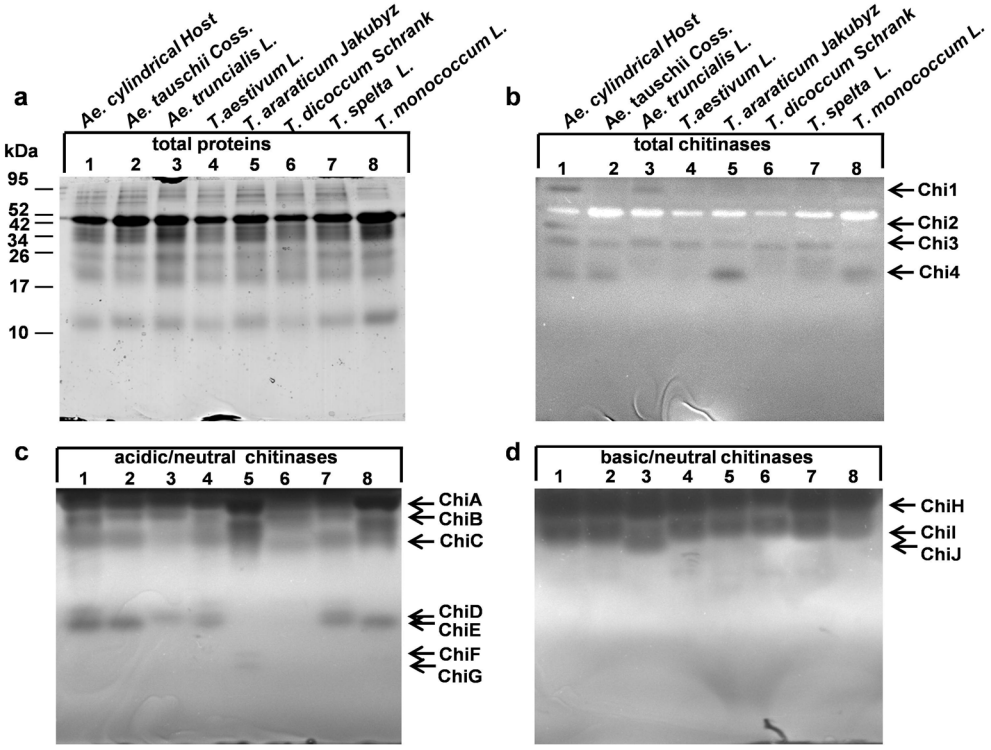

Figure 2. Detection of chitinase activities after separation of crude protein extracts in the SDS-PAGE. Proteins were isolated from plants and their amount and quality was checked upon visualization with Coomassie Brilliant Blue R 250 (a). For activity detection towards glycolchitin as a substrate they were separated under semi-denaturing conditions based on the size (b) and under native conditions in the PAGE for acidic/neutral (c) and basic/neutral proteins (d). Numbers on the left refer to the molecular mass marker. Arrows indicate the detected isoforms.

tivities. These activities were significantly higher in tetra- and hexaploid than in diploid genotypes. The enzymes with hydrolytic activities towards oligomers and chitin polymers possess a potential for production of biologically active substances with antibacterial, antifungal, antitumor or immunity-enhancing effects (Li *et al.* 2016).

CONCLUSIONS

The activities of chitinases in two hexaploid wheat (*T. aestivum* L., *T. spelta* L.) and six their crop relative species (*Ae. tauschii* Coss., *Ae. cylindrical* Host, *Ae. triuncialis* L., *T. monococcum* L., *T. araraticum* Jakubyz, *T. dicoccum* Schrank) were studied. Our results showed that chitinases are active in the leaves of all analysed genotypes, while the gained values were variable and dependent on genotype. The activities in the tetra- and hexaploid genotypes appeared significantly higher than in their diplod counterparts. The gel activity assays revealed up to four fractions of proteins with chitinase activity of which the isoform Chi3 of ~30 kDa was identified in all analysed genotypes. More detailed analyses detected up to seven acidic and three basic

Table 3

Overview of the chitinase activities in wheat and its relative species

	Chitinase isoforms						
	Total*				Acidic/neutral**		
	Chi1 ~95 [kDa]	Chi2 ~42 [kDa]	Chi ~30 [kDa]	Chi4 ~20 [kDa]	ChiA	ChiB	ChiC
Ae. tauschii Coss.	–	–	+	+	+	+	+
T. monococcum L.	–	–	+	+	+	+	+
T. araraticum Jakubyz	–	–	+	+	+	+	+
T. dicocum Schrank	–	–	+	–	+	+	+
Ae. cylindrical Host	+	+	+	+	+	+	+
Ae. truncialis L.	+	–	+	–	+	+	+
T. aestivum L.	–	–	+	–	+	+	+
T. spelta L.	–	–	+	+/	+	+	+

Table 3 continued

	Chitinase isoforms						
	Total*				Basic/neutral***		
	ChiD	ChitE	ChiF	ChiG	ChiH	ChiI	ChiJ
Ae. tauschii Coss.	–	+	–	–	+	+	–
T. monococcum L.	–	+	–	–	+	+	–
T. araraticum Jakubyz	–	–	+	+	+	+	–
T. dicocum Schrank	–	–	–	–	+	+	–
Ae. cylindrical Host	+	+	–	–	+	+	–
Ae. truncialis L.	+	–	–	–	+	+	+
T. aestivum L.	+	–	–	–	+	+	–
T. spelta L.	+	–	–	–	+	+	–

*Size of the isoforms (Chi1-Chi4) detected in the gel after re-naturation of separated proteins in the SDS-PAGE (Figure 2b)

**Fractions detected in the gel after separation of proteins in the PAGE under conditions for acidic/neutral proteins (Figure 2c)

***Fractions detected in the gel after separation of the proteins in the PAGE under conditions for basic/neutral proteins (Figure 2d)

enzyme fractions of which some were specific for individual genotypes. However, a larger set has to be studied to assign them with respect to ploidy level. The presence of additional isoforms in the wild relatives of wheat brings a promise for identifying novel sources of defence compounds potentially interesting for breeding purposes. Besides, wheat chitinases can be studied for potential use in biotechnological programs focused on production of bioactive chitoologosacharides.

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