The Activities of Amylases and α-Amylase Inhibitor in Wide-range Herbicide Resistant Wheat Lines

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Abstract

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The activities of total amylase, α-amylase, and α-amylase inhibitor in the albumin-globulin fractions of isogenic non transgenic control (CY 45) and ppt (phosphinothrichin) resistant transgenic spring wheat (*Triticum aestivum* L.) lines (T106, T116, T117, T124, T128, T129) were studied in two subsequent years. The plants were either sprayed with a selective herbicide Granstar (G), a wide range herbicide Finale 14 SL (F), or were grown without spraying (Q). Samples were obtained from field trial experiments of the Cereal Research Non Profit Co (Szeged, Hungary). Our results showed an increased trend in total amylase activity of untreated transgenic wheat lines in comparison with non transgenic wheat. The herbicide treatments enhanced the total amylase activity in both transgenic and non transgenic wheat samples. The changes in α-amylase inhibitor activity showed the same trend as that observed in total amylase activity in transgenic lines.

Keywords: transgenic wheat; amylolytic activities; α-amylase inhibitor; electrophoresis; specific staining

Plant seeds are rich sources of a large number of different enzymes and inhibitors acting on plant physiology, food technological features, and having importance in human and animal nutrition.

Wheat α-amylase inhibitors (α-AIs) are increasingly studied for their agronomical role as natural defence molecules of plants against the attack of insects and pests (Zoccatelli *et al*. 2007). A number of studies have demonstrated the changes of the amylolytic enzyme system in response to biotic and abiotic stresses (Jacobsen *et al*. 1986; Robertson *et al*. 1989; Sanchez-Hernandez *et al*. 2004.). Kaplan and Guy (2004) have established the induction of β-amylase in response to abiotic stress.

The main criteria making wheat flour suitable for baking is the sufficient content of good gluten quality and moderate enzymatic activity, mainly with regard to α-amylolytic activity. Both high and low α-amylolytic activities in flour have negative organoleptic effects on bread (Klockiewicz-Kaminska *et al*. 1995).

An other important aspect in terms of amylases and amylase inhibitors is their effect on human health. Hypersensitivity reactions have been noted in relation to wheat flour, caused both by inhalation and ingestion (Martín *et al*. 1990, Kusaba-Nakayama *et al*. 2000). Most prominent allergens of wheat flour have been related to the salt-soluble fraction, several 12–16 kD proteins having been

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identified as members of the α-amylase/trypsin inhibitor family (Fränken *et al*. 1991). High dietary intakes of αAIs can cause a number of potentially deleterious alterations in the body metabolism of experimental animals (Grant *et al*. 1995).

Modern gene technologies allow for the expression of recombinant proteins in non-native hosts, thus providing the potential to increase greatly the yield and/or product quality in a variety of globally important plants. The increased use of GM foods for direct human consumption and for feeding to meat and milk producing animals has led to some public concerns (Kuiper *et al*. 2002; EFSA 2006). Besides the intended and useful modifications, there might occur several unintended effects on the level of plant genome and proteome. The gene-insertion (expected effect) occurring during the application of the recombinant technique can cause unexpected gene-expression due to the random insertion (Kuiper *et al*. 2004; Prescott & Hogan 2006). Because of the gene over-working or silencing, there is a higher or lower level of specific protein expression, while in the case of gene-disintegration, modified protein expression could occur. Therefore, the parental and transformed lines must be tested in parallel (Kuiper *et al*. 2000; Nagy *et al*. 2006). It is also important to study the level of marker proteins (anti-nutrients, allergens, and physiologically active substances) which can indicate any altered gene expression affecting the food safety and quality.

In the Cereal Research Non-Profit Company (Szeged, Hungary) GM wheat lines were developed with a broad range of herbicide resistance. Phosphinothrichin is the active substance in the herbicides Liberty, Basta, and Finale. It is a non-selective herbicide that kills a wide range of plants and with little systemic activity. The transgenic wheat containing bacterial-derived (*Streptomyces hygroscopicus*) bar is regulated under the maize ubiquitin promoter and expresses phosphinothrichin acetyltransferase (PAT), which inactivates glyphosinate by acetylation, becoming resistant to the glyphosinate agent family (Pauk *et al*. 2001). The crops of these GM wheat lines were studied from various aspects in Hungary. Nagy *et al*. (2008) made comparative analysis of the main nutrients and some of the marker proteins in parental and transgenic wheat samples harvested in the years of 2000 and 2001. The levels of the marker proteins (anti-nutrients, allergens, and physiologically active substances) can indicate any altered gene expression. Halász *et al*. (2007) compared the total amylase activities of parental and six GM lines harvested in 2003, and found a significantly increased activity of amylases in four of them.

Amylolytic enzymes and alpha-amylase inhibitor as potential marker components were chosen in this study. The objective of the experiments was to assess and compare the activity of amylases and to detect the activity of α-amylase inhibitors in the parental and herbicide resistant transgenic wheat lines treated with Finale 14SL and Granstar, and those without spraying, harvested in the years of 2001 and 2002.

MATERIAL AND METHODS

Wheat samples. The non-transgenic recipient isogenic control spring wheat (*Triticum aestivum* L., CY 45) and six independent transformed wheat lines (T106, T116, T117, T124, T128, T129) without spraying (Q) were investigated in the experiments. All the wheat plants were treated either with a wide-range herbicide (Finale 14SL – F) or the traditional wheat selective herbicide (Granstar – G). The transgenic wheat line contains bacterial-derived alien gene (*bar*) under the corn ubiquitin promoter, which possesses resistance to agents of the glyphosinate (phosphinotrichin) family (Pauk *et al*. 2001).

The samples were obtained from Cereal Research Non-Profit Co. (Szeged, Hungary) following two subsequent field trial experiments (2001 and 2002) on the same location. The agro-technological circumstances were controlled and identical. The climatic conditions were ideal for the spring sowing in 2001. Watering was applied once, at the beginning of June. Because of the drought, the spring wheat was watered 3 times during the growing season in 2002. The conditions of the treatment with herbicides Finale 14 SL (total effect) and Granstar (traditional effect) were the same in both years.

Fractionation of proteins. The wholemeal flour samples were fractionated according to Osborne (1907) with a small modification. The albumin and globulin fractions were exracted in one step. The collected soluble protein fractions were freeze-dried and kept at –20°C until further analysis.

Measurement of total amylase and α-amylase activities. Twenty mg of albumin/globulin fractions of wheat samples were shaken (125 rpm

at 30°C for 30 min) in 10 ml distilled water, and 0.25 ml of the extract was used as crude amylase preparation. The activity of total amylases was determined spectrophotometrically with a slight modification (Decker 1977a, b), using the dinitrosalicylic acid (DNSA) assay. One enzyme unit (U) liberates 1 μmol of reducing sugars (calculated as maltose) per minute at 37°C and pH 5.6 from soluble starch under specified conditions. The crude extract was heated at 70°C for 15 min to inactivate the heat-labile β-amylase and the procedure was repeated to determine α-amylase activity. The results of three parallel measurements were expressed in U/mg DW.

Determination of starch content. Total starch content of the whole grain wheat samples was determined using a Total Starch test kit obtained from Megazyme (K-TSTA 05/2008). In phase I, starch is converted to soluble fragments by the treatment with thermo-stable α-amylase at 100°C, followed by pullulanase/β-amylase at 50°C. In phase II, the starch dextrins are quantitavely hydrolysed with amyloglucosidase to glucose. All tests were made in duplicates and the results were expressed in g/100 g DW.

Detection of α-amylase inhibitor activity. Laemmli's gel system without SDS was used for native PAGE (Laemmli 1970). The separation of proteins was carried out in thin layer gels (75 mm × 83 mm × 0.75 mm) in BIO-RAD Mini-Protean II cell system. The total acrylamide content of the running gel was 10%. α-Amylase inhibitor activity was detected according to Henson and Stone (1988). As α-amylase inhibitor standard, Alpha Trim-W (Nutricepts Inc., St. Burnsville, USA) was used in four concentrations. The gels were incubated at room temperature for 20 min with α-amylase solution (0.14 U/ml α-amylase from porcine pancreas, Sigma-Aldrich, St. Louis, USA) in 40mM Tris buffer (pH 8.0) containing 1mM $CaCl_2$. Then the gels were incubated with 1% soluble starch in the same buffer. After thorough rinsing in distilled water, the gels were stained with KI-I_2 (1mM I_2 and 0.5M KI). For imaging and semiquantitative evaluation, the electrophoretograms were scanned with Biotec-Fischer video-densitometer ((Biotec Fischer, Reisskirchen, Germany).

Statistical evaluation. The effect of genetic modification was evaluated by variance analysis (ANOVA) based on the differences between the mean values of non transgenic control and transgenic wheat lines on the levels of non treated and both treated groups. For comparison, CY45Q was applied in the non treated group while in the Granstar and Finale 14SL treated groups CY45G was used, as the Finale 14SL sprayed control did not survive. The effect of the herbicide treatment was evaluated using variance analysis (ANOVA) based on the differences between the treated and non treated samples, independently from them being transgenic or non transgenic ones. The differences were considered significant at $P = 0.05$; 0.01; 0.001 probability levels using SPSS 11.0 Software.

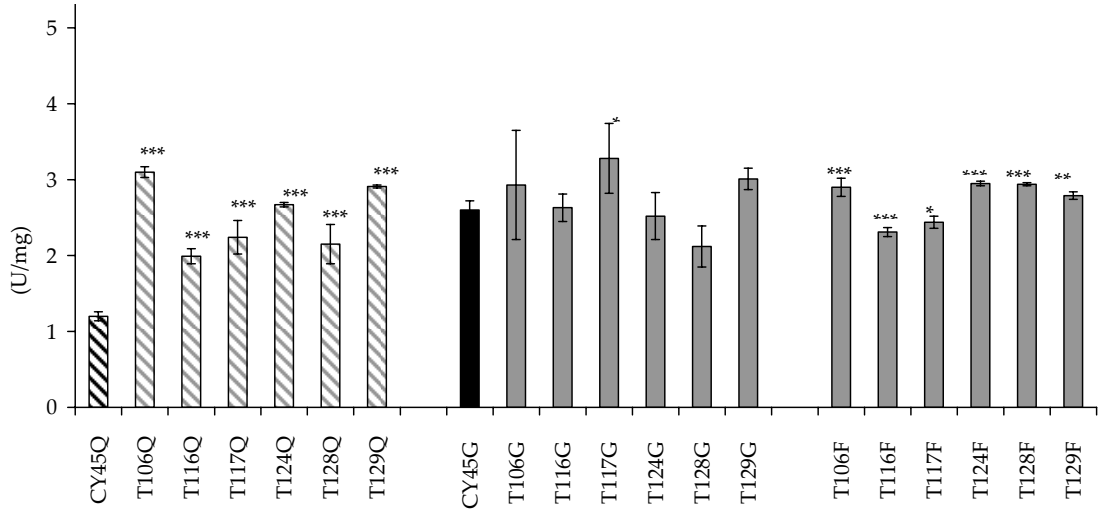
RESULTS AND DISCUSSION

Starch content

The amylolytic state and the content of starch as the substrate affect the bread making quality as published by Every *et al.* (2002). Therefore, the average starch contents of the wheat samples were determined. It was found that the average starch content (58.6 ± 2.5%) in both years did not significantly differ in the investigated samples.

Total amylase activity

The amylase activity was compared for the non transgenic and transgenic wheat samples in three different treatment groups (Q, G, F) to monitor the effect of genetic modification. In the non treated groups of the year 2001, the total amylase activity (Figure 1) in all the six transgenic lines without spraying was significantly higher ($P < 0.001$) than that of the control wheat sample. In the year of 2002, the tendency (Figure 2) was the same for the non treated group, the total amylase activity being the lowest in the isogenic control wheat sample, but the differences were not significant. In the year of 2001, no ignificant differences ($P < 0.001$) were found between the parental and transgenic wheat samples treated by Granstar and the same trend was observed in the year of 2002 except T129G wheat line, where the total amylase activity was significantly lower ($P < 0.001$) in the group. Following the Finale 14SL spraying, the parental wheat decayed and therefore the results of total amylase activity for the transgenic wheat lines were compared to the Granstar sprayed control wheat. In the year 2002, no significant differences occurred following the Finale 14SL spraying.

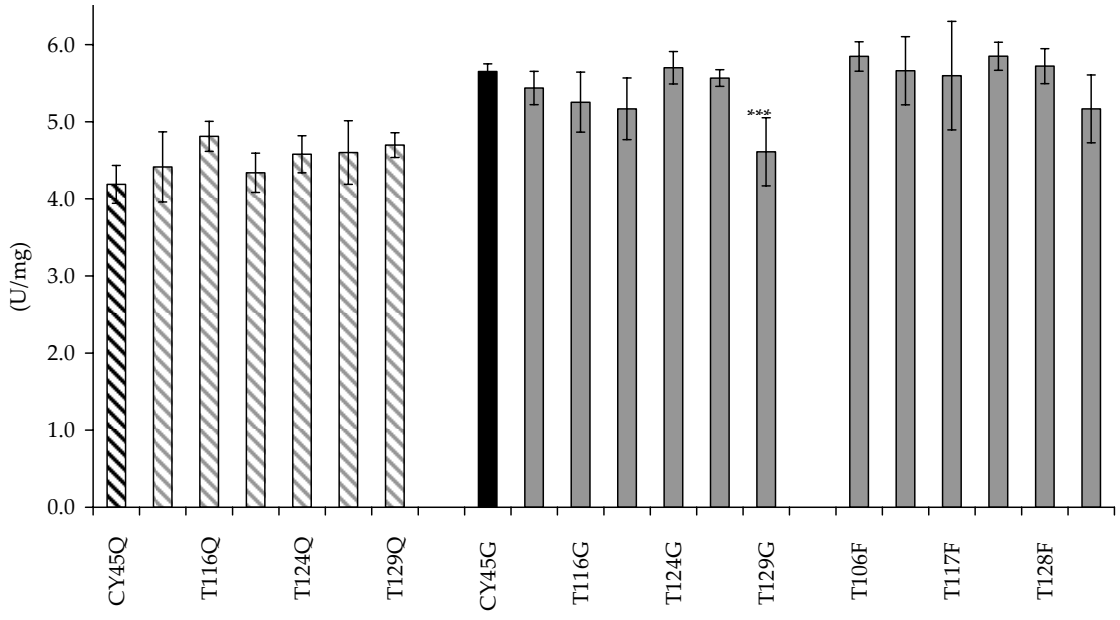


n = 3, probability levels:*P < 0.05, **P < 0.01, ***P < 0.001

Mean values within the columns with the stars are significantly different at the applied probability levels from the mean values of the control wheat. In the non treated group CY 45Q, and in the Granstar and Finale 14SL treated groups CY 45G were used as control wheats

Figure 1. Total amylase activities of non transgenic wheat and transgenic wheat lines grown in 2001 year field experiment without spraying (Q) or sprayed with Granstar (G) and finale 14SL (F)

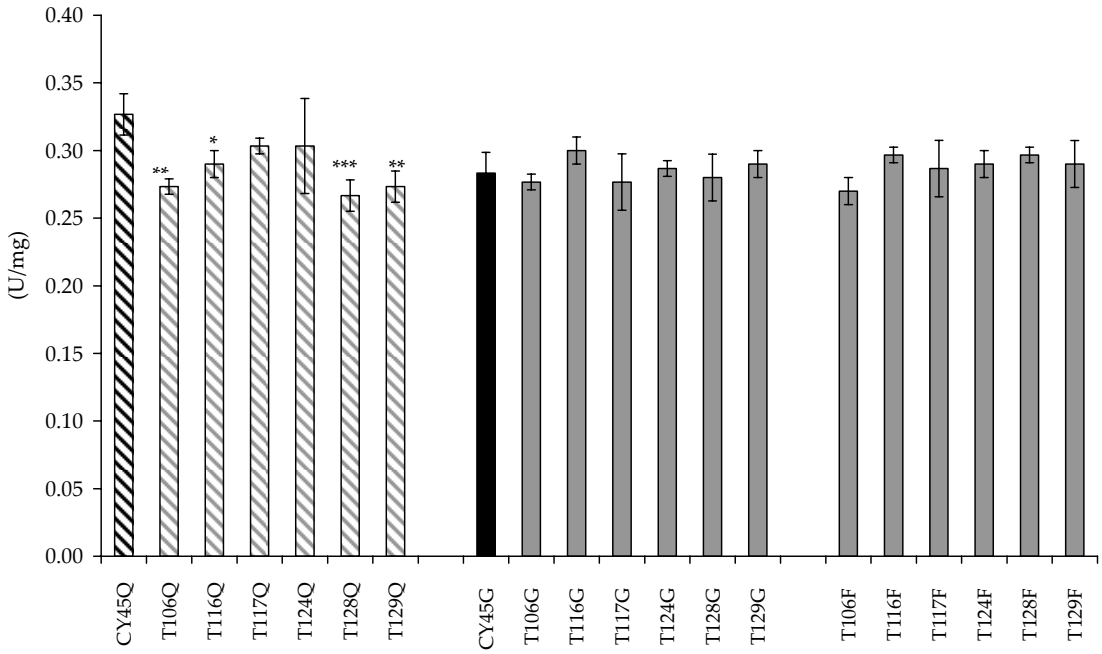
On the other hand, considering the effect of spraying (Finale 14SL, Granstar) in both years, the total amylase activity was increased in the respective samples in comparison with the group without spraying. The effect was significant for the Finale 14SL treated group in the second year.



n = 3, probability levels ***P < 0.001

Mean values within the columns with the stars are significantly different at the applied probability levels from the mean values of the control wheat. In the non treated group CY 45Q, and in the granstar and finale 14SL treated groups CY 45G were used as control wheats

Figure 2. Total amylase activities of non transgenic wheat and transgenic wheat lines grown in 2002 year field experiment without spraying (Q) or sprayed with Granstar (G) and Finale 14SL (F)



n = 3, probability levels:*P< 0.05, **P < 0.01, ***P < 0.001

Mean values within the columns with the stars are significantly different at the applied probability levels from the mean values of the control wheat. In the non treated group CY 45Q, and in the Granstar and Finale 14SL treated groups CY 45G were used as control wheats

Figure 3. α-Amylase activities of non transgenic wheat and transgenic wheat lines grown in 2001 year field experiment without spraying (Q) or sprayed with Granstar (G) and Finale 14SL (F)

The activity of α-amylase

α-amylase activities in both years were relatively low, in the range of 0.25–0.35 U/mg, as also stated in the literature for the non germinated seeds (Nádudvari-Márkus *et al.* 1982). In the year 2001, α-amylase activity showed a lower value in all transgenic lines (Figure 3) than the parental untreated sample (CY45Q). In the year 2002, no significant differences occurred between the samples either in terms of genetic modification or spraying (Figure 4).

Table 1. Densitometric evaluation of the electrophoretograms

Sample	2001		Sample	2002	
	peak area (pixel)	relative value (CY45Q = 100)		peak area (pixel)	relative value (CY45Q = 100)
CY45Q	850	100	CY45Q	1160	100
CY45G	990	116	CY45G	2060	177
T128Q	1050	123	T128Q	1460	126
T128G	1170	138	T128G	1590	137
T128F	1430	168	T128F	2300	198
T129Q	1030	121	T129Q	1670	143
T129G	1410	165	T129G	1830	157
T 129F	1600	188	T 129F	1910	164

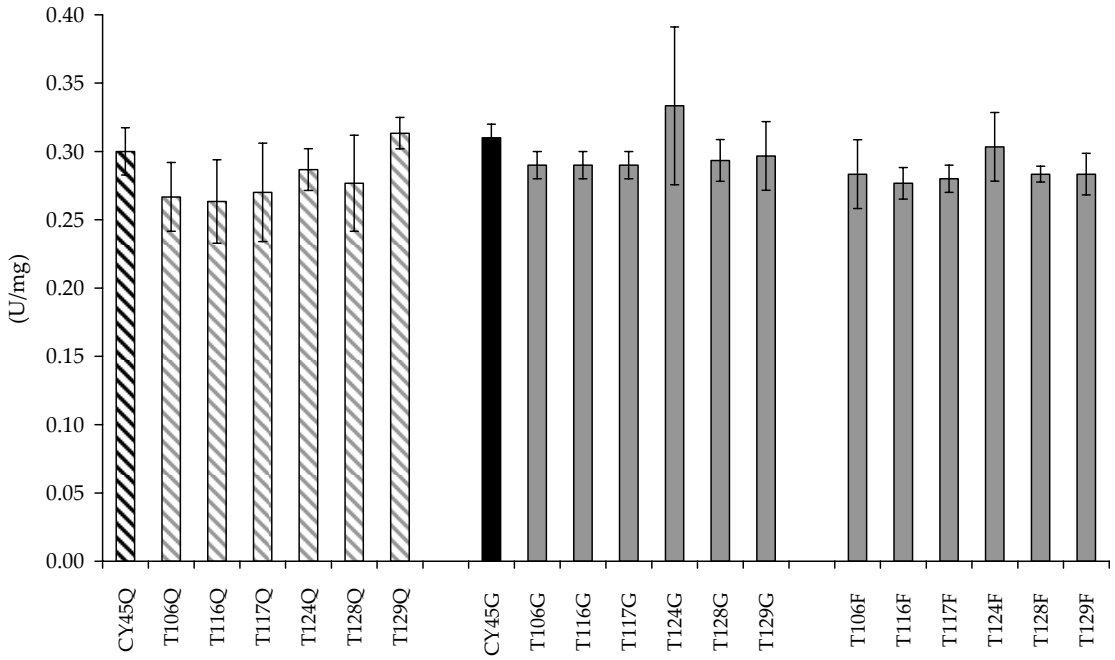


Figure 4. α-amylase activities (n = 3) of non transgenic wheat and transgenic wheat lines grown in 2002 year field experiment without spraying (Q) or sprayed with Granstar (G) and Finale 14SL (F)

The activity of α-amylase inhibitor (AAI)

α-Amylase inhibitor activity of the parental and T128 and T129 wheat lines was characterised in both years using native PAGE followed by specific staining (Figure 5). In the gel incubated in α-amylase and starch solutions, the components with α-amylase inhibitor activities appear as blue zones, following KI-I_2 reaction. The α-amylase inhibitor activities can be characterised by the peak areas

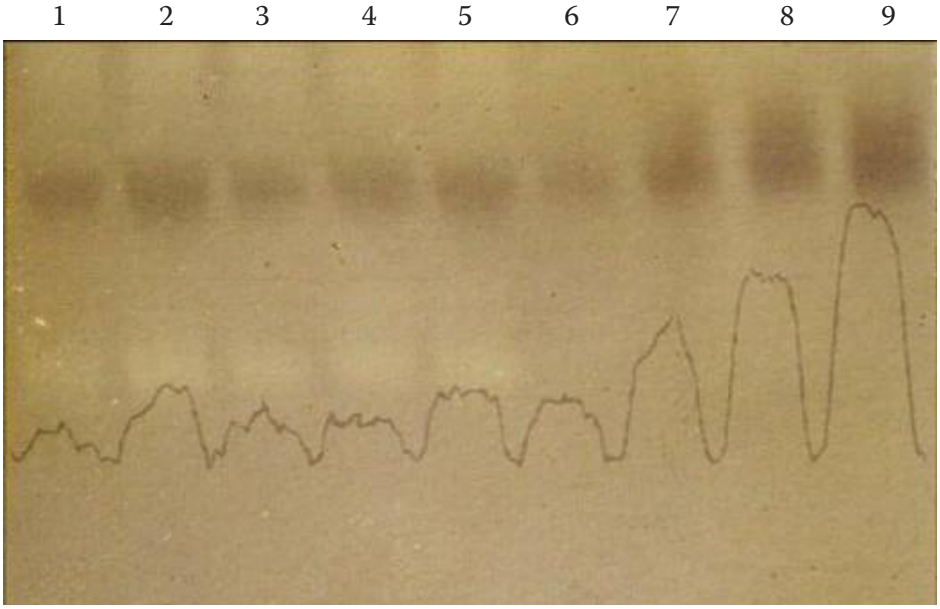


Figure 5. The albumin and globulin fractions of the parental (CY45) and T128 herbicide resistant wheat lines (harvested in 2002) separated in native-PAGE and stained for α-amylase inhibitor activity: lane 1 – CY45Q, lane 2 – CY45G, lane 3 – T128Q, lane 4 – T128G, lane 5 – T128F, lane 6 – 0.1 mg α-amylase inhibitor, lane 7 – 0.13 mg α-amylase inhibitor, lane 8 – 0.16 mg α-amylase inhibitor, lane 9 – 0.19 mg α-amylase inhibitor

of the blue zones. The densitometric evaluation of the gels (Table 1) showed that the zones of small intensity corresponded to the lowest inhibitor activity in the parental lines without spraying. Treating parental line with Granstar resulted in an increase of α-amylase inhibitor activity. The activities of α-amylase inhibitors of transgenic wheat lines treated with herbicides (Finale 14SL, Granstar) were higher than of those without the herbicide treatment. Furthermore, the Finale 14SL treated samples showed zones with the largest area which corresponded to the highest α-amylase inhibitor activity in both years. (Results for T129 are not shown.)

Other research groups arrived at the conclusion that the toxicity and food safety studies of the bar gene inserted into GM plants did not show any adverse effects on the somatic or histopathological alterations using animal feeding experiments (Burks & Fuchs 1996; Rhee *et al*. 2005). At the same time, it cannot not be excluded that the inserted gene induces the over-production of the endogenous gene, which can enhance some unfavourable compounds, for example allergenic proteins. On the basis of the results obtained in two following years, it can be concluded that the genetic transformation and the treatment with herbicides presumably affected the amylolytic enzyme system of wheat lines. The amylases and the α-amylase inhibitor should be taken into consideration in view of technological properties, nutritional and safety aspects. An increase of total amylase and α-amylase inhibitor activities in transgenic and herbicide treated lines was detected in comparison with the parental line being beyond the seasonal variations.

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