BETA-1,3-GLUCANASE ACTIVITIES IN WHEAT AND RELATIVE SPECIES

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Abstract: The (1,3)-β-D-glucan also referred to as callose is a main component of cell walls of higher plants. Many physiological processes are associated with the changes in callose deposition. Callose is synthesised by the callose synthase complex while its degradation is regulated by the hydrolytic enzymes β-1,3-glucanases. The latter one specifically degrade (1,3)-β-D-glucans. This work is aimed to study β-1,3-glucanase activities in the leaves of plants at two leaf stage in two diploids (*Agilops tauschii*, *Triticum monococcum* L.), four tetraploids (*Ae. cylindrica*, *Ae. triuncialis*, *T. araraticum*, *T. dicoccum*) and two hexaploids (*T. aestivum* L, *T. spelta* L.). The leaves were subjected to qualitative and quantitative β-1,3-glucanase activity assays. Our results showed that the total β-1,3-glucanase activities were variable and genotype dependent. No significant correlation between β-1,3-glucanase activities and ploidy level was observed. The gel activity assays revealed a single fraction of ~52 kDa Glu1 that was found in all genotypes. The Glu1 fraction corresponds to a single or two acidic Glu isoforms in dependence on genotype. However, none of the acidic Glu fractions can be assigned as a specific for di-, tetra- or hexaploid genotypes. A single basic GluF isoform was detected and found as present in all genotypes.

Key words: (1,3)-β-D-glucans, β-1,3-glucanases, callose, laminarin, ploidy, wheat

1. Introduction

β-D-glucans are non-starch polysaccharides that are main component of the cell walls of most plants, fungi or microorganisms. Certain β-D-glucans of different origin were studied mainly for their health-promoting effect. They were demonstrated to have hypocholesterolemic and anticoagulant activities; and chemoprotective effect as well. They can also be a potential source of long chain probiotics. Not surprisingly that organisms rich of β-glucans are of economic interest.

The biological activities and application potential of β-D-glucans are mainly determined by their chemical structure. β-D-glucans consist of D-glucose monomers

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linked by (1,3)-β, (1,4)-β or (1,6)-β glycosidic bonds. In higher plants, β-D-glucans are synthesized as linear (1,3)-β-D-glucans, (1,4)-β-D-glucans and mixed (1,3)(1,4)-β-D-glucans. Cellulose or (1,4)-β-D-glucan is the major component of plant cell walls (MINIC *et al.*, 2008) while the mixed (1,3)(1,4)-β-D-glucan is present mainly in the cell walls of starchy endosperm (HAVRLENTOVA and KRAIC, 2006). The (1,3)-β-D-glucans are commonly referred to as callose. During plant growth development it can be found in the cell plate during cell division. Callose deposition is important during pollen development, microsporogenesis or seed germination (PIRSELOVA and MATUSIKOVA, 2013). Besides, callose is synthesized between the cell wall and the plasma membrane after exposure of plants to various (a)biotic stresses (ZAVALIEV *et al*., 2011).

Callose degradation and thus various plant physiological processes are regulated by the β-glucan endohydrolases namely β-1,3-glucanases (EC 3.2.1.39) (PIRSELOVA and MATUSIKOVA, 2013). The β-1,3-glucanases (GH 17) catalyse the hydrolysis of (1,3)-β-D-glucosidic linkages in (1,3)-β-D-glucans but not in (1,3)(1,4)-β-D-glucans (HØJ and FINCHER, 1995). The β-1,3-glucanases have widely been studied for their potential to inhibit fungal pathogen (MORAVCIKOVA *et al.,* 2004), while recently their importance in plant defence against abiotic stresses has also been proven e.g. low temperature (ROMERO *et al*., 2008), drought (GREGOROVA *et al.,* 2015) or heavy metals (PIRSELOVA *et al*., 2011).

Plant β-1,3-glucanases are referred to as "Pathogenesis-related proteins" (PR2). They are grouped into the classes I-IV. The class I are vacuolar basic proteins that are accumulated in mature leaves and roots upon pathogen infection. The classes II and III are acidic extracellular proteins. The class IV is similar to the class II; however, β-1,3-glucanases of the class IV are not inducible upon pathogen attack (LEUBNER-METZGNER, 2003; MINIC, 2008).

Wheat is a plant with complicated genetic information. Due to the evolutionary hybridisation, domestication and/or selection steps; the wheat genome was evolved to diploid, tetraploid and hexaploid genomes. During polyploidization, many duplicated genes were retained and the redundancy conferred by duplicated genes may lead to their novel or divergent functions (CHEN *et al.,* 2007).

In this work, we aimed to study the activities of β-1,3-glucanases in two wheat and six crop relatives species at early growth stages. We studied the plants of two diploids (*Aegilops tauschii*, *Triticum monococcum* L.), four tetraploids (*Ae. cylindrica*, *Ae. triuncialis*, *T. araraticum*, *T. dicoccum*) and two hexaploids (*T. aestivum* L, *T. spelta* L.) They were grown to two leaf stage and the leaves were subjected to qualitative and quantitative β-1,3-glucanase activity assays.

2. Material and Methods

2.1. Plant material and growth conditions

Seeds of wheat and crop relative species (Table 1) were obtained from the Gene Bank of the Slovak Republic (National Agricultural and Food Centre, Pieštany). 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 $\mu E/m^2.s^1$ light intensity for 3 weeks. The leaves of the plants (10 plants/genotype) at two leaf stage (Zadoks stage 12) were collected and used for further analyses.

Table 1. Wheat and crop relatives species used in experiments.

	Gene Bank[a]	**Ploidy**	**Genome**
Triticum monococcum L.	AZESVK2009-84	diploid	AA^m
Aegilops tauschii	ARMEN06-40	diploid	DD
Aegilops cylindrical	ARMEN06-02	tetraploid	CCDD
Aegilops triuncialis	ARMEN06-06	tetraploid	UUCC
Triticum araraticum	AZESVK2009-47	tetraploid	$GGAA^u$
Triticum dicoccum	AZESVK2009-78	tetraploid	$BBAA^u$
Triticum aestivum, L.	Astella	hexaploid	$BBAA^u$ DD
Triticum spelta, L.	Brun 5/9	hexaploid	$BBAA^u$ DD

[a] Accession number in the Gene bank of the Slovak Republic

2.2. Protein extraction

Crude protein extracts were isolated from the leaves 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 spectrophotometrically.

2.3. Detection of β-l,3-glucanase activities in the gel

Protein extracts (30 µg) were separated on 12.5 % (w/v) SDS-containing polyacrylamide slab gels (LAEMMLI, 1970) with 2.5 % (w/v) laminarin from *Laminaria digitata* (Sigma L-9634), as an enzyme substrate. 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, separately) was performed according to KONOTOP *et al.* (2012) using 11 % (w/v) acrylamide gels with 2.5 % (w/v) laminarin. The β-l,3-glucanase activity was visualised by boiling the gel for 10 min in 1 mol/dm^3 NaOH containing 0.1 % (w/v) 2,3,5-triphenyltetrazolium chloride according to the method of PAN *et al.* (1991). After enzyme activity detection, the gels were stained with Coomassie Brilliant Blue R 250.

2.4. β-l,3-glucanase quantitative assays

β-l,3-glucanase activities were assayed spectrophotometrically with laminarin as a substrate (Sigma L-9634) using dinitrosalicylic acid (DNS) method (MILLER, 1959). The absorbance was measured at 500 nm (Ultra spect 100, Pharmacia Biotech). The enzyme activity was expressed in nmol as an amount of released

reducing sugar (D-glucose) per hour per milligram of soluble proteins. All data are the means of three replications. Statistical significance of the experimental results was evaluated by ANOVA/MANOVA and Duncan's tests, using STATISTICA® ver. 7.1.

3. Results and Discussion

Plant β-l,3-glucanases are abundant and highly regulated enzymes that are commonly found throughout the plant kingdom (LEUBNER-METZGNER, 2003). So far, these enzymes were studied mainly for their anti-pathogenic functions (MINIC, 2008) and for their role in reversible deposition of (1,3) β-D-glucans found in the cell-wall sheath surrounding plasmodesmata orifices during plant response to stress (LEVY *et al*., 2007). Their role in plant growth and developmental processes has been also proven (MOROHASHI and MATSHUSHIMA, 2000; LEUBNER-METZGNER, 2003; RUAN *et al.,* 2004). However, they were mainly studied in dicot plants such as *Arabidopsis* (DOXEY *et al*., 2007), tobacco (LEUBNER-METZGNER, 2003), soybean (JIN *et al.*, 1999) or tomato (MOROHASHI and MATSHUSHIMA, 2000). Relatively little is known about β-1,3-glucanases in wheat. So far, the Uniprot/NCBI databases contain up to 11 characterised sequences concerning β-1,3-glucanases only in the hexaploid *T. aestivum* (Table 2). Moreover, β-1,3-glucanases were studied mainly for their induction upon fungal infection.

In this work we aimed to study the activities of β-1,3-glucanases in wheat and crop relatives species with different genome background (Table 1). The seedlings of two wheat and six crop relatives species were grown under controlled conditions up to two leaf stage. Subsequently, the leaves were collected and subjected to the qualitative and quantitative activity assays of the β-l,3-glucanases. The activities were evaluated based on the ability of plant β-l,3-glucanases to hydrolyse laminarin, a linear (1,3)-β-D-glucan with a low degree of glucosyl substitution at 0-6. Even though, there are available other (1,3)-β-D-glucans such as curdlan or pachyman (HRMOVA and FINCHER, 1993), laminarin was found to be the most suitable substrate for plant β-l,3-glucanases (PAN *et al*., 1991; HRMOVA and FINCHER, 1993; PIRSELOVA *et al.,* 2011; ŽUR *et al*., 2013; GREGOROVA *et al*., 2015).

The total β-l,3-glucanase activity detected was variable. Data are summarised in Fig. 1. The highest enzyme activity was detected for genotypes *Ae. tauschii* (4.47 nmol D-glucose/h/mg) and *T. spelta* (4.11 nmol D-glucose/h/mg) while the lowest activity was detected for *Ae. cylindrica* (1.82 nmol D-glucose/h/mg). The β-l,3-glucanase activity was shown to be genotype dependent (at $p \leq 0.001$) (Fig. 1a, Table 2). However, no significant differences (at $p \leq 0.05$) between di-, tetra- and hexaploid genotypes were observed (Fig. 1b). The enzyme activities may be related to diversification of wheat due to domestication, natural hybridization and allopolyploid speciation. For example, the genome of hexaploid *T. aestivum* is composed of three closely-related and independently maintained genomes as a result of hybridization of tetraploid *T. turgidum dicoccum* or emmer wheat (genome AABB) with *Ae. tauschii* (genome DD) (MATSUOKA, 2011). This supports also the fact that even within the group of tetraploid cultivars significant differences (at $p \leq 0.01$) in β-l,3-glucanase activities were detected (Table 2).

Table 2. β-1,3-glucanases in *T. aestivum* L. and their characterisation based on the data in the UNIPROT a NCBI databases (July 2016).

UNIPROT/ NCBI	Name	AA [kDa]	MW [bp]	DNA	Description/function	Literature
P52409/ U30323	β-1,3-glucanase (Glc1)	461	48.9	2072	Abiotic stress (Al^{3+})	CRUZ-ORTEGA *et al*., 1997
Q1ERF8/ AB244641	Endo-β-1,3-glucanase (TaGlb2e)	332	35.0	1150	Abundant in healthy leaves	HIGA-NISHIYAMA *et al.,* 2006
Q1ERF9/ AB244640	Endo-β-1,3-glucanase (TaGlb2d)	336	35.4	1093	Abundant in healthy spikes	HIGA-NISHIYAMA *et al.,* 2006
Q1ERG0/ AB244639	Endo-β-1,3-glucanase (TaGlb2c)	336	35.5	1089	Abundant in healthy spikes	HIGA-NISHIYAMA *et al.,* 2006
Q1ERF7/ AB244642	Endo-β-1,3-glucanase (TaGlb2f)	342	36.1	1181	Abundant in leaves	HIGA-NISHIYAMA *et al.,* 2006
Q1ERG1/ AB244638	Endo-β-1,3-glucanase (TaGlb2b)	340	35.9	1438	Biotic stress *(Erysiphe graminis, Fusarium graminearum)*	HIGA-NISHIYAMA *et al.,* 2006
B5A7B8/ EU816911	β-1,3-glucanase (Glc2)	399	44.2	2152	–	–
Q9XEN7/ AF112967	β-1,3-glucanase (Glb3)	334	34.7	1439	Biotic stress (*Fusarium graminearum*)	LI *et al.,* 2001
Q9XEN5/ AF112965	β-1,3-glucanase (Glb3)	334	34.9	1269	Biotic stress (*Fusarium graminearum*)	LI *et al.,* 2001
Q4JH28/ DQ090946	β-1,3-glucanase	334	35.4	1338	Biotic stress (*Puccinia striiformis*)	–
Q4JK90/ DQ078255	β-1,3-glucanase	334	35.4	1337	Biotic stress (*Puccinia striiformis*)	–

AA – amino acids; MW – molecular weight

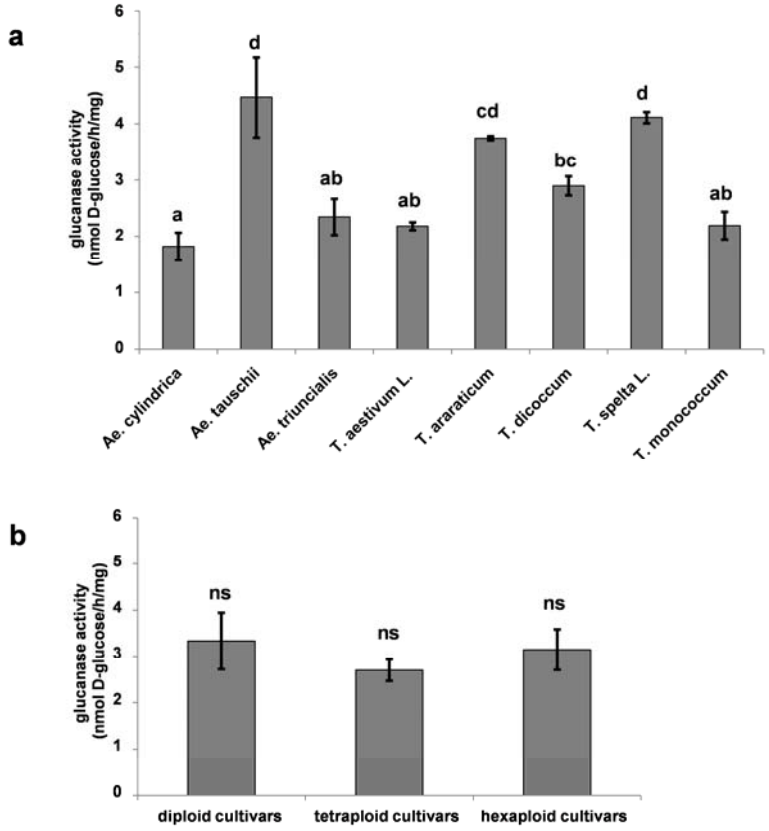

Fig. 1. Total β-1,3-glucanase activities in wheat and crop relatives species (**a)** and in dependence on ploidy level **(b)**. The enzyme activities were assayed spectrophotometrically using laminarin as a substrate. The activity was expressed in nmol of D-glucose released per hour per mg of soluble proteins. Bars represent means ± standard deviations of three replications. Distinct letters denote statistically significant differences with Duncan's test at $p \leq 0.001$, ns – not significant at $p \leq 0.05$.

The total β-l,3-glucanase activities measured can comprise several glucanase isoforms (Fig. 2, Table 3). We identified a single glucanase fraction of a ~52 kDa in all analysed genotypes (Fig. 2b). In literature, wheat β-l,3-glucanases were studied mainly in the context of a/biotic stresses (LI *et al*. 2001; HIGA-NISHIYAMA *et al.,* 2006; PIRSELOVA *et al.,* 2011; GREGOROVA *et al*., 2015). Thus, only data from non-stressed wheat plants might be used for comparison. Besides, these experiments were performed mainly on bread wheat *T. aestivum,* moreover, at different stage of growth. For example, GREGOROVA *et al.* (2015) observed up to four glucanase isoforms of sizes ranging from ~30 kDa to ~150 kDa in the breeding line SK-196 at growth stage after tillering. Similarly, Western blot analyses performed on the control plants of the cv. BobWhite (*T. aestivum* L.) revealed three glucanases

of molecular weight ranging from ~30 kDa to ~60 kDa (JAYARAJ *et al.*, 2004). A higher number of the glucanase isoforms in latter stages of wheat plant might coincide with reversible callose deposition and degradation growth (JAYARAJ *et al.*, 2004; GREGOROVA *et al.,* 2015). RUAN *et al.* (2004) showed the expression of the fibre-specific β-1,3-glucanase gene *GhGluc1* was undetectable when callose was deposited but became evident at the time of callose degradation. They suggested that callose deposition and degradation might correlate with timing of plasmodesmata closure and reopening that is important for division, growth and differentiation of plant cells.

Table 2. Variance analyses.

β-1,3-glucanases vs.	F empirical[a]
Genotype	10.51***
Ploidy level	1.01 [ns]
Teptraploid species	14.07**

[a] statistical significance at *** $p \leq 0.001$; ** $p \leq 0.01$, * $p \leq 0.05$

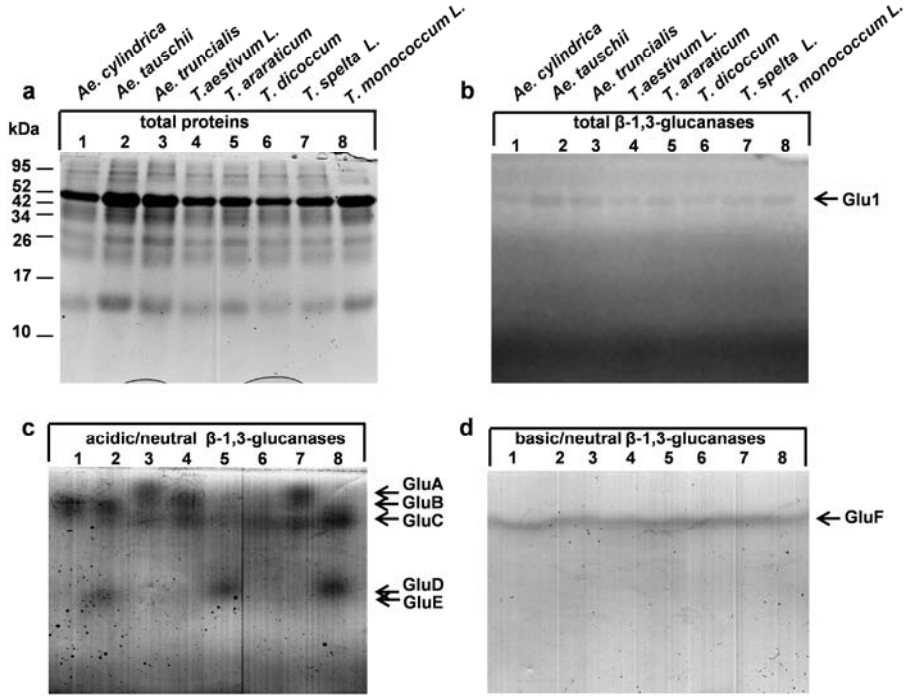

Fig. 2. Detection of β-1,3-glucanase activities after separation of crude protein extracts in SDS-PAGE (**b);** and under native conditions in PAGE for acidic/neutral (**c**); and basic/neutral proteins (**d).** Separated proteins were visualised with Coomassie Brilliant Blue R 250 (**a**). Numbers on the left refer to the molecular mass marker.

The glucanase fraction of a ~52 kDa can comprise up to five acidic (GluA-GluE) (Fig. 2c) and a single basic (GluF) isoforms (Fig. 2d) (Table 3). Neither of the analysed genotypes contained the all five acidic isoforms. In most of them, a combination of two acidic isoforms was found out. However, none of the acidic isoforms can be associated with ploidy level. A single basic GluF was detected in the all analysed genotypes (Table 3). Based on the classification by STINTZI *et al.* (1993) the acidic isoforms are considered as extracellular while basic as vacuolar. However, several authors pointed out that such classification cannot be fully applied for wheat. Analyses performed on apoplastic fluid of wheat leaves revealed accumulation of not only acidic but also basic glucanase fractions (VAN DER WESTHUIZEN *et al.,* 1998).

Our results (Fig. 1, Fig. 2) proved that β-1,3-glucanases are accumulated in the leaves of all studied genotypes. The genotype has significant effect on β-1,3-glucanase activities. However, no significant differences between di- tetra- and hexaploid genotypes were observed. Since, the β-1,3-glucanases might be involved in defence against pathogens, the role of individual β-1,3-glucanase isoforms during biotic stress might be in future evaluated for its use in stress marker assistance breeding or biotechnology programmes.

Table 3. Overview of the β-1,3-glucanase activities in wheat and crop relatives species.

	β-1,3-glucanase isoform						
	Total[a]	Acidic/neutral[b]					Basic/neutral[c]
	Glu1 [~52kDa]	GluA	GluB	GluC	GluD	GluE	GluF
Ae. tauschii	+	-	+	-	-	+	+
T. monococcum L.	+	-	-	+	+	-	+
T. araraticum	+	-	-	-	+	-	+
T. dicoccum	+	-	-	+	-	-	+
Ae. cylindrica	+	-	+	-	-	-	+
Ae. triuncialis	+	+	+		-	-	+
T. aestivum L.	+	-	+	+	-	-	+
T. spelta L.	+	+	-	+	-	-	+

[a] Size of the fraction detected in the gel after re-naturation of separated proteins in the SDS-PAGE (Fig. 2b)
[b] Fractions detected in the gel after separation of the proteins in the PAGE under conditions for acidic/neutral proteins (Fig. 2c)
[c] Fractions detected in the gel after separation of the proteins in the PAGE under conditions for acidic/neutral proteins (Fig. 2d)
+/- presence/absence of fractions with β-1,3-glucanase activities

4. Conclusions

The activities of β-1,3-glucanases in the leaves of two diploids (*Ae.s tauschii*, *T. monococcum* L.), four tetraploids (*Ae. cylindrica*, *Ae. triuncialis*, *T. araraticum*, *T. dicoccum*) and two hexaploids (*T. aestivum* L., *T. spelta* L.) were studied. The total β-1,3-glucanase activities were variable and genotype dependent. We did not observe significant correlation between β-1,3-glucanase activities and ploidy level. The gel activity assays revealed a single ~52 kDa Glu1 fraction that was found in all

genotypes. The Glu1 isoform comprised a single or a combination of two acidic Glu isoforms in dependence on genotypes. Neither of the acidic Glu isoforms could be assigned as specific for di-, tetra- or hexaploid genotypes. The basic GluF isoform was present in all genotypes. A large variability in enzyme activities might be a result of genome evolution. Due to the involvement of β-1,3-glucanases in plant defence, the profile of the activities of these enzymes might be indicative for plant performance in various environmental conditions, thus it deserves further investigations for their use in biotechnology and breeding programmes.

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