Electronic Journal of Biotechnology 30 (2017) 1–5

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

Introduction of a synthetic *Thermococcus*-derived α-amlyase gene into barley genome for increased enzyme thermostability in grains

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ARTICLE INFO

Article history:
Received 10 April 2017
Accepted 8 August 2017
Available online 15 August 2017

Keywords:
Amylopectin
Amylose
Beer
Fermentation
Gene transfer
Glutenin
Hordeum
Promoter
Seed-directed expression
Synthetic gene
Thermotolerant bacteria

ABSTRACT

Background: The enzymes utilized in the process of beer production are generally sensitive to higher temperatures. About 60% of them are deactivated in drying the malt that limits the utilization of starting material in the fermentation process. Gene transfer from thermophilic bacteria is a promising tool for producing barley grains harboring thermotolerant enzymes.
Results: Gene for α-amylase from hydrothermal *Thermococcus*, optimally active at 75–85°C and pH between 5.0 and 5.5, was adapted *in silico* to barley codon usage. The corresponding sequence was put under control of the endosperm-specific promoter *1Dx5* and after synthesis and cloning transferred into barley by biolistics. In addition to model cultivar Golden Promise we transformed three Slovak barley cultivars Pribina, Levan and Nitran, and transgenic plants were obtained. Expression of the ~50 kDa active recombinant enzyme in grains of cvs. Pribina and Nitran resulted in retaining up to 9.39% of enzyme activity upon heating to 75°C, which is more than 4 times higher compared to non-transgenic controls. In the model cv. Golden Promise the grain α-amylase activity upon heating was above 9% either, however, the effects of the introduced enzyme were less pronounced (only 1.22 fold difference compared with non-transgenic barley).
Conclusions: Expression of the synthetic gene in barley enhanced the residual α-amylase activity in grains at high temperatures.

1. Introduction

The enzymes utilized in the process of beer production are generally sensitive to higher temperatures; consequently about 60% of them are deactivated in drying the malt. Such limited utilization of starting material in the fermentation process can be solved by developing new varieties of barley that produce thermostable enzymes, or adding industrially manufactured thermostable enzymes. For the latter, it is desirable to engineer the thermal stability either by mutations of the coding DNA or by transforming genes for thermostable enzymes [1]. Significant source of such genes are thermophilic or thermotolerant bacteria *Bacillus licheniformis* [2] or *Thermococcus* from hydrothermal sulfur springs, as well as fungi such as *Aspergillus tamarii* [3] and *Scytalidium thermophilum* [4].

The α-amylase is an endohydrolase that cleaves (1 → 4)-α-glycosyl linkages of amylose and amylopectin within the molecule. Barley has successfully been transformed with genes for modified α-amylase from *Bacillus* spp. for elevated activity [5]. Tissue specific promoters active in the seed are particularly useful for the expression of desired transgene in cereals. The number of such promoters is, however, rather limited and includes the promoters of B1- and D-hordein active in the endosperm [6], promoter of the *Lem1* gene that is active in the husk and in developing ear [7]. The latter is important, for example, for transfer of resistance genes against *Fusarium* [8]. Other promoters usable for cereals are those driving the glutenin subunits of wheat [9], or the promoters of α-amylase [10] and β-glucanase genes [11] with expression in the grain aleurone layer. Thus, biotechnology can bring promise for production of barley grain with desired characteristics [12,13].

Here we show the feasibility of the α-amylase gene from hydrothermal *Thermococcus* for production of a thermostable enzyme in transgenic barley grains. After modification of its codons, the gene

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2017.08.002

under control of a seed specific promoter was biolostically transferred into barley genome, targeting the model cultivar Golden Promise and three Slovak cultivars Pribina, Levan and Nitran. An active, thermotolerant recombinant enzyme we confirmed in transgenic grains of selected T_0 as well as T_1 analyzed plants. Since α-amylase plays a key modifiable role in starch degradation, its biochemistry, genetics and application in plant biotechnology represent fruitful areas for characterization and exploitation by barley breeders [13].

2. Material and methods

2.1. In silico adjustment, synthesis and cloning of α-amylase gene

The DNA sequence for the artificial *α-amylase* was derived from the *Thermococcus thermophilus* gene published previously [14]. The original start codon of this bacterial sequence was replaced by a signal sequence from the gene for HMW-GS *1Dx5* (GenBank accession no. **JQ867091**) [15] that directs the synthesized protein to endoplasmic reticulum. The codon usage was changed and optimized for expression in barley using the software OPTIMIZER [16]. Finally, the artificial gene sequence was flanked by *Pst* I and *Xba* I restriction sites (respectively) for cloning purposes in subsequent steps. The complete gene was synthesized commercially (MWG Operon, Ebersberg, Germany) and cloned into the plasmid pLRPT (kindly provided by Dr. H. D. Jones, Rothamsted Research Station, Rothamsted, UK) between the present endosperm-specific promoter *1Dx5* of the HMW-GS and the 35S terminator (yielding pLRPT-AMY). Secondary and higher order structural elements in amylase proteins were searched using Phyre^2 program [17] under default settings.

2.2. Plant material, transformation and analyses of transgenic nature

Grains of spring barley cultivars Golden Promise, Pribina, Levan and Nitran (*Hordeum vulgare* L.) were kindly provided by Dr. Klára Križanová from the breeding company Hordeum Ltd. (Sládkovičovo, Slovakia). Immature caryopses were harvested 12–16 days post anthesis and surface-sterilized with 70% (*v*/*v*) ethanol for 2 min, followed by 20 min in commercial bleach (~4% of sodium hypochlorite) and rinsing three times with sterile water.

For transformation experiments, immature embryos were aseptically excised from the caryopses and co-bombarded (gene gun PDS 1000/He, BioRad) with the plasmids pLRPT-AMY and pAHC20 as described previously [18]. The plasmid pAHC20 [19] carried the *bar* gene under control of the constitutive maize ubiquitin promoter. Transformed immature embryos were cultivated in presence of 1 mg·l^{-1} herbicide phosphinotricin (PPT). The regenerated T_0 barley plants were transferred to soil and cultivated to maturity.

Genomic DNA was isolated from the leaves of T_0 barley plants using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). PCR analyses were performed using combination of primers P1 (5′-TCCTCTTTGTGGCGGT AATC-3′) and P2 (5′-ACCAGTGCTGATCCCAAGAC-3′). The PCR reaction mixture (25 μL) contained: 1× PCR buffer, 1.5 mM $MgCl_2$, 10 pM both of primers, 0.2 mM dNTP, 0.5 U Platinum® Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA, USA), and 30 ng of template DNA. The PCR was performed in a Mastercycler®ep (Eppendorf, Hamburg, Germany) using the following conditions: initial heat denaturation at 94°C for 3 min, followed by 35 cycles each consisting of a denaturation step at 94°C for 1 min, annealing at 60°C for 45 s, extension at 72°C for 1 min and a final extension step at 72°C for 10 min.

RT-PCR analyses were performed on immature grains of (transgenic) barley plants. Total RNA was isolated using NucleoSpin® RNA Plant kit (Macherey-Nagel, Düren, Germany). The cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Real-Time PCR (ABI PRISM® 7000, Applied Biosystems, New York, USA) was carried out for artificial α-amylase gene using the primers P1/P2 and conditions described above. The *GAPDH* gene (GenBank accession no. **AK359500**) was used as a positive control house-keeping gene using the primers P3 (5′-GAAGGGCTGCTAGCTT CAACA-3′) and P4 (5′-GGCCATTCCAGTCAACTTTCC-3′). Equal amounts of template cDNA in two dilutions (25 and 50 ng of the total cDNA) were used in the both types of reaction mixtures.

2.3. Enzyme detection

Crude proteins were isolated from barley grains plants using P-PER Plant Protein Extraction Kit (Thermo Fisher Scientific). Naturally present enzymes were inactivated by heating of extracts to 80°C for 10 min, and cooled down to room temperature. Aliquots of extracts (50 μg per line) were separated on 12% (*w*/*v*) SDS-containing polyacrylamide slab gels with incorporated soluble starch (4.7 mg starch per ml of gel solution) as an enzyme substrate. After standard electrophoresis, the proteins in the gels were re-natured by shaking the gels in 2.5% (*w*/*v*) Triton X-100 at 4°C for 1 h. The gels were fixed in 12% (*w*/*v*) trichloracetic acid for 30 min. Amylase activity occurred during gentle shaking in 0.1 mol·l^{-1} phosphate–citrate buffer with 0.05 mol·l^{-1} NaCl (pH 6) for 3 h at 75°C. After staining with iodine solution (3.3 mg·ml^{-1} I_2 in 6.7 mg·ml^{-1} KI) for 10 min the proteins with α-amylase activities were observed on gels as white bands on brown background.

Quantitative α-amylase activity was assayed spectrophotometrically using α-Amylase Assay Kit (Ceralpha Method, Megazyme). The grain protein extracts (200 μg in 50 μl) were incubated with Amylase HR Reagent solution (50 μl) at 40°C, 60°C and 75°C (respectively) for 20 min. The reaction was stopped by adding 750 μl of Stopping Reagent. The absorbance of the samples was measured at 400 nm against distilled water. The α-amylase activity was calculated as Ceralpha Unit (CU) per gram of grain tissue. The residual - relative remaining activity (%) was calculated as activity under heat treatment condition/activity under no-treatment condition.

2.4. Data analyses

The data were processed by analysis of variance (ANOVA). The means were compared by the Fisher's Least Significant Difference (LSD) Method at a significance level of $\alpha = 0.05$ using STAGRAFICS (Centurion XVI.I, StatPoint Technologies).

3. Results

3.1. Creating of the artificial gene

The gene for α-amylase (AF068255) from the hyperthermophilic *Thermococcus hydrothermalis* was selected for this study as a functional template. This gene with molecular characteristics specific to the Archaea encodes a K-amylase of 457 amino acids, including a 22 amino acid putative signal peptide [20]. The recombinant K-amylase with calculated molecular mass 49.236 Da was optimally active at 75–85°C and pH between 5.0–5.5 [20]. In the present work the gene sequence was adjusted *in silico* for expression in barley plants. The complete sequence of the artificial α-amylase (AMY) gene (**KY806739**) differs from its bacterial origin by a total of 19% of nucleotides. Structure prediction using Phyre^2 showed structural similarity to a barley amylase representative (**AAA32925**) by means of typical enzyme domains (Fig. 1) [21].

Prior to synthesis, the start codon of the bacterial amylase gene and its surrounding sequences (a total of 84 base pairs) were replaced *in silico* by a homologous area from the barley gene for D-hordein [15]. This signal sequence is expected to direct the synthesized protein (enzyme) to endoplasmic reticulum. After adding the cloning sites to the ends, the final gene sequence was synthetized commercially.

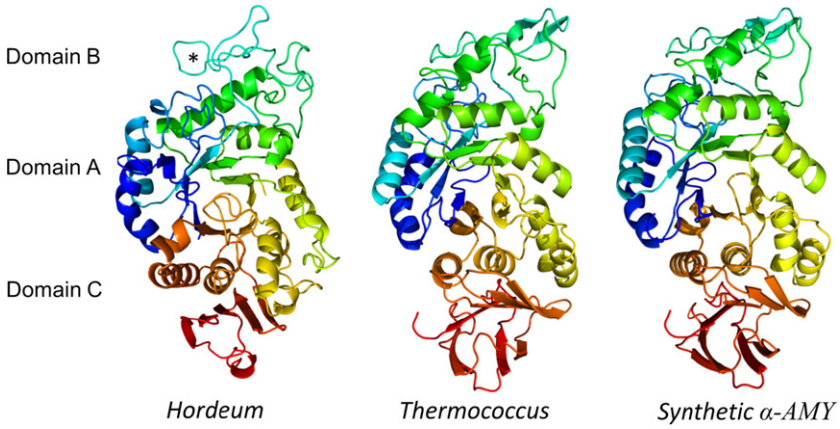

Fig. 1. Three dimensional structures of α-amylases. Protein structures were predicted using Phyre for α-amylases encoded by genes from (a) barley (**AAA32925**), (b) archaebacterium *Thermococcus hydrothermalis* (**AF068255**) and by the synthetic gene (**KY806739**). The structure of barley amylase as described previously [20] consists of a paralell (α/β)8-barell (domain A) and irregular fold stabilized by three Ca^{2+} ions (asterisk) (domain B), and a 5-stranded anti-parallel β-sheet (domain C). The barley structure was chosen to illustrate the most probable structure of archaeal amylases based on sequence similarities and evolutionary relationships [21].

3.2. Barley transformation and recovery of transgenic T_0 plants

The synthetized amylase (AMY) gene was cloned behind the promoter *1Dx5* for specific activity in grain [9,18,22] into the cloning vector pLRPT, yielding the plasmid pLRPT-AMY (Fig. 2a). Immature embryos of four barley cultivars Golden Promise, Pribina, Levan and Nitra were co-transformed with the plasmids pAHC20 and pLRPT *via* biolistic bombardment. The number of calli regenerated in presence of PPT as a selection agent (Fig. 2b) ranged from 28.0% (cv. Golden Promise) to 82.2% (cv. Levan) (Table 1). Not all putative transgenic calli did produce shoots. Overall, 8 T_0 barley plants were obtained (Table 1, Fig. 2c). The presence of the α-amylase gene was confirmed in 4 of them (Table 1). RT-PCR analyses showed corresponding mRNA transcript in 3 out of 4 analyzed T_0 plants (Fig. 2d). Those three T_0 plants were used in further analyses.

3.3. Enzyme analyses of grains from T_0 transgenic plants

The spikes of selected RT-PCR positive T_0 plants shared phenotypes similar to their non-transformed counterparts (Fig. 2c). Activities of α-amylase in the grain extracts of the three obtained T_0 plants were detected after protein separation in starch containing PAGE (Fig. 3), and quantified spectrophotmetrically (Table 2). After exposure of protein extracts to 80°C for 10 min we studied the α-amylase activity in gel activity assays. The zymogram (Fig. 3b) revealed single active fractions in 2 out of 3 grain protein samples of T_0 plants. The mobilities of detected amylase bands correspond to the predicted size of ~50 kDa and indicate un-glycosylated structure. We did not observe any active fraction in non-transgenic samples.

Further, we quantified the α-amylase activity in grains spectrophotometrically after exposure of protein extracts to 40°C, 60°C and 75°C (respectively) (Table 2). At the lower temperatures (40°C and 60°C) the values obtained in transgenic grains were comparable with those of non-transgenic barley counterparts (for all three varieties, respectively) (Table 2). At higher temperature (75°C), however, they significantly dropped indicating to inactivation of the native amylases (Table 2). The share of residual enzyme activity upon 75°C heat treatment in all cultivars was above 9%, which is for cvs. Pribina and Nitran more than 4 times higher compared to non-transgenic controls (Table 2). The effect of the introduced enzyme was less pronounced in the model cv. Golden Promise (only 1.22 fold difference compared with non-transgenic barley), given that

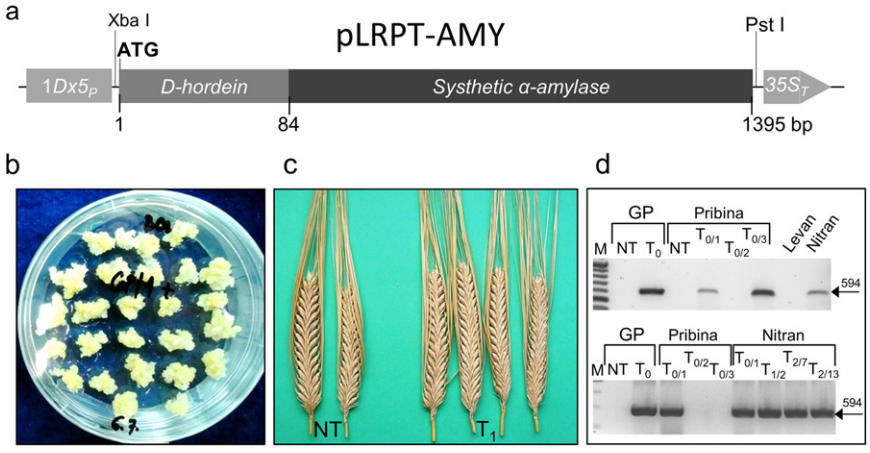

Fig. 2. Introduction of the synthetic α-amylase gene into barley genome. (a) The synthetic gene from *Thermococcus* with adjusted codon usage (GenBank accession no. **KY806739**) and a signal sequence for targeting the protein to endoplasmatic reticulum was synthetized commercially and cloned into the vector pLRPT, between the *1Dx5* promoter and the *35S* terminator. (b) Barley calli were transformed biolistically and regenerated under selection pressure of 1 $mg \cdot l^{-1}$ phosphinotricin. (c) Mature transgenic plants and grains (T_0-1 of the cv. Nitran) showed phenotype as the corresponding non-transgenic controls (NT). (d) The transgene was detected in plants at the level of DNA (upper gel) and RNA (lower gel) as a 596 bp amplicon (arrow). GP – Golden Promise.

Table 1
Overview of barley transformation and molecular analyses of T_0 plants.

Cultivar[a]	Calli[b]	Calli(PPT+)[c]	Calli(PPT+)[%][d]	Transgene (AMY+)[e]	
				PCR	RT-PCR
Golden Promise	250	70	28.0	1(1)	1(1)
Pribina	325	251	77.2	1(5)	0(1)
Pribina	275	149	54.2	1(1)	1(0)
Levan	275	226	82.2	0(0)	–
Nitran	250	84	33.6	1(1)	1(1)
total	1375	780	56.7	4(8)	3(4)

[a] Barley cultivars of which immature embryos were used in transformation experiments.
[b] Number of embryogenic calli used in transformation experiments.
[c] Number of calli regenerated under of selection pressure of 1 $mg \cdot l^{-1}$ phosphinotric (PPT+).
[d] Number of (PPT+) calli as a percentage of total number of calli.
[e] Number of PCR and RT-PCR positive (AMY+) T_0 plants. Genomic DNAs (PCR) and cDNAs (RT-PCR) were amplified with the primers P1/P2 corresponding to the synthetic α-amylase gene. In brackets the total numbers of analyzed T_0 plants are given.

the α-amylase activity was naturally lower comparing with the other two cultivars ($P < 0.05$, ANOVA, LSD) (Table 2).

3.4. Enzyme analyses of transgenic grains of T_1 plants

We studied the activity of the transgene product in the second generation of transgenic barley. The available T_0 plant of cv. Nitran was grown to maturity and seeds were sown. Seven among 20 lines of obtained T_1 plants were selected for presence/activity of transgene at both DNA and RNA levels (respectively; data not shown). Measurement of α-amylase activity in grains showed that 7–9% of activity was retained in samples from each transgenic line at 75°C (Fig. 4), similarly as in the T_0 plants.

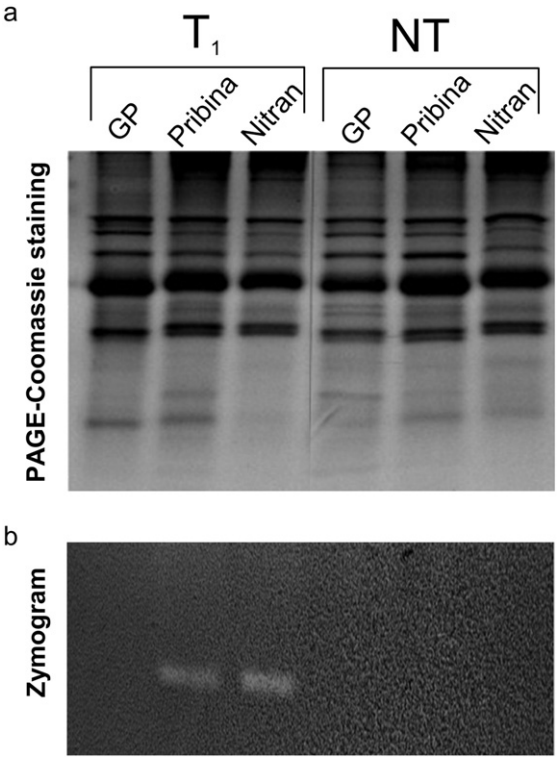

Fig. 3. Detection of α-amylase activity in barley grains. Proteins from grains of transgenic (T1) and non-transgenic control (NT) barley grains were heated to 80°C and separated in SDS-PAGE with incorporated soluble starch as an enzyme subtrate. Protein profile in the gels was visualized with Coomasie staining (a) or assayed for α-amylase activity (b). Corresponding values of the enzyme measurements are given in the Table 2. GP — Golden Promise.

Table 2
Activities of synthetic α-amylase in grain protein extracts of transgenic T_0 and non-transgenic control (NT) barley plants.

Cutivar		Enzyme activity ($CU.\cdot g^{-1}$)[a]					
		40°C	P^b	60°C	P^b	75°C	P^b
Pribina	NT	62.80 ± 0.22	*c*	66.31 ± 0.96	*d*	1.29 ± 0.28	*a*
	T_0-1	63.57 ± 0.14	*c*	70.70 ± 0.19	*e*	5.43 ± 0.19	*d*
Nitran	NT	68.67 ± 0.51	*d*	57.75 ± 0.54	*c*	1.51 ± 0.17	*a*
	T_0-2	60.73 ± 0.14	*b*	70.32 ± 0.07	*e*	5.70 ± 0.23	*d*
Golden Promise	NT	46.18 ± 0.49	*a*	44.81 ± 0.06	*a*	3.44 ± 0.38	*b*
	T_0-3	46.06 ± 0.35	*a*	46.92 ± 0.13	*b*	4.18 ± 0.13	*c*

[a] α-Amylase activities were quantified spectrophotometrically using α-Amylase Assay Kit. The enzyme activity was calculated as Ceralpha Unit (CU) per gram of grain tissue. Data represent mean ± standard deviation.
[b] Probability according to the LSD test. Different letters indicate significant differences at $P < 0.05$.

4. Discussion

Breeding of barley (*Hordeum*) focuses primarily on increasing yields, resistance to disease, and improving nutritional and malting properties, while in the brewing industry the level of malt α-amylase is a key quality parameter. The natural α-amylase activity in grains of the tested cvs. Nitran, Pribina and Golden Promise pointed on variability that falls in the common range of 40–80 $U \cdot g^{-1}$ reported for commercial barley cultivars [23]. At 60°C the measured enzyme activity was still remarkably high in all the cultivars, while in some wild and commercial barley a drop to less than 50% has been reported at 50°C, and to 10–30% at 60°C [23]. At higher temperature (75°C), our measurements showed inactivation of most total α-amylase activity in grains of all three barley varieties tested (Table 2). The corresponding residual activity values (between ~2.0 and 7.5%) were lower than those reported for malt extracts from 78 barley varieties at 72.5°C (9 to more than 60%) [13].

The gene for a thermostable α-amylase from thermophilic bacteria was here modified *in silico* for subsequent expression in barley grains. Though efficiency of transformation was a clear bottleneck in our efforts, single transgenic plants were regenerated for three of four different barley cultivars (respectively). The transgenic plants showed no change in phenotype and expressed the synthetic (trans)gene at RNA as well as protein level. The *1Dx5* wheat promoter ensured expression in the barley grain as has been suggested previously [9]. The zymogram confirmed that α-amylase was still active at 75°C in T_0 transgenic plants of cv. Nitran and Pribina, but more accurate enzyme measurements reflected to ~9% residual amylase activity in transgenic grain samples of all varieties. Similar values were achieved in grains of all T_1 transgenic lines of cv. Nitran. Previosly, Kihara et al. [24] have improved the thermostability of sevenfold-mutant barley β-amylase in transgenic barley seeds. They reached 4.0–67.2% relative remaining activity under 65°C treatment; however, at 70°C it dropped to less than 5%.

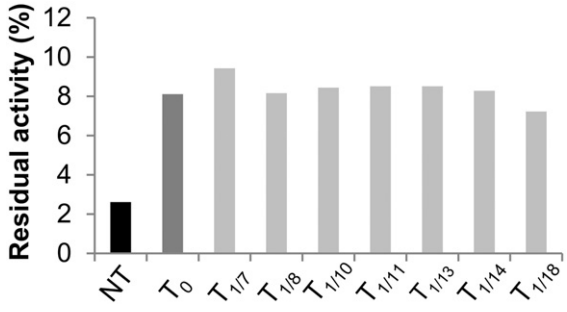

Fig. 4. Measurement of α-amylase activity in transgenic barley grains. Shown are the values of relative residual activity upon 75°C heat treatment, obtained in transgenic grains of cv. Nitran T_0-1 and in the grains of its progeny. For comparison, the activity in the corresponding non-transformed control (NT) is given.

5. Conclusion

Modification of codon usage enabled expression of a functional bacterial gene in barley, while the given wheat promoter was used at first time to drive the transgene product to the barley grain. Genetic transfer of the synthetic α-amylase gene into three barley cultivars resulted in retaining 7–9% of amylase activity after heat-treatment of grain protein samples. Nevertheless, further research is needed to achieve technologically more relevant values. The final outcome of the transfer is apparently influenced by the substantial natural variation in thermostability of α-amylases among the cultivars. Our results confirmed that plant biotechnology can provide a good mean to complement the natural potential of barley varieties for exploitation by barley breeders.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Financial support

This work was financed by the Slovak Research and Development Agency under contracts numbers APVV-16-0051 and APVV-380-12, and the grant VEGA 2/0035/17.

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