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Research article

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

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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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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.