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Transgenic barley producing essential polyunsaturated fatty acids

D. MIHÁLIK[1]*, M. GUBIŠOVÁ[1], T. KLEMPOVÁ[2], M. ČERTÍK[2], K. ONDREIČKOVÁ[1], M. HUDCOVICOVÁ[1], L. KLČOVÁ[1], J. GUBIŠ[1], I. DOKUPILOVÁ[1,2], L. OHNOUTKOVÁ[1], and J. KRAIC[1]

Plant Production Research Center, Bratislavská cesta 122, SK-92168 Piešťany, Slovak Republic[1]
Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, SK-81237 Bratislava, Slovak Republic[2]

Abstract

Polyunsaturated fatty acids (PUFAs) affect diverse physiological processes and human health. Most cereals are poor in n-3 and n-6 PUFAs. Using biolistics, barley (*Hordeum vulgare* L. cv. Golden Promise) was transformed with an artificial gene encoding Δ^6-desaturase (D6D) under an endosperm-specific promoter. This artificial gene was designed from the sequence of *D6D* of the filamentous fungus *Thamnidium elegans*, but codon usage was optimised for cereals. A signal sequence from the gene encoding for high molecular mass glutenin Dx5 was added to a destinate mature protein. Successful transformation was confirmed in T_0 plants at the genomic level and in T_1 seeds at the transcriptomic and metabolomic levels. Transformed plants produced up to 0.141 % of γ-linolenic acid (GLA) and 0.294 % of stearidonic acid (SDA) of the total amount of fatty acids in their grains. Although the content of these fatty acids was relatively low, the current study provides the first evidence that transgenic barley can be a source of GLA/SDA.

Additional key words: biolistic transformation, Δ^6-desaturase, γ-linolenic acid, *Hordeum vulgare,* stearidonic acid

Introduction

Barley (*Hordeum vulgare* L.) is one of the most widely grown cereals in Europe after wheat and maize, with an annual global production of 123 million tons (www.faostat.fao.org). It is used in the malting and brewing industry as livestock feed, and also for baking purposes. In addition to polysaccharides, cereals are a main source of protein in the human diet, but they lack n-3 and n-6 polyunsaturated fatty acids (PUFAs). A diet based primarily on cereals can lead to a nutritional and physiological imbalance. Increasing the content of PUFAs in cereals is a possible way to improve their nutritional value. Changes in the composition of fatty acids in cereal grains are not feasible by classic breeding methods, but an alternative approach to achieve this task may be targeted genetic transformation of barley. Cereals do not produce essential PUFAs, but produce metabolites that are suitable substrates for enzymes catalysing formation of n-6 PUFAs. Δ^6-desaturase (D6D) is an enzyme catalysing the conversion of linoleic acid (LA; C18:2, n-6) to γ-linolenic acid (GLA; C18:3, n-6) (Brenner 1976, Gunstone 1992). D6D also catalyses the conversion of α-linolenic acid (ALA: C18:3, n-3) to stearidonic acid (SDA; C18:4, n-3).

GLA is an important precursor that can be relatively quickly metabolised *in vivo* into dihomo-γ-linolenic acid (DGLA; C20:3 n-6) by an enzyme Δ^6-elongase (Flider 2005). DGLA is then metabolised into prostaglandin PGE 1 which is involved in important physiological processes (Nykiforuk *et al*. 2012). The human body is not able to synthesize essential PUFAs including GLA and must obtain them from food (*e.g*., fish and crustaceans). Plant species accumulating GLA include *Oenothera biennis* (Hudson 1984), *Borago officinallis* (Stymme and Stobart 1986), and *Ribes nigrum* (Traitler *et al*. 1988). GLA is produced in more than 200 plant species that unfortunately are not suitable for industrial production (Ucciani 1995). Important producers of PUFAs also include microorganisms, such as bacteria, algae, yeast, and filamentous fungi that may be used in solid state fermentation and submerge fermentation (Gill and Valivety 1997, Certik and Shimizu 1999, Ratledge 2004, Zhao *et al*. 2008).

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Abbreviations: ALA - α-linolenic acid, D6D - Δ^6-desaturase, DGLA - dihomo-γ-linoleic acid, HMM - high-molecular-mass, GAPDH - glyceraldehyde 3-phosphate dehydrogenase, GLA - γ-linolenic acid, LA - linoleic acid, OA - oleic acid, PUFA - polyunsaturated fatty acid, SDA - stearidonic acid.
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* Corresponding author; fax: (+421) 33 7726 306; e-mail: mihalik@vurv.sk

Nevertheless, plants have the highest production potential for PUFAs. With development of transformation techniques, the number of transgenic plants producing GLA has increased. Reddy *et al*. (1993) were the first who isolated the gene coding D6D in cyanobacterium, later used to transform tobacco plants (Reddy and Thomas 1996). However, the highest production of GLA was only about 1.2 % of the total amount of fatty acids. A higher content of GLA (~ 13 %) was detected in tobacco plants transformed with the *D6D* gene from borage (Sayanova *et al*. 1997). A high content of GLA, 43 % of total fatty acids, was produced by canola plants co-transformed with the gene for D6D and the gene encoding for Δ^{12}-desaturase from filamentous fungus *Mortierella alpina* (Liu *et al*. 2001). The gene encoding D6D from *Echium gentianoides* was used for tobacco transformation, and GLA was detected in transgenic calli (García-Maroto *et al*. 2002). A high content of GLA was later achieved by using a seed-specific promoter regulating the *D6D* gene from *Pythium irregulare* in transgenic *Brassica juncea* (Hong *et al*. 2002). Sato *et al*. (2004) also reported a seed-specific overproduction of GLA in soybean transformed with the gene from borage. Recently, Nykiforuk *et al*. (2012) described the transformation of safflower (*Carthamus tinctorius*) with *D6D* genes from *Saprolegnia diclina* and *Mortierella alpina*. The transgenic plants produced 50 - 70 % of GLA.

Nearly 50 % of fatty acids in cereal grains is represented by LA but no GLA is produced there. Genetic transformation of cereals with appropriate genes may provide an important alternative source of PUFAs, even if relatively low amounts are produced. In this paper, we described the genetic transformation of barley with a synthetic *D6D* gene under an endosperm-specific promoter. The main goal of this work was to ascertain whether barley plants transformed with the gene encoding Δ^{6}-desaturase could produce GLA in their grains.

Materials and methods

Plants: Spring barley (*Hordeum vulgare* L.) cv. Golden Promise was grown in pots under natural conditions. Immature seeds were removed from the spikes 14 - 18 d after anthesis and sterilised in 70 % (v/v) ethanol for 1 min followed by sterilisation for 10 min in commercial bleach containing 4 % (v /v) sodium hypochlorite with gentle shaking. The seeds were then washed three times in sterile distilled water. Immature embryos (1 - 2 mm in diameter) were aseptically excised from the caryopses under a stereo microscope.

Characterisation of recombinant plasmid: Artificial gene *D6D* was prepared by synthesis (*MWG Operon*, Ebersberg, Germany) based on the sequence of *D6D* from *Thamnidium elegans* (Wang *et al*. 2007), but codon usage was optimised for gene expression in cereals using the *OPTIMIZER* software (http://genomes.urv.cat/OPTIMIZER/; Puigbó *et al*. 2007). The start codon of the gene was replaced by the signal sequence of the gene encoding for the high molecular mass (HMM) glutenin subunit 1Dx5 (Anderson *et al*. 1989), and the sequence recognized by the restriction endonuclease *Sal*I was inserted in front of the signal sequence. This signal sequence is capable to destinate a synthesised protein into endoplasmic reticulum where the biosynthesis of fatty acids is located (Gregova *et al*. 2007). After cleavage with restriction enzymes *Sal*I and *Xba*I, the fragment carrying the artificial *D6D* sequence was cloned (unpublished data) into the vector pLRPT (kindly provided by Dr. H.D. Jones, Rothamsted Research Station, Rothamsted, UK) which contains the promoter from the 1Dx5 HMM glutenin subunit (He *et al*. 2005). This endosperm-specific promoter from the HMM glutenin subunit was used as it has been previously demonstrated to be functional in barley (Furtado *et al*. 2009).

Transformation of barley: The entire embryonic axis was removed from each embryo and explants (16 per plate) were placed a scutellum-side up onto a callus induction medium supplemented with 2.5 mg dm^{-3} Dicamba (3,6-dichloro-2-methoxybenzoic acid). The compositions of all media were previously described by Harwood and Smedley (2009), but the callus induction medium was enriched with 1.25 mg dm^{-3} $CuSO_4 \cdot 5\ H_2O$ (Gubisova *et al*. 2011). One to two days after isolation, the explants were transferred onto an osmotic medium which was identical to the callus induction medium supplemented with 72 g dm^{-3} mannitol.

Four hours later, the explants were bombarded using a *PDS-1000/He* biolistic device (*BioRad*, Hercules, CA, USA). Gold particles (1 μm) were coated with the plasmids pLRPT, carrying the *D6D* gene, and pAHC20 (Christensen and Quail 1996), carrying the *bar* gene for the selection of transformed tissue. The proportion of pLRPT to pAHC20 was 3:2, and the coating procedure followed the standard protocol (Harwood and Smedley 2009). The coated gold particles (80 μg) resuspended in 5 mm^3 of 100 % ethanol were used as shot. The parameters of the gene gun were as follows: a stopping screen to the sample 6 cm, a helium pressure 7.58 MPa, and a vacuum 91.43 kPa.

On the following day, the bombarded explants were placed a scutellum-side down onto a callus induction medium without mannitol, but supplemented with 5 mg dm^{-3} phosphinothricin (PPT) for selection of transformed tissue. The cultures were stored in the dark at 25 °C for 2 weeks and then transferred to a fresh medium for another 2 weeks. The induction of regeneration took place on a transition medium supplemented with 2.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg dm^{-3} 6-benzylaminopurine (BAP) (Harwood and Smedley 2009) under a 16-h photoperiod, an irradiance of

50 μmol m^{-2} s^{-1}, and day/night temperatures of 25/20 °C for 2 weeks. For regeneration, the cultures were transferred onto a regeneration medium without growth regulators (Harwood and Smedley 2009) and cultivated under the specified photoperiod. The transition medium as well as the regeneration medium were supplemented with a low dose of PPT (1 mg dm^{-3}). Calli regenerated shoots within four weeks (transferred onto a fresh medium after 2 weeks). The regenerated shoots were rooted for additional 3 weeks in a rooting medium which was the same as the callus induction medium, but casein hydrolysate and Dicamba were omitted and PPT was added to a final concentration of 1 mg dm^{-3}.The rooted plants were transferred to soil, acclimatised to *ex vitro* conditions and grown to maturity. All components of cultivation media were purchased from *Duchefa Biochemie* (Haarlem, The Netherlands).

Genomic DNA extraction and PCR detection of artificial D6D: Total genomic DNA was isolated from 1 - 2 g of leaf tissue of putative transformants and control plants (nT - non-transformed plant regenerated *in vitro*, NC - parental non-transformed plant) 3 to 5 weeks after the transfer to soil using a *DNeasy* plant maxi kit (*Qiagen*, Hilden, Germany). Primers were designed using the *CLC Genomics Workbench* software (Aarhus, Denmark) based on the gene sequence of the GenBank accession No. HM640246. Primer sequences were F: 5'-GGTGGAAGGACAAGCACAAT-3' and R: 5'-CGCCCAGTAATGACCTGAAT-3'. The expected PCR product size was 517 bp. A DNA amplification was carried out in 25 mm^3 of a reaction mixture containing 0.5 U *Platinum® Taq* DNA polymerase (*Invitrogen*, Carlsbad, CA, USA), 2.5 mm^3 of a 10× reaction buffer, 2.5 mm^3 of 50 mM $MgCl_2$, 10 pmol of each primer, 0.125 mm^3 of 10 mM dNTP, and 30 ng of a DNA template. PCR was performed using the following settings: 94 °C for 3 min, 35 cycles at 94 °C for 1 min, at 60 °C for 0.5 min, at 72 °C for 1 min, and final polymerization at 72 °C for 5 min in a *Mastercycler® ep* (*Eppendorf*, Hamburg, Germany). PCR products were separated in a 1 % (m/v) agarose gel and stained with ethidium bromide.

Transcriptomic analysis of transgenic plants: Total RNA from putative transformants and control plants was extracted from 0.2 - 0.5 g of immature seeds (at a milk ripe stage) using a *NucleoSpin®* RNA isolation kit (*Macherey-Nagel*, Düren, Germany). Potential genomic DNA contaminants were removed by a DNase treatment (*Fermentas*, St. Leon-Rot, Germany). A RNA concentration was measured spectrophotometrically, and RNA quality was determined by electrophoresis in a 1.5 % agarose-formaldehyde gel stained with ethidium bromide. First strand cDNA synthesis was done using a *RevertAid First Strand cDNA Synthesis* kit (*Fermentas*). Qualitative analysis of transgene cDNA was performed by PCR with 100 ng of a template. The conditions of PCR and the composition of the amplification mixture were the same as described for the DNA analysis. PCR products were separated in a 1 % agarose gel and stained with ethidium bromide.

Semi-quantitative measurement of transgene expression: Real-time PCR was carried out for *D6D* with *GAPDH* (GenBank acc. No. AK359500.1) as house-keeping gene (Matsumoto *et al*. 2011) using an *ABI PRISM® 7000* (*Applied Biosystems*, New York, USA) and a *SYBR® Green PCR Master Mix* (*Applied Biosystems*). Amplification for both genes was carried out in 25 mm^3 of a reaction mixture containing 12.5 mm^3 of a *SYBR® Green PCR Master Mix*, 0.1 μM both primers, 5 mm^3 of cDNA, and water to a final volume. Primers were different for both genes with the following sequences: D6D primers (F: 5´-CGGGCCTTTCACTCCTTT ATG-3´and R: 5´-CCACCCCACGACTGAGTTG-3´) and barley GAPDH primers (F: 5´-GAAGGGCTGCTA GCTTCAACA-3´ and R: 5´-GGCCATTCCAGTCAA CTTTCC-3´). Analyses were done in triplicate (3 samples per each transgenic or control plant). The sizes of expected PCR products were 101 and 100 bp for *D6D* and *GAPDH*, respectively. Real time-PCR was performed for both genes using the following setting: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and at 65 °C for 1 min. Equal amounts of template cDNA in two dilutions (25 and 50 ng of total cDNA) were used in both types of the reaction mixtures. Isolated fragments of *D6D* (101 bp) and *GAPDH* (100 bp) ranging from 2.5×10^{-4} pg to 2.5×10^{3} pg were used as standards for the calibration curve.

Fatty acid extraction and analysis: Fatty acids from total lipids were converted to their methylesters as previously described (Certik and Shimizu 1999). Samples (three from each transgenic plant) contained two seeds from one spike. The seeds were homogenized in a mortar with a pestle and 50 mg of a homogenised sample was mixed with 1 cm^3 of dichloromethane (containing 1 mg of hepta-decanoic acid as internal standard) and 2 cm^3 of 10 % (v/v) HCl in methanol. After incubation at 50 °C for 3 h, 4 cm^3 of hexane and 1 cm^3 of deionized water were added and transmethylated fatty acids were pre-extracted to hexane with a vortical mixer. The mixture was then centrifuged to separate hexane phases, and fatty acid methylesters were analysed by gas chromatography (*GC-6890 N*, *Agilent Technologies*, Santa Clara, CA, USA) using a capillary column *DB-23* (60 m × 0.25 mm, a film thickness of 0.25 μm; *Agilent Technologies*) and a FID detector (a constant flow, hydrogen 40 cm^3 min^{-1}, air 450 cm^3 min^{-1}, 250 °C) under a temperature gradient: 150 °C held for 3 min; 150 - 175 °C (rate 7 °C min^{-1}); 175 °C held for 5 min; 175 - 195 °C (rate 5 °C min^{-1}); 195 - 225 °C (rate 4.5 °C min^{-1}); 225 °C held for 0.5 min; 225 - 215 °C (rate 10 °C min^{-1}); 215 °C held for 7 min; 215 - 240 °C (rate 10 °C min^{-1}); 240 °C held for 7 min. Hydrogen was used as carrier gas (flow 2.5 cm^3 min^{-1}, velocity 57 cm s^{-1}, pressure 220 kPa) and a split ratio of 1/20 (inlets: heater 230 °C; hydrogen flow 51 cm^3 min^{-1}

for 2 min, then hydrogen flow 20 cm^3 min^{-1}; pressure 220 kPa). The fatty acid methylester peaks were identified based on a C_4 - C_{24} fatty acid methylester standard mixture (*Supelco*, Bellefonte, PE, USA) and evaluated by *ChemStation B 01 03* (*Agilent Technologies*). All analyses were done in triplicate.

Statistical analysis: Data were processed by one-way analysis of variance (*ANOVA*) followed by the Tukey's test ($P < 0.05$) using the *Statgraphics* (*XV.II*) software.

Results

In total, 240 immature barley scutella in 15 Petri dishes were co-transformed by the described biolistic technique with plasmids pLRPT-*D6D* and pAHC20 harbouring the *bar* gene to control resistance to phosphinothricin. Excluding contaminated explants, 224 calli were evaluated for plant regeneration on the selection medium supplemented with PPT. In total, 18 individual plants regenerated from 11 transformed embryos. These putative transformants were rooted, successfully acclimatised to *ex vitro* conditions and grown to maturity. Any morphological differences between the control plants and transformants were not observed (Fig. 1). The plants formed 8 - 16 spikes with 12 - 18 seeds (Fig. 1). During the growth period, the transgene analyses were conducted.

Fig. 1. *A* - Barley plants acclimatised after *in vitro* regeneration: *left* - a transformed plant, *right* - a non-transformed plant; *B* - spikes of barley plants: *left* - a non-transformed plant, *right* - a transformed plant.

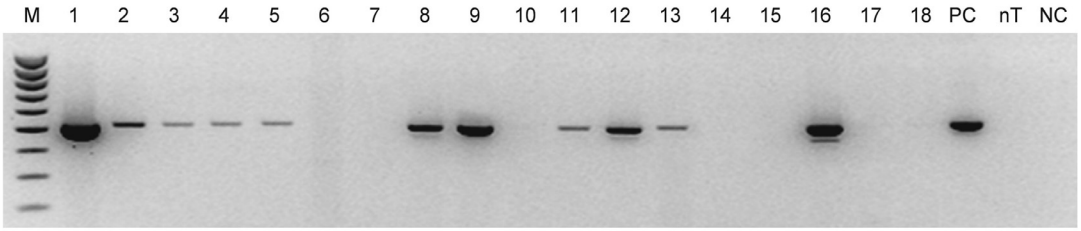

Fig. 2. The PCR detection of *D6D* transgene in putative transformants. DNA fragments were separated in a 1 % agarose gel. *Lanes*: M - a *MassRuler DNA Ladder Mix* (100 bp, *Fermentas*); 1 to 18 - transformed plants; PC - a positive control; nT - a non-transformed plant regenerated *in vitro*; NC - a negative control - a parental non-transformed plant. PCR fragment size 517 bp.

The plants that underwent the selection were analysed for the presence of transgene DNA at the level of T_0 generation. The transgene was detected in 11 of 18 plants (Nos. 1, 2, 3, 4, 5, 8, 9, 11, 12, 13, and 16; Fig. 2), and a transformation efficiency was determined to be 4.9 %. A PCR product of 517 bp was detected in all these samples.

The plants of T_0 generation showing the presence of transgene at the genomic level (excluding plant No. 5 which was sterile) were then analysed at the transcriptomic level. To exclude false positive PCR signals due to possible contamination with genomic DNA, the samples were treated with DNase I. The integrity of extracted RNA was assessed by gel electrophoresis and subsequent analysis of ribosomal RNA bands. At the mRNA level, a positive signal was detected in five samples: plants Nos. 3, 4, 9, 11, and 12 (Fig. 3).

To quantify the expression of the transgene in the immature barley grains of T_1 seed generation, semi-quantitative real-time PCR was used. The quantity of transgene (*D6D*) cDNA was determined based on a calibration curve ranging from 2.5×10^{-4} to 2.5×10^{3} pg of amplified fragments. The plants with an expression below the lowest standard (*e.g.*, plant No. 11 which also had a weak PCR band; Fig. 3) were not evaluated or subjected to further analysis for the presence of metabolites. Only one amplified product was detected by dissociation curves in both the reactions. Measured

values of *D6D* cDNA in the individual transformants ranged from 1.63 to 4.25 pg and the values of *GAPDH* cDNA from 162.0 to 752.25 pg in 50 μg of total RNA. To ensure the objective assessment of transgene expression, the ratio between the amount of transgene cDNA and the amount of cDNA of house-keeping *GAPDH* gene is reported (Table 1).

A fatty acid composition was determined in the mature seeds of four transformed plants that exceeded the expression of a standard in semi-quantitative real-time PCR: plants Nos 4 and 12, which regenerated independently from one transformed embryo, and plants Nos. 3 and 9, which regenerated from other embryos. In all samples, LA and ALA were the most abundant polyunsaturated fatty acids (50 - 54 % and 4.0 - 6.5 %, respectively, of total fatty acids; for the control plant 51.7 and 5.9 %). Each analysed plant contained both GLA and SDA in low concentrations. Yields varied from 0.098 - 0.141 % (v/v) for GLA and from 0.026 - 0.294 % (v/v) for SDA (Table 1). These results suggest that artificially expressed D6D from *Thamnidium elegans* did not have a preference for LA or ALA as substrate since the yields for both products were very similar. The seeds from the control plants contained neither GLA nor SDA. The yields of GLA and SDA correlated with the results of semi-quantitative real-time PCR. Plant No. 4, which showed the highest ratio of *D6D/GAPDH*, contained the highest amount of GLA and SDA.

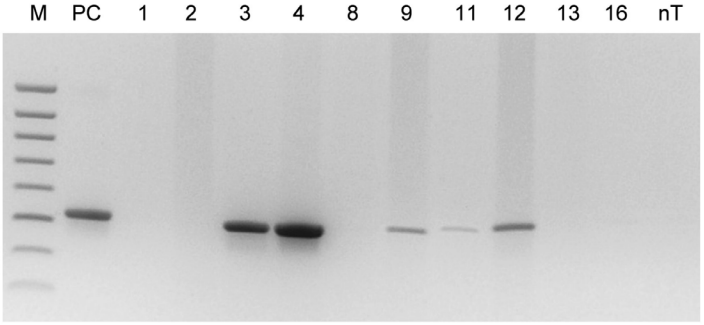

Fig. 3. The detection of D6D-mRNA in T_1 seeds by RT-PCR. Fragments were separated in a 1 % agarose gel. *Lanes*: M - a *MassRuler DNA ladder mix*; PC - a positive control; *lanes* 1, 2, 3, 4, 8, 9, 11, 12, 13, and 16 - designation Nos of transformed plants; nT - a non-transformed plant regenerated *in vitro*.

Table 1. The analysis of a *D6D* gene expression at transcriptomic (second column) and metabolomic (third and fourth columns) levels. The relative amounts of *D6D*-cDNA measured by real-time PCR are expressed by the ratio between cDNA of the transgene and cDNA of house-keeping gene *GAPDH*. The content of GLA and SDA in the transgenic barley plants and control plant are expressed in percentage of the total amount of fatty acids. nT control - a non-transformed plant regenerated *in vitro*; D6D trans - transgenic plants Nos. 3, 4, 9, and 12. Means ± SD, $n = 3$. Different superscripts indicate statistically different values ($P < 0.05$) counted by the Tukey's test.

Samples	mRNA-D6D/GAPDH	GLA [%]	SDA [%]
nT control	0.0000 ± 0.0000^{a}	0.0000 ± 0.0000^{a}	0.0000 ± 0.0000^{a}
D6D trans - 3	0.0126 ± 0.0013^{c}	0.1286 ± 0.0015^{cd}	0.0396 ± 0.0019^{c}
D6D trans - 4	0.0165 ± 0.0031^{d}	0.1436 ± 0.0038^{d}	0.2945 ± 0.0073^{d}
D6D trans - 9	0.0025 ± 0.0006^{ab}	0.0953 ± 0.0171^{b}	0.0257 ± 0.0010^{b}
D6D trans.- 12	0.0058 ± 0.0009^{b}	0.1164 ± 0.0053^{bc}	0.0327 ± 0.0046^{bc}

Discussion

Barley as well as other cereals is rich in proteins and sugars but deficient in essential fatty acids. Barley produces high amounts of LA and limited quantities of ALA. Cereals cannot produce essential PUFAs derived from LA or ALA due to the lack of Δ^6-desaturase catalysing conversion of LA into GLA or of ALA into SDA. Also, cereals are not able to form PUFAs with a longer chain than C20 since biosynthesis of these fatty acids is dependent on the presence of a functional elongase/desaturation system. Although several papers focusing on the production of GLA by plants transformed with genes involved in the biosynthesis of fatty acids have been published (Sayanova *et al*. 1997, Liu *et al*. 2001, Hong *et al* 2002, Chen *et al*. 2005), these reports primarily concern dicotyledonous oleaginous plants with poor agronomical characteristics. On the other hand, no work describing cereals as producer of GLA by either classical plant breeding or gene engineering techniques has been published so far. However, the preparation of cereal-based materials enriched with PUFAs has only

been developed by fungal solid state fermentation (Certik and Adamechova 2009, Certik *et al.* 2010). Therefore, the goal of the current work was to transform barley with the gene encoding Δ^6-desaturase in order to induce production of GLA in barley seeds.

After transformation of monocotyledonous plants, the recovery of fertile plants from transformed explants is very difficult (Sood *et al.* 2011). As barley is one of the best-studied monocot crops (Dahleen and Manoharan 2007, Harwood and Smedley 2009), it was chosen for the current study. The successful transformation with the *D6D* gene was confirmed at genomic, transcriptomic, and metabolomic levels. Since this is the first report dealing with transgenic cereals synthesizing PUFAs, we decided to present already the results achieved at the level of T_1 generation seeds. However, it is surprising that the explicit functionality of the transgene was confirmed not only by the presence of GLA, but also by the presence of SDA. None of these metabolites were detected in the control plants. It was depicted that Δ^6-desaturase as the rate-limiting step in PUFA formation exhibits different specificities for OA, LA, and ALA in oleaginous fungi (Certik *at al*. 1998). Depending on the conditions, it may finally lead to various amounts of n-3, n-6, and n-9 PUFAs being formed. Because the expression of the fungal *D6D* gene in barley resulted in the similar production of both GLA and SDA, the enzyme probably changed its preferable specificity for LA as it was found in the fungus *Thamnidium elegans*. Similar variations in substrate utilization among Δ^6-desaturases from various organisms have been described. For example, functional characterization of the fungus *Mucor rouxii* Δ^6-desaturases in yeast *Saccharomyces cerevisiae* shows that they had a broad specificity on C15 - C18 unsaturated fatty acids with a double bond at the Δ^9 position (Na-Ranong *et al*. 2005). Among the Δ^9-unsaturated C18 substrates, a triene fatty acid (ALA) is the preferred substrate for the *Mucor* enzyme over monoene (OA) and diene (LA) fatty acids, respectively. Thus, the expressed fungal Δ^6-desaturase might equally utilize both LA and ALA in barley regardless of their actual content in this crop. The exact reason for such biochemical behaviour of the Δ^6-desaturase in barley remains to be answered. In addition, the accumulation of newly formed PUFAs in barley was quite low. The content of GLA and SDA in total of fatty acids reached maximum of 0.14 and 0.29 %, respectively. This is in contrast with previously published studies describing relatively higher yields of GLA in other transgenic plants containing *D6D*, such as tobacco (Sayanova *et al*. 1997), canola (Liu *et al*. 2001), *Brassica juncea* (Hong *et al*. 2002), *Lotus japonicus* (Chen *et al*. 2005), and *Echium plantagineum* (Zhou *et al.* 2006). Nevertheless, the transformation of dicotyledonous plants with the *D6D* gene is much simpler compared to monocot plants.

In the current work, we have shown that a properly selected biotechnological approach may provide a way to produce PUFAs in cereals. Although the content of GLA and SDA produced by the transgenic barley was too low to act as an ideal source to supply the recommended daily dose, our results indicate that cereals transformed with the *D6D* gene may be a potential alternative source of PUFAs in the human diet. This is particularly relevant since the current availability of seafood, such as fish and shellfish (the main sources of essential PUFAs for humans), is becoming limited. It is well known that filamentous fungi cultivated under low temperatures increase production of PUFAs and thereby increase membrane fluidity to adapt to changing conditions (Cheawchanlertfa *et al*. 2011). An increased content of PUFA and improved cold tolerance has been reported in tobacco as result of overexpression of the acyl carrier protein that plays an important role in *de novo* synthesis of fatty acids (Tang *et al*. 2012). Based on this, it is expected that a *D6D* gene expression driven by a constitutive promoter will lead to PUFA incorporation into membrane structures, causing increased membrane fluidity and improved cold tolerance. These added characteristics may be extremely beneficial in central Europe, where freezing winter cereals is a common problem during cultivation.

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