The production of recombinant proteins in transgenic barley grains

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The grain of the self-pollinating diploid barley species offers two modes of producing recombinant enzymes or other proteins. One uses the promoters of genes with aleurone-specific expression during germination and the signal peptide code for export of the protein into the endosperm. The other uses promoters of the structural genes for storage proteins deposited in the developing endosperm. Production of a protein-engineered thermostabile (1, 3–1, 4)-β-glucanase with the D hordein gene (Hor3–1) promoter during endosperm development was analyzed in transgenic plants with four different constructs. High expression of the enzyme and its activity in the endosperm of the mature grain required codon optimization to a C+G content of 63% and synthesis as a precursor with a signal peptide for transport through the endoplasmic reticulum and targeting into the storage vacuoles. Synthesis of the recombinant enzyme in the aleurone of germinating transgenic grain with an α-amylase promoter and the code for the export signal peptide yielded ≈1 μg mg⁻¹ soluble protein, whereas 54 μg mg⁻¹ soluble protein was produced on average in the maturing grain of 10 transgenic lines with the vector containing the gene for the (1, 3–1, 4)-β-glucanase under the control of the Hor3–1 promoter.

Barley cultivars have been and are being bred to store large amounts of carbohydrates, oil, and protein during grain development. In germination, the stored amino nitrogen and carbohydrates are mobilized to sustain the development of the embryo and seedling. The stored carbohydrates, oils, and proteins in the kernels are exploited for feed, food, and beverages, both in the form of mature grain and as malt produced in large scale by steeping, germination, and kiln drying (1, 2). Advances in the molecular genetics of barley storage proteins (3, 4), elucidation of mechanisms by which storage proteins are synthesized, targeted, and sequestered for long-term storage in the endosperm cells (5, 6), and establishment of genetic transformation procedures (7–10) provide the basis for studying the production of recombinant proteins in the developing barley grain. The unique capacity of the scutellum and aleurone cells to synthesize hydrolytic enzymes during germination and secrete them into the starchy endosperm (11, 12) provides an additional avenue for the production of recombinant proteins in this cereal (9, 10).

Barley grains of cultivars with a 1,000-grain weight of 30–45 g contain 8–16% protein on a dry-weight basis (1). A thousand grains of barley contain on the average 4.5 g of protein, of which ≈2.3 g is distributed to the endosperm and the remainder to the embryo and aleurone tissue. The promoters and signal peptide codes of the genes encoding the barley prolamins (hordeins) are suitable candidates for deposition of recombinant proteins in the protein bodies of the developing endosperm cells. These isoosparan-soluble proteins account for approximately 50% of the total endosperm protein, comprising the high molecular weight D, the sulfur-poor C, and the sulfur-rich B and γ hordein polypeptides (3, 4). The different hordein proteins are coordinately expressed during endosperm development (13). As diagrammed in Fig. 1 (14), electron microscopic analyses of wild types and mutants deficient in groups or individual hordein polypeptides in combination with biochemical analyses have revealed the following picture of the synthesis and deposition of the hordein polypeptides: the mRNAs of the Hor1, Hor2, Hor3, and Hor5 loci on chromosome 5 encoding the hordein C, B, D, and γ precursor polypeptides (3, 5) are translated on the polysomes of the endoplasmic reticulum (ER) (15). The N-terminal signal peptides direct the nascent polypeptide chains into the lumen of the ER, where they are cleaved off during or immediately after translocation (16). Monoclonal and polyclonal antibodies recognizing individual hordein polypeptides or polypeptide families indicate that they are transported in small vesicles via the Golgi cisternae into the storage vacuoles of the endosperm cells (5). Surrounding the tonoplast, the protein bodies consist of globules (H in Fig. 1) containing C, B, and γ hordein (5, 17, 18) and of a reticulate component, identified as D hordein (19). In certain genotypes and under not clearly defined conditions, hordein polypeptides also accumulate in the cisternae of the ER (Fig. 1 and ref. 5).

This paper describes the expression of a protein-engineered thermostable (1, 3–1, 4)-β-glucanase with the Hor3–1 D hordein gene promoter in transgenic barley plants obtained by Agrobacterium-mediated transformation. The characteristics of and requirements for the synthesis of the recombinant protein with the endosperm-specific promoter are compared with results obtained with transgenic barley plants expressing the same recombinant protein with an aleurone-specific α-amylase promoter.

Materials and Methods

Plasmid Constructions. Methods used for PCR and DNA manipulations were as described (20). Plasmids pJH270 (14.943 kb), pHJ271 (15.003 kb), pJH276 (14.943 kb) and pJH277 (15.003 kb) were constructed in the binary cloning vector pJH2600, which is derived from pBIN19 (21). Plasmid pJH2600 was assembled by cloning a HindIII-SmaI fragment containing the bar gene 3′ to the ubiquitin promoter and a SmaI-EcoRI fragment with the nos terminator from pUBARN (10) into HindIII-EcoRI-digested derivative of pBIN19 plasmid by a three-way ligation.

(1) pJH270. Plasmid pHor-H(A12-M)Y13-GC-NOS contains a translational fusion between the 434-bp D hordein gene promoter, the ATG initiation codon (19), and the heat-stable
The resultant fragment was digested with the two PCR fragments were spliced together by SOE and cloned into pUC18.

(3) pH276. Plasmid pHor-H(A12-M)ΔY13-NOS contains a translational fusion between the 437-bp promoter + initiator codon fragment of the D hordein gene and the heat-stable codon-optimized (1, 3–1, 4)-β-glucanase gene. The fragment was amplified with primer hor3’d13 (5’-CTGTTTGATCTCCGGTGAGACTCT-3’) and the universal forward primer. The heat-stable β-glucanase gene was PCR amplified from plasmid pEII-ATG-H(A12-M)ΔY13-NOS with primer hor/d13 (5’-CGAGATGCAAACAGGCGGATCGTT-3’) and the M13 reverse primer. The two PCR fragments were spliced by SOE and cloned in pUC18.

(4) pH277. Plasmid pHsig-H(A12-M)ΔY13-NOS is similar to pHor-H(A12-M)ΔY13-NOS, except that it contains the coding sequence for the D hordein signal peptide. The four resultant plasmids were sequenced for the entire coding and the D hordein promoter regions. The translational fusions were confirmed, and no nucleotide alteration was found.

The third component of plasmids pH270, 271, 276, and 277 is the synthetic mutant green fluorescent protein gene (sGFP) (ref. 22; plasmid no. 5) under the control of the CaMV 35S promoter and the nos terminator. Plasmid pUC19-35S-sGFP-TYG-NOS was constructed by cloning a BamHI-EcoRI fragment containing the sGFP-TYG gene and the nos terminator into BamHI and EcoRI digested pBI221 (Clontech), 3’ to the 35S promoter. In a three-way ligation, the EcoRI-HindIII fragment from pHor-H(A12-M)ΔY13-GC-NOS and the EcoRI-HindIII fragment from pUC19-35S-sGFP-TYG-NOS were inserted into HindIII-digested pH2600 to give pH270. pH271 (Fig. 2) was correspondingly constructed by using the EcoRI-HindIII fragment from pHsig-H(A12-M)ΔY13-GC-NOS, pH276 by using the EcoRI-HindIII fragment from plasmid pHor-H(A12-M)ΔY13-NOS and pH277 by using the EcoRI-HindIII fragment from plasmid pHsig-H(A12-M)ΔY13-NOS.

Barley Transformation. Transgenic plants of the cultivar Golden Promise were produced by cocultivation of immature zygotic embryos with Agrobacterium tumefaciens strains AGL-1 carrying codon-optimized (1, 3–1, 4)-β-glucanase gene (9). The D hordein gene promoter fragment was PCR amplified from a Hor3–1 genomic clone (19) by using the universal forward primer (5’-CGTCTGATCTCGGTGGACTGCA-3’) and primer dhor3 (5’-GGTTTTCCCAGTCACGAC-3’). The heat-stable β-glucanase gene was PCR amplified from plasmid pEII-αH(A12-M)ΔY13-GC-NOS (10) by using primer hor/glu (5’- CGAGATGCAACGCGCGCGCATCGTC-3’) and the M13 reverse primer (5’-GGTTTTCCAGTCAGCCAGC-3’). These two PCR fragments were spliced together by overlap extension (SOE) (9). The resultant fragment was digested with BamHI and EcoRI and cloned into pUC18 with the same two enzymes.

(2) pH271. Plasmid pHsig-H(A12-M)ΔY13-GC-NOS is similar to plasmid (1) but also contains the coding sequence for the D hordein signal peptide. The D hordein gene fragment including the promoter, 5’ untranslated leader region, and the signal peptide-coding region was amplified from the Hor3–1 genomic clone (19) by using the universal forward primer and primer B (5’-CGGTCTGATCTCGGTGGACTGCA-3’). The heat-stable β-glucanase gene was PCR amplified from plasmid pEII-αH(A12-M)ΔY13-GC-NOS by using primer C (5’-CACCGCTCAGACCACGGCGGCACTAC-3’) and the M13 reverse primer. The two PCR fragments were spliced together by SOE and cloned into pUC18.

Fig. 1. The developing endosperm cell of barley. C, chromatin; E, ER; G, Golgi apparatus; H, hordein in protein body; M, mitochondrion; P, plasmodesma; S, starch grain; V, vacuole. (Reproduced with permission from the Carlsberg Foundation.)
a disarmed Ti plasmid and one of the plasmids pJH270, 271, 276, or 277 essentially according to ref. 8 and described in detail in ref. 23. The transgenic barley plants obtained with the biolistic method have been described in refs. 9 and 10. Southern hybridizations and Western Blot analyses were carried out according to the procedures detailed in ref. 23.

Heat-Stable (1, 3–1, 4)-β-Glucanase Assays. Identification of homozygous or heterozygous transgenic plants producing this enzyme are identified in the following way: grains are germinated for 3 days, heated for 2 hr at 65°C to inactivate the endogenous barley enzyme, cut in half, and placed endosperm-side down on Murashige–Skoog (24) medium containing 2 gL⁻¹ lichenan [(1, 3–1, 4)-β-glucan]. The β-glucanase diffuses out of the endosperm into the medium and degrades the lichenan. The zymogram is developed after a few hours with Congo red, revealing degradation of the substrate as an unstained zone around the grain (Fig. 3). The amount of enzyme produced in transgenic grains is determined in extracts from mature or germinated grains with the method of McCleary (25) as described in refs. 9, 10.

**Results**

**The Production of Recombinant (1, 3–1, 4)-β-Glucanase in Developing vs. Germinating Barley Grains.** Four different types of transformants were generated with plasmids pJH 271 (Fig. 2), pJH 270, pJH 277, and pJH 276. The ORF of the mature heat-stable hybrid (1, 3–1, 4)-β-glucanase gene in plasmid 271 is codon optimized to a G+C content of 63%, fused to the code for the D hordein signal peptide comprising 21 amino acids (MAKRLVLFVAVIVALVALTTA), and provided with the 437-bp Hor3–1 promoter. A CpG island in this promoter prevents the requirement of an endosperm-specific unmethylated state, which has to be established during endosperm development in the case of the B and C hordein gene promoters. The Hor3–1 promoter is 3- to 5-fold more active than the latter promoters (19). Ten transformants were regenerated after cocultivation with Agrobacterium and mature T1 grains analyzed for activity of heat-stable (1, 3–1, 4)-β-glucanase. Recombinant enzyme of up to 40 µg mg⁻¹ soluble protein was obtained in individual kernels (Fig. 4). When samples of five to six segregating T1 grains were germinated for 5 days, amounts of active recombinant enzyme ranging from 6.8 to 24.4 µg mg⁻¹ soluble protein were measured.

In the absence of the Hor3 signal peptide (pJH 270), localization of the recombinant enzyme is expected in the cytosol instead of the protein bodies. Much lower amounts of recombinant enzyme (2–5 µg mg⁻¹ soluble protein) were present in the mature grains of transformants with the gene lacking the signal peptide code. It remains to be determined whether this is caused by a decreased synthesis or by proteolytic degradation during

![Fig. 3. Zymogram assay for identification of homozygous and heterozygous transgenic plants producing heat-stable β-glucanase. Germinated and heated half grains are placed on lichenan-containing agar plates. Congo red binds to lichenan but not to its depolymerized saccharides. (a) Germlings of a homozygous transgenic plant, (b) of a heterozygous transgenic plant, and (c) of a nontransgenic segregant in the F2 of the cross transgenic line 5607 × Ca 803111 (ant-499(Apei) × Alexis). An untransformed germling is placed at the top of each plate.](www.pnas.org)

![Fig. 4. Expression of recombinant heat-stable (1, 3–1, 4)-β-glucanase under the control of the D hordein gene (Hor3) promoter in individual T1 grains. For the codon optimized gene, two individual grains were analyzed, and values are given. The codon optimized gene and a signal peptide for targeting the recombinant protein into endosperm storage protein vacuoles are required for optimal production.](www.pnas.org)
programmed cell death in the last stages of grain maturation. The latter seems to be the case in transgenic barley grains expressing the uidA gene with the D or B1 hordein gene promoter without a signal peptide code, because it is reported that the GUS activities per milligram protein declined during grain filling from 3,800 pmol min⁻¹ at 10–20 days after fertilization to 280 pmol min⁻¹ at maturity (26). Barley transformed with the noncodon-optimized ORF produced small amounts of recombinant enzyme in the mature endosperm when it was synthesized as a precursor with the N-terminal signal peptide. In the absence of the signal peptide, no heat-stable glucanase was synthesized as a precursor with the N-terminal signal peptide. In recombinant enzyme in the mature endosperm when it was synthesized without the signal peptide. In Fig. 6 A and B, the molecular mass of the hybrid (1, 3–1, 4)-β-glucanase synthesized with the hordein gene promoter and signal peptide (lanes 2–4) is compared with that of the unglycosylated recombinant enzyme made in Escherichia coli (P, Mr = 24 kDa) and with the recombinant enzyme expressed with the α-amylase promoter in aleurone tissue (lane 1). The Western blot analysis with the heat-stable β-glucanase-specific antibody reveals the higher molecular mass isoforms (Mr = 28 kDa) of the glycosylated enzyme synthesized with the α-amylase promoter in the germinating transgenic barley grains (lane 1) as well as those produced with the D hordein gene promoter and signal peptide code in the developing endosperm (lanes 2–4). A slower migrating glycosylated isoform can be seen in the Coomassie blue-stained gel (Fig. 6 A, arrow). The recombinant heat-stable (1, 3–1, 4)-β-glucanase synthesized without the signal peptide in the developing grains is not glycosylated because it migrates in the SDS gel like the purified enzyme expressed in E. coli (Fig. 6 C, sharp stained narrow double band; D, single antibody decorated band). In contrast, the enzyme produced as a precursor with the signal peptide shows the slower migrating mature glycosylated isoforms (Fig. 6 E–F).

**Southern Blot Analysis.** In Fig. 7, the hybridization patterns of DNA isolated from T1 seedlings of 10 transformants obtained with the Agrobacterium vector pJH271 (Fig. 2) are presented. The DNA was cut with the EcoRI restriction enzyme and probed with the 620-bp long coding region of the heat-stable (1, 3–1, 4)-β-glucanase lacking an EcoRI restriction site. The vector contains an EcoRI restriction site in the nos terminator of the transgene. The hybridizing restriction fragments of the blot are therefore bordered at one end by this EcoRI site and at the other end by a site in the chromosome near the place of insertion. The different sized bands thus indicate the number of insertions. In the present transformants, they range from one to four copies of the transgene. The EcoRI digest was also probed with the 569-bp coding region of the bar gene identifying in all transformants a single hybridizing fragment with the expected size of 1.43 kb (data not shown).

**Discussion**

We used a protein-engineered heat-stable bacterial (1, 3–1, 4)-β-glucanase as a test protein and found that two conditions were required to obtain a high yield of the recombinant protein: (i) The gene had to be codon optimized to a C+G content of 63%, and (ii) the protein had to be synthesized as a precursor with a signal peptide that will likely deliver it to storage vacuoles in a manner similar to D hordein. The requirement for codon optimization is surprising, because genes for cereal prolamins are generally not especially C+G rich. The C+G content of the promoter of the Hor2–4 gene, a member of the gene family encoding B-hordeins, ranges from 35 to 45% (200 bp window) and that along the ORF from 35 to 52% (19). The promoters of the B and C hordein genes are hypermethylated in the leaf tissues but hypomethylated in the developing endosperm. The high-lysine mutation hys3 prevents establishment of the hypomethylated state in the endosperm (19), and this results in very low

**Table 1. Expression of recombinant (1,3-1,4)-β-glucanase in T2-grains of homozgyous transgenic plants from transgenic lines Hor2SP (A12–ΔY13)CG 271.0.1-10 obtained with vector pJH271**

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>Enzyme, μg·mg⁻¹ soluble protein</th>
<th>x ± error of the mean</th>
</tr>
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<tbody>
<tr>
<td>99-271.0.1</td>
<td>73, 60, 67, 17, 29</td>
<td>49.2 ± 11.1</td>
</tr>
<tr>
<td>0.2</td>
<td>99, 54</td>
<td>76.5 ± 22.5</td>
</tr>
<tr>
<td>0.3</td>
<td>25, 51, 51, 20</td>
<td>36.8 ± 8.3</td>
</tr>
<tr>
<td>0.4</td>
<td>79, 74, 62</td>
<td>71.7 ± 5.1</td>
</tr>
<tr>
<td>0.5</td>
<td>53, 51</td>
<td>52.0 ± 2.9</td>
</tr>
<tr>
<td>0.6</td>
<td>75, 41, 38, 68</td>
<td>55.5 ± 9.4</td>
</tr>
<tr>
<td>0.7</td>
<td>81, 56, 23, 26</td>
<td>46.5 ± 13.7</td>
</tr>
<tr>
<td>0.8</td>
<td>25, 36, 63, 59</td>
<td>45.8 ± 2.9</td>
</tr>
<tr>
<td>0.10</td>
<td>69, 57, 65, 58, 56, 69</td>
<td>62.3 ± 2.5</td>
</tr>
</tbody>
</table>

53.8 ± 2.8

**Fig. 5.** Expression of recombinant heat-stable (1, 3–1, 4)-β-glucanase under the control of a barley high pi α-amylase gene promoter in nine different transgenic lines grown in the field in 3 successive years. GP, Golden Promise.
transcription rates for the B and C hordein genes as well as several other genes. The nucleotide sequence of the D hordein gene promoter and the 5′ part of the coding region has characteristics of CpG dinucleotide islands and differs in this respect from the other hordein gene promoters. Analysis of the methylation pattern by genome sequencing revealed that the D hordein gene promoter remains hypomethylated in the leaf tissues as well as in the developing endosperm. Consequently, transcription and translation of the D hordein gene are not curtailed in the lys3a mutation. It will be of interest to determine whether codon optimization is a requirement for gene expression with the B, C, and γ hordein promoters.

Although direct demonstration of the localization of the recombinant (1, 3–1, 4)-β-glucanase in the protein bodies has to be undertaken, the requirement of the signal peptide for a high content of the recombinant protein in the mature grain and its retention in the endosperm during grain maturation indicates deposition of value-adding proteins in the storage bodies. Targeting the protein into storage vacuoles can provide protection against the extensive proteolytic action that eliminates a large part of the cellular components of the cereal endosperm during the programmed cell death at the final stages of grain maturation. The nucleotide sequence of the coding region for the D hordein gene is 71–79% identical to the high molecular weight (HMW) glutenin genes of wheat, whereas the homology is 85–89% in the promoter region. The close relationship among these genes and the similarity in their tissue-specific expression patterns in wheat and barley endosperms are underscored by the success of transgenic expression of the 1Ax1 and hybrid Dy10:Dx5 wheat genes for HMW glutenin subunits in the cultivar Bob White (30, 31). The transgenic wheat plants synthesized up to 2.3% of the total protein in the endosperm as 1Ax1 glutenin subunit, providing as much as a 71% increase in the glutenin class of prolamins, which are known to be beneficial for dough elasticity and bread-making quality.

In the absence of the signal peptide, only small amounts of the recombinant protein are present in the mature grain, and this may be caused by the synthesis of the recombinant protein on cytosolic ribosomes. Deposition in the cytosol might make it sensitive to proteolytic degradation during the final stages of grain maturation.

The B, C, and γ hordein polypeptides are stored in different parts of the protein bodies (5). These polypeptides are encoded by families of genes that are linked in gene clusters, with each member having its own promoter. These promoters provide additional opportunities for the expression of recombinant proteins in the developing endosperm. In rice, the gene promoters for the endosperm storage proteins glutelin, albumin,

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**Fig. 6.** Identification of heat-stable (1, 3–1, 4)-β-glucanase synthesized with the D hordein gene promoter in maturing grains and with an α-amylase promoter in germinating grains. GP, extract of untransformed Golden Promise; M, molecular marker; P, purified unglycosylated enzyme synthesized in E. coli; M, 24 kDa. (A) SDS/PAGE and Coomassie blue staining; (B) Western blot decorated with specific antibody and visualized by chemiluminescence. Lanes: 1, extract of germinating grains expressing the enzyme with the α-amylase promoter; 2–4, extracts of germinating grain containing the recombinant enzyme produced with the Hor3 promoter during grain maturation. The larger apparent molecular mass M, 28–40 kDa of the barley-produced enzyme is because of N-glycosylation. (C–F) Comparison of the recombinant heat-stable (1, 3–1, 4)-β-glucanase synthesized with the D hordein gene promoter in developing grains with or without the signal peptide. (C and E): SDS/PAGE/Coomassie blue staining; and (D and F) Western blot of extracts containing recombinant enzyme synthesized in transformants with signal peptide (E and F, lanes 1–4) and in transformants without it (C and D, lanes 1–4). The recombinant enzyme synthesized without the signal peptide has an apparent molecular weight characteristic of the unglycosylated protein.

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**Fig. 7.** Southern blot analysis of T1 seedlings transgenic for the heat-stable (1, 3–1, 4)-β-glucanase (lanes 5–9 and 10–14). The transgenic plants were obtained by Agrobacterium-mediated transformation by using vector pJH271 with the transgene driven by the Hor3 promoter and translation effected on the ER with the D hordein signal peptide. Ten micrograms of genomic DNA was digested with EcoRI and probed with the digoxigenin-labeled coding region of the heat-stable (1, 3–1, 4)-β-glucanase (620 bp). Lanes: 1, 2, 3 – 20, 4, and 2 copies of the plasmid cut with EcoRI (10.2 kb); 4, Golden Promise; M, molecular marker.
amino acids to be used as nutrients by the growing germling. The
during germination the mixed-linked cell walls. This has to take place before
is the reason why barley is considered a low-metabolic energy feed
for poultry. The birds do not produce an enzyme in their gastro-
intestinal tract to depolymerize the (1, 3–1, 4)-\(\beta\)-glucan from 1 kg of malt containing high levels of the polymer (10). Thus enzyme in \(\approx 1\) g (40 grams) of transgenic malt of the high-enzyme producing lines in Fig. 5 is expected to depoly-
merize the soluble \(\beta\)-glucan of 1 kg (\(\approx 25,000\) grams) of nontrans-
genic “normal” barley, whereas 200 \(\mu\)g enzyme or 10 g of transgenic malt will depolymerize the 50 g of water-soluble and insoluble (1, 3–1, 4)-\(\beta\)-glucan of 1 kg of normal barley cultivars. Chicken-feeding trials to verify these predictions have been initiated. When the transgenic lines described in this communication have been field propagated, mature grain containing 40 times more recombinant enzyme than is present in current transgenic malt will be available for use as a supplement in broiler chicken feed barley. This will convert barley into a high-metabolic feed grain.

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