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Phytochemistry 65 (2004) 1619–1627
PHYTOCHEMISTRY
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Environmental and transgene expression effects on the barley seed proteome

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Received 12 January 2004; accepted 16 April 2004
Available online 19 May 2004

Abstract

The barley (*Hordeum vulgare*) cultivar Golden Promise is no longer widely used for malting, but is amenable to transformation and is therefore a valuable experimental cultivar. Its characteristics include high salt tolerance, however it is also susceptible to several fungal pathogens. Proteome analysis was used to describe the water-soluble protein fraction of Golden Promise seeds in comparison with the modern malting cultivar Barke. Using 2D-gel electrophoresis to visualise several hundred proteins in the pH ranges 4–7 and 6–11, 16 protein spots were found to differ between the two cultivars. Eleven of these were identified by mass spectrometric peptide mass mapping, including an abundant chitinase implicated in defence against fungal pathogens and a small heat-shock protein. To enable a comparison with transgenic seed protein patterns, differences in spot patterns between field and greenhouse-grown seeds were analysed. Four spots were observed to be increased in intensity in the proteome of greenhouse-grown seeds, three of which may be related to nitrogen availability during grain filling and total protein content of the seeds, since they also increased in field grown seeds supplied with extra nitrogen. Finally, the fate of transgene products in barley seeds was followed. Spots containing two green fluorescent protein constructs and the herbicide resistance marker phosphinothricin acetyltransferase were observed in 2D-gel patterns of transgenic seeds and identified by mass spectrometry. Phosphinothricin acetyltransferase was observed in three spots differing in p*I* suggesting that post-translational modification of the transgene product had occurred.

Keywords: *Hordeum vulgare*; Poaceae; Barley; Proteome analysis; Mass spectrometry; Water-soluble seed proteins; Transgene products; Green fluorescent protein; Phosphinothricin acetyltransferase

1. Introduction

Transformation efficiency of barley (*Hordeum vulgare*) is known to be strongly genotype-dependent and transformation of many agronomically important cultivars has not yet been successful. Therefore the cultivar Golden Promise is commonly used for production of transgenic plants due to its ability to regenerate from callus. Production of proteins in the barley seed is of particular interest. Many studies have utilised this approach to produce barley lines with improved properties such as malting quality due to expression of, e.g., thermotolerant endo-1,4-β-glucanase (Nuutila et al., 1999) or bacterial α-amylase (Tull et al., 2003). Although transformation techniques have also now been developed for other barley cultivars such as the North American 6-rowed malting cultivar Morex (Chang et al.,

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doi:10.1016/j.phytochem.2004.04.018

2003), it would be of value to know how comparable Golden Promise is to barley cultivars that are currently used in agriculture. The widespread use of Golden Promise for malting has decreased as it has been superseded by modern malting cultivars.

Introduction of a heterologous protein or overexpression of a protein in barley seeds may cause pleiotropic effects on seed metabolism, due to the point of insertion of the transgene into the genome. Overexpression of a single protein may affect the expression of the other proteins in the seed, or the transgene product may interact with other seed proteins or metabolites. It is important to detect such effects both for interpretation of experimental results and for possible risk-assessment purposes. Transgenic barley plants are analysed under controlled growth conditions and effects observed under these circumstances may not be reproduced when the same material is grown in the field (e.g., Anand et al., 2003). Thus greenhouse-grown and field-grown seeds cannot be directly compared so it is of additional interest to characterise the differences between them.

When a protein is overexpressed in the plant, it may be modified in different ways. Many different post-translational modifications are known to occur in plants and they may alter the properties of a transgene product. Most previous studies of transgenic plants have not analysed these possible effects. Here, we demonstrate that proteome analysis is ideally suited to address these aspects.

Proteome analysis, involving two-dimensional (2D) gel electrophoresis and mass spectrometry, is a powerful technique for the visualisation of hundreds of proteins in complex mixtures, for identification of proteins and of possible post-translational modifications. Previously, we have undertaken a detailed analysis of the water-soluble proteome of barley seeds during seed development and germination, resulting in the identification of several hundred protein spots in the overlapping p*I* ranges 4–7 (Østergaard et al., 2002; Finnie et al., 2002; Finnie and Svensson, 2003; Østergaard et al., in press) and 6–11 (Bak-Jensen et al., 2004). In this study, a comparative proteome analysis of the barley cultivar Golden Promise is undertaken to analyse: (a) differences in protein spot patterns between Golden Promise and the important modern malting cultivar Barke, (b) differences in the proteome of field-grown and greenhouse-grown seeds and (c) differences in the seed proteome due to the expression of transgenes.

2. Results and discussion

2.1. Cultivar comparison

The 2D-gel spot patterns of water-soluble protein extracts were compared for field-grown seeds of cultivars Barke and Golden Promise. Using identical protein extraction conditions only minimal differences in a few spots on the 2D-gels were observed between extracts of the same cultivar grown in different locations and in different years (A. Jensen et al., unpublished data). Distinct and reproducible differences have been observed between different cultivars, showing that such analyses have potential to distinguish between cultivars (Østergaard et al., 2002; Finnie et al., 2002). Ultimately, spot pattern differences may be correlated with functional properties of cultivars or linked to genetic map data (Thiellement et al., 1999).

Several spot differences were reproducibly observed in the p*I* ranges 4–7 and 6–11 (Fig. 1, Table 1), although the vast majority of protein spots were common to both cultivars. The protein spots observed to vary between Barke and Golden Promise were in some cases also seen to vary among 16 barley cultivars chosen based on their varying properties (Østergaard et al., in press, Bak-Jensen et al., unpublished data). Varying spots were excised from 2D-gels, in-gel trypsin digested and subjected to MALDI-TOF mass spectrometry for peptide mass mapping. In some cases identity of protein spots could be inferred from the identity of the corresponding spot from another barley cultivar. Database searches with peptide mass data resulted in identification of proteins in 11 of the spots (Table 1).

Among the varying protein spots, some appeared to be completely absent in either Barke or Golden Promise (e.g., spot 68 absent in Golden Promise, Fig. 1(f); spot c absent in Barke, Fig. 1(g)). In other cases, a spot of similar intensity and mobility was observed but with an altered p*I* in comparison to the spot in the other cultivar. This implies that the spots

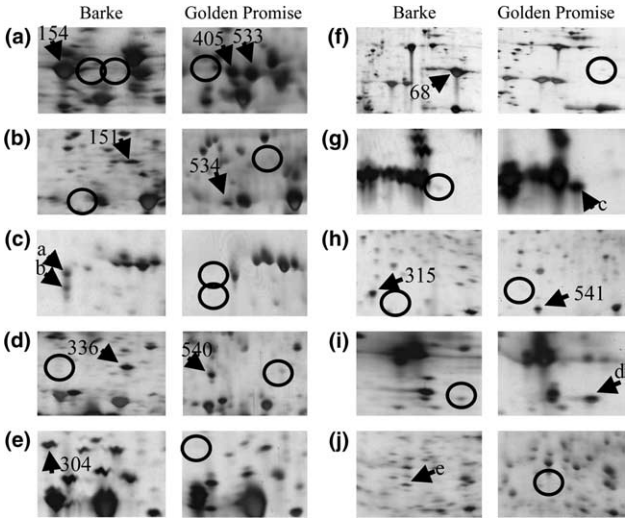

Fig. 1. Protein spots differing between barley cultivars Barke and Golden Promise. 2D-gels are shown containing varying protein spots marked with arrows. Circles indicate the absence of the corresponding spots. Identified spots are indicated by numbers and non-identified spots by letters that refer to Table 1. Observed molecular mass and p*I* of the spots are given in Table 1.

Table 1
Protein spots differing between cultivars Barke and Golden Promise

Spot No.[a]	Identified protein	Accession No.[e]	Observed p*I*	Observed MW (kDa)	Golden Promise	Barke
68[b]	Chitinase	JC5918	8.5	27.6	–	+
405	Serpin Z7	T06183	5.8	49.1	+	–
533[c]	Serpin Z7	T06183	5.8	49.1	+	–
534	Mixture of two proteins similar to Serpin Z7 (ESTs)	AJ481566 (TC98891) BI947801 (TC98890)	5.1	40.1	+	–
151[d]	Serpin Z7	T06183	5.2	43.8	–	+
154[d]	Serpin Z7	T06183	5.7	49.1	–	+
304[d]	Small heat shock protein	CAA69172	5.2	21.1	–	+
336[d]	Phosphogluconolactonase-like protein (EST)	AV940134 (TC57027)	5.7	36.7	–	+
540	Phosphogluconolactonase-like protein (EST)	AV940134 (TC57027)	5.5	36.7	+	–
315[d]	Translationally controlled tumour protein homologue	AAF61933	4.4	23.3	–	+
541	Translationally controlled tumour protein homologue	AAF61933	4.5	21.8	+	–
a	Not identified		4.6	51.1	–	+
b	Not identified		4.6	48.7	–	+
c	Not identified		4.9	11.4	+	–
d	Not identified		6.8	36.7	+	–
e	Not identified		4.8	27.3	–	+

[a] Spot numbers refer to Fig. 1.
[b] Identification from Bak-Jensen et al. (2004).
[c] Identification confirmed by nano-ESI MS/MS.
[d] Identification from Østergaard et al. (in press).
[e] Accession No. refer to GenBank or TIGR databases.

might contain different forms of the same protein. Previously, such a p*I* difference was observed in spots that were shown by peptide mass mapping to contain allelic sequence variants of β-amylase, that caused a p*I* difference in the protein products (Finnie et al., 2002).

Several spots containing the serpin Z7 were observed to differ between the two cultivars. Mass spectrometry enabled different forms of the protein (Accession No. T06183 in spots 151, 154, 405 and 533 and both Tentative Consensus sequences TC98890 and TC98891 in spot 534; Table 1) to be distinguished, however the basis for the different spots containing Accession No. T06183 could not be determined. One of the most prominent differences between the two cultivars was the absence in Golden Promise of a highly abundant spot (spot 68, Fig. 1(f)) containing chitinase. No additional spot of similar molecular mass or intensity was present in the Golden Promise spot pattern, suggesting that the spot absence was not due to a p*I* shift of the protein. This chitinase, synergistically with 1,3-β-glucanase and ribosome-inactivating protein, was shown to inhibit fungal growth in vitro (Leah et al., 1991). Golden Promise is susceptible to several fungal pathogens including *Blumeria graminis*, *Puccinia striiformis*, *Puccinia hordei*, *Rhynchosporium secalis* and *Ustilago nuda* (European Brewery Convention, 1976). This susceptibility could be related to the absence of this abundant chitinase in the seed, although it cannot be excluded that the protein is present in other parts of the plant.

Another property of Golden Promise is salt tolerance (Foster, 2001). A spot identified in Barke to contain a small heat-shock protein (spot 304, Fig. 1(e)) was also absent in Golden Promise. Several other sHSP-containing spots were present in both cultivars (data not shown). These proteins are part of a diverse family involved in responses to related stresses such as heat, salt and dehydration and may be involved in protection against desiccation during seed maturation (Sun et al., 2002). A spot containing a protein homologous to 6-phosphogluconolactonases (spot 336, Fig. 1(d)) and thus predicted to be involved in the pentose phosphate pathway was absent in Golden Promise, but seemed to be replaced by a spot at lower p*I* (spot 540, Fig. 1(d)). Spot 541 was identified by peptide mass mapping to contain the same protein as spot 336. Spot 315 in Barke was replaced by spot 541 in Golden Promise (Fig. 1(h)) both of which were identified to contain the same protein of unknown function. The peptide mass maps obtained could not explain the p*I* differences observed between the spots.

2.2. Comparison of field-grown and greenhouse-grown seeds

Spot patterns were compared for protein extracts made from field-grown seeds and from seeds grown

under two different greenhouse conditions. As expected for the water-soluble protein fraction (Shewry et al., 2001) the overall spot patterns were highly similar. However, a few protein spots were more abundant in greenhouse-grown samples than field-grown samples (Fig. 2). In order to investigate this in more detail, a section of the 2D-gels containing these differences was subjected to image analysis. Four protein spots (spots f, g, 441 and 572; Fig. 2) were confirmed to appear or be increased over fivefold in intensity when comparing five gels from greenhouse-grown samples with five gels from field-grown samples. The spot differences between greenhouse and field-grown seeds were observed both in Golden Promise and Barke, and in Golden Promise seeds grown in two different greenhouse locations (not shown) and therefore are unlikely to be due to local or genotype specific effects, but may be due to general differences between field and greenhouse conditions.

A major difference between greenhouse- and field-grown seeds is in the total protein content. Greenhouse-grown seeds have an increased protein content. A similar effect on protein content can be observed in the field by addition of extra nitrogen fertiliser. This results in an extended period of grain-filling and corresponding increase in protein content. The application of N must be managed carefully, especially for malting barley for which the acceptable protein content is between 9% and 12%. High N levels can result in an excessively high protein content. The increased grain total protein content as a result of increased N is correlated with an increase in the percentage of hordein storage proteins in barley seeds (Shewry et al., 2001), whereas the other protein fractions, including the water-soluble albumin fraction, are relatively unaffected. However, differences in individual proteins in these fractions might occur.

To investigate whether the differences observed between greenhouse- and field-grown seeds might be related to the differences in total protein content or nitrogen availability, Barke seeds were grown under different fertiliser regimes in the field. Total protein content of the mature seeds increased with increased N application, the greenhouse-grown seeds having the highest protein content of all of the samples (Table 2) that was above the acceptable limit for malting purposes. Proteins were extracted from these seeds and run on 2D gels for spot pattern comparison. The four spots observed to be increased in greenhouse-grown seeds also increased with increasing N application and total protein content (Fig. 2). Spot 441 (Østergaard et al., in press was identified as a late embryogenesis abundant (LEA) protein. Two peptides originating from spot 572 were sequenced by MALDI-TOF tandem MS and found to match a wheat EST sequence (Accession No. BQ842244) also similar to LEA proteins. The LEA proteins are likely to be involved in desiccation tolerance of the embryo during seed development and accumulate at the onset of the

Table 2
Protein content of Barke seeds grown under different nitrogen availability

Sample	Nitrogen added kg/hectare	Seed protein content %
Field N1	56	8.4
Field N2	156	9.3
Field N3	206	10.7
Greenhouse	–	15.7

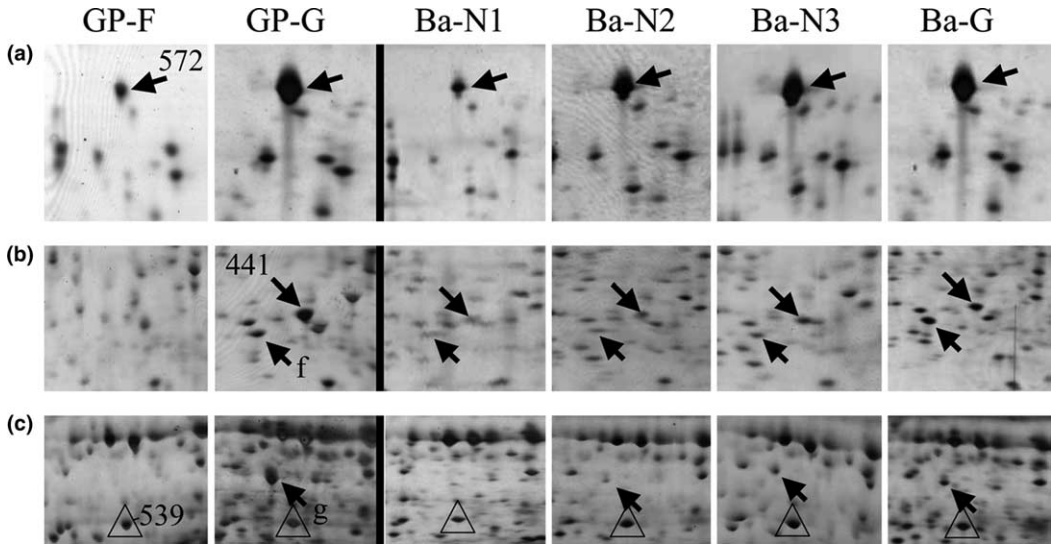

Fig. 2. Spots differing between greenhouse- and field-grown seeds. (a)–(c): Sections of 2D-gels containing varying spots f, g, 441 and 572 (arrows) are shown from field (GP-F) and greenhouse-grown (GP-G) Golden Promise seeds, field-grown Barke seeds with increasing amounts of applied nitrogen fertiliser (Ba-N1, Ba-N2 and Ba-N3; Table 2) and greenhouse-grown Barke seeds (Ba-G). The triangle indicates a spot (spot 539) identified as cytosolic glutamine synthetase that is at a constant level in all gels. Total protein content of Barke seeds is given in Table 2. Spots 441 (Østergaard et al., in press) and 572 were identified by mass spectrometry as Late Embryogenesis Abundant (LEA) proteins.

desiccation stage (Ingram and Bartels, 1996). Interestingly, cytoplasmic glutamine synthetase, a key enzyme in nitrogen metabolism (Miflin and Habash, 2002), was identified in a spot (spot 539) that was at a constant level under the different nitrogen applications (Fig. 2(c)). This protein was identified on the basis of EST (Accession No. BG344204) and Tentative Consensus (Accession No. TC45514) sequences that are predicted to encode a protein highly similar but not identical to a barley glutamine synthetase sequence (Accession No. Q06378).

The other proteins were not identified by peptide mass mapping. However, based on analysis of dissected seed tissues (Finnie and Svensson, 2003), the proteins that were more intense in greenhouse-grown samples and samples with higher protein content originated mainly from the embryo rather than from the aleurone layer or starchy endosperm (data not shown). The reason for this is unclear, although since the grain-filling period is longer in the seeds with additional N, the time available for accumulation of LEA proteins may be extended, leading to a relative increase in their amount. Another possibility is that due to the changed composition of the grain, the embryo proteins become relatively easier to extract under the conditions used. Previous work has suggested that embryo proteins are slightly under-represented in the standard extracts from whole seeds that are used here (Finnie and Svensson, 2003).

2.3. Analysis of transgenic barley seeds

Green fluorescent protein from the jellyfish *Aequorea victoria* (Chalfie et al., 1994) is often used as a marker protein to study gene expression or protein localisation due to its unique fluorescent properties. Transgenic barley lines expressing two different GFP constructs and selective markers commonly used in cereal transformation (Ahlandsberg et al., 1999) were used for investigation of the fate of transgene products in the barley seed proteome. Five transgenic lines were analysed (Table 3). Two lines (L4-3-1 and L4-3-2; Brinch-Pedersen et al., 1996) carried the selective markers phosphinothricin acetyltransferase encoded by the *bar* gene from *Streptomyces hygroscopicus* and β-glucuronidase encoded by the *uidA* gene from *Escherichia coli*. Two lines (SEB122-3-4-1-8 and SEB122-3-4-1-3) were analysed that additionally carried the gene for GFP expressed from the ubiquitin promotor and a single line (SEB110-9-1-1-102) was analysed where GFP was fused to the N-terminal signal sequence of the high p*I* isoform of barley α-amylase to direct the protein into the endoplasmic reticulum (Caspers et al., 2001). Protein spot patterns from the transgenic lines were compared with those from greenhouse-grown wild type Golden Promise seeds (Fig. 3).

Two or three spots were observed to be present in all of the transgenic seed samples (spots b1, b2 and b3; Fig. 3(b)–(d)) with molecular mass of approximately 24 kDa and p*I* of 6.0, 6.2 or 6.4 (Table 4). Spots b1, b2 and b3 were all identified to contain the phosphinothricin acetyltransferase encoded by the *bar* gene suggesting that modification of the protein occurred, resulting in the 0.2 unit p*I* differences between the spots. Similar mass spectra were obtained for the proteins in each of the spots. Higher sequence coverage and MS/MS peptide fragmentation will therefore be required in order to determine the site and/or nature of the modification.

In GFP-transformed samples GFP-3 and GFP-8 two additional spots, g1 and g2 (Fig. 3(c)) were observed close to the position expected based on the predicted molecular mass and isoelectric point of GFP (Table 4). Both spots had the same p*I* and spot g2 had a slightly higher mobility on the 2D-gel suggesting that it might contain a truncated form of the protein in spot g1. Spot g1 was successfully identified as GFP, however no mass spectrum could be obtained for g2, possibly due to the low amount of protein in the spot.

The α-amylase signal sequence –GFP fusion protein was identified in spot g3 (Fig. 3(d)). This protein was detected in a single spot at a higher p*I* than the GFP in GFP-3 and GFP-8 samples, suggesting either that the N-terminal signal peptide remains uncleaved or that a modification of the protein occurs. Peptides covering the α-amylase-derived sequence were not observed in the

Table 3
Transgenic barley lines analysed in this study

Sample	Line	Transgenes introduced	Transgene segregation among seeds of primary transformant[a]
L4-1	L4-3-1	Bar, GUS	3:1[b]
L4-2	L4-3-2	Bar, GUS	– (Homozygous)
GFP-3	SEB122-3-4-1-3	Bar, GUS, hGFP-C3	– (Homozygous)
GFP-8	SEB122-3-4-1-8	Bar, GUS, hGFP-C3	15:1[b]
α-GFP	SEB110-9-1-1-102	Bar, GUS, α-amylase signal sequence-hGFP-C3	3:1[c]

[a] Segregation based on detection of GFP fluorescence (GFP-3, GFP-8 and α-GFP) or GUS activity (L4-3-1 and L4-3-2).
[b] Seeds were not screened to remove null transformants before protein extraction.
[c] Seeds that tested negative for GFP fluorescence were removed before protein extraction.

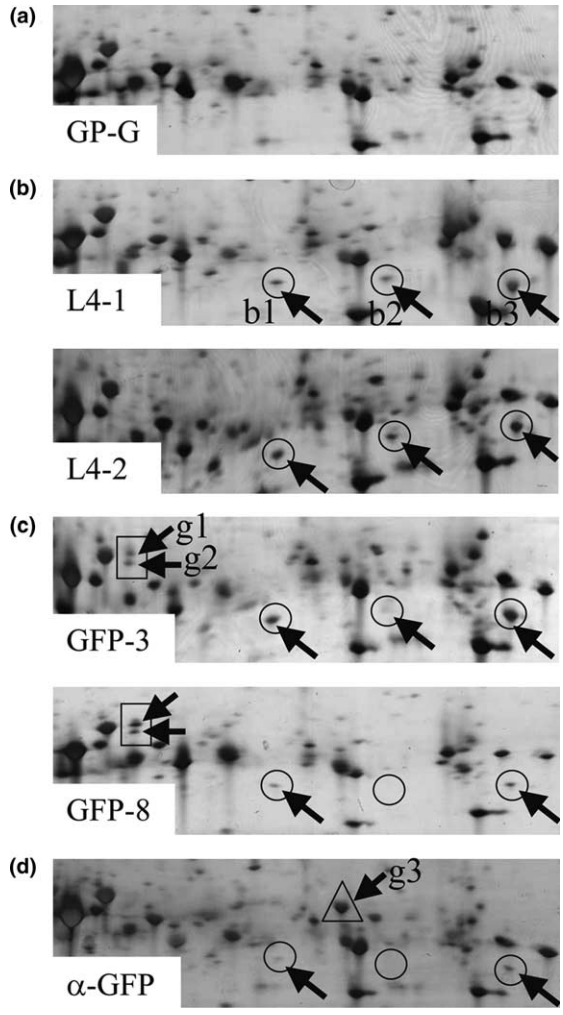

Fig. 3. Transgene products identified on 2D-gels. 2D-gels from (a) greenhouse-grown Golden Promise (b) transgenic samples L4-1 and L4-2, (c) transgenic samples GFP-3 and GFP-8 and (d) transgenic sample α-GFP. Spots identified as phosphinothricin acetyltransferase (b1–b3), green fluorescent protein (g1) and α-amylase-GFP fusion protein (g3) are indicated and refer to Table 4. No mass spectrum was obtained for spot g2 although it is likely also to contain green fluorescent protein.

mass spectrum. No additional spots were observed in the 2D-gels in the region expected based on the predicted molecular mass and p*I* of β-glucuronidase. Small spot pattern differences could be observed between the different transgenic lines, although additional replicates would be required to verify them.

3. Concluding remarks

Previous studies of the barley seed proteome provide the basis for mapping the proteome of the experimentally important cultivar Golden Promise. That the overall spot pattern is conserved between Barke, Golden Promise and other cultivars means that information gained from prior studies is relevant for Golden Promise. The differences observed provide a means of describing the proteome of Golden Promise in comparison to other cultivars. Proteins identified in varying spots may be related to properties of the cultivars involved. In addition, it is shown that heterologous proteins expressed in plants may undergo modifications. Such modifications could potentially affect the function of the transgene products and such a possibility should be considered when analysing transgenic plants. Proteome analysis is a powerful method for detecting such events and to evaluate environmental versus genetic effects on many proteins simultaneously.

4. Experimental

4.1. Seed material and generation of transgenic seeds

Spring barley cultivars Golden Promise and Barke were field grown in the 1998 and 2000 seasons, respectively. Barke seeds grown under differing nitrogen regimes were supplied by Sejet Plantbreeding, Denmark and were grown in the field with the addition of nitrogen fertiliser according to Table 2. Golden Promise seeds were supplied by Risø National Laboratory, Denmark. Plants were also grown in greenhouses at Carlsberg Laboratory and Risø National Laboratory, Denmark. Total protein content of barley seeds was determined by NIT (Near Infrared Transmission) analysis using an Infratec 1221 instrument (Foss Electric A/S, Denmark)

Table 4
Transgene products observed on 2D gels

Spot[a]	Protein identified	Number of peptides	Sequence coverage	p*I* predicted[b]	MW Predicted[b]	p*I* observed	MW observed
b1	Phosphinothricin acetyl transferase	8	43%	6.1	20.6	6.0	24
b2	Phosphinothricin acetyl transferase	11	69%	6.1	20.6	6.2	24
b3	Phosphinothricin acetyl transferase	11	81%	6.1	20.6	6.4	24
g1	Green fluorescent protein	6	30%	5.7	26.7	5.7	27
g2	No peptide mass data	–	–	–	–	5.7	27
g3	α-Green fluorescent protein fusion	10	43%	5.8	29.8	6.1	27

[a] Spot numbers refer to Fig. 3.
[b] Predicted isoelectric point and molecular mass calculated for the full length protein sequecnces.

with centralised artificial neural network-based calibration for barley samples. Transgenic barley lines (Table 3) were generated from Golden Promise as previously described (Brinch-Pedersen et al., 1996). Lines L4-3-1 and L4-3–2 were transformed with pDM803 carrying the *bar* gene and *uidA* gene driven by the actin promotor (Brinch-Pedersen et al., 1996). Lines expressing GFP were generated similarly by cotransformation with constructs carrying a fluorescence-enhanced GFP-variant encoded by the synthetic hGFP-C3 gene (Nielsen et al., 1999) driven by the ubiquitin promotor (lines SEB122-3-4-1-3 and SEB122-3-4-1-8), or with an α-amylase signal sequence–hGFP-C3 fusion (Caspers et al., 2001) driven by the ubiquitin promotor (line SEB110-9-1-1-102). Since seeds from primary transformants were used for analysis, they were tested for segregation of GUS or GFP activity (Table 3). Seed batches from plants showing no segregation or 1:15 segregation were analysed directly without screening. Seeds from line L-4-3-1, although showing 3:1 segregation for GUS activity, were also extracted directly since it was not possible to use seed material for protein extraction after testing for GUS activity. Line SEB110-9-1-1-102 showed 3:1 segregation of GFP activity and before protein extraction the seeds were screened and GFP-negative seeds were discarded as follows. The tip of the seed furthest from the embryo was removed with a scalpel, followed by a thin slice. This slice was placed in a microtitre plate with 50 μL water. GFP fluorescence was observed using a fluorescence microscope (Leica GFP-plus). The remainder of the positive seeds was pooled for protein extraction.

4.2. Protein extraction from barley seeds

Proteins were extracted from 4 g milled barley flour in 20 mL extraction buffer (5 mM Tris–HCl pH 7.5, 1 mM $CaCl_2$) for 30 min at 4 °C, as previously described (Finnie et al., 2002). Extractions were made in duplicate from field-grown seeds, from seeds originating from two different greenhouse environments or from two lines resulting from the same transformation experiment.

4.3. 2D-gel electrophoresis of seed proteins

Protein extracts were applied to 2D-gels under previously described conditions using p*I* ranges 4–7 (Finnie et al., 2002) and 6–11 (Bak-Jensen et al., 2004). Proteins in 100 μL seed extracts (about 80 μg proteins) were precipitated with 4 volumes of acetone at −20 °C for 24 h. For separation of proteins in the pH range 4–7, proteins were resuspended in reswelling buffer (8 M urea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer 4–7 (Amersham Biosciences), 20 mM DTT and a trace bromophenol blue). The first dimension separation in immobilised pH gradient (IPG) 18 cm strips with a linear gradient of p*I* 4–7, was carried out on an IPGphor (Amersham Biosciences) as previously described (Finnie et al., 2002). For separation of proteins in the pH range 6–11, proteins were resuspended in reswelling buffer (8 M urea, 2% (w/v) CHAPS, 2% (v/v) IPG buffer 6–11 (Amersham Biosciences), 20 mM DTT and a trace bromophenol blue). The first dimension separation in IPG 18 cm p*I* 6–11 strips was carried out on a Multiphor II (Amersham Biosciences) as previously described (Bak-Jensen et al., 2004). After isoelectric focusing, IPG strips were equilibrated for 20 min in equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and a trace bromophenol blue) containing 10 mg/mL DTT, followed by 20 min in equilibration buffer containing 25 mg/mL iodoacetamide. Second dimension SDS–PAGE gels (12–14%, 18×24 cm, Amersham Biosciences) were run on a Pharmacia Multiphor II according to the manufacturer's recommendations for pH 4–7 IPG strips and according to Bak-Jensen et al., 2004) for pH 6–11 IPG strips. Proteins were visualised on 2D-gels by silver staining (Heukeshoven and Dernick, 1985) or colloidal Coomassie blue staining (Rabilloud and Charmont, 2000). Spot patterns were reproducible for duplicate gels and for duplicate protein extractions.

4.4. Analysis of spot pattern differences between greenhouse- and field-grown seeds

Image analysis software ImageMaster 2D Elite v3.1 (Amersham Biosciences) was used to analyse spot patterns. A section containing about 350 spots including the areas shown in Fig. 2 was analysed for each gel. Spot detection, background subtraction (mode of non-spot) spot matching and spot normalisation (total spot volume, scaling factor total spot area) was performed for each gel section according to the manufacturer's recommendations. Five gels were analysed each for greenhouse- and field-grown samples that included both Barke and Golden Promise gels. For both sets of five gels, an average gel was constructed that contained the spots present in all five gels. Finally, spot matching was performed with the average gels and spots showing a fivefold or greater change in normalised spot volume were selected.

4.5. Identification of proteins by mass spectrometry

Spots were cut out from gels and subjected to in-gel trypsin digestion (Shevchenko et al., 1996) and tryptic peptides were desalted and concentrated using a homemade nano column (Gobom et al., 1999) as previously described (Finnie et al., 2002). For analysis by MALDI-TOF MS the peptides were eluted with 0.8 μL matrix solution (20 mg/mL α-cyano-hydroxycinnamic acid in 70% CH_3CN, 0.1% trifluoroacetate) and deposited

directly onto the MALDI target. A Bruker REFLEX III MALDI time-of-flight (TOF) mass spectrometer (Bruker-Daltonics, Bremen, Germany) in positive ion reflector mode was used to analyse tryptic peptides. The m/z software (Proteometrics, New York, USA) was used to analyse spectra. Spectra were calibrated using trypsin autolysis products (m/z 842.51 and m/z 2211.10) as internal standards. Protein identification was performed by searching in the National Center for Biotechnology Information (NCBI) or SWISSPROT non-redundant sequence databases using the Profound (http://www.proteometrics.com) and/or Mascot (http://www.matrixscience.com) servers. The following parameters were used for database searches: monoisotopic mass accuracy 80 ppm, missed cleavages 1, allowed modifications carbamidomethylation of cysteine (complete) and oxidation of methionine (partial). For a positive identification over 15% sequence coverage was required including at least three independent peptides with a mass deviation of less than 50 ppm from the theoretical value. If identification failed, the list of peptide masses was re-searched against an expressed sequence tag (EST) database using the Mascot server. Tentative consensus sequences containing matched ESTs were identified by searching the Institute for Genome Research (TIGR) barley gene index (www.tigr.org/tdb/hvgi). Spot 572 was identifed by MALDI-TOF tandem mass spectrometry using an Applied Biosystems 4700 analyser. Certain samples were subjected to nanoelectrospray MS/MS (Wilm et al., 1996) using a Q-TOF hybrid instrument (Micromass, Manchester, UK). The instrument was calibrated using NaI and database searching was performed using the MASCOT search engine.

Acknowledgements

Birger Eriksen and Kurt Hortshjolm (Sejet Plantbreeding, Denmark) are thanked for providing field-grown barley material. Mette Hersom Bien (Carlsberg Laboratory), is gratefully acknowledged for excellent technical assistance and Kate Rafn, Andrea Lorentzen and Peter Roepstorff (University of Southern Denmark, Odense) for assistance with mass spectrometry. Members of the SUE project "The barley proteome: identification of proteins important in malting and nutrient uptake" are thanked for helpful discussions. This research was supported by the Danish Research Agency SUE programme grant no. 9901194.

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