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Barley peroxidase isozymes Expression and post-translational modification in mature seeds as identified by two-dimensional gel electrophoresis and mass spectrometry

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Abstract

Thirteen peroxidase spots on two-dimensional gels were identified by comprehensive proteome analysis of the barley seed. Mass spectrometry tracked multiple forms of three different peroxidase isozymes: barley seed peroxidase 1, barley seed-specific peroxidase BP1 and a not previously identified putative barley peroxidase. The presence of multiple spots for each of the isozymes reflected variations in post-translational glycosylation and protein truncation. Complete sequence coverage was achieved by using a series of proteases and chromatographic resins for sample preparation prior to mass spectrometric analysis. Distinct peroxidase spot patterns divided the 16 cultivars tested into two groups. The distribution of the three isozymes in different seed tissues (endosperm, embryo, and aleurone layer) suggested the peroxidases to play individual albeit partially overlapping roles during germination. In summary, a subset of three peroxidase isozymes was found to occur in the seed, whereas products of four other barley peroxidase genes were not detected. The present analysis documents the selective expression profiles and post-translational modifications of isozymes from a large plant gene family.

Keywords: Barley; Peroxidase; Glycosylation; Truncation; Tissue localization

1. Introduction

Peroxidases utilize hydrogen peroxide to oxidize a variety of organic and inorganic compounds and are present in almost all living organisms (reviewed in Ref. [1]). By convention the peroxidases are divided into two large superfamilies, the mammalian peroxidases and the peroxidases present in prokaryotes, fungi, and plants [2]. The families of prokaryote, fungal and plant peroxidases are evolutionarily related and belong to three classes based on structural differences. Class I comprises intracellular peroxidases of prokaryotic origin and is represented by cytochrome c peroxidase (CcP) [3] and ascorbate peroxidase (APX) [4]. Class II contains extracellular peroxidases of fungal origin mainly involved in lignin degradation [5]. Finally, Class III encompasses the "classical" secretory plant peroxidases occurring in large multigene families of, e.g., 73 and 138 members in *Arabidopsis thaliana* [6,7] and rice (*Oryza sativa*) [8,9], respectively. The extensively studied horseradish peroxidase C (HRPC) (reviewed in Ref. [10]) belongs to Class III. The expression pattern of Class III peroxidases showed tissue variation, a large proportion of genes being preferentially expressed in root tissues, while fewer genes were expressed in all organs of the plant [6–8]. Such structural and spatial occurrence reflected

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the diverse physiological processes in which peroxidases are active; biosynthesis and degradation of lignin in cell walls; auxin catabolism; biosynthesis of secondary metabolites; defensive response to wounding, pathogen and insect attack (reviewed in Ref. [11]).

The enzymatic mechanism of peroxidases is well understood, however, one challenge associated with peroxidases is to assign specific functions to individual isoforms. Most of the functional determinants of classical plant peroxidases are defined on the basis of HRPC [10], but this cannot be extrapolated to all plant peroxidases as shown, e.g., for the barley grain peroxidase (BP1) [12].

It has not been easy to associate certain peroxidase isozymes with specific functions and the biological role of the many of the isozymes is still unresolved. Recently, an exhaustive plant peroxidase gene mapping taking advantage of the completed of the *Arabidopsis* and rice genome sequencing projects emphasized the need to study peroxidases individually in their environment. The present survey of barley grain peroxidases applied a proteomics approach to identify three different peroxidase isozymes: BP1 and BSSP1 [13,14] and one hitherto not reported putative peroxidase protein discovered in the present study. The spatial distribution in the seed and post-translational modifications are described for the three isozymes and their possible roles in grain filling and germination are discussed using data from 16 different barley cultivars comprising malting and feed barleys.

2. Experimental

2.1. Plant material

Sixteen spring barley (*Hordeum vulgare*) cultivars (cvs.) were field grown on Funen, Denmark, in year 2000 [15]. Seeds were collected during grain filling as described [16] and their developmental stage scored using the Zadoks scale [17]. Endosperm, embryo, and aleurone layer from five germinated seeds (cv. Sloop) were dissected and freeze-dried prior to protein extraction as previously described [16]. Non-dormant seeds of the 16 cultivars were germinated [15].

2.2. Protein extraction

Seeds were milled and proteins (from 4 g flour) were extracted in 5 mM Tris/HCl, 1 mM $CaCl_2$, pH 7.5 (20 mL) at 4 °C as described [15]. Extracts from dissected seed tissues (endosperm, aleurone, and embryo) were prepared as described [16].

2.3. 2-D gel electrophoresis

2-Dimensional gel electrophoresis (2-DE) in the pH range pH 6–11 was performed essentially as described earlier [18]. Briefly, to ensure high resolution 2-DE, low molecular weight components interfering with 2-DE were removed using NAP10 desalting columns (Amersham Biosciences). For analytical 2-DE, 100 μL (intact seeds) or 250 μL (dissected seeds) extracts were acetone precipitated and pelleted. Samples containing about 40 μg (mature seeds) and 100 μg (germinated seeds) soluble proteins were suspended in 100 μL rehydration solution (8 M urea, 2% CHAPS, 0.5% IPG 6–11 buffer, 20 mM DTT, trace of Bromophenol Blue) and cup-loaded to the anodic end of 18 cm Immobiline DryStrips pH 6–11 (Amersham Biosciences) for IEF. After IEF proteins in the strips were reduced and alkylated by DTT and iodoacetamide, and SDS-PAGE was run in precast gradient gels (ExcelGel SDS XL (18 cm × 24 cm) 12–14%) in a Multiphor II (Amersham Biosciences) according to the manufacturers recommendations. Analytical gels were stained with Colloidal Coomassie Brilliant Blue [19] or silver [20]. Compatibility of silver staining with mass spectrometry was ensured by omitting glutardialdehyde during sensitization and formaldehyde during reaction with silver nitrate [21].

2.4. In-gel digestion of protein spots and sample preparation for mass spectrometry

Spots were excised and subjected to in-gel digestion according to a published protocol [22] with minor modifications. Gel pieces were swollen in 2–3 μL 50 mM NH_4HCO_3 containing 12.5 ng/μL trypsin (modified porcine trypsin, sequencing grade, Promega, Madison), 50 ng/μL Asp-N (Calbiochem, Darmstadt, Germany) or 12.5 ng/μL Lys-C (Calbiochem, Darmstadt, Germany) on an ice bath. After 30 min the supernatant was removed and discarded, 10 μL 50 mM NH_4HCO_3 was added to the gel and the digestion proceeded overnight at 37 °C. Twenty to thirty percent of the supernatant was then concentrated and desalted on a reversed-phase nano-column as described [23] with some modifications. Briefly, a constricted GELoader tip column (Eppendorf, Hamburg, Germany) was packed sequentially with POROS R1 followed by POROS R2 (Applied Biosystems, Framingham, MA, USA). A syringe was used to force liquid through the column by applying a gentle air pressure. The column was equilibrated with 20 μL 5% formic acid (FA) followed by application of the peptide digest. After a wash with 20 μL 5% FA and bound peptides were eluted directly onto the MALDI target by 0.4 μL matrix solution (20 μg/μL DHB in 70% acetonitrile (ACN) 5% FA).

2.5. Hydrophilic interaction liquid chromatography (HILIC)

Peptide digests were subjected to HILIC essentially as described [24]. Briefly, a short (∼5 mm) column with HILIC resin was packed in a GELoader tip (Eppendorf, Hamburg, Germany) and equilibrated with 20 μL of 80% ACN, 0.5% FA. The sample in 80% ACN, 0.5% FA was applied, washed twice with 20 μL 80% ACN, 0.5% FA and bound peptides were eluted by 10 μL 0.5% FA.

2.6. Deglycosylation

In-gel digested sample was equilibrated with 100 mM ammonium acetate (pH 5.0) and 1 mU of *N*-glycosidase A (Roche

Applied Science, Mannheim, Germany) was added followed by incubation for 12 h at 37 °C.

2.7. Protein identification by peptide mass mapping

Peptide mass mapping was performed on a Bruker REFLEX MALDI-TOF mass spectrometer upgraded to a REFLEX IV (Bruker-Daltonics, Bremen, Germany). Positively charged ions were analyzed in the reflector mode using delayed extraction. All spectra were analyzed with the *m/z* software (Proteometrics, New York, USA) using trypsin autolysis products (*m/z* 842.509 and 2211.104) for internal calibration [25] resulting in a mass accuracy of <50 ppm. Protein identification was performed by searching in a non-redundant protein sequence database at the National Center for Biotechnology Information (NCBI) using the online Mascot program (http://www.matrixscience.com). The search criteria were a monoisotopic mass accuracy < 50 ppm and one missed cleavage site, carbamidomethylation of cysteine (complete), partial oxidation of methionine and pyroglutamic acid as allowed modifications. The peptide mass maps and the protein identifications were evaluated as described [26].

2.8. Peptide sequencing by tandem mass spectrometry

Nano-electrospray (ES) mass spectra were acquired on a Q-TOF mass spectrometer (Waters/Micromass, Manchester, UK) in the positive ion mode. Data acquisition was performed on a Mass Lynx PC data system (Software Version 3.5 for Windows NT). Sodium iodide (~2 μg/μL in propan-2-ol:water (1:1, v/v)) was used for TOF calibration. Mass spectra were searched using the online Mascot program and the sequences were manually confirmed.

2.9. 3D-modeling

A 3D-model of the barley peroxidase homolog (TIGR accession TC29818) was built in the homology module of InsightII (Accelrys Software Inc.) using the crystal structures of HRPC [27] and barley peroxidase BP1 [14] as templates. Homology modeling was followed by an energy minimization in the Discover module. No molecular dynamics simulation was applied.

3. Results

3.1. Identification of barley seed peroxidase spots by peptide mass mapping

Three peroxidase isozymes were identified from mature barley (cv. Sloop) seed extracts in the pH 6–11 range (Fig. 1, Table 1). No additional peroxidase spots were detected by extensive proteome analysis using 2-DE in the pH 4–7 range [28,29]. Peptide mass mapping of spots 1 and 2 (Fig. 1) led to identification of BP1 (barley seed peroxidase 1), while spots 3–11 contained BSSP1 (barley seed-specific peroxidase BP1). Despite 99% sequence identity (Fig. 2), BP1 and BSSP1 were distinguished by unique peaks at *m/z* 2063.0 and 1716.7 (Fig. 3) corresponding to the tryptic pep-

Table 1
Protein identification of peroxidase isozymes in germinated (micromalted) cv. Sloop by mass spectrometry

Spot #	Protein name	Theor. p*I*	Theor. M_w (kDa)	Obs. p*I*	Obs. M_w (kDa)	Sequence coverage %		Cultivar[a] (seed and germinated)	Accession: GenBank, TIGR
						Initial	Final		
1	Barley seed peroxidase 1	6.5	34.3	7.0	39.6	79	100	All except An	gi|2624498
2	Barley seed peroxidase 1	6.5	34.3	7.0	38.0	79	100	All except An	gi|2624498
3	Barley seed-specific peroxidase BP1	7.8	34.3	7.9	39.6	75	99	An, Mo, Sl	gi|82410
4	Barley seed-specific peroxidase BP1	7.8	34.3	7.9	38.0	75	99	An, Mo, Sl	gi|82410
5	Barley seed-specific peroxidase BP1	7.8	34.3	8.0	39.6	68	–	An, Mo, Sl	gi|82410
6	Barley seed-specific peroxidase BP1	7.8	34.3	8.0	38.0	68	–	An, Mo, Sl	gi|82410
7	Barley seed-specific peroxidase BP1	7.8	34.3	8.2	39.6	80	100	An, Mo, Sl	gi|82410
8	Barley seed-specific peroxidase BP1	7.8	34.3	8.2	38.0	80	100	An, Mo, Sl	gi|82410
9	Barley seed-specific peroxidase BP1	7.8	34.3	8.2	37.1	75	100	An, Mo, Sl	gi|82410
10	Barley seed-specific peroxidase BP1	7.8	34.3	8.3	39.6	75	99	An, Mo, Sl	gi|82410
11	Barley seed-specific peroxidase BP1	7.8	34.3	8.3	38.0	75	99	An, Mo, Sl	gi|82410
12	Barley peroxidase homolog	8.0	30.6	8.0	32.5	96	100	All	TC29818
13	Barley peroxidase homolog	8.0	30.6	8.3	33.3	96	–	All	TC29818
14[b]	Barley seed peroxidase 1	6.5	34.3	8.3	38.6	55	–	All except An, Mo, Sl	gi|2624498

Spot numbers correspond to Fig. 1. Theoretical p*I* and M_w values were calculated omitting the signal peptide and the C-terminal vacuolar targeting peptide. Observed p*I* and M_w values were offset from the calculated ones due to lack of accurate internal markers. Sequence coverage obtained using routine method for sample preparation; trypsin digestion and reversed-phase (POROS R2) purification is labelled "initial" while an optimized method combining the routine method with Asp-N digestion and HILIC purification is labelled "final". (–) Signifies no attempts to increase the sequence coverage.

[a] The peroxidase spots are detected in the indicated cultivars.

[b] The spot is shown in Fig. 9.

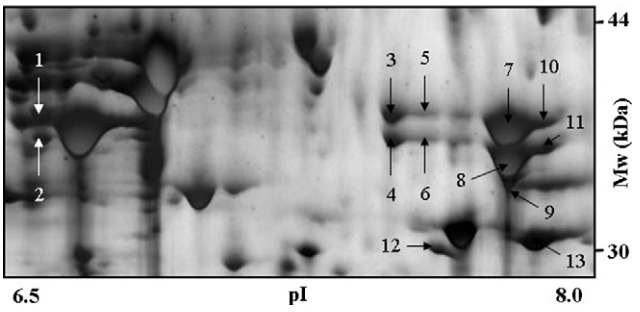

Fig. 1. Close-up of silver stained 2-DE of extract (see Sections 2.2 and 2.3) of mature barley seeds (cv. Sloop) in M_w range 30–44 kDa and p*I* 7.0–8.0. Approximately 40 μg of protein was loaded on the gel. Spot numbers correspond to Table 1.

tides $_{266}$FAQSQQDFFEQFGVSIGK$_{283}$ from BP1 (Fig. 2) and $_{269}$SQQDFFEQFGVSIGK$_{283}$ from BSSP1, respectively. Furthermore, a peak at *m/z* 2338.0 (Fig. 3) was assigned to the N-terminal peptide $_1$AEPPVAPGLSFDFYWQTCPR$_{20}$ of BP1 (Fig. 2), while two peaks at *m/z* 1665.8 and 1821.8 (Fig. 3) matched the tryptic peptides $_1$AEPPVAPGLSFDFYR$_{15}$ and $_1$AEPPVAPGLSFDFYRR$_{16}$ of BSSP1. These marker peptides resulted from substitution of Gln$_{268}$ Trp$_{15}$ and Gln$_{16}$ of BP1 by arginine in BSSP1 (Fig. 2).

Initial peptide mass mapping of spots 12 and 13 (Fig. 1) gave no identification when the mass list was searched against the NCBI protein or DNA databases. However, search against the NCBI EST database (non-mouse, non-human) matched a translated barley sequence (AV926393) for which a BLAST [30] search in the TIGR Barley Gene Index (http://www.tigr.org/tdb/hvgi) identified a tentative consensus sequence (TC29818; Fig. 2). This TC had an open reading frame corresponding to a putative peroxidase with predicted mass of 32.7 kDa and p*I* 8.0, in excellent agreement with experimental data on spots 12 and 13 (Fig. 1; Table 1). This TC sequence further supported the protein identification by matching previously unassigned peaks.

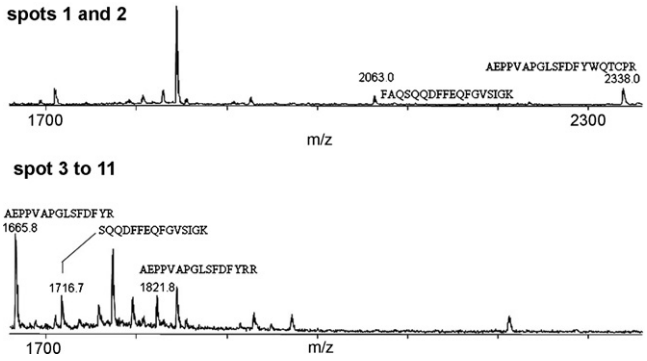

Fig. 3. Section of MALDI-TOF spectra from spots 1 and 2 (top) and spots 3–11 (bottom) in-gel digested by trypsin (see Section 2.4). Peaks at *m/z* 2063.0 and 2338.0 (top) correspond to BP1 tryptic peptides residues 266–283 and 1–20, respectively. These two peptides are modified in BSSP1 (Fig. 2). Peaks at *m/z* 1665.8 and 1821.8 correspond to BSSP1 N-terminal peptide in which $_{15}$ArgArg$_{16}$ replaced TrpGln in BP1. The peak at *m/z* 1716.7 corresponded to the tryptic peptide 269–283 where Glu$_{268}$ in BP1 was replaced by arginine in BSSP1 yielding a cleavage site for trypsin.

3.2. Mass spectrometric analysis of post-translational modifications

The multiple spots for each of the three peroxidase isozymes indicated post-translational modification (PTM), sought to be identified by mass spectrometry. Combined use of trypsin and Asp-N, and two chromatographic resins, reversed-phase (POROS R2) and HILIC, thus resulted in complete or almost complete sequence coverage for the three isozymes (Table 1).

3.2.1. Identification of modification responsible for M_w differences

All spots containing either BP1 (spots 1 and 2) or BSSP1 (spots 3–11) by 2-DE appeared in pairs of essentially equal intensity and p*I*, but with a mass difference of approximately 1 kDa (Fig. 1). BP1 was found earlier to be partly glycosylated at Asn$_{300}$ (Fig. 2) by Man α 1–6(Xyl β 1–2)Man β 1–4GlcNAc

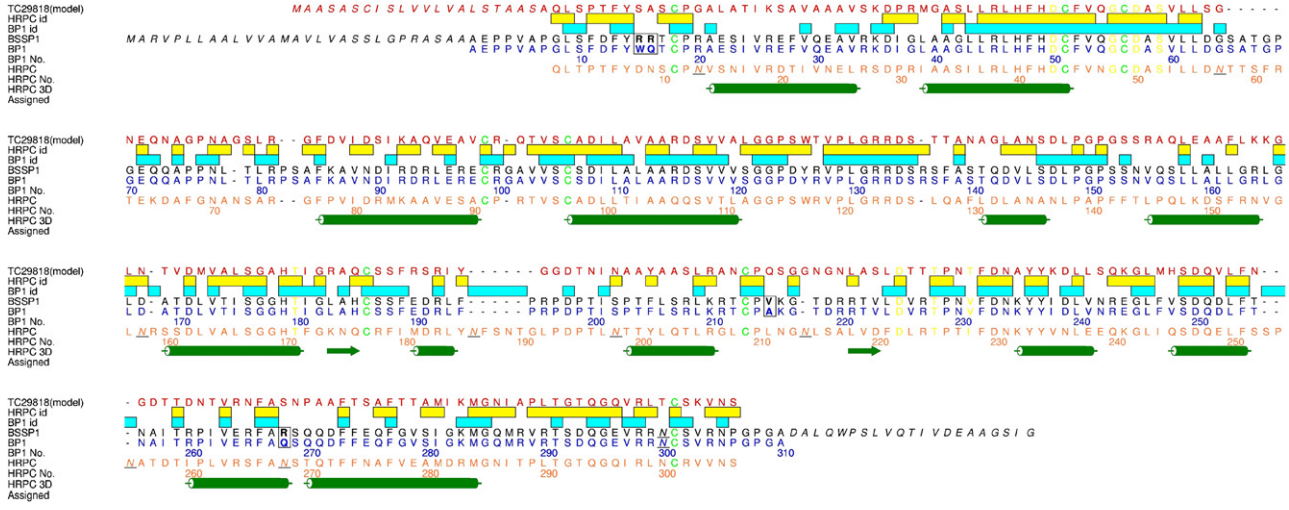

Fig. 2. Structure-guided sequence alignment of barley peroxidases identified in 2-DE patterns compared to HRPC. Cysteine in disulfide bridge in green; Ca^{2+} ligand in yellow; glycosylation site in italics; BP1 amino acid variations in bold; identical positions of sequence gaps are included. N- and C-terminal signal peptides of BP1 are included (in italics). Helices are symbolized by cylinders and β-strands by arrows. Numbering in blue and red are according to BP1 and HRPC, respectively.

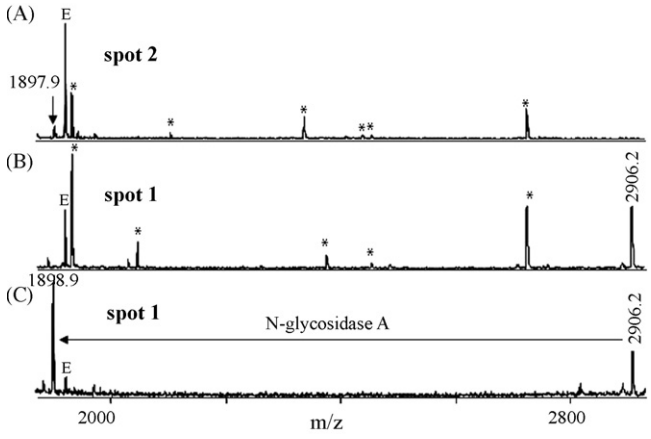

Fig. 4. Section of MALDI-TOF mass spectra of spots 1 and 2 after glycopeptides enrichment using HILIC (see Section 2.5). (A) Peptide mass map of spot 2 showed a peak at *m/z* 1897.9 corresponding to the C-terminal tryptic peptide of BP1 ($_{293}$DQGEVRRNCSVRNPGPGA$_{310}$; Fig. 2). (B) Peptide mass map of spot 1 showing a peak at *m/z* 2906.2 corresponding to the glycosylated C-terminal peptide $_{293}$DQGEVRRNCSVRNPGPGA$_{310}$. (C) The same as (B) but after deglycosylation by *N*-glycosidase A. The deglycosylated peptide appears at *m/z* 1898.9 as expected for the deamidated form. (E) Indicates an enzyme autolysis peptide. Asterisks signify matching unmodified peptides expected from BP1.

β 1–4(Fuc α 1–3)GlcNAc [13]. The M_w of this glucan of 1008.3 was in agreement with the observed mass differences (Fig. 1). In an attempt to apply the Pro-Q Emerald Glycoprotein stain to reveal the glycosylation, no clear results was obtained, however, as the autofluorescence of the plastic support of the gel interfered with this technique. Moreover, this glycosylation site in BP1 and BSSP1 resides in the C-terminal region and was not obtained in tryptic peptide mass mapping and POROS R2 chromatography for sample preparation, presumably due to poor affinity of the resin for the short, hydrophilic glycosylated peptide 300NCSVR304 (Fig. 2).

The problem was overcome by using endoprotease Asp-N [31] generating the longer peptide $_{293}$DQGEVRRNCSVRNPGPGA$_{310}$ combined with peptide purification and concentration by HILIC resin, shown to enrich glycopeptides [24,32]. MALDI mass spectra noticeably for the low M_w spots 2, 4, 6, 8, and 11 contained a peak at *m/z* 1897.9 (Fig. 4A), which was replaced by a peak at *m/z* 2906.2 in spectra for the higher M_w spots 1, 3, 5, 7, and 10 (Fig. 4B). These data suggested the latter peroxidase spots to contain a carbohydrate moiety of 1008.3 Da as proven by *N*-glycosidase A treatment of peptides generated from spot 1 reducing content of 2906.2 Da peptide giving rise to a peptide of *m/z* of 1898.9 (Fig. 4C).

3.2.2. Analysis of the pI difference of BSSP1 forms

The glycosylated and non-glycosylated forms of BSSP1 (Fig. 1; Table 1) showed p*I* variation appearing as two parallel spot "trains" of 39.6 kDa (spots 3, 5, 7 and 10) and 38.0 kDa (spots 4, 6, 8, and 11). Complete sequence coverage was achieved for the abundant spot 8 (Table 1) which contained a full-length, unmodified polypeptide chain. Spot 8 therefore was used as internal p*I* reference in the 2-DE pattern, and all other BSSP1 spots were assumed to have undergone post-translational processing. The nature of the modifications was analyzed for selected spots 4 and 11, showing an acidic and a basic p*I* shift relative to spot 8, respectively (Fig. 1). Peptide mass mapping of both spots gave sequence coverage of 96%, but failed to identify any modification. Since the 4% of the sequence that has not been identified corresponds to 50DCFVQGCDASVLL62 (Fig. 2), it cannot be decided if PTM occurred in that region and deamidation of Gln_{54} to Glu_{54}, belonging to the deamidation consensus sequence -Q-G- [33], may therefore be the cause of the acidic p*I* shift in case of spot 4. A peptide of *m/z* 1371.6 corresponding to this deamidation, was, however, not detected by MALDI-TOF MS. A point mutation could be responsible for the increased p*I* of spot 11 and subjecting the 50DCFVQGCDASVLL62 peptide to BLAST search against barley ESTs, indeed identified several barley ESTs with Gln_{54} changed to asparagine, arginine, or lysine.

No MS peaks, however, matched the corresponding peptides in the MALDI-TOF mass spectrum. Alternatively the p*I* shift can be due to rare modification, such as oxidative deamination of lysine or C-terminal amide formation.

3.2.3. Investigation of proteolytic processing of protein corresponding to TC29818 and of BSSP1

Peptide mass mapping of spots 12 and 13 matching TC29818 (Fig. 1) provided complete sequence coverage of the protein, except for the N-terminal MAASASCISLVVLVALSTAASAQLSPTFYSASCPGALATIK and the C-terminal $_{299}$LTCSKVNS$_{306}$ amino acid residues (Fig. 2). The SignalP Server (http://www.cbs.dtu.dk/services/) predicted MAASASCISLVVLVALSTAAS to be a signal peptide. A BLAST search however, of the TC against plant peroxidase sequences suggested glutamine rather than alanine as the N-terminal residue of the mature protein. Indeed, a peak at *m/z* 1995.0 (Fig. 5) in the MALDI-TOF mass spectra obtained from both spots confirmed the presence of the pyroglutamate form of the N-terminal peptide of the mature protein.

The C-terminal region of the TC sequence (Fig. 2) comprised two short tryptic peptides $_{299}$LTCSK$_{303}$ and $_{304}$VNS$_{306}$ not observable by MALDI-TOF mass spectrometry due to interfering signals from the matrix. Therefore, cleavage was performed by using Lys-C only cleaving peptide bonds C-terminal to lysine residues, but $_{284}$MGNIAPLTGTQGQVRLTCSK$_{303}$ of 2139.09 Da that includes $_{299}$LTCSK$_{303}$ was not detected from spots 12 or 13 (Fig. 2). In contrast four peaks of *m/z* 2004.0, 1916.9, 1756.9, and 1656.9 (Fig. 5B) appeared from spot 12, but not from 13. Detailed analysis revealed that they represented sequential trimming of the Lys_{303}, Ser_{302}, Cys_{301}, and Thr_{300} residues from $_{284}$MGNIAPLTGTQGQVRLTCSK$_{303}$. Moreover, peptides of *m/z* 2020.0, 1932.9, 1772.9, and 1672.9 (Fig. 5B) corresponded to oxidized Met_{284} versions. Although no peptide from the C-terminus was detected in the MALDI-TOF spectrum of Lys-C fragments from spot 13, the apparent M_w of the spot on the 2-D gel suggests that the C-terminus is intact.

Peptide mass map of BSSP1 in spot 9 (Fig. 1; Table 1) lacked the peak comprising the C-terminal peptide suggesting truncation of the protein. Indeed MALDI-TOF MS of the

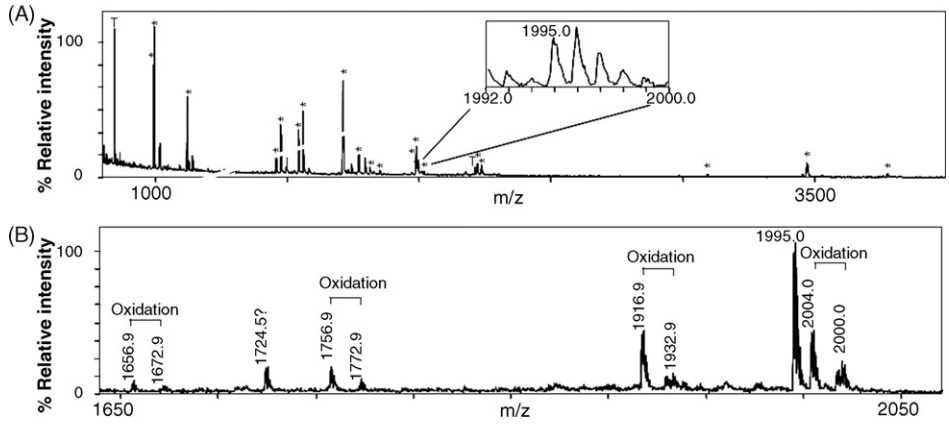

Fig. 5. Processing sites at the N- and C-termini of the putative peroxidase (TC29818). (A) Region of a tryptic MALDI peptide mass map of spot 13 containing a peak of the N-terminal peptide (*m/z* 1995.0) in insert (A) in pyroglutamate form. (B) Region from Lys-C peptide mass map of spot 12 containing the N-terminal peptide (*m/z* 1995.0) and peptides from the C-terminal region (*m/z* 2004.0, 1916.9, 1756.9, 1656.9) including methionine sulfoxide forms (see Section 3.2.3.). Asterisks signify predicted unmodified peptides matching TC29818.

Asp-N digested protein gave a peak of *m/z* 973.48 (not shown) unique to spot 9, which probably corresponded to a C-terminal peptide $_{293}$DQGEVRRN$_{300}$ (Fig. 2). This protein therefore lacked a C-terminal decapeptide present in the full-length form (Figs. 1 and 2; Table 1).

3.3. Structural model of a novel peroxidase

A structural model (Fig. 6) was built for TC29818 by using the crystal structures of BP1 (1BGP, [14]) and horseradish peroxidase HRPC (7ATJ, [27]) as based on the structural alignment (Fig. 2) in which TC29818 possesses all conserved residues characteristic of a Class III plant peroxidase. The model predicts the proximal (Thr$_{171}$, Asp$_{222}$, Thr$_{225}$, Ile$_{228}$) and distal (Asp$_{43}$, Gly$_{48}$, Asp$_{50}$, Ser$_{52}$) Ca^{2+}-binding sites to be intact and the central active site residues Arg$_{38}$, Phe$_{41}$, His$_{42}$, Asn$_{70}$, and His$_{170}$, to be conserved (HRPC numbers). Residues 44–75 (Fig. 2) in the BC loop are important for activity in plant peroxidases as an asparagine (Asn$_{70}$) in this loop is hydrogen bonded to the active site distal histidine (His$_{42}$), thereby orienting the hydrogen-bonding network in the distal cavity and regulating the pK_a value of this histidine. A conserved glutamic acid (Glu$_{64}$) participates in the same hydrogen-bonding network, which also involves the distal Ca^{2+}. The alignment (Fig. 2) indicates that most likely TC29818 is shortened in the region of the structure containing the *N*-glycosylated site of the BC loop of HRPC.

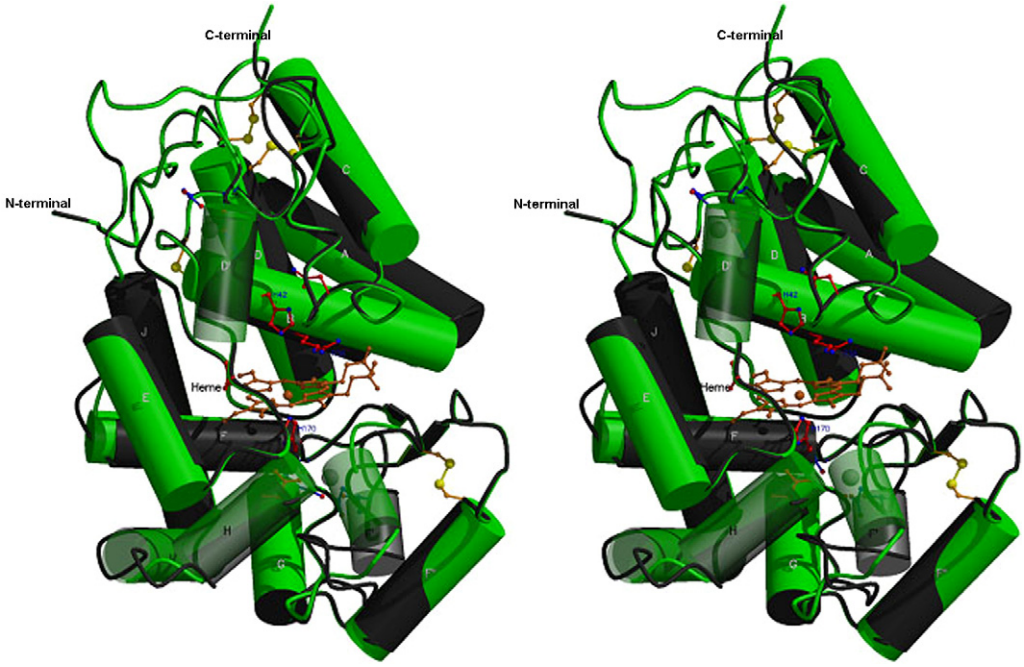

Fig. 6. Three-dimensional model of the putative peroxidase (TC29818). The stereo representation showing a superposition of the energy minimized TC29818 model (green) and horseradish peroxidase C (grey, 7ATJ). Active site residues are coloured red, disulfide bridges are yellow and calcium ligands are blue. Nitrogen atoms are blue and oxygen atoms red. Selected helices are transparent to improve the information given by the figure. Helices are labelled according to convention in the peroxidase field and active site residues are labelled according to the horseradish peroxidase C sequence.

The F′–F′′ area of the TC29818 model corresponds to that of BP1 by having a short loop connecting the F′ and F′′ helices. Furthermore, the substrate-binding site resembles BP1 by lacking the phenylalanine patch characteristic of HRPC, and rather having an open site gated by small and/or hydrophilic residues. Sequence gaps corresponding to those observed in the TC29818 (Fig. 2) are commonly observed between the 73 peroxidase genes characterized in *Arabidopsis* [6].

3.4. Tissue localization of barley seed peroxidase isozymes

The occurrence of peroxidases BP1, BSSP1, and TC29818 in dissected tissues from 3-day germinating barley seeds (cv. Sloop) was monitored by 2-DE (Fig. 7). All multiple forms of the three isozymes (spots 1–13) were present in the endosperm. BP1 spots 1 and 2 were lacking in the aleurone layer and embryo. BSSP1 spots 7 and 8 occurred in low level in aleurone and were abundant in the embryo. Spots 12 and 13 of the putative peroxidase TC29818 were clearly seen by 2-DE in the aleurone layer, whereas only spot 12 was found from the embryo (Fig. 7).

3.5. Changes in peroxidase spot pattern during seed development

Extracts of seeds collected during grain filling (cv. Morex) were analyzed by 2-DE and showed that the three peroxidase isozymes appeared at different developmental stages (Fig. 8). Near-end levels of BP1 (spots 1 and 2), BSSP1 (spots 3–11), and the putative peroxidase (spot 12) were detected prior to seed maturity at Zadoks developmental stages 80, 86, and 80 [17], respectively. Spots 1 and 2 increased dramatically from stage 80 to 85, followed by a steady slight increase until maturity. Well-defined spots 3–11 appeared at stage 86 and increased a few fold thereafter. The C-terminally processed putative peroxidase (spot 12) was detected in low amounts already at stage 80, increased markedly from stage 85 to 86, and was only slightly enhanced until maturity. Interestingly, spot 13 appeared later than spot 12. It was barely detected at stage 86, but increased many-fold until seed maturity (Fig. 8).

3.6. Cultivar-dependent spot patterns and implication of peroxidase multiple forms in germination

The abundance of the three peroxidase isozymes was monitored during 6 days of germination for 16 barley cultivars (Fig. 9). BP1 (spots 1 and 2) essentially remained constant during germination for all 16 cultivars whereas abundance of BP1 was high in 12 of the cultivars including Barke (an excellent malting cultivar), moderate in cvs. Sloop and Harrington, low in cv. Meltan (a feed barley), and lacking in cv. Annabell (Fig. 9). BSSP1 (spots 3–11 of which 7 and 8 were particularly abundant) was exclusively present in cvs. Annabell, Morex, and Sloop (Fig. 9) seemed essentially unaffected by germination. Remarkably, Barke and 12 other cultivars showed a single BP1 spot 14 (Fig. 9) in the 2-DE region where BSSP1 spots 3–11 appeared in other cvs. Annabell, Morex, and Sloop (Fig. 9). However, the

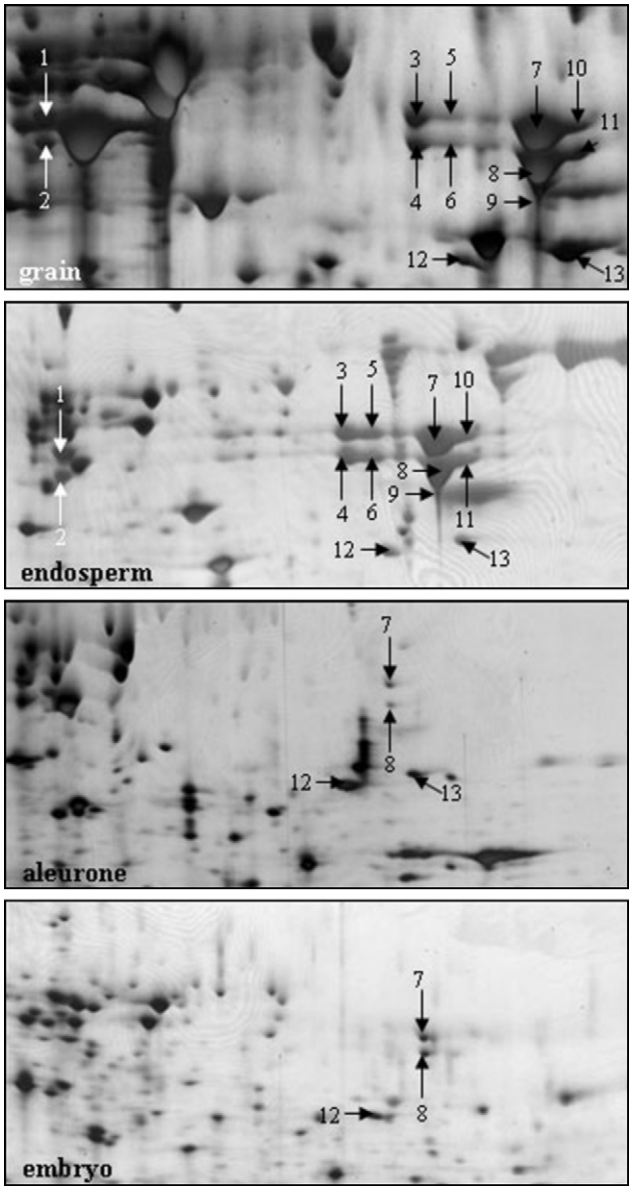

Fig. 7. Tissue localization of peroxidases in dissected 3 day germinated barley seeds (cv. Sloop). The presence of the peroxidase isozymes BP1 (spots 1 and 2), BSSP1 (spots 3–11), and TC29818 (spots 12 and 13) in the whole seed, endosperm, aleurone, and embryo tissues is monitored. Spot numbering corresponds to Fig. 1 and Table 1.

1.3 unit higher p*I* of BP1 spot 14 relative to spots 1 and 2, (which do not carry modifications altering p*I*), could not be accounted for by the obtained mass spectra. Moreover, spot 14 in contrast to spots 1 and 2 decreased in abundance during germination (Fig. 9). Due to its proximity to the BSSP1 spots in the 2-DE, the presence of this form of BP1 in cvs. Annabell, Morex, and Sloop could not be excluded although mass spectra from this 2-DE region for these cultivars contained no peaks specific to BP1. All cultivars throughout germination showed an apparent 1:1 ratio for glycosylated and non-glycosylated peroxidase (e.g., spot 1 relative to spot 2). The TC29818 putative peroxidase in spot 13 was rather abundant at day 0 and decreased during germination for all cultivars in parallel to a C-terminally truncated

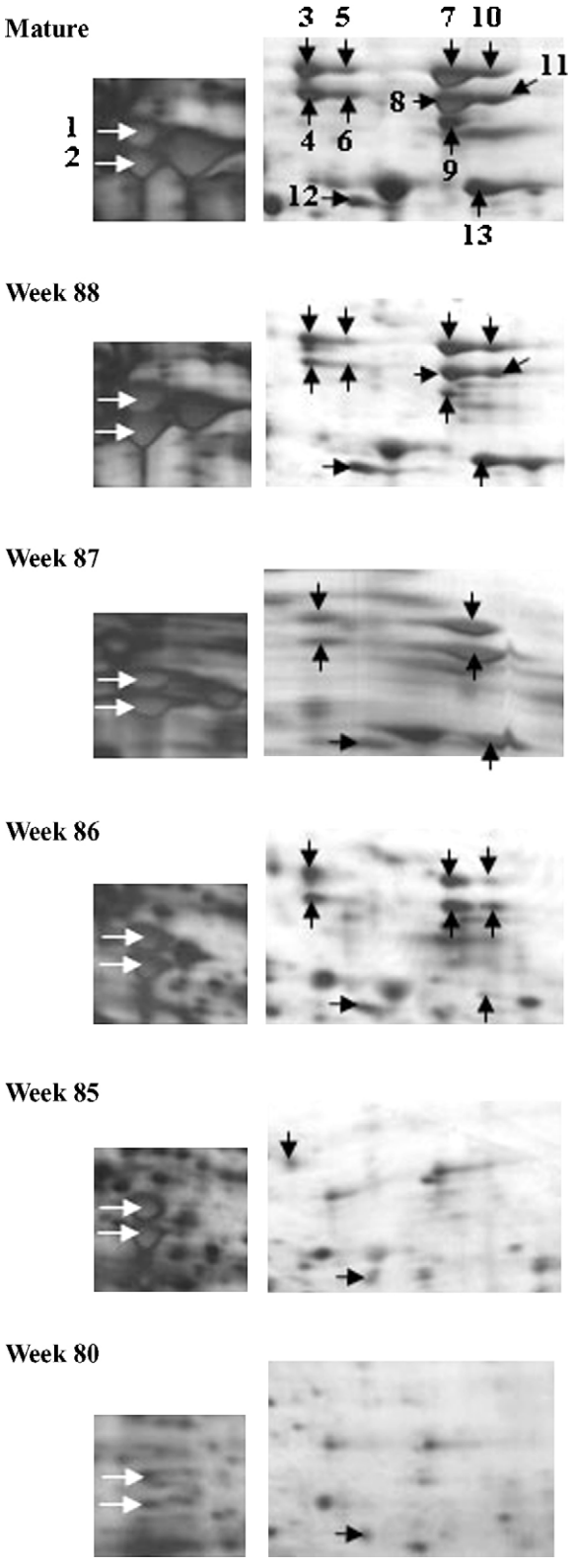

Fig. 8. Monitoring of peroxidase isoforms during seed development (cv. Morex). Patterns at grain filling stages 80, 85, 86, 87, and 88 (according to Zadoks et al. [17]) are shown for BP1 in spots 1, 2, BSSP1 in spots 3–11, and TC29818 in spots 12 and 13. Gel sections correspond to Fig. 1.

form (spot 12) being less abundant at day 0 and increasing during germination.

4. Discussion

It is evident from numerous large-scale protein identification studies that very often more than one protein form originated from a particular gene. Thus, protein profiling of cells and tissues by 2-DE showed characteristic spots "trains" covering a p*I* and/or M_w range. Such spot "trains" can represent multiple forms derived from a single gene product due to post-translational and other processing and/or stem from isoforms of different but closely related genes and allele variants. The present study identified a total of 13 forms derived from three different genes by using 2-DE, mass spectrometry, and database searching. BP1 (gi|2624498) and a putative peroxidase (TC29818) were each found in two spots, while BSSP1 (gi|82410) was found in nine. BP1 is the most extensively characterized barley peroxidase [12–14] and previously found in mature seeds of a barley mutant (Risø 1508) of cv. Bomi.

The present detection of BSSP1 in nine spots and in several cultivars at higher amounts than BP1, was surprising because the corresponding nucleotide sequence was retrieved only from a cDNA library of cv. Bomi; thus the protein was not previously identified. The comparison of 16 cultivars indeed revealed three profiles of BP1 and BSSP1 that divide the cultivars in three groups: Group 1 had only BP1 (13 cultivars; Fig. 9); Group 2 contained only BSSP1 (cv. Annabel, Fig. 9); while in Group 3 both BP1 and BSSP1 were present (cvs. Sloop and Morex; Fig. 9). Thus, the peroxidase profiles outlined by the proteomics based approach revealed that occurrence and absence of certain isozymes might not merely be a matter of polymorphism between different cultivars, as previously suggested [13]. Rather, the proteins appeared as products of two distinct genes and should as a consequence be named individually.

The three isozymes were traced during grain filling in cvs. Morex and Barke in which BP1 appeared two developmental stages earlier than BSSP1 in both of the cultivars (shown for cv. Morex in Fig. 8).

BP1 and BSSP1 spots were always present as doublets in 2-DE of identical p*I* and different apparent M_w. Partial glycosylation of Asn_{300} was reported to result in BP1a (glycosylated) and BP1b (non-glycosylated) forms [13]. The 3D structure suggested this incomplete glycosylation to stem from competition between the glycosylation and formation of an adjacent disulfide bridge [13]. The predicted glycopeptide was indeed present in all spectra obtained for high M_w forms of BP1 and BSSP1. The carbohydrate moiety has not been ascribed a specific biological role; but has been suggested to contribute in solubility [34]. It was situated near the C-terminus of BP1 and BSSP1 and thus far from the active site. Peroxidases, however, are thought to be involved in polymerisation of lignin and lignin-like compounds and the glycosylation perhaps influences substrate specificity in such reactions.

Despite high sequence identity of BP1 and BSSP1, only the latter showed charge variation spot "trains". Analysis at nearly complete sequence coverage for such spots, however, failed to

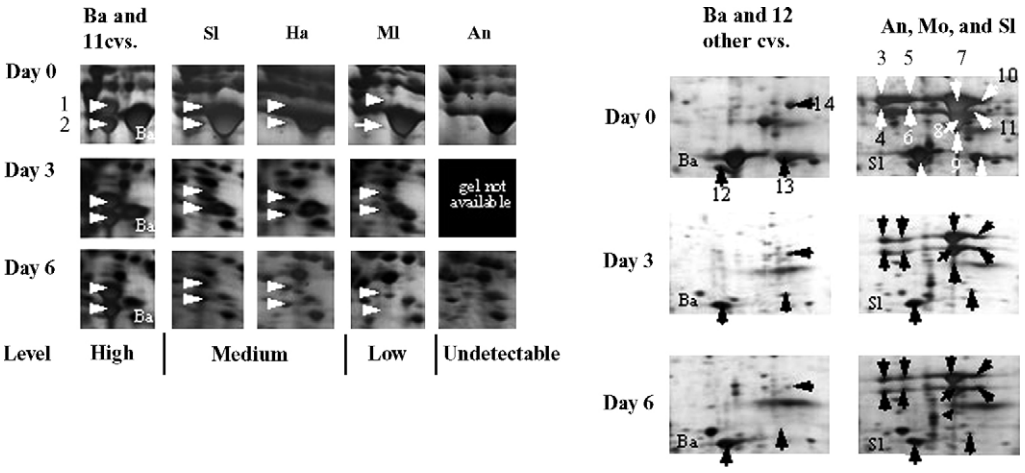

Fig. 9. Monitoring of three barley seed peroxidases during germination of 16 cultivars. BP1 in spots 1 and 2, BP1 in spot 14, BSSP1 in 9 spots (3–11), and TC29818 in spots 12 and 13 at day 0 were overdeveloped during silver staining, thus the real level of spots 14 and 3–11 is roughly constant throughout germinating. The top row contains the glycosylated and the lower row the non-glycosylated forms. An, Annabell; Ba, Barke; Ha, Harrington; Ml, Meltan; Mo, Morex; Sl, Sloop.

identify the underlying modifications of the polypeptide chain. Previously, cases where complete sequence coverage has been obtained and no deamidation was detected for protein charge variants, did suggest that tertiary structure variation during IEF could lead to different electrophoretic mobility [35]. Analysis of EST libraries suggested, however, that charge "trains" for BSSP1 might stem from sequence variations, which were however not confirmed by the present mass spectra.

A putative peroxidase was discovered at the protein level in spots 12 and 13 (Fig. 1) matching an EST that allowed retrieval of a TC29818. This TC sequence has 86% identity to a wheat peroxidase (Q43218) supporting the barley protein to be an active peroxidase. Sequence alignment assigned a signal peptide which was confirmed by mass spectrometric detection of the N-terminal pyroglutamate peptide. Thus, this peroxidase is N-terminally blocked in contrast to the other two barley seed peroxidases. 2-DE demonstrated the new peroxidase in all seed tissues (spot 12, Fig. 7). Similar constitutive expression has been noted for the *Arabidopsis* ATP1 and ATP2 peroxidases [6], suggesting basic metabolic functions for these peroxidases. BP1 and BSSP1 on the other hand, were mainly confined to the endosperm known to be rich in peroxidases [13,36] although it remains an open question which role these peroxidases exert in the metabolically inactive tissue. The putative peroxidase was furthermore present in all 16 cultivars examined. It appeared at the start of dough development and the two forms in spots 12 and 13 differed in tempo-spatial appearance. Spot 12 was in excess of spot 13 during developmental stages 80 through 87 but thereafter the relationship was reversed. This pattern, moreover, was reversed during germination, the 6-day germinated seeds containing only a trace of spot 13 while spot 12 increased. This pattern probably reflected transient synthesis or activation of one or more proteases. Indeed, peptide mass mapping confirmed spot 12 to contain C-terminally processed protein in accordance with a late increase, presumably arising from spot 13. The 3D-model supported this enzyme to be catalytically functional.

Taken together, expression and processing of the peroxidase isozymes differed markedly during seed development and maturation, presumably reflecting different overlapping roles for peroxidases in the seed. Proteome analysis of dissected tissues during grain filling might provide thorough insight on the spatio-temporal expression of the three peroxidase genes and generation of multiple forms through post-translational processing.

The function of various peroxidases in the seed, however, remains obscure, whereas peroxidases in leaves contributed to build a barrier to invading pests, e.g., powdery mildew by cross-linking protein and phenolic components [36]. The repertoire of peroxidase isozymes displayed by different barley cultivars perhaps reflects involvement in the defence against a repertoire of distinct pathogens. Peroxidases have been reported to play a role in the metabolism of oxygen during mashing and also to affect colour and clarity of the wort and probably influence beer flavour [37]. Interestingly, peroxidase isozymes in germinated barley have shown different efficiency in producing these effects [38]. Accordingly, cultivar variations observed in the present study may be directly linked to beer quality parameters. Peroxidases can degrade polyphenols in mashes for which reason hydrogen peroxide is supplied to achieve stabilisation of the beer [39]. Although the hydrogen peroxide decomposes completely to water and oxygen, the treatment may affect flavour stability [40].

5. Conclusion

The present study provides the first detailed map of selective cultivar and spatio-temporal profiles of barley seed peroxidase isozymes occurring in multiple forms due to post-translational modification. Thus, 13 spots by 2-DE of germinated barley seeds were found to contain products from three peroxidase genes encoding two closely related BP1 and BSSP1 and one putative peroxidase. The acquisition of nearly complete or complete sequence coverage was ensured by obtaining different mass spectra datasets by using different proteases for in-gel digestion and sample pre-treatment procedures. MALDI-TOF MS and off-line Q-TOF MS/MS, and carefully searching these spectra against several sequence databases. The structural basis for the

spot "trains" included partial glycosylation on Asn_{300} in BP1 and BSSP1. Monitoring all three peroxidases during grain filling and germination in 16 barley cultivars emphasized that only these three isozymes seemed present in high amounts in barley seeds. Barley contains however at least six peroxidase genes implying that other gene products might be found in other tissues. Possibly the observed differences in seed peroxidases link with important malting (germination) quality parameters, which in turn contribute to defining breeding targets for obtaining superior malting cultivars.

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