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Phytochemistry 65 (2004) 1565–1574
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Proteome analysis differentiates between two highly homologues germin-like proteins in *Arabidopsis thaliana* ecotypes Col-0 and Ws-2

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Received 12 January 2004; received in revised form 20 April 2004
Available online 8 June 2004

Abstract

A proteome approach based on 2-D gel electrophoresis (2-DE) was used to compare the protein patterns of the *Arabidopsis* ecotypes Col-0 and Ws-2. In leaf extracts a pair of protein spots were found to be diagnostic for each of the lines. Both pairs of spots were identified as closely related germin-like proteins differing in only one amino acid by using peptide mass finger printing of tryptic digests and by gaining additional data from post-source decay spectra in the MALDI-TOF analysis. Western blot analysis after separation of protein extracts by 2-DE confirmed results from Coomassie blue-stained gels and revealed additional immunoreactive spots for both ecotypes most likely representing dimers of the spots first identified. Western blot analysis and mass spectrometrical identification of the corresponding weakly stained protein in Coomassie blue-stained gels of the ecotype Col-0 also demonstrated for the first time the occurrence of AtGER3 protein in root extracts. Our results demonstrate the capacity of proteome analysis to analyse and distinguish closely related members of large protein families.

Keywords: *Arabidopsis thaliana* (L.) Heynh; Germin-like proteins; AtGER3; Proteome analysis

1. Introduction

Germins and germin-like proteins (GLPs) comprise a large set of plant proteins encoded by multigene families. Germin proteins were first detected in germinating seeds of cereals and constitute a homogenous group within the branched tree of germin-like proteins. Many germin-like proteins accumulate in the apoplast as oligomers and proved to be quite resistant to degradation. Many GLPs were shown to be heat-stable and SDS-resistant (Vallelian-Bindschedler et al., 1998a; Wei et al., 1998). All GLP sequences have at least one site for glycosylation, but the presence of a glycan moiety has been demonstrated only in few cases such as for a pea GLP with receptor activity for the rhizobial attachment protein rhicadhesin (Swart et al., 1994). A wheat germin was found in two isoforms by SDS–PAGE which differed in the glycosylation pattern (Lane et al., 1986; Berna and Bernier, 1997). Transgenic tobacco plants expressing AtGER1, AtGER2 or AtGER3 produced two proteins with a slight shift on SDS–PAGE most likely representing different glycosylation patterns (Membre et al., 2000).

Although germins and GLPs have been first described in the context of germination, they are expressed in many plant tissues in different developmental stages and their expression level is often controlled by various environmental stimuli. Their expression pattern is consistent with roles in cell expansion and resistance to stress. Fungal infection led to an increased germin and GLP expression in wheat and barley (Dumas et al., 1995; Zhang et al., 1995; Hurkman and Tanaka, 1996; Zhou et al., 2000). Oxalate oxidase activity is associated with germins (Lane et al., 1993; Lane, 1994), which could be important in defence responses. Hydrogen peroxide accumulation has been documented in barley papillae formed after powdery mildew infection (Gregersen

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0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.04.027

et al., 1997), however the presence of oxalate oxidase activity in these papillae has still to be demonstrated. The production of hydrogen peroxide by oxalate oxidase could lead to the reinforcement of the cell wall texture by cross-linking events. Alternatively hydrogen peroxide could participate in signalling cascades triggering defence responses to pathogen attack or have a direct anti-microbial effect.

Transgenic approaches have been used to verify the effects of ectopic expression of oxalate oxidase on disease resistance. Expression of the wheat germin gf-2.8 gene in sunflower led to stimulation of defense responses and in one of the transgenic lines HR-like lesions became visible. The transgenic sunflower lines showed increased resistance against the oxalate generating fungus *Sclerotinia sclerotiorum* (Hu et al., 2003). Ectopic expression of the same wheat germin also made soybean more resistant against this pathogen (Donaldson et al., 2001; Cober et al., 2003) and transgenic corn lines showed reduced herbivory of the European corn borer (Ramputh et al., 2002). Increased resistance by ectopic expression of wheat germin in a tree species was demonstrated in transgenic poplar lines using the poplar pathogenic fungus *Septoria musiva* (Liang et al., 2001). Transient expression of GLPs in epidermal cells of wheat and barley by using a biolistic transient assay system resulted in increased resistance to powdery mildew, whereas transient silencing by RNA interference reduced the basal resistance (Schweizer et al., 1999; Christensen et al., 2004).

Apart from oxalate oxidase, superoxide dismutase activity was also found to be associated with some members of the GLP family. Examples are the tobacco nectarin I (Carter and Thornburg, 2000) and the wheat and barley GLP4 protein, which appears to be an important factor of quantitative resistance (Christensen et al., 2004).

Heat-shock treatment of barley plants led to the insolubilization of GLPs in the apoplast (Vallelian-Bindschedler et al., 1998a; Vallelian-Bindschedler et al., 1998b). The application of thermal stress resulted in an increased resistance against a subsequent challenge of the plants by powdery mildew and the insolubilization might indicate the strengthening of the cell walls. However, not all GLPs are becoming insoluble upon stress exposure (Bernier and Berna, 2001).

Due to the emerging roles of GLPs in plant development and stress defense, a detailed analysis of individual family members and their specific roles is needed. The completion of the first genomic sequences of higher plants opened up new possibilities to study gene families. So far, 59 GLP sequences have been annotated for *Arabidopsis* and 37 for rice, respectively. An earlier study had identified 39 ESTs encoding GLPs in *Arabidopsis*. Alignments of available GLP sequences showed that the wheat and barley germin sequences formed a subfamily with no members from *Arabidopsis* (Carter et al., 1998). All the GLPs shared at least 31% identity on the amino acid level. Within subfamilies, several closely related members were identified with identity of up to 99.5% for GLP3a and GLP3b (Carter et al., 1998).

The progress made in completing the sequencing of whole genomes and the rapid increase in the availability of "expressed sequence tag" (EST) sequences have stimulated the global multiparallel analysis of transcript levels making use of arrays and filters with large sets of specific gene probes (Bevan, 2002). In addition, the availability of nucleotide sequence information now enables the identification of proteins of interest. The technological progress made in the separation of proteins by 2-D gel electrophoresis and the development of mass spectrometric techniques allowing rapid and sensitive analysis of peptides and proteins provides powerful tools combined in proteome approaches.

Here, we report on the differentiation of closely related GLPs on the protein level with a comparative proteome approach based on separation of protein extracts by 2-D gel electrophoresis and subsequent identification of relevant spots using sensitive mass spectrometry techniques. During a comparative proteome study of *Arabidopsis* ecotypes Col-0 and Ws-2, we identified spots diagnostic for each of the lines. Among the diagnostic spots, we identified two germin-like proteins. In the following we describe the part of the proteome study focusing on the analysis of these two GLPs.

2. Results

Arabidopsis plantlets for our analysis were harvested both from cultures on soil and from a newly developed sterile hydroponic system (Schlesier et al., 2003). All the following experiments were carried out on both materials with the exception that roots were analysed only from hydroponic culture. Proteins from leaves and roots were extracted from TCA–acetone-precipitated material and separated on immobilized pH gradients in the first dimension and on SDS–PAGE in the second dimension. A large number of spots were resolved on the gels stained with Coomassie G250. Protein patterns of diverse *Arabidopsis* lines were rather similar and no obvious differences in strongly stained protein spots became visible under both growth conditions (data not shown). However, detailed comparison displayed a number of more weakly stained proteins, which were differentially expressed. Such individual protein spots were excised from the 2-D gels and digested with trypsin, resulting in a set of highly specific tryptic peptides. The masses of extracted peptides were measured by MALDI-TOF MS to obtain peptide mass fingerprints (PMF) and post-source decay spectra (PSD) of selected peptides, and used for protein identification by searching in databases.

2.1. Leaf proteins

The differentially expressed leaf proteins included two pairs of spots designated 1a/1b from Col-0 and spots 2a/2b from Ws-2. After staining with Coomassie blue, the spots 1a/1b can be detected only in Col-0, the spots 2a/2b only in Ws-2 (Fig. 1(a) and (b)). This is additionally demonstrated by a separation of a mixture of both samples on the same gel (Fig. 1(c)) to exclude errors in 2-D-based separation. Spots 1a and 2a were more strongly stained as 1b and 2b and showed a higher electrophoretic mobility (Fig. 1(a) and (b)). Additionally, spots 2a/2b showed a slightly higher mobility in comparison to the protein pair 1a/1b. Whereas the masses of all four proteins were rather similar, the p*I* of spots 1a/1b differed from spots 2a/2b by approximately 0.45 pH units.

To identify the proteins in these four spots, we performed MALDI-TOF measurements. Peptide mass fingerprints of spots 1a and 1b resulted in identical spectra. Similarly the protein from spot 2a produced the same peptide pattern as the protein from spot 2b. Most remarkably, the peptide fingerprint patterns of spots 1a/1b were similar to those of spots 2a/2b (Fig. 2). Particularly, the masses with $m/z = 1181$, 2075, 2090 and the high intensity mass $m/z = 2187$ were common to all spectra. The spectra from spots 2a/2b exhibited two additional masses at $m/z = 1852$ and 3015. Searching for *Arabidopsis thaliana* proteins in the latest version of the non-redundant NCBI database using the Mascot search engine resulted in highly homologous germin-like proteins as the best matching entries. Spots 1a and 1b were identified as the germin-like protein AtGER3 (gi|15242028). However, this hit was not statistically significant by peptide mass fingerprinting, because only four of 14 observed mass values matched although the sequence coverage was 25%. Spots 2a and 2b were also identified as germin-like protein (gi|1755154). From these two protein spots, six of 14 observed masses including the additionally masses $m/z = 1852$ and 3015 matched and lead to a statistically significant hit with a sequence coverage of 33%. To get additional information for an unambiguous protein identification, we acquired post-source decay spectra of selected peptides. The intensities of the peptides with masses $m/z = 1181$, 1852 and 2187 were sufficient to accumulate such data sets. The results from PSD measurements are summarized in Table 1. MS/MS search of PSD data against the NCBInr database resulted in the peptide sequences given in the table. All the single PSD measurements gave as top hit the germin-like sequences already obtained by peptide mass fingerprinting. Although the individual hits were not statistically significant, the data supported the results from PMF analysis. A combined search using PMF and PSD data resulted in statistically significant hits and confirmed the identification of the proteins of spots 1a and 1b from Col-0 as germin AtGER3 (gi|15242028), and the proteins of spots 2a and 2b from Ws-2 as a very similar germin-like sequence (gi|1755154).

The unique difference between both proteins is an exchange in position 59 in which the Col-0 GLP contains a Thr residue, whereas the Ws-2 GLP possesses a Lys in this position. The Lys residue introduces an additional tryptic cleavage site and, in agreement with the predictable peptide masses, signals of $m/z = 1852$ and 3015 were present only in the PMF spectra from the Ws-2 GLP spots 2a and 2b. The observation of these peptides enables the discrimination between these highly homologues germin-like proteins and from other germin-like proteins. An alignment of the five most similar sequences out of 59 germin-like proteins from *Arabidopsis* against germin gi|1755154 is given in Fig. 3. The peptides observed by mass spectrometry of spots 1 and 2 are underlined. A signal peptide from 1 to 23 was predicted by both algorithms implemented in the program SignalP. The amino acid exchange at position 59 between sequences gi|1755154 and gi|15242028 is shaded. It is evident from the alignment that only three sequences agree with the MALDI-TOF data. Sequences gi|1755188 and gi|15242028 result in the same mature protein. The p*I*s were calculated for both mature germins from Col-0 and Ws-2. They differ by 0.45 pH units, which is identical with the experimentally value estimated by measuring the distance of the spots and recalculation of the difference in pH units as described in Section 4.

In addition, we performed immunoblotting after separation of proteins by 2-D gel electrophoresis to verify the results and to see whether additional spots would be detected indicating the presence of other germin-like proteins in the extracts with high homology to the spots 1 and 2. Western blot analysis also allowed us to monitor the occurrence of oligomeric forms of the spots 1 and 2, as germin-like proteins are well known for being highly resistant to denaturing agents such as urea or SDS (Vallelian-Bindschedler et al., 1998b; Wei et al., 1998) being present in our buffer systems. We used an antiserum raised against a unique peptide sequence from a variable region of AtGER3 present in no other known GLP. The position of this sequence is given in Fig. 3. The very clear immunoblots from 2-D gels show mainly signals corresponding with the spots 1a and 1b of the Col-0 germin (Fig. 1(d)) and the spots 2a and 2b of the Ws-2 germin (Fig. 1(e)). Co-electrophoresis of both samples demonstrated the different positions of both proteins in the total protein pattern consistent with the pattern observed by Coomassie blue-staining (Fig. 1(f)). Some other weak signals can be detected in the extracts from both ecotypes. Faint staining was observed at positions with the same p*I* but at about the twofold mass when compared with the spots 1 and 2, respectively.

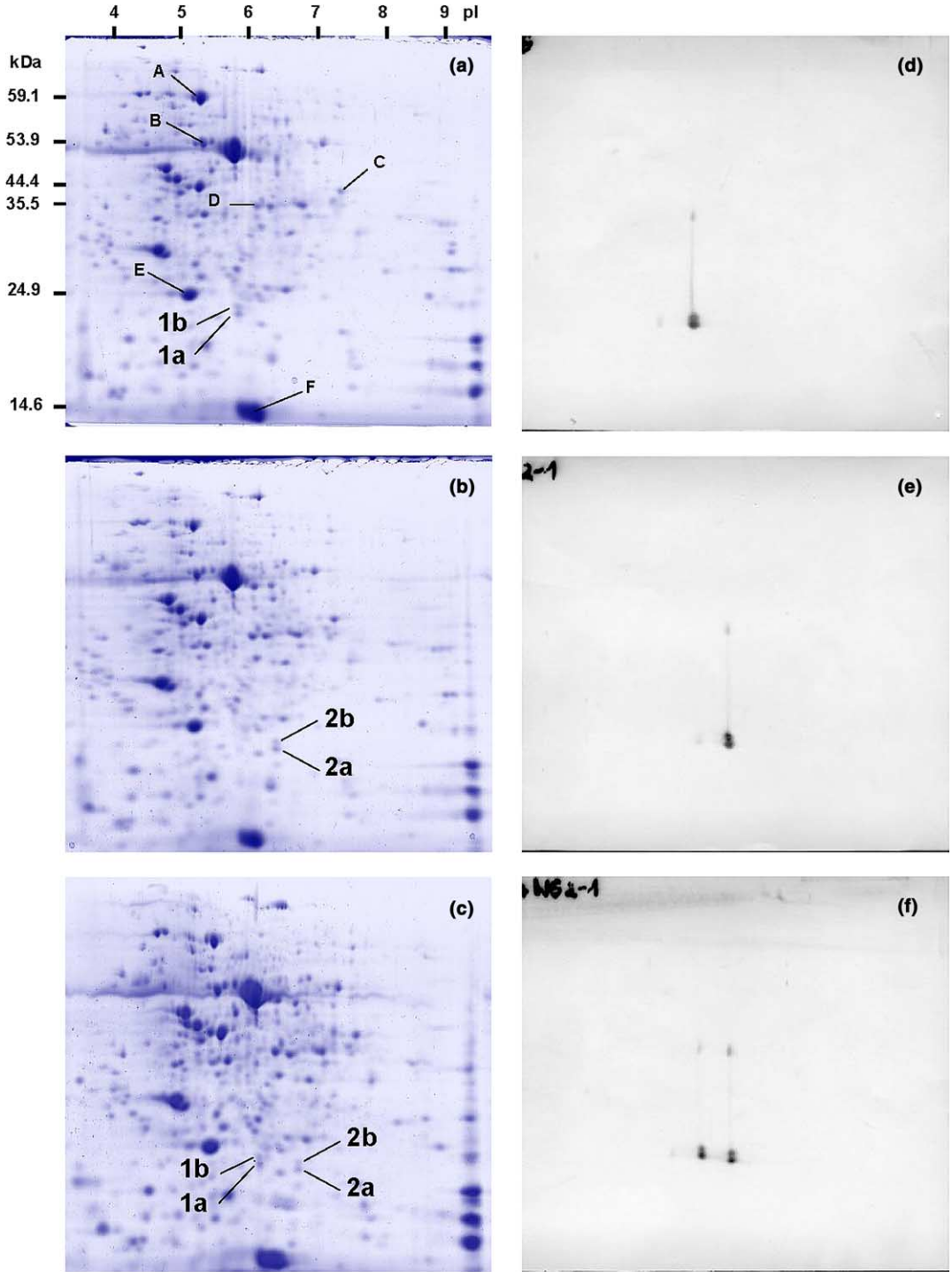

Fig. 1. Two-dimensional gel analysis of leaf proteins from *A. thaliana* ecotypes Col-0 and Ws-2. After separation of 50 μg proteins from Col-0 (a), Ws-2 (b) or a mixture of each 38 μg from Col-0 and Ws-2 (c), gels were stained by colloidal Coomassie G250. AtGER3 and its closest homologue GLP were also detected by immunoblotting using an antiserum against a peptide characteristic for both sequences (d–f). For the Western blot analysis, 5 μg proteins from Col-0 (d) and Ws-2 (e) and a mixture of each 5 μg from Col-0 and Ws-2 (f) were separated. The GLP spots are indicated by 1a,b (Col-0) and 2a,b (Ws-2). Masses of mature proteins used as internal markers are myrosinase (A, 59.0 kDa, gi|15239563), ATPase β subunit (B, 53.9 kDa, gi|7525040), aminomethyltransferase (C, 44.4 kDa, gi|15221119), malate dehydrogenase (D, 35.5 kDa, gi|15219721), photosystem II oxygen-evolving complex (E, 24.9 kDa, gi|15222166) and ribulose bisphosphate carboxylase small chain (F, 14.6 kDa, gi|13926229).

Another week signal can be seen at the same mass but about 0.5 pH unit lower as the main spot. Altogether the Western blot analysis confirmed the results obtained from 2-D protein gels but revealed the presence of minor additional spots which could not be clearly detected in Coomassie blue-stained gels.

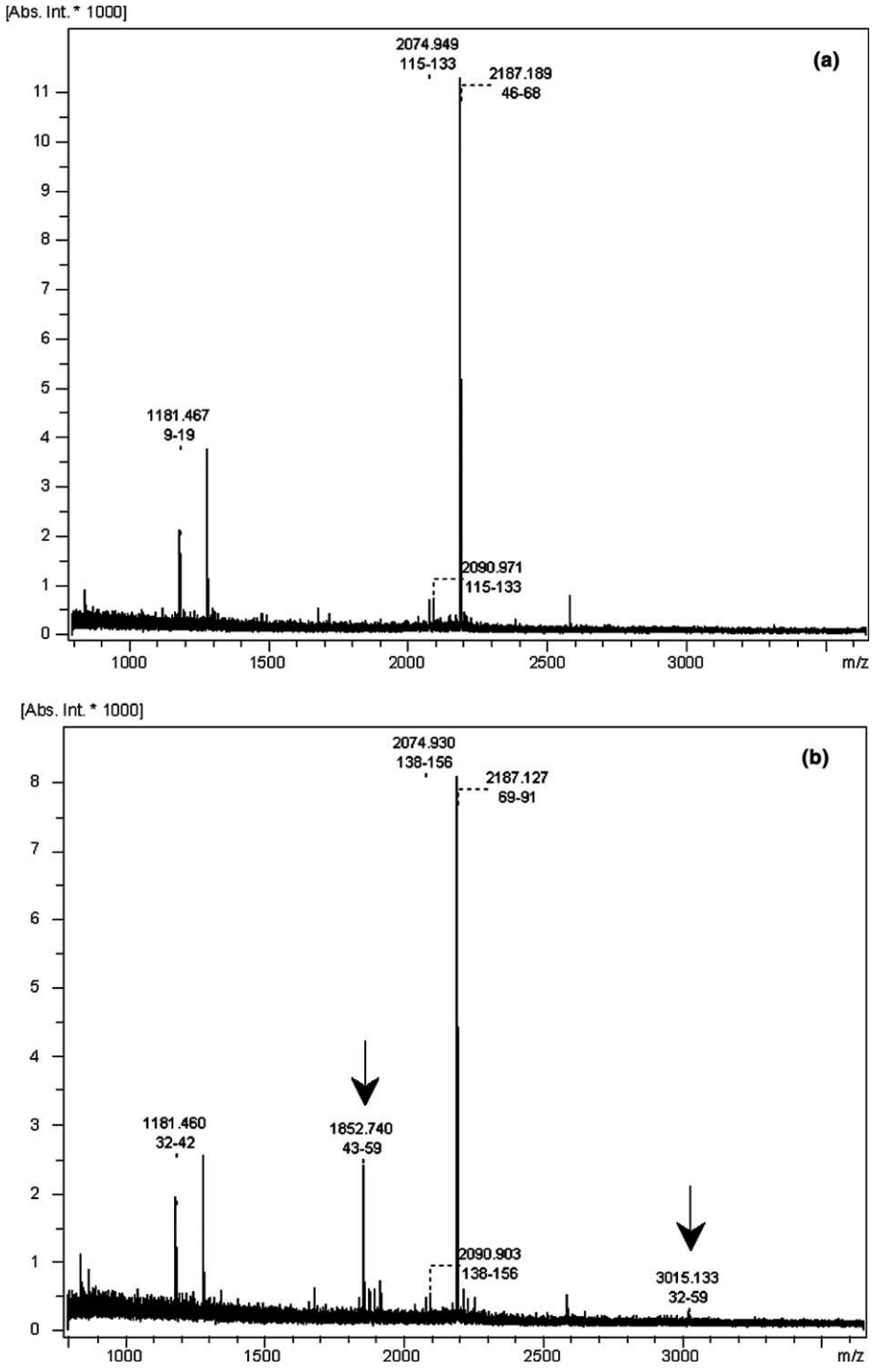

Fig. 2. Peptide mass fingerprints of GLPs from Col-0 (a) and Ws-2 (b).

2.2. Root proteins

Earlier analysis of the tissue specificity of AtGER3 expression by Northern blotting had shown no signals for root tissues (Membre et al., 1997). As the levels of transcripts and proteins do not correlate very strictly, we also performed 2-D gel electrophoresis and 2-D Western blotting experiments from root protein extracts (Fig. 4). As we were not able to harvest undamaged roots from soil grown plants, this analysis was done only using roots from plantlets grown in our hydroponic system. Remarkably we received a signal at the same position as in leaf protein patterns on immunoblots from Col-0 but not from Ws-2 (Fig. 4(b)). Careful inspection of Coomassie blue-stained gels from root proteins and comparison with protein patterns from leaves pointed to a

Table 1
Peptide masses and PSD spectra used for identification of germins from Col-0 and Ws-2

	Col-0	Ws-2	
Accession	gi|15242028	gi|1755154	
MW[a]	19250	19233	
p*I*a	5.86	6.31	
Peptides	1181[b]	1181[b]	GPQSPSGYSCK + 1 Propionamide (C)
	Absent	1852[b]	NPDQVTENDFAFTGLGK
	2075	2075	GDSMVFPQGLLHFQLNSGK
	2091	2091	GDSMVFPQGLLHFQLNSGK + 1 Oxidation (M)
	2187[b]	2187[b]	AAVTPAFAPAYAGINGLGVSLAR
	Absent	3015	GPQSPSGYSCKNPDQVTENDFAFTGLGK

[a] Mature protein.
[b] Results from PSD spectra.

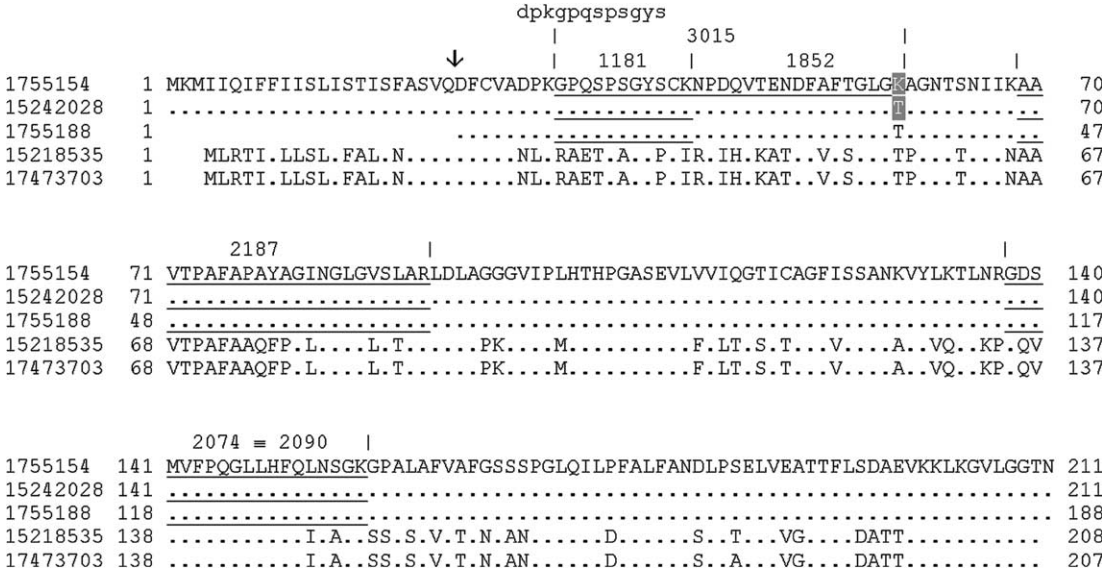

Fig. 3. Alignment of top five of 59 amino-acid sequences of GLPs from *A. thaliana*. The sequences were aligned using the BLASTP (2.2.6) program. The cleavage site for signal peptides of sequences gi|1755154 and gi|15242028 was predicted using the SignalP (2.0) program and is indicated by "↓" . Peptides contributing to the identification of GLPs by Mascot Peptide Mass Fingerprint Search are underlined and annotated by their m/z values. The amino acid exchange in position 59 of sequences gi|1755154 and gi|15242028 is shaded. Small letters denotes the peptide sequence used for raising the antibodies used in the blotting experiments.

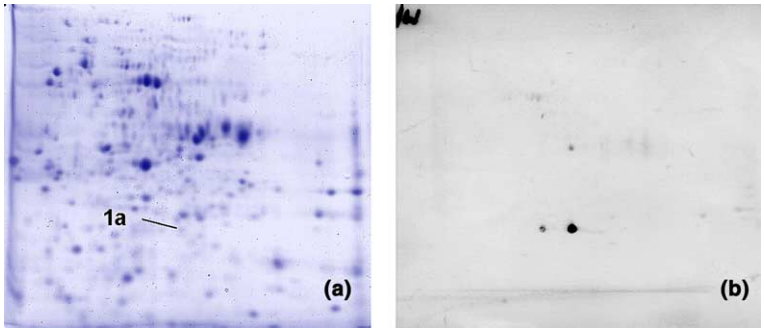

Fig. 4. Two-dimensional gel analysis of root proteins from *A. thaliana* ecotype Col-0. For Coomassie blue-staining, 50 μg of protein was applied (a). For detection of AtGER3 GLP by immunoblotting, 25 μg of protein was separated (b). The protein spots from ecotype Col-0 are indicated by 1a,b.

faint protein spot probably responsible for the signal in the Western blot and putatively representing AtGER3 (Fig. 4(a)). In contrast to the leaf extracts, the root protein pattern showed only a single spot and not the doublets. This spot is located in close proximity to a spot which we identified both from leaf and root pro-

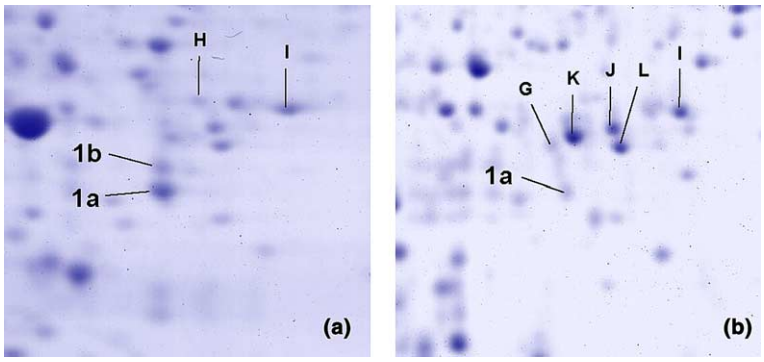

Fig. 5. Selected area of 2-D separation on medium-sized gels of each 150 μg of leaf proteins (a) and root proteins (b) from ecotype Col-0. The AtGER3 GLP protein spots are indicated by 1a,b. Other identified proteins in this part of the gels are germin-like protein GLP9 (G, gi|15233510), glutathione *S*-transferase (H, gi|2554769), glutathione *S*-transferase (I, gi|15224581), glutathione *S*-transferase 6 (J, gi|12643792), quinone reductase (K, gi|15239652) and manganese superoxide dismutase (L, gi|15228407).

teins as glutathione *S*-transferase (gi|2462929). This glutathione *S*-transferase is part of a pattern occurring both in leaf and root protein extracts. As the putative AtGER3 spot was hardly to be seen on minigels of root proteins due to its low amount and the low resolution of this gel format (Fig. 4(a)), we used medium-sized gels to separate extracts from leaves and roots (Fig. 5). Loaded with three times the amount of root proteins in comparison to the minigels, this weak spot was clearly separated from neighbouring spots and could be excised easily (Fig. 5(b)). MALDI-TOF analysis of the tryptically digested putative germin-like protein spot from roots resulted in low signal intensities corresponding to the low abundance of the protein, yet the peptide ions at $m/z = 1181$, 2075, 2091 and 2187 were clearly detected allowing the identification of the protein as AtGER3 by database searches. From Coomassie blue-stained gels, the amount of this germin in roots was estimated to be 10–20% of the content in leaves (Fig. 5). This agrees with results from Western blotting. In order to obtain comparable staining intensities of germins in the immunoblots for roots as for leaves we had to apply about five times the amount of root proteins on 2-D gels as for leaf protein extracts (data not shown).

3. Discussion

Arabidopsis has become an important resource as a model plant in many aspects of plant biology due its small genome size, its short life cycle and ease of transformation (Somerville and Koornneef, 2002). One of the important features for functional genomics is the availability of a large set of ecotypes reflecting broad natural variations (Alonso-Blanco et al., 1999; Somerville and Koornneef, 2002). The development of novel tools for the analysis of metabolites, proteins and transcripts opens up new possibilities for the functional characterization of plant resources, such as accessions from the spectrum of ecotypes. Here, we compared the protein patterns of two of the ecotypes Columbia and Wassilewskija, which are used as standard lines in many laboratories and from which a large collection of mutants and transgenic lines have been derived. One of the obvious features were pairs of proteins in Coomassie blue-stained gels of approximately the same molecular mass and being diagnostic for both lines.

To reveal the identity of the proteins, spots were excised and tryptically digested for further analysis by MALDI-TOF mass spectrometry. Both from PMF and PSD spectra, we obtained the germin sequences as the top hit after searching the NCBInr database, but only in a few data sets out of a range of parallel experiments, statistically significant scores were received. Several factors might be responsible for this limitation. First of all, some proteins only yield a low number of tryptic peptides. In the case of the two germin-like proteins, the sequences gi|15242028 and gi|1755154 should yield 14 and 15 tryptic peptides, respectively, with m/z of 500 or higher, assuming complete cleavage and including the propionamide modified cysteine containing peptides as well as methionine oxidation. Only eight masses would be expected for the protein gi|15242028 and 9 masses for gi|1755154, respectively, which fit in the range from m/z 800 to 3600 routinely used for accumulation of the PMF spectra. Experimentally, we detected only four and five, respectively, of these predicted masses also when we expanded the detection range to 500–5000 m/z. However, an additional mass (3015 m/z) was detected, which corresponds to a peptide of gi|1755154 with one missing cleavage site. Obviously not all the expected peptides were ionisable and the low number of masses was insufficient for unambiguous protein identification by peptide mass fingerprinting. In these cases, additional information on the protein is required for its unequivocal identification. In order to receive sequence-dependent data, we accumulated PSD spectra of selected peptides. Although PSD spectra often show only

incomplete ion series and the fragment masses have only low intensities, the information from such spectra can be helpful for identification of proteins (Gevaert et al., 2001). In our case, similar to the results from PMF, the PSD spectra alone resulted in GLP sequences as top hits, but without statistically significant scores. Compiling the information from PMF and PSD spectra in Mascot generic files and searching this against the database resulted in substantially higher and statistically significant scores and allowed to clearly identify the two GLPs differing in only one amino acid.

The quality of the analysis was also influenced by the preceding 2-D gel separation. The immunological detection of the two germin-like protein doublets was always successful after separation on IPG strips with a pH range from 3 to 10. After running identical amounts of leaf proteins from Col-0 and Ws-2 on pH 4–7, the germin spots from Ws-2 showed a distinctly lower immunological reaction in comparison to separations at pH 3–10. Additionally, the PMF spectra from such spots from Coomassie blue-stained gels were very poor and did not allow any identification. These spot migrated on pH 4–7 IPG strips in a position close to the cathode and damage or modifications of proteins might occur in the vicinity of the electrodes.

The Western blot analysis of the leaf and root extracts using an antiserum against a peptide present only in both identified GLPs confirmed the 2-D gel analysis, but the presence of additional faint immunoreactive spots became visible (see Figs. 2 and 4). Weak signals were consistently found corresponding to proteins with the same p*I*, but twofold higher masses. Most likely these spots represent dimers of the germin-like proteins designated 1 and 2. Under native conditions, GLPs form oligomers and the occurrence of dimeric forms in the 2-D gels can be best explained with the known resistance of germin-like proteins against denaturing agents (Vallelian-Bindschedler et al., 1998b; Wei et al., 1998). The isoforms a and b of spots 1 and 2 correspond to the doublets observed in SDS–PAGE of many GLPs and most likely represent different glycosylation patterns (Bernier and Berna, 2001). Remarkably, the isoforms of spot 2 showed a slightly higher electrophoretic mobility (Fig. 2), although the proteins differ only in one amino acid. The influence of single amino acid changes on the electrophoretic mobility in SDS–PAGE separations has occasionally been reported (de Jong et al., 1978).

Two-dimensional gel Western blot analysis also revealed the presence of immunoreactive spots in root tissue. The main signal corresponds to a single spot and not from a pair as in leaf extracts. Results from Western blot analysis using 2-D gel-based separation enabled to pinpoint a corresponding spot in Coomassie blue-stained 2-D gels and by mass spectrometry data the presence of the AtGER3 protein in root extracts could be clearly demonstrated (this paper). Previously, Northern blotting had indicated the absence of AtGER3 transcripts in roots (Membre et al., 1997). The difference with the present results might be explained by the low abundance of *AtGER3* transcript after accumulation of the protein.

In conclusion, our paper demonstrates the power of proteome analysis based on 2-D gel electrophoresis and mass spectrometry to distinguish between highly homologues proteins even differing in only one position. The comparison of protein patterns after staining of 2-D gels or after additional Western blotting contains valuable information when analysing plant genetic resources or different tissues and developmental stages. It can therefore be anticipated that proteome approaches (Cánovas et al., 2004) will significantly contribute to define the occurrence and roles of individual family members of proteins in plants.

4. Experimental

4.1. Plant material

Arabidopsis thaliana ecotypes Col-0 and Ws-2 were used in these experiments. Plants were cultivated in two different ways. Leaves and roots were harvested after three weeks from our new developed sterile hydroponic culture (Schlesier et al., 2003). For this purpose, plantlets were grown in one-quarter-strength Murashige and Skoog medium including Gamborg B5 vitamins and supplemented with 0.5% sucrose at 22 °C under a white fluorescent lamp using a light period of 12 h per day with a light intensity of 120 $\mu E/s/m^2$. Additionally leaves were harvested after seven weeks from soil culture under a light/dark regime of 12/12 h at 21/18 °C and a light intensity of 120 $\mu E/s/m^2$. Immediately after harvest the material was placed between two sheets of nylon mesh (100 μm), and residual liquid from hydroponic culture or water from washing the leaves from soil culture was removed with filter paper. After this the material was frozen in liquid nitrogen and stored at −80 °C prior to protein extraction.

4.2. Protein extraction

Plant material was ground under liquid nitrogen in a mortar. About 1 g of this fine powder was precipitated by 10 ml of 10% (w/v) TCA in acetone containing 0.07% (w/v) 2-ME according to Damerval et al. (1986). Aliquots (1.8 ml in Eppendorf tubes) of suspension were first chilled in liquid nitrogen for 15 s and then transferred in a refrigerator at −20 °C for 45 min. After 5, 10 and 15 min, the suspension was mixed. Precipitated material was collected by centrifugation. After washing two times with acetone containing 0.07% (w/v) 2-ME the precipitate was dried in a vacuum centrifuge. Proteins

were dissolved from dried precipitate using 50 μl/ mg of lysis-buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer and 20 mM DDT). Insoluble material was pelleted by centrifugation (15 min, RT, 32,000*g*) using a micro22R centrifuge (Hettich, Germany). The supernatant was additionally clarified by centrifugation through a 0.45-μm filter unit (ULTRAFREE-MC, Millipore). Protein concentrations of these samples were measured using the 2-D Quant Kit (Amersham Biosciences) and BSA as standard.

4.3. Two-dimensional gel electrophoresis

Protein was applied by rehydration to immobilized pH gradient 7 cm 3–10 IPG strips. Isoelectric focusing was performed on an IPGphor unit (Amersham Biosciences) by increasing the voltage in three steps: 60-min gradient to 500 V, 30-min gradient to 3000 V and 280-min gradient to 3000 V for a total of about 15 kV/h. After IEF, strips were equilibrated for 15 min in equilibration buffer (50 mM Tris–HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol; 2% (w/v) SDS; 20 mM DTT and 0.01% (w/v) bromphenol blue). Second dimension SDS gels (11.25%, 7 cm × 10 cm × 0.1 cm) with stacking gel (Laemmli, 1970) were run in a Mini-PROTEAN II Cell (Bio-Rad) at constant voltage (30 min 75 V and 60 min 150 V). In the same cases, we used medium-sized gels with 13-cm IPG strips and a total of 24 kV/A for the first dimension and 14 cm distance for resolution in the second dimension (Hoefer SE600 apparatus, Amersham Biosciences). After separation, gels were washed twice with water and once with 5% phosphoric acid each for 10 min. Gels were stained with colloidal Coomassie G250 solution (GelCode, PIERCE) for 1 h, washed with water for 5 min and stored in water. Molecular weights were estimated using some identified common protein spots from leaf protein extract (marked in Fig. 1(a)). Isoelectric points of proteins were estimated from their focusing position using the graph of the pH gradient for the IPG strips (Amersham Biosciences).

4.4. Immunoblotting for AtGER3

Immunoblotting was performed onto nitrocellulose membrane BA85 (Schleicher & Schuell) using the Mini Trans-Blot Cell (Bio-Rad). The primary polyclonal antibodies against germin AtGER3 were raised against peptide DPKGPQSPSGYS coupled to keyhole limpet hemacyanine (Membre et al., 2000, and references therein). The antiserum was diluted 1:10,000 for immunostaining the blots. Antibody binding was visualized using anti-rabbit IgG alkaline phosphatase conjugates (Boehringer) in a dilution of 1:1000 and alkaline phosphatase color development reagents 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride.

4.5. MALDI-TOF MS

Gel pieces of 1.5 mm in diameter were excised manually from the 1-mm thick gels and washed for 30 min at room temperature under vigorous shaking with 400 μl of 10 mM ammonium bicarbonate solution containing 50% (v/v) acetonitrile. After removing the supernatant, gel pieces were dried for 15 min in a vacuum concentrator. For digestion, 5 μl trypsin solution (Sequenzing grade modified trypsin, Promega, Madison and 10 ng/μl in 5 mM ammonium bicarbonate/5% acetonitrile) was added to each sample. After incubation for 5 h at 37 °C, the reaction was stopped by adding 1 μl of 1% TFA. For better extraction of peptides, the samples were stored overnight at 5 °C. Without further purification, 1 μl supernatant was mixed with 2 μl of matrix solution (5 mg α-cyano-4-hydroxycinnamic acid in 40% [v/v] acetone, 50% [v/v] acetonitrile, 9.9% [v/v] water and 0.1% [w/v] TFA in water). From this mix, 1 μl was deposited onto the MALDI target.

Tryptic peptides were analysed with a REFLEX III MALDI-TOF mass spectrometer (Bruker-Daltonics, Germany) in positive reflectron mode. Peptide mass fingerprint spectra were calibrated using trypsin autolysis products ($m/z = 842.51$ and 2211.10) as internal standards. Background ions from trypsin autolysis and contamination by keratins were removed from mass lists. Protein identification was performed by searching for *A. thaliana* proteins in the latest version of the non-redundant NCBI database using the Mascot search engine (Perkins et al., 1999). The following parameters were applied: monoisotopic mass accuracy, 100 ppm; missed cleavages, 1; allowed variable modifications, oxidation (Met) and propionamide (Cys). Fragmentation of selected peptides was measured using the PSD mode. The mass lists were used for MS/MS search applying the database and search engine as mentioned above.

Acknowledgements

Financial support from the Deutsche Forschungsgemeinschaft, Bonn, to H.-P.M. (Mo 479/4–1 and Mo 479/4–2) is gratefully acknowledged. We thank Dr. Patrick Schweizer, I.P.K. Gatersleben, for critical reading of the manuscript and Annegret Wolf for excellent technical assistance.

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