ligands, which requires special facilities and careful handling to avoid potential hazard. Frequently used ^{125}I-labeled ligands are also unstable and difficult to make. Additionally, radiolabeled ligands are useful for the study of receptor–ligand interaction but difficult to apply for the purification of the receptor molecule, except to trace a radioactive receptor–ligand complex during the purification process (Scheer and Ryan 2002, Yamaguchi et al. 2006).

The use of biotinylated compounds as non-radioactive ligands, especially for affinity biotinylation, potentially has several advantages for the study of putative receptor molecules. These compounds can be used without the problems associated with radiolabeled ligands and the 'biotin-tagged' receptor molecule can be traced with the commercially available horseradish peroxidase (HRP)–avidin or corresponding antibodies similar to conventional immunochemical analyses. In addition, it should be possible to purify the biotin-tagged receptor molecule by affinity chromatography by taking advantage of the high affinity of biotin for avidin. The use of biotinylated ligands for the study of plant receptors has been applied to the analysis of the BRI1–brassinosteroid interaction (Kinoshita et al. 2005) and to the purification of the fusicoccin binding protein (Korthout et al. 1994). However, there is no report discussing its use for receptor characterization from the analysis of receptor–ligand interaction to the purification and identification of the receptor molecule. In this work, we use the chitin elicitor binding protein CEBiP (Kaku et al. 2006) and an RLK for flagellin, FLS2 (Gomez-Gomez and Boller 2000, Takai et al. 2008) as models to demonstrate how biotinylated ligand can be used for a comprehensive analysis of such a receptor.

Results

Preparation of biotinylated *N*-acetylchitooctaose

A biotinyl group was introduced into *N*-acetylchitooctaose (GN8) by reductive amination with biocytin hydrazide according to the modified method used for the preparation of the aminophenylethylamine (APEA) conjugate of GN8 (**Fig. 1A**) (Shibuya et al. 1993). The synthesized derivative, biotinylated *N*-acetylchitooctaose (GN8-Bio) was purified by gel filtration and reverse phase chromatography (**Fig. 1B**). MALDI–TOF/MS analysis of the purified GN8-Bio gave a major ion peak ($m/z = 2035$) corresponding to $[GN8\text{-}Bio + Na]^+$ (data not shown). The yield of the preparation was 0.9 mg GN8-Bio from 5 mg GN8. The purified GN8-Bio showed elicitor activity comparable to that of *N*-acetylchitoheptaose (GN7), an oligosaccharide that corresponds to the intact sugar moiety of GN8-Bio, in the induction of reactive oxygen generation (**Fig. 1C**). This result indicates that the biotinylation of GN8 at the reducing end did not affect the elicitor activity, similar to the case of APEA/AzPEA (azidophenylethylamine) derivatives that were previously used for binding assay and affinity labeling (Ito et al. 1997).

Application of the biotinylated ligand for the analysis of receptor–ligand interaction

Affinity biotinylation with GN8-Bio and a chemical cross-linker, glutaraldehyde or ethylene glycol *bis*[succinimidyl succinate] (EGS), were used to identify and characterize chitin binding proteins in the membrane fractions from several plant species. Affinity biotinylation of the membrane proroteins followed by detection with an anti-biotin antibody showed the presence of a binding protein in the microsomal as well as plasma membrane preparations from suspension-cultured rice cells (**Fig. 2A**). The incorporation of GN8-Bio into the binding protein was completely inhibited with 100-fold excess of GN8, indicating the specific nature of the binding. The position of the biotinylated band matched the band of chitin elicitor binding protein CEBiP detected by anti-CEBiP antibody (**Fig. 2B**). In addition, no biotinylated protein was observed in the microsomal preparation from a *CEBiP*-RNAi line (**Fig. 2C**). These results indicate that the GN8-Bio binding protein was CEBiP (**Fig. 2B–D**). The biotinylated protein could also be detected by HRP–avidin and ECL reagent (data not shown), though we used the anti-biotin antibody in the present work because of the higher sensitivity and reproducibility. A GN8-Bio binding protein, similar in size to the chitin binding protein of carrot reported previously, was also detected in the membrane preparation from carrot cultured cells (**Fig. 2E**; Okada et al. 2002).

The binding characteristics of GN8-Bio to CEBiP were evaluated by analyzing the concentration dependency of the binding and its inhibition by various oligosaccharides. Specific binding of GN8-Bio to the microsomal membrane was dependent on the ligand concentration and saturated within the range 150–300 nM (**Fig. 3A, B**). The inhibitory potency of the *N*-acetylchitooligosaccharides for binding was dependent on the size of the oligosaccharides, which is in good agreement with previous results obtained using ^{125}I-labeled *N*-acetylchitooctaose derivative (**Fig. 3C**; Shibuya et al. 1996, Ito et al. 1997). The binding specificity also showed good agreement with the elicitor activity of these oligosaccharides on suspension-cultured rice cells for various cellular responses, such as phytoalexin biosynthesis and defense gene expression (Yamada et al. 1993, Minami et al. 1996).

Purification and identification of the biotinylated protein

The biotinylated ligand, GN8-Bio, was also used for the purification of the chitin elicitor binding protein. The binding protein was affinity biotinylated, captured on a pre-packed column of avidin beads and subsequently released by spin-down elution (**Fig. 4**). 3,3′-Dithio*bis*[sulfosuccinimidyl propionate] (DTSSP), which was shown to be similarly effective to glutaraldehyde as a cross-linker, was used for this experiment because of the cleavability of the link it creates with thiol reagents for elution from the avidin beads. The plasma membrane preparation from suspension-cultured rice cells was affinity labeled with GN8-Bio and DTSSP, and subsequently solubilized with 0.5% Triton X-100.

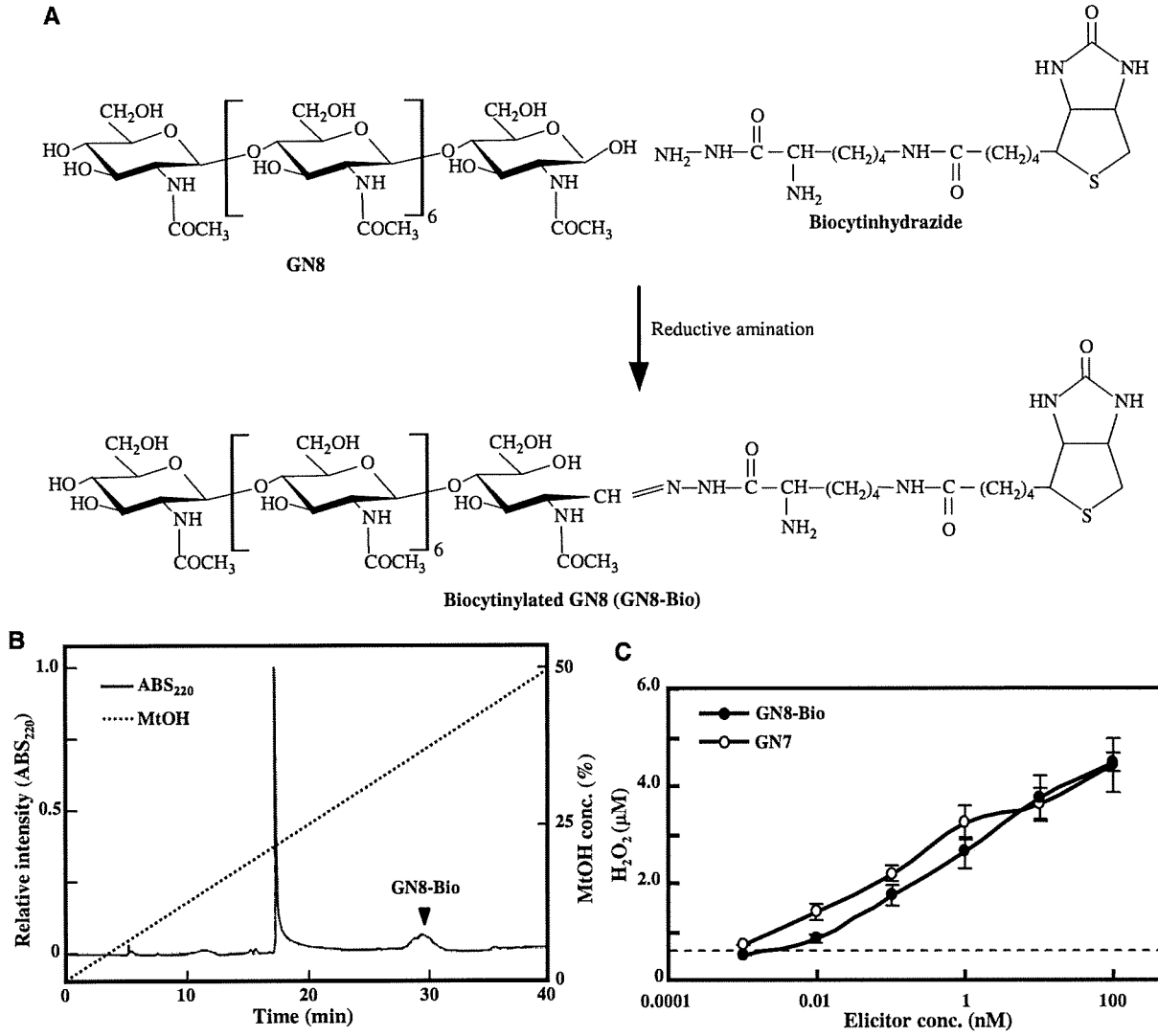

Fig. 1 Preparation and characterization of GN8-Bio. (A) Coupling of biocytin hydrazide to the reducing end of GN8. (B) Purification of GN8-Bio fraction by reverse phase chromatography. Methanol elution profile (0–50%) of GN8-Bio at a flow rate of 0.5 ml/min was monitored by UV absorption at 220 nm. (C) ROS generation induced by GN8-Bio. Reactive oxygen species (ROS) generation was measured at 120 min after treatment with GN8-Bio or GN7 at the concentrations indicated. ROS generation by GN8-Bio was compared with that by GN7 because the latter compound carries the same intact heptaose unit as GN8-Bio. The dotted line is the background level of ROS, which was measured for the rice cells treated with water for 120 min.

The solubilized fraction was applied to the avidin–agarose column and eluted with a buffer containing 0.5% dithiothreitol (DTT), which cleaves the disulfide linkage between the binding protein and biotinylated ligand and liberates the protein from the avidin beads. The eluted fraction was concentrated by ultrafiltration and analyzed by SDS–PAGE. A protein band corresponding in size to CEBiP was detected by silver staining (**Fig. 5A**). The purified protein was identified as CEBiP by its reactivity with an anti-CEBiP antibody (**Fig. 5B**). In a control experiment without GN8-Bio, the antibody did not detect any protein (**Fig. 5B**). To further confirm the identity of the protein and to assess the applicability of the method for the identification of unknown receptor molecules, the purified protein was analyzed by LC-MALDI-MS/MS after tryptic digestion. Four tryptic peptides were identified as CEBiP fragments by search algorithms based on GPS Explorer software and MASCOT (**Fig. 5C**). Coverage of the identified peptides was 19.4% of the CEBiP sequence and the probability score was 384 ($P < 0.05$), which was significantly higher than the value for the second candidate, heat shock protein, 101 (score = 25). Probability scores of other candidate proteins were very low (10–18). These results indicated that the present method can be applied to the identification of CEBiP-like molecules obtained from 100 μg of a plasma membrane preparation.

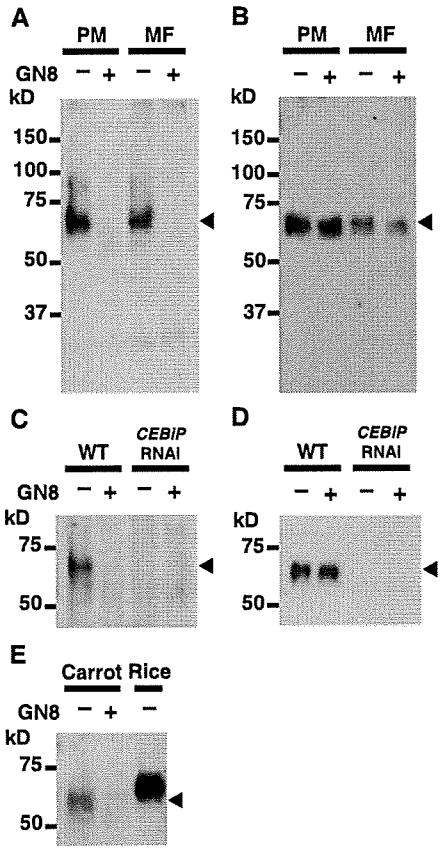

Fig. 2 Detection of high-affinity binding proteins for chitin oligosaccharides. (A) Affinity cross-linking of GN8-Bio to the plasma membrane (PM) and microsomal (MF) proteins from suspension-cultured rice cells. GN8-Bio (0.4 μM) was mixed with the plasma membrane (2 μg protein) or microsomal (10 μg protein) preparations obtained from the non-transgenic rice cells in the presence (+) or absence (−) of excess (40 μM) unlabeled GN8 as a competitor. Cross-linking with glutaraldehyde and the detection with anti-biotin antibody were performed as described in the Materials and Methods section. (B) Detection of CEBiP in the PM and MF preparations from non-transgenic rice cells using anti-CEBiP antiserum. (C) Decrease of the elicitor binding activity in the microsomal preparation from *CEBiP*-RNAi rice cells. The bands were detected with anti-biotin antibody. (D) Western blot analysis of the microsomal proteins from *CEBiP*-RNAi and non-transgenic (WT) cell lines with anti-CEBiP antiserum. (E) Detection of chitin oligosaccharide binding proteins in the microsomal proteins from cultured carrot cells. GN8-Bio (0.4 μM) was mixed with the microsomal preparations (200 μg protein) from the carrot cells or rice cells (control) and treated with glutaraldehyde. Arrowheads indicate the position of CEBiP (A–D) or CEBiP-like protein (E).

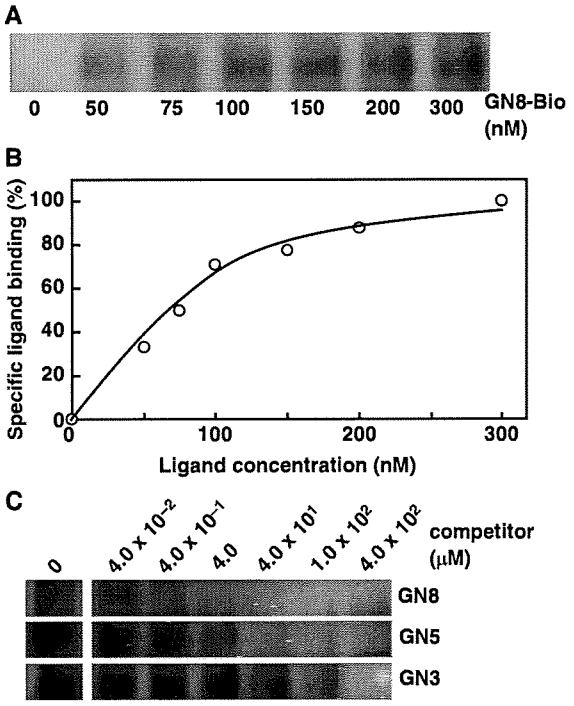

Fig. 3 Binding characteristics of CEBiP to chitin oligosaccharides. (A) GN8-Bio saturation curve. Varying amounts of GN8-Bio were mixed with the microsomal protein fraction from rice cells (10 μg protein) and cross-linked with EGS. Biotinylated proteins were detected with anti-biotin antibody after SDS–PAGE. (B) The intensities of biotinylated bands were quantitatively analyzed using Scion Image (Scion Corp., Frederic, MD, USA). Specific ligand binding to the CEBiP was calculated by subtracting the background intensity for samples incubated in the presence of unlabeled GN8. The percentage of specific ligand binding is calculated on the plateau region being 100%. (C) Inhibition of GN8-Bio binding to the receptor by unlabeled chitin oligosaccharides. Binding of microsomal proteins from rice cells (10 μg protein) to GN8-Bio (0.4 μM) in the presence of varying amounts of each oligosaccharide was monitored.

Application for the identification of RLKs

We also used this method for the identification of RLKs with ligand binding ability. FLS2 is a well-characterized RLK responsible for the perception of flg22, a fragment of bacterial flagellin that induces defense responses in Arabidopsis, tomato and rice (Gomez-Gomez and Boller 2000, Robatzek et al. 2007, Takai et al. 2008). Affinity labeling with radiolabeled flg22 showed that FLS2 binds flg22 directly. The presence of an FLS2 orthologue in rice, OsFLS2, was also reported. Thus, we tried to detect FLS2/OsFLS2 by affinity biotinylation. Biotinylated flg22 was synthesized by adding a biotin at the N-terminus of flg22, as it was previously shown that the addition of an extra tyrosine at the N-terminus did not affect the biological activity of flg22 (Meindl et al. 2000). Affinity cross-linking with the biotinylated flg22 and the microsomal membrane preparation from Arabidopsis cultured cells, did not allow the detection of any specific biotinylated bands (data not shown). However, when the membrane preparations from transgenic rice cells overexpressing OsFLS2 (OsFLS2ox) were used, detection of a biotinylated band was possible. This band was also detected with an anti-OsFLS2 antibody (**Fig. 6**). The biotinylated band was not detectable in the presence of excess amount of unlabeled flg22. In addition, this band was not detected in the membrane

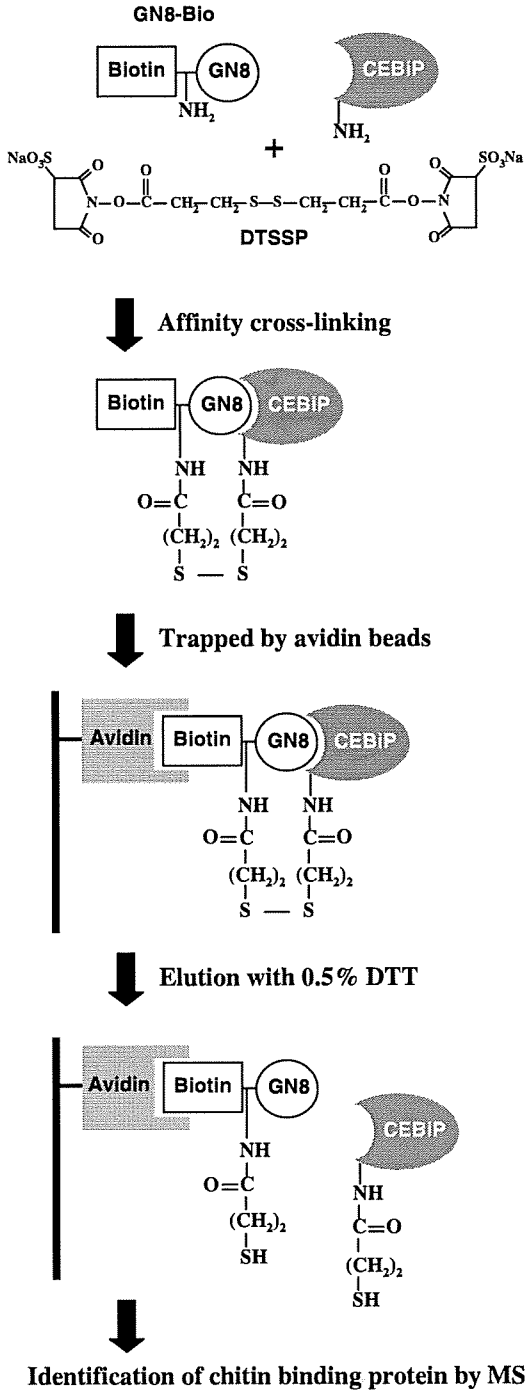

Fig. 4 Purification scheme of biotin-tagged CEBiP by trapping on avidin–agarose beads.

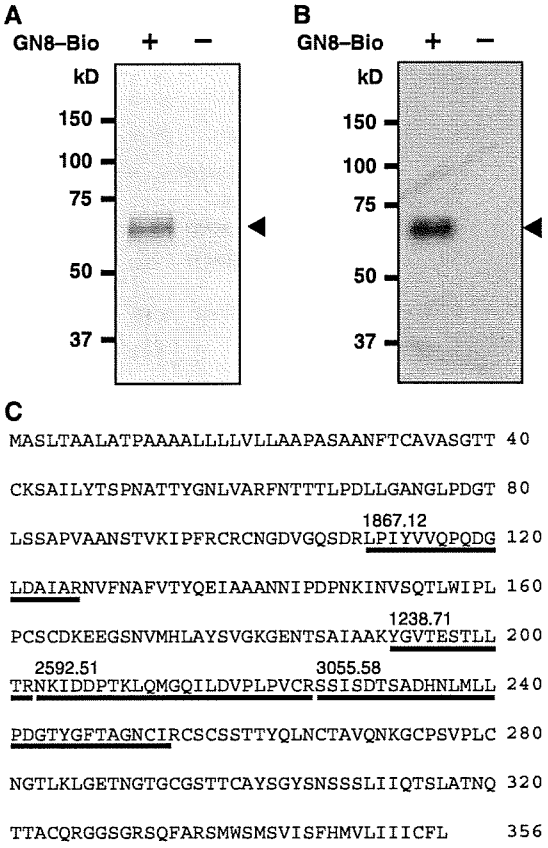

Fig. 5 Immunochemical and mass-spectral identification of CEBiP recovered from the avidin–agarose column. The plasma membrane proteins treated with DTSSP in the presence (+) or absence (−) of GN8-Bio were solubilized from the membrane and applied to an avidin–agarose cartridge column. The protein eluted from the column with a solution containing 0.5% DTT was subjected to SDS–PAGE and detected by silver staining (A) or with anti-CEBiP antiserum after blotting (B). A faint band detected by silver staining in the control sample prepared in the absence of GN8-Bio seemed a non-specific one because it was not detected with the anti-CEBiP antiserum. (C) Mass-spectral analysis of the tryptic peptides generated from the purified protein. The four tryptic peptides of CEBiP identified by LC-MALDI-MS/MS are underlined and their molecular masses are indicated.

preparation from the non-transformed rice cells that were used as a negative control because the expression level of OsFLS2 is low in rice cultured cells (Takai et al. 2008). These results indicated that affinity labeling with biotinylated ligands can be applied to the study of RLKs, if they are present in amounts suitable for detection and the corresponding ligand can be cross-linked to the RLK successfully.

Discussion

In this study, we demonstrated the usefulness of affinity cross-linking with biotinylated ligands for the characterization and identification of specific receptor molecules. A biotinylated ligand, GN8-Bio, was successfully applied to the detection of the chitin binding protein, CEBiP, in plasma/microsomal membranes from suspension-cultured rice cells (**Fig. 2**). Binding characteristics of GN8-Bio to CEBiP showed good agreement

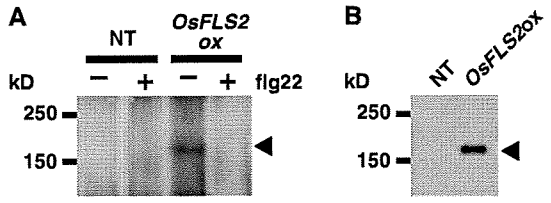

Fig. 6 Detection of OsFLS2 by affinity cross-linking of biotinylated flg22. (A) Affinity cross-linking of biotinylated flg22 to the microsomal proteins from OsFLS2-overexpressing rice cells and non-transgenic rice cells. Affinity labeling with biotinylated flg22 (10 μM) was performed with EGS in the presence (+) or absence (−) of excess flg22 (500 μM) as a competitor. The bands were detected with anti-biotin antibody. (B) Detection of OsFLS2 in microsomal preparations from OsFLS2-overexpressing rice cells and non-transgenic rice cells using anti-OsFLS2 antiserum.

with the results obtained previously by the use of radiolabeled ligands and also the elicitor activity of the chitin oligosaccharides. This indicates that biotinylation of the elicitor does not affect its binding properties with its receptor in this case. It is expected that biotinylated ligands can be used similarly for the isolation and characterization of other putative receptors or binding proteins.

The fact that biotinylation of GN8 at the reducing end did not affect its biological activity coincided well with the previous observation for the synthesis of ^{125}I-labeled GN8 derivative, where derivatization of GN8 at the reducing end also did not affect its elicitor activity (Ito et al. 1997). A reducing end sugar residue was also used for the synthesis of radiolabeled hepta-β-glucoside for the study of glucan elicitor binding proteins in leguminous plants (Cheong and Hahn 1991), indicating the possible use of this portion for the synthesis of various oligosaccharide derivatives for receptor study.

While ligand-based affinity chromatography is a very effective technique in the purification of receptor proteins, the affinity purification of receptors based on the avidin–biotin interaction has several advantages. In the case of a purification scheme based on the interaction of the biotin-tagged receptor and immobilized avidin, the receptor molecule does not need to remain active after affinity biotinylation. Also, the fact that the affinity of the avidin–biotin interaction is higher than the affinities of most ligand–receptor interactions and avidin itself is a very stable protein makes it possible to use very harsh conditions to remove non-specifically bound proteins from the affinity matrix. These situations are very different from the traditional ligand-based affinity purification of corresponding receptors, in which the solubilization conditions of the membrane fractions should be carefully determined to get active binding protein and the whole purification steps should be performed under conditions that prevent the inactivation of the receptor molecule. For example, in the previous purification of CEBiP from rice plasma membrane, approximately 70% of the binding activity was lost at the solubilization step, even with the best combination of detergent and solubilization conditions (Kaku et al. 2006). In addition, complex combination of affinity columns consisting of a main column with immobilized chitin oligosaccharide and 'dummy' columns to prevent the contamination of non-specifically adsorbing proteins were required for purification of the receptor. In contrast, in the present study the biotin-tagged proteins were solubilized with a simple detergent solution at room temperature, adsorbed to the avidin beads and eluted by splitting the cross-linkage between the ligand, GN8-Bio, and the tagged protein with DTT. This one-step purification yielded CEBiP preparation suitable for further characterization (**Figs. 4, 5**).

The question was whether the protein obtained by this purification scheme is suitable for structural characterization by mass-spectrometry, because several amino acid residues of the protein are modified by cross-linking with DTSSP, even after the cleavage of cross-linkage with DTT. However, our analysis indicated that unmodified peptides, suitable for mass-spectrometric identification, were generated by tryptic digestion of the DTSSP-treated protein. Three out of four tryptic peptides obtained from the purified CEBiP were not modified by DTSSP. The modified peptide was detected as a mixture of both modified and unmodified forms. Such DTSSP-modified peptides should also provide structural information useful for the identification of target proteins with the use of a program for the identification of the modified peptides.

We also demonstrated that the method is applicable to the analysis of RLKs such as FLS2. Affinity labeling with biotinylated flg22, which could be synthesized easily, detected the presence of OsFLS2 in the membrane preparation of transgenic cells that overexpress OsFLS2, but it was not possible to detect it in the membrane preparation of wild type *Arabidopsis thaliana*. This indicates that the approach is applicable for the analysis of this group of receptor molecules but we need to improve the sensitivity of immunological detection for the analysis of less-abundant RLKs. In the case of Arabidopsis CERK1, a RLK essential for chitin elicitor signaling, we previously reported that affinity labeling with GN8-Bio and Arabidopsis membrane preparations did not detect any band that binds specifically to chitin oligosaccharides (Miya et al. 2007). This work confirms our previous interpretation of these experiments, i.e. CERK1 might not bind chitin oligosaccharides or the amount of protein is not sufficient to be detected.

Affinity labeling with biotinylated ligands has several advantages compared with traditional binding assays; however, it should be noted that the success of this approach depends on the effectiveness of the cross-linking reactions, which is determined by the topological arrangement of the biotinylated ligand and reactive amino acid residues as well as the size and shape of the cross-linking agent. The affinity of the ligand should also be high enough to ensure specific labeling. With respect to the choice of cross-linkers, glutaraldehyde, DTSSP, DSP and EGS could be used for affinity biotinylation of CEBiP with similar efficacy.

The work described here helps in designing a strategy for the identification and characterization of the receptor for

a ligand of interest. The ligand-based receptor fishing strategy described here appears to be complementary to the recently proposed immobilized receptor-based strategy for ligand fishing (Shinohara and Matsubayashi 2007) and is expected to contribute to the study of plant receptor function in the post-genome sequencing era.

Material and Methods

SDS–PAGE and Western blotting

SDS–PAGE was carried out on 7.5% or 10% gels according to the method of Laemmli (Laemmli 1970). Western blotting was performed with an Immun-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA) and a semi-dry blotting apparatus (Bio-Rad) according to the manufacture's protocol. Detection of biotinylated proteins was performed by using a rabbit antibody against biotin (Rockland, Gilbertsville, PA, USA) as a primary antibody and a horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon International, Inc., Temecula, CA, USA) as a secondary antibody. Detection of biotinylated proteins was also performed using avidin–HRP (Pierce, Rockford, IL, USA). For the detection of CEBiP and OsFLS2, the proteins transferred to the PVDF membrane were detected with antisera against CEBiP (Kaku et al. 2006) and OsFLS2 (Takai et al. 2008), respectively, and a horseradish peroxidase-conjugated goat anti-rabbit IgG was used as secondary antibody. After treatment with the antibodies, the target proteins were detected by chemiluminescence with the Immobilon Western Detection reagents (Millipore Corporation, Billerica, MA, USA). Silver staining was performed using EzStain Silver (Atto, Tokyo, Japan) according to the manufacturer's protocol.

MALDI–TOF/MS analysis of GN8-Bio

GN8-Bio was analyzed by MALDI–TOF/MS (Voyager-DE PRO; Applied Biosystems, Foster City, CA, USA) operated with an acceleration energy of 20 kV under positive-ion detection mode. Samples for MS analysis were prepared according to the method described previously (Shinya et al. 2006) using 2,5-dihydroxybenzonic acid (2,5-DHBA, 20 μg/ml in 60% acetonitrile) as a matrix.

Synthesis of biotinylated ligand

GN8-Bio (**Fig. 1A**), the conjugate of biocytin hydrazide and *N*-acetylchitooctaose, was prepared by reductive amination as follows: 5 mg of *N*-acetylchitooctaose was dissolved in 5 ml of H_2O, 25 mg of biocytin hydrazide, and 6.25 mg of $NaCNBH_3$ were added to the mixture, which was heated at 80°C for 1 h on a heating block and and left overnight at room temperature. The reaction mixture was lyophilized and then washed several times with water by centrifugation (12 000 rpm, 5 min) until GN8-Bio became not detectable by MALDI–TOF/MS in the supernatants. The supernatant fractions containing GN8-Bio were collected and lyophilized. The lyophilized product was dissolved in water, applied to a 1.0×18.5 cm Bio Gel P-2 gel filtration column (Bio-Rad), equilibrated and eluted with water to remove low molecular weight reagents. The elution was monitored by UV absorption of *N*-acetylchitooligosaccharide at 220 nm. The product was further purified by HPLC using an Inertial ODS-3 column (4.6×250 mm; GL Sciences Inc., Tokyo, Japan). The column was eluted with a linear gradient of 0–50% methanol in 40 min at a flow rate of 0.5 ml/min. The peak fractions were analyzed by MALDI–TOF/MS. Flg22 derivative biotinylated at the N-terminal residue was synthesized at Shimadzu Biotech (Kyoto, Japan).

Affinity biotinylation

Binding experiments were performed according to the modified protocol of affinity labeling with the ^{125}I-labeled ligand (Ito et al. 1997). Briefly, plasma or microsomal membrane fraction was mixed with biotinylated ligand in the presence/absence of unlabeled ligand in the presence of binding buffer. After incubation for 1 h on ice, 1/10 volume of cross-linker (2.5% glutaraldehyde, 3.0% DTSSP or 3.0% EGS solution) was added to the mixture and left to stand for 30 min. After the reaction was stopped by the addition of 1 M Tris–HCl, proteins were concentrated by ethanol precipitation with trypsin inhibitor (50 μg) as a carrier. In some experiments, the ethanol precipitation step was omitted. The precipitate, or the solution without ethanol precipitation, was mixed with SDS–PAGE sample buffer, boiled for 5 min and directly used for SDS–PAGE. In the case of carrot cells, the membrane–GN8-Bio mixture was ultracentrifuged for 1 h (100 000 *g*) before glutaraldehyde treatment to ensure the removal of excess ligand. The detailed conditions of each affinity biotinylation experiment are described in **Supplementary data**.

Purification of the biotinylated protein by avidin bead trapping

Plasma membrane (100 μg protein) from the cultured rice cells was mixed with GN8-Bio (7.2 μg) and cross-linked by adding 1/10 volume of 3% DTSSP for 30 min. The membrane proteins were solubilized by treatment with 0.5% Triton X-100 for 30 min at room temperature. The solubilized proteins were applied to a Streptavidin HP SpinTrap column (GE Healthcare UK Ltd, Little Chalfont, UK) in the presence of 0.4 M guanidine–HCl and allowed to stand for 120 min at room temperature. After extensive washing with 0.5% Triton X-100/0.4 M guanidine–HCl, the gel was mixed with 0.5% Triton X-100 containing 0.5% DTT and 0.4 M guanidine–HCl and heated at 40°C for 1 h. The eluate was desalted and concentrated with an ultrafiltration apparatus (Microcon, Millipore Corporation) of mol. wt 10 000 cut-off.

Identification of the chitin binding protein by LC-MALDI-MS/MS analysis

The affinity-purified proteins were subjected to SDS–PAGE (10% polyacrylamide gels) and stained with EzStain Silver (Atto).

The major protein band (~65 kDa) was manually cut off and subjected to in-gel trypsin digestion (Promega, Madison, WI, USA). The tryptic peptides were then analyzed by LC-MALDI-MS/MS. The peptides were analyzed by a DiNa Direct Nano-flow LC system equipped with a MALDI-plate spotter (DiNa MaP; KYA Tech, Japan), a reverse-phase (RP) trap column (HiQ Sil C18-3, 0.8 mm id×3 mm) and an analytical column (HiQ Sil C18-3, 0.15 mm id×50 mm), which was operated with a gradient elution of acetonitrile (5–80% in water). The RP column effluent was mixed with the MALDI matrix solution (4 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 70% acetonitrile containing 0.1% TFA and 80 μg/ml dibasic ammonium citrate) flowing at a rate of 1400 nl/min at the outlet, spotted directly onto the AB 4800 MALDI plates, and analyzed by MALDI-MS/MS. Mass spectra were acquired using an AB 4800 Proteomics Analyzer (Applied Biosystems). All MS/MS spectra were combined and a single database search was performed against rice protein sequences in the Swiss-Prot database using GPS Explorer software (Applied Biosystems) and MASCOT as a search algorithm. Search parameters included modifications by DSP at K residues for DTSSP modification, oxidation of M, propionamide of C and one missed cleavage; the maximum peptide rank was set at 2.

Details of plant materials, chemicals, membrane preparation and analysis of reactive oxygen species are described in **Supplementary Data**.

Supplementary Data

Supplementary Data are available at PCP Online.

Acknowledgments

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References

Cheong, J.J. and Hahn, M.G. (1991) A specific, high-affinity binding site for the hepta-β-glucoside elicitor exists in soybean membranes. *Plant Cell* 3: 137–147.

Gomez-Gomez, L. and Boller, T. (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Mol. Cell* 5: 1003–1011.

Ito, Y., Kaku, H. and Shibuya, N. (1997) Identification of a high-affinity binding protein for *N*-acetylchitooligosaccharide elicitor in the plasma membrane of suspension-cultured rice cells by affinity labeling. *Plant J.* 12: 347–356.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., et al. (2006) Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl Acad. Sci. USA* 103: 11086–11091.

Kinoshita, T., Cano-Delgado, A., Seto, H., Hiranuma, S., Fujioka, S., Yoshida, S., et al. (2005) Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. *Nature* 433: 167–171.

Korthout, H., Van Der Hoeven, P., Wagner, M.J., Van Hunnik, E. and De Boer, A.H. (1994) Purification of the fusicoccin-binding protein from oat root plasma membrane by affinity chromatography with biotinylated fusicoccin. *Plant Physiol.* 105: 1281–1288.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Matsubayashi, Y., Ogawa, M., Morita, A. and Sakagami, Y. (2002) An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine. *Science* 296: 1470–1472.

Meindl, T., Boller, T. and Felix, G. (2000) The bacterial elicitor flagellin activates its receptor in tomato cells according to the address-message concept. *Plant Cell* 12: 1783–1794.

Minami, E., Kuchitsu, K., He, D.Y., Kouchi, H., Midoh, N., Ohtsuki, Y., et al. (1996) Two novel genes rapidly and transiently activated in suspension-cultured rice cells by treatment with *N*-acetylchitoheptaose, a biotic elicitor for phytoalexin production. *Plant Cell Physiol.* 37: 563–567.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., et al. (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *Proc. Natl Acad. Sci. USA* 104: 19613–19618.

Okada, M., Matsumura, M., Ito, Y. and Shibuya, N. (2002) High-affinity binding proteins for *N*-acetylchitooligosaccharide elicitor in the plasma membranes from wheat, barley and carrot cells: conserved presence and correlation with the responsiveness to the elicitor. *Plant Cell Physiol.* 43: 505–512.

Robatzek, S., Bittel, P., Chinchilla, D., Kochner, P., Felix, G., Shiu, S.H., et al. (2007) Molecular identification and characterization of the tomato flagellin receptor LeFLS2, an orthologue of Arabidopsis FLS2 exhibiting characteristically different perception specificities. *Plant Mol. Biol.* 64: 539–547.

Scheer, J.M. and Ryan, C.A., Jr (2002) The systemin receptor SR160 from *Lycopersicon peruvianum* is a member of the LRR receptor kinase family. *Proc. Natl Acad. Sci. USA* 99: 9585–9590.

Shibuya, N., Ebisu, N., Kamada, Y., Kaku, H., Cohn, J. and Ito, Y. (1996) Localization and binding characteristics of a high-affinity binding site for *N*-acetylchitooligosaccharide elicitor in the plasma membrane from suspension-cultured rice cells suggest a role as a receptor for the elicitor signal at the cell surface. *Plant Cell Physiol.* 37: 894–898.

Shibuya, N., Kaku, H., Kuchitsu, K. and Maliarik, M.J. (1993) Identification of a novel high-affinity binding-site for *N*-acetylchitooligosaccharide elicitor in the membrane-fraction from suspension-cultured rice cells. *FEBS Lett.* 329: 75–78.

Shinohara, H. and Matsubayashi, Y. (2007) Functional immobilization of plant receptor-like kinase onto microbeads towards receptor array construction and receptor-based ligand fishing. *Plant J.* 52: 175–184.

Shinya, T., Menard, R., Kozone, I., Matsuoka, H., Shibuya, N., Kauffmann, S., et al. (2006) Novel β-1,3-, 1,6-oligoglucan elicitor

from *Alternaria alternata* 102 for defense responses in tobacco. *FEBS J.* 273: 2421–2431.

Shiu, S.H., Karlowski, W.M., Pan, R., Tzeng, Y.H., Mayer, K.F. and Li, W.H. (2004) Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. *Plant Cell* 16: 1220–1234.

Takai, R., Isogai, A., Takayama, S. and Che, F.S. (2008) Analysis of flagellin perception mediated by flg22 receptor OsFLS2 in rice. *Mol. Plant Microbe Interact.* 21: 1635–1642.

Yamada, A., Shibuya, N., Kodama, O. and Akatsuka, T. (1993) Induction of phytoalexin formation in suspension-cultured rice cells by *N*-acetylchitooligosaccharides. *Biosci. Biotechnol. Biochem.* 57: 405–409.

Yamaguchi, Y., Pearce, G. and Ryan, C.A. (2006) The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in Arabidopsis, is functional in transgenic tobacco cells. *Proc. Natl Acad. Sci. USA* 103: 10104–10109.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T., et al. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. *Cell* 125: 749–760.

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Research Article

Transgenic peas expressing an α-amylase inhibitor gene from beans show altered expression and modification of endogenous proteins

Differential in-gel electrophoresis showed contrasting effects of the transgenic expression of an α-amylase inhibitor from beans on the proteomes of two pea cultivars. One cultivar showed minor changes relative to its non-transgenic parent with only one protein changing by more than about twofold. Changes in the abundance of certain endogenous proteins in the other cultivar were of greater number and magnitude with some endogenous proteins undetected while some new protein spots appeared in the transgenic proteome. The sets of proteins with altered expression were generally different between the two cultivars. Some of the proteins that were differentially expressed were identified by MS. Most were seed storage globulins, which are sited together with the transgenic product. Some of the changes may be due to alterations in expression levels but there were also changes due to post-translational processing.

Keywords:
α-Amylase inhibitor / Bean / Pea weevils / Proteome / Transgenic pea
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1 Introduction

Inhibitors of digestive enzymes are an important class of naturally occurring proteins known to have insecticidal properties. Transgenic peas *Pisum sativum*, expressing the α-amylase inhibitor (α-AI)-1 gene from the common bean, *Phaseolus vulgaris*, were developed using seed-specific promoters [1]. Field evaluations of some of these lines in Australia have shown that α-AI is highly effective in protecting field peas from the pea weevil (*Bruchus pisorum*), an insect that causes significant economic loss [2].

It is sometimes assumed that the expression of a transgene would have negligible effects on the expression of endogenous genes. However, examples from canola, potato, rice and other plants have shown various unanticipated effects. For example, the expression of the soybean glycinin gene was associated with a 50% increase in the vitamin B6 content of rice grain and a 16–88% increase in the glycoalkaloid content of potato tubers [3, 4]. The extent of other changes in the transgenic rice remains unclear and it is also unclear whether the changes were due directly to the expression of the glycinin gene. In potato, however, the increase in alkaloid content also occurred in controls transformed with an empty vector so that the changes do not appear to be due to the expression of glycinin [3].

Proteomic analysis has become an important tool for quantifying and identifying proteins that are differentially expressed between genetically modified (GM) organisms and their non-genetically modified parents. Corpillo *et al.* [5] compared protein expression in two types of tomato plants containing a virus resistance trait introduced by genetic engineering and did not observe significant differences, either qualitative or quantitative between GM and non-GM plants. Since then, proteomic approaches have been utilised to assess unintended effects of transgenes in crops such as rice [6] and potato [7]. Furthermore, we previously found structural differences between the α-AI natively expressed in the bean and transgenically expressed in the pea [8]. These differences were explained entirely by minor variations of glycosylation and removal of C-terminal amino acid residues.

In this study, differential in-gel electrophoresis (DIGE) was used to compare the proteomes of two non-transgenic pea cultivars (Excel and Laura) with corresponding transgenic lines expressing the α-AI gene. For Excel we found only minor differences. The Laura lines showed more differences of greater magnitude, including quantitative differences and differences in post-translational modification.

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Abbreviations: **α-AI**, α-amylase inhibitor; **DIGE**, differential in-gel electrophoresis; **GM**, genetically modified

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2 Materials and methods

2.1 Plant materials

The construction of the chimeric gene encoding the α-AI was described in detail elsewhere [8]. The *Agrobacterium*-mediated transformation and regeneration procedure were used to introduce the α-AI gene into peas (*P. sativum*) cultivars Excel and Laura and single-copy transgenic lines have been used. The transgenic Excel line was reported elsewhere [8]. Plants containing a homozygous insert were selected by Southern analysis. Parental non-transgenic lines were used for comparison. Plants were grown under glasshouse conditions with natural light (day/night temperature of 24/12°C). Seeds were collected from the plants at maturity as described earlier [2, 8]. Two DIGE experiments were performed, one for each cultivar, to compare transgenic peas with non-transgenic peas. The transgenic and non-transgenic plants of the same cultivar were grown concurrently. There was fourfold biological replication and each biological replicate consisted of seeds pooled from four plants grown in a single pot.

2.2 Sample cleaning and protein extraction

Seed protein samples were prepared as described previously [9]. Portions of fine seed powder (400 mg) were taken into pre-chilled Falcon tubes (13 × 51 mm) with pre-chilled glass beads (about 200 mg, 0.04 mm, Grade-20, HLS Scientific, Sydney, Australia). Freshly prepared cold 10% TCA in acetone containing 0.07% DTT (5 mL) was added. After a brief vortexing, tubes were placed on dry ice and sonicated (MSE 100 probe sonicator, Thomas Optical and Scientific, Sydney, Australia) in six bursts of 10 s each with 60 s intervals. Solutions were then placed at −20°C for 1 h, centrifuged (30 000*g*, 15 min, 4°C), and supernatants were discarded. The pellets were crushed using a clean and pre-chilled glass rod and resuspended in another 5 mL of cold 10% TCA in acetone containing 0.07% DTT. Precipitates, after being treated two times with TCA/acetone, were washed three times with cold acetone containing 0.07% DTT followed by centrifugation. The final pellets were freeze-dried for 10 min in Flexi-Dry μP™ (FTS Systems, Stone Ridge, NY, USA). Protein concentrations were determined by the Bio-Rad (Bradford) Protein Assay Kit, and the final yields were about 40 mg of protein from 400 mg of dry seed powder.

2.3 2-D fluorescence DIGE

The concentrations of four biological replicate samples of transgenic or non-transgenic pea protein were determined using the Bio-Rad (Bradford) Protein Assay Kit, and adjusted to 5 μg/μL by adding an extraction buffer (30 mM Tris/HCL, pH 8.5, 2 M thiourea, 7 M urea, 4% w/v CHAPS). Fifty microgram (10 μL) of protein from each of the samples was separately labelled with 400 pmol of spectrally resolvable fluorescent dyes prior to electrophoresis, two replicates with Cy3, two with Cy5 for each sample type (DIGE labelling kit, GE Bioscience). An equal pool of aliquots from the four transgenic and the four non-transgenic samples was labelled with Cy2 dye for use as an internal standard on each gel. The labelling reactions were incubated in the dark on ice for 30 min, followed by addition of 2 μL of 10 mM lysine with further incubation on ice for 20 min. Four mixtures of samples were made, each comprising an aliquot of the Cy2-labelled internal standard (50 μg), a transgenic sample with Cy3 or Cy5 and a non-transgenic sample with Cy5 or Cy3, respectively, and added to 400 μL of rehydration buffer (2 M thiourea, 7 M urea, 4% w/v CHAPS, 2 mg/mL DTT, 1% v/v IPG 3–10 buffer (GE Bioscience)). Each sample was then applied to a 24 cm IPG strip (pH 3–10, GE Bioscience) with rehydration loading in an IPGphor apparatus (GE Bioscience) for 12 h, then focussing for 1 h with the voltage increasing from 500 to 1000 V, increasing to 6000 V for 1 h, then held at 6000 V for a total of about 98 KVh. The four strips were equilibrated first with 30% v/v glycerol, 6 M urea, 50 mM Tris/HCl, pH 8.8, 2% w/v SDS and 1% w/v DTT for 10–15 min, then with 4.5% w/v iodoacetamide and a trace of bromophenol blue in place of DTT for a further 10–15 min. Strips were applied to SDS–polyacrylamide gels (12%T/0.45%C resolving, 5%T/2.7%C stacker) in an Ettan DALT apparatus and developed overnight with 1–2 W/gel. Protein spots from the internal standard and the two samples on each gel were imaged using the appropriate fluorescence settings on a Typhoon scanner (GE Bioscience). Following fluorescence imaging the gels were stained with colloidal Coomassie G250 to enable manual spot picking.

To detect possible phosphoproteins, 2D-PAGE was performed as above except that the proteins were not labelled with Cy-dyes and two gels were prepared, one with 400 μg of protein from one of the non-transgenic Laura samples and the other with 400 μg from one of the transgenic Laura samples, followed by staining with the Pro-Q Diamond Phosphoprotein Gel Stain (Molecular Probes) reagent according to the manufacturer's instructions. Conclusions for phosphoproteins were based on two biological replicates.

2.4 Image analysis

Following scanning, the 2-DE gel patterns were analysed using Decyder 5.2 software (GE Bioscience). Spot variation was considered significant if the spots appeared in all four gels and showed an increase or decrease of at least 50% and a p-value <0.005.

2.5 Identification of proteins by MS

Individual spots were excised from one of the 2D-PAGE gels from the DIGE experiment with the Laura cultivar. Spots

were washed twice by shaking for 15 min with 200 μL of acetonitrile/water at ratio of 1:1, and then with acetonitrile followed by evaporating to complete dryness. Each spot was rehydrated in 12.5 ng/μL of trypsin (sequencing grade, Boehringer Mannheim, Germany) with 100 mM ammonium bicarbonate (20 μL) containing 5 mM $CaCl_2$. After 20 min, excess trypsin solution was removed, 50 mM ammonium bicarbonate (20 μL) was added, and the incubation continued for 15 h at 37°C. The peptides released from the gel by trypsin digestion were desalted with a pre-column, LC Packing Pepmap (330 μm id × 1 mm) then separated on an analytical column, LC Packing Pepmap (75 μm id × 150 mm). Peptides were eluted by a gradient of increasing acetonitrile concentration in water with 0.1% v/v formic acid directly into an ESI Q-TOF mass spectrometer (Micromass, UK) under the following analytical conditions: cycle time (s), 2.10; scan duration (s), 2.00; retention window, 0.00–70.00; ionisation mode, ES^+; mass range, 400–1800. Eight parent masses were selected for MS/MS analyses with collision energy of 20–30 eV. Data acquisition was performed through MassLynx and BioLynx (Micromass) then matched against entries in the NCBInr database through the Mascot web interface.

3 Results

3.1 Differentially expressed proteins

One of the proteins consistently present in the transgenic samples and absent from the non-transgenic samples was, not surprisingly, the product of the transgene. The position of spot 39 (Fig. 1) on the 2-DE gel and the tryptic peptides recovered are consistent with beta chain of the mature α-AI protein (~17 kDa) and its identity was also confirmed by Western blotting from one of the gels (data not shown). The

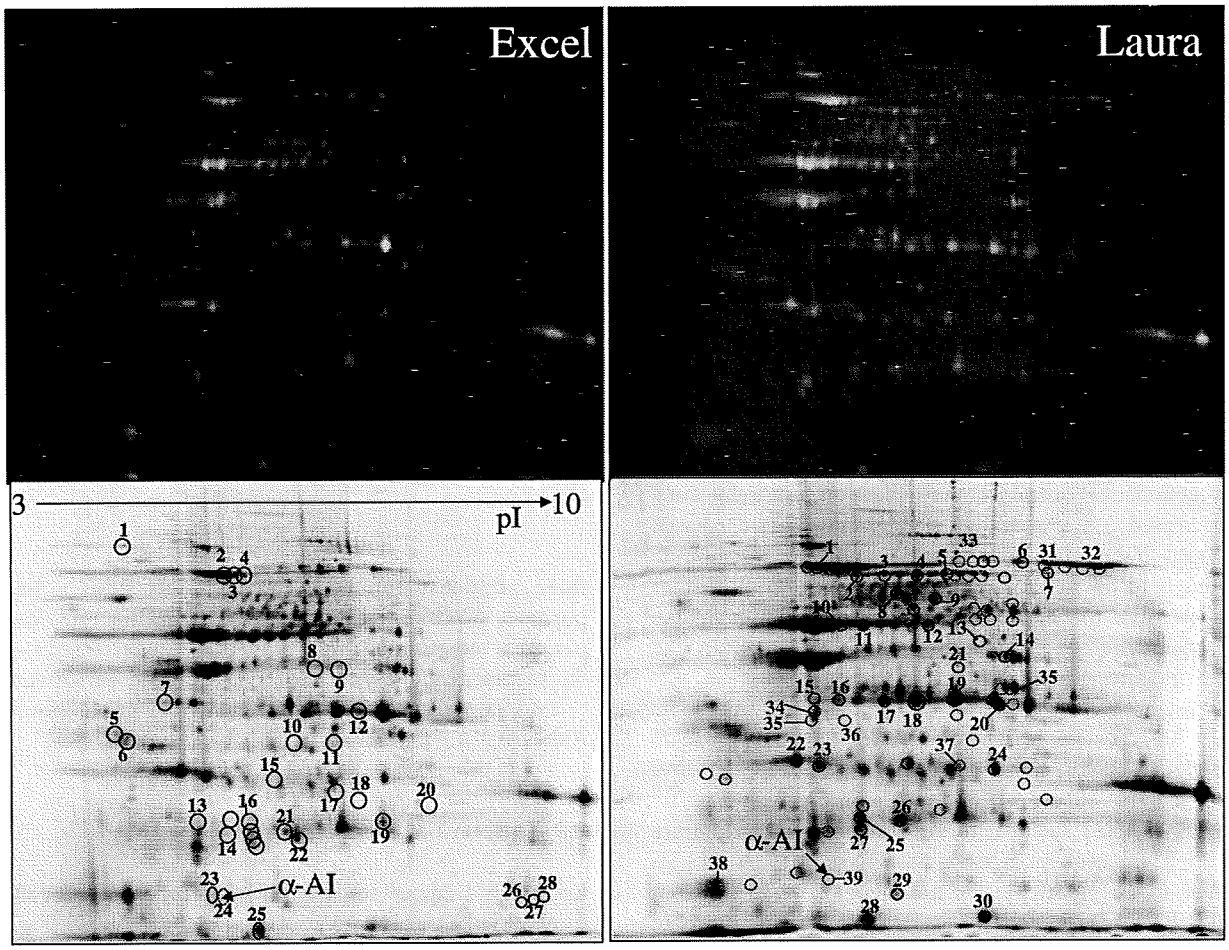

Figure 1. Differential 2-D display of pea seed proteins. Upper panel: Non-transgenic seed proteins were labelled with Cy5 (red) and transgenic with Cy3 (green). Yellow spots indicate no change in spot abundance, whereas a green or red spot indicates a change in spot abundance. The gels shown are each one from a set of four from an experiment performed with the Laura or Excel cultivar. Lower panel: Coomassie-stained gels corresponding to the analytical gels in the upper panel. Protein spots showing significant quantitative changes on the analytical gels are numbered and listed in Table 1 (black circles). Spots present in the transgenic and absent from the non-transgenic are circled with green and the converse with red, to correspond with the DIGE image. The proteins numbered with blue (bottom right block) were identified with genes by MS and are listed in Table 2.

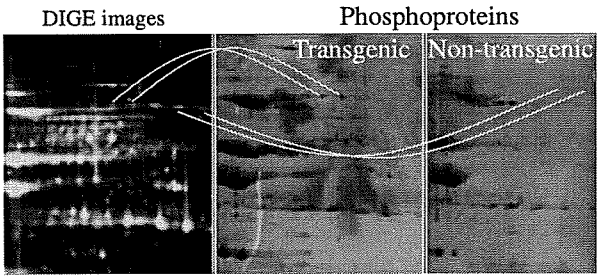

Figure 2. Staining with Pro-Q Diamond Phosphoprotein stain (Molecular Probes). The left-most panel shows a portion of one gel from the set of four from the DIGE experiment performed with pea seed proteins from the Laura cultivar. On this particular gel the labelling of the transgenic and non-trangenic samples was reversed relative to the example used for Fig. 1; here, transgenic is red, non-transgenic is green. The middle and right panels show corresponding transgenic and non-transgenic materials stained with the Pro-Q Diamond reagent.

alpha chain of the mature protein would be too small (~11.3 kDa) to be resolved on these gels. α-AI proteins account for about 4% of total protein based on purification yields [8].

We resolved approximately 600 proteins with molecular masses ranging from 15 to 100 kDa and p*I*s between 3 and 10 (Fig. 1). For the particular gels shown in Fig. 1 the transgenic samples were labelled with Cy3 (green) and the non-transgenic samples with Cy5 (red) but the reverse applies for Fig. 2. In either case yellow spots indicate proteins that do not differ between the samples. Changes between transgenic and non-transgenic samples can be seen for both cultivars but it is obvious from the relative abundance of red or green spots that more dramatic changes occurred in the Laura cultivar than the Excel cultivar.

In Excel, 28 spots in the transgenic line changed their abundance compared with the non-transgenic line, 15 increased (up to 4.2-fold) whereas 13 spots decreased (up to 2.1-fold) (Table 1). In Laura, 19 spots increased (up to fivefold) and 11 decreased (up to 3.6-fold) but a further 19 spots from the non-transgenic line disappeared in the transgenic line whereas 17 new spots appeared in the transgenic line.

3.2 Identification of differentially expressed proteins

The blue-labelled spots in the lower panel of Fig. 1 were identified with sequences in the NCBInr database by MS. In addition to the product of the transgene, 17 spots showing significant changes in the Laura cultivar were identified as products of eight endogenous genes. Five of these proteins belong to the seed storage protein class. Two of the storage proteins, convicilin and albumin 2, appeared (or increased) in certain positions on the 2-D gels while disappearing (or decreasing) from other positions suggesting differences of the post-translational modification of these proteins. A third storage protein, vicilin, decreased by about 2.4-fold from three positions and disappeared entirely from a fourth position, while a fourth storage protein, legumin, disappeared at one position and a pea lectin also decreased (−3.6-fold). Superoxide dismutase (+3.7-fold) and peroxiredoxin (−2.6-fold) are involved in protection from oxidative stress. Aldolase (−1.6-fold) is an enzyme of glycolysis.

Table 1. Quantitative protein changes in transgenic (t) pea seeds expressing a bean α-AI gene relative to non-transgenic (nt) peas. See Fig. 1 for spot locations on 2D-PAGE

Pea cv. Excel			Pea cv. Laura		
Spot no.	Av. ratio(t/nt)	*P* value	Spot no.	Av. ratio(t/nt)	*P* value
1.	+1.9	0.0024	1	+2.6	3.5e−010
2.	−1.5	3.4e−006	2	−1.6	4.8e−007
3.	−1.5	1.4e−006	3	+1.8	3.1e−006
4.	−1.5	6.1e−007	4	−2.4	7.6e−005
5	−1.5	2.6e−005	5	−2.2	1.1e−005
6	−1.6	5.3e−006	6	−2.6	1.5e−005
7	−1.6	2.9e−007	7	−1.7	0.00030
8	−1.5	0.00066	8	−1.8	5.2e−008
9	−2.1	9.4e005	9	+1.7	2.6e−007
10	+1.6	3.5e−006	10	+1.9	1.3e−006
11	+1.7	6.5e−006	11	+1.9	8.0e−006
12	+1.5	0.0029	12	+2.9	5.6e−006
13	+1.7	6.4e−005	13	−3.5	4.8e−007
14	+2.2	4.2e−006	14	−1.6	5.6e−007
15	+1.6	0.00026	15	−2.1	3.6e−006
16	+1.7	9.8e−007	16	−1.9	1.00036
17	−1.8	2.1e−005	17	−1.7	1.7e−007
18	−1.9	2.5e−005	18	−2.5	0.00004
19	−1.8	2.1e−005	19	−2.4	6.7e−009
20	−1.5	0.0032	20	+2.1	6.6e−008
21	−1.7	9.3e−006	21	+1.6	0.00047
22	+2.1	2.3e−006	22	+3.1	9.8e−010
23	+4.2	9.3e−006	23	+5.0	5.2e−011
25	+1.6	1.3e−007	24	−2.6	1.7e−009
26	+2.3	3.2e−006	25	−2.4	7.1e−009
27	+2.3	9.0e−005	26	−3.4	5.5e−008
28	+2.4	0.00055	27	−3.6	3.7e−008
			28	−1.8	1.1e−006
			29	+3.7	1.8e−006
			30	−2.3	3.3e−007

3.3 Differential modification of proteins

Convicilin disappeared (spot 32) and reappeared (spot 33) in transgenic Laura seeds, a shift of p*I* from 6.2 to 5.5 (Fig. 1). Staining with the ProQ Diamond reagent suggests that convicilin may have been phosphorylated at the lower p*I* in the Laura transgenic pea seed but not at the higher p*I* in the corresponding non-transgenic (Fig. 2). Interestingly, the corresponding region of the gels for the Excel cultivar (Fig. 1) shows a train of spots with no difference between transgenic and non-transgenic.

In addition to the identified proteins some red/green pairs of vertically aligned spots in Figs. 1 and 2 suggest that other modifications might alter size but not p*I*.

4 Discussion

Our analyses show that two lines of single-copy α-AI transgenic peas have altered protein profiles when compared with their non-transgenic parents (Fig. 1 and Table 1). In the Excel cultivar, the changes in protein abundance were slight and only one protein exceeded a change of 2.4-fold. In contrast, in the Laura cultivar, more proteins changed by three- to fivefold and some proteins disappeared while other proteins appeared in the protein display (Fig. 1 and Tables 1 and 2). Alterations of the protein profiles in the transgenic representatives of the two cultivars shared little in common. Also, it is noteworthy that the non-transgenic representatives of the two cultivars showed considerable obvious differences though these were not analysed (Fig. 1).

We then identified some of the proteins differentially expressed between transgenic and non-transgenic line seeds from the Laura cultivar. Over half of the identified proteins are seed storage proteins, vicilin, convicilin, legumin, a lectin and albumin 2. Albumin 2 shows limited sequence similarity to the superfamily of seed proteins that includes protease inhibitors, amylase inhibitors and a diverse range of other low-molecular-weight albumins [10]. It is not obvious how the presence of the bean α-AI would have altered the post-translational modification of some of these and reduced the expression of others but it is noteworthy that these storage proteins, in common with α-AI, are processed through the endoplasmic reticulum and Golgi before being deposited in storage vacuoles [11]. It is possible that α-AI competes with these endogenous proteins for resources such as transport and processing enzymes or limiting amino acids and glycans [6] and the expression of these seed storage proteins is certainly very plastic in response to other perturbations such as limiting sulphur or nitrogen [12]. However, it is also noteworthy that these changes were not seen in Excel although the two transgenic lines express bean α-AI to similar levels [8].

The presence of the recombinant α-AI might interfere with the processing of endogenous proteins in only one of the lines due to some pre-existing difference between the two cultivars. Focussing on the convicilin spots (Fig. 2) we found that the transgenic rather than non-transgenic Laura more closely resembles non-transgenic Excel. Presumably various perturbations occur following the selection of desirable traits in particular cultivars. Perhaps, the perturbations in Laura due to transgenic expression of α-AI resembled perturbations that had already been caused in Excel by some other selection and the addition of α-AI caused little further change. The relative amounts of the various storage proteins are the characteristics of different lines of peas and are readily varied by only moderate stresses [13]. On the other hand, we cannot exclude the possibility that the differences in the effect of transgenic expression of α-AI might reflect differences in the site of insertion of the transgene for which the identity of the transgene may be irrelevant; the insertion in Laura may have affected some gene with a general role in protein modification.

The genetic modification of peas with α-AI, a protein from edible beans, very effectively conferred the intended

Table 2. Identification of differentially expressed proteins in transgenic pea seeds. Individual spots were excised from two-dimensional gels (Fig. 1) and identified by tandem mass spectrometry of tryptic peptides. A-appears, D-disappears in the transgenic line. All identifications are from Pisum sativum except where indicated

Spot no.	Protein identified	Av. ratio	Mascot Score	No. of peptides matched	Mr from seq.	pI	Sequence Coverage (%)	Accession No.
2	Convicilin	−1.6	450	14	66949	6.3	26	gi|313670
14	Fructose-1,6-bisphophate aldolase	−1.6	606	12	38467	6.8	44	gi|927505
18	Vicilin 47 kD proteins	−2.6	892	26	49592	5.5	45	gi|297170
20	Vicilin 47 kD proteins	−2.4	105	31	49592	5.5	50	gi|297170
23	Albumin 2 (PA2)	+5.0	855	22	26222	5.2	76	gi|113570
24	1-cys peroxiredoxin [*Medicago truncatula*]	−2.6	185	5	24398	6.0	18	gi|49618728
25	Vicilin 47 kD protein	−2.4	438	12	49592	5.5	27	gi|297170
27	Pea Lectin	−3.6	403	10	19930	5.3	56	gi|29726273
29	copper zinc superoxide dismutase	+3.7	207	5	15314	5.6	36	gi|60360880
32	Convicilin	D	208	5	66949	6.2	6	gi|7339551
33	Convicilin	A	150	5	72020	5.5	10	gi|7339551
34	Albumin 2 (PA2)	D	540	16	26222	5.1	68	gi|113570
36	Albumin 2 (PA2)	A	219	6	26222	5.1	31	gi|113570
37	legumin (minor small)	D	359	10	64833	5.4	23	gi|2578438
38	Vicilin 47 kD protein	D	291	6	49592	5.5	10	gi|297170
39	α-Al beta chain [*Phaseolus vulgaris*]	A	80	2	16527		22	gi|126150

insect resistance [1, 2] but differences in post-translational modifications, particularly glycosylation, between α-AI when expressed transgenically in peas rather than natively in the bean, were linked with an immune reaction in a mouse model [7] and CSIRO discontinued commercial development (www.pi.csiro.au/GMpeas/GMpeas.htm).

In addition to unintended effects on the product of the transgene, complex transcriptional, post-transcriptional and/or post-translational perturbations of endogenous proteins can occur as illustrated here. The differences in the effects on the two cultivars expressing the same transgene could be due to differences in their genetic backgrounds, or differences in the insertion sites and integration events of the α-AI gene, or both. However, the results presented here suggest that these differences are of a similar magnitude to those that occur between cultivars that are not subject to the same level of scrutiny by regulatory bodies. This study suggests that a precautionary approach for the development of transgenic food crops could include preference for lines that show the fewest differences from their non-transgenic parents by proteome display even where no harmful consequence has been demonstrated or suspected.

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The authors have declared no conflict of interest.

5 References

[1] Morton, R. L., Schroeder, H. E., Bateman, K. S., Chrispeels, M. J., Armstrong, E., Higgins, T. J. V., *Proc. Natl. Acad. Sci. USA* 2000, *97*, 3820–3825.

[2] Sousa-Majer, M., Turner, N. C., Hardie, D. C., Morton, R. L., Lamont, B., Higgins, T. J. V., *J. Exp. Bot.* 2004, *55*, 497–505.

[3] Hashimoto, W., Momma, K., Yoon, H., Ozawa, S., Ohkawa, Y., Ishige, T., Kito, M. *et al.*, *Biosci. Biotechnol. Biochem.* 1999, *63*, 1942–1946.

[4] Momma, K., Hashimoto, W., Ozawa, S., Kawai, S., Katsube, T., Takaiwa, F., Kito, M. *et al.*, *Biosci. Biotechnol. Biochem.* 1999, *63*, 314–318.

[5] Corpillo, D., Gardini, G., Vaira, A., Basso, M., Aime, S., Accotto, G. P., Fasano, M., *Proteomics* 2004, *4*, 193–200.

[6] Islam, N., Upadhyaya, N. M., Campbell, P. M., Akhurst, R., Hagan, N., Higgins, T. J. V., *Phytochemistry* 2005, *66*, 2534–2539.

[7] Lehesranta, S. J., Davies, H. V., Shepherd, L. V. T., Nunan, N., McNicol, J. W., Auriola, S., Koistinen, K. M. *et al.*, *Plant Physiol.* 2005, *138*, 1690–1699.

[8] Prescott, V. E., Campbell, P. M., Moore, A., Mattes, J., Rothenberg, M. E., Foster, P. S., Higgins, T. J. V. *et al.*, *J. Agric. Food Chem.* 2005, *53*, 9023–9030.

[9] Islam, N., Lonsdale, M., Upadhyaya, N. M., Higgins, T. J., Hirano, H., Akhurst, R., *Proteomics* 2004, *4*, 1903–1908.

[10] Shewry, P., Tatham, A., Halford, N., Barker, J., Hannappel, U., Gallois, P., Thomas, M. *et al.*, *Transgenic Res.* 1994, *3*, 3–12.

[11] Vitale, A., Hinz, G., *Trends Plant Sci.* 2005, *10*, 316–323.

[12] Tabe, L. M., Hagan, N. D., Higgins, T. J. V., *Curr. Opin. Plant Biol.* 2002, *5*, 212–217.

[13] Spencer, D., Rerie, W. G., Randall, P. J., Higgins, T. J. V., *Aust. J. Plant Physiol.* 1990, *17*, 355–363.

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RESEARCH ARTICLE

Proteomic analysis of membrane proteins expressed specifically in pluripotent murine embryonic stem cells

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Embryonic stem cells (ESCs) are established from the inner cell mass of preimplantation embryos, are capable of self-renewal, and exhibit pluripotency. Given these unique properties, ESCs are expected to have therapeutic potential in regenerative medicine and as a powerful tool for *in vitro* differentiation studies of stem cells. Various growth factors and extracellular matrix components regulate the pluripotency and differentiation of ESC progenies. Thus, the cell surface receptors that bind these regulatory factors are crucial for the precise regulation of stem cells. To identify membrane proteins that are involved in the regulation of pluripotent stem cells, the membrane proteins of murine ESCs cultured with or without leukemia inhibitory factor (LIF) were purified and analyzed by quantitative proteomics. 2-D PAGE-based analysis using fluorescently labeled proteins and shotgun-based analysis with isotope-labeled peptides identified 338 proteins, including transmembrane, membrane-binding, and extracellular proteins, which were expressed specifically in pluripotent or differentiated murine ESCs. Functions of the identified proteins revealed cell adhesion molecules, channels, and receptors, which are expected to play important roles in the maintenance of murine ESC pluripotency. Membrane proteins that are expressed in pluripotent ESCs but not in differentiated cells such as Slc16a1 and Bsg could be useful for the selection of the stem cells *in vitro*.

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Abbreviations: AP, alkaline phosphatase; **ESC,** embryonic stem cell; **IPG,** immobiline pH gradient; **LIF,** leukemia inhibitory factor; **ZIC-HILIC,** zwitterionic hydrophilic interaction LC

1 Introduction

Embryonic stem cells (ESCs) have the abilities to self-renew and differentiate into all cell types of adult tissues, including germ cells [1–3]. Therefore, ESCs have therapeutic potential

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in regenerative medicine and represent a powerful tool to elucidate the mechanisms of cell differentiation *in vitro* [4, 5]. Murine ESCs are derived from the inner cell mass of blastocyst-stage embryos, and are able to grow and maintain their pluripotency on monolayers of mitotically inactivated murine embryonic fibroblast feeder cells in culture medium supplemented with LIF [6, 7]. Since ESCs can differentiate into various cell types in response to growth factors and extracellular signals, they are assumed to have specific subsets of cell surface proteins that recognize extracellular stimuli and regulate intracellular signaling [8]. These membrane proteins are considered to play important roles in the maintenance of undifferentiated ESCs and differentiation into specific cell types [9, 10]. Although transcription factors, such as Nanog, Sox2, and Oct-3/4, are known to play indispensable roles in the pluripotency of murine ESCs [10], the membrane proteins and extracellular proteins specifically expressed in pluripotent murine ESCs are not fully elucidated. For example, LIF is required for the maintenance of pluripotent murine ESCs *in vitro*. However, gene-targeted mouse embryos that lack LIF can develop to a stage subsequent to murine ESC derivation [11]. These findings suggest the involvement of an alternative pathway in the regulation of pluripotency *in vivo*. Therefore, identification of the membrane proteins that regulate the pluripotency and differentiation of ESCs is an important issue in understanding the regulatory mechanisms of murine ESCs both *in vivo* and *in vitro*.

Approximately 20–30% of the genes in vertebrates encode integral membrane proteins [12]. These membrane proteins and associated peripheral membrane proteins are involved in central cellular processes and account for the major pharmaceutical drug targets [13]. Despite their biological significance, the investigation of membrane proteomics is technically limited by the relatively high molecular masses and hydrophobicities of these proteins [14].

To identify proteins from complex samples, a proteomics approach involving 2-DE followed by the identification of isolated proteins by MS has been used [15]. For quantitative analysis, 2-D DIGE has been established [16]. In this method, protein samples prepared from cells grown under two different conditions are labeled with different fluorescent CyDyes, combined, and resolved by 2-DE. Quantification of the proteins is based on the fluorescence intensities of the protein spots [16].

Another approach to the identification of proteins in a mixture is the shotgun method [17]. With this method, the protein samples are initially digested with a protease, such as trypsin, and the resulting peptide mixtures are separated by 2-D LC and analyzed by MS [18, 19]. For quantitative analysis with the shotgun method, isobaric tags for relative and absolute quantitation (iTRAQ) was recently introduced as a labeling reagent for peptide fragments [20]. The iTRAQ reagents represent an improved version of a prototypical stable-isotope labeling reagent, isotope-coded affinity tags (ICAT) [21]. Although ICAT labels only cysteine residues, iTRAQ employs primary amine reactive isobaric tags to derivatize peptides at the N-termini and lysine side-chains. Therefore, theoretically, iTRAQ labels most of the peptide fragments in a digested mixture. Although the peptides labeled with any of the iTRAQ reagents are indistinguishable in single MS analysis, tag fragmentation during MS/MS analysis produces reporter ions (m/z = 114, 115, 116, and 117), which provide quantitative information upon integration of the peak areas [20].

In the present study, we applied the quantitative proteomics to generate a list of membrane proteins that are putatively involved in the maintenance of pluripotency in murine ESCs. For this purpose, 2-D DIGE with CyDye labeling reagents and a shotgun method with the iTRAQ reagent were used. We identified more than 300 membrane proteins and membrane-associated proteins that were differentially expressed between pluripotent murine ESCs and their differentiated progenies. We discuss the possible functions of the identified membrane proteins in the maintenance of pluripotency.

2 Materials and methods

2.1 Culture of ESCs

The murine ESC line D3 was cultured in a 0.1% gelatin (Sigma)-coated dish in DMEM (High-Glucose; Gibco BRL) that was supplemented with 15% heat-inactivated ES-qualified FCS (Gibco BRL), 0.1 mM β-mercaptoethanol (Wako), 1 × nonessential amino acids (Sigma), 0.1 mg/mL penicillin/streptomycin (Gibco BRL) and 1000 U/mL LIF (ESGRO; Chemicon). All cells were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2. To prepare membrane protein samples from pluripotent and differentiated cells, murine ESCs were cultured for 7 days in the above medium with or without LIF, respectively.

2.2 Biochemical analyses

2.2.1 Alkaline phosphatase (AP) staining

Murine ESCs cultured for 7 days with or without LIF were fixed with 3.7% formaldehyde in PBS for 5 min at room temperature, and then incubated with an AP substrate, BM purple AP substrate (Roche), for 30 min at room temperature to visualize the enzyme activity.

2.2.2 Immunofluorescence staining

Murine ESCs were washed with PBS, fixed in 3.7% formaldehyde in PBS for 30 min at room temperature, and then permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. The cells were blocked with 5% FCS in PBS for 1 h at room temperature, and then incubated with primary antibodies. After washing three times with 5% FCS

in PBS for 10 min, the cells were incubated with fluorescently labeled secondary antibodies for 30 min at 4°C. The cells were washed with 5% FCS in PBS and mounted in an anti-photobleaching mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). The cells were observed under a fluorescent microscope or a confocal fluorescent microscope.

2.2.3 Preparation of whole cell extracts

Whole cell extracts were prepared from murine ESCs cultured for 7 days with or without LIF, respectively. The cells were lysed in extraction buffer that contained 150 mM NaCl, 1 mM EDTA, 2% Triton X-100, protease inhibitor cocktail (Complete, Roche), and 20 mM Tris-HCl (pH 7.4). After centrifugation at 27 000 × *g* for 5 min at 4°C, the supernatants were collected as whole cell extracts. The protein concentrations were measured with a protein assay kit (BioRad Laboratories).

2.2.4 Western blotting

Samples (20 μg) of the whole cell lysates from undifferentiated and differentiated murine ESCs were resolved by SDS-PAGE and transferred to PVDF membranes (Applied Biosystems). The PVDF membranes were blocked with 5% skim milk in TBS/Tween-20 (TBS-T, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20), and incubated with primary antibody for 16 h at 4°C. After washing with TBS-T, the PVDF membranes were incubated with secondary antibody for 1 h at room temperature. The protein bands were detected using the SuperSignal West Femto system (Pierce).

2.2.5 Antibodies

The primary antibodies used were rabbit polyclonal anti-Oct3/4 (sc-9081; Santa Cruz Biotechnology), mouse monoclonal anti-Oct-3/4 (611203; BD Biosciences), rabbit polyclonal anti-Nanog (RCAB0001P; ReproCELL), mouse monoclonal anti-SSEA-1 (TM13; Kyowa Medex), rabbit polyclonal anti-Mct1 (AB3540P, Slc16a1; Chemicon), mouse monoclonal anti-CD147 (ab666, Bsg; Abcam), mouse monoclonal anti-ErbB4 (sc-8050; Santa Cruz Biotechnology), rabbit polyclonal anti-Glut1 (AB1340; Chemicon), rabbit polyclonal anti-Akt2 (07–372; Upstate Biotechnology), mouse monoclonal anti-α-tubulin (T5293, Sigma), goat polyclonal anti-E-cadherin (sc-1500; Santa Cruz Biotechnology), and rabbit polyclonal anti-EphA2 (sc-924; Santa Cruz Biotechnology).

2.3 Proteomic analyses

2.3.1 Purification of membrane proteins for 2-DE

Murine ESCs were grown in 0.1% gelatin-coated dishes (15 cm) with or without LIF. The cells were split before reaching full confluency (approximately 95%). After culturing for 7 days with or without LIF, the cells were harvested and the membrane proteins were purified. Briefly, the cell surface membrane proteins were labeled *via* biotinylation by culturing the cells with membrane-impermeable sulfo-NHS-SS-Biotin (3 μM; Pierce), as described previously [22]. After quenching the reagents by the addition of 0.2 mM lysine, the cells were washed with PBS, scraped from the dish, and collected by centrifugation at 270 × *g* for 5 min at 4°C. The cell pellets were swollen in a hypotonic buffer (1.5 mM $MgCl_2$, 10 mM KCl, 1 mM NaF, protease inhibitor cocktail, 10 mM Tris-HCl (pH 7.4)) for 30 min on ice, disrupted in a Dounce homogenizer (tight pestle) for 30 strokes on ice, and the nuclear fractions were removed by centrifugation at 1000 × *g* for 10 min at 4°C. The supernatant was layered onto sucrose buffer (60% sucrose, protease inhibitor cocktail, 10 mM Tris-HCl (pH 7.4)) and centrifuged at 46 000 × *g* for 1 h at 4°C. A crude plasma membrane fraction was collected as a band on the surface of the sucrose layer, resuspended in ice-cold hypotonic buffer, and pelleted again by centrifugation at 46 000 × *g* for 20 min at 4°C. The pellet was resuspended in hypotonic buffer. The membrane fraction was rotated with Dynabeads M-280 streptavidin (Dynal Biotech) for 1 h at 4°C and purified using a magnetic device. The affinity-purified plasma membrane was solubilized in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, protease inhibitor cocktail, 30 mM Tris-HCl (pH 6.8)). The protein concentration was determined using the BioRad Protein Assay Kit.

2.3.2 2-D DIGE analysis

All the chemicals and instruments used for 2-D DIGE were from GE Healthcare. Membrane proteins prepared from two sets of 7-day cultures of murine ESCs with or without LIF were labeled with Cy3 or Cy5, respectively. The membrane proteins from another two sets of murine ESCs culture were labeled with the opposite combination of these dyes. All the protein samples were combined and labeled with Cy2; this was used as an internal control to normalize the protein quantities across independent experimental gels. For the fluorescence labeling of samples, 50 μg of membrane proteins were incubated with 400 pmol of CyDye for 30 min on ice. Then, 50 μg of the labeled proteins from undifferentiated and differentiated cells (Cy3- and Cy5-labeled) and 50 μg of Cy2-labeled internal control protein were combined. Prior to IEF, an equal volume of sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% DTT, 2% Pharmalytes at pH 3–11) was added to the labeled sample mixture and incubated for 10 min on ice. Then, rehydration buffer (8 M urea, 4% CHAPS, 2% DTT, and 1% Pharmalytes at pH 3–11) was added to the mixture, giving a final volume of 460 μL. First-dimensional separation by IEF was carried out on Immobiline pH Gradient (IPG) Dry Strips (pH 3–11, NL 24 cm) using Ettan IPGphor II according to the manufacturer's protocol. Briefly, after rehydration of the IPG strips with rehydration buffer containing the labeled samples for 12 h at 20°C, focusing was carried out at 500 and 1000 V for 1 h each

and 6000 V for a total of 90 kV·h. After IEF, each strip was incubated in equilibration buffer (6 M urea, 2% SDS, 30% glycerol, 0.5% DTT, 50 mM Tris-HCl (pH 6.8)) for 15 min at room temperature with shaking, followed by alkylation (carbamidomethylation) buffer (6 M urea, 2% SDS, 30% glycerol, 4.5% iodoacetamide, one grain of BPB, 50 mM Tris-HCl (pH 6.8)) for 15 min at room temperature with shaking. The IPG strips were then loaded on 12.5% polyacrylamide Laemmli gels. The gels for second-dimensional electrophoresis were run at 13 mA *per* gel at 20°C until the front of the BPB dye reached the bottom of the gel. Gel images were captured by the Typhoon 9400 (GE Healthcare) fluorescence gel scanner for each fluorescence dye. DeCyder software version 5.02 (GE Healthcare) was used for detection, normalization, and comparative statistical analyses between the gels.

2.3.3 MS analysis and database searching for the 2-D gel-based method

Protein spots were excised from SYPRO Ruby-stained 2-DE gels, and in-gel digestion was performed as previously described [23]. Briefly, the gel pieces were washed three times with 60% ACN that contained 100 mM NH_4HCO_3, and then dried completely. The dried gel pieces were incubated with 100 mM NH_4HCO_3 that contained 2.5 μg trypsin (Trypsin Gold, MS Grade; Promega) for 16 h at 37°C. After digestion, peptides were extracted with 60% ACN. For further recovery of peptides from the gel pieces, a second extraction was performed with 0.1% formic acid/0.01% TFA. The peptide extracts filtered by Ultrafree-MC with 0.22 mm Durapore (Millipore) were desalted using the capillary LC nano-LC system equipped with PepMap C18 (320 mm × 1 mm; LC Packing), and applied continuously to MonoCap for Peptide (100 mm × 250 mm; GL Science). The peptide fragments were eluted with a gradient of ACN and 0.1% formic acid/0.01% TFA, then injected directly into Q-TOF MS (Q-TOF Micro; Micromass) through a nano-LC probe (ESI) under the following conditions: cycle time, 2.10 s; mass range, 400–1800 Da. Four parent masses were selected for MS/MS analysis with collision energy of 20–30 eV. The peptide mass fingerprints were analyzed using the MASCOT search program (Matrix Science, www.matrixscience.com) at the National Center for Biotechnology Information (NCBI; ftp.ncbi.nih.gov/blast/). The search conditions were defined as tryptic peptides and one missing cleavage was allowed. Carbamidomethylation at cysteine residues and oxidation at methionine residues were selected as variable modifications. Precursor error tolerance and MS/MS fragment error tolerance were set to 200 ppm and 0.5 Da, respectively. Only the top-ranked peptide matches were taken into consideration for protein identification. Samples having total ion scores of less than 30 were rejected. Functional and subcellular location data on identified proteins were obtained from the NCBInr and Bioinformatic Harvester online databases (http://harvester.fzk.de/harvester/), and predictions of transmembrane regions were performed using the SOSUI software (http://bp.nuap.nagoya-u.ac.jp/sosui/). The raw data of single peptide-based identification from MASCOT search results are shown in Supporting Information Table 3.

2.3.4 Preparation of membrane proteins for shotgun method

The murine ESCs grown as described above were harvested without biotinylation of cell surface proteins. Disruption of the cells and removal of nuclear fractions were performed as described above. Centrifugation was performed with the sucrose buffer at 46 000 × *g* for 1 h at 4°C, and the band that contained the membrane fraction was collected and resuspended in hypotonic buffer. The membrane fraction was washed twice by centrifugation at 46 000 × *g* for 20 min at 4°C. The resulting pellet was extracted with an extraction buffer that contained 7 M urea, 2 M thiourea, 0.2% SDS, 10 mM NaF, 2 mM Na_3VO_4, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail, and 20 mM HEPES (pH 7.4). After centrifugation, the protein extracts were desalted by methanol precipitation. For the labeling of peptides with iTRAQ (Applied Biosystems), the proteins were dissolved, reduced, and alkylated according to the manufacturer's instructions. The proteins were then tryptically digested and labeled with the iTRAQ kit as follows: for the undifferentiated murine ESC sample, iTRAQ 114; and for the differentiated murine ESC sample, iTRAQ 117. The labeled samples were combined, and then separated by HPLC, as described below.

2.3.5 2-D LC and MALDI-TOF/MS/MS analysis

Samples (100 μg) of iTRAQ-labeled peptides were fractionated by zwitterionic hydrophilic interaction LC (ZIC-HILIC) (Sequant, 100 mm × 2.1 mm, 5 μm, 200 Å) as the first-dimensional separation, and then by RPLC (MonoCap C18, 300 mm × 0.1 mm; Kyoto Monotech) as the second-dimensional separation. The separations were run on the Micro-LC system (LC-10AD; Shimadzu) at 40°C and at a flow rate of 100 μL/min. Digested peptides were subjected to ZIC-HILIC using a gradient of binding solvent (81% ACN, 19% 15 mM KH_2PO_4 (pH 4.5)) and elution solvent (30% ACN, 70% 20 mM KH_2PO_4 (pH 4.5)). After a 5-min wash with binding solvent, a 35-min linear gradient (0–60% elution solvent) was followed by a 10 min wash with elution solvent. Absorption of peptides was monitored at 220 nm using the SLC-10A UV–VIS detection system (Shimadzu). The column fractions (1 min each) were collected automatically, and each fraction was further separated by RPLC with a gradient of buffer A (5% ACN containing 0.1% TFA) and buffer B (90% ACN containing 0.1% TFA) using a three-step linear gradient elution: 0–1 min, linear gradient to 15% of B; 1–24 min, linear gradient to 45% of B; 24–28 min, linear gradient to 60% of ACN concentration. Eluted peptides were detected at a wavelength of 214 nm in the UV–VIS MU701

(GL Science). The eluent was automatically mixed with matrix solution (5 mg/mL of α-CHCA/50% ACN containing 0.1% TFA) and spotted onto a MALDI targeting plate using the LC spotting system (AccuSpot; Shimadzu) and analyzed in a MALDI-TOF/TOF mass spectrometer (4700 Proteomics Analyzer; Applied Biosystems). For analysis of the data from the iTRAQ experiments and identification of labeled peptides, the GPS Explorer software version 3.0 (Applied Biosystems) was used to create and search files with the MASCOT software version 2.0 (Matrix Science) used to identify iTRAQ-labeled peptides. The search conditions were defined as tryptic peptides and one missing cleavage was allowed. iTRAQ labeling at the N-terminus and lysine residues and blocking at cysteine residues (MMTS) were selected as fixed modifications. iTRAQ labeling at tyrosine residues and oxidation at methionine residues were selected as variable modifications. Precursor error tolerance and MS/MS fragment error tolerance were set at 200 ppm and 0.5 Da, respectively. Only the top-ranked peptide matches were taken into consideration for protein identification. The peptide mass fingerprints, subcellular localizations, and calculations of transmembrane regions were analyzed as described above.

3 Results

3.1 Preparation of protein samples from undifferentiated and differentiated murine ESCs

To generate a list of membrane proteins that are expressed in pluripotent murine ESCs but not in differentiated cells, we performed a quantitative proteomics analysis (Fig. 1). As murine ESCs spontaneously differentiate in the absence of LIF [6, 7], we prepared membrane protein samples from pluripotent and differentiated murine ESCs cultured for 7 days with or without LIF, respectively. The differentiation of murine ESCs was evaluated using the stem cell surface marker of AP activity [24]. Although murine ESCs cultured in the presence of LIF appeared as rounded colonies and had high AP activity, murine ESCs cultured without LIF for 7 days exhibited a flattened morphology and lower AP activity (Fig. 2A). Immunofluorescence analyses of other markers for pluripotent stem cells, Oct-3/4 [10] and SSEA-1 [25], also showed remarkable decreases in the expression of these markers, indicating that these cells had lost pluripotency (Fig. 2B). The membrane fractions of these differentiated cells and pluripotent murine ESCs were used in the subsequent quantitative proteomics analyses of membrane proteins.

3.2 2-D gel-based analysis

Membrane proteins prepared from murine ESCs cultured for 7 days with or without LIF were labeled with Cy3 or Cy5, respectively, and analyzed using a gel-based 2-D DIGE

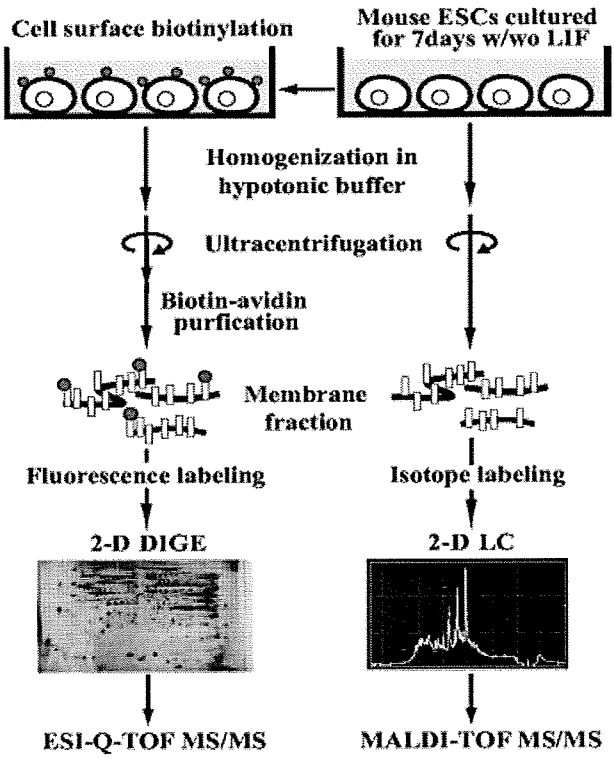

Figure 1. Scheme for sample preparations and proteomics analyses. Purified membrane proteins from murine ESCs were subjected to 2-D gel-based and shotgun-based analyses.

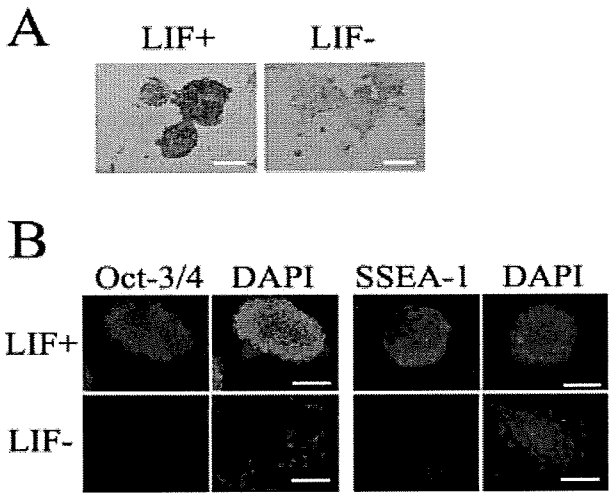

Figure 2. Expression profiles of undifferentiated markers in pluripotent murine ESCs. (A) AP activities of murine ESCs cultured for 7 days with or without LIF. Murine ESCs were fixed with 3.7% formaldehyde in PBS for 5 min, and then incubated with AP substrate for 30 min at room temperature. The reaction products displayed a blue color for AP. (B) Immunofluorescence staining for Oct-3/4 and SSEA-1 in murine ESCs cultured with or without LIF. Murine ESCs cultured as in (A) were fixed with 3.7% formaldehyde in PBS for 30 min and stained immunofluorescently for Oct-3/4 (red) and SSEA-1 (green), as described in Section 2. The nuclei were stained with DAPI (blue). Scale bars, 200 μm.

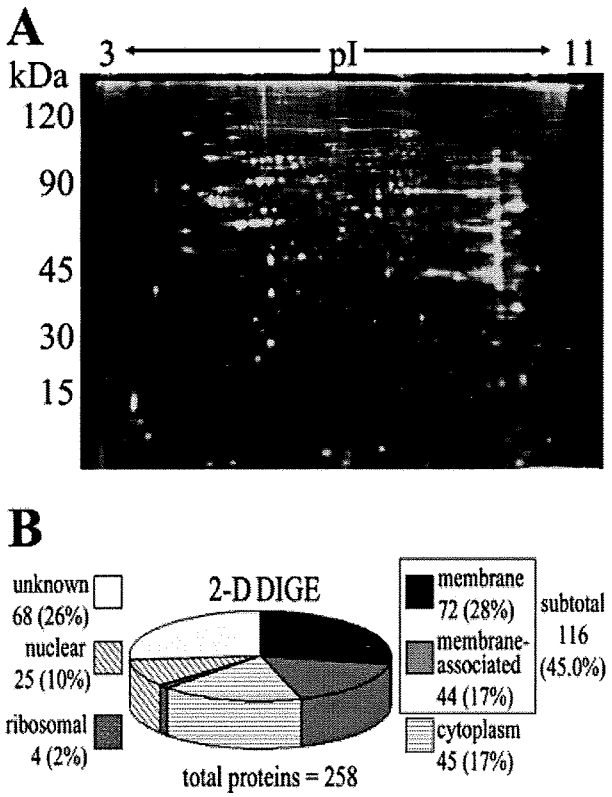

Figure 3. 2-D DIGE analysis of membrane proteins of pluripotent and differentiated murine ESCs. (A) A representative 2-D gel image. The green and red spots indicate undifferentiated and differentiated state-specific proteins, respectively. (B) Subcellular localization of proteins identified in the 2-D DIGE analysis.

technique (Fig. 3A and Supporting Information Fig. 1). Analysis with a fluorescence scanner detected 2488 spots. MS analysis of most of the differentially expressed protein spots and some of the other spots identified 258 unique proteins. The number of peptides used to identify a single protein ranged from 1 to 33, with an average of 6.1 peptides/protein, and 184 of the proteins (71%) were identified by multiple peptide assignments. The molecular masses (Mw) of the identified proteins ranged from 2.8 to 540 kDa, and the p*I* values ranged from 4.28 to 11.88. Some of the identified proteins were found in more than one spot, corresponding to proteins with similar Mw but different p*I* values, implicating the presence of post-transcriptional modifications, such as phosphorylation. After protein identification, the molecular and biological functions and subcellular localizations were annotated by searching the NCBI and Bioinformatic Harvester databases, and the numbers of transmembrane regions and potential secondary structures were predicted by the SOSUI software. A membrane protein has a signal sequence and/or more than one transmembrane region. In contrast, an intracellular membrane-associated protein has neither a signal sequence nor a transmembrane region, but it associates with membrane proteins. An extracellular membrane-associated protein has a signal sequence but no transmembrane region. Using these criteria, we classified 116 proteins as membrane and membrane-associated proteins (Fig. 3B and Supporting Information Table 1).

3.3 Shotgun-based analysis

For the quantitative analysis of membrane proteins derived from two different sources, isobaric labeling methods, including iTRAQ reagents, can be employed. We applied this method for the analysis of pluripotency-specific membrane proteins in murine ESCs. The purified membrane proteins were initially digested with trypsin, and the resulting peptides were labeled with iTRAQ, separated by HPLC, and subjected to MS/MS analysis. Although the peaks derived from two of the isotopic tags were indistinguishable in the MS analysis, upon tag fragmentation in MS/MS, peaks derived from the reporter tags (m/z = 114 and 117) were distinguished (Fig. 4A). The relative quantities of peptides prepared from the two different sources were compared based on the integration of peak areas.

For the efficient separation of iTRAQ-labeled peptide mixtures, we used 2-D LC with a combination of zwitterionic chromatography (ZIC-HILIC) and RPLC. In the first analysis, 10 315 MS/MS spectra were generated, while in the second round of analysis, 3500 MS/MS spectra were generated in which the first peak list was excluded. In these two analyses, the spectra were assigned to 2158 unique peptides by sequential MASCOT searching. All of these peptides were labeled with iTRAQ reagents. A total of 13 815 peptides were attributed to 659 unique proteins (Supporting Information Table 2). The number of peptides used to identify a single protein ranged from 1 to 32, with an average of 3.0 peptides/protein, and 339 of the proteins (51%) were identified by multiple peptide assignments. The Mw of identified protein sequences ranged from 5.3 to 876.5 kDa, and the p*I* values ranged from 4.18 to 12.57. Using the Bioinformatic Harvester database and the SOSUI software, 273 proteins were classified as membrane and membrane-associated proteins (Fig. 4B). In total, among 831 proteins identified by 2-D DIGE and 2-D LC methods, 338 proteins were classified as membrane proteins or membrane-associated proteins (Fig. 4C).

3.4 Verification of identified proteins

Some of the pluripotency-specific membrane proteins identified by MS (shown in Table 1) were verified by biochemical analyses. Whole lysates of murine ESCs cultured for 7 days with or without LIF were analyzed by Western blotting. As shown in Fig. 5A, the expression levels of various membrane proteins and membrane-associated proteins, such as Slc16a1, Akt2, ErbB4, E-cadherin, Bsg, EphA2, and Glut1, were decreased when the ESCs were cultured without LIF. Culturing in the absence of LIF also decreased the protein levels of the undifferentiated state-specific nuclear transcriptional factors, Oct-3/4 and Nanog. Under these conditions,