Protein sorting into protein bodies during barley endosperm development is putatively regulated by cytoskeleton members, MVBs and the HvSNF7s

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Cereal endosperm is a short-lived tissue adapted for nutrient storage, containing specialized organelles, such as protein bodies (PBs) and protein storage vacuoles (PSVs), for the accumulation of storage proteins. During development, protein trafficking and storage require an extensive reorganization of the endomembrane system. Consequently, endomembrane-modifying proteins will influence the final grain quality and yield. However, little is known about the molecular mechanism underlying endomembrane system remodeling during barley grain development. By using label-free quantitative proteomics profiling, we quantified 1,822 proteins across developing barley grains. Based on proteome annotation and a homology search, 94 proteins associated with the endomembrane system were identified that exhibited significant changes in abundance during grain development. Clustering analysis allowed characterization of three different development phases; notably, integration of proteomics data with in situ subcellular microscopic analyses showed a high abundance of cytoskeleton proteins associated with acidified PBs at the early development stages. Moreover, endosomal sorting complex required for transport (ESCRT)-related proteins and their transcripts are most abundant at early and mid-development. Specifically, multivesicular bodies (MVBs), and the ESCRT-III HvSNF7 proteins are associated with PBs during barley endosperm development. Together our data identified promising targets to be genetically engineered to modulate seed storage protein accumulation that have a growing role in health and nutritional issues.

After differentiation, fully developed cereal endosperm makes up to 75% of the grain weight and covers four major cell types: aleurone, starchy endosperm, transfer cells, and the cells of the embryo surrounding region. The starchy endosperm thereby is characterized as a storage site, accumulating starch and seed storage proteins (SSPs). The aleurone layer plays essential roles during seed germination and mobilizes starch and SSP reserves in the starchy endosperm by releasing hydrolytic enzymes that are responsible for the degradation of stored nutrients in the endosperm. Contrary to the persistent endosperm of cereals, the cellular endosperm of Arabidopsis thaliana (A. thaliana) supports the developing and growing embryo, resulting in a gradually depleted endosperm as the embryo grows. Finally, the massive A. thaliana embryo is only accompanied by a single peripheral layer, the aleurone layer, in mature seeds. Consequently, A. thaliana cannot be used as a model system to study the endomembrane system in grain endosperm.

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In cereals, SSPs, which account for more than 50% of the grain protein content, accumulate in the outer layer of the endosperm, in the subaleurone, and in the starchy endosperm, the latter in parallel with starch granules. In barley, for instance, globulins and prolamins comprise the major endosperm SSPs.

The SSP trafficking routes depend on the cereal species. Endosperm layer and development stage. Endosomes are involved in the sorting and transport of SSPs. Recent research has highlighted the role of endosomal sorting complexes required for transport (ESCRT)-III proteins in the process of SSP trafficking.

In barley, endosperm development is characterized by a series of distinct events including the formation of protein deposition or trafficking organelles and endomembrane remodeling. This work aimed to map the endomembrane system during barley endosperm development, using integrative cell biology experimental approaches. Label-free proteomics approaches of four different stages of grain development allowed the quantification of 1,822 proteins. Among these, 94 proteins could be associated with the endomembrane system. We identified cytoskeleton members, ESCRT proteins, and MVBs as putative organelles associated with early and mid-development. In this context, confocal and transmission electron microscopy analysis of HvSNF7 and MVBs at the periphery of PBs and later within PBs, a putative role in protein sorting to PBs at mid-development. These results pave the way to exploit the endomembrane system to modulate SSP accumulation and/or to improve the production of recombinant proteins.

Materials and Methods

Plant material and growth conditions. Barley (Hordeum vulgare) wild-type variety Golden Promise (GP) and transgenic lines (TIP (Tonoplast intrinsic protein)3-GFP, p6U::SNF7.1-mEosFP) were cultivated as described in17 and in the supplementary material. In detail, caryopses were harvested at different stages of grain development including 6–8, 10, 12–18 and ≥20 days after pollination (DAP) (designated as 6, 10, 12 and ≥20 DAP) of three biological replicates.

Data processing and protein identification. Sample preparation for proteomics analyses and Mass spectrometry (MS) was performed as previously described in16 and in the supplementary material. RAW files were processed with MaxQuant 1.5 (http://www.maxquant.org) and the Andromeda search algorithm in16–20 on the barley UniProt database (http://uniprot.org). Peptide identification was performed as previously described in16. Label-free quantification was done at the MS1 level with at least two peptides per protein. PTXQC was used to assess data quality and statistical analysis was performed with Perseus 1.5 software in21,22. Protein annotation was performed with the MERCATOR tool (http://mapman.gabipd.org/de) in21. Unknown proteins were identified by using BLAST at the UniProt homepage searching for the most identical cereal protein. Proteins were classified to " compartment-specific proteins" (functional associated with a specific subcellular endomembrane pathway or organelle), and " trafficking regulators" (functional associated with several organelles) based on published data. In the final dataset, representative proteins were quantified in at least 9 of the 12 samples analyzed. Data were first Log2 transformed prior Z-scores (zero mean, unit variance) and were finally used to calculate the relative protein abundance. A one-way analysis of variance (ANOVA) and Student’s t-tests were performed with Perseus 1.5 software. Cluster analysis was performed with fuzzy-c means algorithm implemented in GProX in24. Generation of protein-protein interaction (PPI) networks was conducted via the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database for known and predicted protein-protein interactions (http://string-db.org/) with default parameters in21,22. The MS proteomic data have been deposited to the ProteomeXchange Consortium in21 via the PRIDE (Vizcano et al., 2016) partner repository with the dataset identifier PXD009722.

Cloning of constructs. Bimolecular Fluorescence Complementation (BiFC). The backbone of all vectors (MK4_5SPYCE and MPK3_SPYNE, kindly provided by Dr. Andrea Pitzschke) used in this study contain a pA5S promoter (Cauliflower mosaic virus 35 S promoter), 5′UTR (untranslated region from tobacco etch virus), our
genes of interest (HvSNF7.1), C-terminal or N-terminal sequence of YFP (SPYCE or SPYNE, t35 (Cauliflower Mosaic virus 35S terminator)), HA-tag (Human influenza hemagglutinin) or c-MYC-tag, and a kanamycin antibiotic resistance sequence. HvSNF7.1 (according to HvSNF7a.1 described in12) was cloned into the vector pCR2.1 (+K200001, Thermo Fisher Scientific, Massachusetts, USA) using HvSNF7.1_Ncol-F and HvSNF7.1_NotI-R as primers, digested by Ncol and NotI and inserted into previously digested MKK4_SPYCE and MPK3_SPYNE, respectively. To obtain a pSPYCE vector without insert for control reactions, plasmids were cut (Ncol/NotI), blunted using Klenow fragment and re-ligated. All the clones were verified by sequencing.

Yeast two-hybrid (Y2H). The restriction sites Ndel and Sfil were introduced into HvSNF7.1 by PCR using the primers pGADT7_SNF7F and pGADT7_SNF7R3. Using Ndel and Sfil, HvSNF7.1 was ligated into the vector pGEM-T Easy (#A1360, Promega, Madison, Wisconsin, United States). All the clones were verified by sequencing and finally cloned into the target vectors pGADT7 AD (#630442, Clontech Laboratories, Mountain View, United States) and pGBK77 DNA-BD (#630443, Clontech Laboratories, Mountain View, California, United States). Positive clones were verified by sequencing.

Transformation of barley endosperm cells. Barley (GP) transformation was carried out using particle bombardment17,28. T1 plants surviving hygromycin selection were used as previously described13. ER-Tracker Green (BODIPY FL Glibenclamide; #E34251, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and LysoTracker Red DND-99 (#L7528, Thermo Fisher Scientific, Waltham, Massachusetts, USA) were used to visualize ER and acidic compartments, respectively. ER-Tracker Green was used as previously described13. In detail, at least three randomly selected transgenic and GP grains were harvested at 6, 12 and ≥20 DAP, sectioned, washed, and stained as follows: ER-Tracker Green, 1 h (final concentration, 2 µM, from 1 mM DMSO stock with water); LysoTracker Red, 30 min (final concentration, 2 µM, from 1 mM DMSO stock with water). Mock treatments included the final DMSO concentration in water. Sections were mounted in tap water and immediately imaged by the Leica SP5 CLSM using sequential scans with filter settings for GFP (excitation 488 nm, emission 500–530 nm), LysoTracker Red (excitation 561 nm, emission 570–630 nm), and ER-Tracker Green (excitation 488 nm, emission 500–531 nm). Transgenic p6U::SNF7.1-mEosFP grains were chipped, mounted in tap water and analyzed by CLSM with excitation at 488 nm, emission 508–540 nm.

Bimolecular Fluorescence Complementation (BiFC). A single colony of transformed Agrobacterium tumefaciens was inoculated in 5 mL of YEB-Medium (0.5% beef extract, 0.5% sucrose, 0.1% yeast extract, 0.05% MgSO4·7H2O) containing appropriate antibiotics and incubated at 28 °C overnight. In the morning, 5 mL of the same medium was re-inoculated with 1 mL of the pre-culture. Cells were collected by centrifugation at 5000 g for 5 min and the pellet was washed with 1 mL infiltration buffer (10 mM MES pH 5.7, 10 mM MgCl2, 100 µM acetosyringone). Washed cells were re-collected by centrifugation (5000 g, 5 min) and washed two additional times with 500 µL infiltration buffer and finally adjusted to an OD600 of 0.3. The resuspended bacteria containing the corresponding binary expression vectors for BiFC were mixed in a ratio of 1:1 and incubated for 3 h in darkness. Nicotiana benthamiana plants were cultivated in the greenhouse on soil, maintained at 60% humidity, with a 14 h light period and a 25 °C day/19 °C night temperature cycle. The entire leaf area of two leaves per Nicotiana benthamiana plant was infiltrated with the bacterial solution through the abaxial side using a 1 mL syringe. After infiltration, the plants were kept in a tray with a hood at 25 °C. After 2–3 days, the detection of protein–protein interaction by BiFC was performed using confocal microscopy (Leica SP5 CLSM). The excitation wavelength was 514 nm (argon laser) and emission was detected between 525–600 nm and 680–760 nm for YFP and autofluorescence detection, respectively. Red channels were visualized in magenta.

Histological and immunofluorescence studies. At least three randomly selected GP grains were harvested at 6, 12 and ≥20 DAP and fixed, embedded and sectioned as described in13,15. The 1.5 µm sections on glass slides were stained with toluidine blue (0.1%) for 30 s at 80 °C on a hot plate and rinsed with distilled water. Immunofluorescence microscopy of developing barley grains was performed as described by25 using: polyclonal rabbit anti-V-ATPase antibody (#AS 07 213, Agrisera, Vännäs, Sweden), raised against Arabidopsis thaliana At4g11150, specific for higher plants including Hordeum vulgare), dilution 1:100; polyclonal rabbit anti-actin antibody (#AS 13 2640, Agrisera, Vännäs, Sweden, raised against Arabidopsis thaliana actin-1/2/3/4/5/7/8/11-12, specific for higher plants including Hordeum vulgare), dilution 1:50; polyclonal rabbit anti-tubulin-α antibody (#AS 10 680, Agrisera, Vännäs, Sweden, raised against Arabidopsis thaliana tubulin alpha-1/2/3/4/5/6-chain, specific for higher plants including Hordeum vulgare, dilution 1:50); polyclonal rabbit anti-VSR1 antibody.
| Nr. | Uniprot Accession | Protein Name                                      | Pathway                  |
|-----|-------------------|--------------------------------------------------|--------------------------|
| 1   | M0XY35            | Coatomer subunit delta (RET2p) COPI               | secretory pathway        |
| 2   | M0UY14            | Golgin candidate 5                               | secretory pathway        |
| 3   | A0A287KUM9        | Coatomer subunit beta (COPI)                     | secretory pathway        |
| 4   | F2E4V3            | Coatomer subunit epsilon                         | secretory pathway        |
| 5   | A0A287HD61        | SEC. 31 homolog B (COPII)                        | secretory pathway        |
| 6   | F2CX0             | Endoplasmic reticulum vesicle transporter protein | secretory pathway        |
| 7   | A0A287H313        | Transmembrane emp24 domain-containing protein 10 | secretory pathway        |
| 8   | F2CQ85            | Protein transport protein Sec. 61 subunit beta    | secretory pathway        |
| 9   | A0A287T0X1        | Putative ADP-ribosylation factor GTPase-activating protein AGD8 (COPI) | secretory pathway        |
| 10  | A0A287NDD5        | SEC. 24 like (COPII)                             | secretory pathway        |
| 11  | A0A287N3A5        | CASP                                             | secretory pathway        |
| 12  | F2DH14            | SEC. 13 homolog B (COPII)                        | secretory pathway        |
| 13  | F2DF14            | Signal recognition particle subunit SRP72        | secretory pathway        |
| 14  | A0A287J9F8        | Gamma-soluble NSF attachment protein             | secretory pathway        |
| 15  | F2CRB3            | Ras-related protein RIC1 - ARA5                  | secretory pathway        |
| 16  | A0A287WFAD7       | Peroxisome biogenesis protein 5 PEX5             | peroxisome               |
| 17  | A0A287QYS2        | Proton pump-interactor 1                          | PM                       |
| 18  | M0UEQ6            | Nicastrin                                         | PM                       |
| 19  | F2CWF3            | Putative voltage-gated potassium channel subunit beta | PM                       |
| 20  | A0A287RX4         | Proton pump-interactor 1                          | PM                       |
| 21  | F2C46             | redox.ascorbate and glutathione; Membrane steroid-binding protein 1 | PM                       |
| 22  | A0A287QB60        | SEC. 1 family transport protein SLY1             | sorting                  |
| 23  | A0A287ZU3         | CLC2 (CCV)                                       | sorting                  |
| 24  | A0A287MQ9         | CLC1                                             | sorting                  |
| 25  | A0A287Y199        | EHS (TPLATE)                                     | sorting                  |
| 26  | F2D106            | VPS20.1                                          | sorting                  |
| 27  | A0A287NK0         | TOL3                                             | sorting                  |
| 28  | M0X08            | TOL2                                             | sorting                  |
| 29  | M0XC79            | TOL1                                             | sorting                  |
| 30  | A0A287X2J4        | TOL8                                             | sorting                  |
| 31  | A0A287RJU8        | CHC1                                             | sorting                  |
| 32  | A0A287FFS1       | SH3PH                                            | sorting                  |
| 33  | M0XLE4            | non-specific serine/threonine protein kinase      | sorting                  |
| 34  | A0A287QP21        | Auxilin-related protein 1                        | sorting                  |
| 35  | A0A287FUM0        | SNX2b                                            | sorting                  |
| 36  | A0A287UA9         | VPS29                                            | sorting                  |
| 37  | A0A287K255        | SKD1                                             | sorting                  |
| 38  | A0A287H7X6        | VSR1                                             | sorting                  |
| 39  | A0A287N2SS        | VSR1                                             | sorting                  |
| 40  | A0A287RIU7        | VSR1                                             | sorting                  |
| 41  | F2DS44            | SNX1                                             | sorting                  |
| 42  | A0A287R803        | SNF7.1                                           | sorting                  |
| 43  | A0A287XAB9        | SNF7.2                                           | sorting                  |
| 44  | A0A287RZ89        | PUX 8.1                                          | transport                |
| 45  | A0A287WLG4        | PUX 8.2                                          | transport                |
| 46  | A0A287G0M8        | Patellin1                                         | transport                |
| 47  | A5CFY5            | Tubulin beta chain                               | transport                |
| 48  | A5CFY9            | Tubulin beta chain                               | transport                |
| 49  | A0A287FF9         | Actin-2                                           | transport                |
| 50  | F2DY31            | Actin-depolymerization factor 4                  | transport                |
| 51  | A0A287MS88        | Myosin-like protein                               | transport                |
| 52  | M0ZYX8            | Autophagy-related protein 8C                     | degradation              |
| 53  | A0A287FQD8        | Autophagy-related protein 3                      | degradation              |
| 54  | A0A287UDR1        | Vacuolar processing enzyme 1                     | vacuolar processing      |
| 55  | A0A287IX5         | Vacuolar processing enzyme 2b                    | vacuolar processing      |

Continued
dilution 1:100, kindly provided by Dr. Liwen Jiang, raised against *Pisum sativum* BP80, specific for *Pisum sativum*, *Arabidopsis thaliana* and BY2-cells) and polyclonal rabbit anti-SNF7 antibody (dilution 1:100, kindly provided by D. Teis, raised against *Saccharomyces cerevisiae* SNF7, specifically recognizing SNF7). Goat Anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (#A-11008, Thermo Fisher Scientific, Waltham, Massachusetts, USA) (dilution 1:30) was used as a secondary antibody. The specificity of the first antibodies for GP was proved by western blot analyses (Supplementary Fig. 1). At least three sections were analyzed, and pictures captured by Nikon Eclipse Ni. Images were processed using Leica confocal software version 2.63, ImageJ and Adobe Photoshop CS5. Negative controls show sections incubated only with the secondary antibody.

**Table 1.** Identified proteins classified corresponding to their involvement within the endomembrane pathway and to their diverse endomembrane functions.

| Nr. | Uniprot Accession | Protein Name | Pathway |
|-----|-------------------|--------------|---------|
| 56  | A0A2871XX4        | Vacuolar processing enzyme 2c | vacuolar processing |
| 57  | A0A2871XM3        | Vacuolar processing enzyme 2d | vacuolar processing |
| 58  | A0A287RKR9        | Vacuolar processing enzyme 4 | vacuolar processing |
| 59  | A0A287TV00        | Legumain | vacuolar processing |
| 60  | A0A287GR50        | Dynamin-related protein 1 A | dynamins |
| 61  | A0A287N3N7        | Dynamin-related protein 1 C, putative | dynamins |
| 62  | A0A287WH654       | Dynamin-2A | dynamins |
| 63  | A0A287MCV3        | Dynamin-related protein 3 A | dynamins |
| 64  | A0A287GAT9        | NSF | SNARE |
| 65  | A0A287GJC4        | SYP71 protein | SNARE |
| 66  | A0A287N7N5        | ERO1 | Disulfide-generating enzyme and carrier |
| 67  | A0A287WES7        | HvPDIL2-1 | Disulfide-generating enzyme and carrier |
| 68  | A0A287NWQ9        | HvPDIL1-1 | Disulfide-generating enzyme and carrier |
| 69  | A0A287RLW1        | HvPDIL2-2 | Disulfide-generating enzyme and carrier |
| 70  | A0A287P669        | HvPDIL5-1 | Disulfide-generating enzyme and carrier |
| 71  | A0A287TS03        | HvPDIL1-3 | Disulfide-generating enzyme and carrier |
| 72  | M0XFC8            | Vacular proton-ATPase subunit A | ATPase |
| 73  | F2DCK0            | V-type proton ATPase subunit B 1 | ATPase |
| 74  | A0A287L85C5       | YLP; Vacular ATP synthase subunit E | ATPase |
| 75  | F2EFW5            | Pyrophosphate-energized vacuolar membrane proton pump | ATPase |
| 76  | A0A287J931        | RABA1d/d | GTPase |
| 77  | A0A287EG08        | AtRABD1 | GTPase |
| 78  | A0A287KE00        | RABD2a | GTPase |
| 79  | A0A287GB68        | RABGEF | GTPase |
| 80  | A0A287HZ99        | Ras-related protein RABH1b | GTPase |
| 81  | A0A287WHY3        | Signal recognition particle receptor beta subunit | GTPase |
| 82  | A0A287HAL5        | Signal recognition particle 54 kDa protein | GTPase |
| 83  | A0A287UM33        | ADP-ribosylation factor GTPase-activating protein AGD12 | GTPase-activating protein |
| 84  | A0A287FE46        | GDH1/2 | RAB regulator |
| 85  | F2GQ27            | GTP binding nuclear protein | GTP binding protein |
| 86  | A0A287P5H8        | Ran-binding protein | GTP binding protein |
| 87  | M0ZCE0            | Ran-binding protein 1 | GTP binding protein |
| 88  | M0ZLC9            | Ran-specific GTPase-activating protein 2 | GTP binding protein |
| 89  | A0A287QN80        | GTPase SAR1A | GTP binding protein |
| 90  | M0X1Z2            | Ran GTPase activating protein | GTP binding protein |
| 91  | F2CFW2            | GTP-binding protein SAR1A | GTP binding protein |
| 92  | A0A287H404        | GTP-binding protein | GTP binding protein |
| 93  | F2DYD4            | GTP-binding protein SAR1A | GTP binding protein |
| 94  | M0YT49            | ADP-ribosylation factor homolog1 | GTP binding protein |

Transmission electron microscopy (TEM). The coat was removed from grains harvested at 6, 12 and ≥ 20 DAP, chopped and immediately fixed in 4% (w/v) paraformaldehyde plus 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, overnight at 4°C. After washing with sodium cacodylate buffer, the samples were immersed in
Additionally, Cluster Two and Three contain HvPDIL1–1 and HINs15,16. ≥DAP and increasing at between the early (6 DAP) and late development stages (≥20 DAP) shows that PC1 (76.7% of variance) separated the sample based on the different development stages, particularly involved in the early development stage discrimination. Protein loadings on each principal component are indicated in Supplementary Table S2. To assess the proteome dynamics during barley grain development, both PCA and hierarchical bi-clustering analysis (HCA) were performed. Thereby identical clusters of stage-specific groups (Supplementary Table S2). To determine the proteins that were significantly changed along the grain development, we applied a one-way ANOVA analysis, corrected with a permutation-based false discovery rate (FDR) (p < 0.05). Among the 1,822 quantified proteins, the abundance of 1,544 proteins was significantly changed (Supplementary Table S2). To assess the proteome dynamics during barley grain development, both PCA and a hierarchical bi-clustering analysis (HCA) were performed. Thereby identical clusters of stage-specific groups related to development stages of the barley grain were identified (Fig. 1a,b). An investigation of the PCA results shows that Cluster Two and Three exhibit higher expression levels at ≥20 DAP than at 6 DAP, corresponding to the end of mid-stage and beginning of the late stage, where the accumulation of storage reserves can be observed in the endosperm (Fig. 1c and Supplementary Fig. S3a). Finally, the 456 proteins associated with Cluster Two and Three exhibit higher expression levels at ≥20 DAP, corresponding to the end of mid-stage and beginning of the late stage, where the accumulation of storage reserves can be observed in the endosperm (Fig. 1c and Supplementary Fig. S3a). Using LC-MS/MS, all 20 identified SSPs showed significantly higher abundance at ≥20 DAP (Supplementary Fig. S3b,c).

Results

Multivariate statistics unravel proteome reorganization across multiple biological processes during barley grain development. To identify proteins that are localized at the endomembrane system and/or are functionally associated with the rearrangement of the endomembrane system in barley, grains of different development stages including 6, 10, 12 and ≥20 DAP were harvested as previously described6. We used liquid chromatography–mass spectrometry (LC-MS/MS) to identify a total of 3,005 proteins. Our label-free quantification (LFQ) data obtained by MS was validated: first, by the comparison of the relative intensity of the HvF1a and HvVSR1 to a semi-quantitative Western blot (Supplementary Fig. S2b, Supplementary Table S2) that both show the stable and increasing trend of protein accumulation, respectively. Second, the measured protein abundances were highly reproducible with an average Pearson's correlation coefficients of >0.95 between biological replicates (Supplementary Fig. S2c). Additionally, the performed Principal Component Analysis (PCA) supports these results and shows that samples grouped and clustered in a stage-specific manner (Fig. 1a). However, two samples of 10 DAP cluster together with samples of 6 and 12 DAP, respectively. The high distribution of the standard deviation of the normalized LFQ intensities at 10 DAP (Supplementary Fig. S2d) point to a physical variability at 10 DAP. Additionally, the seed weight between the stages are significantly different except the stage between 6 and 10 DAP (Supplementary Fig. S2e). These results are in line with the previously published microscopic data where the most dramatic endomembrane rearrangements could be observed between 8 and 12 DAP23.

A total of 1,822 out of 3,005 were quantified in at least 9 out of the 12 analyzed biological samples (Supplementary Table S2). To determine the proteins that were significantly changed along the grain development, we applied a one-way ANOVA analysis, corrected with a permutation-based false discovery rate (FDR) (p < 0.05). Among the 1,822 quantified proteins, the abundance of 1,544 proteins was significantly changed (Supplementary Table S2). To assess the proteome dynamics during barley grain development, both PCA and a hierarchical bi-clustering analysis (HCA) were performed. Thereby identical clusters of stage-specific groups related to development stages of the barley grain were identified (Fig. 1a,b). An investigation of the PCA results shows that Cluster One (76.7% of variance) separated the sample based on the different development stages, particularly between the early (6 DAP) and late development stages (≥20 DAP). PC2 (13.7% of variance) was rather more involved in the early development stage discrimination. Protein loadings on each principal component are indicated in Supplementary Table S2. As expected, SSPs were among the highest loadings on PC1. Interestingly, most of the detected changes occurred at ≥20 DAP (Fig. 1b).

To refine our protein expression pattern analysis, unsupervised clustering was performed with GproX software to partition the temporal profiles of 1,544 significantly changed proteins measured at all time points. Six clusters based on a fuzzy-mean clustering process24 were detected (Fig. 1c and Supplementary Table S2): Proteins presenting a higher expression level at 6 than at ≥20 DAP belong to Clusters One, Five and Six. Those three clusters account for most of the significantly changed proteins (in total, 962 proteins). It is known that endosperm development involves cell division, cellular differentiation events and the deposition of SSPs between early, mid and late development6,51. These findings were confirmed by toluidine-stained sections prepared at 6, 12 and ≥20 DAP that revealed fully cellularized endosperm including three aleurone cell layers at 6 DAP (Supplementary Fig. S3a). Cluster Four accounts for 126 proteins and shows its highest protein abundance between 10 and 12 DAP, which corresponds to mid-stage, where the differentiation of aleurone, subaleurone, and starchy endosperm is finalized (Fig. 1c and Supplementary Fig. S3a). Finally, the 456 proteins associated with Cluster Two and Three exhibit higher expression levels at ≥20 than at 6 DAP, corresponding to the end of mid-stage and beginning of the late stage, where the accumulation of storage reserves can be observed in the endosperm (Fig. 1c and Supplementary Fig. S3a). Using LC-MS/MS, all 20 identified SSPs showed significantly higher abundance at ≥20 DAP (Supplementary Fig. S3b,c).

Toluidine staining of sections prepared at 6, 12 and ≥20 DAP confirmed protein accumulation starting at 12 DAP and increasing at ≥20 DAP in subaleurone as well as in the starchy endosperm (Supplementary Fig. S3a). Additionally, Cluster Two and Three contain HvPDIL1–1 and HINs15,16.

Development phase I contains proteins of high abundance associated with endocytosis and cytoskeleton, plasma membrane proteins, and ATPases. Based on a BLAST search, 94 proteins related to the endomembrane system (out of the 1,544 significantly changed ones) could be identified in total, where 59 compartment-specific proteins (secretory pathway, peroxisome, plasma membrane, sorting, transport, degradation, and vacuolar processing) and 35 trafficking regulators were defined (dynamins, SNAREs, disulfide-generating enzyme and carrier, ATPase, GTPase, GTPase-activating protein, RAB regulator, and GTP binding protein) (Table 1). The abundance of all identified endomembrane related proteins at 6, 10, 12, and ≥20
DAP were visualized by a heat map categorizing the protein expression pattern based on Pearson correlation (Supplementary Fig. S4). Compartment-specific proteins and trafficking regulators were categorized in pink and blue, respectively (Supplementary Fig. S4).

The 94 proteins could be associated with the three main development phases (Supplementary Fig. S4): 39 proteins present a higher expression during the development phase I (green cluster), 15 proteins presented an expression peak at the development phase II (yellow cluster), and 40 proteins are associated to the development phase III (red cluster). Within each phase, compartment-specific proteins and trafficking regulators were categorized (Table 1, Supplementary Fig. S5a,b). Taken together, these data present molecular regulators for the endomembrane system in developing barley grain.

**Figure 1.** Proteome profiling during barley grain development. (a) PCA was conducted on logarithmically transformed protein intensities; each dot corresponds to a single biological replicate (n = 3). (b) Hierarchical cluster analysis of quantified proteins along barley grain development was performed with Perseus after Z-score transformation of the data21. Clustering of proteins was done based on Euclidian distance while samples’ clustering is based on Pearson correlation. (c) Cluster of proteins dynamics along the grain development. Quantified proteins were subjected to unsupervised clustering with the fuzzy c-means algorithm implemented in GproX24. Cluster distribution indicates the number of proteins in each cluster. Membership value represents how well the protein profile fits the average cluster profile.
Among the 39 proteins identified in the development phase I, proteins related to the secretory pathway (ER, Golgi, Golgi – ER), plasma membrane, sorting pathway (endocytosis, ESCRT), transport (vesicle-mediated transport, cytoskeleton), degradation (Autophagy-related protein 3) and to vacuolar processing as well as trafficking factors (dynamins, SNAREs, disulfide-generating enzymes and carriers, ATPase, GTPase, GTPase-activating protein and GTP binding protein) were enriched at 6 and 10 DAP (Fig. 2a).

More precisely, plasma membrane-associated proteins such as putative voltage-gated potassium channel subunit beta (F2CW3F), Membrane steroid-binding protein 1 (F2CS48), Nicastrin (M0UEQ), 6 and Proton pump-interactor 1 (A0A287RSX4) present decreasing abundance in concomitance to proteins associated to the endocytosis processes such as dynamins (A0A287GK50, A0A287N3M7, A0A287W654, A0A287MCV3), tubulin (A5CFY5, A5CFY9), actin (A0A287FFF9), myosin (A0A287MS88), EH2 (A0A287Y199) and CLC2 (A0A287XZU3). In line with the constant need of energy necessary for endocytosis and plasma membrane remodeling processes, three vacuolar-ATPase subunits were identified (A: M0XF8, B1: F2DCK0, E: A0A287L8C5). All three subunits showed a decreased expression, indicating an acidification process at the early development stage (Supplementary Table S2). Using STRING, a functional association between dynamins and cytoskeleton-related proteins was visualized (Fig. 2b), both necessary for plant endocytic processes32,33. Additionally, STRING revealed a functional correlation between several ATPases that were abundant at 6 and 10 DAP (Fig. 3b). The functional association between Golgin candidate 5, RABH1b and further GTPases shown by STRING points to an active protein sorting in the trans-Golgi or at the trans-Golgi network (TGN)41. Taken together, the proteins that are highly abundant at 6 DAP point to the necessity of cytoskeleton, acidification, and sorting during the development phase I.

We performed immunofluorescence studies of actin and tubulin to follow the subcellular appearance of the cytoskeleton during barley endosperm development. We observed strong signals within PBs at 6 DAP, becoming weaker at 12 and ≥20 DAP, respectively (Fig. 3a–c). PBs are identified in the bright field as small spherical structures as recently published13,15,16. Interestingly, a faint actin signal was observed at the plasma membrane at ≥20 DAP, whereas the signal of tubulin at the plasma membrane was strong at 6 DAP but was reduced at 12 and ≥20 DAP (Fig. 3a–c). No signal could be detected in the negative controls for immunofluorescence for all stages (Supplementary Fig. 6b).

Recently it was shown that PBs in maize are acidified and can be visualized by live cell imaging of fluorescent organelle markers35. To determine if the identified and functionally associated ATPases are putatively involved in the acidification of PBs in barley, we first used LysoTracker Red to detect acidic compartments in the transgenic TIP3-GFP line that visualizes PSVs. At 6 DAP, acidic compartments could be observed within PSVs in the starchy endosperm (Fig. 3d). Large vacuoles are most prominent in the starchy endosperm at 6 DAP35 when the accumulation of PBs has just started (Supplementary Fig. 3)35, indicating that the LysoTracker Red-labelled compartments represent PBs. Indeed, co-labelling of ER and acidic compartments in starchy endosperm cells at 12 DAP revealed LysoTracker Red- and ER-Tracker Green-positive PBs (Fig. 3e).

To determine the time-dependent subcellular distribution of ATPase, we used immunofluorescence microscopy with anti-V-ATPase subunit epsilon antibody on sections at 6 and ≥20 DAP (Fig. 3f). V-ATPases are described to localize at the membranes of PSVs as well as in mature PB in developing pea cotyledons36. Whereas at 6 DAP a punctate structure was observed at the plasma membrane and a strong signal within PBs, a weaker labelling could be observed at the periphery of starch granules and within PBs at 12 DAP (Fig. 3f). At ≥20 DAP, an additional strong signal appeared in aleurone (putatively at the tonoplast of PSVs), whereas the signal was weak within PBs and at the periphery of starch granules in the starchy endosperm (Fig. 3f). No signal could be detected in the negative controls for immunofluorescence for 6, 10, 12 and ≥20 DAP (Supplementary Fig. 6b). Overall, our proteomics and in situ microscopic results point to a high abundance of proteins involved in cytoskeleton regulation and acidification of PBs at early barley grain development stage.

Sorting-associated proteins preferentially accumulate at development phase II. Among the 15 proteins associated with development stage II (Fig. 4a, Supplementary Fig. S4, yellow tree), we found one peroxisome protein, PEX5 (A0A287WFD7); one cytoskeleton protein, Actin-depolymerizing factor 4 (F2DY31); proteins related to endocytosis, CHC1 (A0A287R3U8), Auxilin-related protein 1 (A0A287QP21); as well as proteins of ESCRT machinery, vacuolar processing enzymes (VPEs), GTP-binding proteins, GTPases, and ATPases (Fig. 4a). Again, three ESCRT proteins, TOL4 (A0A287NWKO), NSF7,1 (A0A287R803) and NSF7,2 (A0A287XAB9) were identified, pointing to sorting processes including MVBs already detected in development phase I.

Using STRING, a functional association between the ESCRT TOL4, clathrin, and an auxilin-related protein appeared (Fig. 4b). TOLs have putative clathrin-binding motifs37 and auxilin-related protein 1 functions as clathrin-uncoating factor38, and both were described to be involved in endocytosis. Interestingly, proteins involved in sorting, transport, and degradation have been already identified in the development phase I.

Compartment-specific proteins and trafficking regulators participating in storage protein targeting, transport, and deposition are accumulating at development phase III. In total 40 proteins were associated with development phase III, most of them highly abundant at ≥20 DAP (Fig. 5a).

Interestingly, 11 proteins involved in the secretory pathway (e.g., COPII/COPI), and 9 proteins that function in the protein sorting (e.g., VSR1, retromer, and ESCRT) were identified. Additionally, proteins involved in degradation (autophagy-related 8c, vacuolar processing enzyme1) were highly abundant at ≥20 DAP. Among other proteins, one SNARE was identified as well as 11 trafficking regulators associated with the ATPase, GTPase or the GTP-binding group. Strikingly, endomembrane-associated proteins characterizing the phase III presented the highest interconnectivity within the STRING database compared to phases I and II (Fig. 5b).
As expected, important ER markers such as the disulfide isomerase protein (HvPDIL1-1 and ERO1) were found in this group, being in line with the accumulation of SSPs (Supplementary Fig. S2). The STRING network indicates that the group of ER markers is linked with proteins associated with the secretory pathway and their associated factors such as a RAB regulator GDI1/2 (A0A287FZ46), COPII (F2D1J4, A0A287HD61,}

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**Figure 2.** Identification of proteins that are highly abundant at development phase I of developing barley grains. (a) Data-matrix heat map representing Z-score values of 6, 10, 12 and $\geq$20 DAP. Heat map was prepared using Microsoft Excel. Scale: grey = smallest value; blue = 50% quantile; pink = highest value. (b) Proteins present in this stage were analyzed using the STRING database. STRING default parameters were used$^{25}$, protein names are indicated. PBs are identified in the bright field as small spherical structures as recently published$^{13,15,16}$.
Figure 3. *In situ* microscopical analyses of the cytoskeleton and acidification of PBs in development phase I. (a–c) Immunofluorescence studies of 1.5 µm prepared sections of 6, 12 and ≥20 DAP using antibodies for anti-actin and anti-tubulin-α showing a strong signal at PBs (arrowheads), respectively. PBs are identified in the bright field as small spherical structures as recently published. Note the signal at the plasma membrane with anti-tubulin-α (arrow). The fluorescence signal intensity is weaker at 12 and at ≥20 DAP. Note the additional signal at the periphery of the starch granule at ≥20 DAP using anti-actin (black–white arrowhead). (d) LysoTracker Red (lysoR) accumulation (arrowheads) within TIP3-GFP labelled vacuoles (arrows) at 6 DAP. (e) ER-Tracker Green (ERg)-labelled compartments (arrows) accumulate LysoTracker Red (lysoR) positive PBs (arrowheads) at 12 DAP. (f) Immunofluorescence studies of 1.5 µm sections of 6, 12 and ≥20 DAP using anti-V-ATPase antibody showing no positive signal at aleurone at 6 DAP whereas strong signal could be detected in aleurone at ≥20 DAP. In starchy endosperm, anti-V-ATPase antibody labels strongly PBs (arrowheads) and was found weaker at the plasma membrane (arrows). At 12 DAP, signal appeared at PBs in subaleurone and
starchy endosperm. Note the specific signal at the PBs (arrowheads), at the periphery of starch granules (black-white arrowhead) and the weak labelling of vesicles at the plasma membrane (arrow). At ≥20 DAP, the anti-V-ATPase antibody labels strongly PBs in subaleurone (arrowhead), but to lesser extent in the starchy endosperm. s = starch granule. Bars = 5 µm in a–e and 10 µm in f, except at ≥ 20 DAP where the bar represents 100 µm in the overview picture.

A0A287NDD5) as well as COP1 proteins (A0A287KUM9, A0A287T0X1) and other regulators (F2E4V3, A0A287HI31). Interestingly, this core set of proteins are functionally associated with diverse proteins including ESCRT related SKD1 protein (A0A287K2S5), autophasy-related protein 8c (M0YZY8), sorting nexins, as well as regulatory factors related proteins (GTPases, GTP binding proteins).

However, some of the identified functional groups clustered within each development phase of barley grain development, suggesting a temporal regulation of the identified mechanisms. For example, VSR1, which participates in vacuolar sorting of 12S globulins and 2S albumins in A. thaliana seeds39, was strongly localized in the aleurone layer instead of the starchy endosperm at ≥20 DAP (Supplementary Fig. 7a), indicating predominantly functional activity in the aleurone layer during development or during germination. The vacuolar processing enzymes (VPEs) are known to be involved in PCD40–42 as well as in processing SSPs in seeds43–45. Similarly, ESCRT-related proteins contained members of each development phase, indicating tissue-specific functions of proteins.

ESCRT-III HvSNF7 associates to MVBS at development phase I and both localize to and within PBs at development phase II and III. Proteomic analysis identified eight proteins related to ESCRT-0, ESCRT-III and SKD1 complex. Interestingly, they showed different expression patterns and subsequently belonged to different clusters (Fig. 6a).

To gain insight into the expression behavior of identified ESCRT members, we analyzed the transcript levels of HvTOLs, HvSNF7s, HvVPS20.1, and HvVPS4 during barley grain development by RT-qPCR (Fig. 6b). RNA was isolated from whole grains harvested at 6, 10, 12 and ≥ 20 DAP and the previously characterized most stable genes were used to normalize the ESCRT transcripts16. A high correlation between transcript and protein abundances for all identified HvESCRT members was observed, except for HvVPS4. Even though the HvVPS4 transcript follows the same trend as HvSNF7 transcripts and proteins, HvVPS4 protein increased, suggesting a delay of the response or a fine-tuning of HvVPS4 translation (Fig. 6b). These results indicate that the expression of HvESCRT is temporally regulated during barley grain development.

ESCRT originally refers to a protein–protein interaction network in yeast and metazoan cells that coordinates sorting of ubiquitinated membrane proteins into intraluminal vesicles (ILVs) of the MVB46–48. Specifically, ESCRT-III is known to be necessary for membrane remodeling that drives the biogenesis of MVBs49,50. Recent electron tomography studies in A. thaliana revealed that intraluminal vesicles form as large networks of interconnected or concatenated vesicles. AtSNF7 was detected in the intervesicle bridges, suggesting that ESCRT-III proteins remain trapped inside the vesicle cluster in MVBS and are finally delivered together with the cargo into the vacuole45–53. So far, no observations of HvSNF7 and MVBSs have been reported in barley endosperm tissues. Thus, the subcellular localization of HvSNF7 and the identification of MVBSs was performed. To elucidate the subcellular localization of HvSNF7, we first studied the localization of HvSNF7 in vivo at the early development stage using the transgenic line p6U::SNF7.1-mEosFP. Confocal live cell imaging of the p6U::SNF7.1-mEosFP transgenic line revealed punctual structures and few agglomerations around PBs at 6 DAP (Fig. 7a). AtSNF7.1 is known to form homodimers and thus can possibly lead to agglomerations54. Indeed, bimolecular fluorescence complementation (BiFC) and Yeast Two-Hybrid (Y2H) analyses revealed homodimerization of HvSNF7.1 (Supplementary Fig. 8a,b). We used an anti-SNF7 antibody to analyze the localization by immunofluorescence microscopy at 6 DAP where the observations of small punctual structures and few agglomerations from the live cell imaging could be confirmed (Fig. 7a). Transmission electron microscopy (TEM) analyses showed that grains at 6 DAP contained MVBSs in proximity to starch granules and PBs, as representatively displayed in Fig. 7a. As live cell imaging in developing endosperm is limited to early- and mid-development stages55, immunofluorescence analyses of sections prepared from 12 and ≥ 20 DAP were analyzed. Strong signals within PBs at 12 and ≥ 20 DAP could be detected (Fig. 7b). The punctured structures that could be observed inside the PBs possibly appeared by fusing of several smaller PBs (Fig. 7b). Additionally, a weak signal could be observed at the periphery of starch granules at 12 and ≥ 20 DAP (Fig. 7b). Indeed, TEM analyses identified MVBSs associated with PBs at 12 DAP and within fused PBs at ≥ 20 DAP (Fig. 7b). These findings indicate that HvSNF7 is first localized at vesicular structures (putatively at MVBSs) and later associated with single PBs and finally localized inside fused PBs.

Discussion

Proteomics and in situ microscopic analyses enable the mapping of the endomembrane system of developing barley endosperm. Many proteomic analyses in barley grain allow us to identify and understand the molecular composition of developing barley grain56–57. However, less is known about proteins regulating the endomembrane system in developing barley grains. Only one of the proteins identified in our study, Membrane steroid-binding protein 1 (F2CS48), was recently characterized in barley58. Recent studies have shown that the endomembrane system and SSP trafficking are spatio-temporally regulated in developing barley endosperm between 8 and 12 DAP13,15. This time-dependent regulation was confirmed by PCA analysis of our proteomics data, which revealed stage-specificity of protein expression during grain development.

A detailed analysis of the 94 endomembrane related proteins associated them with three development phases: I, II and III. Altogether with previously published work, correlation of proteomics data with in situ microscopic analyses allowed us to provide the first temporal map of endomembrane-related proteins involved in stage-specific
regulation of different endomembrane processes (Fig. 8): in development phase I, prolamin and glutelin RNAs are localized to two subdomains of the cortical ER, and targeted to the Golgi or to PBs by RNA-binding proteins and the cytoskeleton. Additionally, development phase I displays endocytic activities involving plasma membrane rearrangement. Such processes have been shown to be associated with specific cytoskeleton dynamics: besides the necessity of the plant cytoskeleton in vesicle trafficking and organelle movement, actin is required for the auxin-dependent convolution and deconvolution of the vacuole. Specifically, SNAREs, actin and its associated motor protein myosin shape the vacuole by actin-dependent constrictions. While the protein accumulation starts at 6 DAP with observable PBs at 8 DAP, the size of PSVs decreases between 8 and 10 DAP. As we could observe actin as well as tubulin associated with PBs, we conclude that the cytoskeleton proteins present at early stages of barley endosperm development regulate the PBs formation/trafficking and the size of PSVs. Concurrent development with the endocytic activity, microscopic analyses indicate an increase of the acidity within PBs. During development phase II, processes associated with the sorting system seem to be highly active. Recently, MVBs were discussed to be taken up into the vacuole by autophagy. Additionally, studies in A. thaliana root cells have shown that central vacuoles were derived from MVB to small vacuole transition and subsequent fusions of small vacuoles. Thus, we propose that MVBs loaded with HvSNF7 possibly contribute to PSV rearrangement events, resulting in large PSVs containing PBs associated with MVBs and HvSNF7, as it is known that PBs are taken up by PSVs at 10 DAP. Finally, during development phase III, PBs, MVBs, and HvSNF7 were found in proximity to the protein matrix at the periphery of starch granules, while the presence of the cytoskeleton was reduced.

Here, we point out the necessity to correlate proteomics data with microscopic analysis for reasons of spatio-temporal specificity. Although the bulk of the barley grain is mainly occupied by the starchy endosperm, we cannot exclude that identified proteins can reflect different spatial activity. For example, VPE4 was described to be most expressed in the pericarp of barley between 8 and 10 DAP, and to be necessary for programmed cell death execution in the developing pericarp, thereby being responsible for the grain size, starch and lipid content. As our proteomics data identified VPE4 to be most abundant at 6 and 10 DAP and subsequently to be grouped into development phase I, we assume the main function of VPE4 is in the pericarp. In contrast, VPE2b, c, and d, which are most abundant between 10 and 12 DAP and were grouped to development phase II, have been described to be involved in mucellar PCD. Thus, proteomics from dissected sections would be useful to identify tissue-specific proteins. In order to refine our analysis to a spatio-temporal level, we compared our dataset with previously published LMD-based proteomics analysis. However, only four out of our 94 identified endomembrane related proteins could be detected (F2DY31, Actin-depolymerization factor 4; A0A287NWD9, HvPDIL1-1;
This indicates a detection range limitation of proteomic analyses of dissected samples prepared for laser microdissection. Consequently, a combined analysis of proteomics together with microscopy appears as the most appropriate strategy.

It is worth mentioning that our data provide comprehensive coverage of the endomembrane-associated proteins involved in the rearrangement of the endomembrane system and protein trafficking. However, we cannot entirely exclude additional proteins involved in these processes or organelle-specific proteins involved in other
processes. For example, even though the importance of the Golgi in protein trafficking was shown previously in wheat in the transition between stage I and II69–71, only two Golgi-associated proteins could be found (Golgin 5 and CASP), both grouped to development phase I.

In order to define if Golgi-associated proteins are underrepresented in our dataset, we compared our data with previously identified proteins in A. thaliana72. Interestingly, we could explicitly identify more than 10 Golgi-resident proteins such as cell wall synthesis-associated proteins (e.g., UDP-glucuronic acid decarboxylase, A0A287H8Z0), which are parts of the glycosylation processes (Supplemental Table 2). It is possible that these identified proteins are active in aleurone, as previous data characterized the barley aleurone N-glycoproteome, in which numerous N-glycosylation sites were identified that play key roles in protein processing and secretion73.

How do ESCRTs and MVBs contribute to PB formation? Although MVBs were reported to be responsible for targeting proteins to the storage vacuole in maize aleurone cells74,75, and ESCRTs, proteins from the retromer complex, mediating the recycling pathway at the TGN and at the MVB76. Additionally, our proteomics data identified eight ESCRT proteins (four from ESCRT-0, three from ESCRT-III and one from the SKD1 complex) quantified over all three development stages. MVBs have been suggested to arise by TGN maturation77,78 with the support of Rab GTPases79. It is worth mentioning that several Rab GTPases were detected over all three different development stages, possibly supporting MVB maturation.

Additionally, ESCRT are known to be necessary to drive the formation of ILVs in MVBs46–48. Only few studies concerning ESCRT proteins are described in cereals: in maize, supernumerary aleurone layer1 (Sal1), that encodes the maize homolog of VPS46/CHMP1 (ESCRT-III associated), restricts aleurone cell identity to the outer cell layer of endosperm80. SAL1 preserves the proper plasma membrane concentration of DEFECTIVE KERNEL1 (DEK1) and CRINKLY4 (CR4), both involved in aleurone cell fate specification, by regulating the internalization and degradation of SAL1 positive endosomes81. The rice AAA ATPase LRD6-6, which is homologous to the AAA ATPase VPS4/SKD1, was identified as an interactor with OsSNF7b/c (Os06g40620/Os12g02830) and OsVPS2.2
(Os03g43860), supporting its putative MVB-mediated vesicular trafficking function. OsLRD6-6 is described to be localized at MVBs, to be required for MVB-mediated vesicular trafficking and to inhibit the biosynthesis of antimicrobial compounds for the immune response in rice.

Recently, the overexpressed ESCRT-III-associated component HvVPS60 was shown to be involved in protein targeting in developing barley endosperm. Here, seven of the identified ESCRT proteins were most expressed at development phase I and II, indicating a possible involvement in MVB body formation. The high abundances of the ESCRT-0 proteins, HvTOL1, HvTOL2 and HvTOL8 in the development stage I indicate early steps of cargo endocytic events to the vacuole: originally, nine Tom1 (target of Myb1) proteins with a domain structure similar to the VHS domain of ESCRT-0 were identified and it was speculated that these proteins are responsible to load the ESCRT machinery. Tom1 proteins were further characterized as members of the A. thaliana TOL family (TOM1-LIKE), which are able to bind ubiquitin directly and participate in the endocytic trafficking of plasma membrane proteins, such as the auxin efflux facilitator PIN2. HvTOL3, which was detected at development phase II, may have an additional/different function, as it was already previously speculated that AtTOLs can participate in different pathways of distinct endosomal systems of plants.
Our proteomics, RT-qPCR, microscopic and biochemical analyses showed a highly similar protein expression and transcription behaviour of both HvSNF7.1/2, which is reasonable as interaction studies showed heterodimerization. Snf7/VPS32 induce membrane curvature at MVBs by assembling into long spiral filaments and are discussed to be involved in corralling the ESCRT cargo at the vesicle bud. Given the transgenic line p6U::SNF7.1-mEosFP, which showed punctate structures and are most probably labelling MVBs, and the TEM analyses, we were able to detect MVBs around PBs at early barley endosperm development stages, and later within fused PBs.

To conclude, our proteomic approach combined with in situ microscopy, which focused on endomembrane-associated proteins isolated from four different stages of barley grain development, provide only a snapshot of the endomembrane system dynamics. Future studies will be needed to verify the temporal protein-protein interactions identified by the STRING analysis. Furthermore, it would be of interest to study the endomembrane system remodeling under abiotic stress conditions that are likely to affect the SSPs synthesis and/or the production of recombinant proteins. Finally, experimental proof of the involvement of cytoskeleton-related proteins, MVBs and ESCRT in sorting proteins to the PBs, ideally obtained by investigating mutant barley lines impaired in ESCRT function using proteomics and in situ microscopy, has yet to be provided. Nevertheless, the proteins that have been identified in association with the endomembrane system are useful targets for genetic engineering to modulate SSPs accumulation.

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**Author contributions**

V.R., J.H., S.R. and V.I. designed the experiments. V.R., J.H., M.W., S.R., A.S., C.G., B.D., G.D., P.-J.R., M.S. and V.I. conducted the experiments and analyzed data. E.S. discussed data and results. V.R. and V.I. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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