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Arabidopsis Plasmodesmal Proteome

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Abstract

The multicellular nature of plants requires that cells should communicate in order to coordinate essential functions. This is achieved in part by molecular flux through pores in the cell wall, called plasmodesmata. We describe the proteomic analysis of plasmodesmata purified from the walls of Arabidopsis suspension cells. Isolated plasmodesmata were seen as membrane-rich structures largely devoid of immunoreactive markers for the plasma membrane, endoplasmic reticulum and cytoplasmic components. Using nano-liquid chromatography and an Orbitrap ion-trap tandem mass spectrometer, 1341 proteins were identified. We refer to this list as the plasmodesmata- or PD-proteome. Relative to other cell wall proteomes, the PD-proteome is depleted in wall proteins and enriched for membrane proteins, but still has a significant number (35%) of putative cytoplasmic contaminants, probably reflecting the sensitivity of the proteomic detection system. To validate the PD-proteome we searched for known plasmodesmal proteins and used molecular and cell biological techniques to identify novel putative plasmodesmal proteins from a small subset of candidates. The PD-proteome contained known plasmodesmal proteins and some inferred plasmodesmal proteins, based upon sequence or functional homology with examples identified in different plant systems. Many of these had a membrane association reflecting the membranous nature of isolated structures. Exploiting this connection we analysed a sample of the abundant receptor-like class of membrane proteins and a small random selection of other membrane proteins for their ability to target plasmodesmata as fluorescently-tagged fusion proteins. From 15 candidates we identified three receptor-like kinases, a tetraspanin and a protein of unknown function as novel potential plasmodesmal proteins. Together with published work, these data suggest that the membranous elements in plasmodesmata may be rich in receptor-like functions, and they validate the content of the PD-proteome as a valuable resource for the further uncovering of the structure and function of plasmodesmata as key components in cell-to-cell communication in plants.
cytoskeleton (actin, myosinVIII, centrin [14,19,22,24,26,27,29]), ER-located calreticulin [12], and remorin [23]. For the latter, cell walls have proven to be an effective fraction enriched for PD. Hence, a cell wall fraction from tobacco led to the identification of a PD-located kinase [20], and 1-D gel electrophoresis of salt-eluted proteins from maize mesocotyl cell walls, identified a class 1 41 kDa reversibly glycosylated polypeptide (C1RGP2), which associated with PD following ectopic expression as a fluorescently-tagged fusion protein [25,28]. C1RGP2 is also a Golgi-associated protein; no function has yet been identified [15,28].

2-D gel separations of sub-cellular fractions from two cell types of Chara, differentiated by the presence and absence of PD, also identified a tropomyosin-like protein and RGP2 as PD-located proteins [18,30]. In a refinement of the earlier cell fractionation approaches, the Epel group [21] released PD from Arabidopsis cell wall fractions using cellulase, separated extracted proteins by 1-D gel electrophoresis, and identified a 45 kDa β-1,3 glucanase (named A. thaliana beta-1,3-glucanase_putative Pd-associated protein; AtBG_ppap) using in-gel proteolysis and ion-trap mass spectrometry.

Figure 1. Isolation of plasmodesmata. The basic structure of plasmodesma (PD) is illustrated in Panel A. In addition to the key physical elements of PM, ER, desmotubule in the wall, a speculative arrangement of actin spiralled around the desmotubule is shown. Panel B shows a negatively stained electron micrograph of membranous PD (pellet P2 in M&M) collected after release from the cell wall following cellulase digestion, while Panel C shows contamination of the PD with residual cell wall fibres, observed very occasionally. Scale bars = 100 nm. Panel D – Immunoblot analysis of fractions harvested during PD isolation procedure. Proteins extracted from whole cells, cell walls (pellet P1 in M&M) and purified PD (pellet P2 in M&M) were analysed using antibodies to the PD marker PDLP1, BIP (ER), Membrane11 (Golgi), PMA2 (PM) and P16 (chloroplast thylakoid envelope). While PDLP was enriched through the isolation procedure, the other proteins diminished and were virtually undetectable in the final PD preparation.

Total cell extract: proteins extracted from 6 μl of Arabidopsis cell suspension lysate (corresponding to 0.6 μl of purified cell wall). Cell wall extract: proteins extracted from 75 μl of purified cell walls (pellet P1). PD extract: proteins extracted from 375 μl of purified PD (pellet P2).
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In our previous work [13], we established the proteome for cell walls isolated from a culture of rapidly dividing *Arabidopsis* suspension cells, using two-dimensional liquid chromatography tandem mass spectroscopy (2D-LC MS/MS) of total extracted proteins. The proteome, which included secreted and non-secreted proteins, identified both known (e.g. AtBG_papp, RGP2, calreticulin) and unknown PD proteins. From a total of 69 PM-targeted membrane proteins lacking ER-retention signals [13], new PD proteins were identified and comprised two families of membrane-associated proteins, called PD-located proteins (PDLP; [31]) and PD-callose-binding proteins (PDCB; [11]). These were targeted to PD as fluorescently tagged protein fusions and modified molecular flux through the channel following altered protein accumulation. In this paper we describe the outcome of a combination of our previous strategy with that described by Levy et al [21], to purify and characterise PD from *Arabidopsis* suspension cells. After using a more sensitive nano-LC ion-trap MS/MS method, we report a list of identified proteins that best describes to date the structural and functional proteome of PD from *Arabidopsis*. The size and content of the list indicates that the very sensitive technologies still reveals the presence of contaminant proteins but also that the list contains many proteins with known or inferred association with PD. Also, again focussing on membrane-associated proteins and using subcellular targeting as the criterion, we report the identification of several new putative PD proteins. These include several receptor-like kinases and a tetraspanin and, together with the identification of receptor-like properties of the PDLP proteins [31], suggest that PD may represent a membrane domain rich in receptor functions.

**Results**

**PD isolation**

The value of proteomics is strongly correlated with the purity of the target in the samples analysed. For PD, this is a major challenge since the membrane-rich structures and callose collars are integral to the structure of the insoluble wall matrix. Previously, we used isolated cell walls from *Arabidopsis* suspension cultures as samples enriched for PD with respect to extraneous cellular components. To achieve a higher level of PD enrichment in this work, suspensions of cell walls were digested with a commercial unpurified cellulase preparation and the released membranous components collected by differential centrifugation. Cell wall digestion gave approximately 70% digestion of cell wall mass. This could not be increased by higher concentrations of enzyme or longer digestion periods. Addition of pectin degrading enzymes (e.g. polygalacturonase) reduced slightly the amount of residual cell wall but gave no measurable improvement in protein recovery (data not shown). Centrifugation of the digested mixture at ~6000g nevertheless separated the remaining visible insoluble material from small particulate material retained in the supernatant, which could be collected using higher speed centrifugation. Transmission electron microscopy of negatively stained samples of the smaller material revealed vesicle-like structures of 50–100 nm (Fig. 1B), which appeared to be composed of limited numbers of concentric membrane layers. Occasionally, samples were contaminated with residual fibrillar material, probably remnants of cell wall microbrils (Fig. 1C). Immunoblot analysis of samples collected sequentially during the PD isolation procedure showed that the membranous sample was substantially free of contaminant proteins representative of the endoplasmic reticulum (BiP), plasma membrane (PMA2), Golgi (membrane11), and chloroplast (thylakoid P16), whilst showing a corresponding increase in the abundance of PDLP1 (Fig. 1D).

**PD Proteomics**

As previously [13], we applied a LC-MS strategy to preparations of total protein extracted from purified PD; nano-LC-MS/MS experiments were performed on an LTQ-Orbitrap™ mass spectrometer. Since the aim was to determine the total protein compliment, consecutive runs were made until the novel protein detection was minimized (i.e. close to ~ >95% saturation). This series of runs (13 in total) also included minor modifications to the sample preparation (e.g. protease digestion conditions and length of LC separation) and several biological and technical replicates. For any one condition, reproducibility between technical replicates was approximately 60–70% and between biological replicates, approximately 50–70%. Protein identification was achieved by reference to the TAIR 8 database using MASCOT, SEQUEST and SCAFFOLD software. Using the criteria of greater than 99.0% probability of correct protein identification, for proteins identified with at least two unique peptides, the total number of proteins identified from these samples was 1341 (Table S1). We refer to this as the PD-proteome.

**Analysis of the PD-proteome**

To analyse the list of 1341 proteins, we used a number of bioinformatic tools, databases and literature sources, to obtain information about predicted subcellular localizations, and functional domains. Because of the high sensitivity of the Orbitrap mass spectrometer used, we anticipated the detection of PD proteins and a number of contaminant proteins. Given the acidic composition of the extracellular matrix and that our enriched PD fraction still contained very small amounts of undigested wall it was possible that cytoplasmic proteins, bound to the cell wall through ionic interactions, may also contribute to a pool of potential contaminant proteins. Although classifying a protein as a contaminant necessarily makes assumptions about the requirements for PD function, we judged that proteins from plastids, mitochondria, nuclei and some classes of cytoplasmic proteins would qualify. On this basis almost 35% of proteins were predicted to be contaminants, with chloroplast proteins being the most abundant (Fig. 2A). More than 10% (136) of all the proteins were ribosomal, which could have originated from cytoskeleton-bound polysomes anchored to the PM via actin filaments [32,33].

From information recorded in the Plant Proteome Database (PPDB) we have observed that almost 75% of the proteins in the PD-proteome have been described previously in other proteomic studies. Approximately 40% are represented in PM proteomes [34–40] and 12% in cell wall proteomes [13,41–43] (Table S2). In our and other proteomes, a significant number of proteins were recorded as being derived from multiple subcellular locations (Table S2).

The PD-proteome was analysed with respect to gene ontology (GO) terms for predicted functional categorization (represented by three main subcategories: ‘GO Cellular components’, ‘GO Molecular function’ and ‘GO Biological processes’) (Figure S1 and Table S3). To get a broad descriptive comparison with the Arabidopsis cell wall proteome, the Cellular Component subcategory of the GO was divided broadly into classes representing cell wall proteins, membrane proteins associated with the secretion pathway and potentially targeted to the cell periphery (Golgi, ER, PM and PD – secretory membrane proteins), cytoplasmic proteins (including, plastids, mitochondria, nuclei, cytosolic etc), and a group for which no prediction could be made (unclassified). These classes were compared with the cell wall proteome from *Arabidopsis* suspension culture cells [13] (Fig. 2B). GO classifications for single proteins may overlap between classes and so quantitative comparisons between classes could not be made. In comparison...
with our published Arabidopsis cell wall data, the PD-proteome showed a lower frequency of cell wall proteins and a higher proportion of membrane proteins, consistent with the removal of the cell wall by digestion before PD purification (Fig. 2B). Surprisingly, despite additional washes associated with isolating PD from digested cell walls the proportion of cytoplasmic proteins was similar to that found for purified cell walls. Overall, 53% of proteins present in the CW proteomic list were also present in the PD-proteome. The overlap contained 30% contaminants and ~20% membrane-associated proteins. The latter group included known PD proteins, PDLP1 [31], and AtBG_ppap [At5g42100; [21]].

Since PD represent membrane-rich structures, we analysed the predicted membrane proteins in more detail with respect to their domain structures and functions. Based upon prediction softwares (TMHMM [44] and MEMSAT-SVM [45] and searches in publicly available algorithms [e.g. Aramemnon, TAIR, Expasy and NCBI sites], and excluding contaminant proteins, there are 279 membrane proteins (proteins with one or more transmembrane domains (TMD) excluding the hydrophobic signal peptide, or a GPI anchor) in the PD-proteome, 21% of total proteins. The group of membrane proteins potentially targeted to the PM (i.e. with a signal peptide but lacking ER-retention signal) was subdivided into type I, type II, multiple TMD, and GPI-anchored proteins (Figure 3A). The most abundant sub-grouping was the multiple TMD proteins (38%), followed by Type II proteins (26%), Type I (23%) and GPI-anchor proteins (13%). For the type I class of membrane proteins, 49% are receptor-like molecules (many being receptor-like kinases; RLKs) and only 11% are involved in transport (Fig. 3B).

As yet we can make few predictions as to the functional categories of proteins that might occur tightly associated with PD. Connected with our increased understanding of the nature of molecules that transit the channel we might anticipate the presence of chaperones for proteins and nucleic acids and the potential for activities to provide energy for the transport process. However, in reality we have very little idea as to which molecular functions should be present.

Validation of the PD-proteome

The PD-proteome comprises 1341 proteins, a larger number than might have been predicted from parallels drawn between PD and the nuclear pore complex [46]. Despite the further purification of PD away from the cell wall, and the enrichment for membranous structures, we detected a significant number of apparent contaminant proteins derived from cytoplasmic components (including plastids, mitochondria, nuclei etc; Fig. 2A; Table S2). However, our proteome analysis was qualitative, not quantitative, and therefore does not reflect relative abundance. We reasoned that the low variability between replicates might be attributed to the large number of proteins detected with few (two or three) peptides, which in turn reflected the sensitivity of the Orbitrap detector and the presence of large numbers of proteins with low abundance. For these classes of proteins, detection might be stochastic and therefore variable between runs. Since the overall objective was to use purified PD to reveal the spectrum of novel proteins associated with PD, we also predicted that the proteome should contain known PD proteins and that these might be represented by the more abundant proteins. In theory, these should have been amongst those proteins detected with the largest number of tryptic peptides. The proteome contains a number of known PD proteins, i.e. PDLP1 and PDLP6 [31], β1-3 glucanase (AtBG_ppap; [21]) calreticulin [12,47] and remorin [23]. Surprisingly, these showed no correlation with the number of detected peptides (Fig. 4) indicating that, despite PD purification, these proteins may have been very different in their abundance in our suspension cells relative to the tissues in which they were first identified.
Previously, we focussed our attention on secreted membrane proteins lacking an ER-retention signal, mostly type I membrane- and GPI-anchored proteins, as an entry point for our search for novel PD proteins. For this work, in the absence of other indicators, we validated the authenticity of the PD-proteome by sampling a limited number of candidates from the class of membrane proteins theoretically targeted to the cell periphery for their potential to target to PD as fluorescent fusion proteins. We chose initially to sample a GPI-anchored β1-3 glucanase, following the precedent set by AtBG_gppap, and a number of the receptor-like proteins since these were abundant in the PD-proteome and the precedent set by AtBG_ppap, and a number of the receptor-to YFP or RFP following stable transformation into expressed, using the CaMV 35S promoter, as translational fusions expansin (At3g45970), which targeted to the apoplast and vacuole, or unknown functions was also tested (Table 1). Except for structure see [48]). The RLK1-like protein, respectively (For a review of RLK class class VIII RLK, an S-domain RLK and a Catharanthus roseus illustrated for At1g56145 in Fig. 5A). These genes encode LRR connections with the subtending mesophyll cells (example cases, maximum projections of CSLM image stacks revealed the interface between the lower epidermal wall and the subtending mesophyll cell were examined. At this latter location a face-on view of PD clusters in pit-fields is possible. Of the seven sampled proteins, three showed a pattern of fluorescence consistent with PD targeting (Table 1; Fig. 5). Of the six RLKs, three showed uniform labelling of the PM (Table 1; Fig. 5 with PM labelling with At5g59700 illustrated as an example); At4g27300 was targeted to the ER. In contrast, a punctate pattern of fluorescence, often combined with PM labelling, was identified for three of the RLKs: At1g56145, At4g21380, and At5g24010 (Fig. 5). In each case, the punctate fluorescence pattern showed co-localisation with callose (Fig. S2). These were proteins encoded by At3g15480 and At3g15600, At3g15480 has three TMDs included in a recognised domain (DUF 1218), but with no assigned function. At5g45600 encodes a tetraspanin TET3; TET3 has four TMD domains and has been implicated in the formation of specialised domains (tetraspanin webs) on the PM of animal cells [49].

**Discussion**

PD present particular challenges when it comes to their molecular characterisation. Their location, embedded in the cell wall matrix, their functional and structural diversity associated with different symplastic boundaries in complex tissues, and their essential nature in maintaining co-ordinated growth and development, makes their study recalcitrant to a range of biochemical and genetic approaches. We have found it effective to exploit the physical and developmental simplicity of rapidly dividing and readily dispersing suspension cells as a way of characterising relatively uniform populations of primary PD in purified cell walls [50]. PD released from cell walls after digestion of the wall matrix showed the membrane-rich nature of the structures, represented by the PM and ER components. Surprisingly, while immunoblot analysis revealed enrichment of PDLP1 in the purified PD fraction it did not detect significant amounts of marker proteins for PM or ER. This indicates that while the PM and ER provide membrane continuity between cells, the nature of these membranes within the PD might be distinct. The PM in PD has been defined elsewhere [23] as a domain with similarity to membrane rafts (or microdomains) characterised by the presence of remorin and GPI-anchor proteins that preferentially reside in sterol-rich membrane domains. The ER may also be distinct in that it is very tightly appressed, excluding the much of the lumen.

The low abundance of other marker proteins (for Golgi, chloroplasts etc. detected by immunoblotting) showed that the biochemical strategy followed was an effective method for purifying PD. It was surprising then that proteomics detected such a large number of proteins, many of which appeared to be contaminants (e.g. derived from other cytoplasmic compartments). One likely explanation is the higher sensitivity of the Orbitrap technical platform and the qualitative nature of the assay, where abundant and rare protein species are listed equally. An
alternative factor, however, is related to our relatively poor understanding of the nature and operation of PD. Only a few proteins have been shown to reside in PD and very few are believed to be uniquely associated with PD. Hence, although we have shown that PDLP1 is strongly localised to PD in leaves [31], we also find that it has a more dispersed localisation pattern in roots (unpublished data). Also, membrane proteins such as PDLP may arrive at PD via the secretory pathway [51] and therefore associate with the ER and Golgi in transit. Calreticulin and CTRGP2 are targeted to PD but are also associated with the Golgi [25]; remorin is similarly found in PD but is also distributed in patches along the PM [23]. In addition to the physical association with PD, there is a much larger selection of proteins that have a functional association with PD but are found predominantly at other subcellular locations. For example, the RNA helicase proteins ISE1 and ISE2, which both affect trafficking through

Figure 4. Distribution of known PD proteins in the total PD-proteome. In the hope of identifying potential PD proteins on the basis of the ease of proteomic detection (number of signature peptides), known PD proteins were placed upon a plot of the frequency of identified proteins against detected peptides. No positive correlation was found indicating that PD proteins are very variable in the abundance in PD.
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PD, and PD ontology [52], are found in mitochondria [53] and
cytoplasmic RNA granules respectively [54]. Also THIOREDI-
OXIN-m (TRXm), which profoundly influences PD gating and
development, is a plastidial enzyme [55]. It remains possible that
in particular cell types such proteins may have a transient
association with PD. Lastly, non-cell-autonomous proteins (e.g.,
some transcription factors; [56]) may have a transient interaction
with PD and may be captured when cells are plasmolysed prior to
cell wall and PD isolation. In summary, there is no a priori
reason why any protein in the PD-proteome list should be counted
initially as irrelevant in the context of PD structure/function,
although some (e.g. ribosomal proteins) intuitively may be less
likely candidates.

Encouragingly, the PD-proteome list contains a number of
the proteins, or likely orthologous proteins, for which experimental
evidence shows a physical protein association with PD; identifi-
cation of true orthologues, however, must remain speculative
pending appropriate experimental data (Table 2). Hence, PDLP1
and PDLP6, AtBG_papp, calreticulin, remorin, type III peroxi-
dases, several actins, and myosinVIII were identified in purified
PD. Additional proteins, identified using complementary biolog-
ical/experimental systems, have functions in common with
representatives in the PD-proteome. For many of these, evidence
shows a physical protein association with PD; identifi-
ability of some members of this family found that they interact with PD
and a role in the cell-to-cell and systemic transmission of redox
signals have been suggested [59,60]. The finding of these proteins
in the PD-proteome strengthens the hypothesis that cell redox
homeostasis is important for PD formation and function.

We are also increasingly appreciating the importance of redox
control in the regulation of callose at PD and its impact on cell-to-
cell communication [53,55]. While the size exclusion limit and
development have been shown to be regulated indirectly by redox
status mediated by proteins located in plastids (GAT1; [55]) and
mitochondria (ISE1, [53]), a more direct effect mediated by PD-
located type III peroxidases has been suggested [58]. Peroxidases
have been found by immunolocalization in the vicinity of PD and
their location correlates with the presence of H2O2 [58]. The PD-
proteome includes several class III peroxidases (AtPer12,
At1g71695; AtPer30, At3g21770; AtPer44, At4g26010; AtPer45,
At4g30170; AtPer57, At5g17820; AtPer69, At5g64100) which
potentially are candidates to function as ROS generators in PD.

A number of other proteins with the potential to regulate cell
redox status are also found in the PD-proteome. The list is
extensive and includes oxygenases, oxidases, oxidoreductases and
thiol redoxins. For example, we found two type II thioredoxins
(TRXH5; At1g5145 and TRXH3; At2g12980). Previous studies of
some members of this family found that they interact with PD
and a role in the cell-to-cell and systemic transmission of redox
signals have been suggested [59,60]. The finding of these proteins
in the PD-proteome strengthens the hypothesis that cell redox
homeostasis is important for PD formation and function.

Protein trafficking to and through PD requires the support of
molecules with chaperone like activity. HSP70 homologues
isolated from pumpkin [61] have been shown to contain a short
variable region (SVR) at the C-terminus at which the lack of a
threonin seems to be responsible for their translocation through
the PD [61]. A closterovirus–encoded HSP70 homologue
(HSP70h) is also essential for protein translocation through
PD [62]; HSP70- Arabidopsis homologues AtHSC70.1 and
AtHSC70.3 are present at the PD proteome (At5g02500 and
At3g09440 respectively) and they also lack the threonnine
aminocid at the SVR, showing higher homology with those
pumpkin HSC70 proteins that are able to facilitate transport
through PD. Very recently, a chaperonin protein was identified from a genetic screen for molecules that assist in the intercellular

PD deposition and turnover in the near-cell wall is central to
the regulation of PD size exclusion limit (SEL). Hence, some β,1,3
glucanases have a physical association with PD. Callose synthase 10
(GSL8) is specifically involved in callose deposition at PD
[7]. The PD-proteome contains callose synthases (At1g05570,
At1g03530, At2g38650), β,1,3 glucanases (AtBG_PPAP At5g12100,
At5g58090) and other enzymes described as participating in the
callose synthase complex (UDP-glycosyl transferases, At3g46650 &
At4g14090) [57].

| AGI      | MW      | Description                                      | Localisation |
|----------|---------|--------------------------------------------------|--------------|
| AT1G56145 | 112 kDa | LRR RLK                                          | PM and PD    |
| AT1G73650 | 34 kDa  | Hypothetical protein - predicted oxidoreductase  | PM           |
| AT3G15480 | 19 kDa  | Hypothetical protein containing a DUF1218        | PM and PD    |
| AT3G25290 | 43 kDa  | Auxin responsive family protein                  | PM and ER    |
| AT3G45600 | 32 kDa  | Membrane protein of unknown function - tetraspanin | PM and PD    |
| AT3G45970 | 29 kDa  | Expansin protein (ATEXLA1)                       | Apoplast and vacuole |
| AT4G16120 | 73 kDa  | ATSEB1 – GPI anchored                            | ER           |
| AT4G21380 | 96 kDa  | S-domain RLK                                     | PM and PD    |
| AT4G27300 | 92 kDa  | S-domain RLK                                     | ER           |
| AT3G14030 | 21 kDa  | Translocon-associated protein beta (TRAPB) protein | ER           |
| AT5G24010 | 92 kDa  | CrRLK-like                                       | PM and PD    |
| AT5G58090 | 52 kDa  | β-1,3-glucanase – GPI anchored                   | ER           |
| AT5G59700 | 92 kDa  | CrRLK-like                                       | PM           |
| AT5G60320 | 75 kDa  | Lectin RLK                                       | PM           |
| AT5G61790 | 60 kDa  | Calnexin1                                        | ER           |

*4LRR: Leucine rich repeat, RLK: Receptor-like kinase, DUF: Domain of unknown function, CrRLK: Catharanthus roseus RLK; ER: Endoplasmic reticulum, PD: Plasmodesmata; PM: Plasma membrane.

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transport of homeodomain containing proteins [31] raises the interesting prospect that receptors with the potential to sense extracellular signals, through their extracellular DUF26 domains, may influence the extent and/or specificity of cell-to-cell communication through PD. Although PDLP proteins lack an integral symplastic signalling module [e.g. an active kinase domain] they could signal into the PD by interaction with partner molecules providing the ancillary function. DUF26 receptor-like kinases have been shown to be responsive to salicylic acid [64] and DUF26 kinases are present in the PD-proteome (PDLP1, PDLP6 and a novel receptor-like kinase, At1g70520), although At1g70520 has not yet been tested for PD-targeting.

Other RLKs are also present in the PD-proteome and a limited survey of the potential for some of these to be PD-located proteins has identified three (At1g56145, At4g21380 and At5g24010) that target to PD when expressed transgenically as protein fusions to fluorescent markers. These proteins represent three new PD proteins to add to the current very limited list of PD components. The frequency (from a very limited survey) with which these proteins were identified suggests that the PD may represent a receptor-rich domain and points to a previously unrecognised potential for cell-to-cell communication to be influenced by factors in the extracellular environment. Very recently, Jo et al [65] reported preliminary evidence for the existence of six RLKs at PD in rice suspension culture cells. These RLKs comprise two wall-associated kinases, a lectin kinase and three LRR-kinases. None of these kinases were direct homologues of the proteins identified in this study. However, they do reinforce the view that PD represent a receptor-rich domain. Unfortunately, there is no evidence in the literature or from public collections of experimental data to indicate what the ligands for any of these receptors might be.

From our sampling of the membrane complement of the PD-proteome we also identified At3g15480 and TET3 as novel PD proteins. At present there are no indications as to the function of the protein encoded by At3g15480. In contrast, tetraspanins have been proposed in animal systems to define PM microdomains, called tetraspanin webs [66-68]. If equivalent structures also occur in plants, this may further indicate that PM in PD has a highly
specialised organisation. From work with remorin (also present in the PD-proteome), we know that the PM passing through PD may also contain membrane raft microdomains [23,69] and it has been proposed that membrane microdomains may provide the correct environment for clustering of receptor-like activities [49,66,68,70]. The ER membrane contained within the desmotubule may also be defined by the presence of specific proteins. Reticulons are proteins that are associated with ER morphology, specifically in

### Table 2. List of previously described PD proteins and their related proteins in the PD-proteome.

| Std. Annotation | Acc. No. | No. Unique peptides | % Protein Coverage |
|-----------------|----------|---------------------|-------------------|
| **Proteins and orthologous proteins of known PD proteins** | | | |
| PDLP1 | At5g43980 | 8 | 28.4 |
| PDLP6 | At2g01660 | 4 | 12.8 |
| AtBG_PAP | At5g42100 | 5 | 19.6 |
| Callose synthase 10 (GSL10) | At2g36850 | 16 | 11.5 |
| Actin 1 | At2g37620 | 2 | 42.4 |
| Actin 3 | At3g53750 | 2 | 42.4 |
| Actin 7 | At5g09810 | 16 | 55.7 |
| Actin 8 | At1g49240 | 6 | 48.5 |
| Actin 11 | At3g12110 | 2 | 48.8 |
| Myosin VIIIA | At1g50360 | 2 | 3.12 |
| Myosin IXK | At5g20490 | 3 | 3.69 |
| **PD-related proteins with functions in common with representatives in the PD proteome** | | | |
| Calreticulin | At1g56340 | 3 | 8.25 |
| AtPME1 (Pectin methyl esterase) | At1g53840 | 2 | 4.44 |
| Pectinesterase putative | At2g47030 | 2 | 3.06 |
| AtPME26 (Pectin methyl esterase) | At3g14300 | 2 | 3.1 |
| Pectinesterase putative | At4g19410 | 2 | 10.2 |
| Pectinesterase putative | At5g45280 | 2 | 6.22 |
| AtPAP10 (Purple acid phosphatase) | At2g16430 | 20 | 53.6 |
| AtPAP14 (Purple acid phosphatase) | At2g46880 | 5 | 16.1 |
| Acid phosphatase class B | At1g04040 | 14 | 52.4 |
| Acid phosphatase class B | At5g44020 | 7 | 40.8 |
| HSC70.1 (Heat shock cognate 70) | At5g02500 | 22 | 39.2 |
| HSC70.3 (Heat shock cognate 70) | At3g09440 | 5 | 27.6 |
| CalS1 (Callose synthase 1) | At1g05570 | 2 | 8.26 |
| ATGSL5 (Glucan synthase-like) | At4g03550 | 11 | 7.81 |
| Glycosyl hydrolase 17 protein | At3g55430 | 2 | 6.46 |
| eIF4A-1 | At3g13920 | 2 | 29.4 |
| eIF4A-2 | At1g54270 | 15 | 36.7 |
| UDP-glucuronosyl (glycosil transferase) | At3g46650 | 2 | 8.22 |
| UDP-glucuronosyl (glycosil transferase) | At4g14090 | 2 | 5.26 |
| AtPer12 (Class III peroxidase) | At1g71695 | 5 | 18.4 |
| AtPer30 (Class III peroxidase) | At3g21770 | 2 | 10.9 |
| AtPer44 (Class III peroxidase) | At4g26010 | 6 | 21.3 |
| AtPer45 (Class III peroxidase) | At4g30170 | 3 | 10.2 |
| AtPer57 (Class III peroxidase) | At5g17820 | 7 | 32.3 |
| AtPer69 (Class III peroxidase) | At5g64100 | 2 | 9.37 |
| Thioredoxin H3 | At5g42980 | 2 | 28 |
| Thioredoxin H5 | At1g45145 | 2 | 16.9 |
| **DUF26 domain proteins** | | | |
| PDLP1 | See above | | |
| PDLP6 | See above | | |
| Protein kinase | At1g70520 | 2 | 4.93 |

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the constriction of ER tubules [71] and the identification of reticulons B3 and B6 (At1g64090 and At3g61560) in the PD-proteome raises the hypothesis that these proteins play a role in the constriction of the desmotubule. Further fractionation of PD into its constituent membrane components (ER, PM and membrane rafts) would be a feasible practical strategy for more formal testing of these hypotheses.

Value of the PD-proteome

PD have been notoriously difficult to dissect with respect to their protein constituents. The existence of actual, inferred and experimentally validated PD proteins in the PD-proteome is testament to its potential in helping to overcome this barrier to understanding the structure/function properties of PD. For our experimental validation we selected a subset of 15 proteins to test for subcellular targeting. This selection was not completely random so does not allow extrapolation to the wider range of proteins with respect to the abundance of actual PD proteins. Nevertheless, the frequency of new PD proteins is highly encouraging. Our experimental analysis focussed on membrane proteins although membrane proteins constituted only 21% of the total. Our definition of membrane proteins was one that required an integral association and it seems very likely that some non-membrane proteins or loosely associated membrane proteins could also reside in PD, especially if they form complexes with integral membrane proteins. The value of the proteome data is extended through the use of alternative sources of complementary data. For example, by using publically available resources for gene expression (http://attd.jp; [72]) and protein-protein interaction (AtPID, http://atpid.biosino.org/) data new functional networks of proteins can be proposed that raise testable hypotheses. In summary, this PD-proteome provides the community with a valuable resource for cross-referencing from other PD-related experimentation or for the generation of new hypotheses about the functioning of these important cellular structures.

Materials and Methods

Preparation of plasmodesmata

Cell wall fractions from a rapidly dividing Arabidopsis thaliana (ecotype Landsberg erecta) cell suspension cultures [50] were treated with cell wall-degrading enzymes as described by Levy et al. [21] with modifications. Briefly, purified cell walls [50] were digested (1 ml per g of cell culture) with 0.7% w/v of cellulase R10 (Karlan) in digestion buffer (10 mM MES, pH 5.5, 4.4% mannitol) [21] and a cocktail of protease inhibitors (Sigma) for 2 h at 37°C with 100 rpm shaking. After centrifugation at 5860 g for 5 min at 4°C, the supernatant and pellet (P1) fractions were collected separately. P1 was washed in digestion buffer and the two supernatants combined before centrifugation at 75600 g for 40 min. The pellet was washed (10 mM MOPS, pH 7.5, 4.4% mannitol) and the final pellet (P2) resuspended in a minimal volume of buffer.

Immunoblot analysis

Proteins from total cell homogenates and PD fraction were directly solubilised by boiling in 1X Laemmli buffer [73] for 5 min. Proteins from suspension culture cell walls were extracted sequentially in aqueous- and phenol-based buffers, as described previously [50]. Precipitated proteins were recovered by centrifugation, washed twice with 100 mM ammonium acetate in methanol and four times with 80% acetone. The protein pellet was left to air dry, then resuspended into 1X Laemmli buffer for 5 min. Proteins were separated using 10% SDS-polyacrylamide gel electrophoresis then blotted to PVDF membranes and analysed with anti-serum specific for PDLP1 (1/1250; [31]), immunoglobulin-binding protein (IbP) (1/8000; [74]), Membrane11 (1/1400; antibody provided by A.Hocquellet, L. Maneta-Peyret & P. Moreau.), plasma membrane H+-ATPase (PM2) (1/16000; [75]) and P16 (1/20000; [76]). Specific binding was visualised by standard techniques.

Proteomic analysis

The protein pellet following extraction from isolated PD was dissolved in either a minimal volume of 8M urea, 0.1 M Tris-HCl, pH 8, or 0.5% Rapigest (Waters), 50 mM ammonium bicarbonate. Rapigest samples were heated in a boiling water bath for 5 min. All samples were reduced, alkylated, and digested with trypsin according to standard procedures. Digestion was halted by addition of trifluoroacetic acid and 0.5%. Rapigest was removed according to the manufacturer’s protocol. Samples, digested in urea, were purified using OMIX® C18 tips (Varian Inc., Santa Clara, USA) before loading to the nanoLC.

Nano-LC-MSMS experiments were performed on an LTQ-Orbitrap™ mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA 02454, USA). For nanoLC, two different systems were used: an Accela™ HPLC (Thermo) with a flow splitter or a nanoAcquity UPLC™ (Waters, Manchester, UK). The LC systems were run at a flow rate of 250 nL min⁻¹ and coupled to the mass spectrometer via an ion source (Proxeon, Odense, Denmark) with a nanospray emitter (SilicaTips™, 10 μm, New Objective, Woburn, MA 01801, USA). Samples were dissolved in 0.1% TFA and, on the nanoAcquity system, peptides were trapped using a pre-column (Symmetry® C18, 5 μm, 180 μm × 20 mm, Waters) which was then switched in-line to an analytical column (BEH C18, 1.7 μm, 75 μm × 250 mm, Waters). Other runs were performed with the Accela™ HPLC (Thermo) equipped with a trap column (C18 PrepTip™, Dionex, Camberley, UK) and a self-packed analytical column (BEH C18, 1.7 μm, Waters, 75 μm × 200 mm). Peptides were separated and eluted with a gradient of 5–45% acetonitrile in water/0.1%formic acid at a rate of 0.2% min⁻¹.

Mass spectrometry was operated in positive ion mode at a capillary temperature of 200°C. The source voltage and focusing voltages were tuned for the transmission of MRFA peptide (m/z 524) (Sigma-Aldrich, St. Louis, MO). Data-dependent analysis was carried out in Orbitrap-IT parallel mode using CID fragmentation on the seven most abundant ions in each cycle. Collision energy was 35, and an isolation width of two was used. The Orbitrap was run with a resolution of 30,000 over the range of m/z 350 to m/z 2000 with an MS target of 10⁶ and 1 s maximum scan time. The MS2 was triggered by a minimal signal of 2000 with an AGC target of 3 × 10⁶ ions and 100 ms scan time.

For selection of 2+ an 3+ charged precursors, charge state and monoisotopic precursor selection was used. Dynamic exclusion was set to 1 count and 30 s exclusion time with an exclusion mass window of ±20 ppm. MS scans were saved in profile mode while MSMS scans were saved in centroid mode.

Tandem mass spectra were extracted by BioWorks version 3.3.1 and mgf files were generated using a perl script (Matrixscience). All samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot 2.2) and Sequest (ThermoFinnigan, San Jose, CA; version 27, rev. 15).

Both Sequest and Mascot were set up to search the TAIR8 (20080413, 33024 entries) database, and both searches were done with a parent ion mass tolerance of 5.0 ppm and a fragment ion mass tolerance of 0.50 Da. Iodoacetamide derivative of cysteine was specified in Mascot and Sequest as a fixed modification.
Oxidation of methionine was specified in Mascot and Sequest as a variable modification. Trypsin was designated as the protease and up to two missed cleavages were allowed. Tair 8 uses database entries for the Col-0 ecotype. Although our biological source material was L-er ecotype, database entries for the L-er ecotype are substantially fewer. L-er was selected for the suitability of the suspension culture for the biochemical purification of cell walls. The small sequence differences between Col-0 and L-er could have resulted in slightly fewer peptides being identified but this was outweighed by the benefit of the utility of the L-er suspensions and the larger database resources for Col-0.

Scaffold (version Scaffold_2.04.00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [77]. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [78]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. To calculate false discovery rates (FDR), the file was loaded into Scaffold version 3.00.3, and with the specified settings a protein FDR of 0.1% and peptide FDR of 5.3 was obtained. This Scaffold file has been lodged with TRANCHE (https://proteomecommons.org/tranche/). RAW Mascot files have been lodged with TRANCHE, and data is available in the PRIDE database [79] (www.ebi.ac.uk/pride). The data was converted using PRIDE converter [80] (http://code.google.com/p/pride-converter).

**Protein sequence feature prediction**

Feature predictions for protein sequences in the proteomic output were automated using local installations of several software packages and Perl scripts. Because of the importance to this study of identifying likely transmembrane domains we used two independent programs; TMHMM [44] for fast processing of candidates and then a second evaluation of TMHMM-positives using the MEMSAT-SVM [45] tool. This has been shown to be more accurate [45] but is more computationally intensive, relying on a PSI-BLAST [81] search (versus UniProt [82]). MEMSAT-SVM explicitly attempts to identify signal peptides, and in conjunction with results of SIGNALP-HMM [83,84] these helped to highlight possible false-positive TM regions near the N-terminus. Both TMHMM and MEMSAT-SVM predict not only positions of TM-domains, but also their topology; the end of each predicted TM-segment is predicted to be ‘inside’ (cytoplasmic) or ‘outside’ (extracellular, or in the ER lumen depending on the context). Here, we use ‘Type I’ to denote those proteins with a single predicted TM domain with the N-terminus outside and ‘Type II’ to denote those predicted single-TM domain proteins with the N-terminus inside. ‘Multiple TMD’ denotes those with multiple TM domains. Additionally, we applied programs to predict subcellular location (TARGETP [85]) and chloroplast transit peptides in particular (CHLOROP [86]), and GPI-anchoring signals (DGPI [87]). We also used our own Perl script to search for C-terminal tetrapeptides (HDEL, KDEL, REEL) indicating possible ER-retention. For individual candidates, especially those with a GPI-anchor where fluorescent reporters were inserted internally in the coding region, additional information was collected using Aramemnon and tools available through TAIR, Expasy and NCBI.

We obtained further functional annotations of our dataset from the MapMan [88] and Gene Ontology (GO) [89] resources. Each protein was placed in one of the MapMan “bins”, using the online search facility of the Plant Proteome Database (PPDB, [90]). Note that terms attached by the GO Consortium to genes/proteins summarize what is known from published experimental and/or computational studies, as well as the results of automated electronic annotation. It is therefore possible for seemingly contradictory terms to be attached to the same protein, even when supported by experimental evidence (for example, when a protein has been identified in independent published studies of two different organelles). Assessing GO terms of our proteins is nevertheless useful for obtaining an overview of the functional and spatial profile of a large dataset. To this end, we used the GO Plant Slim developed by The Arabidopsis Information Resource [91], rather than the highly detailed, complete Gene Ontology when comparing proteome data sets.

**Comparison of the PD-proteome with other published proteomes**

At a basic level, the online search facility of the Plant Proteome Database (PPDB, [90]) was used to compare proteins identified in the plasmodesmal proteome with proteins listed within the Proteomic Publications collection. For specific comparisons with our previously published [15] Arabidopsis cell wall proteome (89 secreted proteins from a total of 792 proteins) we compared amino acid sequences since the databases use different identifiers for the same sequence. We looked for matching proteins by aligning pairs of proteins between sets using NEEDLE from the EMBoss package [92] with a conservative global pairwise identity threshold of 95%.

**Gene cloning and expression**

Clones for the transient and transgenic expression of *Arabidopsis* genes were generated using Gateway technology (Invitrogen). Gene sequences were amplified by PCR using Phusion DNA polymerase (NEB) from a genomic DNA or cDNA made from the aerial tissues of *Arabidopsis thaliana* Col-0 plants, using Gateway adapter primers; primer sequences are available upon request. Resulting DNA fragments were recombined into the entry vector pDONR207 (Invitrogen). The sequence of the resulting pDONR clone was verified by automated sequencing. Validated entry clones were recombined with binary destination vectors pB7FWG2.0, pB7RWG2.0 or pB7YWG2.0 clone [93] providing expression from *Agrobacterium* T-DNA, using the cauliflower mosaic virus 35S promoter upstream of coding fusions to green fluorescent protein (GFP), red fluorescent protein (RFP) or yellow fluorescent protein (YFP), respectively. GPI-anchored proteins were tagged internally with m-Citrine following published protocols [94]. Binary clones in *Agrobacterium tumefaciens* GV3101 were used for plant transformation [95].

**Confocal microscopy**

Plant tissue was imaged at room temperature using a Zeiss LSM510 confocal microscope with an Argon ion laser. GFP and YFP were excited at 488 nm, and the emitted light was captured at 495–520 nm and 525–650 nm respectively. RFP was excited using 561 nm and emitted light captured at 590–630 nm. Images were captured digitally and handled using the Zeiss LSM image browser software. For callose staining, seedlings or mature leaves were infiltrated with 0.1% aniline blue solution. Aniline blue fluorochrome was excited at 405 nm and emitted light captures at 420–480 nm. Sequential scanning was used to image aniline blue with YFP or RFP.

**Supporting Information**

Figure S1 Gene ontology (GO) terms for the predicted functional categorization of the PD-proteome. The three
main subcategories are represented: Cellular components (A), Molecular function (B) and Biological processes (C).

**Figure S2** Colocalisation of fluorescent puncta with callose. Leaf tissues stably expressing fluorescent protein fusions (left panel) were stained with aniline blue (centre panel) to identify sites of callose deposition. Colocalisation of the fluorescence (right panel) supports these fluorescent puncta as the location of PD on the wall. Similar patterns of staining were seen for proteins encoded by At1g56145 (A), At3g15480 (B), At3g15600 (C), At4g21380 (D) and At3g24010 (E). Bar = 10 μm.

**(TIF)**

**Table S1** Complete list of PD-proteome sequence identities (1341) with associated the proteomic information. *It should be noted that when paralogous proteins could not be distinguished, all were included.*

**(XLS)**

**Table S2** PD-proteome with MapMan Bin functional categories and predicted information on subcellular localisation and description of proteins in the Public Proteome collection (PPDB) (ProteomicsPub. Column). a CHLORIL = Chloroplast protein; MIT = Mitochondrial protein; VACUOL = Vacuolar protein; S = Secreted; SM = Secretory membrane; NSnoTM = Nonsecreted no transmembrane protein; NSTM = Non secreted transmembrane protein; GPI = GPI anchor protein.

**Table S3** PD-proteome with Gene Ontology descriptions. *Comp = Cellular component; Proc = Biological processes; Func = Functional categories. RAW Mascot files have been lodged with TRANCHE (https://proteomecommons.org/tranche/), and protein and peptide identifications with associated spectra have been lodged with ‘PRIDE (http://www.ebi.ac.uk/pride/easySubmitData.do).***

**(XLS)**

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**Author Contributions**

Conceived and designed the experiments: LF-C CF GS EB YA AM. Performed the experiments: LF-C CF GS EB YA AM. Analyzed the data: LF-C CF JW GS EB YA AM. Contributed reagents/materials/analysis tools: LF-C CF JW GS EB YA AM. Wrote the paper: LF-C CF YA AM.

**References**

1. Lucas WJ, Ham IK, Kim JY (2009) Plasmodesmata - bridging the gap between neighboring plant cells. Trends Cell Biol 19: 495–503.
2. Opara KJ (2004) Getting the message across: how do plant cells exchange macromolecular complexes? Trends Plant Sci 9: 33–41.
3. Faulkner C, Maule A (2011) Opportunities and successes in the search for plasmodesmal proteins. Proteoplasma 248: 27–38.
4. Overall RL, Blackman LM (1996) A model of the macromolecular structure of plasmodesmata. Trends Plant Sci 1: 307–311.
5. Borba CE, Cross RI (2000) Towards reconciliation of structure with function in plasmodesma-whose is the gatekeeper? Micron 31: 713–721.
6. Delmer DP, Volokita M, Solomon M, Fritz U, Delphendahl W, et al. (1993) A monoclonal antibody recognizes a 65 KD higher plant membrane polypeptide which undergoes cytokinesis-dependent association with callose synthesis in vitro and colocalizes with site of high callose deposition. Proteoplasma 176: 35–42.
7. Guseman JM, Lee JS, Bogenschutz NL, Peterson KM, Virata RE, et al. (2010) Dysregulation of cell-to-cell connectivity and stomatal patterning by loss-of-function mutation in Arabidopsis CHORUS (GLUCAN SYNTHASE-LIKE 9). Development 137: 1731–1741.
8. Levy A, Gueoune-Gelbart D, Epel BL (2007) Beta-1,3-glucanases: Plasmodesmatal Gate Keepers for Intercellular Communication. Plant Signal Behav 2: 494–497.
9. Northcote DH, Davve R, Lay J (1989) Use of antiser to localize callose, xylan and arabinogalactan in the cell-plate, primary and secondary walls of plant cells. Planta 178: 355–366.
10. Radford JE, Vesk M, Overall RL (1997) Callose deposition at plasmodesmata. Proteoplasma 201: 30–37.
11. Simpson C, Thomas C, Findlay K, Bayer E, Maule AJ (2009) An Arabidopsis GPI-Anchor Plasmodesmal Neur Protein with Callose Binding Activity and Potential to Regulate Cell-to-Cell Trafficking. Plant Cell 21: 581–594.
12. Baluska F, Sajmi J, Napier R, Volkmann D (1999) Maize calreticulin localizes preferentially to plasmodesmata in root apex. Plant J 19: 481–488.
13. Bayer EM, Bottrill AR, Walshaw J, Vigooroux M, Naldret MJ, et al. (2006) Arabidopsis cell wall plasmodesma defined using multidimensional protein identification technology. Proteomics 6: 301–311.
14. Blackman LM, Overall RL (1998) Immunolocalisation of the cytoskeleton to plasmodesmata of Chara corallina. Plant J 14: 733–741.
15. Dhugga KS, Tiscari SC, Ray PM (1997) A reversibly glycosylated polypeptide (RGPI) possibly involved in plant cell wall synthesis: Purification, gene cloning, and trans-Golgi localization. Proc Natl Acad Sci U S A 94: 6769–6774.
16. Ding B, Kwon MO, Warnberg L (1996) Evidence that actin filaments are sites of callose deposition. Colocalisation of the fluorescence (right panel) supports these fluorescent puncta as the location of PD on the wall. Similar patterns of staining were seen for proteins encoded by At1g56145 (A), At3g15480 (B), At3g15600 (C), At4g21380 (D) and At3g24010 (E). Bar = 10 μm.
17. Ding XS, Liu J, Cheng NH, Fominov A, Hou YM, et al. (2004) The Tobacco mosaic virus 126-kDa protein associated with virus replication and movement suppresses RNA silencing. Mol Plant Microbe Interact 17: 583–592.
18. Faulkner CR, Blackman LM, Cordwell SJ, Overall RL (2005) Proteinic identification of putative plasmodesmatal proteins from Chara corallina. Proteomics 5: 2866–2873.
19. Golomb L, Abu-Ahied M, Belauoev E, Sadot E (2008) Different subcellular localizations and functions of Arabidopsis myosin VII. BMC Plant Biol 8: 3.
20. Lee JY, Taoka K, Yoo BC, Ben-Nissan G, Kim DJ, et al. (2005) Plasmodesmal-associated protein kinase in tobacco and Arabidopsis recognizes a subset of non-cell-autonomous proteins. Plant Cell 17: 2817–2831.
21. Levy A, Erlander M, Rosenthal M, Epel BL (2007) A plasmodesma-associated beta-1,3-glucanase in Arabidopsis. Plant J 49: 669–682.
22. Radford JE, White RG (1998) Localization of a myosin-like protein to plasmodesmata. Plant J 14: 743–750.
23. Raffaele S, Bayer E, Lafarge D, Chazet S, German Retana S, et al. (2009) Remorin, a solanaceae protein resident in membrane rafts and plasmodesma, impairs potato virus X movement. Plant Cell 21: 1541–1555.
24. Reichli S, Knight AE, Hodge TP, Baluska F, Samaj J, et al. (1999) Characterization of the unconventional myosin VIII in plant cells and its localization at the post-cytokinetin cell wall. Plant J 19: 553–567.
25. Sagi G, Katz A, Gueoune-Gelbart D, Epel BL, (2005) Class I reversibly glycosylated polypeptides are plasmodesmal-associated proteins delivered to plasmodesmata via the Golgi apparatus. Plant Cell 17: 1780–1800.
26. Van Gesteld K, Slegers H, Von Wirsich M, Samaj J, Baluska, F, et al. (2003) Immunological evidence for the presence of plant homologues of the actin-related protein Arp3 in tobacco and maize: subcellular localization to actin-enriched pit fields and emerging root hairs. Protoplasma 222: 45–52.
27. White RG (1994) Actin associated with plasmodesmata. Protoplasma 180: 169–184.
28. Zavavev R, Sagi G, Gera A, Epel BL (2010) The constitutive expression of Arabidopsis plasmodesmal-associated class 1 reversibly glycosylated polypeptide impairs plant development and virus spread. J Exp Bot 61: 131–142.
29. Blackman LM, Harper JD, Overall RL (1999) Localization of a centrin-like protein to higher plant plasmodesmata. Eur J Cell Biol 78: 297–304.
30. Faulkner CR, Blackman LM, Collings IA, Cordwell SJ, Overall RL (2009) Anti-tropomyosin antibodies co-localise with actin microfilaments and label plasmodesmata. European J Cell Biol 88: 357–369.
31. Thomas CL, Bayer EM, Ritzenenthaler C, Fernandez-Calvino L, Maule AJ (2008) Specific targeting of a plasmodesmal protein affecting cell-to-cell communication. Plos Biology 6: 180–190.
32. Heidke J, Jodar D, Johannesen A, Partridge K, Pryme I, et al. (1996) Enrichment of specific mRNAs in cytoplasmic and membrane-bound polysomes in Chinese hamster ovary cells. Biochem Soc Trans 24: 1878.
33. Medalia O, Weber I, Frangakis A, Nacastro D, Gerich J, et al. (2002) Macromolecular architecture in eukaryotic cells visualized by cryoelectron tomography. Science 298: 1209–1213.
34. Benschop JJ, Mohammed S, O’Flaherty M, Heck AJ, Slijper M, et al. (2007) Towards reconciliation of structure with function in plasmodesma-whose is the gatekeeper? Micron 31: 713–721.
35. Thomas CL, Bayer EM, Ritzenenthaler C, Fernandez-Calvino L, Maule AJ (2008) Specific targeting of a plasmodesmal protein affecting cell-to-cell communication. Plos Biology 6: 180–190.
36. Heidke J, Jodar D, Johannesen A, Partridge K, Pryme I, et al. (1996) Enrichment of specific mRNAs in cytoplasmic and membrane-bound polysomes in Chinese hamster ovary cells. Biochem Soc Trans 24: 1878.
36. Elortza F, Mohammed S, Bunkenborg J, Foster LJ, Nuhse TS, et al. (2006) Modification-specific proteomics of plasma membranes: identification and characterization of glycosylphosphatidylinositol-anchored proteins released upon phospholipase D treatment. J Proteome Res 5: 953–963.

37. Marmagne A, Ferro M, Meunier T, Brulé C, Kuhl L, et al. (2007) A high content in lipid-modified peripheral proteins and integral receptor kinase features in the arabidopsis plasma membrane proteome. Mol Cell Proteomics 6: 1869–1966.

38. Marmagne A, Rouet MA, Ferro M, Rolland N, Alcon C, et al. (2004) Identification of new intrinsinc proteins in Arabidopsis plasma membrane proteome. Mol Cell Proteomics 3: 675–691.

39. Nuhse TS, Stembhalle A, Jensen ON, Peck SC (2003) Large-scale analysis of in vitro phosphorylated membrane protein complexes from modified Arabidopsis thaliana cell suspension cultures. Plant Physiol 131: 1434–1446.

40. Boudart G, Janet E, Lafitte C, Rossignon M, Jaunaye A, et al. (2003) Proteomics of loosely bound cell wall proteins of Arabidopsis thaliana cell suspension cultures. Phytochemistry 64: 3412–3432.

41. Boudart G, Janet E, Rossignon M, Lafitte C, Boudart G, et al. (2005) Cell wall proteins in apoplastic fluids of Arabidopsis thaliana rosettes: identification by mass spectrometry and bioinformatics. Proteomics 5: 212–221.

42. Miniz J, Janet E, Negroni L, Arseny de Garbadan P, Zivy M, et al. (2007) A sub-proteome of Arabidopsis thaliana thaliana stomata trapped on Concanavalin A is enriched in cell wall hydroxyl glycosides. J Exp Bot 58: 2503–2512.

43. Krog A, Larsson B, von Heijne G, Somnhammer ELL (2001) Predicting transmembrane protein topology with a hidden Markov model: Application to complete genomes. J Mol Biol 305: 567–580.

44. Nugent T, Jones DT (2009) Transmembrane protein topology prediction using support vector machines. BMC Bioinformatics 10: 159.

45. Minic Z, Jamet E, Negroni L, Arsene der Garabedian P, Zivy M, et al. (2007) A sub-proteome of Arabidopsis thaliana mature stems trapped on Concanavalin A is enriched in cell wall hydroxyl glycosides. Protoplasma 225: 93–102.

46. Opatka KJ (1994) Structure and function of the receptor-like protein kinases of higher plants. Plant Mol Biol 26: 1599–1609.

47. Charrin S, le Naour F, Silvie O, Milhiet PE, Boucheix C, et al. (2009) Lateral transfer as epi to the callosome complexes. Nat Rev Mol Cell Biol 10: 95–97.

48. Chen MH, Tian GW, Gafni Y, Citovsky V (2005) Effects of calreticulin on viral cell-to-cell movement. Plant Physiol 139: 1066–1076.

49. Walker JC (1994) Structure and function of the receptor-like protein kinases of higher plants. Plant Mol Biol 26: 1599–1609.

50. Charrin S, Le Naour F, Silvie O, Milhiet PE, Boucheix C, et al. (2009) Lateral organization of membrane proteins: tetraspanspin their web. Biochem J 410: 133–154.

51. Levy S, Shehata T (2005) The tetraspanin web modulates immune-signalling complexes. Nat Rev Immunol 5: 136–140.

52. Yanez-Mo M, Barreiro O, Gordon-Alonso M, Sala-Valdes M, Sanchez-Madrid F (2009) Tetraspanin-enriched microdomains: a functional unit in cell biology. Plant J 59: 434–446.

53. Mengrand S, Stanislas T, Reyner EM, Lherminier J, Simon-Plas F (2010) Plant membrane rafts in plant cells. Trends Plant Sci 15(12): 656–663.

54. Zappel NF, Panstruga R (2008) Heterogeneity and lateral compartmentalization of plant plasma membranes. Current Opin Plant Biol 11: 632–640.

55. Benitez-Alfonso Y, Jackson D (2009) Redox homeostasis regulates plasmodesmal transport through plasmodesmata. Planta 205: 12–22.

56. Elortza F, Mohammed S, Bunkenborg J, Foster LJ, Nuhse TS, et al. (2006) Heat shock cognate 70 chaperones carries a motif that facilitates trafficking through plasmodesmata. J Virol 82: 2836–2843.

57. Karimi M, Inze D, Depicker A (2002) GATEWAY (TM) vector system for plant transformation. Plant Cell 14: 1911–1921.

58. Charrin S, Le Naour F, Silvie O, Milhiet PE, Boucheix C, et al. (2009) Lateral organization of membrane proteins: tetraspanspin their web. Biochem J 410: 133–154.