

Arabidopsis leaf plasma membrane proteome using a gel free method: Focus on receptor-like kinases

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Abstract

The hydrophobic proteins of plant plasma membrane still remain largely unknown. For example in the *Arabidopsis* genome, receptor-like kinases (RLKs) are plasma membrane proteins, functioning as the primary receptors in the signaling of stress conditions, hormones and the presence of pathogens form a diverse family of over 610 genes. A limited number of these proteins have appeared in protein profiles. The detection of these proteins and thus the determination of their dynamics and tissue specificity, is technically challenging due to their low abundance and association to a lipid membrane. To identify new putative membrane proteins especially receptor systems, we used a gel free proteomic strategy based on mass spectrometry analyses of a plasma membrane fraction enriched in hydrophobic proteins. We produced from *Arabidopsis* leaf a highly purified plasma membrane fraction with the aqueous two-phase partitioning technique. By separating the proteins in the plasma membrane fraction with ion exchange and reverse phase chromatography and analyzing the resulting fractions on a MALDI-TOF mass spectrometer, over 900 proteins were detected. The plasma membrane proteome generated by this approach contains numerous plasma membrane integral proteins, one-third displaying at least four trans-membrane segments. An *in silico* analysis shows a correlation between the putative functions of the identified proteins and the expected roles for plasma membrane in transport, signaling, cellular traffic and metabolism. Of these proteins, 304 were annotated as membrane proteins, 69 were RLKs, distributed among the different receptor families in proportions reflecting the distribution in the genome. Of the RLKs that were identified, most are reported for the first time at the protein level and will constitute interesting targets for further functional studies.

Key word: *Arabidopsis*, Plasma Membrane, Protein, Receptor-Like Kinase.

Introduction

About 20–30% of all genes in an organism code for membrane proteins including plasma membrane (PM). The plasma membrane forms the

barrier between the cell and the environment also forming the passageway for information from the outside to the inside of the cell. In plant cells, as well as in animal cells, the PM is involved in many primary cellular functions, such as

metabolite and ion transport, endocytosis, cell differentiation and proliferation. Integral plasma membrane proteins are also involved in the perception of abiotic and biotic challenges to the plant. Consequently, to achieve these functions, a large variety of proteins is necessary and exists in plasma membrane. Plasma membrane proteins can have very different chemical-physical properties and can be present in highly distinct abundances. For example proteins such as aquaporins or the proton pumping ATPase (H^+ -ATPase) are highly abundant and proteins, such as receptors or other regulatory proteins, are typically very low abundant proteins. Estimates indicate that there can be protein abundance differences of 3 to 4 orders of magnitude (Eriksson and Fenyö 2007). The low abundance proteins such as receptor-like kinase or phosphatases are usually suppressed by the more abundant ones in protein identification and profiling. In the protein identification of complex protein samples by high-resolution 2-DE or by gel-independent techniques, usually only the most abundant proteins are identified by subsequent mass spectrometry. Because the limited resolution of the available separation techniques, applied in protein profiling and expression analysis, proteins with a low copy number can only be revealed after using additional fractionation processes at the protein or peptide level. In order to achieve this goal, methods such as protein or peptide affinity purification (Hara 2009), chromatographic protein pre-fractionation (Mc Nulty and Annan 2009), zoom gels of narrow pH ranges for 2-DE and preparative protein isoelectrofocusing (Righetti *et al.* 2004), as well as subcellular fractionation (Haynes and Roberts 2007) or multidimensional peptide separations (Fournier *et al.* 2007) are used. These techniques are used for purification or enrichment of proteins and characterization of proteomes of organelles, such as nucleus, mitochondria, the golgi apparatus, lysosomes, exosomes, and plasma membranes in different organisms (Warnock *et al.* 2004, Marmagne 2008, Fagioni *et al.* 2009). Nevertheless, to date the published proteomics data on the plasma membrane of *Arabidopsis* only

cover subsets of the full set of plasma membrane proteins.

It is illustrative that, based on the analysis of *Arabidopsis* genome there are more than 610 RLKs (Shiu and Bleeker 2001), while only a limited number of the proteins of these RLK genes have appeared in protein profiling studies up till now. Marmagne *et al.* (2008) identified 49 RLKs using two different washing methods for preparing the hydrophobic plasma membrane derived from an *Arabidopsis* cell suspension culture. Before their work, only 23 RLKs had been reported in proteomic studies (Alexandersson *et al.* 2004, Dunkley *et al.* 2006).

In the present study, we used two dimensional liquid chromatography Ms/MS(2DLC-MS/MS) for *Arabidopsis* leaf plasma membrane in order to obtain a comprehensive plasma membrane protein profile, specifically aimed at the detection of low abundance proteins such as RLKs.

Material and Methods

Plant material preparation

Arabidopsis plants were grown under controlled conditions in soil at a short day regime, (8 hours light/16h dark) and a daily temperature at 21 °C and night temperature at 19 °C. Leaf material was collected after 6 weeks.

Plasma membrane isolation

Plasma membranes were purified essentially by aqueous polymer two-phase partitioning as described by Larsson *et al.* (1987) with the flowing change. The whole preparation procedure was performed at 4°C. The plasma membrane pellet, containing 1.5-2 mg of protein, was resuspended in 0.5 ml of resuspension medium, frozen in liquid nitrogen and stored at -80° C until further use. Protein concentration was determined according to Bradford (1977) with bovine serum albumin (BSA) as standard. The aqueous two-phase partitioning method (Larsson *et al.* 1987) is well-established technique for the isolation of a plasma membrane fraction. Nevertheless, cross-contamination with membranes from other compartments or non-membrane (cytosolic) proteins will always occur to some degree. A

washing step with NaOH or sodium bicarbonate could be used to remove soluble proteins and enrich the hydrophobic membrane protein fraction. In the current procedure this additional washing step was omitted as it might also remove, low abundant plasma membrane proteins like RLKs that have just one transmembrane domain and possess relatively big extracellular and intracellular domains.

Two dimensional liquid chromatography and MALDI-TOF-MS/MS

In two dimensional liquid chromatography (2DLC), strong cation exchange and reverse phase chromatography were used. For off-line peptide pre-fractionation, a silica-based Polysulfoethyl Aspartamide strong cation exchange column was used (PolyLC Inc., Columbia USA). The separation was run on an Ettan-MDLC system (Amersham Biosciences AB, Uppsala, Sweden) at a flow rate of 200 μ L/min. Buffer A: 10 mM $\text{KH}_2\text{PO}_4\text{-H}_3\text{PO}_4$, pH 2.7, 25% acetonitrile and buffer B: 10 mM $\text{KH}_2\text{PO}_4\text{-H}_3\text{PO}_4$, pH 2.7, 25% acetonitrile, 1 M KCl was used for elution. Labelled samples were resuspended in buffer A prior to loading. Peptide elution was performed with a step gradient from 3 to 12% B in 12 column volumes, followed by 12 to 30% B in 3 column volumes. Fractions were collected every 45 sec in 96-well plates. Eluted peptides were concentrated in a vacuum centrifuge and diluted with TFA to a final concentration of 0,1%. Depending on the complexity, either separate fractions or pools of two fractions were analyzed by RP-LC MALDI-TOF as described above, with the only exception that no internal standard were used for MS spectra calibration.

Protein identification and data mining

Protein identification was carried out using Mascot (version 2.1, Matrix Science, London, UK) and ProteinPilot (Applied Biosystems, Foster City, CA, USA) searching against a the TAIR7 release of the *Arabidopsis* proteome (www.arabidopsis.org), combined with reversed entries for all protein sequences. Peptide tolerance was set to 0.2 and 0.4 Da for MS and MS/MS

respectively, allowing for 1 missed trypsin cleavage. Oxidation of methionine residues, deamidation of asparagine and glutamine were specified as variable modifications. Protein identifications based on at least 2 peptides, identified independently with probability higher than 95%, were accepted.

Predictive methods

For functional annotation databases search were performed using both PPDB (ppdb.tc.cornell.edu/), and *Arabidopsis* TAIR database (ftp.arabidopsis.org/home/tair/). Information such as; GRAVY, molecular weights and signal peptide from TargetP annotations were retrieved from PPDB (ppdb.tc.cornell.edu/). Predictions for membrane-spanning regions were achieved by using the TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>). Robust predictions of GPI anchors were retrieved from the ARAMEMNON database that compiles three prediction programs (big-PI, DGPI, and GPI-SOM). Localization data based on green fluorescent protein/yellow fluorescent protein experiments were obtained from SUBA (<http://www.suba.bcs.uwa.edu.au>).

Results and Discussion

Identified proteins and general characteristics

With a confidence level > 99%, more than 900 proteins were identified (Due to limited pages, data were not shown), Distribution of total identified proteins based on transmembrane domain presence and the function distribution was shown in figure1. 762 were identified with a confidence level > 99.9% by a minimum of two unique peptides, each with more than 95% probability. The remaining proteins were identified with a confidence level > 99%, mostly with two or more peptides. When we compare the plasma membrane proteins identified in the current study with published datasets (Alexandersson *et al.* 2004, Marmagne *et al.* 2004, Marmagne *et al.* 2008, PPDB database) the two phase partition plasma membrane purification followed by 2DLC results in the most comprehensive profile so far, even though some of

the proteins identified by other methods are not present in our profile. With more than 900 proteins that were identified, this study has yielded the largest dataset on the plant plasma membrane proteome so far. Although contamination with endomembrane derived proteins was apparent, the largest proportion of proteins was likely of plasma membrane origin.

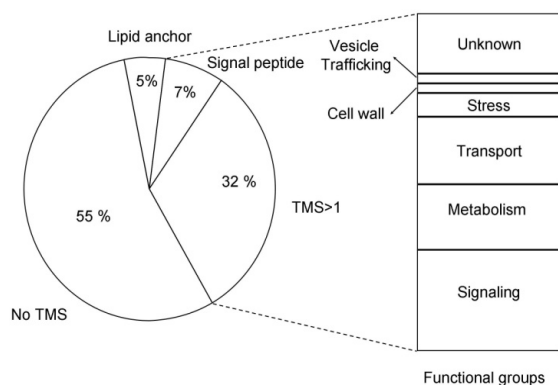


Figure 1. Distribution of total identified proteins based on transmembrane domain presence and the function distribution. More than 900 proteins were identified. Proteins were annotated using PPDB and TAIR database. Abbreviation: TMS= transmembrane segment

Using on TargetP (www.cbs.dtu.dk/services/TargetP/), 304 of the identified proteins are predicted to be membrane proteins. Among them 177 are indicated to be secreted proteins. 66 are mitochondrial and 61 are chloroplastic proteins. According to SUBA (<http://www.plantenergy.uwa.edu.au/suba2/>) 143 proteins of all identified proteins in this study have been used previously in GFP/YFP-based (green florescent protein / yellow florescent protein) localization experiments. More than 40% of these proteins were localized in the plasma membrane (figure 2).

Based on sequence homologies with known proteins and on identified functional domains, 75% of the proteins could be assigned to a putative functional class and/or biological process. According to prediction analysis and annotated information, all identified proteins could be placed in three large functional groups: transport,

signaling and metabolism (data were not shown). Using the TMHMM prediction programs (<http://www.cbs.dtu.dk/services/TMHMM/>), 32% of all the identified proteins had at least one (but up to 16) transmembrane domain (figure 3).

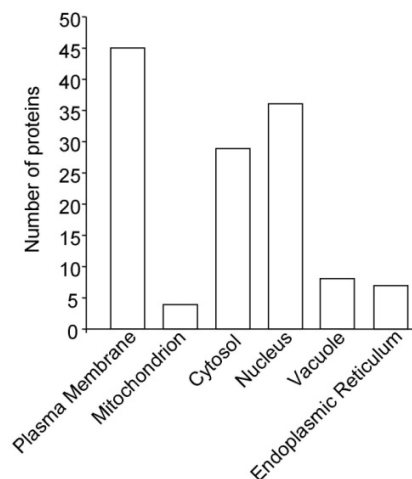


Figure 2. Cellular distribution of the identified membrane proteins. The identified proteins were compared with data retrieved from SUBA (<http://www.plantenergy.uwa.edu.au/suba>)

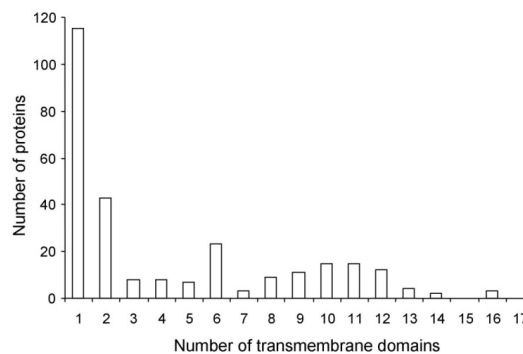


Figure 3. Number of transmembrane domains present in the identified membrane proteins. The number of transmembrane domains was predicted using the TMHMM prediction programs (<http://www.cbs.dtu.dk/services/TMHMM/>).

Of the more than 900 identified proteins in the obtained profile, more than 30% are predicted to be integral membrane proteins and have at least one transmembrane domain.

Proteins that were identified in the profile, but have no transmembrane domain, might be anchored to the PM through myristoylation or prenylation (GTP-binding proteins) or by other uncharacterized means like for instance P24 (Marmagne *et al.* 2004). The fairly high number (146) of unknown proteins is an illustration of our still sketchy knowledge of the plasma membrane composition, mainly due to the technical difficulties in extraction, separation and identification of hydrophobic proteins by mass spectrometry. As mentioned before in general, we can classify the proteins that were identified into

three main groups: 1) signal transduction and cellular trafficking proteins, 2) transport proteins and 3) proteins involved in metabolism.

Receptor-like kinases, other signaling and cellular traffic components

More than 140 protein kinases were totally identified in the profile. Among them 69 could be identified with high confidence (at least 2 peptides and >99 % confidence of protein identification) as an RLK protein (table 1). The distribution of identified RLKs over the different subfamilies correlates with the distribution of RLKs found in

Table 1. List of identified RLKs with confidence level $\geq 99\%$.

No.	Accession number	Common name	RLKs class	M.W.	Number of unique peptides
1	AT2G23200	-	CrRLK1L-1	93	6
2	AT3G46290	-	CrRLK1L-1	91	6
3	AT5G38990	-	CrRLK1L-1	98	12
4	AT5G54380	-	CrRLK1L-1	93	2
5	AT1G70520	-	DUF26	72	4
6	AT1G70530	-	DUF26	72	2
7	AT4G23140	CRK6	DUF26	75	3
8	AT4G23180	CRK10	DUF26	74	16
9	AT4G23250	EMB1290	DUF26	112	8
10	AT1G15530	-	L-Lectin	73	4
11	AT2G37710	-	L-Lectin	76	13
12	AT4G02420	-	L-Lectin	75	3
13	AT2G37050	-	LRR I	104	5
14	AT4G33430	BAK1	LRR II	68	4
15	AT1G48480	RKL1	LRR III	71	3
16	AT2G26730	-	LRR III	72	11
17	AT3G02880	-	LRR III	68	16
18	AT3G08680	-	LRR III	69	5
19	AT3G17840	RLK902	LRR III	70	12
20	AT5G16590	-	LRR III	67	11
21	AT5G67200	-	LRR III	74	2
22	AT1G66150	TMK1	LRR IX	102	2
23	AT2G01820	-	LRR IX	102	2
24	AT3G23750	-	LRR IX	-	8
25	AT1G53730	-	LRR V	78	2
26	AT3G14350	-	LRR V	78	6
27	AT1G06840	-	LRR VIII-1	104	3
28	AT5G49760	-	LRR VIII-1	105	23
29	AT1G07650	-	LRR VIII-2	113	4
30	AT1G53430	-	LRR VIII-2	114	16
31	AT1G53440	-	LRR VIII-2	115	2
32	AT1G56120	-	LRR VIII-2	115	4
33	AT1G56140	-	LRR VIII-2	114	2
34	AT3G14840	-	LRR VIII-2	109	14
35	AT1G27190	-	LRR X	65	7
36	AT3G28450	-	LRR X	67	6
37	AT4G39400	BRI1	LRR X	131	3
38	AT5G48380	-	LRR X	69	7

Table 1. continued

No.	Accession number	Common name	RLKs class	M.W.	Number of unique peptides
39	AT2G31880	-	LRR XI	71	7
40	AT5G65700	BAM1	LRR XI	109	2
41	AT4G08850	-	LRR XII	115	15
42	AT5G46330	FLS2	LRR XII	129	4
43	AT2G23770	-	LysM	67	2
44	AT3G21630	-	LysM	67	4
45	AT1G52290	-	PERK-L	56	3
46	AT3G24550	ATPERK1	PERK-L	69	9
47	AT1G52540	-	RLCK	40	3
48	AT3G51550	-	RLCK	98	7
49	AT4G23220	-	RLCK	61	2
50	AT5g20050	-	RLCK	115	2
51	AT4G35230	-	RLCK II	57	10
52	AT5G59010	-	RLCK II	55	3
53	AT5G25440	-	RLCK III	35	2
54	AT1G01540	-	RLCK V	52	5
55	AT4G02630	-	RLCK V	55	3
56	AT5G18500	-	RLCK V	54	2
57	AT2G17220	-	RLCK VII	46	3
58	AT5G13160	PBS1	RLCK VII	50	2
59	AT5G47070	-	RLCK VII	47	6
60	AT1G06700	-	RLCK VIII	40	9
61	AT1G48210	-	RLCK VIII	40	7
62	AT2G30740	-	RLCK VIII	41	14
63	AT2G47060	-	RLCK VIII	40	5
64	AT3G59350	-	RLCK VIII	46	7
65	AT3G26700	-	RLCK XI	42	2
66	AT1G11330	-	SD-1	94	6
67	AT1G11350	-	SD-1	93	2
68	AT2G19130	-	S-domain2	92	4
69	AT1G21250	WAK1	WAKL	81	10

the *Arabidopsis* genome, an indication that the gel-free procedure used potentially allows the identification of all different classes of RLKs and has no bias towards certain classes (figure 4).

Based on sequence homology the *Arabidopsis* genome codes for more than 610 RLKs, but at the protein level, only few RLKs have been characterized. In the previous studies only 23 RLKs had been reported in proteome studies (Alexandersson *et al.* 2004, Dunkley *et al.* 2006). Marmagne *et al.* (2008) identified 49 RLKs, including 26 previously not detected ones in the plasma membranes of an *Arabidopsis* cell suspension culture. In the present study 69 RLKs were identified in the plasma membrane of *Arabidopsis* leaves (table 1). These identified RLKs include the well-characterized RLKs,

BAK1 (bril-associated receptor kinase), BRI1 (brassinosteroid Insensitive 1), FLS2 (Flagellin sensitive 2) and TMK1 (transmembrane kinase 1).

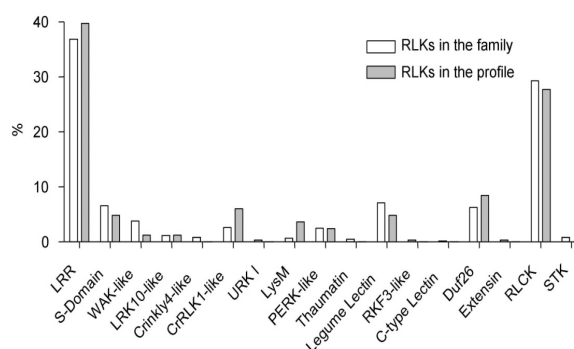


Figure 4. Distribution of identified RLK subfamilies in the profile and in the RLK family. Each RLK subfamily was retrieved from paper Shiu and Blecker (2001).

The plasma membrane localization of these well-characterized receptors has been confirmed by GFP fusions. However, for most of the identified RLKs there is no information about their role, tissue specificity, developmental stage-dependent production or intracellular localization. The distribution of identified RLKs over the distinctive subfamilies in the profile corresponds to the distribution over the subfamilies in the genome (figure 4), indicating that the preparation of the samples does not exclude the identification of certain types of RLKs.

Four calcium-dependent protein kinases (CDPKs) were also identified, several of which are known to be associated with the plasma membrane (Dammann *et al.* 2003). Several groups of soluble proteins, such as CDPKs, which are putative regulators of plasma membrane proteins (Alexandersson 2004), were found. We identified about 25 ribosomal proteins. It is suggested that polysomes are linked to actin filaments, which in turn are associated with the plasma membrane (Medalia *et al.* 2002).

Several identified proteins are involved in membrane trafficking between the Golgi apparatus and the plasma membrane. Syntaxins are involved in the formation of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE). Of the syntaxins the following members were identified: SYP121, 122 and 132 (three syntaxins of the SYP1 subfamily) SYP22 and SYP71. By using a GFP-tag, these syntaxins were shown to be associated with the plasma membrane in *Arabidopsis* cell suspension cultures (Uemura *et al.* 2004). Alpha-SNAP2, also identified in the protein profile, is involved in disassembling the SNARE complex after membrane fusion has occurred. Also, other proteins, which are considered to be involved in membrane trafficking were identified: ADP-ribosylation factor 1 (ARF1), the nodulin-like protein, clathrin heavy and light chains, ADL3 and SC3 (Alexandersson *et al.* 2004)

Transport proteins

As expected, transporters, pumps, and channels were identified in the plasma membrane. Of the P-

type H⁺-ATPase, (one of the most prominent integral proteins in the plasma membrane) five of its isoforms, AHA1, AHA2, AHA3, AHA6 and AHA11, were detected with high confidence. The AHA3 isoform, present in the current profile, was earlier demonstrated in leaf tissue (Arango *et al.* 2003), where it is located in the plasma membrane of phloem companion cells. However, AHA4 that was identified in the plasma membrane proteome by Alexandersson *et al.* (2004) could not be detected in the present study.

Out of the 13 plasma membrane aquaporins (PIPs) present in the *Arabidopsis* genome, 9 were identified. The localization of PIP1-2, PIP2-1 and PIP2-7 to the plasma membrane have been confirmed by GFP fusion proteins. Some transport proteins that are not endogenous to the plasma membrane such as the H⁺-PPase (AVP1), two ABC-transporters (PDR8 and MDR11), V-ATPase subunits and one TIP isoform (TIP2), usually regarded as vacuolar membrane proteins (Heazlewood *et al.* 2004), were also identified in our plasma membrane profile.

Metabolism

Enzymes involved in primary or secondary metabolism were also identified in the profile, but only a few of these have transmembrane domains. Some are associated with the plasma membrane (e.g. enolase and glyceraldehyde-3-phosphate dehydrogenase), but others are highly abundant, soluble proteins and therefore might be contaminants in the plasma membrane fraction. Most of the enzymes in plasma membrane fraction are biotic and abiotic stress-regulated enzymes. For example, six ERD (early response to dehydration) gene products were found (Froehlich *et al.* 2003).

Conclusion

In general conclusion the analysis resulted in the identification of 69 RLK proteins with a high confidence level, most of which were detected at the protein level for the first time and for most there is no functional annotation present. The data exemplify that the combination of plasma membrane enrichment, peptide purification with

strong cation exchange and reverse phase chromatography and analysis of the resulting fractions on a MALDITOF mass spectrometer, is a powerful tool for retrieving RLKs.

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