**Title:** Integrating RNA-seq and plasma membrane proteomics upon flagellin perception in *Arabidopsis* reveals novel regulators of innate immunity

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**Abstract (~200 words)**

The plant plasma membrane (PM) is a central regulator of plant responses to biotic and abiotic stress. We employed RNA-seq and high-resolution shotgun proteomics of PM-enriched fractions to study temporal changes over a twelve hour period in *Arabidopsis* leaf tissue after activation of the FLS2 immune receptor. A large percentage of (>25%) of the leaf transcriptome was mobilized within one hour of flg22 treatment. Over 4600 proteins were consistently identified in PM fractions across multiple samples, representing the largest dataset of plant PM-associated proteins to date. In contrast with the transcriptome, protein changes at the PM were gradual, with around 6% of proteins changing abundance in PM fractions by twelve hours after flg22 treatment. The receptor-like kinase (RLK) superfamily and membrane transporters represent the two largest classes of differentially expressed genes and proteins. Temporal expression clustering identified temporal waves of gene expression after FLS2 activation. Using a combination of protein-protein interaction network analysis and phosphorylation mapping we identified and functionally characterized novel proteins that regulate plant immunity, including a cysteine-rich receptor kinase and a calcium binding protein. This study represents one of the largest-scale proteomic investigations of biotic stress responses in any organism and serves as a resource for future hypothesis-driven inquiries of plant immune responses.

**Introduction**

Every plant is a potential host for a variety of microorganisms and the plant innate immune system is a critical regulator of plant growth and survival. Some of the largest gene families encoded in plant genomes are largely devoted to pathogen detection and many of these immune receptors localize to the plasma membrane (PM) ([Dodds and Rathjen, 2010](#_ENREF_24)). In addition to the primary receptor, other proteins associated with the PM, either integrally or peripherally, function in immediate downstream signaling and the sustained defenses that limit pathogen spread ([Dodds and Rathjen, 2010](#_ENREF_24)). Taken together with the fact that many classes of plant pathogens complete their lifecycle outside the host cell membrane, the PM plays an essential role in plant-microbe interactions.

Plants recognize conserved molecular patterns present in the apoplast of infected tissue using PM-localized receptors, often with receptor-like kinase (RLK) domain architecture ([Dodds and Rathjen, 2010](#_ENREF_24)). Activation of these pattern recognition receptors (PRRs) leads to rapid (<15 min) induction of a conserved set of immune markers. These early defense outputs include transient induction of mitogen-activated protein kinase (MAPK) cascades, accumulation of reactive oxygen species (ROS), cytosolic ion fluxes (particularly Ca2+), and activation of Ca2+-dependent protein kinases ([Tena et al., 2011](#_ENREF_108); [Monaghan and Zipfel, 2012](#_ENREF_78)). MAPK signaling cascades converge at the nucleus, where MPK3/6 phosphorylate WRKY transcription factors to up-regulate the expression of defense-associated genes within 30 minutes of pathogen perception ([Asai et al., 2002](#_ENREF_4); [Tena et al., 2011](#_ENREF_108)). Later responses include vesicle trafficking to sites of pathogen perception, secretion of antimicrobial compounds, and formation of papillae containing callose (a β-1,3-glucan) and protein in the cell wall ([Underwood and Somerville, 2013](#_ENREF_109)). All major plant hormones have been implicated in controlling the plant immune response at multiple levels and evidence exists for a variety of sophisticated positive and negative feedback networks ([Pieterse et al., 2009](#_ENREF_86); [Kim et al., 2014](#_ENREF_51)). Collectively, this suite of responses, termed pattern-triggered immunity (PTI), suppresses pathogen growth in infected tissue.

The *Arabidopsis* PTI receptor Flagellin-sensing 2 (FLS2) is a model for studying immune responses activated by plant cell surface receptors. FLS2 is a leucine-rich repeat RLK (LRR-RLK) that directly binds a conserved epitope of bacterial flagellin (flg22). Co-receptors from the Somatic embryogenesis receptor kinase (SERK) family contribute to FLS2 signaling ([Roux et al., 2011](#_ENREF_94)), with biochemical and structural studies demonstrating that Brassinolide-insensitive 1-associated kinase 1 (BAK1/SERK3) rapidly heteromizes with the flg22-bound FLS2 ([Chinchilla et al., 2007](#_ENREF_15); [Heese et al., 2007](#_ENREF_36); [Sun et al., 2013](#_ENREF_102)). The FLS2-BAK1 complex phosphorylates the receptor-like cytoplasmic kinase (RLCK) Botrytis-induced kinase 1 (BIK1) to initiate intracellular signaling ([Lu et al., 2010](#_ENREF_69); [Lin et al., 2014](#_ENREF_67)). BAK1 and BIK1 are required for immune signaling activated by multiple PRRs ([Monaghan and Zipfel, 2012](#_ENREF_78); [Liu et al., 2013](#_ENREF_68)). The requirement of BAK1/BIK1, the large overlap in immediate downstream signaling events, and similar defense gene expression activated by different receptors in response to diverse ligands suggests that 1) the plant immune system rapidly converges on a common set of responses and 2) several aspects of FLS2 function may be generalized to signaling activated by other PRR RLKs. Although recent work has revealed a detailed mechanism of FLS2 activation at the PM, how downstream signaling proteins interface with early immune outputs has remained elusive.

Mass spectrometry(MS)-based approaches have been instrumental in identifying protein complex constituents and phosphorylation dynamics and of the hormone receptor BRI1 ([Tang et al., 2010](#_ENREF_105); [Clouse, 2011](#_ENREF_19)) and the immune receptors FLS2 and EFR ([Roux et al., 2011](#_ENREF_94); [Kadota et al., 2014](#_ENREF_44); [Li et al., 2014](#_ENREF_63); [Lin et al., 2014](#_ENREF_67)). Phosphoproteomics profiling of PM fractions after FLS2 activation identified global important modified residues on proteins that control cell membrane potential and the apoplastic ROS burst ([Benschop et al., 2007](#_ENREF_8); [Nuhse et al., 2007](#_ENREF_84)). In addition, analysis of detergent-resistant PM membrane (DRM) fractions after flg22 treatment demonstrated that several RLKs including FLS2 rapidly (<15 min) associate with DRM preparations, suggesting dynamic changes in membrane protein compartmentalization contribute to early immune signaling ([Keinath et al., 2010](#_ENREF_50)). While these studies have been instrumental in deciphering specific PM protein regulatory events in response to pathogen perception, to date there has been no global investigation into how PM protein signaling unfolds over time in response to activation of PTI.

Here we present the results of temporal profiling of gene expression using RNA-seq and PM-associated protein abundance using high resolution LC-MS/MS over a twelve hour window following activation of FLS2-PTI. Our deep profiling of PM fractions identified over 4900 proteins, representing the largest inventory of PM-associated proteins for any plant. We provide evidence for transcription-dependent and -independent protein regulation at the PM. Two-thirds (over 400 members) of the RLK superfamily in *A. thaliana* were identified by MS/MS with most of the differentially expressed RLKs belonging to certain subfamilies. In addition to RLKs, many membrane transporters are differentially expressed during FLS2-PTI. Expression-based protein-protein interaction network analysis identified known modulators of FLS2 signaling in addition to novel expression-activated RLK complexes. Finally we report preliminary functional characterization of two proteins, Cysteine-rich receptor kinase 14 and a Calmodulin-like protein 12, with previously unknown roles in plant immunity.

**Results**

**Time course to determine appropriate temporal sampling after flg22 application**

FLS2-PTI responses were investigated in leaf tissue of four week-old *Arabidopsis thaliana* plants. In order to determine timing of early, middle and late PTI markers in this experimental setup, leaves were sprayed with 10μM flg22 peptide or water containing the surfactant Silwett L-77. MAPK activation was observed within 5 minutes of flg22-treatment, strongly sustained from 10 to 30 minutes and returned to basal levels within 90 minutes (Fig 1B). To monitor changes in gene transcription we analyzed expression of the flg22-induced receptor kinase (FRK1) (Figure 1A) ([Asai et al., 2002](#_ENREF_4)). FRK1 expression increased dramatically within 60 minutes of flg22 application, and continued to be up-regulated relative to water treatment through 720 minutes (12 hours) of sampling. To monitor later phases of the defense response, leaf tissue was assayed for callose deposition. ([Adam and Somerville, 1996](#_ENREF_2)). We could reliably observe callose deposition via aniline blue staining and fluorescence microscopy by 720 minutes (Figure 1C).

Based on the above analyses, time points were selected to study flg22-induced cellular changes. For RNAseq, leaves were collected at 10, 60, 180, 360, and 720 minutes after flg22 application (Figure 1A). Current proteomic approaches cannot quantify all proteins in a cell at a given time and therefore subcellular fractionation is often required to profile low-abundance proteins. We focused on analyzing the plant plasma membrane (PM) with significant depth. For PM-associated protein sampling, leaf tissue was collected at 10, 180, and 720 minutes after flg22 application, corresponding to peaks in the activation of early (MAPK activation), middle (transcriptional reprogramming), and later (callose deposition) PTI markers (Figure 1A). For every time point, three biological replicates of flg22-treated and control tissue were harvested and analyzed. This sampling strategy facilitates a detailed temporal analysis of plant PTI responses over a 12 hour window following FLS2 activation.

**Experimental overview**

For PM protein enrichment, tissue was immediately processed after harvesting and PM vesicles were purified by aqueous two-phase partitioning ([Larsson et al., 1994](#_ENREF_58)). PM vesicles were treated with the Brij-58 detergent to release trapped cytosolic contaminants ([Larsson et al., 1994](#_ENREF_58)). A previous analyses demonstrated that this approach results in the isolation of relatively pure PM preparations ([Elmore et al., 2012](#_ENREF_27)). PM samples were solubilized and subjected to 1D SDS-PAGE to enhance protein identifications by dividing each sample into 15 fractions for LC-MS/MS analysis (Supplemental Figure 1B). Raw spectra were matched to the complete TAIR10 proteome using the X!Tandem spectrum-to-sequence modeler ([Craig and Beavis, 2004](#_ENREF_21)) and peptide and protein identifications were modeled in Scaffold 4 (Proteome Software) (Supplemental Tables 1-2). With a minimum of two unique peptides, 4932 proteins were identified in total (Figure 2A, Table 1). Spectral counting was used to assess differential protein abundance as previously described (Supplemental Table 3) ([Choi et al., 2008](#_ENREF_17); [Elmore et al., 2012](#_ENREF_27)). To our knowledge, this dataset represents one of the largest inventories of PM-associated proteins described to date for any organism.

RNA-seq was performed on cDNA generated from total leaf RNA using the Illumina Hi-Seq 2000 platform with paired-end reads and 100 cycles (Supplemental Figure 1A). After read processing and alignment using GENE-counter ([Cumbie et al., 2011](#_ENREF_22)), an average of 19000 genes were identified per sample (Table 2, Supplemental Table 4). RNA-seq data were filtered to remove genes with a low number of counts (<10 reads) (Table 2, Figure 2A, Supplemental Table 5). After filtering, transcripts corresponding to 16,355 (16091 protein-coding) genes were identified at least one sampling time across the experiment. The NBP-Seq package was used for differential expression analysis of the filtered gene identifications ([Di et al., 2011](#_ENREF_23)) (Table 1, Supplemental Tables 6-10).

**Complement of gene and protein identifications highlights functional specialization of the plant PM**

A substantial overlap in total RNA-seq gene identifications and total MS/MS protein identifications was observed, with only 2% of detected proteins having no corresponding mRNA (Figure 2A, left). Somewhat unexpectedly, almost 10% of filtered protein identifications (i.e. proteins consistently identified in PM fractions) had very low mRNA detected (not present in mRNA filtered identifications) (Figure 2A, right). Similar to observations in other species, rank abundance plots of normalized relative RNA and PM protein abundance values demonstrate an overall low correlation of RNA and protein levels in the dataset (Supplemental Figure 2) ([Vogel and Marcotte, 2012](#_ENREF_110); [Walley et al., 2013](#_ENREF_111)). The proteins with no corresponding mRNA detected in our dataset belong to a range of classes including RLKs, membrane transporters, and ribosomal proteins (Supplemental Table 11). Manual inspection of a subset of these proteins confirmed that confidently-assigned unique peptides were identified, indicating that inappropriate MS/MS spectrum matching did not occur (data not shown). It is likely that mRNA expression of some PM proteins cycles temporally and/or the proteins persist at the PM while the corresponding mRNA is turned over more rapidly ([Schwanhausser et al., 2011](#_ENREF_96); [Vogel and Marcotte, 2012](#_ENREF_110); [Walley et al., 2013](#_ENREF_111)).

We next sought to characterize the biological processes performed by RNA-seq identified genes and MS/MS identified PM-associated proteins relative to the entire *Arabidopsis thaliana* genome. BioMart-retrieved Gene Ontology (GO) slim terms ([Ashburner et al., 2000](#_ENREF_5); [Durinck et al., 2009](#_ENREF_25); [Kinsella et al., 2011](#_ENREF_52)) associated with the filtered identifications revealed significant differences between RNA-seq and MS/MS for most GOslim biological process and molecular function terms in the annotation database (Figure 2B, Figure 2C, Supplemental Table 12). Generally, the RNA-seq identifications associated with each GOslim term more closely resembled the *A. thaliana* genome background. In contrast, the PM proteome is highly enriched in proteins with kinase and membrane transporter activity and biological process terms associated with signaling and stress responses (Figure 2B, Figure 2C). These results demonstrate that the PM-associated proteome is highly specialized in nature, enriched in processes and functions consistent with the role of the cell membrane.

**Characterization of the PM-associated proteome**

As expected, the PM-associated MS/MS identifications were highly enriched in the GOslim Cellular Component terms "plasma membrane" (p = 0) and "cytosol" (p= 2.24 x 10-273) relative to the background of annotations within the *A. thaliana* genome (Figure 3A, Supplemental Table 12). To further characterize the PM association of identified proteins, sequence-predicted and experimental evidence of protein transmembrane domains ([Krogh et al., 2001](#_ENREF_54)) and the protein post-translational modifications myristoylation ([Tchieu et al., 2003](#_ENREF_107); [Podell and Gribskov, 2004](#_ENREF_87)), palmitoylation ([Hemsley et al., 2013](#_ENREF_37)), prenylation ([Maurer-Stroh and Eisenhaber, 2005](#_ENREF_73); [Maurer-Stroh et al., 2007](#_ENREF_74)) and GPI-anchoring ([Schwacke et al., 2003](#_ENREF_95)) associated with membrane targeting were analyzed. Although no membrane stripping was employed to enrich specifically for integral membrane proteins, 32% (n=1501) of the filtered protein identifications contain at least one transmembrane domain (Figure 3C and Supplemental Table 13). This result is comparable to other Arabidopsis membrane proteomics studies that found purified membrane fractions to contain 20-50% integral membrane proteins ([Alexandersson et al., 2004](#_ENREF_3); [Nelson et al., 2006](#_ENREF_81); [Marmagne et al., 2007](#_ENREF_72); [Mitra et al., 2007](#_ENREF_76); [Huang et al., 2013](#_ENREF_39)). In addition to transmembrane domains, 15% (n=672) of the filtered protein identifications have predicted or experimental evidence of being membrane associated via lipid post-translational modifications (Supplemental Figure 13).

Other available evidence was also used to determine PM localization of the identified proteins. Due to the complementary nature of different databases, we also compared the filtered protein identifications to SUBA3 database consensus predictions ([Tanz et al., 2013](#_ENREF_106)) and AtSubP predictions ([Kaundal et al., 2010](#_ENREF_49)). The SUBA3 consensus predictions use a Bayesian approach based on experimental and predicted evidence of subcellular location ([Tanz et al., 2013](#_ENREF_106)). AtSubP uses a machine-learning technique for subcellular predictions based on the presence of multiple protein sequence features ([Kaundal et al., 2010](#_ENREF_49)). In total, 48% (n=2208) of the identified proteins have evidence of PM association in at least one of these databases (Supplemental Figure 03, Supplemental Table 13). Only 21% (n=991) of the identified proteins had evidence of PM localization in two or more databases, indicating that the annotations within a single database are likely incomplete (Supplemental Figure 03). Taken together, these results demonstrate that our PM protein preparations are significantly enriched in PM proteins.

**RNA/protein correspondence and evidence for transcription-independent PM protein changes**

From an initial analysis of the differential expression data, it was evident that most of the transcriptome changes in response to flg22 treatment occurred by 60min while most of the PM-associated protein changes occurred after 180min (Figure 4A). As expected. scatterplots of all Log2 mRNA and protein fold change values indicated a low but significant correlation in overall expression patterns (r=0.26-0.37) (Figure 4B). Stronger correlations were observed for genes that were detected as differentially expressed at both the mRNA and protein levels (r=0.65-0.83) (Figure 4C). Slightly higher correlations were detected for proteins annotated as PM-associated depending on the database used (r=0.68-0.89) (data not shown). The strongest correlations between mRNA and protein expression were observed between the mRNA-60min/protein-180min and the mRNA-180min/protein-720min sampling times, likely reflecting a 2-9 hour lag phase from transcription to translation and trafficking of a functionally localized protein at the PM. These observations are in line with a previous study in yeast finding that protein abundance changes at six hours were most highly correlated with mRNA changes at one and two hours post-treatment ([Fournier et al., 2010](#_ENREF_29)).

Next, hierarchical clustering of all DE proteins and their corresponding RNA fold changes was used to partition all differentially expressed PM proteins (n=384) by their combined mRNA and protein expression patterns (Figure 4D). The resulting dendrogram identified sub-clusters of proteins exhibiting transcription-dependent and -independent control. Proteins residing in specific clades were queried for Gene Ontology term enrichment (Figure 5D and Supplemental Table 14). Proteins in several of these clusters are known to dynamically associate with the cell membrane, such as exocyst complex components ([Frey and Robatzek, 2009](#_ENREF_30)). Other proteins in these clades likely originate from contaminating organelles as evidenced by proteins associated with plastoglobules and ribosomes, however their differential abundance in flg22-treated PM preparations relative to water-treated controls suggests that important modifications to these membrane sub-proteomes likely occur during FLS2-PTI (Figure 4D).

**Protein kinases and membrane transporters are the two largest classes of DE proteins at the PM**

In order to characterize the cellular changes associated with flagellin perception over the course of the experiment, differentially expressed (DE) mRNA and protein at each time point were analyzed for over-representation of GO slim terms (Figure 5, Supplemental Table 15). Within 60 min of flg22 treatment biological processes associated with the immune system, stress responses, protein targeting, signal transduction, and transport were significantly up-regulated (Figure 5A). Down-regulated mRNA at 60-180 min includes genes involved in growth, development and various primary and secondary metabolic processes. This down-regulation likely reflects the known trade-off between plant growth and immune system activation ([Gómez-Gómez et al., 1999](#_ENREF_32)) and the metabolic reprogramming necessary to mount a defense response ([Bolton, 2009](#_ENREF_10)).

Up-regulated genes at multiple timepoints were enriched in GO molecular function terms associated with transcription factors, kinase activity, and transmembrane transporter activity (Figure 5B). The latter two, kinases and transmembrane transporters, represent the two major up-regulated functional protein classes at the PM in response to flg22 (Figure 5B, Supplemental Table 15). Interestingly, the major transcriptome changes in response to flg22 appear to be concentrated at the cell periphery, with genes located at the plasma membrane, cell wall, and extracellular region significantly changing 60-720 minutes post-flg22 treatment (Figure 5C). Collectively, these results highlight the major cellular reprogramming that occurs in response to activation of FLS2, with substantial changes in the kinase and transporter complement of the PM proteome.

**Membrane transporters differentially expressed during FL2-PTI**

Multiple transmembrane transporters were differentially expressed in PM fractions (Supplemental Table 16). ABC transporters belonging to the ABCB and ABCG subfamilies were up-regulated 180-720 minutes post-flg22. ABCB14 is a malate importer that can affect stomatal movement by controlling guard cell osmotic pressure ([Lee et al., 2008](#_ENREF_60)). Other significantly changing ABC transporters include those implicated in auxin transport ([Kamimoto et al., 2012](#_ENREF_46)), abscisic acid (ABA) import ([Kang et al., 2010](#_ENREF_47)), and export of sporopollenin precursors involved in pollen exine formation ([Quilichini et al., 2010](#_ENREF_90)). In addition, several members of the NITRATE TRANSPORTER1/PEPTIDE TRANSPORTER (NPF) family that have been reported to transport nitrate, auxin, and ABA also increased in abundance in response to flg22 ([Léran et al., 2014](#_ENREF_62)). As members of the ABC transporter and NPF families can transport a range of substrates, some of these transporters likely facilitate hormone transport as well as the export of antimicrobial or cell wall-reinforcing compounds into the apoplast upon pathogen perception ([Kang et al., 2011](#_ENREF_48); [Léran et al., 2014](#_ENREF_62)). Autoinhibited Ca2+ ATPase 12 (ACA12) was highly up-regulated in PM fractions at 180 and 720 minutes post-flg22. In contrast with other ACAs, ACA12 has recently been demonstrated to be a hyperactive Ca2+ pump that lacks autoinhibition and calmodulin regulation ([Limonta et al., 2014](#_ENREF_66)), and its expression pattern at the PM suggests that it could be involved in cytosolic calcium ion homeostasis during PTI signaling.

**Temporal expression clustering of RNA-seq reveals regulatory waves of gene activation during PTI**

**I am still working on this analysis. This section and Figure 6A will change, but I would like to include it in some capacity if I find something interesting.**

To better understand the temporal expression dynamics of the FLS2-activated immune response, we used Bayesian hierarchical clustering implemented in the Splinecluster package([Heard et al., 2005](#_ENREF_35)). We clustered all differentially expressed mRNA (n=5612) based on their Log2 fold change difference relative to water-treated controls (Figure 6 and Supplemental Figures 4). Using our clustering parameters, the RNA-seq DE genes were divided into 23 clusters based on their overall expression pattern and magnitude of differential expression (Supplemental Figure 4). We then combined these clusters into groups based on the time of first differential expression and each cluster's nearest neighbors, leading to the classification of 13 dominant expression patterns in the RNA-seq data (Figure 6A). To gain insights into how these expression groups are regulated, we looked for the presence of transcription factors and over-represented cis-elements in the promoter regions (-2kb upstream of the transcription start site) of genes within each group....

**Expression clustering of MS/MS data highlights protein families enriched at the PM during FLS2-PTI**

Temporal clustering of differentially expressed proteins resulted in 16 distinct clusters identified with Splinecluster (Supplemental Figure 5), representing seven major expression patterns of differential changes at the PM (Figure 6B). In order to further characterize the changes in the complement of PM-associated proteins, we looked for over-representation of specific protein domains in these expression groups using Interpro annotations retrieved from BioMart (Figure 6B, Supplemental Table 19) ([Durinck et al., 2009](#_ENREF_25); [Kinsella et al., 2011](#_ENREF_52)). Most of the enriched Interpro domains are associated with proteins known to be either peripherally, integrally, or transiently membrane localized (Figure 6B). Three proteins (NOI8/10/11) with homology to the plant immune regulator RPM1-interacting 4 (RIN4) that harbor the IPR008700 domain were up-regulated 180-720 min post-flg22. This domain is targeted by the pathogen effector protease AvrRpt2 ([Chisholm et al., 2005](#_ENREF_16); [Elmore et al., 2012](#_ENREF_27)), suggesting that in addition to RIN4, these proteins might have immune system function.

We also detected an increase in proteins (Flotillin 1/2/3 and Hypersensitive-induced reaction protein 2) with the IPR001107 Band 7/SPFH domain at 180-720 min (Figure 6B). Band 7 proteins localize to PM microdomains and have roles in vesicle trafficking and membrane scaffolding ([Qi et al., 2011](#_ENREF_89); [Li et al., 2012](#_ENREF_65)). Flotillins are involved in a clathrin-independent endocytic pathway in *Arabidopsis* and have multiple roles in membrane trafficking in other eukaryotes ([Haney and Long, 2010](#_ENREF_33); [Stuermer, 2010](#_ENREF_101); [Haney et al., 2011](#_ENREF_34); [Li et al., 2012](#_ENREF_65)). In *Medicago truncatula*, FLOT4 is required for symbiosis and co-localizes with LYSIN MOTIF RECEPTOR-LIKE KINASE3 at PM microdomains in response to perception of *Sinorhizobium meliloti* ([Haney and Long, 2010](#_ENREF_33); [Haney et al., 2011](#_ENREF_34)). Further, HIR2 can associate with the plant disease resistance protein RPS2 and contribute to plant immunity ([Qi et al., 2011](#_ENREF_89)). Up-regulation of SPFH proteins indicates that PM microdomains have an important role in plant defense responses that occur several hours after initial flg22 perception. Taken together, this analysis highlights protein families that are differentially expressed at the PM during FLS2-PTI and provides a starting point to dissect the contributions of specific gene family members to plant immunity.

**Receptor-like kinases represent the largest class of differentially expressed PM proteins**

We consistently identified 411 RLKs by MS/MS, representing 66% of all RLKs encoded in the *A. thaliana* genome. Further, 60 (15%) of the identified RLKs were differentially expressed at one or more time points. Due to this dynamic regulation of RLKs in PM fractions, we decided to focus our efforts on identifying novel RLKs regulating plant immunity and understanding their contribution to plant defense. To better understand RLK changes in the context of the evolutionary history of this large gene family, phylogenies were constructed based on the kinase domain of each RLK in the *Arabidopsis thaliana* Col-0 TAIR10 genome ([Shiu et al., 2004](#_ENREF_98)). For visualization, we divided the RLK superfamily into LRR-RLKs (Supplemental Figure 6) and non-LRR RLKs with extracellular domains (Supplemental Figure 7). We also plotted the relative abundance (mean NSAF ± sd) of each identified RLK in PM-enriched fractions averaged across all treatment and control samples ([Paoletti et al., 2006](#_ENREF_85)). Differential protein expression of LRR-RLKs was largely limited to specific subfamilies, namely the LRR-Ia, LRR-XI, and LRR-XII groups ([Shiu et al., 2004](#_ENREF_98)). Furthermore, a large dynamic range (10^4 difference in mean NSAF values) in LRR-RLK abundance at the PM was observed. Interestingly, members within individual sub-clades exhibited similar levels of relative abundance (for example, the SERK and BIR family proteins) (Supplemental Figure 6). Six members of the Impaired-oomycete susceptibility 1 (IOS1) RLK family were up-regulated 180-720 min post-flg22 and are clustered on Chromosome 1. Plants defective in IOS1 are more resistant to downy mildew disease, suggesting that IOS1 is a negative regulator of defense against Hyaloperonospora arabidopsidis ([Hok et al., 2011](#_ENREF_38)). A similar analysis of non-LRR RLKs showed that most differentially expressed proteins belonged to the L-Lectin, SD-1b (S-domain), and DUF26/Gnk2-like domain-containing subfamilies (Supplemental Figure 7).

**PPI Network Analysis**

To investigate potential RLK signaling networks involved in FL2-PTI, we constructed a custom protein-protein interaction (PPI) network derived from publically available sources, recent large-scale *Arabidopsis* protein interactions studies, and some manually curated annotations from recent literature ([Lalonde et al., 2010](#_ENREF_56); [Consortium, 2011](#_ENREF_20); [Klopffleisch et al., 2011](#_ENREF_53); [Li et al., 2011](#_ENREF_64); [Mukhtar et al., 2011](#_ENREF_80); [Chen et al., 2012](#_ENREF_11); [Wang et al., 2012](#_ENREF_112)). From this global PPI network, a RLK network containing all *Arabidopsis* RLKs and their nearest neighbors was generated using only evidence of physical interactions. Proteins were clustered according to the mRNA and protein Log2-transformed fold change values at each time point using Autosome clustering to identify putative expression-activated protein complexes (Supplemental Figure 8) ([Newman and Cooper, 2010](#_ENREF_82)). The Autosome algorithm uses self-organizing maps to cluster the input network by its underlying structure and node attributes ([Newman and Cooper, 2010](#_ENREF_82)).

Three major expression-activated sub-networks were identified using this approach (Supplemental Figure 8), one of which was a BAK1 interaction network (Figure 7). Although up-regulated at the mRNA level, we did not detect significant changes in protein abundance for BAK1. However, we did detect differential protein expression changes for the BAK1-interacting proteins EFR, FLS2, and PEPR1 (up-regulated at 720 min) and BRI1 (down-regulated at 720 min) (Figure 7). We also observed complex regulation of Plant U-box 12 (PUB12) at the mRNA and protein level (Figure 7). PUB12 is an E3 ubiquitin ligase that directly targets FLS2 for degradation and its decrease at the PM 180-720 min likely enables FLS2 to accumulate to higher levels ([Lu et al., 2011](#_ENREF_70)). Many of the differential protein expression changes we detected within the BAK1 sub-network have been documented independently by other groups, validating our approach to detect novel proteins with immune system function.

**Identification of a Ca2+-binding protein TCH3/CAL4/CML12 with a role in plant immune signaling**

The PPI network analysis identified a sub-network containing several RLKs and two calcium-binding calmodulin-like proteins, CML9 and TCH3/CML12 (Figure 8A-C). Cytosolic Ca2+ signaling has a pronounced role in abiotic and biotic stress signaling ([McCormack et al., 2005](#_ENREF_75)). CML9 has recently been implicated in plant immune responses ([Leba et al., 2012](#_ENREF_59))**.** Gene expression of TCH3/CML12 is induced by mechanical stimulation ("touch") and a variety of other stimuli ([Sistrunk et al., 1994](#_ENREF_99)). We obtained a T-DNA line with an insertion in the TCH3 coding region and assayed it for altered immune responses (Supplemental Figure 9). The *tch3-1* knock-out plants exhibited enhanced disease susceptibility to *Pseudomonas syringae* pv. tomato strain DC3000 (*Pto*) using spray inoculation, indicating that TCH3 is a positive regulator of the Arabidopsis immune response (Fig 8D). We had previously noted that TCH3 abundance increases in PM fractions during ETI responses activated by the intracellular NLR RPS2 and immunoblot signals using an antibody recognizing TCH3 migrated as two to three distinct bands ([Elmore et al., 2012](#_ENREF_27)). We next tested whether the TCH3 protein is modulated in response to flg22 treatment. **Will complete based on results next week**

**Identification of Cysteine-rich receptor kinases and potential regulatory mechanisms that contribute to plant immunity**

From the previous analyses (Figure 6B and Supplemental Figure 7), we noted that multiple (n=7) members of the Ginkbilobin-2 (Gnk2)-homologous/DUF26 domain-containing RLK subfamily were drastically up-regulated at the PM during FLS2-PTI. The Gnk2-homologous domain (IPR002902) is a cysteine-rich domain present in antifungal proteins found in the seeds of *Ginkgo* and Spruce trees ([Wang and Ng, 2000](#_ENREF_113)). Different CRK family members have been previously implicated in activation of plant immunity ([Chen et al., 2003](#_ENREF_12); [Chen et al., 2004](#_ENREF_13); [Acharya et al., 2007](#_ENREF_1)) and responses to various abiotic factors like water and ozone stress ([Wrzaczek et al., 2010](#_ENREF_115); [Tanaka et al., 2012](#_ENREF_104); [Idänheimo et al., 2014](#_ENREF_41)), indicating that the CRK family might have multiple functions in plant stress responses.

An unrooted phylogeny of the TAIR-annotated CRK family was created using the complete amino acid sequence of each protein (Figure 9A). After aligning with a heatmap of differential RNA and protein expression values, it was clear that while a majority of the CRK subfamily is up-regulated, differential protein abundance was only detected within a few specific subclades (Figure 9A). In order to establish if additional CRK proteins had roles in plant immunity, we ordered T-DNA insertion lines for previously uncharacterized CRKs. Of the tested KOs, a line with an insertion in CRK14 (At4g23220), was more susceptible to *Pto* (Figure 9B). These results indicate that CRK14 is a positive regulator of plant immune responses to phytopathogenic bacteria.

Due to the importance of protein phosphorylation in plant immunity, we searched our MS/MS data for phosphorylation at serine (S), threonine (T), and tyrosine (Y) residues, identifying over 1500 phosphopeptide spectra corresponding to 762 proteins at relatively stringent peptide identification thresholds (Supplemental Table 19). Several phosphopeptides mapped to different CRK proteins (Supplemental Table 19). We aligned the amino acid sequences of the corresponding CRKs to check if any of these identified phosphorylated residues were conserved across family members (Figure 9C and Supplemental Figure 10). Five identified phosphopeptides with a total of eight potentially modified residues exhibited conserved S/T/Y or aspartic acid D/ glutamic acid (E) residues for most proteins in the alignment (Figure 9C and Supplemental Figure 10). To assay the role of these conserved phosphorylation sites on CRK function, we cloned CRK13, a CRK implicated in plant immunity, which elicits cell death when over-expressed in *Arabidopsis* ([Acharya et al., 2007](#_ENREF_1)) and *Nicotiana tabacum* (Figure 9D). PCR-based site-directed mutagenesis was then performed on CRK13 to convert the identified residues to alanine on a subset of the conserved sites. These phosphonull mutants were then assayed for cell death elicitation upon *Agrobacterium*-mediated transient expression in *N. tabacum* (Figure 9D). The CRK137A and CRK137E mutations, with seven residues mutated to alanine or glutamic acid (phosphomimetic), respectively, did not elicit cell death although CRK137E could not be detected by immunoblotting. Wild-type CRK13 and the CRK13S389A mutation elicited cell death around 24 hour post-infiltration, while the CRK13T392A and CRK13S438A mutations abolished cell death. Proteins with single point mutations accumulated to similar levels as CRK13WT (Figure 9D). Together, these results suggest that conserved, phosphorylated residues on CRKs are important for cell death activation *in planta* (Figure 9D).

**Discussion**

**Towards a complete list of PM-associated proteins**

The FLS2 immune receptor is a major contributor to plant disease resistance against phytopathogenic bacteria in multiple monocot and dicot plant species ([Zipfel et al., 2004](#_ENREF_118); [Robatzek et al., 2007](#_ENREF_92); [Takai et al., 2008](#_ENREF_103); [Zeng and He, 2010](#_ENREF_116)). Here, we sought to uncover temporal phases of the FLS-PTI response with a focus on dynamic changes in the PM proteome. To our knowledge, the in-depth profiling of PM fractions presented here represents one of the largest inventories of PM-associated proteins for any organism. In contrast with other studies seeking highly pure membrane fractions ([Marmagne et al., 2007](#_ENREF_72); [Huang et al., 2013](#_ENREF_39)), no membrane stripping using carbonate treatment was employed with hopes to preserve sensitive, yet important protein-protein interactions at the PM. Nevertheless, 1501 (32%) of the identified proteins contain a TM domain and 672 (14%) are predicted to harbor a membrane-targeted post-translational modification. In total, about half of the proteins we identified have evidence for PM association (Supplemental Figure 3). However, we did observe GOslim subcellular component terms associated with intracellular membranes like the vacuole and endoplasmic reticulum were also enriched in PM fractions (Figure 3A), likely representing 1) co-purification of other intracellular membranes in PM preparations and 2) proteins which have been localized to more than one cellular compartment. Thus, although our dataset is highly enriched in PM proteins, contaminants exist in biochemical fractionations from complex tissues, and therefore localization of individual proteins should be validated through independent approaches.

**RNA/protein correspondence**

While the global correlation in RNA/PM protein changes as well as RNA/PM protein relative abundance were low (Figure 4B and Supplemental Figure 2), these observations agree with previous studies which reported a similar low correspondence between RNA and protein levels in animal cells, yeast, and plants ([Maier et al., 2009](#_ENREF_71); [Fournier et al., 2010](#_ENREF_29); [Lan et al., 2012](#_ENREF_57); [Ning et al., 2012](#_ENREF_83); [Vogel and Marcotte, 2012](#_ENREF_110); [Walley et al., 2013](#_ENREF_111)). In developing maize leaves, several abundant proteins with no corresponding mRNA are under circadian control ([Walley et al., 2013](#_ENREF_111)). A study employing parallel metabolic labeling of mRNA and protein in mammalian cells demonstrated that while mRNA/protein levels correlated better than previous studies, their cellular half-lives varied substantially with virtually no correlation, and proteins were on average five times more stable than mRNA ([Schwanhausser et al., 2011](#_ENREF_96)). Directly comparing RNA and protein relative abundance levels is complicated in this study because we profiled a specific subcellular compartment which contains a distinct complement of proteins relative to the rest of the cell (Figure 2). The strongest correlations we observed for RNA and protein expression changes were between mRNA-60min/protein-180min and mRNA-180min/protein-720min (Figure 4C), which suggests a 2-9 hour interval between transcriptome changes and appearance of functionally-localized PM proteins. This window is likely exploited by pathogens that deliver effectors into host cells to disrupt immune signaling networks before the host can mount an effective defense.

**TCH3/CML12 and calcium signaling in plant defense**

Calcium has a multi-faceted role in plant responses to microbes ([McCormack et al., 2005](#_ENREF_75)). Calcium sensors in plants include calmodulins, calmodulin-like (CML) proteins, calcineurin B-like proteins, calcium-dependent protein kinases, and additional enzymes that are regulated directly through Ca2+ binding ([McCormack et al., 2005](#_ENREF_75)). TCH3/CML12 increases in PM fractions during plant immune signaling activated by FLS2 and the resistance protein RPS2 (Figure 8) ([Elmore et al., 2012](#_ENREF_27)). Here we report that *tch3-1* mutant plants are more susceptible to virulent *Pto*. Most CMLs contain two Ca2+-binding EF-hand motifs; TCH3/CML12 is unique in that it contains three EF-hand domains ([McCormack et al., 2005](#_ENREF_75)). TCH3/CML12 can interact with multiple RLKs *in vitro* ([Popescu et al., 2007](#_ENREF_88)), suggesting that TCH3 might be involved in bridging Ca2+ and RLK signaling during PTI. Consistent with a role for TCH3 in plant defense, altered glucosinolate hydrolytic product profiles were observed in *tch3* knockout lines ([Humphry et al., 2010](#_ENREF_40)). Interestingly, TCH3 antibody recognizes 2-3 distinct protein bands in leaf tissue extracts....([Elmore et al., 2012](#_ENREF_27))**. Will complete based on results next week**

**PPI network analysis identifies proteins involved in plant immune signaling**

Subcellular quantitativeproteomics is inherently advantageous to apply to PPI network analysis because it provides simultaneous information on both protein co-expression and co-localization for potential interacting proteins. Our RLK-focused network analysis uncovered both known and previously undocumented expression-activated RLK sub-networks whose members function in plant immune responses (Figures 7-8). The BAK1-interacting protein network highlights the tradeoff between plant growth and development and defense responses (Figure 7) ([Gómez-Gómez et al., 1999](#_ENREF_32)). Although BAK1 gene expression is up-regulated at the RNA-level, we were unable to detect any significant changes in BAK1 or SERK protein levels at the time points assayed (Figure 7, Supplemental Figure 6). However, major known BAK1-interacting RLKs are modulated at the PM, with immune-related PRRs up-regulated 180-720 min and the brassinosteroid receptor BRI1 down-regulated by 720m post-flg22 (Figure 7 and Supplemental Figure 6). These results suggest that a decrease in BRI1 levels might allow more PRRs to interact with BAK1 to quickly activate the immune response in case of subsequent pathogen attack. Most known PRRs have a relatively low estimated relative abundance at the PM (Supplemental Figures 6-7), suggesting that PRR levels could be a major limiting factor during initial plant immune signaling activation. Determining the number of PRRs required for effective immune system activation and how receptor levels are correlated with the timing and magnitude of the defense response will be an interesting topic for future research. Pre-treatment of plants with flg22 primes the plant immune system so that it can elicit a more robust defense response when challenged with pathogens ([Zipfel et al., 2004](#_ENREF_118)). The up-regulation of many known PRRs (FLS2/EFR/PEPR1/WAK1, Supplemental Figures 6-7) around 720 min post-flg22 supports a model where the primary recognition event stimulates the cell to increase the number of pathogen receptors at the PM which allows the cell to more rapidly activate a robust defense response against subsequent pathogen attack. Further, the similar expression patterns of known PRRs strongly implicate uncharacterized RLKs with the same expression profile as additional plant immune receptors.

**PM receptor turnover during PTI**

Upon activation, FLS2 undergoes ligand-induced endocytosis, trafficking through early and late endosomes, and degradation resulting in a decrease in receptor levels 60 min post-flg22 ([Robatzek et al., 2006](#_ENREF_91); [Lu et al., 2011](#_ENREF_70); [Beck et al., 2012](#_ENREF_7); [Choi et al., 2013](#_ENREF_18); [Smith et al., 2014](#_ENREF_100)). Blocking endocytosis with chemical inhibitors like wortmannin and Tyrphostin A23 (TyrA23) can disrupt FLS2 internalization and lead to altered an ROS response ([Beck et al., 2012](#_ENREF_7); [Smith et al., 2014](#_ENREF_100)). We observed an increase in the levels of a direct target of TyrA23, the adaptor protein complex 2 (AP-2), at 180m post-flg22 (Figure 6B) ([Banbury et al., 2003](#_ENREF_6)). The AP-2 complex is involved in loading transmembrane proteins carrying the internalization motif YXXɸ (ɸ = bulky hydrophobic residue) into clathrin coated vesicles at the PM ([Banbury et al., 2003](#_ENREF_6); [Geldner and Robatzek, 2008](#_ENREF_31); [Smith et al., 2014](#_ENREF_100)). Although FLS2 does not contain a YXXɸ motif, many other RLKs do, indicating that plant receptor endocytosis plays an important role in receptor turnover ([Ron and Avni, 2004](#_ENREF_93); [Geldner and Robatzek, 2008](#_ENREF_31)). Many receptors with known or implied roles in plant development and contain the YXXɸ motif are down-regulated at the PM in response to flg22 treatment (Supplemental Figures 6-7). However, many RLKs that are up-regulated at the PM also contain the same motif ([Geldner and Robatzek, 2008](#_ENREF_31)), so it will be interesting to uncover how the plant cell discriminates between PRRs and growth-related RLKs for protein turnover during the defense response.

**CRKs in plant defense signaling**

Proteins with Ginkbilobin-2 (Gnk2)-homologous domains in Arabidopsis comprise ~60 small secreted proteins and ~42 non-LRR cysteine-rich RLKs (CRKs), all of which contain 1-2 copies of the C-8X-C-2X-C amino acid motif ([Chen, 2001](#_ENREF_14)). The structure of GNK2 has been solved, demonstrating that the cysteine residues form intramolecular disulfide bridges important for protein structural stability ([Miyakawa et al., 2009](#_ENREF_77)). Around 20 of the CRKs are present in a tandem array on chromosome 4 of the *Arabidopsis thaliana* Col-0 genome and nearly all of these genes are transcriptionally activated in response to elicitor or ozone treatment ([Lehti-Shiu et al., 2009](#_ENREF_61); [Wrzaczek et al., 2010](#_ENREF_115)). Nearly 30% of *Arabidopsis* RLKs are present in tandem arrays and genomic duplications tend to be highly expressed during biotic stress ([Lehti-Shiu et al., 2009](#_ENREF_61)). While most of the CRK family is up-regulated at the mRNA level, we only detected changes in the protein levels of CRK11, CRK13, CRK14, CRK18, CRK22, CRK28, and CRK29, suggesting that this family is under post-transcriptional and/or translational control (Figure 9A).

Several CRKs including CRK4, CRK5, CRK13, CRK19, and CRK20 have a role in bacterial resistance and/or cell death activation ([Chen et al., 2003](#_ENREF_12); [Chen et al., 2004](#_ENREF_13); [Acharya et al., 2007](#_ENREF_1)). CRK10, which is present at the PM at levels 3-4 times higher than the next most abundant CRK, does not activate cell death when over-expressed *in planta* ([Chen et al., 2003](#_ENREF_12)), suggesting that it might function in distinct cellular pathways from other CRKs. Plants with reduced expression of CRK6 and CRK7 are more sensitive to ozone treatment, suggesting a role for CRKs in ROS signaling ([Idänheimo et al., 2014](#_ENREF_41)). The presence of disulfide bonds in the GNK2 protein domain suggests that CRKs might be sensitive to apoplastic redox states. It is tempting to speculate that CRK proteins are up-regulated at the PM during PTI in order to monitor the apoplast for subsequent signs of pathogen recognition e.g. the ROS burst activated by PRRs ([Wrzaczek et al., 2013](#_ENREF_114); [Smith et al., 2014](#_ENREF_100)). Thus, future investigations into the connection between CRKs, ROS signaling, and immunity could yield mechanistic insights into the regulation of plant stress responses.

**Materials and Methods**

**Plant growth conditions and flg22 treatment**

*Arabidopsis thaliana* Col-0 plants were grown in soil in a controlled environment chamber at 24˚C with a 10h-light/14h-dark photoperiod with a light intensity of 85μE/m2/s. Plants were sprayed with 10μM flg22 peptide (>85% purity, synthesized by GenScript USA Inc.) in water containing 0.025% Silwett L-77 surfactant using a Preval 267 compressed air sprayer. Col-0 plants sprayed with H2O containing 0.025% Silwett L-77 were used as a negative control. Plants were incubated for the indicated time period before harvesting tissue for protein and RNA isolations. Three biological replicates of plants grown and harvested at different times were performed. Four to five week-old plants were used for all experiments.

**Plasma membrane isolation and In-gel Digestion**

PM enrichment was performed using three rounds of aqueous two-phase partitioning as in ([Larsson et al., 1994](#_ENREF_58)). The final upper phase fraction containing enriched plasma membrane vesicles was incubated with 0.02% Brij-58 detergent on ice for 10min to invert vesicles and release cytosolic contaminants ([Johansson et al., 1995](#_ENREF_43)). Samples were then diluted 20 times with water and centrifuged at 90000g for 60min to pellet PM vesicles. Membrane pellets were frozen in liquid N2 and stored at -80˚C. Membrane pellets were thawed on ice and solubilized in 2X Laemmli buffer containing 6M urea and 4% SDS. Protein samples were quantified using the 660nm Protein Assay with Ionic Detergent Compatibility Reagent (Pierce). Samples (250ug total protein) were subjected to 1D SDS-PAGE using an 8-16% Precise Protein Gradient Gel (Thermo Scientific). The entire sample lane was excised and cut into 15 pieces of equal size using a disposable grid cutter (The Gel Company). In-gel digestions were performed with Promega sequencing-grade modified trypsin according to ([Shevchenko et al., 2006](#_ENREF_97)). Digested peptides were dried using a vacuum concentrator then solubilized in 60μL 2% acetonitrile/0.1% trifluoroacetic acid and frozen at -80C.

**LC-MS/MS**

Separate LC-MS/MS analyses were performed on peptides derived from individual gel pieces with a total of 15 LC-MS/MS runs per sample. The LC-MS/MS system configuration consisted of a CTC Pal autosampler (LEAP Technologies) and Paradigm HPLC (Michrom BioResources) coupled to a QExactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific) with a CaptiveSpray ionization source (Michrom BioResources). LC was performed by injecting 20μL of each peptide sample onto a Zorbax300SB-C18 trap column (5μm, 5x0.3mm, Agilent Technologies) and desalted online. Peptides were then eluted from the trap and separated on a reverse-phase Michrom Magic C18AQ (200μm x 150mm) capillary column at a flow rate of 2 μL/min using a 120min gradient (2 to 35% buffer B 85min, 35 to 80% buffer B 25min, 2% buffer B 10min; buffer A=0.01% formic acid in H2O, buffer B=100% acetonitrile). The mass spectrometer was operated in data-dependent acquisition mode using a standard Top15 method.

**Protein identification and quantitative analysis**

Tandem mass spectra were extracted to MZML format using Proteome Discoverer v1.3.0.0 and analyzed using the X!Tandem GPM-XE Cyclone version 2012.10.01.2 spectrum modeler (http://www.thegpm.org) ([Craig and Beavis, 2004](#_ENREF_21)). X!Tandem was configured to search the TAIR10 Arabidopsis complete proteome sequence (TAIR10\_pep\_20101214.fasta, 35386 entries) with a common repository of adventitious proteins database (cRAP version 1.0, 112 entries). A reversed and concatenated database served as a decoy sequence database to determine peptide and protein false discovery rates (FDR) ([Kall et al., 2008](#_ENREF_45)). X!Tandem was configured to allow parent ion mass error of 20ppm and fragment mass error of 20ppm. Data were searched using fixed modification of +57 (carbamidomethyl) modification on C residues and the following variable modifications: -18 on n (Glu->pyro-Glu), -17 on n (Ammonia-loss), -17 on n (Gln->pyro-Glu), +1 on NQ (Deamidated), +16 on MW (Oxidation), +32 on MW (Dioxidation), +42 on Kn (Acetyl), +80 on STY (Phospho) while allowing one missed cleavage. X!Tandem search results were imported into Scaffold 4.0.3 (Proteome Software) with all MS runs corresponding to the same sample merged. The probability of peptide identifications was modeled using a local FDR algorithm with discriminant scoring with a naïve Bayes classifier. Protein identifications required two unique peptides, 99.8% protein probability, and 20% peptide probability. Using these identification thresholds, the dataset contained 6227230 spectra and 4932 *Arabidopsis* proteins at 0.7% peptide FDR and 4.7% protein FDR. Shared spectral count distributions was performed within Scaffold similar to ([Zhang et al., 2010](#_ENREF_117)) using each protein's cumulative unique peptide identification probability as the distribution factor. Spectral counts of protein isoforms mapping to the same genomic locus were summed before differential expression analysis. The Scaffold protein report containing total spectra, number of exclusive spectra, and all protein identification probabilities is available in Supplemental Table 02. The QSpec/QProt statistical framework v1.2.2 (http://sourceforge.net/projects/qprot/) ([Choi et al., 2008](#_ENREF_17)) was used for differential expression analysis with a criterion FDR ≤ 0.05 and minimum abundance change ± 50%.

**RNA-seq**

Total leaf RNA was extracted using the Trizol reagent (Invitrogen) according to manufacturer’s instructions. Library preparation of total RNA and sequencing was done using the TruSeq RNA kit and the Illumina HiSeq 2000 (Paired-end, 100 cycles). The GENE-counter configuration, processing, and assessment tools were used to process and align the reads to the *Arabidopsis thaliana* TAIR10 reference genome and transcriptome ([Cumbie et al., 2011](#_ENREF_22)). Read alignment was performed using CASHX v1.3 ([Fahlgren et al., 2007](#_ENREF_28)). Processing paired-end reads through GENE-counter was done using *ad hoc* scripts. Prior to differential expression analysis at each time point, the RNA-seq data were filtered to remove genes with a low number of counts (<10 reads) across all replications of a time point. Differential expression and regression analysis was done using the R package NBP-Seq version 0.1.8 ([Di et al., 2011](#_ENREF_23)). Genes were identified as differential expressed if they had a p-value ≤ 0.05, q-value ≤ 0.05, and -1 ≥ Log2 fold change ≥ 1. After filtering, transcripts corresponding to 16,355 (16091 protein-coding) genes were identified at least one sampling time across the experiment.

**Quantitative RT-PCR**

Total RNA was extracted using the Trizol reagent (Invitrogen) and incubated with RNase-free DNase I (Invitrogen) to remove DNA contamination. Each biological sample comprised three leaves from 1-2 plants; 2ug of RNA was used as a template for cDNA synthesis with Superscript II reverse transcriptase (Invitrogen) in the presence of 0.5ug/ul oligo(dT) primers. Equal amounts of first-strand cDNAs were used as templates for RT-PCR amplification. Quantitative real-time PCR reactions used Bio-Rad SsoFast EvaGreen Supermix according to manufacturer’s directions with 6-fold diluted cDNA using a CFX96 Touch (Bio-Rad). Thermocyling parameters began with a first step at 95°C for 30s and 39 cycles afterwards alternating between 5s at 95°C and 15s at 60°C. A melting curve followed the final cycle and ran 5 s at 65°C and 5 s at 95°C. Gene expression was normalized against Elongation factor 1-alpha (At5g60390).

**Hierarchical clustering and visualization of heatmaps**

Cluster 3.0 (http://bonsai.hgc.jp/~mdehoon/software/cluster/) ([Eisen et al., 1998](#_ENREF_26)) was used to cluster genes according to mRNA and protein DE fold change values using hierarchical clustering with average linkage (Figure 4D). Heatmaps was visualized in Java TreeView v.1.1.6r4 (http://jtreeview.sourceforge.net).

**Gene Ontology (GO) analysis**

The ClueGO v.2.0.8 plugin ([Bindea et al., 2009](#_ENREF_9)) within Cytoscape version 3.0.2 was used for GO biological process (v2014.02.10) term enrichment using kappa score grouping (threshold =0.4) of terms that shared 67% gene overlap (Figure 4D, Supplemental Table XX). Over-represented terms (Benjamini-Hochberg corrected p-value ≤ 0.1) were identified using a hypergeometric test. For Figures 2 and 5, GO Slim term annotations were retrieved from Ensembl Plants 21 (EBI UK) *A. thaliana* genes (2010-09-TAIR10) within the BioMart database system ([Durinck et al., 2009](#_ENREF_25); [Kinsella et al., 2011](#_ENREF_52)). Statistical enrichment was calculated using the hypergeometric test with Benjamini-Hochberg multiple test correction (p-value ≤ 0.1) within R using the background sets of the *Arabidopsis* genome, filtered RNA (n=16355) or protein (n=4649) annotations as indicated.

**Temporal expression clustering**

mRNA: All Log2 fold change values for genes differentially expressed at one or more time points (n=5612) were input into Splinecluster ([Heard et al., 2005](#_ENREF_35)) and clustered using the following settings: normalisetargets=1, priorprecision=0.0001, reallocationsweeps=10000, gramschmidt=1, gammaparameter=0.5, gammaparameterb=0.5. The resulting clusters (n=23) were grouped according to similar expression patterns (i.e. DE across multiple time points), resulting in thirteen major expression groups. These expression groups were queried for *Arabidopsis* transcription factor genes using the PlantTFDB 3.0 database ([Jin et al., 2014](#_ENREF_42)).

PM protein: All Log2 DE fold change values for proteins differentially expressed at one or more time points (n=384) were input into Splinecluster and clustered using the following settings: normalisetargets=1, priorprecision=0.1, reallocationsweeps=10000, gramschmidt=1, gammaparameter=0.1, gammaparameterb=0.1. Non-significant fold change values were set to 0 prior to Splinecluster analysis. The resulting clusters (n=16) were grouped according to similar expression patterns (i.e. DE across multiple time points), resulting in seven major expression groups.

**Protein domain analysis**

Interpro domain assignments were retrieved from the Ensembl Plants 21 within the BioMart database system ([Durinck et al., 2009](#_ENREF_25); [Kinsella et al., 2011](#_ENREF_52)). The frequency of individual protein domains identified within the sample set was compared to the frequency of the domain in the background set. Statistical enrichment was calculated using the hypergeometric test with Benjamini-Hochberg multiple test correction (p-value ≤ 0.1) within R using the background set of all protein (n=4649) identifications.

**Phylogenetic analysis**

Clustal X 2.1 (http://www.clustal.org/) was used to align RLK kinase domains ([Shiu et al., 2004](#_ENREF_98)) or complete protein sequences (TAIR10) and to bootstrap the resulting trees with the neighbor-joining method using default settings. Trees were visualized in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

**PPI Network analysis**

Custom PPI network was constructed from the following databases: ANAP ([Wang et al., 2012](#_ENREF_112)), AI-1 ([Consortium, 2011](#_ENREF_20)), PPIN-1 ([Mukhtar et al., 2011](#_ENREF_80)), AtGSID ([Klopffleisch et al., 2011](#_ENREF_53)). The RLK plus first neighbor physical interaction network was clustered using mRNA 10-720 min and protein 10-720 min significant fold change attribute values with the Autosome algorithm ([Newman and Cooper, 2010](#_ENREF_82)) within the Cytoscape v2.8.3 application 'clustermaker' v1.10 ([Morris et al., 2011](#_ENREF_79)).

**Immunoblotting**

SDS-PAGE and immunoblotting were performed according to standard procedures. The following antibodies were used at the indicated concentrations: anti-Phospho-p44/42 MAPK (Erk1/2) (Cell Sgnaling #9101), 1:2000; anti-TCH3, 1:2000 ([Sistrunk et al., 1994](#_ENREF_99)). Secondary goat anti-rabbit IgG-HRP (Biorad) conjugates were used at a concentration of 1:3000 for detection using enhanced chemiluminescence (Pierce).

**Pathogen and callose assays**

*Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000) carrying the broad-host range plasmid pVSP61 ([Kunkel et al., 1993](#_ENREF_55)) was sprayed onto 4 week-old Arabidopsis plants at a concentration of 1x 108 cfu/mL in 10mM MgCl2 containing 0.025% Silwett L-77 surfactant. Leaf tissue (~1 cm2 per leaf) was harvested at four days, homogenized, and dilution plated to assay pathogen growth. Aniline blue staining and fluorescence microscopy of callose deposition was performed according to ([Adam and Somerville, 1996](#_ENREF_2)).

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