**Title:** Temporal profiling of the *Arabidopsis* plasma membrane proteome and transcriptome reveals dynamic expression of Receptor-like kinases and transporters in response to bacterial flagellin

**Authors:** J. Mitch Elmorea, Koste A. Yadetaa, Allison L. Creasonb, Jessica Y. Francoa, Yanming Did, José S. Rufiáne, Carmen R. Beuzóne, Brett S. Phinneyf, Jeff H. Changb,c, and Gitta L. Coakera#

**Author Affiliations:**

1. Department of Plant Pathology, University of California at Davis, Davis, CA
2. Department of Botany and Plant Pathology and the Molecular and Cellular Biology Program, Oregon State University, Corvallis, OR
3. Center for Genome Research and Biocomputing, Oregon State University, Corvallis, OR
4. Department of Statistics, Oregon State University, Corvallis, OR
5. Instituto de Hortofruticultura Subtropical y Mediterránea, Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Málaga, Spain
6. Genome Center Proteomics Core Facility, University of California at Davis, Davis, CA

#address correspondence to: [glcoaker@ucdavis.edu](mailto:glcoaker@ucdavis.edu)

**Running Title:** Membrane proteomics during flagellin perception

**ABBREVIATIONS:**

CRK Cysteine-rich Receptor-Like Kinase

LRR-RLK Leucine-Rich Repeat Receptor-Like Kinase

MAPK Mitogen-Activated Protein Kinase

PAMP Pathogen-Associated Molecular Pattern

PM Plasma Membrane

PRR Pattern Recognition Receptor

PTI Pattern-Triggered Immunity

RLK Receptor-Like Kinase

**SUMMARY**

The plasma membrane is a central regulator of plant responses to biotic and abiotic stresses. We employed high-resolution shotgun proteomics of plasma membrane-enriched fractions coupled with RNA-seq to investigate cellular changes occurring in *Arabidopsis* over a 12h periodupon activation of the FLS2 immune receptor with the flagellin peptide flg22. Over 4600 proteins were consistently identified across multiple samples, including two-thirds of the receptor-like kinases encoded in the *Arabidopsis* genome. A large percentage (>25%) of the leaf transcriptome exhibited differential expression 1h after elicitor treatment. Protein changes were more gradual, with 6% of proteins changing abundance 12h after flg22 treatment. A delayed correlation between transcript and protein expression was observed. We provide evidence that relatively small (<2-fold) changes in transcript abundance can lead to significant changes at the protein level. Biological process enrichment emphasized the tradeoff between plant growth and pathogen defense. Receptor-like kinases and membrane transporters were the two largest classes of differentially expressed proteins in plasma membrane fractions. Expression co-clustering of differentially expressed transcripts and proteins revealed temporal waves of the plant immune response and highlighted protein families that are coordinately expressed at the plasma membrane including proteins involved in oxidative stress responses, membrane microdomain formation, and vesicle trafficking. Functional analysis of the cysteine-rich receptor like kinase family identified multiple conserved phosphorylated residues controlling protein activity. Together, this study identifies novel components of the plant immune response and underscores the tradeoff between plant growth and pathogen defense.

**INTRODUCTION**

Every plant is a potential host for pathogenic microorganisms and some of the largest gene families function as immune receptors for pathogen detection ([Dodds and Rathjen, 2010](#_ENREF_21)). Many of these receptors localize to the plasma membrane (PM) and can recognize both external and endogenous signs of pathogen attack. Additional PM-associated proteins can control receptor abundance or propagate signals immediately downstream of the activated receptor. As many pathogen classes complete their lifecycle outside the plant cell membrane, PM proteins also participate in the host defenses that limit pathogen spread in infected tissue ([Dodds and Rathjen, 2010](#_ENREF_21)). Thus, the plant PM performs multiple roles in plant-microbe interactions.

Upon attack by pathogens, plants can recognize conserved molecular patterns in the apoplast of infected tissue using PM-localized transmembrane receptors, often with receptor-like kinase (RLK) domain architecture ([Dodds and Rathjen, 2010](#_ENREF_21)). Activation of these pattern recognition receptors (PRRs) leads to rapid (<15min) 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_77); [Monaghan and Zipfel, 2012](#_ENREF_60)). MAPK signaling cascades converge in the nucleus, where MPK3/6 phosphorylate transcription factors to up-regulate the expression of defense associated genes within 30min ([Asai et al., 2002](#_ENREF_4); [Tena et al., 2011](#_ENREF_77)). Later responses (>30min) include vesicle trafficking at 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 ([Dodds and Rathjen, 2010](#_ENREF_21); [Underwood and Somerville, 2013](#_ENREF_78)). This suite of responses, termed pattern-triggered immunity (PTI), suppresses pathogen growth in infected tissue.

The *Arabidopsis thaliana* PTI receptor Flagellin-sensing 2 (FLS2) is a model for immune responses activated by plant cell surface receptors. FLS2 is a leucine-rich repeat RLK (LRR-RLK) that directly binds a 22 amino acid epitope of bacterial flagellin called flg22. Co-receptors from the Somatic embryogenesis receptor kinase (SERK) family also contribute to FLS2 signaling ([Roux et al., 2011](#_ENREF_67)). Biochemical and structural studies demonstrate that Brassinolide-insensitive 1-associated kinase 1 (BAK1/SERK3) rapidly heterodimerizes with flg22-bound FLS2 ([Chinchilla et al., 2007](#_ENREF_13); [Heese et al., 2007](#_ENREF_28); [Sun et al., 2013](#_ENREF_74)). The FLS2-BAK1 complex phosphorylates the kinase Botrytis-induced kinase 1 (BIK1) to initiate intracellular signaling ([Lu et al., 2010](#_ENREF_53); [Kadota et al., 2014](#_ENREF_35); [Li et al., 2014](#_ENREF_48); [Lin et al., 2014](#_ENREF_51)). BAK1 and BIK1 are required for immune signaling activated by multiple PRRs and different PRRs can elicit similar changes in gene expression, indicating that multiple aspects of FLS2 signaling can be generalized to other immune receptors([Monaghan and Zipfel, 2012](#_ENREF_60)). While much progress has been made in deciphering the early molecular events that control pathogen perception, there is less understanding of how PM protein signaling unfolds in the hours following PTI activation.

Here we present temporal profiling of plant innate immunity over a 12-hour period after elicitation of leaf tissue with flg22. We coupled high-resolution liquid chromatography tandem mass spectrometry (LC-MS/MS) of PM protein fractions with RNA-Seq to profile changes in the proteome and transcriptome, respectively. Transcriptional changes peaked at 1h post-FLS2 activation, whereas protein changes gradually increased over 12h. Accordingly, we observed a delayed correlation in transcript and protein changes at the PM. Temporal co-clustering of transcript and protein expression revealed that slight changes in gene expression can lead to significant changes in protein abundance. We focused on two of the largest classes of differentially expressed proteins: membrane transporters and RLKs. Two-thirds (over 400 members) of the RLK superfamily in *A. thaliana* were identified by MS/MS and coordinate regulation of specific RLK subfamilies was observed. Multiple members of the Cysteine-rich Receptor Kinase (CRK) family were upregulated in response to flg22. Conserved CRK phosphorylated residues were identified and their importance for CRK activity validated. Together, this study revealed the contribution of previously unknown components to the PTI response at the plant cell membrane.

**EXPERIMENTAL PROCEDURES:**

**Plant growth conditions and elicitor treatment**

*A. 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. Four to five week-old plants were used for all experiments. Three hours after the onset of light, plants were sprayed with 10μM flg22 peptide (>85% purity, synthesized by GenScript USA Inc.) in 18.2 MΩ·cm-1 water containing 0.025% Silwet L-77 surfactant using a Preval-267 compressed air sprayer. Col-0 plants sprayed with 18.2 MΩ·cm-1 water containing 0.025% Silwet 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. Flg22-treated and water-treated control samples were processed in parallel for all replications. Aniline blue staining and fluorescence microscopy of callose deposition was performed as previously described ([Adam and Somerville, 1996](#_ENREF_2)).

**Plasma membrane enrichment and processing**

PM enrichment was performed on 30-40g of leaf tissue using three rounds of aqueous two-phase partitioning as described previously ([Larsson et al., 1994](#_ENREF_44); [Elmore et al., 2012](#_ENREF_22)) with minor modifications. Homogenization buffer was supplemented with 50mM sodium pyrophosphate, 25mM sodium fluoride, 1mM sodium molybdate, 1mM Sodium orthovanadate, and 25mM β-glycerophosphate**.** 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_33)). Samples were diluted 20 times with H2O and centrifuged at 90000g for 60min to pellet plasma membrane vesicles. Membrane pellets were frozen in liquid N2 and stored at -80˚C. Gel-based multidimensional separation was performed on each sample similar to ([Elmore et al., 2012](#_ENREF_22)), with each treatment and control sample pair processed on the same gel. Briefly, protein samples were solubilized in 2X Laemmli buffer with 6M urea and quantified using the RCDC Protein Assay (Biorad). Samples (300ug protein) were fractionated by 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 trypsin ([Shevchenko et al., 2006](#_ENREF_70)). 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**

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) which was desalted online. Peptides were 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 quantitation**

Tandem mass spectra were extracted to mzML format using Proteome Discoverer and analyzed with the X!Tandem GPM-XE Cyclone version 2013.02.01.2 spectrum modeler ([Craig and Beavis, 2004](#_ENREF_16)) using the TAIR10 *Arabidopsis* complete proteome (TAIR10\_pep\_20101214.fasta, 35386 entries) with a common repository of adventitious proteins (cRAP database 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_36)). 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 of cysteine 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/MS runs corresponding to the same sample merged. The probability of peptide identifications was modeled using a local FDR algorithm and discriminant scoring with a naïve Bayes classifier. Protein identifications required two unique peptides, 99.8% protein probability, and 20% peptide probability resulting in a 0.7% peptide decoy FDR and 4.7% protein decoy FDR. Shared spectral count distributions was performed within Scaffold similar to ([Zhang et al., 2010](#_ENREF_84)) 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 and proteins identified in only one of three biological replicates were removed prior to differential expression analysis. The QSPEC/QPROT statistical framework v1.2.2 (http://sourceforge.net/projects/qprot/) ([Choi et al., 2008](#_ENREF_15)) was used for differential expression analysis. Protein spectral counts were normalized for protein length and overall sample abundance within QSPEC/QPROT. Multiple testing correction was performed by the FDR estimation procedure implemented in QSpec/QProt v1.2.2 ([Bayer-Santos et al., 2012](#_ENREF_7)). Proteins with a criterion FDR ≤ 0.05 and minimum abundance change ±50% in two of three biological replicates were classified as differentially expressed (Supplemental Table 1). Phosphopeptide identifications required a peptide probability >= 99%, X!Tandem -log(e) score >= 1.5, and parent mass error <= 6ppm resulting in a 0.03% peptide decoy FDR for phosphorylated spectra.

**RNA-seq and quantitative analysis**

For RNA-seq analysis, plants were grown and treated in the same way as above. Total RNA was extracted from 4-5 leaves using the Trizol reagent (Invitrogen) according to manufacturer’s instructions. The TruSeq RNA kit (Illumina) was used to enrich for mRNA and prepare cDNA libraries which were sequenced on 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* TAIR10 reference genome (http://www.arabidopsis.org/) ([Cumbie et al., 2011](#_ENREF_17)). Read alignments were performed using CASHX v1.3 ([Fahlgren et al., 2007](#_ENREF_23)). Reads aligning to more than one gene locus were excluded. Genes with less than 10 reads were filtered out prior to analysis of differential expression. Differential expression and regression analysis were performed using the R package NBP-Seq version 0.1.8 ([Di et al., 2011](#_ENREF_20)). Transcripts with a p-value ≤ 0.05, q-value ≤ 0.05, and -1 ≥ Log2 fold change ≥ 1 were classified as differentially expressed (Supplemental Tables 2-6).

**Gene Ontology (GO) analysis**

GOslim term annotations were retrieved from Ensembl Plants 21 (EBI UK) At genes (2010-09-TAIR10) within the BioMart database system ([Kinsella et al., 2011](#_ENREF_41)). Tests for statistical enrichment were performed using the hypergeometric distribution with Benjamini-Hochberg correction (p-value ≤ 0.1) within R using the background set of the *A. thaliana* TAIR10 genome, filtered RNA-seq, or filtered protein sets as indicated.

**Temporal expression clustering and analysis**

Splinecluster was used for temporal expression co-clustering of differentially expressed proteins and their corresponding transcripts as described previously ([Heard et al., 2005](#_ENREF_27)). Temporal expression groups were tested for Interpro domain enrichment using the hypergeometric distribution with Benjamini-Hochberg correction (p-value ≤ 0.1) within R using BioMart annotations ([Kinsella et al., 2011](#_ENREF_41)).

**Phylogenetic analysis**

Clustal X 2.1 (http://www.clustal.org/) was used to align RLK kinase domain sequences ([Shiu et al., 2004](#_ENREF_71)) or complete protein sequences (TAIR10) and to bootstrap the resulting trees with the neighbor-joining method using default settings. Aminoglycoside 3'-phosphotransferase (P0A3Y6) was used as the outgroup for the kinase domain phylogenies ([Shiu et al., 2004](#_ENREF_71)). Trees were visualized in FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

**Immunoblotting**

SDS-PAGE and immunoblotting were performed according to standard procedures. Phospho-p44/42 Erk1/2 (MAPK) (Cell Signaling Technology #9101) and monoclonal ANTI-FLAG M2 (Sigma #F1804) antibodies were used a concentration of 1:2000. Secondary goat anti-rabbit (BioRad #170-5046) or goat anti-mouse (BioRad #170-6516) IgG-HRP conjugates were used at a concentration of 1:3000 for detection using enhanced chemiluminescence (Pierce).

**PCR**

Quantitative real-time PCR reactions used Bio-Rad SsoFast EvaGreen Supermix according to manufacturer’s directions using a CFX96 Touch (Bio-Rad). Gene expression was normalized against ELONGATION FACTOR 1-ALPHA (AT5G60390) and flg22-induced gene expression was compared to water-treated controls using the comparative C(t) method (Schmittgen and Livak, 2008). Site-directed mutagenesis used the QuickChange kit (Stratagene). All oligonucleotide primers are listed in Supplemental Table 10.

**Transient expression and analysis of cell death**

Agrobacterium-mediated transient expression was performed as described previously ([Leister et al., 2005](#_ENREF_46)). *A. tumefaciens* C58C1 carrying the pMD1 binary vector expressing wild-type CRK13 and site-directed mutants under control of the 35S promoter were syringe-infiltrated into 4 week-old *Nicotiana benthamiana* or *N. tabacum* plants at a concentration of OD600=0.4. Cell death was monitored 24-72 hours post-infiltration. *N. benthamiana* leaves were stained with trypan blue to visualize cell death at 40 hours post-infiltration ([Heese et al., 2007](#_ENREF_28)).

**RESULTS**

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

FLS2-mediated immune responses were investigated in leaf tissue of four-week-old *Arabidopsis thaliana* plants. Previously characterized early, middle and late PTI markers were used to determine appropriate times of sampling for the profiling experiments. Leaves were sprayed with 10μM flg22 peptide or water containing the surfactant Silwet L-77 (Figure 1A). MAPK activation was observed within 5min of flg22-treatment, was strongly sustained from 10 to 30min and returned to basal levels within 90min (Fig 1B). The marker gene *FLG22-INDUCED RECEPTOR KINASE* (*FRK1*) peaked at 60min and remained up-regulated relative to the water treatment for the duration of the experiment (Figure 1A). We could reliably observe callose deposition, a late PTI marker, in flg22-treated leaves by 720min (Figure 1C).

For PM-associated protein sampling, leaf tissue was collected at 10, 180, and 720min after flg22 application, corresponding to activation of early (MAPK activation), middle (transcriptional reprogramming), and late (callose deposition) markers, respectively (Figure 1). We focused on analyzing plasma membrane (PM) fractions to increase the resolution of low-abundance proteins in this important subcellular compartment. For RNA-seq, leaves were collected at 10, 60, 180, 360, and 720min after flg22 application (Figure 1A). For every time point, three biological replicates of flg22-treated and control tissue were harvested and analyzed. This sampling strategy facilitated a detailed temporal analysis of plant PTI responses over a 12h window.

**Protein and transcript identifications and differential expression analysis**

PM vesicles were purified using aqueous two-phase partitioning and treated with the Brij-58 detergent to release trapped cytosolic contaminants ([Larsson et al., 1994](#_ENREF_44)). The MS/MS dataset contained 6,227,230 spectra identifying 4,932 *A. thaliana* proteins (Figure 2A). This total set was filtered to remove proteins identified in only one replicate of a time point (Figure 2B). Differential expression analysis using spectral counting was performed on the filtered protein set (n= 4649) using the QSPEC/QPROT statistical framework with an FDR threshold of 0.05 and a minimum fold change of ±50% in at least two of three biological replicates ([Choi et al., 2008](#_ENREF_15)) (Supplemental Table 1). RNA-seq was also performed, resulting in an average of 19,000 genes identified per sample. Genes with a low number (<10) of aligned reads were further filtered out, leaving a total of 16,355 (16,091 protein-coding) gene identifications across all time points (Figure 2B). The genes that passed filtering were analyzed using the NBP-Seq package for differential expression ([Di et al., 2011](#_ENREF_20)) (Supplemental Tables 2-6). For proteins and transcripts, differential expression analysis was performed at each time point individually using the three biological replicates of flg22-treated and water-treated control samples.

A substantial overlap was observed between RNA-seq and MS/MS identifications. Only 2% of detected proteins had no corresponding RNA-seq reads (Figure 2A). Somewhat unexpectedly, ~10% of the proteins had very low read counts in the RNA-Seq dataset (Figure 2B). 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 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_69); [Vogel and Marcotte, 2012](#_ENREF_79); [Walley et al., 2013](#_ENREF_80)). Unless noted otherwise, the filtered protein and RNA-seq gene sets are used for subsequent analyses.

**Functional specialization of the plant plasma membrane**

Next, we investigated the molecular functions of identifications from RNA-seq and MS/MS approaches. Analysis of Gene Ontology (GO) terms ([Ashburner et al., 2000](#_ENREF_5); [Kinsella et al., 2011](#_ENREF_41)) revealed significant differences between the RNA-seq and MS/MS identifications and the *A. thaliana* genome for most GOslim molecular function terms (Figure 2C). The spectrum of GO terms associated with the RNA-seq data more closely resembled the background *A. thaliana* genome. In contrast, the GO terms associated with the PM-associated proteome were highly enriched in proteins with kinase and membrane transporter activity relative to the *A. thaliana* genome (Figure 2C). These results highlight functional specialization within the cellular membrane, which is enriched in signaling receptor-like kinases and membrane transporters.

**Characterization of the plasma membrane-associated proteome**

The analyzed PM-enriched fractions are expected to contain *bona fide* PM proteins and PM-associated cytosolic proteins. Previous experiments demonstrated that our PM isolation protocol resulted in the isolation of relatively pure PM preparations ([Elmore et al., 2012](#_ENREF_22)). It is important to note that membrane stripping was not performed, thus enabling the detection of PM-associated cytosolic proteins. Because biochemical purification strategies rarely attain 100% purity, it is also expected that there will be some degree of contamination of proteins localized to other organelles.

To evaluate the purity of PM fractions, the SUBcellular location database for Arabidopsis proteins (SUBA3) consensus predictions were used to assign a single, most-probable cellular location for all features identified in the MS/MS data, RNA-seq data, and the *A. thaliana* genome (Figure 3) ([Tanz et al., 2013](#_ENREF_76)). Relative to the entire *A. thaliana* genome, PM-associated proteins were highly enriched in the SUBA consensus locations "plasma membrane" (p = 2.7 x 10-74) and "cytosol" (p= 2.3 x 10-43) (Figure 3A). PM fractions were also enriched in proteins predicted to localize to the endoplasmic reticulum (p = 4.2 x 10-55) and depleted in proteins predicted to localize to the nucleus (p = 2.7 x 10-92) and extracellular region (p = 3.4 x 10-106) (Figure 3A). To further evaluate the presence of contaminating proteins from other organelles across the experiment, normalized spectral abundance factors (NSAF) were calculated for each protein and averaged across all samples ([Paoletti et al., 2006](#_ENREF_63)). Cumulative NSAF values were then calculated for each cellular compartment based on the SUBA consensus predictions for individual proteins. As expected, the majority of NSAF values are predicted to be derived from plasma membrane (42%) and cytosolic proteins (31%) (Figure 3B). Taken together, these results indicate that the analyzed PM fractions are highly enriched in the targeted proteomes (plasma membrane and cytosol). However, independent experimental methods should be used to establish the localization of individual proteins prior to detailed analyses.

Identified proteins were also analyzed for predicted and experimentally-supported evidence of transmembrane domains ([Krogh et al., 2001](#_ENREF_42)) and post-translational modifications associated with membrane targeting such as: myristoylation ([Podell and Gribskov, 2004](#_ENREF_64)), palmitoylation ([Hemsley et al., 2013](#_ENREF_29)), prenylation ([Maurer-Stroh et al., 2007](#_ENREF_57)) and GPI-anchoring ([Schwacke et al., 2003](#_ENREF_68)). Although no membrane stripping was employed to specifically enrich for integral membrane proteins, 32% (n=1501) of the proteins are predicted to contain at least one transmembrane domain (data not shown). In addition to transmembrane domains, 14% (n=672) of the proteins have predicted or experimental evidence of membrane association via lipid post-translational modifications (data not shown). This result is comparable to other *A. thaliana* membrane proteomics studies that found 20-50% integral membrane proteins in membrane-enriched fractions ([Alexandersson et al., 2004](#_ENREF_3); [Nelson et al., 2006](#_ENREF_61); [Marmagne et al., 2007](#_ENREF_56); [Mitra et al., 2007](#_ENREF_58); [Huang et al., 2013](#_ENREF_30)).

**Delayed correlation in the expression dynamics of the transcriptome and plasma membrane-associated proteome**

The majority of transcriptional changes in response to flg22 treatment occurred by 60min while most of the PM-associated protein changes occurred after 180min (Figure 4A). To further analyze the correspondence between changes at the mRNA and protein level, pairwise comparisons were made for each time point. Scatterplots of all protein and associated mRNA Log2 fold changes indicated a low but significant correlation in expression patterns (r=0.26-0.37) (data not shown). Next, we focused solely on analyses of differentially expressed genes and proteins. Significantly stronger correlations were observed for genes that were detected as differentially expressed at both the mRNA and protein levels (r=0.49-0.81) (Figure 4B). The strongest correlations between mRNA and protein expression were observed between the mRNA-60min/protein-180min (r=0.81) and the mRNA-180min/protein-720min (r=0.72) sample pairs. This delayed correlation likely reflects a 2-9 hour lag phase from transcription to translation and trafficking of a PM-localized protein. 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_24)).

**Biological process enrichment highlights the trade-off between plant growth and defense**

In order to characterize the cellular changes associated with flagellin perception over the course of the experiment, differentially expressed transcripts and proteins at each time point were analyzed for over-representation of GOslim biological process terms (Figure 5). Within 60min of flg22 treatment, up-regulated transcripts were enriched in biological processes associated with the immune system, stress responses, protein targeting, signal transduction, and transport (Figure 5). Down-regulated transcripts at 60-180min were enriched in terms associated with growth, development and various primary and secondary metabolic processes. Consistent with the delayed correlation observed between transcript and protein (Figure 4B), many of the significant biological processes observed at the transcript level were reflected at later time points at the protein level. Biological process terms associated with the immune system, protein targeting, response to stress, signal transduction, transmembrane transport, and vesicle transport were significantly over-represented in up-regulated proteins at 180-720min post-flg22 (Figure 5). Down-regulated processes at the protein level include cell differentiation, cell wall organization, developmental maturation, and growth. These cellular changes reflect the known trade-off between plant growth and immunity ([Lozano-Durán et al., 2013](#_ENREF_52); [Huot et al., 2014](#_ENREF_31)) and the metabolic reprogramming necessary to mount a defense response ([Bolton, 2009](#_ENREF_8)).

**Membrane transporters that are differentially expressed during FLS2 activation**

Multiple membrane transporter proteins were differentially expressed 180-720min after flg22 treatment (Table 1). ATP-binding cassette (ABC) transporters belonging to the ABCB and ABCG subfamilies were up-regulated 180-720min post-flg22. ABCB14 is a malate importer that can affect stomatal movement by controlling guard cell osmotic pressure ([Lee et al., 2008](#_ENREF_45)). Other significantly changing ABC transporters include those implicated in auxin transport ([Kamimoto et al., 2012](#_ENREF_37)), abscisic acid import ([Kang et al., 2010](#_ENREF_38)), and export of sporopollenin precursors involved in pollen exine formation ([Quilichini et al., 2010](#_ENREF_66)). In addition, several members of the Nitrate transporter1/Peptide transporter family (NPF) also increased in abundance during PTI ([Léran et al., 2014](#_ENREF_47)). Members of the ABC and NPF families can transport a range of substrates ([Kang et al., 2011](#_ENREF_39); [Léran et al., 2014](#_ENREF_47)). Thus, these proteins likely contribute to hormone transport as well as the export of antimicrobial or cell wall-reinforcing compounds into the apoplast upon pathogen perception. We identified 14 of 15 P-type Ca2+ ATPases in *A. thaliana*, but only the Autoinhibited Ca2+ ATPase 12 (ACA12) was differentially expressed in PM fractions. 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_50)). The strong up-regulation of ACA12 at 180-720min post-flg22 treatment suggests that it could be involved in cytosolic calcium ion homeostasis during PTI.

**Temporal expression clustering highlights protein families coordinately regulated during FLS2 activation**

To better understand temporal expression dynamics during FLS2 signaling, we used Bayesian hierarchical co-clustering implemented in the Splinecluster package ([Heard et al., 2005](#_ENREF_27)). Clustering of differentially expressed proteins (n=333) and their corresponding transcripts over the course of the experiment resulted in 11 clusters representing distinct patterns of expression (Figure 6, Supplemental Figure 1, Supplemental Table 7). Clusters 1 and 3 are comprised of proteins that were down-regulated at 720min and 180min, respectively, but whose transcripts were filtered prior to differential expression analysis due to low or no RNA-seq reads. Clusters 6 and 9 are comprised of proteins that were up-regulated at 180min and 720min, respectively, with only minor changes at the transcript level (<2 fold). Similarly, Clusters 7 and 8 include proteins down-regulated at 720min, but the majority of the corresponding transcripts exhibit minor differences in expression. These observations suggest that significant changes in protein abundance can occur in the absence of major changes in gene expression at commonly used thresholds, e.g. a 2-fold change in transcript abundance.

To identify protein families that exhibit coordinate regulation during PTI, we tested the temporal clusters for over-representation of Interpro protein domains in each group (Table 2, Supplemental Table 8). The majority of the over-represented Interpro domains are associated with membrane-localized proteins and over half are associated with receptor-like kinases (RLKs) or membrane transporters (Table 2). Other protein families of interest and with two or more members present in the temporal clusters include the IPR008700 and IPR001107 domain families. Proteins carrying the IPR008700 domain share homology with the plant immune regulator RPM1-interacting 4 (RIN4). This domain can be targeted by the pathogen effector protease AvrRpt2 ([Chisholm et al., 2005](#_ENREF_14)), suggesting that in addition to RIN4, other IPR008700 proteins may function in regulating immune responses. This observation is consistent with the "zig-zag" model of plant-pathogen interactions which predicts that the host targets of pathogen effectors participate in pattern-triggered immunity ([Jones and Dangl, 2006](#_ENREF_34)).

The IPR001107 Band 7 domain is also known as the stomatin/prohibitin/flotillin/HflK/C (SPFH) domain. SPFH proteins localize to PM microdomains and have roles in vesicle trafficking and membrane scaffolding ([Stuermer, 2010](#_ENREF_73); [Qi et al., 2011](#_ENREF_65); [Li et al., 2012](#_ENREF_49)). Flotillins are involved in a clathrin-independent endocytic pathway in *Arabidopsis* and have multiple roles in membrane trafficking in other eukaryotes ([Stuermer, 2010](#_ENREF_73); [Li et al., 2012](#_ENREF_49)). SPFH proteins have important roles in symbiosis and immunity in diverse plants ([Haney and Long, 2010](#_ENREF_26); [Qi et al., 2011](#_ENREF_65)). Taken together, this analysis highlights protein families that are differentially expressed at the PM during PTI and provides a starting point to dissect the contributions of specific family members to plant immunity.

**Receptor-like kinases (RLKs) represent the largest class of differentially expressed proteins**

The high-resolution proteomics strategy facilitated the detection of numerous RLKs in leaf tissue. We identified 411 RLKs by MS/MS, representing ~66% of all RLKs encoded in the *A. thaliana* genome. Furthermore, 60 (15%) of the identified RLK proteins were differentially expressed over the course of the experiment. To understand the regulation of this large gene family in the context of its evolutionary history, the RLK superfamily was divided into LRR-RLKs and non-LRR RLKs and phylogenetic trees were constructed based on their kinase domain sequences (Supplemental Figures 2 and 3) ([Shiu et al., 2004](#_ENREF_71)). A heatmap of differential mRNA and protein expression was aligned to the RLK phylogenies to evaluate the expression patterns of related proteins (Supplemental Figures 2 and 3). Differential protein expression of LRR-RLKs was primarily detected in the LRR-Ia, LRR-XI, and LRR-XII subfamilies ([Shiu et al., 2004](#_ENREF_71)). Notably, Six members of the LRR-Ia IMPAIRED-OOMYCETE SUSCEPTIBILITY 1 (IOS1) family were up-regulated 180-720min post-flg22. Several members of the LRR-XI subfamily with known or putative roles in development were down-regulated, consistent with the growth/defense antagonism previously described. The immune receptors EFR and FLS2 as well as two additional members of the LRR-XII RLK subfamily were up-regulated 180-720min after flg22 treatment. Analysis of non-LRR RLKs showed that most differentially expressed proteins belonged to the L-Lectin, SD-1b (S-domain), and DUF26/IPR002902 domain subfamilies (Supplemental Figure 3).

To visualize the relative protein abundance of RLKs, the mean NSAF ± standard deviation was calculated across all treatment and control samples (Supplemental Figures 2 and 3) ([Paoletti et al., 2006](#_ENREF_63)). A large dynamic range (104 difference in mean NSAF values) in estimated LRR-RLK abundance was observed (Supplemental Figure 2). Interestingly, members within certain sub-clades exhibited similar levels of relative abundance (for example, the SERK and BIR families) (Supplemental Figure 2). Spectral count assignments for shared peptides were weighted according to the number of unique peptides identified for each protein, suggesting that the coordinate expression of these families is biologically relevant. However, protein inference from bottom-up peptide analysis can be challenging for homologous proteins and expression of individual family members should be validated using independent approaches. Nevertheless, these analyses provide unique and previously unreported information about the relative levels of RLKs in PM fractions from leaf tissue.

**Regulation of the BAK1/FLS2 protein interaction network**

Bacterial flagellin is perceived by the FLS2 immune receptor, which also functions in concert with its co-receptor BAK1. Because we profiled temporal changes occurring after flagellin perception, differential regulation of the BAK1/FLS2 network was investigated. This network of BAK1/FLS2 associated/interacting proteins was generated based on previously published reports (Supplemental Figure 4). Although BAK1 was up-regulated at the transcript level, we did not detect significant changes in BAK1 protein abundance. However, differential protein expression was detected for the BAK1-interacting proteins EFR, FLS2, IOS1, and PEPR1 (all up-regulated at 720min) and BRI1 (down-regulated at 720min) (Supplemental Figure 4). Complex patterns of regulation of the Plant U-box 12 (PUB12) at the transcript and protein level were detected (Supplemental Figure 4). PUB12 is an E3 ubiquitin ligase that directly targets FLS2 for degradation and its decrease at the PM 180-720min post-FLS2 activation likely enables FLS2 to accumulate to higher levels, amplifying the signal of bacterial perception in the event of significant bacterial titers ([Lu et al., 2011](#_ENREF_54)). We also detected an increase in the protein levels of Respiratory burst oxidase homologue D (RBOHD), which has recently been shown to reside in complex with FLS2 and EFR and is activated by BIK1 to regulate production of reactive oxygen species in response to pathogen perception ([Kadota et al., 2014](#_ENREF_35); [Li et al., 2014](#_ENREF_48)). The down-regulation of the developmental RLK BRI1 and up-regulation of several immune-related RLKs likely contribute to the antagonistic relationship between plant growth and immunity. Further, the composition of BAK1/SERK protein complexes appear to be altered not only in the seconds to minutes following receptor activation but sustained over several hours.

**Cysteine-rich receptor kinases (CRKs) are dynamically expressed during PTI and regulated by phosphorylation**

Among the differentially regulated RLKs (Table 2 and Supplemental Figure 3), we noted that seven members of the DUF26/IPR002902 domain family were up-regulated at the protein level and the majority of this family was transcriptionally mobilized upon flagellin perception (Figure 7A). In plants, these proteins are known as Cysteine-rich receptor kinases (CRKs) and typically contain two extracellular cysteine-rich Ginkbilobin-2 (Gnk2)-homologous/DUF26 domains ([Wang and Ng, 2000](#_ENREF_81)). Different CRK family members have been previously implicated in the activation of plant immunity ([Chen et al., 2003](#_ENREF_10); [Chen et al., 2004](#_ENREF_11); [Acharya et al., 2007](#_ENREF_1)) and responses to various abiotic factors like water and ozone stresses ([Wrzaczek et al., 2010](#_ENREF_83); [Tanaka et al., 2012](#_ENREF_75); [Idänheimo et al., 2014](#_ENREF_32)), indicating that the CRK family plays an important role in stress responses. An unrooted phylogeny of the CRK family was created using complete amino acid sequences (Figure 7A). A heatmap of differential transcript and protein expression and a bar plot of estimated relative PM abundance were constructed (Figure 7A). While the majority of the CRK subfamily is transcriptionally up-regulated, differential protein abundance was only detected in a few subclades (Figure 7A).

Due to the importance of protein phosphorylation in plant immunity, the MS/MS data were searched for peptides with phosphorylated serine (S), threonine (T), or tyrosine (Y) residues. Based on stringent peptide identification thresholds (see EXPERIMENTAL PROCEDURES), we identified 17081 phosphopeptide spectra that correspond to 930 unique peptides derived from 546 proteins, (Supplemental Table 9). Several phosphopeptides mapped to CRK proteins (Supplemental Table 9). We used an alignment of the amino acid sequences of the CRK family to identify conserved phosphorylated residues. Three phosphopeptides corresponding to the modified sites CRK2S309, CRK10S662, and CRK41S653 exhibited conserved S/T residues for most CRK family members. We also identified several lower confidence phosphopeptide spectra with conserved, potentially modified residues.

We examined the role of a subset of these conserved residues in CRK13 (Figure 7B), which elicits defense responses and cell death when over-expressed in *A. thaliana* ([Acharya et al., 2007](#_ENREF_1)). *Agrobacterium*-mediated transient expression of a 35S:CRK13:3XFLAG construct in *Nicotiana benthamiana* and *N. tabacum* also elicits cell death (Figure 7C). Site-directed PCR mutagenesis was performed on CRK13 to substitute the candidate phosphorylated residues to alanine (primers available in Supplemental Table 10). We also mutated the highly conserved lysine residue CRK13K386 in the ATP-binding site to generate a "kinase-dead" mutant ([De Bondt et al., 1993](#_ENREF_18)). The CRK13 mutants CRK13S345A (corresponding to CRK2S309), CRK13K386N, CRK13S389A, CRK13T392A, CRK13S395A, CRK13S66A (corresponding to CRK10S662 and CRK41S653) were assayed for cell death elicitation in *N. benthamiana* and *N. tabacum* (Figure 7C). Wild-type CRK13 (CRK13WT), CRK13S345A,and CRK13S389A elicited similar levels of cell death starting around 24 hours post-infiltration, resulting in complete tissue collapse within 48 hours. The CRK13K386A and CRK13T392A mutations completely abolished cell death activity in both *N. benthamiana* and *N. tabacum* (Figure 7C). The CRK13S395A mutant exhibited reduced cell death (50-75% of infiltrated leaves) compared to CRK13WT in *N. benthamiana* while the majority of CRK13S395A infiltrations in *N. tabacum* were similar to CRK13WT. The CRK13S666A mutant consistently (85-90% of infiltrated leaves) showed reduced cell death compared to CRK13WT in *N. benthamiana and N. tabacum* (Figure 7C). All proteins accumulated to similar levels as CRK13WT (Figure 7C). Importantly, CRK13K386A did not induce cell death, indicating that kinase activity is essential for the cell death phenotype. Together, these results suggest that kinase activity and conserved, phosphorylated residues on CRK13 are important for cell death activation *in planta*.

**DISCUSSION**

In this study we sought to uncover temporal phases of the FLS2-mediated PTI responses with a focus on dynamic changes in the PM proteome. In contrast with other studies seeking highly pure membrane fractions ([Marmagne et al., 2007](#_ENREF_56); [Huang et al., 2013](#_ENREF_30)), no membrane stripping was employed to preserve important protein-protein interactions at the PM. Accordingly, the majority of spectral counts are derived from predicted PM and cytosolic proteins (Figure 3B). Furthermore, 1501 (32%) of the identified proteins are predicted to contain a transmembrane domain and 672 (14%) are predicted to harbor a membrane-targeting post-translational modification. The in-depth profiling of PM fractions presented here greatly expands the repertoire of putative PM-associated proteins in *Arabidopsis* leaves and highlights important areas for future investigations of immune signaling.

Gene expression is controlled at multiple steps before and after transcription and translation. This multi-tiered regulation allows a cell to fine-tune its responses to stimuli. Accordingly, it is possible that changes observed at the transcript level are not translated into similar changes in protein abundance. Direct comparison of transcript and protein regulation was complicated in this study because a specific subcellular compartment was profiled containing distinct proteins relative to the rest of the cell (Figure 2). The global correlations between mRNA and protein expression were low (data not shown), which might be attributed to the chosen sampling times or the differences in the sensitivity of our transcript and protein quantification methods. However, our observations agree with previous studies which reported a similar low correspondence between transcript and protein levels in animal cells, yeast, and plants ([Maier et al., 2009](#_ENREF_55); [Fournier et al., 2010](#_ENREF_24); [Lan et al., 2012](#_ENREF_43); [Ning et al., 2012](#_ENREF_62); [Vogel and Marcotte, 2012](#_ENREF_79); [Walley et al., 2013](#_ENREF_80)). Stronger correlations (r = 0.7-0.8) were observed for genes detected as differentially expressed at the mRNA and protein level (Figure 4B). The strongest correlations we observed for transcript and protein expression were between mRNA-60min/protein-180min and mRNA-180min/protein-720min sample pairs (Figure 4B). The data suggest a 2-9 hour interval between transcriptome changes and appearance of functionally-localized PM proteins. This delay could be exploited by pathogens that deliver effectors into host cells to disrupt immune signaling networks before the host can mount an effective defense.

**RLK dynamics highlight the tradeoff between plant growth and defense priming**

Biological process analysis of differentially expressed proteins indicated that plant growth and development processes are down-regulated during activation of immune responses (Figure 5). The BAK1/FLS2 protein network highlights this tradeoff between plant growth, development and defense responses (Supplemental Figure 4). Although *BAK1* transcription was up-regulated, we were unable to detect any significant changes in BAK1 or SERK proteins (Supplemental Figures 2 and 4). However, known BAK1-interacting RLKs were modulated at the protein level, with the immune-related kinases EFR, FLS2, IOS1, and PEPR1 up-regulated 180-720min and the brassinosteroid receptor BRI1 down-regulated by 720min post-flg22 (Supplemental Figure 4). Up-regulation of IOS1 during PTI is consistent with its role in regulating FLS2/EFR association with BAK1 and priming of defense responses ([Chen et al., 2014](#_ENREF_9)). The decrease in BRI1 levels likely facilitates the interaction between immune signaling PRRs and BAK1 enabling rapid activation of immunity in response to subsequent pathogen attack as well as positive feedback loops to sustain PTI.

Several RLKs with known or predicted roles in plant development are down-regulated at the PM in response to flg22 treatment, often with subtle transcriptional changes (Supplemental Figure 2). This observation indicates that in addition to FLS2, receptor turnover at the PM may play an important role in RLK dynamics during immune signaling ([Geldner and Robatzek, 2008](#_ENREF_25); [Lu et al., 2011](#_ENREF_54); [Smith et al., 2014](#_ENREF_72)). Accordingly, we observed an increase in the levels of the adaptor protein complex 2 (AP-2), at 180min post-flg22 (Supplemental Table 1) ([Banbury et al., 2003](#_ENREF_6)). The AP-2 complex is involved in loading transmembrane proteins (for example BRI1) carrying an internalization motif into clathrin coated endocytic vesicles at the PM ([Banbury et al., 2003](#_ENREF_6); [Geldner and Robatzek, 2008](#_ENREF_25); [Di Rubbo et al., 2013](#_ENREF_19)). In addition, flotillin proteins involved in clathrin-independent endocytic pathways and microdomain formation were up-regulated in PM fractions after flg22 treatment, suggesting a restructuring of membrane compartments during PTI ([Keinath et al., 2010](#_ENREF_40); [Li et al., 2012](#_ENREF_49)). These observations support previous reports that both endocytic pathways and membrane microdomains play an important role in regulating membrane signaling during immune responses ([Geldner and Robatzek, 2008](#_ENREF_25); [Keinath et al., 2010](#_ENREF_40)).

Most known PRRs are present at relatively low levels in PM fractions (Supplemental Figures 2 and 3). PRR abundance is likely tightly controlled in order to avoid inappropriate activation. Pre-treatment of plants with flg22 primes the plant immune system to elicit robust defense responses upon pathogen challenge ([Zipfel et al., 2004](#_ENREF_85)). The up-regulation of many known PRRs (Supplemental Figures 2 and 3) around 720min post-flg22 supports a model where the primary recognition event stimulates an increase in pathogen receptors at the PM, leading to stronger activation of defense responses against subsequent pathogen attack. The increase in PRRs recognizing different microbial ligands may allow the plant to more rapidly achieve the signaling threshold required for activation of defense. Moreover, the expression patterns of known PRRs strongly implicate uncharacterized RLKs with similar expression profiles as additional plant immune receptors or regulatory proteins.

**CRKs in plant defense signaling**

Proteins with IPR002902/Gnk2-homologous domains in *Arabidopsis* comprise ~60 small secreted proteins and ~42 non-LRR cysteine-rich RLKs (CRKs) ([Chen, 2001](#_ENREF_12)). The presence of disulfide bonds in the Gnk2-homologous domain suggests that CRKs might be sensitive to apoplastic redox states ([Miyakawa et al., 2009](#_ENREF_59)). Thus, CRK proteins may be up-regulated during PTI in order to monitor the apoplast for subsequent signs of pathogen recognition or to amplify reactive oxygen species-mediated immune signaling ([Wrzaczek et al., 2013](#_ENREF_82); [Smith et al., 2014](#_ENREF_72)). Around 20 of the CRK-encoding genes are present in a tandem array on chromosome 4 of the *A. thaliana* Col-0 genome and nearly all are transcriptionally activated in response to elicitor or ozone treatment (Figure 7A) ([Wrzaczek et al., 2010](#_ENREF_83)). While most of the CRK family is up-regulated at the transcript level, we only detected changes in the protein levels of CRK11, 13, 14, 18, 22, 28, and 29. These results suggest that 1) the CRK family is under post-transcriptional and/or translational control or 2) limitations in MS/MS quantification or sampling times prevented detection of changes in additional CRK proteins (Figure 7A). Analysis of phosphopeptides resulted in the identification of conserved phosphorylated amino acids that impact CRK-mediated cell death *in vivo*. Importantly, a kinase-dead mutant of CRK13 lost the ability to elicit cell death and a conserved, phosphorylated residue (S666)in its C-terminal cytoplasmic tail impaired the ability of CRK13 to activate cell death (Figure 7B-C). These results indicate that both kinase activity and phosphorylation at key residues play an important role in CRK regulation. Future investigations into the connection between CRKs, ROS signaling, and immunity should yield mechanistic insights into the regulation of plant stress responses.

**CONCLUSION**

The in-depth MS/MS analysis of plasma membrane-enriched fractions identified major alterations occurring within the membrane-associated proteome during pattern-triggered immune signaling. Detailed analyses of differentially expressed proteins will likely uncover novel mechanisms of pathogen recognition, RLK regulation, and transporter-mediated plant defense at the plasma membrane.

**ACKNOWLEDGEMENTS**

This work is funded through NSF grant number MCB-1054298 and NIH grant number RO1GM092772 awarded to GC. JME is supported in part by the NSF CREATE-IGERT graduate research training program (DGE-0653984). Protein sample analyses were performed at the UC Davis Genome Center Proteomics Core Facility. This was work also funded in part by a grant awarded to JHC and YD (NIH R01GM104977). RNA-seq was performed at the Center of Genome Research and Biocomputing at Oregon State University. We thank Shin-Han Shiu for sharing kinase domain sequences and Hyungwon Choi, Nicholas Heard, and Sebastian Maurer-Stroh for helpful discussions regarding the QProt, Splinecluster, and PrePs software, respectively.

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**FIGURE LEGENDS:**

**Figure 1. Experimental overview and timing of flg22-activated responses.**

1. Overview of the sampling strategy. Four-week-old *Arabidopsis* Col-0 plants grown in soil were sprayed with 10μM flg22 peptide or H2O. Rosette leaf tissue was harvested for plasma membrane (PM) protein enrichment or RNA extraction at the indicated time points. The line graph depicts expression of the *FLG22-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*) marker gene relative to H2O-treated controls (mean ± SD). Expression values were normalized to Elongation factor-1α. Blue and red arrows indicate the sampling times for RNA and protein extractions, respectively.
2. Robust mitogen-activated protein kinase (MAPK) activation is observed 10-15min post-flg22 treatment. Plants were treated as in (A). Total leaf protein was extracted from tissue harvested at the indicated time points and subjected to SDS-PAGE and immunoblotting using an antibody recognizing phospho-p44/p42 MPK. Signals corresponding to MPK3 and MPK6 are indicated with arrows.
3. Reproducible callose deposition is observed 720min (12h) post-flg22 treatment as determined by aniline blue staining and fluorescence microscopy. Plants were treated as in (A). Scale bar = 100μm.

**Figure 2. RNA and protein identifications highlight functional specialization of the plasma membrane-enriched proteome.**

1. Overlap in identifications in the unfiltered RNA-seq and MS/MS datasets. Genes with at least one unique RNA-seq read or proteins with two unique peptides present in at least one sample across the experiment were considered identified.
2. Overlap in identifications in the filtered RNA-seq and MS/MS datasets. The filtered datasets were used for differential expression analysis. The filtered RNA-seq data include genes with ten or more reads in all three replicates of either treatment (flg22) or control (H2O) at a given time point. The filtered MS/MS data include proteins identified in at least two replicates of either treatment or control at a given time point.
3. Comparison of Gene Ontology (GO) Molecular Function term annotations in filtered data compared to the complete *Arabidopsis* genome. GO slim annotations were tested for differences using the hypergeometric test with Benjamini-Hochberg multiple test correction. The most significant terms (p < 0.001) for the protein and RNA-seq identifications are indicated with asterisks (\*) and hashes (#), respectively. Proteins with kinase and transmembrane transporter activities are enriched in the MS/MS data.

**Figure 3. The majority of proteins identified by MS/MS are predicted to be associated with the plasma membrane and cytosol.**

1. SUBcellular localization database for Arabidopsis (SUBA) consensus predictions in filtered identifications compared to the complete *Arabidopsis* genome. The bars represent the percentage of identified genes or proteins predicted to localize in each cellular compartment relative to all filtered genes or proteins, respectively. The most significant terms (p < 0.001) for the identified proteins are indicated with asterisks (\*).
2. The majority of MS/MS spectral counts are derived from proteins predicted to localize to the plasma membrane and cytosol. Protein normalized spectral abundance factor (NSAF) values were summed for all proteins predicted to localize to each subcellular compartment to estimate the relative abundance of each compartment in plasma membrane-enriched fractions.

**Figure 4. Delayed correlation in the expression dynamics of the transcriptome and plasma membrane-associated proteome.**

1. An overview of the percentage of genes that are differentially expressed in the RNA-seq and MS/MS data.
2. Moderate correlations in mRNA/protein regulation were observed for genes differentially expressed at both the mRNA and protein level.Pairwise scatter plots depict the correlation of mRNA and protein log2 fold changes for all genes identified as differentially expressed at each timepoint. The red dotted line is the linear regression of the plotted data. Pearson correlation coefficients are reported above each plot*.*

**Figure 5. Biological process enrichment of flg22 responses highlight the tradeoff between growth and development.**

Stacked bar charts depicting the number of differentially expressed (DE) genes and proteins with their corresponding GOslim biological process annotations. Astericks (\*) indicate time points where the corresponding term is significantly enriched (hypergeometric test with Benjamini-Hochberg correction, p<0.1) relative to the background sets of mRNA and protein identifications, respectively. Down-regulated proteins are significantly enriched in processes related to cell differentiation, development, and growth. Up-regulated proteins are significantly enriched in processes related to protein modification, immunity, protein targeting, signal transduction, and transport.

**Figure 6. Temporal clustering of differentially expressed proteins and their corresponding transcripts highlights unique and disparate patterns of regulation in response to flg22.**

Differentially expressed proteins (n=333) were clustered according to their observed RNA and protein expression patterns (log2 fold change) over the course of the experiment. The Splinecluster algorithm was used to partition the proteins into eleven discrete clusters. The line graphs depict the RNA (red) and protein (blue) expression patterns of individual genes (thin lines) in each cluster. The thick lines represent a fitted LOESS regression line with standard error depicted in the shaded region. The number of genes in each cluster is indicated in the top right corner of each expression plot.

**Figure 7. Identification of important phosphorylated residues on Cysteine-rich receptor-like kinases (CRKs) that impact function.**

1. Most CRK family members are up-regulated transcriptionally, but only a seven are up-regluated at the protein level following FLS2 activation. Left: Unrooted phylogeny of the *Arabidopsis* CRK family generated from full-length protein sequences. Middle: Heatmap of RNA and protein differential expression. Right: Bar chart of relative expression in plasma membrane enriched fractions. #, kinase domain only; \*, no kinase domain present.
2. Several identified phosphopeptides are conserved across multiple CRKs. Conserved residues selected for mutagenesis are indicated with red arrows on the CRK13 domain architecture.
3. Functional analysis of the role of CRK13 phosphorylation in inducing cell death. Wild-type *35S:CRK13:3XFLAG* induces cell death after transient expression in *Nicotiana benthamiana* and *N. tabacum.* Phosphorylated residues were mutated to alanine (phosphonull) and the resulting mutant proteins were assayed for the ability to induce cell death*.* A kinase-dead version of CRK13 (K386N) was also tested. Cell death was visualized in *N. benthamiana* by trypan blue staining 40 hours post-infiltration. Pictures were taken 72 hours post-infiltration. Anti-FLAG immunoblots detecting CRK13 expression are shown below.

**Table 1. Transporters exhibiting differential expression in the MS/MS data.**

Transporter families were annotated according to the Aramemnon plant membrane protein database v8.0 (http://aramemnon.botanik.uni-koeln.de/). \*, differentially expressed; ND, not determined.

**Table 2. Enriched protein domains in temporal expression clusters (accompanies Figure 6)**

Interpro domains present in each cluster were tested for over-representation using the hypergeometric test with Benjamini-Hochberg multiple test correction (p < 0.1). The filtered protein identifications served as the background set. Protein domains corresponding to RLKs and membrane transporters are indicated with a 'X'.

**Supplemental Figure 1. Heatmap of the temporal expression clusters depicted in Figure 6**

Differentially expressed proteins (n=333) were clustered according to their observed RNA and protein expression patterns (log2 fold change) over the course of the experiment. The Splinecluster package was used to partition the proteins into eleven discrete clusters (indicated on the x-axis). Blue and yellow boxes indicate down- and up-regulation relative to controls at the indicated time points. A dendrogram representing cluster relatedness is shown at the top.

**Supplemental Figure 2. Differential expression and protein relative abundance of LRR-RLKs**

Left: Phylogenetic tree of all TAIR10 annotated LRR-RLKs generated from kinase domain amino acid alignments. RLK subfamily membership is indicated after the Arabidopsis Gene Identifier on the tree. Major subfamily clades are indicated in orange. Middle: Heatmap of mRNA and protein differential expression patterns after FLS2 activation. Non-significant fold changes were set to zero on the log2 scale. Right: Bar chart of LRR-RLK relative protein abundance in PM-enriched protein fractions (normalized spectral abundance factors, NSAF). Mean NSAF values were averaged across all MS/MS samples. Error bars represent standard deviation. Specific RLKs or RLK families are indicated in black. Note modified scale to highlight differences in lower abundance proteins.

**Supplemental Figure 3. Differential expression and protein relative abundance of non-LRR RLKs**

Left: Phylogenetic tree of all TAIR10 annotated non-LRR-RLKs generated from kinase domain amino acid alignments. RLK subfamily membership is indicated after the Arabidopsis Gene Identifier on the tree. Major subfamily clades are indicated in orange. Middle: Heatmap of mRNA and protein differential expression patterns after FLS2 activation. Non-significant fold changes were set to zero on the log2 scale. Right: Bar chart of RLK relative protein abundance in PM-enriched protein fractions (normalized spectral abundance factors, NSAF). Mean NSAF values were averaged across all MS/MS samples. Error bars represent standard deviation. Specific RLKs or RLK families are indicated in black.

**Supplemental Figure 4. Expression of the FLS2/BAK1 signaling network after flagellin treatment**

A protein interaction network was generated according to published experimental data. Proteins are represented by ovals and are colored based on the up- (yellow) or down- (blue) regulation observed in the MS/MS data. Connections represent physical protein-protein interactions. The line graphs depict RNA (red) and protein (blue) expression (log2 fold change) for the indicated genes over the course of the experiment. Astericks (\*) indicate significant changes in expression at the indicated timepoint.