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SHORT COMMUNICATION

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An unbiased nuclear proteomics approach reveals novel nuclear protein components that participates in MAMP-triggered immunity

Zainab Fakih, Md Bulbul Ahmed, Claire Letanneur, and Hugo Germain

Department of Chemistry, Biochemistry and Physics and Groupe de Recherche en Biologie Végétale, Université du Québec à Trois-Rivières, Trois-Rivières, QC, Canada

ABSTRACT

(MAMP)-triggered immunity (MTI) is the first layer of molecular defense encountered by pathogens. Genetic screens have contributed to our knowledge of MTI, but are limited to phenotype-causing mutations. Here we attempt to identify novel factors involved in the early event leading to plant MTI by comparing the nuclear proteomes of two Arabidopsis genotypes treated with chitosan. Our approach revealed that following chitosan treatment, *cerk1* plants had many nuclear accumulating proteins in common, but also some unique ones, when compared with Col-0 plants. Analysis of the identified proteins revealed a nuclear accumulation of DNA-modifying enzymes, RNA-binding proteins and ribosomal proteins. Our results demonstrate that nuclear proteomic is a valid, phenotype-independent approach to uncover factor involved in cellular processes.

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Plants have evolved a multilayered system to detect and defend against potentially harmful pathogenic microbes. Beyond structural defenses, the first molecular layer is composed of transmembrane pattern recognition receptors (PRR) that detect slowly-evolving microbial components.[1] These microbe-associated molecular patterns (MAMPs), also known as pathogen-associated molecular patterns (PAMPs) include, among many others, the bacterial flagellin (flg22) and elongation factor Tu.[2] MAMP recognition by PRRs triggers ion fluxes, oxidative bursts[3] and mitogen-activated protein kinase (MAPK) pathways activation[4] leading to the transcriptional reprogramming of over 1,200 genes[5] and to the induction of required basal defense responses.[1] The importance of MTI is best illustrated by the pressure exerted by the pathogen to suppress it. One striking example is the HopF2 effector which directly supresses MTI at two different levels of the MAMP-activated MAPK cascades. It can directly target BAK1, which is required for the full elicitation of pathogen-induced defense responses,[6] at the plasma membrane, thereby acting upstream of the MEKK1-MKK1/2- MPK4 pathway. It can also directly block MKK5 of the MEKK-MKK4/5-MPK3/6 cascade.[6]

The chitin receptor is one of the MAMP receptors that has been investigated with some success. Chitin, a major component of the fungal cell wall, is a β-1,4-linked N-acetyL-glucosamine polymer that has long been recognized as a potent MAMP in plant-fungus interactions.[7] In *Arabidopsis*, it is mostly detected by the CHITIN-ELICITED RECEPTOR KINASE 1 (CERK1). *cerk1* knock-out plants lose their response to chitin elicitor, including MAPK activation, reactive oxygen species (ROS) generation and induction of gene expression.[8] Indeed, CERK1 phosphorylates after exposure to chitin or chitosan (acetylated chitin) and can homodimerize when binding to chitin monomers to activate its kinase domain.[9] However, chitin signaling seems to require co-receptors: two additional LysM receptor kinases, AtLYK4 and AtLYK5, are also involved in chitin recognition.[10,11] Supporting the co-receptor theory is the fact that AtLYK5 binds chitin with high affinity and can dimerize with CERK1 in a chitin-dependent manner.[10] Other receptors may also be implicated but are masked by the dominant effect of CERK1.

Despite the importance of MTI, the intracellular modulation that takes place after MAMP recognition, which involves transcriptional reprogramming, is still somewhat unclear. More precisely, the chitin-elicited nuclear proteins involved in the establishment of basal defense responses are not fully known. Two MAPK pathways have been shown to be activated downstream of MAMP signaling. One elicits the activation of the MAPKs MPK3 and MPK6[4] and the second leads to MPK4 activation.[12] Recently, MPK1, MPK11 and MPK13 were also found to be phosphorylated upon flg22 treatment.[13] The absence of MTI defect in these three MAPKs knockout lines suggests functional redundancy, so many more components acting downstream of receptor activation may be missed in phenotype-based screening.

In the present study, we sought to discover proteins that participate in MTI but have escaped phenotype-based screening. Toward this end, we took an unbiased approach based on protein mass spectrometry (MS) of the nuclear proteome of young *Arabidopsis* plants subjected or not to chitosan treatment. Chitosan is known to also bind CERK1[9] and triggers a transcriptional response that overlaps with the response to chitin.[14] Using high performance liquid chromatography-electrospray

CONTACT Hugo Germain hugo.germain@uqtr.ca
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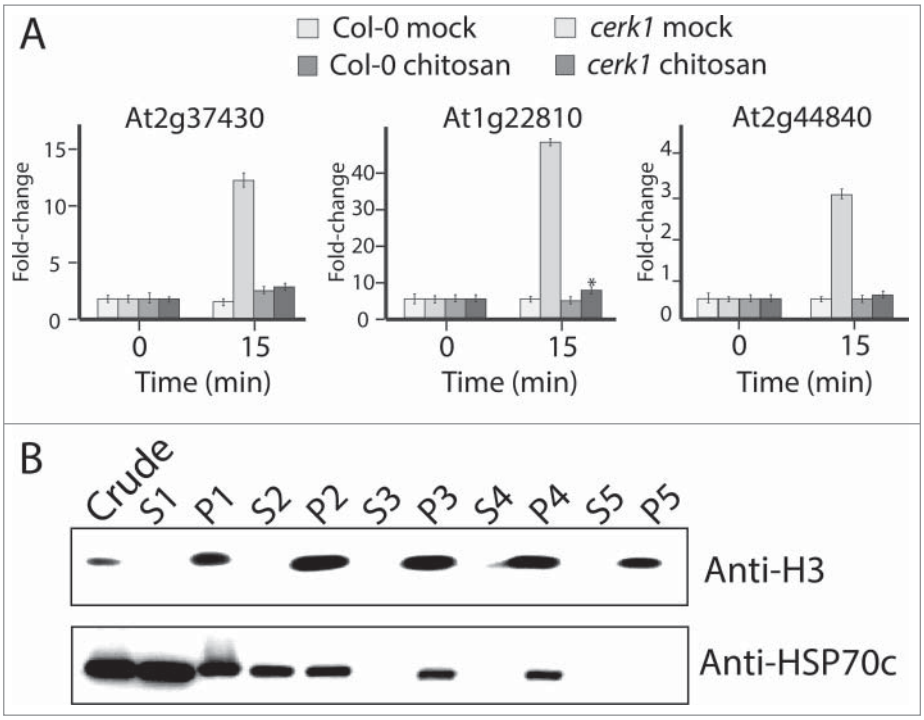

Figure 1. Chitosan treatment elicits MTI responsive gene in planta. (A) Expression of the MAMP-triggered immunity responsive marker genes At2g37430, At1g22810, At2g44840 in Col-0 and *cerk1* following chitosan or mock treatment. Q-RT-PCR was performed on soil grown three-weeks-old plants. ACT1 was used to normalize the transcript levels. (B) Quality control of the fractionation procedure by western blotting using HSP70c and histone H3 as cytosolic and nuclear markers respectively. Crude indicates crude extract, S = supernatant, P = pellet and number indicate the wash number.

ionization tandem mass spectrometry (HPLC-ESI-MS-MS), we identified several plant proteins that accumulate in the nucleus exclusively after chitosan treatment of *Arabidopsis* Columbia-0 (Col-0) or *cerk1* plants.

Before proceeding with the nuclear proteome MS analysis, we assessed if chitosan treatment was efficient in triggering a MAMP-like response. Three genes that are among the most up-regulated after chitin treatment[15] were analyzed by RT-qPCR: At2g37430 (C2H2-ZF), At1g22810 (AP2/ERE) and At2g44840 (AP2/ERE). All three genes were upregulated after chitosan treatment of Col-0 plants, showing respectively 13-fold, 51-fold and 3-fold induction 15 min post-treatment (Fig. 1A). We also observed that At1g22810 was slightly upregulated following chitosan treatment of *cerk1* plants albeit at much lower level than in Col-0 (5-fold).

We assessed the purity of our nuclear fractions by using the cytosolic marker HSP70c and nuclear marker histone H3. HSP70c could not be detected by Western blotting in the nuclear fraction corresponding to pellet five, while the nuclear marker anti-histone H3 was still clearly visible, hence this nuclear fraction was sent for mass spectrometry analysis. Tandem MS identified 1,372 different *Arabidopsis* proteins among a total of 31,416 spectra from our eight samples (duplicates of *cerk1* or Col-0 plants treated or not with chitosan) (PRIDE repository with the dataset identifier PXD003821 and 10.6019/PXD003821). We set very conservative criteria for our analyses: all proteins identified needed a minimum of two spectra to be considered, and all proteins that were present in only one of the duplicates were also rejected.

Our first analysis of the proteomic results was to compare the functional categorization of the 232 proteins found in the nucleus after chitosan treatment (in Col-0 and *cerk1*) with the 182 proteins from the nuclear proteome of cold-treated plants, one of the few studies of *Arabidopsis* nuclear proteomes that can relate to our investigation.[16] In parallel, we performed similar analysis with the SUBA database using only proteins predicted to be nuclear by SUBA bioinformatics tools or confirmed to be nuclear by GFP-tagging (total of 4,421 proteins). Finally, we compared our data to findings on the cytosolic proteome published by Ito et al. (2011) (Fig. 2A). The first observation from this categorization based on predicted cellular components is that only 26% of the nuclear proteins from the SUBA data set were annotated as nuclear proteins by TAIR's gene ontology (GO) annotator (Fig. 2A). In other words, the remaining 74% may be nuclear at some point, but the nucleus was not deemed to be their primary localization in GO. This reflects the fact that proteins may have several putative locations and underlines the weakness of bioinformatic to predict protein localization. The nuclear proteomes of chitosan and cold-treated plants contained only 11% and 16% of predicted nuclear proteins while the cytosolic experimental proteome still showed 9% of nuclear predicted proteins (Fig. 2A). Based on the discrepancies observed with the SUBA dataset, we can assume that a significant proportion of proteins annotated as non-nuclear by GO in these three experimental data sets were indeed at some point nuclear.

In the search for proteins that participate in MTI, categorization by molecular function (Fig. 2B) enables us to identify proteins that have the capacity to modulate transcription or translation during defense responses. Our chitosan-induced nuclear proteome contains 19% of DNA- or RNA-binding proteins, which could alter gene expression through

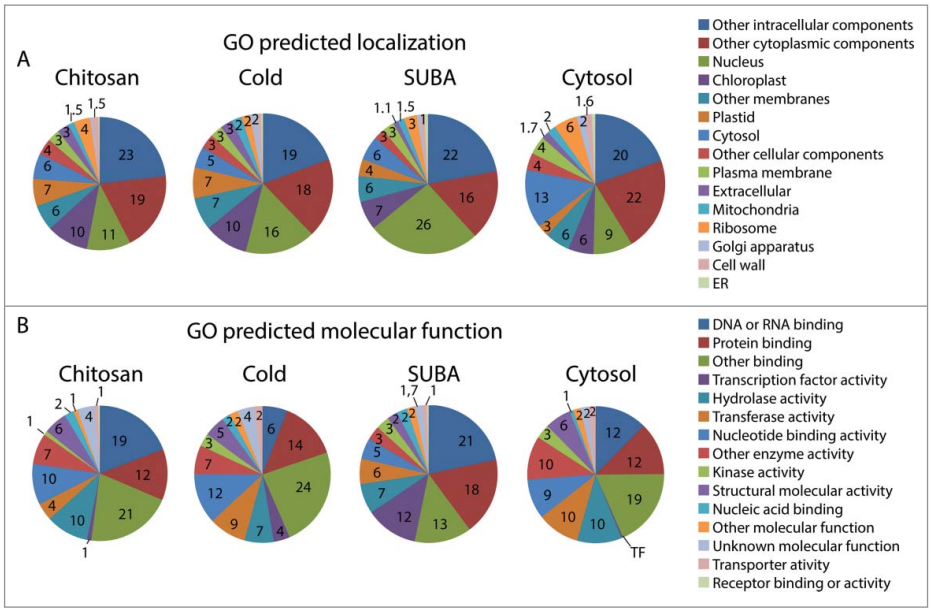

Figure 2. Gene ontologies in chitosan treated plants compared to other datasets. (A) GO predicted subcellular localization. (B) GO predicted molecular function.

DNA-binding, mRNA-processing, and mRNA-export, or could impact translation through mRNA nuclear segregation. Fewer of these proteins (12%) were found in the cytosolic dataset.[17] Proteins with transcription factor activity were most abundant in the SUBA nuclear data set (12%) but still represented 1%, 4% and 0.2% of proteins in chitosan, cold and cytosolic proteomes respectively, confirming that nuclear enrichment does indeed enrich transcription factors. It should also be noted that empirically-obtained proteomes are biased toward abundant proteins which could mask less abundant proteins. Therefore, signaling components such as transcription factors may be under-represented in LC-MS-MS proteomes, as demonstrated by their abundance in the SUBA dataset relative to the three other data sets.

We constructed a Venn diagram comparing the proteins found in each treatment group (Control is the combination of both Col-0 and *cerk1* plants treated with water) (Fig. 3). We identified eight proteins specifically localized to the nucleus of Col-0 plants after chitosan treatment (listed in Table 1). Although most of these are not obvious MTI components, a clear trend toward ribosomal proteins and translation is obvious. Proteins 1 (S19E family ribosomal protein), 4 (ribosomal protein l6), 5 (S19E family ribosomal protein) and 7 (RNAse Z activity involved in tRNA processing) are all involved in translation. Protein 8 (DNA-binding transcriptional regulator) is engaged in transcription regulation while protein 6 (small nuclear ribonucleoprotein G) binds RNA and could be involved in either transcription or translation. Most of these proteins have been reported to be modulated at the transcription level after biotic or abiotic stress, but have not previously been linked with the MAMP response.[18-21]

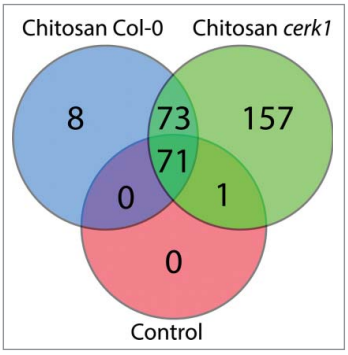

Figure 3. Venn diagram displaying the number of proteins identified in the nucleus for each condition.

157 proteins were only detected in the nucleus of *cerk1* plants after chitosan treatment (reported in Table S2). It is striking that so many protein are unique to *cerk1* as it has an impaired sensing of chitin[8] and as we observed only a weak transcriptional reprogramming in our RT-qPCR results (Fig. 1). On the other hand, it is known that while chitin and chitosan responses largely overlap, 33% of chitosan elicited genes are not elicited by chitin.[14] Table 2 groups the proteins possessing the molecular functions most likely to affect early MTI responses (transcription factor and DNA/RNA-binding protein) and excludes those from metabolisms. Many of those may regulate gene expression or mRNA metabolism, as several additional proteins are RNA helicases that may influence transcription or translation. Interestingly, one resistance protein of the Toll/Interleukin receptor (TIR) family (At4g16990) was found: it is known as RLM3 and is required for resistance to *Leptosphaeria maculans* and other necrophytic pathogens.[22]

We also analyzed the proteins common between Col-0 and *cerk1* nuclei after chitosan treatment (presented at the intersection in Fig. 3). A total of 73 proteins were identified and most of these were either DNA/RNA-binding proteins or ribosomal proteins. Table 3 shortlists the proteins sorted by molecular function, uncovering several DNA/RNA-binding proteins linked with chromatin remodeling and RNA maturation (see full list in Table S3). Receptor for activated C kinase 1 A (RACK1A) was one of the few proteins in Table 3 that was neither ribosomal nor DNA/RNA-binding. This protein was recently shown to act as a scaffold protein

Table 1. Nuclear localized proteins identified by LC-MS-MS in Col-0 plants following chitosan treatment.

Protein description	Uniprot ID	AGI
Ribosomal protein S19e family protein	D7KGE2	AT5G61170
HAD superfamily, subfamily IIIB acid phosphatase	Q9ZWC4	AT1G04040
Galactose mutarotase-like superfamily protein	Q8LFH1	AT3G47800
Ribosomal protein L6 family protein	Q8L9N4	AT1G18540
Ribosomal protein S19e family protein	D7MUI1	AT5G61170
Probable small nuclear ribonucleoprotein G	O82221	AT2G23930
Encodes a protein with RNase Z activity suggesting a role in tRNA processing	Q8L633	AT2G04530
DNA-binding storekeeper protein-related transcriptional regulator	O23063	AT4G00390

in a new immune signaling pathway.[23] The subset common between the two genotypes (Col-0 and *cerk1*) and the two treatments (water and chitosan) – those at the intersection of the three circles (listed in Table S4) – mostly contained proteins from the chloroplasts and mitochondria as well as many enzymes from primary metabolism that likely contaminated the nuclear preparations, which explains that they were found in all genotypes and treatments. This set of proteins also contained some constitutive nuclear components, such as nucleoporins, spliceosome assembly proteins and polymerases, but few RNA- or DNA-binding proteins and ribosomal components, strengthening the results obtained in chitosan-treated plants in which we observed some specificity among RNA- or DNA-binding proteins and ribosomal components.

The MTI response depends on the recognition of conserved molecular pathogen patterns at the cell surface by pathogen recognition receptors.[1] Genetic screening has largely contributed to our understanding of plant defense[24] and to the molecular dissection of the defense signaling pathways.[25] We used HPLC-ESI-tandem MS, a phenotype-independent approach to discover components participating in the establishment of defense responses resulting from MAMP recognition.

Interestingly, proteins that were either part of the ribosome or actively participated in translation were over-represented following chitosan treatment in both genotypes (Table 1, 2, 3). Since ribosomes are assembled in the nucleus, it is not surprising to observe many ribosomal proteins in our nuclear proteomes, but it is interesting that their identity differed in different genotypes and whether the plants had been exposed to chitosan or not. It is well-known that ribosome composition is highly heterogeneous and varies during plant development to ensure translational regulation.[26] Hence, we could speculate that ribosome subunits, which are highly heterogeneous,[27] may disassemble and reassemble after elicitor detection and triggering of MTI. As is observed in development, such reassembly could promote MTI oriented translational regulation. Recently, JIP60, a barley protein that mediates a translational switch toward stress and defense protein synthesis in the presence of jasmonate and at senescence, was discovered.[28] More recently the ribosomal coding genes *RPL12* and *RPL19* were shown to be involved in nonhost disease resistance in *Nicotiana* and

Table 2. Subset of nuclear localized proteins identified by LC-MS-MS in *cerk1* plants following chitosan treatment.

Protein description	Uniprot ID	AGI
Transcription factor or transcriptional regulator		
MED16, Mediator of RNA polymerase II transcription subunit 16, positive regulation of SAR	F4JGZ1	AT4G04920
Small RNA degrading nuclease 3, regulation of transcription	F4K3N3	AT5G67240
ACT domain-containing small subunit of acetolactate synthase protein	Q93YZ7	AT2G31810
Trihelix transcription factor ASIL2, sequence-specific DNA binding transcription factors	Q9LJG8	AT3G14180
VERNALIZATION INDEPENDENCE 5, regulation of transcription, DNA binding	D7KW58	AT1G61040
Sequence-specific DNA binding transcription factors	Q8LF33	AT3G11100
Short life 1, PHD finger and BAH motif containing putative transcription factor	F4JV93	AT4G39100
Mediator of RNA polymerase II transcription subunit 32	Q84VW5	AT1G11760
CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 2, CAMTA2	Q6NPP4	AT5G64220
EARLY BOLTING IN SHORT DAYS, chromatin assembly or disassembly	O65462	AT4G22140
RNA-binding protein		
NUCLEOSTEMIN-LIKE 1, nucleolar GTP- binding protein involved in RNA methylation	Q93Y17	AT3G07050
RPT2a encodes the 26S proteasome subunit, regulate gene silencing via DNA methylation	Q9SZD4	AT4G29040
EMBRYO DEFECTIVE 2770, RNA-directed DNA methylation, mRNA splicing	Q9ZT71	AT4G03430
Serine/arginine-rich SC35-like splicing factor	Q8L3X8	AT3G55460
RZ1B, Putative RNA-binding involved in cold tolerance	O22703	AT1G60650
WD-40 protein involved in histone deacetylation in response to abiotic stress	Q9FN19	AT5G67320
TOUGH, Interacts with TATA-box binding protein 2. RNA binding	Q8GXN9	AT5G23080
THO complex subunit 7B, component THO/TREX complex	Q9M8T6	AT3G02950
Small RNA degrading nuclease 3, regulation of transcription	F4K3N3	AT5G67240
RNA binding (RRM/RBD/RNP motifs), RNA processing	F4J9U9	AT3G12640
mRNA splicing factor, Cwf18	Q9MAB2	AT3G05070
SWI/SNF complex subunit SWI3C, ATP-dependent chromatin-remodeling complex	Q9XI07	AT1G21700
Splicing factor U2af large subunit B, Necessary for the splicing of pre-mRNA	Q8L716	AT1G60900
Small nuclear ribonucleoprotein	Q9SUM2	AT4G30220
Small nuclear ribonucleoprotein family protein, mRNA splicing	Q9C6K5	AT1G76860
nuclear cap-binding protein, mRNA metabolism	Q9XFD1	AT5G44200
RNA-binding protein-related	F4JM55	AT4G28990

Table 3. Subset of nuclear localized proteins identified by LC-MS-MS in both *cerk1* AND Col-0 plants following chitosan treatment.

Protein description	Uniprot ID	AGI
Miscellanous		
Homologous to the co-chaperon DNAJ protein	Q94AW8	AT3G44110
EPITHIOSPECIFIER MODIFIER 1, defense response to bacterium	Q9LJG3	AT3G14210
RECEPTOR FOR ACTIVATED C KINASE 1 A, MAP-kinase scaffold activity	O24456	AT1G18080
DNA-RNA binding proteins		
Nuclear RNA binding protein A-like protein	Q8LDQ7	AT5G47210
GLYCINE-RICH RNA-BINDING PROTEIN 7, DNA binding, RNA binding	C0Z2N6	AT2G21660
mRNA splicing factor	B3H6J5	AT3G49601
RNA BINDING PROTEIN, RNA modification, RNA processing, RNA stabilization	Q04836	AT4G24770
RNA polymerase I-associated factor PAF67	F4JY76	AT5G25754
ATWTF1, RNA recognition domain	A0MFS5	AT4G01037
GENERAL REGULATORY FACTOR 3, 14-3-3 gene	P42644	AT5G38480
COPPER RESPONSE DEFECT 1, putative ZIP protein, DNA binding	Q9M591	AT3G56940
Histone deacetylase HDT2	Q56WH4	AT5G22650
MAR-binding filament-like protein 1, DNA-binding protein	Q9LW85	AT3G16000
Nucleosome assembly protein 1-like 1	B3H684	AT4G26110
Emsy N Terminus and plant Tudor-like domain, defense response to fungus	Q9C7C4	AT3G12140
Histone deacetylase HD2A	F4J378	AT3G44750
Serine/arginine-rich SC35-like splicing factor	Q9LHP2	AT3G13570
U2 SMALL NUCLEAR RIBONUCLEOPROTEIN B, splicing	O22922	AT2G30260
DEK domain-containing chromatin associated protein	Q84JB7	AT5G63550
ATGRP8, glycine-rich protein with RNA binding domain at the N-terminus.	B9DFJ8	AT4G39260
MLP-LIKE PROTEIN 423, defense response, mRNA modification	Q93VR4	AT1G24020
Involved in translation		
LOS1, translation elongation factor 2	Q9ASR1	AT1G56070
Ribosomal protein L4/L1 family	F4KDU5	AT5G02870
EMBRYO DEFECTIVE 2184, structural constituent of ribosome	Q9FWS4	AT1G75350
Eukaryotic translation initiation factor 3 subunit E	Q9C5Z3	AT3G57290
Eukaryotic translation initiation factor 3 subunit B	F4K4D5	AT5G27640
40S ribosomal protein S3a-1	Q9CAV0	AT3G04840
40S ribosomal protein S16-3	A8MRX2	AT5G1838
Ribosomal protein L19	Q8W101	AT1G02780
40S ribosomal protein S20-1	P49200	AT3G45030
Translation elongation factor EF1B/ribosomal protein S6	D7KNE3	AT5G19510
Elongation factor 1-β 2	Q9SCX3	AT5G19510
Ribosomal protein S10p/S20e family protein	Q9LK61	AT3G13120
Ribosomal protein L10 family protein	B5X0P0	AT5G13510
50S ribosomal protein L19-2	Q8RXX5	AT5G47190
TRANSLATION INITIATION FACTOR 3 SUBUNIT H1	Q9C5Z2	AT1G10840
RIBOSOMAL PROTEIN S10E B	Q9FFS8	AT5G41520
60S ribosomal protein L36-2	Q9M352	AT3G53740
60S ribosomal protein L17-1	Q93VI3	AT1G27400
40S ribosomal protein S24e	Q9SS17	AT3G04920
Elongation factor 1B β	A8MRC4	AT1G30230

Arabidopsis and also play a minor role in basal resistance against virulent pathogens.[29]

Another type of proteins abundantly observed in our study were DNA-modifying enzymes that have the capacity to affect chromatin remodeling and in doing so to further impact transcription. The role of chromatin remodelling proteins in regulating *Arabidopsis* defense responses has been reviewed by Berr et al.[30] Mutation of chromatin-remodeling enzymes results in pleiotropic phenotypes not specifically associated with MTI or ETI but in which prominent players in transcriptional repression and activation at the onset of these processes are affected.

Various families of RNA-binding proteins, including proteins linked to mRNA splicing, export and maturation, were also identified after elicitation by chitosan. RNA export defects have previously been shown to suppress NB-LRR-mediated immunity,[31,32] basal responses[32] and response to abiotic stress,[33] suggesting that even more proteins involved in RNA metabolism may participate in defense responses.

As reviewed by Boller and Felix (2009), many molecular events unfold during the first 15 min of MAMP recognition and they set a point of no return upon which cells commit to the massive transcriptional reprogramming required for the establishment of the basal response. Consequently, we chose to concentrate our analysis on early nuclear recruitment of molecular components following MAMP detection. While the MTI response is clearly dependant on MAPK pathways, our data indicate that ribosome reorganization, DNA modification and RNA maturation could play major roles during the early MAMP response. Specific proteins affecting translation or switching it to defense mode need to be investigated further. Similarly, the participation of chromatin-remodeling and RNA-modifying enzymes should be studied. Our results demonstrate that nuclear proteomic is a valid, phenotype-independent approach to uncover factors involved in various cellular processes.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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