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. 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. MAMP recognition by PRRs triggers ion fluxes, oxidative bursts and mitogen-activated protein kinase (MAPK) pathways leading to transcriptional reprogramming of over 1,200 genes and to the induction of required basal defense responses. 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 suppresses MTI at two different levels of the MAMP-activated MAPK cascades. It can directly target BAK1, which is required for the establishment of basal defense responses. 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 and the second leads to MPK4 activation. Recently, MPK1, MPK11 and MPK13 were also found to be phosphorylated upon flg22 treatment. 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 and triggers a transcriptional response that overlaps with the response to chitin. Using high performance liquid chromatography-electrospray...
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 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. 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
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. 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 andcerk1 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

157 proteins were only detected in the nucleus ofcerk1 plants after chitosan treatment (reported in Table S2). It is striking that so many protein are unique tocerk1 as it has an impaired sensing of chitin8 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 toLeptosphaeria maculans and other necrophytic pathogens.22

We also analyzed the proteins common between Col-0 andcerk1 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     | AT5G66170 |
| HAD superfamily, subfamily IIIB acid phosphatase                                     | Q9ZWC4     | AT1G04040 |
| Galactose mutarotase-like superfamily protein                                       | Q8L7F1     | AT3G47800 |
| Ribosomal protein L6 family protein                                                  | Q8L9N4     | AT1G16540 |
| Ribosomal protein S19e family protein                                               | D7U8J1     | AT5G66170 |
| Probable small nuclear ribonucleoprotein G                                          | Q82221     | AT2G23930 |
| Encodes a protein with RNase Z activity suggesting a role in RNA processing          | Q8L633     | AT2G04530 |
| DNA-binding storekeeper protein-related transcriptional regulator                   | O23063     | AT4G00390 |

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

| Protein description                                                                 | Uniprot ID | AGI     |
|-------------------------------------------------------------------------------------|------------|---------|
| MED16, Mediator of RNA polymerase II transcription subunit 16, positive regulation of SAR | F4GJZ1     | AT4G04920 |
| Small RNA degrading nuclease 3, regulation of transcription                          | F4K3N3     | AT5G67240 |
| ACT domain-containing small subunit of acetylactate synthase protein                | Q93Y7Z     | AT2G31810 |
| Trihelix transcription factor ASIL2, sequence-specific DNA binding transcription factors | Q9LJG8 | AT3G14180 |
| VERNALIZATION INDEPENDENCE 5, regulation of DNA binding                             | D7KWS8     | AT1G61040 |
| Sequence-specific DNA binding transcription factors                                  | Q8LF33     | AT3G11100 |
| Short life 1, PHD finger and BAH motif containing putative transcription factor     | F4VJ93     | AT4G39100 |
| Mediator of RNA polymerase II transcription subunit 32                              | Q84VWS     | AT1G17600 |
| CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 2, CAMTA2                                | Q6NPP4     | AT5G64220 |
| EARLY BOLTING IN SHORT DAYS, chromatin assembly or disassembly                     | O6S5462    | AT4G22140 |
| NUCLEOSTEMIN-LIKE 1, nucleolar GTP- binding protein involved in RNA methylation      | Q93Y17     | AT3G07050 |
| RPT2a encodes the 26S proteasome subunit, regulates gene silencing via DNA methylation | Q95SD4     | AT4G29040 |
| EMBRYO DEFECTIVE 2770, RNA-directed DNA methylation, mRNA splicing                  | Q927T1     | AT4G03430 |
| Serine-arginine-rich SC35-like splicing factor                                       | Q8LB3X8    | AT3G55460 |
| RZ1B, Putative RNA-binding involved in cold tolerance                                | O22703     | AT1G60650 |
| WD-40 protein involved in histone deacetylation in response to abiotic stress       | Q9NF91     | AT5G67320 |
| TOUGH, Interacts with TATA-box binding protein 2, RNA binding                      | Q8GNX9     | AT5G23080 |
| THO complex subunit 7B, component THO/TREX complex                                   | O299M66    | AT4G30950 |
| Small RNA degrading nuclease 3, regulation of transcription                         | F4K3N3     | AT5G67240 |
| RNA binding (RRM/RBD/RNP motifs), RNA processing                                    | F4IJ9U9    | AT3G12640 |
| mRNA splicing factor, Cwf18                                                         | Q9MAB2     | AT3G05070 |
| SWI/SNF complex subunit SWI3C, ATP-dependent chromatin-remodeling complex          | Q9X07      | AT1G21700 |
| Splicing factor U2af large subunit B, Necessary for the splicing of pre-mRNA       | Q8L716     | AT1G60900 |
| Small nuclear ribonucleoprotein                                                     | Q9SUJM2    | AT4G30220 |
| Small nuclear ribonucleoprotein family protein, mRNA splicing                      | Q9C6Q5     | AT1G67860 |
| nuclear cap-binding protein, mRNA metabolism                                        | Q9XFD1     | AT5G44200 |
| RNA-binding protein-related                                                        | F4JM55     | AT4G28990 |
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.

References
1. Boller T, Felix G. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition

| Protein description | Uniprot ID | AGI |
|---------------------|-----------|-----|
| Homologous to the co-chaperon DNAJ protein | Q9AW8 | 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 | COZ2N6 | AT2G21660 |
| mRNA splicing factor | B3H6J5 | AT3G49601 |
| DNA-BINDING PROTEIN, RNA modification, RNA processing, RNA stabilization | Q04836 | AT4G24770 |
| RNA polymerase I-associated factor PAF67 | F4JY76 | AT5G25754 |
| ATWT1, RNA recognition domain | A0MF55 | AT4G01037 |
| GENERAL REGULATORY FACTOR 3, 14-3-3 gene | P42644 | AT5G38480 |
| COPPER RESPONSE DEFECT 1, putative ZIP protein, DNA binding | Q9MS91 | AT3G56940 |
| Histone deacetylase HDT2 | Q56WH4 | AT5G22650 |
| MAR-binding filament-like protein 1, DNA-binding protein | Q3LWB5 | AT3G16000 |
| Nucleosome assembly protein 1-like 1 | B3JH64 | AT4G62110 |
| Emsy N Terminus and plant Tudor-like domain, defense response to fungus | Q9FC7C4 | AT3G12140 |
| Histone deacetylase HD2A | F4J378 | AT3G44750 |
| Serine/arginine-rich SC35-like splicing factor | Q9LHP2 | AT3G13570 |
| U2 SMALL NUCLEIC PROTEIN, splicing | O22922 | AT2G30260 |
| DEK domain-containing chromatin associated protein | Q8A4J7 | AT5G63550 |
| ATGRPS, glycine-rich protein with RNA binding domain at the N-terminus. | B9DFJ8 | AT4G39260 |
| MLP-LIKE PROTEIN 423, defense response, mRNA modification | Q9VR94 | AT1G24020 |

**Table 3.** Subset of nuclear localized proteins identified by LC-MS-MS in both cerk1 AND Col-0 plants following chitosan treatment.
receptors. Ann Rev Plant Biol 2009; 60:379-406; PMID:19400072; http://dx.doi.org/10.1146/annurev.apt.032908.103546

2. Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G. The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell 2004; 16:3496-507; PMID:15548740; http://dx.doi.org/10.1105/tpc.104.026765

3. Gao Y, Liang Y, Tanaka K, Nguyen CT, Jedrzejczak RP, Joachimiak A, Jones JD, Felix G, Boller T. Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 2004; 448:497-500; PMID:17625569; http://dx.doi.org/10.1038/nature05999

4. Asai ST, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Mas H, Boller T. Arabidopsis kinase, is essential for chitin elicitor signaling in Arabidopsis. Proc Natl Acad Sci U S A 2007; 104:19613-8; PMID:18042724; http://dx.doi.org/10.1038/nature14243

5. Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, Felix G, Boller T. Nuclear proteome and its response to cold stress. Plant J 2003; 36:652-69; PMID:12962913; http://dx.doi.org/10.1046/j.1365-3104.2003.01907.x

6. Zhou J, Wu S, Chen X, Liu C, Sheen J, Shan L, He P. The Pseudomonas syringae effector HopE2 suppresses Arabidopsis immunity by targeting BAK1. Plant J 2014; 77:235-45; PMID:24237140; http://dx.doi.org/10.1111/tjp.12381

7. Wan J, Zhang X, Li J, Niu Y, Zhang XC, Woody OZ, Xiong Y, Djonović T. A putative nucleoporin 96 Is required for both basal and acquired immunity in Arabidopsis. J Plant Res 2011; 124:619-29; PMID:21559232.2014.976155

8. Ascencio-Ibáñez JT, Sozzani R, Lee T-J, Chu T-M, Wolfsong RD, Cella R, Hanley-Bowdoin L. Global analysis of arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant Physiol 2008; 148:436-54; PMID:18650403; http://dx.doi.org/10.1104/pp.108.121038

9. Ditt RF, Kerr KF, de Figueiredo P, Delrow J, Comai L, Nester EW. The Arabidopsis thaliana transcriptome in response to agrobacterium tumefaciens. Mol Plant-Microbe Interact 2006; 19:665-81; PMID:16776300; http://dx.doi.org/10.1094/MPMI-19-0665

10. Sharma N, Gram D, Huebert T, Zhou N, Parkin IAP. Exploiting the wild crucifer Thlaspi arvense to identify conserved and novel genes expressed during a plant’s response to cold stress. Plant Mol Biol 2006; 63:171-84; PMID:16972165; http://dx.doi.org/10.1007/s11103-006-9080-4

11. Charon J-BF, Oueltet F, Houde M, Sarhan F. The plant Apolipoprotein D ortholog protects Arabidopsis against oxidative stress. BMC Plant Biol 2008; 8:86; PMID:18671872; http://dx.doi.org/10.1186/1471-2229-8-86

12. Glazebrook J, Rogers EE, Ausubel FM. Use of arabidopsis for genetic dissection of plant defense responses. Ann Rev Genet 1997; 31:547-69; PMID:9442907; http://dx.doi.org/10.1146/annurev.genet.31.1.547

13. Monaghan J, Germain H, Weihmann T, Li X. Dissecting plant gene signal transduction: Modifier of snc1 in Arabidopsis. Canadian J Plant Pathol 2010; 32:35-42; http://dx.doi.org/10.1080/07060660103621001

14. Horiguchi G, Van Lijssebetten M, Candela H, Micol IL, Tsukaya H. Ribosomes and translation in plant developmental control. Plant Cell 2012; 191-24-34; PMID:22682562; http://dx.doi.org/10.1016/j.plantsci.2014.02.008

15. Giavalisco P, Wilson D, Kreitler T, Lebrach K, Jombom J, Fucini P. High heterogeneity within the ribosomal proteins of the Arabidopsis thaliana 80S ribosome. Plant Mol Biol 2005; 57:577-91; PMID:15821981; http://dx.doi.org/10.1111/j.1103-005-0699-3

16. Rustgi S, Pollmann S, Buhr F, Springer A, Reinothe C, von Wettstein D, Reinothe C. JIP60-mediated, jasmonate- and senescence-induced molecular switch in translation toward stress and defense protein synthesis. Proc Natl Acad Sci U S A 2011; 114:14181-6; PMID:22522401; http://dx.doi.org/10.1073/pnas.1415690111

17. Nagaraj S, Senthil-Kumar M, Ramu VS, Wang K, Mysore KS. Plant ribosomal proteins, RPL12 and RPL19, play a role in nonhost defense resistance against bacterial pathogens. Front Plant Sci 2015; 6:1192; PMID:26779226; http://dx.doi.org/10.3389/fpls.2015.01192

18. Berr A, Menard R, Heitz T, Shen WH. Chromatin modification and remodelling: a regulatory landscape for the control of Arabidopsis defence responses upon pathogen attack. Cell Microbiol 2012; 14:829-39; PMID:22405188; http://dx.doi.org/10.1111/j.1462-5822.2012.01785.x

19. Germain H, Qu N, Cheng YT, Lee E, Huang Y, Dong OX, Gannon P, Huang S, Ding P, Li Y, et al. MOS11: a new component in the mRNA export pathway. PLoS Genetics 2010; 6:e1001250; PMID:21203492; http://dx.doi.org/10.1371/journal.pgen.1001250

20. Zhang Y, Li X. A putative nucleoporin 96 Is required for both basal defense and constitutive resistance responses mediated by suppressor of npr1-1, constitutive 1. Plant Cell 2005; 17:1306-16; PMID:15772285; http://dx.doi.org/10.1105/tpc.104.029926

21. Dong CH, Hu X, Tang W, Zheng X, Kim YS, Lee BH, Zhu JK. A putative Arabidopsis nucleoporin, AtNUP160, is critical for RNA export and required for plant tolerance to cold stress. Mol Cell Biol 2006; 26:9533-43; PMID:17030626; http://dx.doi.org/10.1128/MCB.01063-06