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RESEARCH ARTICLE

Bacterial outer membrane vesicles induce a transcriptional shift in arabidopsis towards immune system activation leading to suppression of pathogen growth in planta

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Abstract

Gram-negative bacteria form spherical blebs on their cell periphery, which later dissociate from the bacterial cell wall to form extracellular vesicles. These nano scale structures, known as outer membrane vesicles (OMVs), have been shown to promote infection and disease and can induce typical immune outputs in both mammal and plant hosts. To better understand the broad transcriptional change plants undergo following exposure to OMVs, we treated *Arabidopsis thaliana* (Arabidopsis) seedlings with OMVs purified from the Gram-negative plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris* and performed RNA-seq analysis on OMV- and mock-treated plants at 2, 6 and 24 h post challenge. The most pronounced transcriptional shift occurred at the first two time points tested, as reflected by the number of differentially expressed genes and the average fold change. OMVs induce a major transcriptional shift towards immune system activation, upregulating a multitude of immune-related pathways including a variety of immune receptors. Comparing the response of Arabidopsis to OMVs and to purified elicitors, revealed that OMVs induce a similar suite of genes and pathways as single elicitors, however, pathways activated by OMVs and not by other elicitors were detected. Pretreating Arabidopsis plants with OMVs and subsequently infecting with a bacterial pathogen led to a significant reduction in pathogen growth. Mutations in the plant elongation factor receptor (EFR), flagellin receptor (FLS2), or the brassinosteroid-insensitive 1–associated kinase (BAK1) co-receptor, did not significantly affect the immune priming effect of OMVs. All together these results show that OMVs induce a broad transcriptional shift in Arabidopsis leading to upregulation of multiple immune pathways, and that this transcriptional change may facilitate resistance to bacterial infection.

KEYWORDS
Arabidopsis thaliana, bacterial infection, extracellular vesicles, OMVs, outer membrane vesicles, plant immunity, RNA-seq, Xanthomonas campestris pv. campestris

Laura Chalupowicz and Gideon Mordukhovich contributed equally to this work.

1 | INTRODUCTION

Plants are constantly confronted with harming microbes that thrive on its tissues and hinder normal growth. An efficient defense response depends greatly on rapid and accurate detection and identification of the invading microbe. For this purpose, plants utilize broad surveillance systems to monitor for pathogen invasion (Cook et al., 2015). It is speculated that the first line of the plant's surveillance system, or the first cellular interface where plants and microbes interact, is the intercellular space, the apoplast. There, recognition of invading microbes is mediated by membrane-bound, extracellularly exposed pattern recognition receptors (PRRs) (Boutrot & Zipfel, 2017; Couto & Zipfel, 2016). PRRs recognize microbial determinants that are widely present and conserved among many microbes and are known as, microbe- or pathogen-associated molecular patterns (MAMPs) (Ranf et al., 2016).

Since microbes undergo mutagenesis at a fast rate, evolutionary useful immune receptors are adapted to detect highly conserved regions of crucial microbial components that cannot be easily discarded or mutated because of a serious fitness cost. For example, bacterial flagellin is a crucial element in the physiology of many microbes including pathogens and is currently one of the best studied MAMPs (Felix et al., 1999; Zipfel et al., 2004). Perception of flagellin, or the synthetic epitope flg22 (comprised of highly conserved 22 amino acids at the N-terminus of the flagellum building block, flagellin), by the cognate plant immune receptor flagellin sensing 2 (FLS2), leads to a major transcriptional change, followed by an effective immune response that halts infection (Chinchilla et al., 2007; Gómez-Gómez & Boller, 2000).

Many known MAMPs are associated with the microbe's cell wall. For example, fungal chitin (Fesel & Zuccaro, 2016), bacterial peptidoglycan (PG) (Erbs et al., 2008; Gust et al., 2007), bacterial lipopolysaccharides (LPS) (Dow et al., 2000; Silipo et al., 2005), flagellin (Boutrot & Zipfel, 2017; Felix et al., 1999), and more. Nevertheless, it is not quite clear how these cell-wall associated components interact with their cognate immune receptors *in planta*. Whether this occurs due to cell death and/or degradation of the cell wall, or via active release of components such as the flagellum, is a topic in need of further investigation (Bahar, 2020).

An example of the active release of cell-wall fragments by Gram-negative bacteria is the detachment of extracellular vesicles (EVs) that bleb and pinch off from the outer membrane into the surrounding environment (Kulp & Kuehn 2010; Théry et al., 2018). These bacterial EVs are commonly termed outer membrane vesicles (OMVs), and we will henceforth adhere to this nomenclature (Schwechheimer & Kuehn, 2015). The process of OMV release occurs continuously and under various environmental conditions, including during host colonization (Gurung et al., 2011; Ionescu et al., 2014; Jin et al., 2011). In addition to integral outer membrane molecules such as outer membrane (OM) proteins, LPS, and lipids, OMVs encapsulate periplasmic fluids, consisting of a diverse array of molecules such as proteins, cell wall degrading enzymes, polysaccharides, and nucleic acids (Kuehn & Kesty, 2005). Since OMVs are released during host colonization, and since their cargo consists of MAMPs, it is tempting to speculate that they act as carriers of immune elicitors delivering the eliciting molecules in close proximity to their cognate immune receptors (Bahar, 2020; Katsir & Bahar, 2017). Indeed, OMVs have been shown to induce both the mammalian and the plant immune systems when presented to their hosts (Bahar et al., 2016; Ellis & Kuehn, 2010; Janda et al., 2021; McMillan et al., 2021). While in mammalian cells both the LPS and protein components of OMVs act as immune elicitors (Ellis et al., 2010), in plants, it is not yet clear which OMV molecules are the prime immune elicitors.

In addition to modulating the host immune response, OMVs were also shown to carry virulence factors, and to be involved in a multitude of processes. This includes cell-cell communication (Deatheragea & Cooksona, 2012; Mashburn & Whiteley, 2005; Raposo & Stahl, 2019), delivery of toxins to target cells (Ellis & Kuehn, 2010; Kadurugamuwa & Beveridge, 1996), biofilm formation (Schooling & Beveridge, 2006), quenching of antimicrobial compounds (Manning & Kuehn, 2011), response to stress (MacDonald & Kuehn, 2013), horizontal gene transfer (Fulsundar et al., 2014; Velimirov & Ranftler, 2018) and virulence (Ellis & Kuehn, 2010; Kunsmann et al., 2015). While most of these examples come from studies of mammalian bacterial pathogens, recent studies with plant pathogenic bacteria also support that OMVs promote bacterial virulence and plant colonization. Ionescu *et al.* (Ionescu et al., 2014) showed that OMV production by the plant pathogen *Xylella fastidiosa* during xylem vessel colonization inhibits bacterial attachment to the water conducting elements of the plant (the xylem), tilting the balance between the sessile and mobile forms of the pathogen towards the mobile form. This form is believed to promote cell dispersion in the xylem, leading to a faster decline of the plant (Ionescu et al., 2014). Two other studies have shown that virulence factors such as type II-secreted lipases/esterase and xylanase, and type III-secreted effectors, are secreted in association with OMVs (Chowdhury & Jagannadham, 2013; Sidhu et al., 2008; Solé et al., 2015) suggesting that OMV may have an important role in bacterial virulence.

The molecular complexity of OMVs, along with its dual and possibly opposing functions in the host (inducing immunity and promoting virulence), prompts us to study the broader transcriptional response of *Arabidopsis thaliana* (Arabidopsis) plants to OMV challenge and to test whether this transcriptional change would induce resistance, or susceptibility to subsequent bacterial infection.

2 | MATERIALS AND METHODS

2.1 | Plant material and growth conditions

Arabidopsis thaliana (Arabidopsis) wild type Col-0 line as well as the following mutant lines: *bak1-5* (Schwessinger et al., 2011) and *fls2 efr1* (Nekrasov et al., 2009) were used in this study. Arabidopsis seeds were surface sterilized and sown on Murashige and Skoog (MS) agar plates as described (Bahar et al., 2016). Plates were kept in the dark at 4°C for 2–4 days and then moved to 22°C for germination for 5–8 days. Germinated seedlings of similar size were transferred into 24-well plates (two seedlings per well) containing 1 ml of MS medium with 1% (w:v) of sucrose (Duchefa Biochemie) and grown for another 8–10 days at the same conditions before challenged with elicitor as described below.

For priming assays, seeds of Arabidopsis wild type Col-0 line and mutant lines (*fls2 efr1* and *bak1-5*) were germinated as described above and then transplanted into 7 × 7 × 6 cm pots (1 seedling /pot) containing mix soil Green #7611 (Evenari, Ashdod, Israel) and grown at a 9.5 h photoperiod at 22–24°C. Plants were irrigated twice a week and fertilized using an NPK mix (6:2:4, Deshen Gat, Israel) once a week.

2.2 | Bacterial outer membrane vesicles purification

Glycerol stocks of *Xanthomonas campestris* pv. *campestris* (*Xcc*) 33913 were streaked on Nutrient Agar (Difco, NA, Becton, Dickinson and Company) plates and grown for 2–5 days at 28°C. Single colonies were collected and used to inoculate a 3-mL YEB (yeast extract broth) starter containing 10 μg/ml cephalexin hydrate (Cp, Sigma-Aldrich). Starters were grown overnight at 28°C with 185–200 rpm shaking and then used to inoculate 500 ml of PSB (peptone sucrose broth) medium with antibiotics (as described above) in 2-L Flasks at a ratio of ~1:1000 (v:v). Cultures were grown as describe above to an OD_{600} of 0.6–0.8 and then bacterial cells were spun down and OMVs were extracted from the supernatant as described (Mordukhovich & Bahar, 2017). The crude OMV preparation was then subjected to Optiprep gradient centrifugation to obtain purified OMVs, as described (Bahar et al., 2016; Mordukhovich & Bahar, 2017). Each OMV batch was purified from 1.5-L bacterial culture and was finally resuspended in 1 ml of PBS (pH 7.3). Purified OMVs were used immediately or stored at 4°C up to 7 days before use. OMVs size distribution was measured using a dynamic light scattering device (Zetasizer Nano ZS, Malvern Panalytical, Worcestershire, UK) and had a mean diameter of 121.7 ± 55.43 nm (SD). Particle concentration was similarly measured and is provided in each experiment description below.

2.3 | Arabidopsis seedling challenge with OMVs

To examine the transcriptional response of Arabidopsis to OMV challenge, Col-0 seedlings grown in 24-well plates as described above were used. The day before OMV challenge, MS medium was withdrawn from plates and replaced with 250 μl of sterile dH_2O and plates were left on the bench overnight. The morning after, 20 μl of purified OMVs (30 μg per ml corresponding to 1.44×10^9 particles per well), or sterile dH_2O as mock, were added to each well. Seedlings were collected 2, 6, and 24 h after challenge, blotted dry on paper and snap frozen with liquid nitrogen in 2-mL Eppendorf Safe-Lock tubes (Hamburg, Germany). At each time point, four OMV-treated and four mock-treated wells were collected, representing four biological replicates for each treatment at each time point.

2.4 | RNA purification

RNA was extracted from Arabidopsis seedlings using the TRIzol reagent (Invitrogen) according to the manufacturer instructions. RNA was further purified by using the Turbo DNA-free Kit (Ambion, Thermo Fisher Scientific), and the RNA Clean-Up and Concentration Kit (Norgen Biotek) according to the manufacturer's instructions. Purified RNA samples were subjected to concentration and quality analyses using a TapeStation 2200 machine (Agilent Technologies), RNA Screen Tape and RNA Screen Tape Sample Buffer (Agilent Technologies), according to the manufacturer instructions and then kept at –80°C until used.

2.5 | RNA library construction and sequencing

For each treatment and time point, two samples showing the highest purity were selected for analyses. TruSeq mRNA libraries (Illumina) with PolyA capture were prepared from the selected RNA samples at the Crown Institute of Genomics at the

Weizmann Institute of Science (Rehovot, Israel). Each sample was tagged, and a pool was prepared from the samples. This pool was then loaded inside two NGS lanes, and run in Illumina HiSeq sequencing machine, at high output run mode, single read (SR) 60 (v4).

2.6 | Sequencing reads initial processing

The raw sequence reads were cleaned with Trimmomatic software v 0.36 (Bolger et al., 2014), removing low quality reads and remaining adapter sequences. The clean reads were mapped to the reference Arabidopsis TAIR10 reference genome (Lamesch et al., 2012) using bowtie2 (Langmead & Salzberg, 2012) and quantification of genes expression was done using RSEM (Li & Dewey 2011).

Principal component analysis and sample correlation matrix were calculated with the function cor() and precomp(), respectively, of the R base package version 3.6.1. DEGs were determined using the DESeq2 tool (Love et al., 2014). The FDR (false discovery rate) cutoff chosen was FDR < 0.05. The LogFC (Log of the fold change) cutoff for the up-regulated and the down-regulated genes, was > 1 and < -1, respectively. Venn diagrams were built with the use of Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/).

2.7 | Gene ontologies and gene descriptions

Gene Ontologies (GO) were retrieved by using TAIR's GO Annotations (http://www.arabidopsis.org/tools/bulk/go/index.jsp). Gene descriptions, according to the gene models, were obtained by using TAIR's gene description search (http://www.arabidopsis.org/tools/bulk/genes/). GO enrichment was calculated using the AgriGO web tool (http://bioinfo.cau.edu.cn/agriGO/index.php) using Arabidopsis genome locus (TAIR10) as reference

2.8 | Quantitative-PCR and RNA-seq validation

To validate RNA-seq data, RNA samples of mock- and OMV-challenged seedlings were used for cDNA synthesis, followed by quantitative-PCR (qPCR) using gene-specific primers (Supp. Table S4), as described (Bahar et al., 2016). Overall, 17 DEGs from the RNA-seq dataset were tested, using four biological replicates of RNA of OMV- or mock-treated seedlings. Relative expression of the tested genes was compared with ubiquitin expression, using a 7500 Fast real-time PCR machine (Applied Biosystems), as described (Bahar et al., 2016).

2.9 | Comparing Arabidopsis transcriptional response to MAMPs and to OMVs

Our OMV-induced dataset was compared with available transcriptomes of Arabidopsis challenged with flg22, (Denoux et al., 2008) elf26 (Zipfel et al., 2006), PGN (Willmann et al., 2011), OG (Davidsson et al., 2017) and LPS (Livaja et al., 2008). For data comparison, GO terms enrichment was performed and the induced GOs were visualized by Venn diagrams as described above.

2.10 | Arabidopsis priming experiments

To test the priming effect of *Xcc* OMVs on Arabidopsis infected with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), we followed the procedure described by Zipfel et al. (2004). In brief, leaves of 6–8 weeks-old Arabidopsis plants, grown as described above, were infiltrated with 50–100 μl of purified *Xcc* OMVs (30 μg/ml, corresponding to 3.6-7.2 $\times 10^9$ particles per leaf), 1 μM flg22, or water using a needle-less syringe. For each treatment, 5 leaves/plant and three plant replicates were used. *Pst* inoculum was prepared by culturing the bacterium on King's B medium plates (20 g/L peptone, 1.5 g/L $MgSO_4$ x 7 H_2O, 10 ml/L glycerol and 15 g/L agar) at 28°C for 2–3 days, and then resuspending colonies with water and adjusting the inoculum concentration to 10^5 CFU/ml. *Pst* inoculum was infiltrated into primed leaves 24 h following priming, using a needle-less syringe (approximately 100 μl were infiltrated to each leaf). Bacterial growth was determined at 0 (1 h post inoculation) and 2 days post inoculation (dpi) by collecting and weighing the inoculated leaves, macerating them in 1 ml of 10 mM $MgCl_2$ and plating 10-fold serial dilutions on King's B agar plates. The number of CFU on each plate was determined 2 days later and calculated per g leaf.

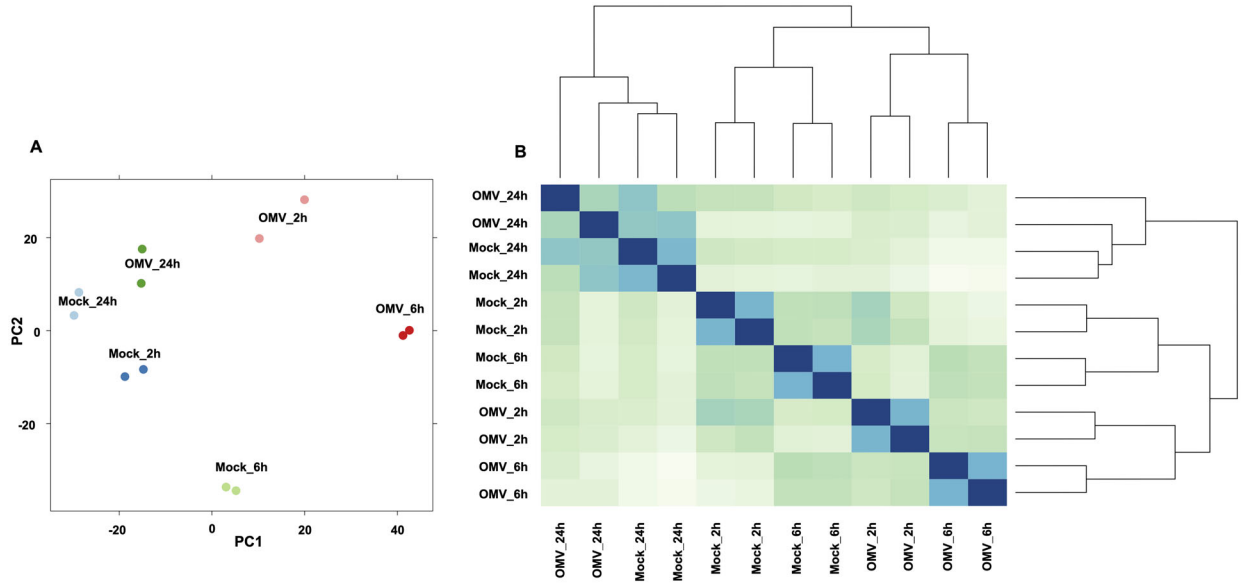

FIGURE 1 Arabidopsis transcriptional response to OMVs at 2, 6 and 24 post challenge. Principal component analysis **(A)** and sample correlation matrix **(B)** of Arabidopsis seedlings transcriptional response to OMV, or mock, at 2, 6 and 24 h post challenge

2.11 | In vitro bacterial growth assays

To assess the effect of purified *Xcc* OMVs on *Pst* growth in vitro, *Pst* starters were grown in King's B liquid medium for 24 h at 28°C and then used to inoculate three different cultures containing 12 ml of King's B medium each, in 50-mL Falcon tubes at a ratio of 1:100. Bacterial cultures were amended with 30 μg/ml OMVs (1:50 or 1:100, corresponding to 1.44×10^9 or 7.2×10^8 particles per ml, respectively), or PBS as control, and incubated for 20 h at 28°C. Bacterial growth was measured using a spectrophotometer (Amersham Biosciences) at optical density (OD) of 600 nm over 22 h.

3 | RESULTS

3.1 | RNA-seq analysis reveals a large set of Arabidopsis genes differentially expressed in response to OMV challenge

To study the transcriptional change in Arabidopsis following OMV challenge, we treated Arabidopsis seedlings with OMVs purified from the bacterial pathogen *Xanthomonas campestris* pv. *campestris* 33913 (*Xcc*) and collected plant RNA at 2, 6 and 24 h post challenge (hpc). RNA from OMV- and mock-treated samples was then sequenced and analyzed as described in Materials and Methods.

Principal correlation (Figure 1A) and sample correlation matrix analyses (Figure 1B) show that the biological replicates in each treatment cluster closely together, an indication of the overall transcriptional response similarity between biological replicates. The OMV treated samples at 2 and 6 hpc cluster together, indicating that the OMV treatment had a greater effect on the transcriptional response than sampling time (Figure 1B). On the other hand, OMV treated samples cluster together with their respective mock treatment at 24 hpc, indicating that the transcriptional change induced by OMVs at this time point had decreased and was similar to that of mock-treated plants.

At each time point tested, OMV-treated seedlings were compared with mock-treated seedlings and differentially expressed genes (DEGs) were extracted. At all time points combined, a total of 984 and 175 genes were found to be significantly (Log fold-change > 1 or $\leftarrow 1$, p value and FDR < 0.05) up- or down-regulated, respectively, in response to OMV challenge (Figure 2A; Supp. Table S1). Gene expression Log fold-change (LogFC) ranged from a maximum of 9.08 (AT1G26410, 6 hpc), which corresponds to over 500-fold change, to -5.73 (AT3G17520, 24 hpc). The highest number of DEGs was found at 2 and 6 hpc, where a total of 647 and 876 DEGs, respectively, were identified (up- and down-regulated combined). At 24 hpc, 121 DEGs were found. More than 50 % of the up-regulated genes at 2 and 6 h post OMV challenge were shared between them (Figure 2B).

To examine the temporal gene expression change, we extracted all the up-regulated DEGs that were found at all time points (37 genes) and compared their fold-change over time (Figure 2C). The fold-change expression of these genes was significantly different among the different time points (one-way ANOVA; $F_{2,108} = 8.1633$, $p = 0.0005$). A post hoc comparison using the Tukey

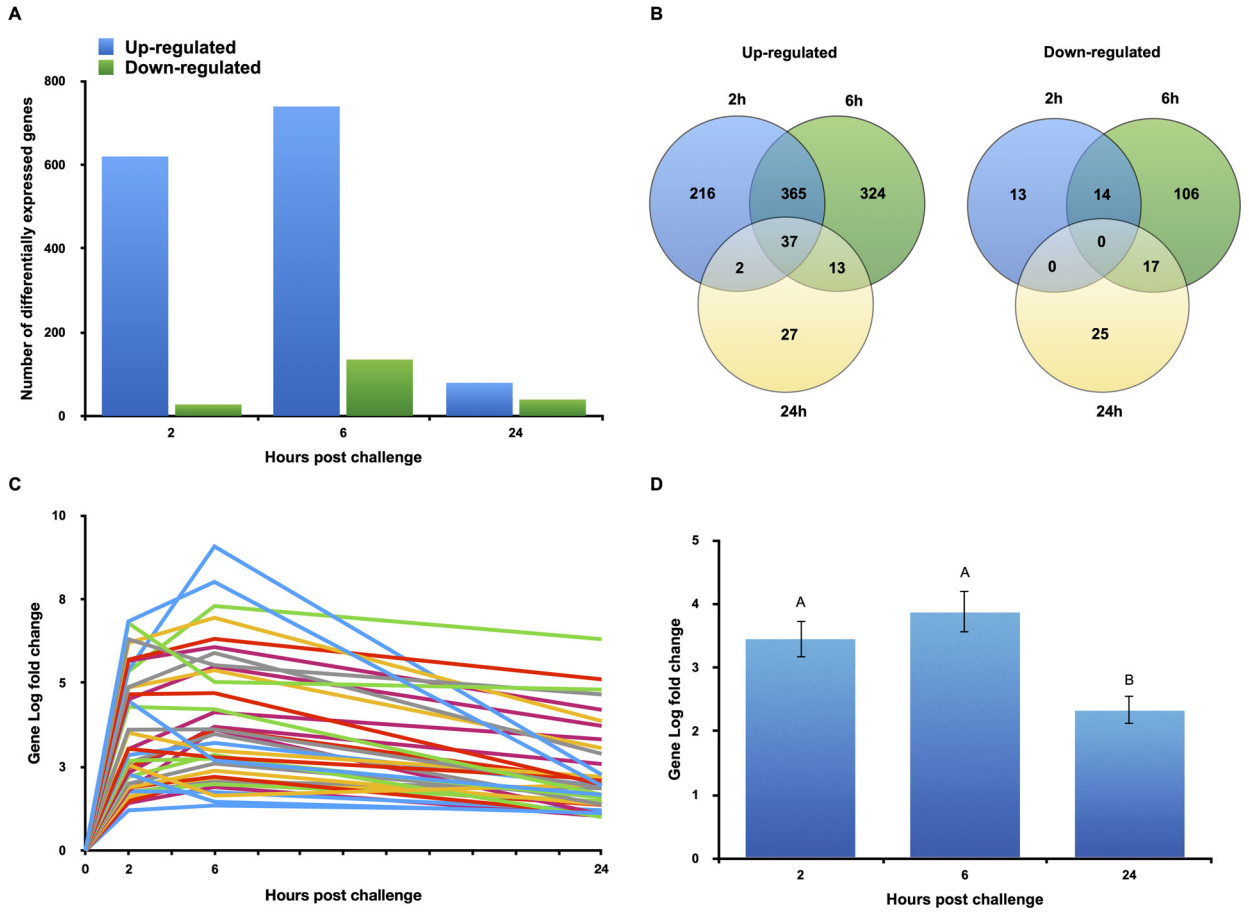

FIGURE 2 Arabidopsis differentially expressed genes in response to OMV challenge. **(A)** Total number of differentially expressed genes (DEGs) (up- or down-regulated, LogFC > 1 or ← 1, p value and FDR < 0.05) at 2, 6, and 24 h post challenge. **(B)** Overlap between DEGs at different time points (left, up-regulated; right, down-regulated). **(C)** Up-regulated genes found in all three time points were plotted on a LogFC expression graph, showing gene expression over time. **(D)** LogFC average of all DEGs at different time points. Different letters indicate statistical difference at $p < 0.05$ by the Tukey-Kramer HSD test

Kramer HSD test indicated that the LogFC at both 2 (M = 3.45, SD = 1.72) and 6 hpc (M = 3.87, SD = 1.98) was significantly higher than at 24 hpc (M = 2.32, SD = 1.31) ($p = 0.0145$ and $p = 0.0005$, respectively) (Figure 2D). While the mean LogFC expression at 6 hpc was higher than at 2 hpc, it was not statistically different ($p = 0.5413$). Hence, when considering the number of DEGs and the overall gene LogFC at the three time points tested, we can conclude that the most significant transcriptional change occurred at 2 and 6 h post OMV challenge.

To examine the validity of the RNA-seq results, the expression of 17 up-regulated genes was determined using quantitative-PCR (qPCR) with specific primers. Fourteen of the tested genes displayed the same pattern as in the RNA-seq analysis and were significantly up-regulated compared with mock. Three of the tested genes had a higher relative expression but were not significantly different from mock by this method (Supp. Figure S1).

3.2 | Arabidopsis responds to OMVs with a transcriptional shift towards activation of the immune system

To identify Arabidopsis pathways significantly affected by OMV challenge, we used the AgriGO web tool (Du et al., 2010; Tian et al., 2017). We identified 333 and 55 significantly (FDR < 0.05) up- and down-regulated gene ontology (GO) terms, respectively, at all time points combined in response to OMV challenge. Nearly 25 % of the up-regulated GOs were related to plant response to stimulus (Figure 3A). Within the 'response to stimulus' category, the most dominant GO terms were associated with response to stress, to biotic stimulus, to chemicals and to endogenous stimulus (Figure 3B; Supp. Table S2).

The AgriGO tool also identified 103 significantly up-regulated molecular functions including 'transferase activity', kinase activity', 'transcription factor activity', 'ion and metal ion binding', 'carbohydrate binding', 'protein binding', 'catalytic activity', 'Adenyl nucleotide binding', 'transmembrane receptor activity' and more binding functions (Supp. Table S2). The cellular location of the

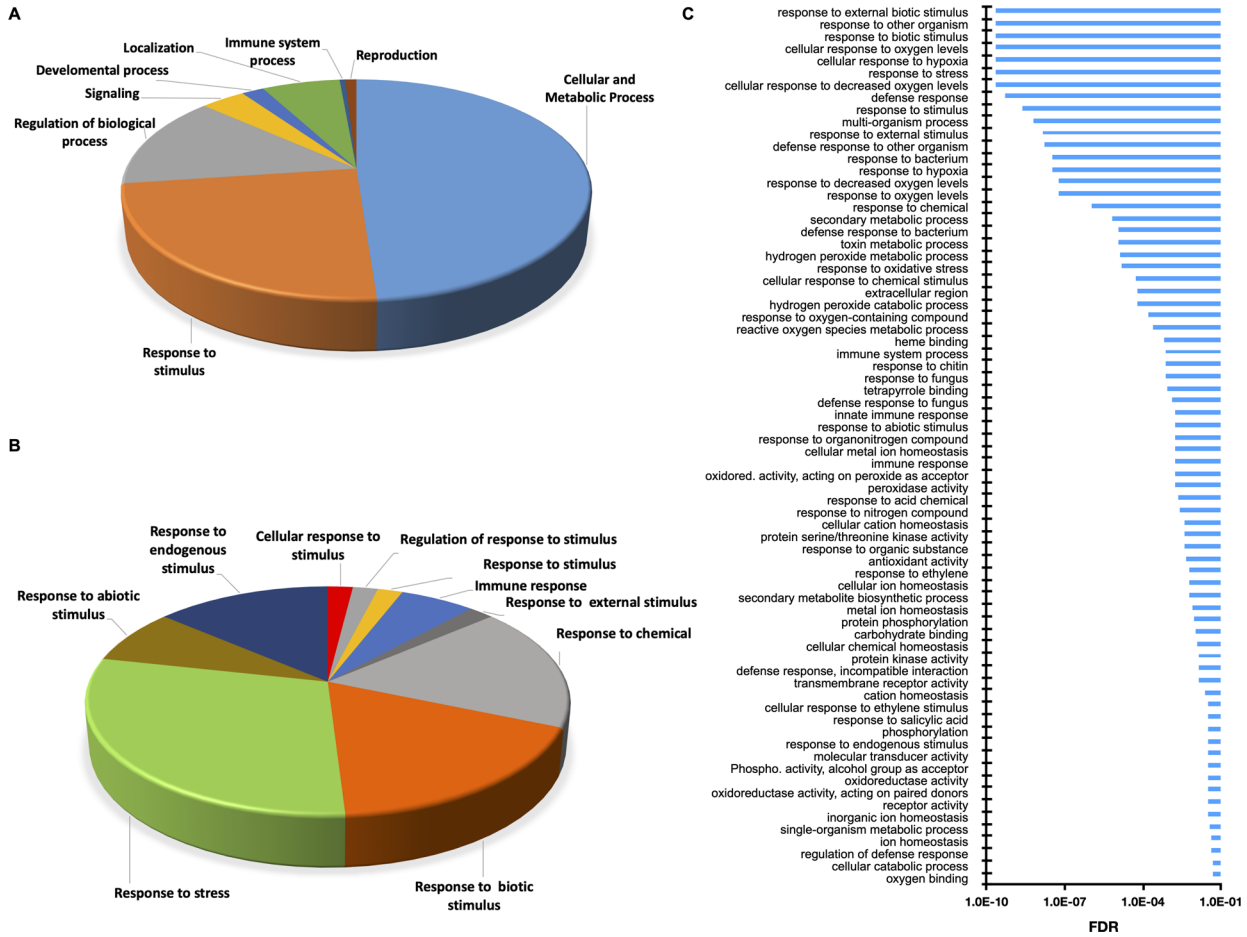

FIGURE 3 Arabidopsis gene ontology (GO) terms enriched in response to *Xcc* OMV challenge. Pie chart representations of the category distribution in up-regulated Biological Process (A) and Response to stimulus (B) GO terms. (C) List of Arabidopsis genes with a LogFC of > 4 in response to OMV challenge. Genes were filtered from the complete dataset of DEGs and used to identify enriched GO terms using AgriGo webtool. FDR cutoff < 0.05

significant terms was in different compartments of the cell including the nucleus, vacuole, and endomembrane system, but was most notably associated with the cell periphery and included 'plasma membrane', 'extracellular region', 'cell wall' and 'apoplast' ontologies (Supp. Table S2).

The significantly down-regulated GOs, on the other hand, included the terms 'toxin catabolic and metabolic process', 'response to water and water deprivation', 'lipid transport and localization' and others (Supp. Table S2). GOs related to response to biotic stimulus were not enriched in the down-regulated genes. Significantly down-regulated molecular functions included many redox terms such as 'oxidoreductase activity', 'heme binding', 'iron ion binding', 'lipid binding', 'oxygen binding' and more oxygen-related functions (Supp. Table S2). Down-Regulated terms were also located to the extracellular region.

To determine to which GO terms DEGs with the highest expression belong, we filtered the original DEGs list by selecting genes that had a LogFC higher than 4, or smaller than -4 (corresponding to 16-fold difference). We identified 117 genes that met these criteria, of which 115 were up-regulated and 2 down-regulated at all time points combined. Because of the small number of down-regulated genes with a LogFC of less than -4, no significantly repressed GOs were identified. On the other hand, 72 significantly up-regulated GOs were identified, of which the most significant ones are related to 'response to external biotic stimulus', 'response to other organisms', 'cellular response to oxygen levels', 'defense response', 'response to stress' and more immune related GOs (Figure 3C).

3.3 | OMV challenge led to up-regulation of immune receptors

MAMP sensing and plant response to MAMPs are largely mediated by membrane-bound PRRs that are mediate pathogen perception and efficient mitigation of infection. PRRs are commonly classified in two groups receptor kinases (RKs) and receptor-like proteins (RLPs) (Boutrot & Zipfel, 2017). To identify PRRs that were differentially expressed in response to OMVs, we compared

our DEG set with previously established lists of Arabidopsis RKs (Kemmerling et al., 2011; Mott et al., 2016) and RLPs (Wang et al., 2008). We have identified in our dataset 33 and 10 up-regulated RKs and RLPs, respectively, at all time points combined (Table 1). No RKs or RLPs were found in our list of 175 down-regulated genes. Kemmerling et al. (2011) defined a list of 49 RKs, whose expression was significantly induced by MAMPs such as flg22 and NLP (necrosis and ethylene -inducing peptide 1- like protein), or pathogen treatment. We compared this list with the up-regulated RLKs from our experiment and found that 45 % of the RKs defined by Kemmerling et al. (2011) were also induced in response to OMVs. Among those, noteworthy are FRK1, SOBIR1, SERK4, RLK/IKU2, PSKR1, HAESA, EFR, BIR and IOS1 (Table 1). Among the RK group, FRK1 had the highest expression at both 2 and 6 hpc with a LogFC of 7.11 and 5.2, respectively, while the average LogFC of all RKs was 2.11 and 2.13 at 2 and 6 hpc, respectively. While membrane bound RKs and RLPs mostly mediate extracellular sensing of invading microbes, nucleotide-binding site–leucine-rich repeat (LRR) receptors (NLRs), are intracellular immune receptors. We found 7 different NLR genes up-regulated in response to OMV-challenge at 2 and 6 hpc, none were found at 24 hpc (NLR list was extracted from TAIR, 102 genes) (Table 1).

3.4 | OMVs induce the expression of multiple WRKY transcription factors

WRKY transcription factors (TFs) have been found to play roles in plant immune responses, participating in both MAMP-triggered immunity (MTI) and effector-triggered immunity (ETI) responses (Birkenbihl et al., 2018; Rushton et al., 2010). OMV challenge led to up-regulation of 20 different WRKY TFs (list extracted from TAIR, 70 genes) only at 2 and 6 hpc (Table 1). WRKY TFs were absent from our down-regulated gene set. Another family of TFs affected by the OMV challenge are MYB domain-containing proteins (Tsuda & Somssich, 2015), known to be involved in multiple processes including biotic and abiotic stresses (Ambawat et al., 2013). Overall, 9 different MYB TFs (list extracted from TAIR, 211 genes) were differentially-expressed in response to OMV challenge, 5 up-regulated and 4 down-regulated (Table 1). Additional classes of differentially-expressed TFs that were detected are listed in Table 1.

3.5 | Comparing Arabidopsis transcriptional response to OMV with response to purified MAMPs

To learn about the differences in Arabidopsis response to purified elicitor versus a crude and molecularly complex structure – OMVs, we compared our RNA-seq data with existing transcriptomic data of Arabidopsis response to known MAMPs including flg22, (Denoux et al., 2008) elf26 (Zipfel et al., 2006), PGN (Willmann et al., 2011), OGs (Davidsson et al., 2017) and LPS (Livaja et al., 2008). Enriched GOs were extracted from the above-mentioned datasets as describe above (Supp. Table S3) and compared with GOs enriched following OMV challenge. Generally, Arabidopsis GO terms induced by OMVs were similar to those induced by single, proteinaceous and non-proteinaceous MAMPs, sharing 56, 51 and 47 % of OMV-induced GOs with GOs induced by flg22, elf26 and PGN, respectively. On the other hand, a lower overlap in induced GOs was seen with LPS and OGs, sharing 24 and 33 %, respectively, with OMV-induced GOs (Figure 4A). Notably, the pathogenesis-related 1 (PR1) gene (At2g14610), a hallmark of LPS-induced immune responses (Silipo et al., 2005, 2008), was absent from the OMV-induced gene list at all the time points tested. Forty-one GOs were found to be induced by OMV and not by any of the other MAMPs tested here (Figure 4B). This list included GOs related to 'apoptosis', 'response to drug', 'drug transport and 'multi-drug transport', and 'lipase activity'(Supp. Table S3, denoted by asterisks and bold font).

3.6 | OMVs induce Arabidopsis resistance to bacterial infection

Here and previously (Bahar et al., 2016), we have provided evidence demonstrating that the Arabidopsis immune system is induced by OMV challenge. To examine whether this OMV-mediated immune induction is translated into an effective immune response, we used an *in planta* bacterial growth test (Zipfel et al., 2004) in which Arabidopsis plants are pretreated with OMVs followed by bacterial inoculation. A significant decrease of more than 10-fold in *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) CFU/g leaf was observed in both OMV- and flg22-pretreated plants compared with mock pretreatment, two days after inoculation (Figure 5A). In vitro growth of *Pst* was not negatively affected by addition of OMVs to the medium, suggesting that the reduced *Pst* growth *in planta* is related to the priming effect of OMV, and is not a direct effect of OMVs on the bacteria (Supp. Figure S2). To test whether the OMV-induced resistance to *Pst* is mediated by FLS2, EFR or BAK1 we repeated this experiment with Col-0, *bak1-5* mutant and the double mutant line *fls2 efr1*. With both mutant lines, OMV pretreatment resulted in a significant reduction in *Pst* CFU/g leaf compared with mock treated plants (Figure 5B-C). As expected, *fls2 efr1* and *bak1-5* mutant lines treated with flg22 had similar *Pst* titers as the untreated plants as they are known to be irresponsive to flg22. To compare the relative reduction in pathogen titer in Col-0 versus the immune receptor mutant lines *fls2 efr1* and *bak1-5* in primed plants, we calculated the difference in *Pst* titer in OMV- and mock-treated plants in three independent experiments (Supp. Figure S3). The

TABLE 1 OMV-induced RKs/RLPs and immune-related transcription factors in Arabidopsis seedlings

		Gene expression (LogFC) per time point (h)			
Gene family	**Gene ID**	**2**	**6**	**24**	**Gene name**
RKs					
	AT2G02220	1.07			PSKR1
	AT4G28490	1.14			HAESA
	AT5G48380	1.30			BIR
	AT5G20480	1.12			EFR
	AT1G09970	1.40	1.03		RLK7/IKU2
	AT1G51800	3.90	3.08		IOS1
	AT1G51850	3.50	2.46		SIF2
	AT1G74360	2.50	2.63		NIRL1
	AT2G13790	1.60	1.16		SERK4
	AT2G19190	7.11	5.20		FRK1
	AT2G31880	1.62	1.53		SOBIR1
	AT4G08850	1.89	1.63		MIK2
	AT5G25930	2.57	2.06		–
	AT1G51790	3.00	2.02		–
	AT1G35710	2.28	1.46	1.12	–
	AT1G07560	1.03			–
	AT1G56140	1.34			–
	AT2G13800	1.39			–
	AT2G28960	1.74	2.06	1.86	–
	AT2G28970	1.60	1.23		–
	AT5G49780	1.96	2.31		–
	AT5G42440	1.26			–
	AT3G53590		3.16		–
	AT1G05700		3.97		–
	AT1G51820		2.33		–
	AT1G51860		1.53		–
	AT1G51890		3.52		–
	AT5G59680		3.53	1.78	–
	AT5G37450		2.24		–
	AT5G49770		2.41		–
	AT3G47090		2.05		–
	AT5G44700		1.58		GSO2
	AT3G09010		1.94		–
RLPs					
	AT2G32680	3.68		2.79	RLP23
	AT2G25470	3.44	2.41		–
	AT3G23120	2.19			RLP38
	AT1G71400	1.68	1.48		RLP12
	AT3G05660	1.15			RLP33
	AT1G47890		3.92		RLP7
	AT3G28890		2.50		RLP43
	AT5G25910		2.04		RLP52
	AT3G11080		1.96		RLP35

(Continues)

TABLE 1 (Continued)

Gene family	Gene ID	Gene expression (LogFC) per time point (h)			Gene name
		2	6	24	
	AT3G05360		1.63		RLP30
NLRs					
	AT4G14370	2.49	2.45		–
	AT5G45000	2.00	1.99		–
	AT5G45240	1.74	1.60		–
	AT5G45220	1.03			–
	AT5G41750		2.60		–
	AT2G17050		1.73		–
	AT5G41740		1.33		–
WRKY					
	AT5G24110	4.65	6.24		WRKY30
	AT1G66600	3.92	6.11		WRKY63
	AT4G23810	3.10	3.60		WRKY53
	AT2G38470	2.90	2.16		WRKY33
	AT1G80840	2.55	1.64		WRKY40
	AT4G22070	2.18	2.13		–
	AT5G15130	1.85	1.68		–
	AT2G23320	1.46			WRKY15
	AT4G18170	1.36			WRKY28
	AT4G31550	1.06			–
	AT4G01250	1.02			WRKY22
	AT2G30250	1.02			WRKY25
	AT1G62300	1.44	1.31		WRKY6
	AT2G40740		4.37		–
	AT5G22570		4.29		WRKY38
	AT5G01900		3.80		WRKY62
	AT1G29860		2.99		WRKY71
	AT2G40750		2.42		WRKY54
	AT5G13080		2.05		WRKY75
	AT5G49520		1.24		WRKY48
MYB					
	AT3G23250	4.18	5.11		MYB15
	AT4G37780	3.56			MYB87
	AT1G18570	3.53	2.28		MYB51
	AT1G74080		2.90		MYB122
	AT4G12350		2.36		MYB42
	AT5G07690	-1.12	-2.05		MYB29
	AT4G05100		-1.58		MYB74
	AT3G24310		-1.85		MYB305
	AT3G30210			-3.87	MYB121
AP2/ERF					
	AT1G71520	4.11			–
	AT3G23240	4.05			–
	AT5G64750	3.26			ABR1
	AT5G61890	3.17	4.04		ERF114
	AT5G47230	2.69	1.97		ERF102

(Continues)

TABLE 1 (Continued)

Gene family	Gene ID	Gene expression (LogFC) per time point (h)			Gene name
		2	6	24	
	AT1G28370	2.54			–
	AT4G17490	2.47	1.45		ERF103
	AT5G51190	2.20	1.26		ERF105
	AT5G47220	2.06			ERF2
	AT2G33710	1.74			–
	AT4G17500	1.57	1.05		ERF100
	AT3G50260	1.51	1.00		–
	AT1G72360	1.48			ERF73
	AT3G25730	1.28			–
	AT5G61600	1.14			ERF104
	AT5G13330	1.07	1.07		–
bHLH					
	AT5G56960	4.82			–
	AT2G43140	1.36	1.78		BHLH129
	AT3G56980			2.57	BHLH39
	AT4G28790		-1.35		–
	AT1G51140		-1.44		BHLH3
	AT1G71200		-1.53	-2.55	BHLH160
	AT4G29930		-2.19	-1.38	–
C2H2	AT2G37430		3.9		ZAT11
	AT3G49930		-1.40		–
bZIP	AT1G42990	1.04			BZIP60

average reduction in *Pst* titer in OMV-pretreated *bak1-5* plants was smaller than that observed in Col-0 plants (0.89 vs.1.14 Log CFU/gr leaf reduction for *bak1-5* and Col-0, respectively, one-way ANOVA; $F_{2,4} = 4.3781$, $p = 0.0523$). We did not see a similar reduction with the *fls2 efr1* mutant line (1.26 vs. 1.32 Log CFU reduction for *fls2 efr1* and Col-0, respectively, one-way ANOVA: $F_{1,4} = 0.0352$, $p = 0.5698$) (Figure 5D-E).

4 | DISCUSSION

Bacterial outer membrane vesicles (OMVs) are complex nanostructures originating from the bacterial outer membrane and are composed of hundreds of proteins and other cell wall components. It was previously shown that Arabidopsis plants respond to OMV challenge by activating typical immune responses such as ROS burst, immune marker gene expression and medium alkalization (Bahar et al., 2016). In this study we examined the broader transcriptional response of Arabidopsis to bacterial OMVs, and its effect on subsequent infection.

The overarching conclusion from the RNA-seq data analyses performed in this study, is that the Arabidopsis immune system is primed following the exposure to *Xanthomonas campestris* pv. *campestris* (*Xcc*) OMVs. This conclusion is supported by distinct and complementary analyses. First, gene ontology (GO) enrichment in plants exposed to *Xcc* OMVs clearly show that OMVs are perceived by Arabidopsis as stressors. The cellular location of the plant response was associated primarily with the cell periphery suggesting outer cellular perception of the challenging material, OMVs. This provides further support to the notion that OMVs, and their constituents are sensed by extracellular receptors, similarly to many known MAMPs. Secondly, we noticed a large suite of RKs and RLPs are upregulated in response to OMV challenge. Many of these receptors are known to mediate pathogen perception or were previously shown to associate with plant immune response. FLG22-induced receptor-like kinase 1 (FRK1) was the most highly induced receptor. This is interesting since we could not detect flagellin in our *Xcc* OMV proteomics analysis (data not shown). It is known that FRK1 is also induced by other immune elicitors, but it is intriguing why its expression is so much higher than the rest of the RKs up-regulated here. Elongation factor receptor (EFR) expression on the other hand, was only significantly up-regulated at the 2 h time point, and had a LogFC of 1.12, even though EF-Tu is found in *Xcc* OMVs

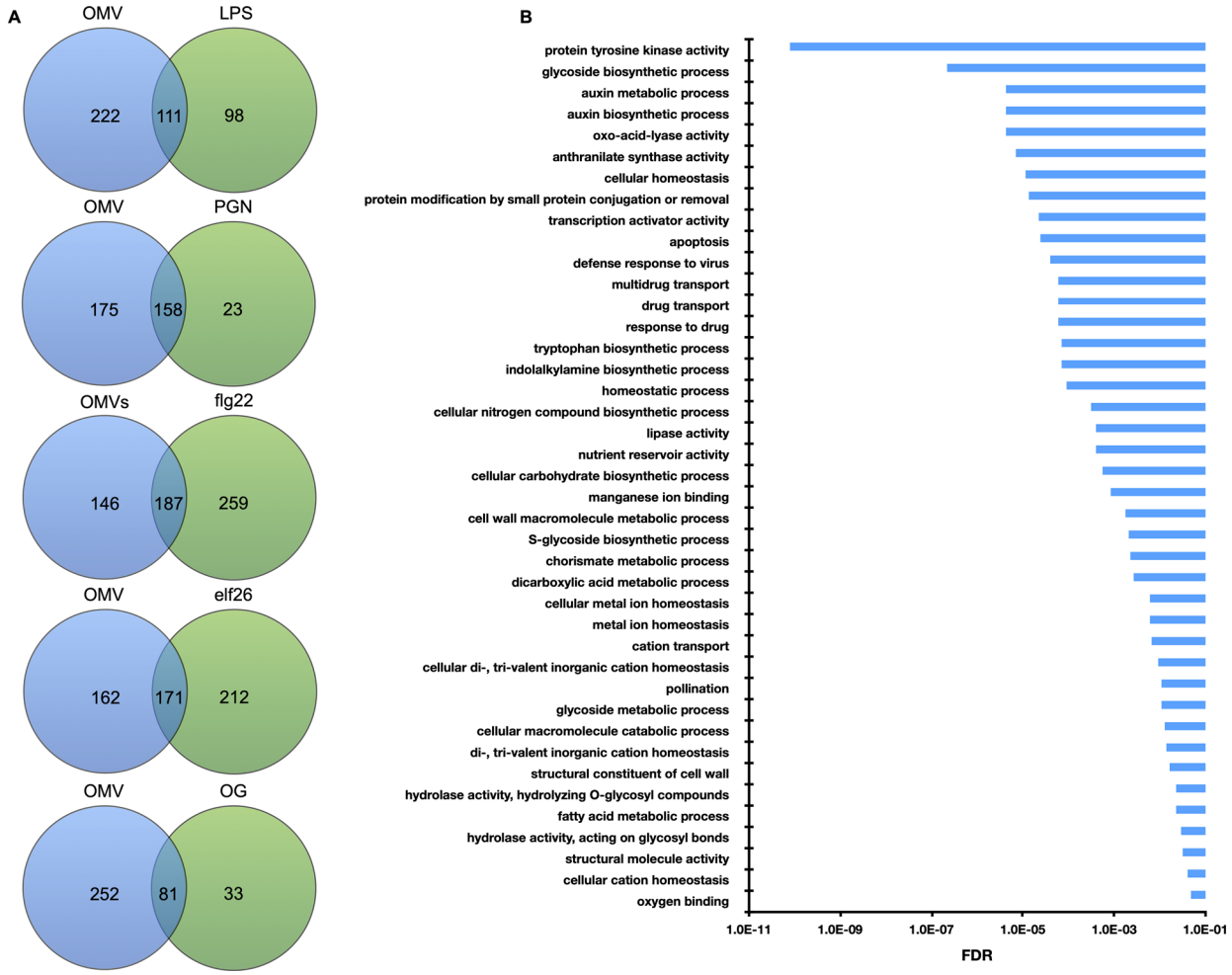

FIGURE 4 Comparison of enriched Gene ontology (GO) terms in response to OMV and to single purified MAMPs. Arabidopsis expression datasets in response to MAMP challenge (elf26, flg22, OGs, PGN and LPS; see Materials and Methods section for references) were used to extract enriched GOs using the AgriGo web-tool. Enriched GO sets of each MAMP were compared with the enriched GO list in response to OMVs using Venny (**A,** and Supp. Table S3). GO terms enriched only in the OMV datasets are shown in (**B**) sorted by their FDR value

(Bahar et al., 2016). We also found a few NLR genes up-regulated in response to OMV challenge, half of which are annotated as disease resistance proteins, yet their function in plant immunity has not been described. While we do not hypothesize NLRs are directly involved in OMV perception, they could be induced downstream of RK/RLPs sensing of OMV molecules as was also observed in response to purified MAMPs such as flg22, elf18 and LPS (Denoux et al., 2008; Livaja et al., 2008; Zipfel et al., 2006). Thirdly, many immune-related transcription factors, WRKY, MYB and others, were significantly upregulated by OMVs (Bjornson et al., 2021).

In our study, the main transcriptional change in response to OMV occurred at the first two time points (2 and 6 hpc). This was illustrated by both a significantly larger number of differentially expressed genes (DEGs) and a significantly higher Log fold-change (LogFC) at 2 and 6 hpc. Nevertheless, out of a total of 121 DEGs found at 24 hpc, almost half (52) were not found at 2 or 6 hpc. This suggests that half of the DEGs at 24 hpc are late-regulated genes, whose expression was up- or down-regulated later than 6 hpc. Indeed, Arabidopsis genes with different expression dynamics following elicitor challenged were previously identified (Bjornson et al., 2021).

The rapid and mostly transient gene expression pattern we have seen here is in accordance with other studies that have tested the temporal response of Arabidopsis to MAMPs. For example, Denoux et al. (2008) and Bjornson et al. (2021) have shown that the transcriptional change in Arabidopsis in response to various MAMPs occurs within minutes to hours, and in most cases, DEGs are back to base levels ~24 h following plant challenge. Unlike interactions between a plant and a pathogen, where the interaction is dynamic and ongoing, when challenged with a non-living sample, such as a purified MAMPs or with OMVs- it can be expected that the plant response, at least on a transcriptional level, would be transient and not sustained over days.

Intensive research in the past three decades have revealed multiple plant immune receptors responsible for microbe recognition. Many of these receptors have the capacity to detect single microbial features and are being studied in detail to better

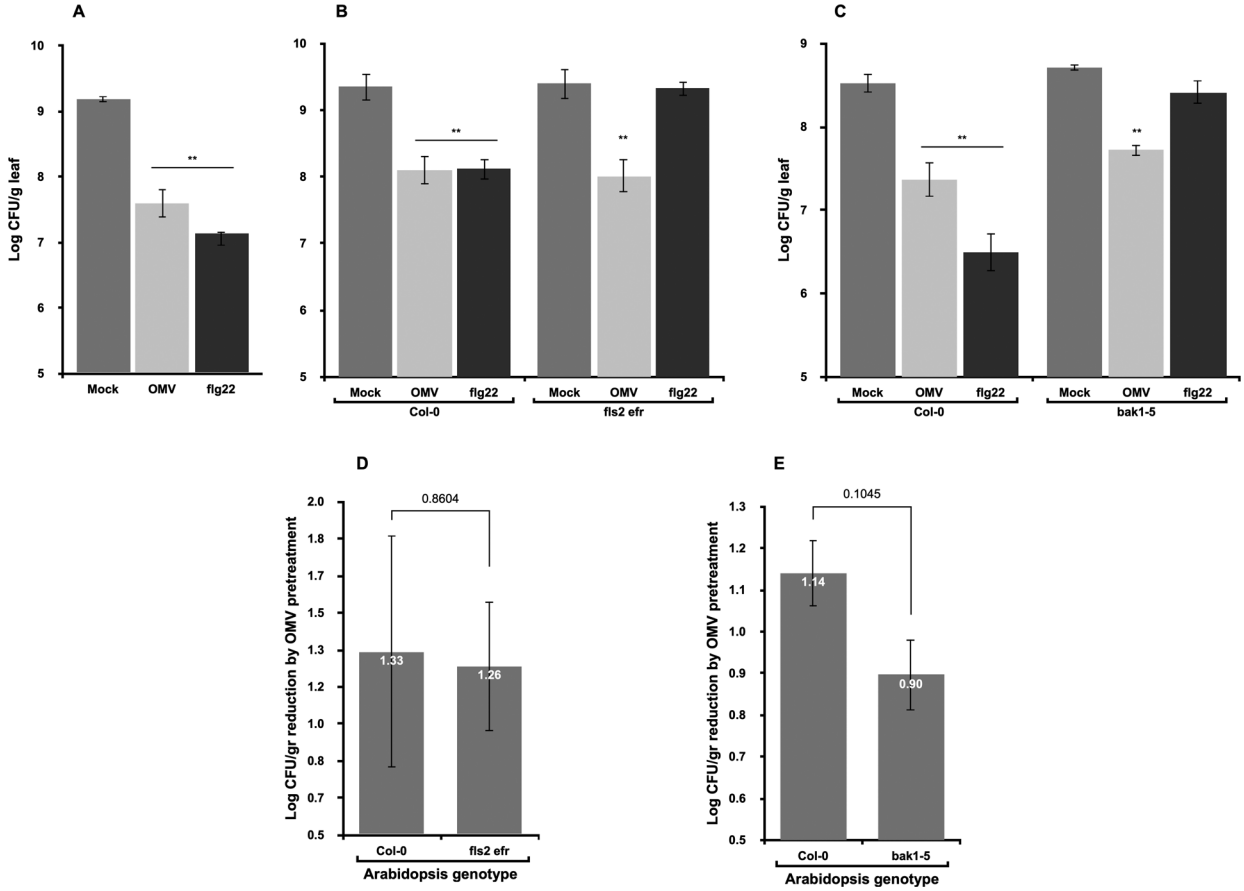

FIGURE 5 Pretreating Arabidopsis leaves with OMVs induce resistance to subsequent bacterial infection. Col-0 plants **(A)** were pretreated with OMVs, water (mock) or flg22 as controls, and 24 h later inoculated with a 10^5 CFU/ml suspension of *Pst* DC3000 using needleless syringe infiltration. *Pst* DC3000 cell titer in the inoculated leaves was determined 48 h after inoculation by serial dilution platings. Arabidopsis Col-0 and *fls2 efr* **(B),** or *bak1-5* **(C)** plants were tested in a similar experiment as described in (A). The mean Log *Pst* DC3000 CFU/gr reduction following OMV pretreatment (compared with untreated plants) in Col-0 and *fls2 efr* **(D),** and Col-0 and *bak1-5* **(E),** was compared. Each bar represents the mean Log *Pst* DC3000 CFU/gr reduction from three independent experiments (data of the independent experiments is presented in Supp. Fig. S3). Differences were not statistically significant (Two-tail student t-test. *p* values are indicated above the graph bars). Experiment A, B, and C were conducted at least three times with similar results (3 plants/replicates per treatment in each experiment). Asterisks (**) indicates significant difference compared with mock (Dunnet's test $p < 0.001$)

understand pathogen perception, immune system signaling and response of model and crop plants. However, plants are simultaneously exposed to multiple microbial features from different sources, which add complexity to immune perception and response. We were interested to examine the differences in the transcriptional response of Arabidopsis to a singular purified MAMPs versus OMVs, which represent a more natural and complex microbial structure but is a degree of complexity removed from a microbe itself. OMVs carry virulence factors, degradative enzymes, toxins and other biomolecules that could have a functional role for bacterial growth in planta, and therefore it was interesting to test if OMV challenge induces unique GOs that are not induced by synthetic MAMPs.

For our transcriptomic comparisons, we assembled data from studies with similar experimental conditions, i.e., in plants and at similar time points. A significant overlap in GO enrichment was seen in Arabidopsis response to OMV and to flg22, elf26 and PGN. This is not unexpected as it is known that many of the defense pathways activated upon pathogen sensing are similar, regardless of the specific elicitor or its source (Bjornson et al., 2021; Zipfel et al., 2006). Nevertheless, some unique GOs were found to be upregulated by OMVs and not by the other MAMPs we have surveyed. Among these are GOs related to cells wall degradation such as 'lipase activity' and 'hydrolase activity acting on glycosyl bonds', which may indicate that the plant defense system is targeting OMV degradation. Interestingly, three GOs related to drug transport were also found to be uniquely upregulated by OMV challenge. This may indicate that plants are faced with toxic compounds being delivered into their cells, perhaps by OMV-mediated delivery.

Unlike Arabidopsis response to immune eliciting peptides and PGN, we observed relatively little overlap between Arabidopsis response to OMVs and to OGs and LPS. This small overlap, especially with LPS, is somewhat surprising, considering that in mammalian cells, LPS are well acknowledged as potent contributors to the host immune response induced by OMVs (Ellis et al., 2010). Furthermore, the fact we could not find upregulation of the LPS immune hallmark PR1 (Silipo et al., 2005), could

suggests that LPS is not a major elicitor of the plant immune interaction with bacterial OMVs . This, however, remains to be more thoroughly examined.

OMVs were shown to contribute to bacterial colonization of both mammalian and plant hosts and in some instances to bacterial virulence. On the other hand, OMVs activates the host immune system hence, acting as a double-edged sword, promoting bacterial survival and virulence on the one hand, and feeding the host surveilling system and activating host immunity on the other (McMillan & Kuehn, 2021). Our priming assays showed that OMV challenge led to a significant inhibition of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) growth *in planta*, similarly to the priming effect seen with synthetic MAMPs (Jung et al., 2009). Hence, in this instance, the pre-administration of OMVs to the inoculated tissue did not promote bacterial colonization, but rather primed the plants to induce an effective immune response that inhibited pathogen growth. This result is in line with our transcriptional data and with our previous study, supporting the notion that OMVs induce a robust and effective immune response in Arabidopsis. Two recent studies have also shown that Arabidopsis pretreatment with OMVs from a pathogenic or a commensal *Pseudomonas* species, suppressed subsequent *Pst* infection (Janda et al., 2021; McMillan et al., 2021). These results cumulatively indicate that under the conditions tested, OMV infiltration do not facilitate pathogen infection.

In a previous study we have shown that multiple immune receptor mutants maintain WT responsiveness to *Xcc* OMVs. These mutants included known PRRs, recognizing either proteinaceous (FLS2, EFR, RLPReMAX) or non-proteinaceous MAMPs (LYM1/LYM3) (Bahar et al., 2016). Interestingly, Janda et al. (2021) reported that when the FLS2 receptor mutant line of Arabidopsis (*fls2*) was challenged with OMVs from *Pst*, FRK1 expression was unchanged and was similar to mock-treated plants, suggesting that FLS2 mediates the response to *Pst* OMVs. It is possible that flagellin is more abundant in *Pst* OMV preparations than it is in *Xcc* 33913 OMVs, and therefore the removal of the flagellin receptor had a more pronounced effect on plant response to *Pst* than to *Xcc* OMVs.

It has been shown that different plant immune assays may yield different results, leading to allegedly contradicting conclusions. For example, we have shown that in a leaf-disc ROS burst assay, the response of Arabidopsis to OMVs was dependent on the EFR receptor, however, in immune marker gene expression assay with Arabidopsis seedlings, the *efr1* mutant line was as responsive to OMVs as the WT. McMillan et al. (2021) showed that different physical treatments applied to OMVs abolished certain activities such as seedling growth inhibition. However, it did not alter others immune outputs such as plant priming. Hence, it is important to combine a variety of assays to test different immune outputs to obtain as broad as possible view on the plant immune response to a given elicitor.

To further examine the involvement of some of the known PRRs and co-receptors, we have tested the *fls2 efr* and the *bak1-5* mutant lines using the plant priming assay. Our results show that the Arabidopsis double mutant line *fls2 efr* was similarly primed by *Xcc* OMV as were WT plants, supporting the notion that MAMPs other than flagellin and EF-Tu are also present in *Xcc* 33913 OMVs. Based on immune marker gene expression assays, we previously suggested that the BAK1 co-receptor is involved in OMV perception and/or response (Bahar et al., 2016). In this study, we revisited this suggestion using the priming assay. Here, the *bak1-5* mutant line was primed by OMV pretreatment, but to a slightly lesser extent than WT Col-0 plants. While this margin was not statistically significant it was greater than that seen with the double *fls2 efr* mutant line. This result is also in line with a recent study that showed that the *bak1-4* mutant line was responsive to OMV as WT plants in immune priming experiments (Tran et al., 2021). Overall, this may suggest that while BAK1 is involved in OMV perception, other immune perception and signaling pathways are primed by OMV, leading to an effective immune response and suppression of pathogen growth.

The involvement of the co-receptors BAK1 and SOBIR1 in response to OMVs (Bahar et al., 2016) led us to assume that multiple immune receptors, likely PRRs, are involved in OMV perception. However, a recent study suggested that plant immune activation by OMVs may be MAMP-independent and results from physio-chemical changes in the plant plasma membrane induced by OMV integration (Tran et al., 2021). This is an intriguing hypothesis that remains to be further addressed. Interestingly, McMillan et al. (2021) reported that OMVs treated with proteinase K retained their immune priming capacity, indicating that this activity may be independent of the OMV proteinaceous cargo. While this result may support the MAMP-independent immune activation hypothesis of Tran et al. (2021), other, non-proteinaceous MAMPs present in OMVs such as LPS and PGN may activate MTI (Bahar et al., 2016; McMillan et al., 2021). Additionally, proteinase K-treated OMV retained their ability to induce seedling growth inhibition, indicating that growth inhibition is dependent on the proteinaceous cargo of OMVs (McMillan et al., 2021). All together, these results further emphasize the complexity of plant response to OMVs and the importance of using a variety of outputs to test the involvement of a particular elicitor in specific pathways.

During 2021, four independent studies including this one (that have likely taken place simultaneously), reported that bacterial OMVs modulate the plant immune system and induce an effective response against pathogen infection (Janda et al., 2021; McMillan et al., 2021; Tran et al., 2021). These exciting results position OMVs as a new and important player in plant-microbe interactions, where there is still much to be learned. In this study, we provide a broader view on the transcriptional response of Arabidopsis to *Xcc* OMV. Complemental research approaches will be needed to further understand the components and mechanisms involved in plant perception of OMVs.

AUTHOR CONTRIBUTIONS

Laura Chalupowicz: Data curation; Formal analysis; Investigation; Methodology; Writing – original draft; Writing – review & editing. Gideon Mordukhovich: Data curation; Formal analysis; Investigation; Methodology; Validation; Writing – review & editing. Nofar Assoline: Formal analysis; Writing – review & editing. Leron Katsir: Formal analysis; Methodology; Writing – review & editing. Noa Sela: Formal analysis; Writing – review & editing. Ofir Bahar: Conceptualization; Data curation; Funding acquisition; Project administration; Supervision; Writing – original draft; Writing – review & editing

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CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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SUPPORTING INFORMATION

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