DOCTOR OF SCIENCES

Study of the mode of action of COS-OGA, a new class of elicitors of plant innate immunity

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Award date:
2017

Awarding institution:
University of Namur

CHAPTER 5

How COS-OGA antagonizes *Phytophthora infestans* on *Solanum tuberosum* – an RNA-seq study

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Abstract

P. infestans, the causal agent of potato late blight, is a strongly adaptable pathogen essentially controlled on susceptible cultivars (CVs) such as Bintje by considerable amounts of fungicides. A possible alternative to fungicides is the stimulation of plant innate immunity, preferably by molecules from natural origin. The combination between chitosan- (COS) and pectin-derived (OGA) oligomers called COS-OGA stimulates plant innate immunity and a first commercial product FytoSave® is registered in Europe for use against powdery mildews in various crops. Preliminary studies showed that FytoSave® reduced late blight disease in controlled conditions but another COS-OGA composition called FytoSol proved to be particularly efficient. The study of their mode of action demonstrated that FytoSave® triggers salicylic acid (SA) and SA-associated transcript accumulation while FytoSol effect did not seem to rely directly on SA or jasmonic acid and probably included stimulation of other hormonal defense pathways. An RNA-seq study was therefore performed on potato leaves pretreated with FytoSol and collected 24 h after inoculation with *P. infestans*. The data revealed upregulation of genes encoding peroxidases, glutathione S-transferases, pathogenesis-related proteins and enzymes involved in cell wall turnover. FytoSol also upregulated transcription of genes coding for several receptor-like kinases (RLKs) essential for PAMP perception, among which several wall-associated kinases. FytoSol enhanced up to variable levels abscisic acid, ethylene and oxylipin pathways. Compared to other hormonal pathways, SA appeared only minimally regulated. These findings challenge the current dogma based on the model species *Arabidopsis thaliana* stating that SA-related defenses are most efficient against biotrophic and hemibiotrophic plant pathogens.

1. Introduction

Phytophthora infestans is a phytopathogenic oomycete causing potato (*Solanum tuberosum* L.) late blight estimated to cost more than $6 billion in losses and control strategies each year worldwide. Massive amounts of fungicides are necessary to manage the disease, which induced severe resistance of some strains to the systemic fungicide metalaxyl. A decrease in sensitivity towards other major fungicides is also observed, requiring higher doses. The disease expands exponentially and a single lesion can produce several thousands of sporangia. Sexual recombination between A1 and A2 mating types leads to higher genetic diversity and potentially more aggressive strains. Potato breeders have long sought to obtain late blight resistant varieties by the introgression of resistance (R). However, a single *P. infestans* strain can easily bypass several R-genes, making even R-gene stacking inefficient. Indeed, sequencing the *P. infestans* genome revealed more than 500 RXLR genes encoding potential effectors located in genomic regions where a high rate of mutation is expected (Fry, 2008; Fry *et al.*, 2015; Haverkort *et al.*, 2008).

The stimulation of basal immunity could be a complementary or even an alternative strategy to lower our dependence on chemical fungicides. The stimulation of plant immunity based on the recognition of pathogen-associated molecular patterns (PAMPs) leading to PAMP-triggered immunity has the reputation to be more lasting as it is based on the perception of conserved molecules less subject to mutation than effectors. COS-OGA, a new active substance that stimulates PTI, combines chitosan oligomers (COS) with pectin-derived oligogalacturonides (OGAs), thereby mimicking fungal presence and cell wall degradation. The commercial product FytoSave® is now registered in Europe for uses against powdery mildew in cucurbits and *Solanaceae* as well as against powdery and downy mildew in grapevine (van Aubel *et al.*, 2014). FytoSave® and a related composition, called FytoSol (FytoFend SA, Belgium) both containing 12.5 g/l COS-OGA were assessed in controlled conditions against late blight on potato CV Bintje that does not possess any R gene (Armstrong *et al.*, 2005). Leaf protection against *P. infestans* was partial after three applications with FytoSave® but complete with FytoSol (Clinckemaillie *et al.*, 2016; van Aubel *et al.*, 2017). First studies in potato and tomato leaves showed that FytoSave® mainly triggered SAR concomitant with a cumulative increase of salicylic acid (SA). FytoSol triggered transcription of defense-related genes but did not induce any accumulation of SA or jasmonic acid (JA) and its derivatives (van Aubel *et al.*, 2017).

The mode of action of FytoSol remains elusive but the advent of next-generation sequencing (NGS) techniques allows a much better understanding of transcription using sequencing-based transcriptome study (RNA-seq). On the one hand, recent whole transcriptome studies focus on the differences between compatible and incompatible interactions of potato with late blight using varieties that possess R-genes. These studies indicate that resistance against *P. infestans* relies mainly on a faster defense gene induction in incompatible interactions compared to compatible ones. (Gao and Bradeen, 2016; Gao *et al.*, 2013; Gyetvai *et al.*, 2012; Restrepo *et al.*, 2005). On the other hand, effective defense pathways involving PTI in potato are still not well understood. Some authors claim both JA and SA pathways to be important for potato basal defense against *P. infestans* (Halim *et al.*, 2009) while others defend that protection does not rely on SA, JA and ET (Smart *et al.*, 2003). The aim of this study is to compare the transcriptomes of control and inoculated potato plants CV Bintje w/without FytoSol pretreatment to start deciphering PTI in a potato variety lacking any R-gene (Kim *et al.*, 2012).

2. Methods

2.1. Plant material and RNA extraction

The late blight susceptible potato CV Bintje was maintained in a growth room at 24°C with a 16 h/8 h (day/night) regime. *In vitro* plantlets grown on MS medium were cut in stem pieces including one node and rooted in loam under saturated humidity during three weeks. After acclimation, plants were re-potted in larger containers watered three times a week with nutrient solutions (FloraSeries, Eurohydro). After three weeks, plants were sprayed seven, three and one day before inoculation till run off on both sides of the leaves with either water or FytoSol (FytoFend SA) at 1:200 dilution (0.5% v/v) containing 12.5 g/L COS-OGA oligosaccharides. The day of the treatment, plants were watered and kept for 24 h at 20°C and 90% relative humidity (RH) in a growth cabinet to maintain stomata open.

The day of the inoculation, sporulation from *P. infestans* (strain 10-022, mating type A2, provided by CRA-W Gembloux, Belgium) restored in its pathogenicity (van Aubel *et al.*, 2017) was scraped from leaves with distilled water containing 0.0025% (v/v) Tween 20 and adjusted at $1.5*10^4$ sporangia/ml and kept for 2 h at 4°C before inoculation. Plants were then sprayed with either distilled water containing 0.0025% (v/v) Tween 20 (mock inoculated or −Pi) or with *P. infestans* inoculum (inoculated or +Pi) on both sides of the leaves till run off. Plants were then stored at 20°C, 99% RH and a 16 h/8 h day/night regime.

Three leaves per plant located below the fifth last expanded leaves were harvested on liquid nitrogen at 24 hpi with 6 samples per condition: C−Pi (water-sprayed, mock-inoculated), C + Pi (water-sprayed, Pi-inoculated), F−Pi (FytoSol-sprayed, mock-inoculated) and F + Pi (FytoSol sprayed, Pi-inoculated). Total RNA was extracted from 75 mg per sample of frozen-ground leaves (Retsch MM400) with NucleoSpin®RNA Plant (Macherey-Nagel) including on-column DNA digestion. Plants were kept for one week after inoculation and symptoms of late blight were well observed on the sole group of C+Pi starting from 72 HPI, indicating a successful inoculation procedure.

2.2. Sequencing experiment

RNA concentration and purity were determined using Nanodrop ND-1000 (Nanodrop Technologies) and RNA integrity assessed with Bioanalyzer 2100 (Agilent). One µg total RNA per sample was used for Illumina TruSeq® Stranded mRNA Sample Prep Kit (protocol version 15031047, Revision E, October 2013). Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. First strand cDNA was obtained using random primers and converted into double-stranded cDNA. The cDNA fragments were extended with a single 'A' base to the 3' ends of the blunt-ended cDNA fragments and multiple indexing adapters were ligated to introduce barcodes before PCR enrichment. Sequencing-libraries of each sample were equimolarly pooled and sequenced on an Illumina NextSeq 500 instrument (High Output, 75 bp, Single Reads, v2) at the VIB Nucleomics core (Belgium).

2.3. RNA-seq read mapping and analysis of differentially expressed genes

RNA-seq reads were first preprocessed. Low quality ends and adapter sequences were trimmed off from the Illumina reads with FastX 0.0.14 from Hannon Lab (HannonLab., 2010) and Cutadapt 1.7.1 (Martin, 2011). Subsequently, small reads (length < 35 bp), polyA-reads (more than 90% of the bases equal A), ambiguous reads (containing N), low-quality reads (more than 50% of the bases < Q25) and artifact reads (all but three bases in the read equal one base type) were filtered using FastX 0.0.14 and ShortRead 1.24.0 (Morgan *et al.*, 2009). With Bowtie 2.2.4 we identified and removed reads that align to phix_illumina (Langmead and Salzberg, 2012).

The preprocessed reads were aligned with Tophat v2.0.13 (Trapnell *et al.*, 2009) to the reference genome of *S. tuberosum* (DMv4.04) (Hardigan *et al.*, 2016). Default Tophat parameter settings were used, except for 'min-intron-length = 15', 'max-intron-length = 15000', 'no-coverage-search' and 'read-realign-edit-dist = 3'. Using Samtools 1.1, reads with a mapping quality smaller than 20 were removed from the alignments (Li *et al.*, 2009).

The annotation came from the representative genes v4.03 released by the PGSC consortium (Hirsch *et al.*, 2014; PGSC consortium, 2016) and annotations for the additional ChrUn of the v4.04 from the Buell Lab (Hardigan *et al.*, 2016; Bluell Laboratory., 2016) were also added. The number of reads in the alignments that overlap with gene features were counted with featureCounts 1.4.6 (Liao *et al.*, 2014). Following parameters were chosen: -Q 0 –s 2 –t exon –g gene_id. We removed genes for which all samples had less than one count-per-million. Raw counts were further corrected within samples for GC-content and between samples using full quantile normalization, as implemented in the EDASeq package from Bioconductor (Risso *et al.*, 2011).

The statistical analysis was performed with the R software, version 3.2.3 (R Core Team, 2014) using the edgeR package (Robinson *et al.*, 2010). Only genes with an expression value higher than 0.5 cpm (5 read counts) in at least 6 samples were retained. TMM normalization (Robinson *et al.*, 2010) was applied using the calcNormFactors function and dataset variability was assessed with MDS (multidimensional scaling) plot. Trended negative binomial dispersion parameters were estimated with the default Cox-Reid method based on a no intercept model with main effects of condition using the estimateDisp function and down-weighting outlying genes. A quasi-likelihood negative binomial regression model (Lund *et al.*, 2012) was then used to model the overdispersed counts for each gene separately as implemented in the function glmQLFit using the above-described model. Five contrasts of interest were estimated using empirical Bayes quasi-likelihood F-tests. p-values were corrected using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995). The five contrasts were F - Pi *vs* C - Pi, C + Pi *vs* C - Pi, F + Pi *vs* C - Pi, F + Pi *vs* C + Pi, F + Pi *vs* F - Pi. All edgeR functions were applied with default values. A gene was called differentially expressed (DE) when the FDR values were < 0.05 and the absolute value of the $\log_2$ fold change > 1.

2.4. Assignment of functional category and MapMan analysis of differentially expressed genes

We used the mapping files provided by Gao and Bradeen (2016) who previously adopted the MapMan ontology (Thimm *et al.*, 2004) by using the Mercator annotation pipeline to assign potato genes into functional categories (bin). DE potato genes were functionally assigned and then analyzed using the MapMan (v3.6) pathway analysis tool.

3. Results

3.1. RNA-seq reads mapping on the reference genome

RNA-seq was performed to understand the mode of action of the COS-OGA elicitor FytoSol against late blight on potato leaves. Six week old potato plants from CV Bintje were sprayed three times at 7, 3 and 1 day before inoculation with either water (control or C) or with FytoSol (F) at 0.5% (v/v). Plants were then split in mock-inoculated (-Pi) and inoculated with the same solution containing *P. infestans* at $1.5*10^4$ sporangia/ml (+Pi). Leaves from six biological replicates of the four conditions (C-Pi, C+Pi, F-Pi, F+Pi) were collected 24 h post-inoculation (HPI) and a total of 24 RNA samples extracted.

Illumina NextSeq 500 sequencing yielded more than 418 million single end reads of 75 nucleotide length with on average 17 million reads per sample. After read preprocessing and quality trimming 417 million remaining reads (99.7%) were mapped to the reference genome of the doubled monoploid *S. tuberosum* (DM v4.04) released by the PGSC consortium (PGSC consortium, 2016). Approximatively 353 million (84.7%) of the reads could be mapped and after quality assessment 326 million reads (78.72%) were kept for feature annotation. We identified 39,428 genes, 39,428 transcripts and 141,847 exons meaning that genes had on average 3.6 exons and one associated transcript. Results were thus comparable to those obtained in a RNA-seq experiment where transcriptome was followed at different time points after *P. infestans* inoculation (Gao *et al.*, 2013).

3.2. Differentially expressed genes

Five contrasts were considered in the analysis of DE genes (Table 5.1). At 24 HPI, the effect on potato transcriptome of three preventive FytoSol treatments (F-Pi *vs* C-Pi) was much larger than *P. infestan*s inoculation (C+Pi *vs* C-Pi).

Even more, the contrast between FytoSol treatment alone and inoculation of FytoSol-pretreated plants (F+Pi v*s* F-Pi) was the less significant one resulting in 105 DE genes only. The two most significant effects were first the combined effect of the elicitor plus the pathogen (F+Pi *vs* C-Pi) which yielded 1,104 DE genes and second, the effect of FytoSol in a inoculated background (F+Pi *vs* C+Pi) which gave 962 DE genes (Table 5.1). In other words, FytoSol had a much larger impact on potato gene expression than inoculation with *P. infestans*.

Table 5.1: Summary of DE expressed genes for the five contrasts considered in the statistical analysis

Contrast	FDR < 0.05	FDR < 0.001	$\|\log_2(FC)\|>1$ and FDR < 0.05
C+Pi vs C-Pi	391	142	297
F-Pi vs C-Pi	1,256	343	643
F+Pi vs C-Pi	2,502	667	1,104
F+Pi vs C+Pi	2,057	618	962
F+Pi vs F-Pi	164	35	105

This first observation was confirmed by MDS plot (Fig. 5.1) where biological replicates from the same group clustered together. Water-pretreated plants (C) are located on the left side of the MDS plot while FytoSol-pretreated plants (F) occupy the right part. The effect of *P. infestans* inoculation is less important in FytoSol- than in water-treated plants. All FytoSol-pretreated samples cluster together despite a possible outlier (F-Pi4) while inoculation clearly separates control plants vertically on the plot.

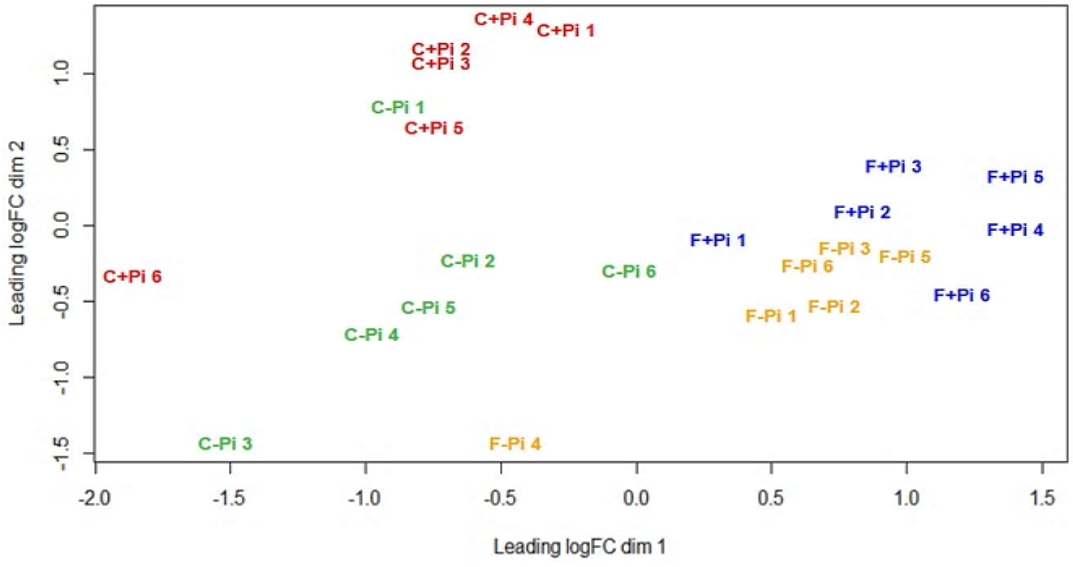

Fig. 5.1: MDS plot based on pairwise distances calculated on the basis of the top 500 DE genes. Green are mock-infected water-pretreated samples (C-Pi); red, the *P. infestans*-infected water-pretreated samples (C+Pi); orange, the mock-infected FytoSol-pretreated samples (F-Pi); blue, the *P. infestans*-infected FytoSol-pretreated samples (F+Pi).

We first decided to focus on the three ratios C+Pi *vs* C-Pi, F-Pi *vs* C-Pi and F+Pi *vs* C-Pi that represent the *P. infestans* effect alone, the FytoSol effect alone and the combined effects of both *P. infestans* and FytoSol, respectively. The Venn diagrams of the DE genes (Fig. 5.2) show that *P. infestans* mostly downregulated potato transcription with 258 genes down- and only 39 genes upregulated. On the contrary, FytoSol treatment upregulated about one-half more genes than the downregulated ones. The combination between the pathogen and the elicitor showed a perfectly balanced effect with 548 genes down- and 556 genes up-regulated.

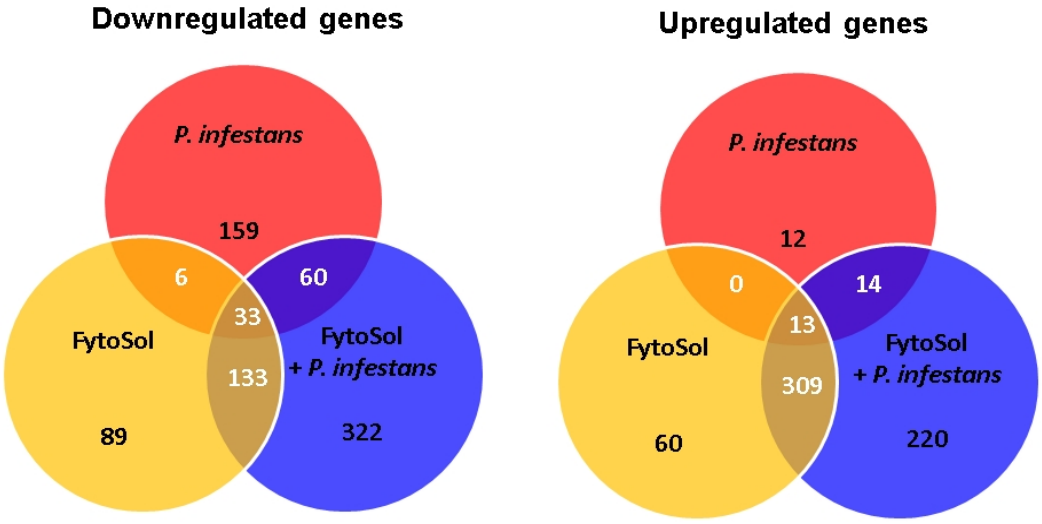

Fig. 5.2: Venn diagram of the DE genes (Log_2 FC > 1, FDR > 0.05) for each experimental condition compared to the control sample (water-pretreated, mock-inoculated).

3.3. MapMan pathway analysis of differentially expressed genes

Once DE genes are assigned to functional categories or bins, MapMan allows data treatment such as heatmaps or cluster analysis to find co-regulated genes. We first observed the number of DE genes for all the functional MapMan categories for three ratios of particular interest (C+Pi *vs* C-Pi, F-Pi *vs* C-Pi and F+Pi *vs* C-Pi). *P. infestans* essentially downregulated potato transcription (blue) while the pathogen plus elicitor combination regulated the greatest number of genes (red). The *P. infestans* effect was clearly counteracted by FytoSol and by the combination FytoSol + *P. infestans*: the very same functional categories were regulated, but in an opposite direction. Among the 35 categories defined, the bins involved in secondary metabolism, hormone metabolism, stress, miscellaneous, RNA, protein, signaling, development, transport and those not assigned were mainly regulated (Fig. 5.3).

Lots of genes are still assigned to unknown functions because the potato genome is not completely annotated and all annotations cannot always be successfully retrieved by the Mercator annotation tool. However, we could compare our three ratios using the "Biotic Stress" pathway tool from MapMan which contains most of the highly regulated bins identified here.

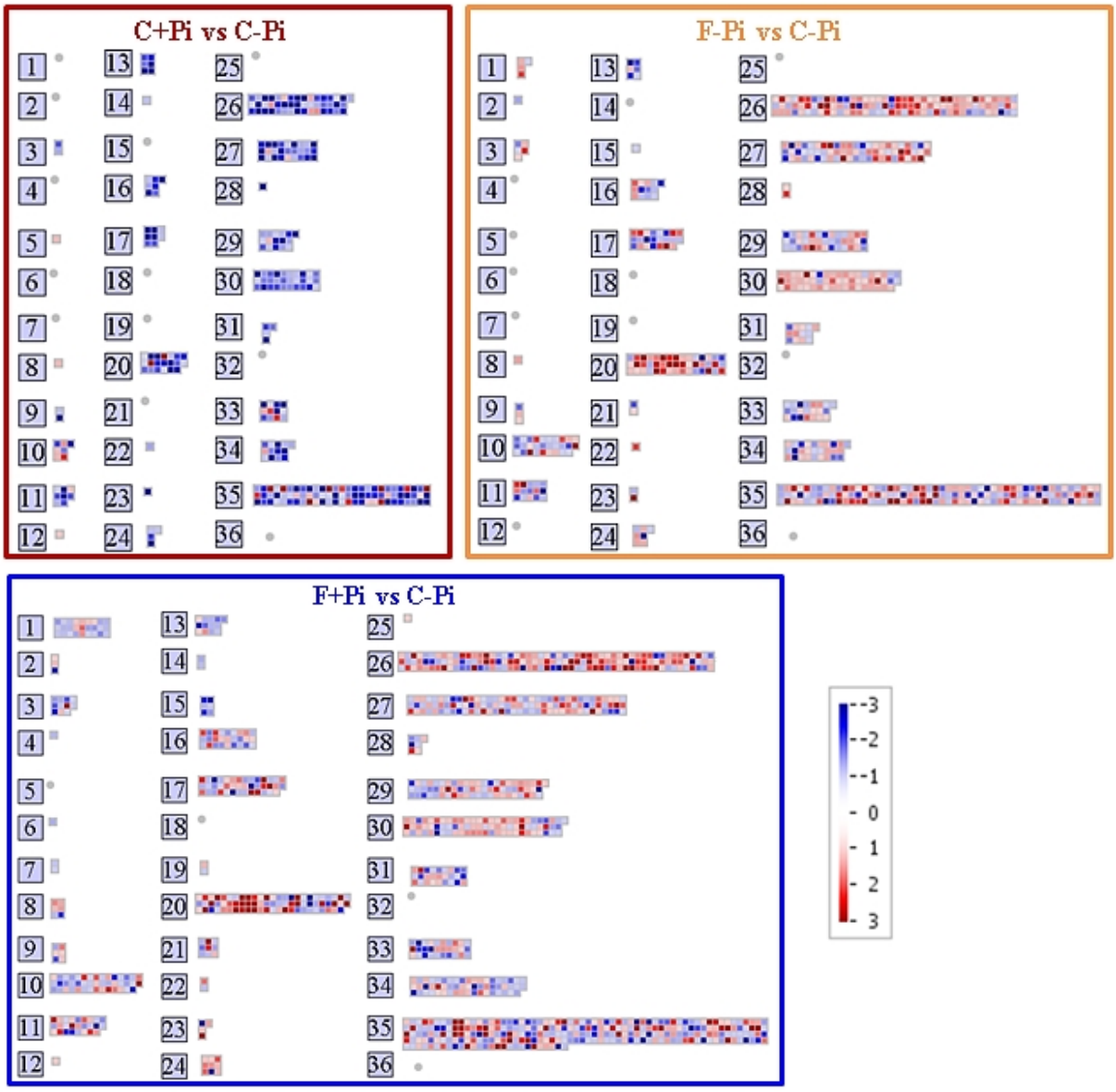

Fig. 5.3: General overview of the functional categories of the DE genes with MapMan pathway analysis tool.

Each number represents a specific bin: 1, photosynthesis; 2, major CHO metabolism; 3, minor CHO metabolism; 4, glycolysis; 5, fermentation; 6, gluconeogenesis/glyoxylate cycle; 7, pentose phosphate pathway; 8, TCA cycle; 9, mitochondrial electron transport/ATP synthesis; 10, cell wall; 11, lipid metabolism; 12, N-metabolism; 13, amino acid metabolism; 14, S-assimilation; 15, metal handling; 16, secondary metabolism; 17, hormone metabolism; 18, co-factor and vitamin metabolism; 19, tetrapyrrole synthesis; 20, stress; 21, redox; 22, polyamine metabolism; 23, nucleotide metabolism; 24, biodegradation of xenobiotic; 25, C1-metabolism; 26, miscellaneous; 27, RNA; 28, DNA; 29, protein; 30, signaling; 31, cell; 32, micro RNA, natural antisense; 33, development; 34, transport; 35, not assigned.

The effect of *P. infestans* on the biotic stress pathway led to a massive downregulation of all classes of genes (Fig. 5.4). The pathogen downregulated gene transcription linked to auxin and ET signaling as well as genes encoding peroxidases and glutathione-S-transferases (GSTs) which were the most downregulated ones with $\log_2FC < -6$. The transcription of pathogenesis-related (PR) proteins and proteins involved in proteolysis and signaling was also downregulated. This last bin comprised the highest number of significantly regulated genes and the most downregulated ones were homologs of pattern recognition receptors (PRRs) such as *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 2 (SERK2)*, *EF-TU RECEPTOR (EFR)* and *FLAGELLIN-SENSITIVE 2 (FLS2*). *P. infestans* also mainly induced the repression of genes encoding ET-responsive factors (ERF) and WRKY transcription factors (TFs) often related to the SA-pathway. The SA-specific bin was not affected by *P. infestans* but the secondary metabolism pathways whose genes mostly code for enzymes linked to the phenylpropanoids and especially lignin synthesis were notably repressed. The only bins clearly upregulated were related to cell wall and PR proteins. The most upregulated wall gene was the *α-expansin EXP8* ($\log_2FC = 2.3$) and the most repressed one was a *pectin methylesterase inhibitor* (*PMEI*, $\log_2FC = -2.7$). The only accumulated transcripts from PR protein class encode two endochitinases ($\log_2FC = 4.1$ and 3.8) and the two most depleted ($\log_2FC = -4.3$ and -3.5) code for transcripts highly similar to the MILDEW RESISTANCE LOCUS O 6 (MLO6).

Then the effect of FytoSol pretreatments alone on DE potato genes involved in the biotic stress pathway was examined (Fig. 5.5). The most regulated bin in hormonal signaling was ET, showing both positive and negative effects. JA and abscisic acid (ABA) were mainly upregulated and brassinosteroid (BR) genes were downregulated. Genes from SA- and auxin-linked pathways were not affected by FytoSol. Interestingly, the genes regulated in the JA pathway were lipoxygenases (LOX) 1 ($\log_2FC = 1.9$) having a 9-LOX activity involved in the production of oxylipins but not JA (Vellosillo *et al.*, 2007). Similarly in the abiotic stress bin, the most upregulated gene encoded α-dioxygenase (DOX) 1 ($\log_2FC = 3.4$) also related to the oxylipins pathway. In this class the ABA receptor PYL4 ($\log_2FC = 2.3$) was also upregulated.

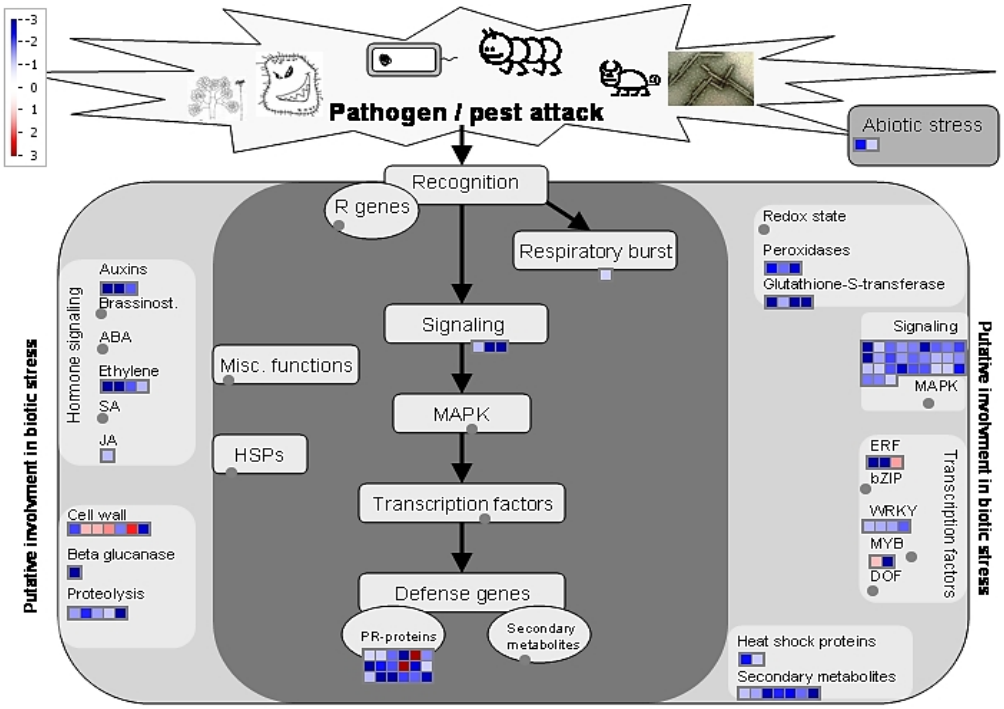

Fig. 5.4: DE genes involved in the biotic stress pathway (MapMan) following the *P. infestans* effect on *S. tuberosum* CV Bintje leaves (C+Pi *vs* C-Pi).
Scale is expressed in $\log_2$FC, blue-colored squares represent significantly downregulated genes and red-colored squares are associated with significantly upregulated genes ($|\log_2(FC)| > 1$, FDR < 0.05, n = 6).

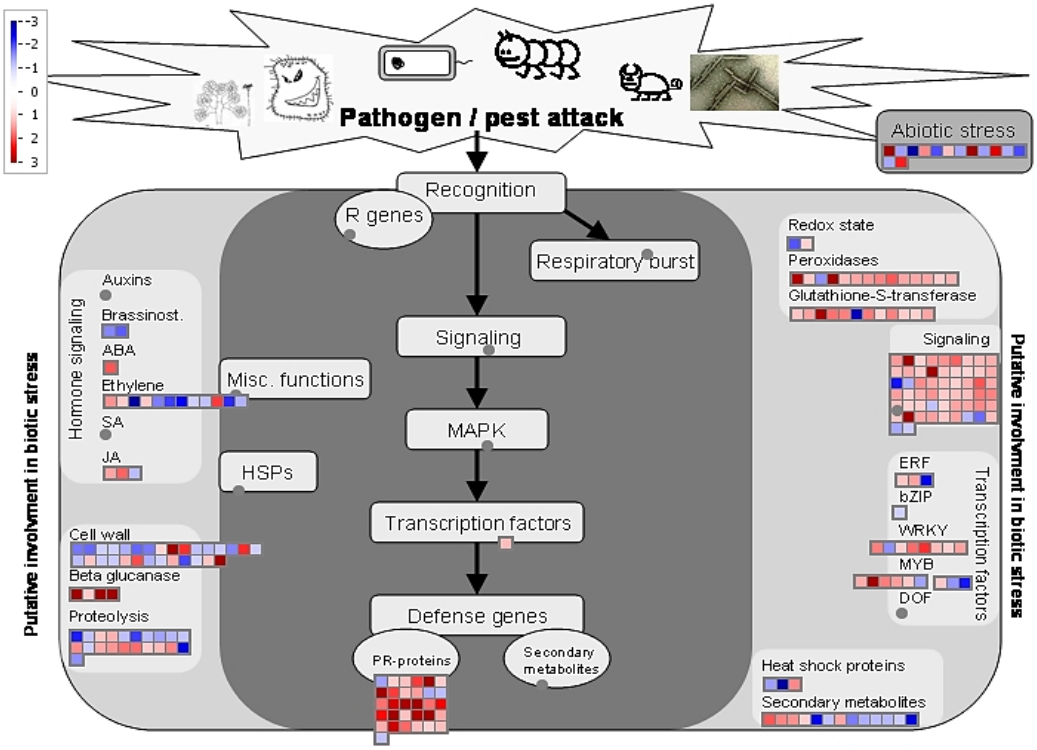

Fig. 5.5: DE genes involved in the biotic stress pathway (MapMan) following the FytoSol effect on *S. tuberosum* CV Bintje leaves (F-Pi *vs* C-Pi).
Scale is expressed in $\log_2$FC, blue-colored squares represent significantly downregulated genes and red-colored squares are associated with significantly upregulated genes ($|\log_2(FC)| > 1$, FDR < 0.05, n = 6).

Among the many regulated genes linked to cell wall metabolism (Fig. 5.5), the four most positively regulated encoded a pectin acetylesterase homolog (PAE, $log_2FC = 3.2$), an extensin ($log_2FC = 3.1$), an endo-1,4-β-glucanase ($log_2FC = 2.2$) and the pectate lyase P18 ($log_2FC = 2.2$). The four most downregulated genes encoded cellulose synthases (CESAs) including two homologs of CESA8, CESA9 and two homologs of the CESA-like protein CSLA09. The *α-expansin EXP8* upregulated by *P. infestans* was downregulated by FytoSol ($log_2FC = -1.1$) while the *PMEI* downregulated by *P. infestans* was upregulated by FytoSol ($log_2FC = 1.4$). The strongly upregulated genes encoding β-glucanases, also sometimes referred to as PR2 proteins, contained three 1,3-β-glucanases ($log_2FC = 3.4$, 3.3 and 1.1) and a lichenase ($log_2FC = 3.4$). The class of PR proteins contained a high number of highly upregulated genes encoding six homologs of the *Arabidopsis* osmotin 34 (AtOSM34, $2 < log_2FC < 4.1$), four chitinases also known as PR3 ($2.1 < log_2FC < 4.5$), three PR1s ($2.9 < log_2FC < 4.4$) and two PR4s or wound-induced hevein-like proteins ($log_2FC = 1.5$ and 1.9). In *Arabidopsis*, *PR3* and *PR4* expression is linked to JA and ET (Hu *et al.*, 2009) while *PR1* and *PR2* expression is related to SA (Caarls *et al.*, 2015).

For what concerns redox regulation (Fig. 5.5), numerous genes encoding peroxidases and GSTs were highly upregulated. Among the peroxidase genes upregulated by FytoSol, two of them were downregulated by *P. infestans* inoculation on control plants. The *GSTs* downregulated by the pathogen were different from the *GST TAU 8* upregulated by FytoSol. Many genes encoding signaling proteins had increased transcription after FytoSol treatment, among which genes encoding calmodulin and RLKs mainly from the leucine-rich repeats (LRR) and the DUF26 class. Some transcripts encoding PRRs and their associated co-receptors were slightly increased like the BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1 (BAK1) and the FLS2 ($1.1 < log_2FC < 1.9$). Transcripts encoding receptor-like proteins (RLPs) including RLP6, RLP7, RLP13, RLP34, RLP19 and RLP43 were increased following FytoSol treatment ($1.1 < log_2FC < 1.9$).

In the TF category (Fig. 5.5), the transcription of most *WRKYs* and *MYBs* were upregulated. If WRKYs are generally associated with SA, MYBs are linked with the regulation of several hormonal pathways like SA, JA and ABA (Tsuda and Somssich, 2015). Several transcripts related to the synthesis of secondary metabolites were also increased and belong almost exclusively to the terpene metabolism with two sesquiterpene synthases ($log_2FC = 1.6$ and 1.7) and one β-amyrin synthase ($log_2FC = 2$) (Singh and Sharma, 2015).

The downregulated genes in the secondary metabolism were linked to the phenylpropanoid pathway and more precisely to the flavonoid and anthocyanin synthesis and encode for an anthocyanin acyltransferase, a chalcone synthase, two dihydroflavonol 4-reductases, a caffeoyl-CoA O-methyltransferase and two isoflavone reductases (Ferrer *et al.*, 2008).

In the biotic stress pathway, genes involved in the auxin and BR signaling are downregulated after FytoSol pretreatments followed by pathogen inoculation (F+Pi *vs* C-Pi, Fig. 5.6). As observed for FytoSol alone, the 9-cis-epoxycarotenoid DOX gene involved in ABA synthesis was strongly upregulated ($log_2FC = 2.5$). ET signaling contained most of the regulated transcripts in the hormonal pathways followed by JA. The most increased transcript was the *ethylene-responsive factor 1* (*ERF1*, $log_2FC = 2.9$) in the ET bin and *LOX1* ($log_2FC = 3.4$) in the JA bin. These two genes were also the most regulated ones in the corresponding bins of the FytoSol effect but here at higher levels. Among the four most upregulated transcripts related to cell wall metabolism, we still found the three same transcripts encoding the PAE ($log_2FC = 3.4$), the endo-1,4-β-glucanase ($log_2FC = 2.2$) and the pectate lyase P18 ($log_2FC = 2.3$) as in the case of FytoSol alone. The gene encoding the α-expansin EXP8 upregulated by *P. infestans* was also upregulated here ($log_2FC = 1.8$), beside a novel DE *expansin* known as the major pollen allergen Ory s 1 ($log_2FC = 2.7$). The combined treatment still reduced the transcription of CESA and CESA-like genes but the two most downregulated genes were the *pectinesterase PME3* ($log_2FC = -2.6$) and the *xyloglucan endotransglucosylase/hydrolase 6* ($log_2FC = -2.4$). This last enzyme was recently shown to modify hemicellulose to promote tissue softening (Han *et al.*, 2016). The β-glucanases were more strongly upregulated by the combined effect of FytoSol and *P. infestans* than with FytoSol only. The three genes encoding for 1,3-β-glucanases ($log_2FC = 4.1$, 3.5 and 1.8) and the lichenase ($log_2FC = 4.3$) were even more upregulated than by individual treatments. Three others novel genes encoding for 1,3-β-glucanases were also regulated: two positively ($log_2FC = 1.8$ and 1.7) and the third one negatively ($log_2FC = -2.1$).

The combination of FytoSol and *P. infestans* regulated a higher number of transcripts involved in proteolysis than each of them individually (Fig. 5.6). The proteolysis bin mainly contained three classes of apoplastic proteases, ring finger proteins from C_3HC_4-type and E3 ubiquitin-protein ligases. The two last were mostly positively regulated while the apoplastic proteases including serine and aspartic proteases were either up or downregulated. Ring finger proteins possess a DNA-binding motif and a RING domain which mediates the transfer of ubiquitin to target proteins (Wu *et al.*, 2014c).

E3 ubiquitin ligases take actively part in the regulation of plant defense, notably by modulating the turnover of PRR and nucleotide-binding LRR receptors (Duplan and Rivas, 2014).

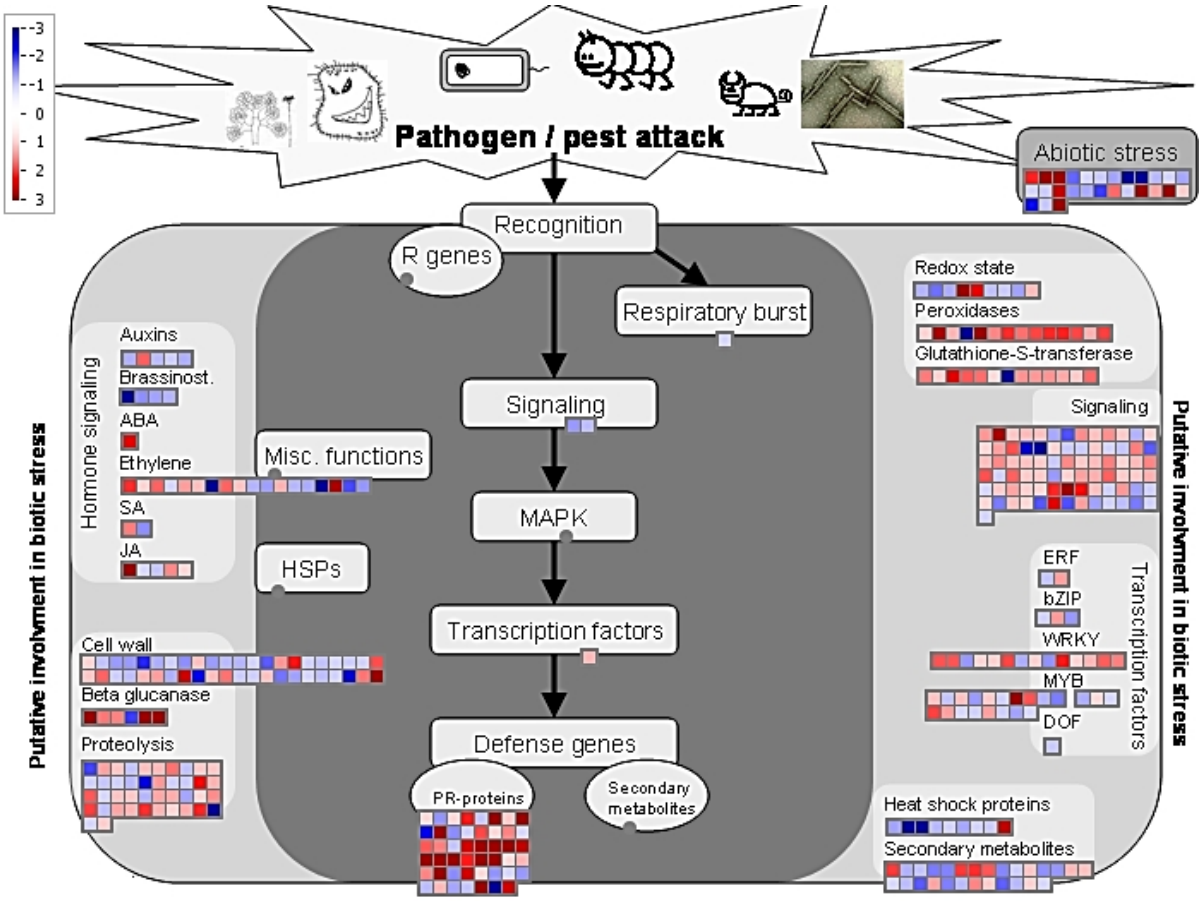

Fig. 5.6: DE genes involved in the biotic stress pathway (MapMan) following the combined effect of FytoSol and *P. infestans* on *S. tuberosum* CV Bintje leaves (F+Pi *vs* C-Pi).
Scale is expressed in $\log_2$FC, blue-colored squares represent significantly downregulated genes and red-colored squares are associated with significantly upregulated genes ($|\log_2(FC)| > 1$, FDR < 0.05, n = 6).

The transcripts encoding PR proteins were still strongly increased and the top overexpressed genes were overall the same as those observed in the ratio F-Pi/C-Pi but at a higher level (Fig. 5.6). For example, the transcripts encoding the osmotin homolog of AtOSM34, the PR1 and the chitinase peaked at a $\log_2$FC of 5.2, 5.7 and 6, respectively. Similarly in the bin linked to abiotic stress we found three *α-DOXs* ($\log_2$FC = 2.3, 3.2 and 3.8) and two *ABA receptors PYL4* ($\log_2$FC = 1.9 and 3.5) which are here more strongly upregulated than after elicitor pretreatment only.

Concerning the redox state category (Fig. 5.6), the two most accumulated transcripts encoded a glutaredoxin ($log_2FC = 2.5$) and a thioredoxin ($log_2FC = 4.8$). But more largely the classes of peroxidases and GSTs contained the greatest number of upregulated transcripts. Surprisingly, almost no upregulation of the respiratory burst oxidase homologs (RBOH) and the mitogen-associated kinases were observed, whether after FytoSol alone or after the combined elicitor plus pathogen treatment. The only effect was a slight downregulation of a RBOHA ($log_2FC = - 1$).

Elicitation plus inoculation increased the transcription of genes encoding calmodulins, PRRs, RLKs and RLPs like after the sole FytoSol treatment but the variety of RLPs and the PRRs overexpressed increased (Fig. 5.6). The two most regulated classes of TFs were the *WRKYs*, especially *WRKY71*, and the *MYBs*, once again *MYB105*, as after FytoSol only pretreatments but again at somewhat higher levels. The heat shock protein genes were mostly downregulated as well as the ones encoding for the secondary metabolism at the exception of terpene synthesis genes that were upregulated.

All these comparisons were performed using the water-treated and mock-inoculated (C-Pi) control. But another interesting comparison is the MapMan diagram showing the ratio F+Pi *vs* C+Pi which gives the most contrasted results (Fig. 5.7) and where the specific upregulating effect of FytoSol on genes in the combined treatment is amplified compared to the downregulating effect of *P. infestans* inoculation on control plants. This leads to the observation of a major specific upregulation by FytoSol of almost all the bins involved in the biotic stress regulation.

When looking to the hormonal signaling (Fig. 5.7), BR and auxin appeared mainly downregulated while ABA, ET, SA and JA were upregulated. The hormonal pathway that included the most regulated genes was again the one associated to ET and the SA pathway was the less regulated one. SA pathway only included the upregulation of a single gene encoding a putative S-adenosyl-L-methionine: carboxyl methyltransferase which converts SA into its volatile counterpart methyl salicylate (Park *et al.*, 2007).

The most positively regulated TF class (Fig. 5.7) was the *WRKYs* followed by *ERFs*. Most *MYBs* were not highly regulated at the exception of *MYB43*, *MYB15* and *MYB105*. A major upregulation was observed for downstream defense-responsive genes coding for peroxidases, GST, β-glucanases and PR proteins of utmost importance for restricting pathogen growth. In the secondary metabolism, the overexpression of genes involved in

terpene metabolism was still observed but the downregulation of phenylpropanoids synthesis genes appeared less contrasted than before.

Genes involved in cell wall metabolism were once again regulated (Fig. 5.7). Overall, the upregulated genes were the same and the four most regulated ones included a *PMEI* ($log_2FC = 3.4$), the *PAE* ($log_2FC = 3$), the *pectate lyase P18* ($log_2FC = 2.5$) and the *endo-1,4-β-glucanase* ($log_2FC = 2.1$), all previously shown to be upregulated by the FytoSol pretreatments. The most downregulated transcript encoded a PME followed by several transcripts encoding CESA or CESA-like proteins, seven of them being DE: five down and two up.

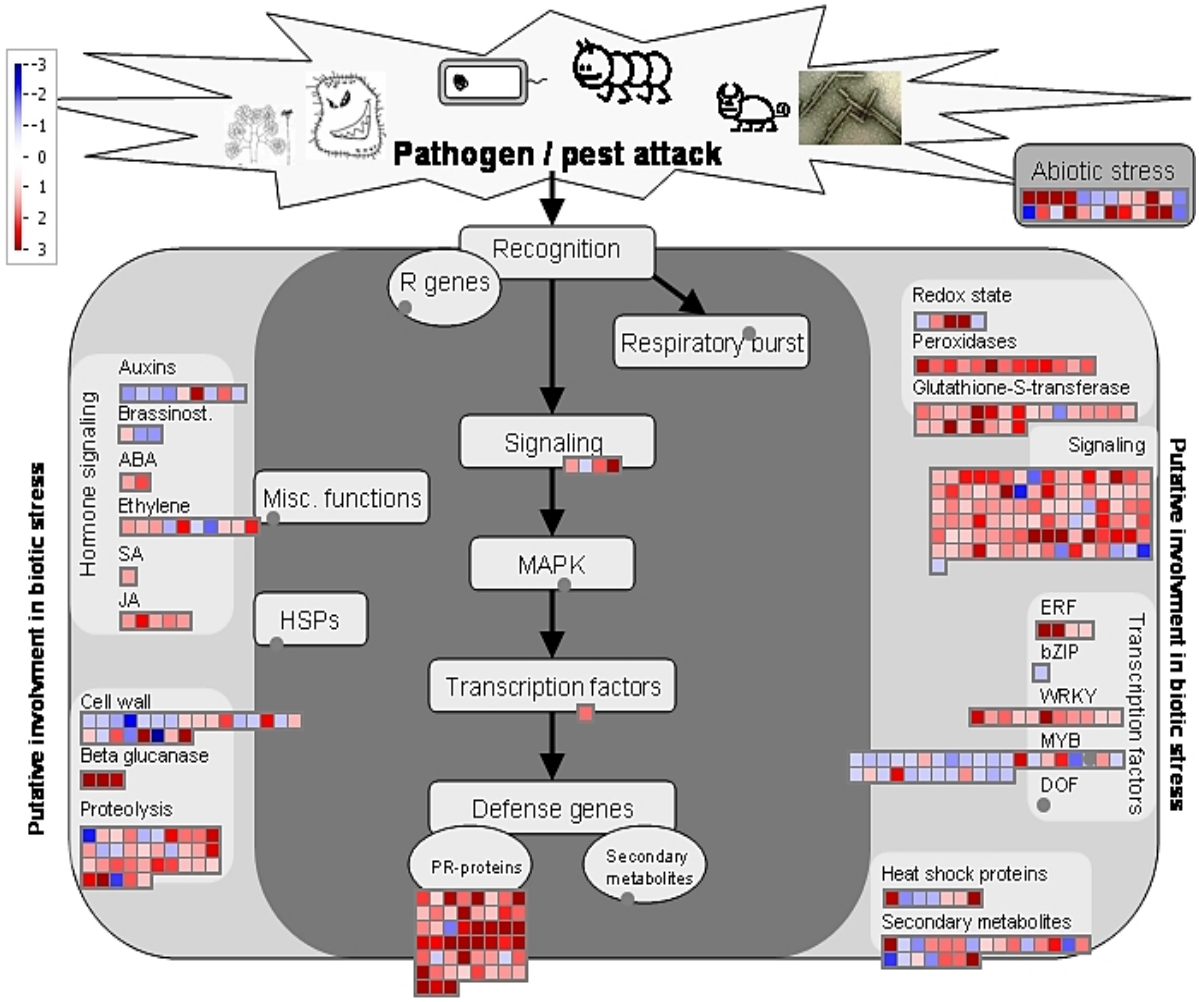

Fig. 5.7: DE genes involved in the biotic stress pathway (MapMan) following the specific effect of FytoSol in the combined FytoSol plus *P. infestans* treatment on *S. tuberosum* CV Bintje leaves (F+Pi *vs* C+Pi).
Scale is expressed in log_2FC, blue-colored squares represent significantly downregulated genes and red-colored squares are associated with significantly upregulated genes ($|log_2(FC)| > 1$, FDR < 0.05, n = 6).

3.4. WAK can FytoSol do against *P. infestans* on potato plants?

The bin including the largest number of DE genes in all the MapMan diagrams related to biotic stress, was the one linked to signaling which contained a high number of RLKs and RLPs. Another pathway to analyze with MapMan is the one specifically linked to RLKs which includes part of the transcripts already presented in the signaling bin (30) but encompassing also the category of "protein.postranslational modification.kinase.receptor like cytoplasmatic kinase VI" (bin 29.4.1.57). Since wall-associated kinase 1 (WAK1) is an OGA receptor (Brutus *et al.*, 2010; Decreux and Messiaen, 2005), we investigated WAKs and wall-associated kinase-like (WAKLs) transcripts (Table 5.2). Several DE genes presented homology with known WAKs or WAKL proteins in *Arabidopsis* and rice, two of the best-annotated genomes. Results show that *P. infestans* inoculation on potato plants led to WAK gene homologs downregulation while FytoSol pretreatments whether followed by mock- or *P. infestans*-inoculation led to WAK genes upregulation. Seven putative WAK gene homologs were upregulated in inoculated leaves pretreated with FytoSol, compared to pretreatment with water. This suggests that both pathogen and elicitor affect cell wall turnover and probably wall integrity sensing.

Table 5.2: DE genes presenting homology with WAK or WAKL proteins in the present RNA-seq experiment.

Ratio	Description	Log_2FC
C+Pi *vs* C-Pi	ATP binding protein | highly similar to AtWAK2	- 3.7
	ATP binding protein | highly similar to AtWAK2	- 2.8
	Wall-associated kinase | moderately similar to OsWAK1	- 1.1
F-Pi *vs* C-Pi	Kinase | moderately similar to AtWAKL22 | RFO1 (RESISTANCE TO FUSARIUM OXYSPORUM 1)	1.4
	Serine-threonine protein kinase | weakly similar to OsWAK53a	2.1
F+Pi *vs* C-Pi	Kinase | moderately similar to AtWAKL22 | RFO1 (RESISTANCE TO FUSARIUM OXYSPORUM 1)	1.3
	Serine-threonine protein kinase | weakly similar to OsWAK53a	1.5
F+Pi *vs* C+Pi	Protein kinase | weakly similar to OsWAK53a	1.0
	Serine-threonine protein kinase | weakly similar to OsWAK74	1.1
	Serine-threonine protein kinase | moderately similar to OsWAK10a	1.2
	Kinase | moderately similar to AtWAKL22 | RFO1 (RESISTANCE TO FUSARIUM OXYSPORUM 1)	1.3
	Serine-threonine protein kinase | weakly similar to OsWAK53a	2.7
	ATP binding protein | highly similar to AtWAK2	3.2
	ATP binding protein | highly similar to AtWAK2	3.7

4. Discussion

We used RNA-seq on potato leaves to study the mode of action of the COS-OGA elicitor FytoSol on plants challenged by *P. infestans*. The experimental setup addressed two factors: leaf pretreatments (FytoSol or water) and inoculation (*P. infestans* or mock). With six biological replicates per sample (Schurch *et al.*, 2016), we processed a total of 24 samples. We focused on 24 HPI because the pathogen appeared to be still in its biotrophic phase (van Aubel *et al.*, 2017).

The first question arising from the previous study (van Aubel *et al.*, 2017) was the hormonal pathway regulated by FytoSol and by the interaction of potato leaves with *P. infestans*. FytoSol and FytoSave® being both COS-OGA elicitors, it was surprising to observe that FytoSol did not induce any cumulative SA increase in potato like with FytoSave®. Considering the much higher protection induced by FytoSol against late blight, we suspected a difference in the elicitor mode of action (van Aubel *et al.*, 2016; van Aubel *et al.*, 2017). Here we confirm that almost no regulation of SA synthesis and SA signaling pathways could be observed after FytoSol treatment. The only DE gene upregulated was SA methyl transferase which converts SA to methyl salicylate, one of the putative critical mobile signal for systemic acquired resistance (SAR) (Park *et al.*, 2007; Pieterse *et al.*, 2014). However, even in absence of any SA increase, we observed a strong transcription of several *PR1* and *PR2* genes, well-known SAR markers (Fu and Dong, 2013). We previously reported on the absence of *PR1* regulation by FytoSol (van Aubel *et al.*, 2017) but the *PR1* sequences we highlighted here do not align with the one we choose from the work of Gallou *et al.* (2009).

FytoSol induced a dramatic increase of transcripts coding for thioredoxin, glutaredoxin, peroxidases and especially GSTs, all involved in redox homeostasis. SA-dependent downstream responses are activated notably by redox-dependent monomerization of NON EXPRESSOR OF PR1 (NPR1) that moves to the nucleus and interacts with the reduced form of the TGA1 transcription factor to allow transcription of defense genes. Exogenous application of GSH but not GSSH is sufficient to mimic SA in inducing *PR1* expression and the effect is dependent on NPR1 reduction and relocation to the nucleus (Foyer and Noctor, 2011). The FytoSol-induced SAR could therefore be redox- rather than SA-induced, especially considering that potato has constitutively high SA levels (Navarre and Mayo, 2004). This could explain the observed increase in transcription of PR1 and PR2 genes

in absence of SA increase. It will be interesting to quantify NPR1 monomerization after FytoSol treatment.

Concerning the other hormonal pathways, we observed a major regulation of the ET bin. Recently, Gao and Bradeen (2016) performed an RNA-seq analysis to compare the susceptible potato CV Russet Burbank with its transgene expressing a very efficient late blight R-gene. They concluded that ET rather than SA bin was the major differentiating factor between the resistant and the susceptible lines in both leaves and tubers. They aimed to study the ETI background but ETI and PTI likely share the same signaling components and downstream defense mechanisms (Thomma *et al.*, 2011). Gao and Bradeen (2016) also mention the regulation of the JA bin, something we also observed, but we did not see any upregulation of genes encoding enzymes directly involved in JA synthesis, which is consistent with the previously reported absence of accumulation of JA, JA-Ile or OPDA precursor following FytoSol treatment (van Aubel *et al.*, 2017). Instead, FytoSol induced transcription of LOX1 having a 9-LOX activity and α-DOX 1, both involved in oxylipin synthesis. Both genes were regulated by the elicitor and their relative expression levels were even further increased after *P. infestans* inoculation. Reverse genetic approaches in *Arabidopsis* show that these enzymes participate additively to local and systemic defense. The products of 9-LOXs and α-DOXs protect against the hemibiotrophic bacterial pathogen *Pseudomonas syringae*. They participate in plant defense by modulating hormonal response, especially the one associated with ABA (Vicente et al., 2012).

ABA is a terpenoid compound and FytoSol application upregulated number of genes involved in terpene synthesis as well as the ABA receptor PYL4 and the 9-cis-epoxycarotenoid dioxygenase (NCED), the rate-limiting enzyme in ABA synthesis (Finkelstein, 2013). Study of the transcription of defense hormone marker genes following one unique FytoSol application previously revealed that the most upregulated one was the ABA marker (van Aubel *et al.*, 2017). Before concluding that FytoSol directly acts through ABA, independent confirmation is needed using mutants or direct quantification of the hormone. ABA is indeed subject to feedback regulation (Finkelstein, 2013) meaning that a decrease in ABA level can lead to an increased transcription of ABA synthesis genes.

It is clear from the MapMan diagrams that transcripts related to cell wall turnover were regulated by the pathogen as well as by the elicitor and the number of DE genes increased under the combined effect of FytoSol and *P. infestans*. The same trend was

observed with the cell wall sensors WAK and WAKL, highlighting the importance of the cell wall in the mode of action of FytoSol against late blight. Several WAK2 homologs were DE and this wall integrity sensor expressed at organ junctions, abscission zones and meristems is probably activated by pectin de-esterification (Kohorn, 2016). Pectins are indeed more sensitive to enzymatic degradation under a de-methylesterified form which is consistent with the fact that *P. infestans* repressed a *PMEI* and induced an *EXP8* encoding an α-expansin involved in cell wall loosening (Cantu *et al.*, 2008). On the contrary, FytoSol downregulated the same *EXP8* and upregulated the *PMEI*.

FytoSol also induced the overexpression of a PAE and downregulated homologs of *Arabidopsis CESA8*, *CESA9* and *CSLA09*. *Arabidopsis* mutants for genes encoding *CESA8* and *CESA9* are more resistant towards the bacteria *Ralstonia solanacearum* and the necrotrophic fungus *Plectosphaerella cucumerina* and double mutant studies indicate that CESA-mediated resistance is independent of SA, JA and ET but probably involves ABA (Hernández-Blanco et al., 2007). The biological significance of pectin deacetylation is not yet clear, however it has been observed that transformed *Brachypodium distachyon* and *Arabidopsis* expressing a PAE from *Aspergillus nidulans* lead to the constitutive expression of defense responses leading to enhanced resistance towards the pathogens *Botrytis cinerea* and *Bipolaris sorokiniana* (Pogorelko *et al.*, 2013b). The perception of cell wall alterations through integrity sensors can lead to the downregulation of pathways involved in growth regulation such as BR and auxin (Peaucelle *et al.*, 2012; Sénéchal *et al.*, 2014) which is coherent with the regulation observed on MapMan diagrams and likely explains FytoSol crosstalk with these hormonal pathway.

From the pathogen side, cell wall integrity should also be affected as FytoSol treatment dramatically increased transcription of plant genes coding for 1,3-β-glucanases and lichenase that degrade β-glucans abundant in oomycete cell walls. The elicitor treatment also strongly overexpressed genes encoding chitinases and PR1. Currently it is not established that *P. infestans* cell wall contains chitin but the polymer was found in other closely related oomycetes such as *Plasmopara viticola*. Recently, it was demonstrated that two PR1s from tobacco and tomato with strong antimicrobial activities against oomycetes were in fact sterol-binding proteins. These proteins sequester sterols and make them completely unavailable for pathogens (Gamir *et al.*, 2017). Sterols are essential components of oomycete membranes and since *P. infestans* cannot synthesize them, they must be diverted from the host plant (Gaulin *et al.*, 2010).

To summarize, FytoSol antagonized the effect of *P. infestans* on potato transcriptome as the pathogen mainly downregulated biotic stress-related transcripts while FytoSol overexpressed them. The inoculation of *P. infestans* after FytoSol pretreatments did not drastically change the set of DE genes but amplified the response obtained by the sole FytoSol applications. In particular, the elicitor induced the upregulation of PRR genes including RLKs and RLPs essential for PAMP perception and fast response to a pathogen when it is effectively present. Such faster and higher defense response is known as the priming phenomenon (Conrath et al., 2015). But FytoSol also directly induced the expression of transcripts with direct adverse effects on *P. infestans* like pathogen cell wall-degrading enzymes β-glucanases (*PR2*), chitinases (*PR3*), osmotin (*PR5*) that lyses microbial plasma membranes, *PR1* that decreases pathogen's plasma membrane stability by sterol-sequestration and hevein-like proteins (*PR4*), directly toxic for oomycetes (Kumar *et al.*, 2016; Stotz *et al.*, 2013; van Loon *et al.*, 2006b). Such an upregulation of PR transcripts is often linked to SAR and SA accumulation (Pieterse *et al.*, 2009) but the SA signaling pathway was the less regulated one by FytoSol. However the elicitor upregulated transcripts involved in redox balance such as peroxidases, GSTs, glutaredoxin and thioredoxin that could contribute to establish a reducing environment necessary for NPR1 monomerization (Frederickson Matika and Loake, 2014) and transcriptional activation of the downstream SA-responsive genes discussed above.

However, the two hormonal defense signaling pathways most regulated by FytoSol were linked to ET and JA represented by oxylipins, followed by ABA. Chitosan and β-aminobutyric acid (BABA), both efficient against potato late blight in controlled conditions, also have a mode of action that partially relies on the ET, JA and ABA triad. Chitosan activates JA/ET pathways and induces callose synthesis mediated by ABA. The effects of BABA on potato leaves inoculated with *P. infestans* were investigated at proteomic and transcriptomic levels and the defense stimulation involved the ABA as well as JA and ET pathways (Atia *et al.*, 2005; Bengtsson *et al.*, 2014; Malerba and Cerana, 2016). It is therefore quite possible that an efficient defense against the hemibiotrophic *P. infestans* in potato necessitates the involvement of other pathways than SA. These observations challenge the current dogma of phytopathology based on the model species *Arabidopsis thaliana* stating that SA defenses are efficient against biotrophic and hemibiotrophic pathogens while JA/ET protect against necrotrophic invaders (Pieterse *et al.*, 2012).

5. Acknowledgments

This work was financially supported by the Service Public de Wallonie, Belgium (DGO 3 research grant No. D31-1339). We thank Nicolas Dauchot for its competent advices in the bioinformatics analysis.