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Research article

Xanthomonas campestris lipooligosaccharides trigger innate immunity and oxidative burst in Arabidopsis

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ABSTRACT

Plants lack the adaptive immunity mechanisms of jawed vertebrates, so they rely on innate immune responses to defense themselves from pathogens. The plant immune system perceives the presence of pathogens by recognition of molecules known as pathogen-associated molecular patterns (PAMPs). PAMPs have several common characteristics, including highly conserved structures, essential for the microorganism but absent in host organisms. Plants can specifically recognize PAMPs using a large set of receptors and can respond with appropriate defenses by activating a multicomponent and multilayered response.

Lipopolysaccharides (LPSs) and lipooligosaccharides (LOSs) are major components of the cell surface of Gram-negative bacteria with diverse roles in bacterial pathogenesis of animals and plants that include elicitation of host defenses. Little is known on the mechanisms of perception of these molecules by plants and the associated signal transduction pathways that trigger plant immunity.

Here we addressed the question whether the defense signaling pathway in *Arabidopsis thaliana* was triggered by LOS from *Xanthomonas campestris* pv. *campestris* (*Xcc*), using proteomic and transcriptomic approaches. By using affinity capture strategies with immobilized LOS and LC-MS/MS analyses, we identified 8 putative LOS protein ligands. Further investigation of these interactors led to the definition that LOS challenge is able to activate a signal transduction pathway that uses nodal regulators in common with salicylic acid-mediated pathway. Moreover, we proved evidence that *Xcc* LOS are responsible for oxidative burst in Arabidopsis either in infiltrated or systemic leaves. In addition, gene expression studies highlighted the presence of gene network involved in reactive oxygen species transduction pathway.

1. Introduction

In an environment that is rich in potentially harmful microorganisms, the survival of higher eukaryotic organisms depends on efficient pathogen sensing and rapidly mounted defense responses (Erbs and Newman, 2011). Plants possess an innate immune system that perceives the presence of pathogens by recognition of molecules known as microbe- or pathogen-associated molecular pattern (MAMPs or PAMPs, respectively) or by sensing effectors proteins that are secreted by the host during plant-pathogen interactions. The term MAMPs was introduced to indicate that these elicitor molecules are not restricted to pathogens, but can also be found in non-pathogenic or saprophytic organisms (Erbs and Newman, 2011). Early interactions between PAMPs/MAMPs and cell surface receptors (Pathogen Recognition Receptors or PRRs) lead to appropriate defenses by activating multicomponent and multilayered responses. The establishment of defense is triggered by several pathways that can involve Ca2+ influx, generation of reactive oxygen and nitrogen species (ROS and RNS, respectively) and the synthesis of phytohormones such as jasmonic acid (JA), salicylic acid (SA) and ethylene (ET), which act as signal molecules (Pieterse et al., 2009).

In bacteria, the most important MAMPs are represented by conserved cell-surface structures like flagellin, lipopeptides, peptidoglycanes and lipopolysaccharides (LPSs), which are unique to bacteria (Livaja et al., 2008). These elicitors are essential structures

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Abbreviations

APS	ammonium persulfate
DCF	dichlorofluorescein
$DCFH_2$-DA	2′-7′-dichlorodihydrofluorescein diacetate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ET	ethylene
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid
HPLC-Chip/Q-TOF	High Performance Liquid Chromatography-Chip/ Quadrupole- Time Of Flight
HRP	horse radish peroxidase
JA	jasmonic acid
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOSs	lipooligosaccharides
LPSs	lipopolysaccharides
LRRs	leucine-rich repeats
MAMPs	microbe-associated molecular patterns
MetOH	methanol
PAMPs	pathogen-associated molecular patterns
PBS	phosphate saline buffer
PR	pathogenesis-related
PVDF	polyvinylidene fluoride
ROSs	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
TEMED	*N*,*N*,*N*′,*N*′-tetramethylethylenediamine
TFs	transcription factors
Xcc	*Xanthomonas campestris* pv. *campestris*

for microbe survival, hence are conserved among microbes. LPSs are ubiquitous and vital components of the cell surface of Gram-negative bacteria and apparently have diverse roles in bacterial pathogenesis of plants. As major components of the outer membrane, LPSs are involved in the protection of bacterial cell, contributing to reduce membrane permeability, thus allowing growth of bacterium in the unfavorable conditions of the plant environment. They are amphiphilic macromolecules composed of a hydrophilic heteropolysaccharide (comprising the core oligosaccharide and the O-specific polysaccharide or O-chain) covalently linked to a lipophilic moiety termed lipid A, which anchors these macromolecules to the outer membrane. LPSs not possessing the O-chain are generally termed lipooligosaccharides (LOSs) (Silipo et al., 2005). In comparison with animal and human cells, little is known about the mechanisms of LPS/LOS perception by plants and the cognate signal transduction pathway. Recent studies revealed that LPSs from various pathogenic and non-pathogenic bacteria induce generation of ROS and defense-related gene expression in rice and that the machinery recognizing LPSs is evolutionary conserved in monocots and dicots (Desaki et al., 2006). LPSs also exert their effects on cell wall inducing callose deposition and on Pathogenesis-Related (PR) gene induction (Silipo et al., 2005; Zeidler et al., 2004).

Recent findings have suggested that the lipid A moiety may be at least partially responsible for LPS perception by the model plant *Arabidopsis thaliana*, leading to a rapid burst of nitrogen oxide (NO), a hallmark of innate immunity in animals (Zeidler et al., 2004). Using synthetic O-antigen polysaccharides (oligorhamnans), it has been shown that the O-chain of LPS is recognized by Arabidopsis and that this recognition leads to elicitation of a specific gene transcription response associated with defense (Bedini et al., 2005).

Xanthomonas campestris pv. *campestris* is the causative agent of black rot, a disease of cruciferous crops that is of worldwide importance. *Xcc* can also infect non-crop crucifers, such as Arabidopsis. Both LPS and LOS have been described in *Xcc*, with LOS being the predominant form in some strains. Silipo et al. (2005) determined the complete structure of purified LOS from *Xcc*, investigating the activity of these (structurally-defined) components in defense gene induction in Arabidopsis. *Xcc* LOS induced the defense-related *PR1* and *PR2* genes in Arabidopsis leaves in two temporal phases; the core oligosaccharide induced gene expression only in the early phase, whereas the lipid A moiety was found to be active only in the latter phase. These findings suggested that although both *Xcc* lipid A and core oligosaccharide are active in defense gene induction, they might be recognized by different plant receptors (Silipo et al., 2010).

The present work highlights the defense-signaling network in Arabidopsis triggered by LOS from *Xcc*, using proteomic and transcriptomic approaches. Moreover, gene network implicated in LOS-induced oxidative burst has been investigated.

Firstly, we reported the identification of protein complex specifically binding the *Xcc* LOS by a pull-down strategy in which biotin derivatized-LOS were immobilized on avidin-coated beads and used as bait to identify specific LOS interactors in Arabidopsis total proteic extract by mass spectrometry analysis (Giangrande et al., 2013). On the basis of proteomic data, RT-PCR time course expression analysis were performed leading to the definition of the putative signaling cascade activated by *Xcc* LOS.

Moreover, we proved evidence that *Xcc* LOS are responsible for oxidative burst in Arabidopsis either in infiltrated or systemic leaves. In addition, gene expression studies highlighted the presence of a gene network involved in ROS transduction pathway.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of Arabidopsis accession Col-0 were sown in quartz sand. After 2 weeks, seedlings were transferred to 60 ml pots containing sand/potting soil mixture that was autoclaved twice for 20 min with a 24 h interval. Plants were cultivated in a growth chamber with a 12/12 h (200 $\mu E\ m^{-2}\ s^{-1}$) day/night cycle, temperature of 21 °C and 70% relative humidity for another 3 weeks. Plants were watered on alternate days.

2.2. LOS purification from X. campestris pv. campestris

Lipooligosaccharide was purified from *Xcc* cells as described (Galanos et al., 1969). Briefly, freeze-dried cells were extracted three times with a mixture of aqueous 90% phenol/chloroform/petroleum ether (2:5:8, v/v/v). After removal of the organic solvents under vacuum, the LOS fraction was precipitated from phenol with water, washed first with aqueous 80% phenol and then three times with cold acetone and lyophilized. To remove all the cell contaminants, the LOS fraction was further subjected to enzymatic hydrolysis with RNase, DNase, and proteinase K followed by a size-exclusion chromatography on Sephacryl S-300 in 50 mM NH_4CO_3 (yield 2.7% of dried cells). LOS was analyzed for its carbohydrate and fatty acid content by published method (De Castro et al., 2010).

2.3. LOS biotinylation

Two mg of LOS were solubilized with 200 μl of pyridine (Romil) and then 6 mg of biotin-p-nitrophenyl-ester (Sigma Aldrich) were added (the amount of reagent was estimated considering LOS/biotin-p-nitrophenyl-ester 1:3 (w/w) as the mass ratio). The reaction was carried out for 2 h at 80 °C. After that, the mixture was dried under a nitrogen flow and then resuspended in water. The excess of reagents was removed by gel filtration on a PD-10 column (GE-Healthcare). The recovered fractions were lyophilized.

The reaction was controlled by SDS-PAGE. The sample was dissolved in 2% SDS (w/v), 80 mM Tris-HCl (pH 6.8), 10% glycerol (v/v), 0.002% bromophenol blue (w/v) and 50 mM DTT. Samples and prestained markers were then loaded on a 12.5% polyacrylamide gel (30% acrylamide (w/v), 0.8% bisacrylamide (w/v), 0.4 M Tris-HCl (pH 9.2), 0.1% SDS (w/v), 0.1% APS (w/v), 0.001% TEMED (v/v)). The run was performed using a running buffer (0.1 M Tris-glycine (pH 8.3)) at an electrical current of 25 mA. After run, the gel was soaked in transfer buffer (48 mM Tris, 30 mM glycine (pH 8.5–9.5), 20% MetOH (v/v), 0.1% SDS (w/v)) for 15 min. At the same time, a PVDF membrane was activated with MetOH, wet with water and soaked for 30 min in transfer buffer. Transfer was obtained by "Semi-dry transfer blot" system (Bio-Rad) and was performed for 45 min at 15 V. After the transfer, the membrane was incubated overnight at 4 °C with a blocking solution (1X PBS, 5% milk (w/v), 0.05% Tween 20). The PVDF membrane was then washed for three times with washing buffer (PBS 1X, 0.05% Tween 20) and finally incubated for 2 h with a solution of streptavidin protein conjugated to horseradish peroxidase (1 mg/ml) dissolved in blocking solution. After incubation, the membrane was again subjected to repeated washes and then it was finally treated according standard procedures of exposure and processing of photographic plates to reveal the peroxidase chemiluminescence.

2.4. Fishing for partners strategy

Dried avidin-agarose resin (0.2 ml) was subjected to extensive washes with binding buffer (0.1 M Na_3PO_4, 0.3 M NaCl (pH 7.2)) containing protease inhibitors and divided in two equal aliquots. One was incubated with the biotinylated lipooligosaccharide preparation, considering a ratio of 3 mg of LOS per 1 ml of resin, whereas the other one was incubated with 20 mg of Arabidopsis protein extract (pre-clearing step). For both samples, incubation was carried out overnight at 4 °C. After incubation, the biotinylated LOS-avidin resin was washed 5 times with binding buffer to remove the excess of biotinylated LOS and then incubated with the extract coming from the pre-clearing step (overnight at 4 °C). The resin samples coming from the last incubation step and the pre-clearing step were repeatedly washed with binding buffer and elution was achieved by boiling the samples for 20 min in 8 M guanidine, 20 mM EDTA (pH 1.5). After elution pH was adjusted to 7.5. The affinity capture experiment was repeated twice.

2.5. Reduction, alkylation and trypsin digestion

Both samples were reduced with DTT 10 mM in 50 mM Ammonium Bicarbonate (AmBic) (pH 7.8) for 45 min at 56 °C, and alkylated with iodoacetamide 55 mM in 0.1 M AmBic for 30 min at room temperature, respectively. Samples were desalted by gel filtration chromatography using PD-10 columns. One hundred μl of a trypsin solution (1 ng/nl) in 100 mM AmBic were added to the collected samples and digestion was carried out overnight at 37 °C.

2.6. LC-MS/MS analyses

The resulting peptide mixtures were lyophilized and dissolved in 10 μl of 0.1% formic acid (v/v) and subsequently analyzed by an HPLC-Chip/Q-TOF 6520 (Agilent Technologies). After loading, mixtures were concentrated and washed at 4 μl/min in a 40 nL enrichment column with 0.2% (v/v) formic acid in 2% (v/v) acetonitrile. Fractionation was carried out on a C-18 reverse phase column (75 μm × 43 mm) at a flow rate of 0.4 nL/min with a linear gradient of eluent B (95% acetonitrile (v/v) and 0.2% formic acid (v/v)) in eluent A (2% acetonitrile (v/v) and 0.1% formic acid (v/v)) from 7% to 80% in 51 min. Mass spectrometry (MS) analyses were performed using data dependent acquisition MS scans (mass range 300–2400 m/z), followed by MS/MS scans (mass range 100–2000 m/z) of the four most intense ions of a chromatographic peak. Raw data from LC–MS/MS were converted to m/z data, and searched against the SwissProt database using the licensed version of Mascot 2.1 (Matrix Science).

2.7. Arabidopsis total protein extract

Five-week-old *A. thaliana* ecotype Col-0 leaves were grounded to fine powder in a pre-chilled mortar in the presence of liquid nitrogen. Extraction buffer, containing 50 mM HEPES (pH 7.2), 150 mM NaCl, 1 mM EDTA, 1% polyvinylpolypyrrolidone (w/v) and a cocktail of protease inhibitors, was added to the powder. The buffer extract was then centrifuged at 9000 rpm for 20 min at 4 °C and the clear supernatant was added with 2% glycerol (v/v) and then used for fishing for partners experiment.

2.8. LOS treatment of Arabidopsis plants

LOS were dissolved in water (50 μg/ml) and infiltrated into 5-week-old central leaf rosettes of Arabidopsis ecotype Col-0. Treatment was performed by applying pressure against the lower side of a leaf lamina with a syringe containing the solution but lacking the needle. A control set of plant was inoculated with water. The plants were placed in the growth chamber as above. Four inoculated and systemic leaves were harvested 4, 8, 24, and 48 h after treatment from five plants for each time-point, immediately frozen in liquid nitrogen, then stored at −80 °C prior to further analysis.

2.9. Gene expression analyses

Leaf samples were grounded to fine powder in a pre-chilled mortar in the presence of liquid nitrogen and the RNA extraction was performed from 70 mg of the powder with a "NucleoSpin® RNA Plant" (Macherey-Nagel) kit following the manufacturer's instructions. The quality and the concentration of RNA samples were examined by GelRed-stained (Bioline) agarose gel electrophoresis and spectrophotometric analysis, respectively.

One μg of total RNA was reverse-transcribed using oligo-$(dT)_{20}$ primers (Invitrogen), 0.5 mM dNTPs and 200 U of ImProm-II™ reverse transcriptase (Promega) according to the manufacturer's instructions.

qPCR analysis was performed in optical 96 well plates using the CFX 96 Real-Time System (BIORAD) and SYBR® Green to monitor to synthesis of double-stranded DNA. A standard thermal profile was used: 30 s at 95 °C for denaturation and 40 cycles of 5 s at 95 °C and 30 s at the annealing temperature of 57 °C. Melting curves were recorded after cycle 40 by heating from 65 °C to 95 °C with a ramp speed of 0.5 °C every 5 s. Transcript levels were calculated relative to the reference gene At1g13320, coding for protein phosphatase 2A, using the $2^{-\Delta\Delta CT}$ formula.

For semi-quantitative RT-PCR analysis, cDNA amplification was performed over 25 cycles in a Progene Termal cycler using BIO-TAQ™ (Bioline) DNA Polymerase according to the manufacturer's instructions under the following conditions: denaturation at 95 °C for 1 min, annealing for 1 min at different temperatures depending on the primers used and elongation at 72 °C for 1 min. To normalize the quantity of RNA used for RT-PCR, cDNA was amplified with specific primers for ubiquitin (*UBI 10*), according to the above protocol. *UBI 10* primers were also used to identify possible contamination of RNA by genomic DNA. All the products of RT-PCR amplification were analyzed on agarose gel 1.5% (w/v).

The specific primers were designed according to their known sequence (Table 1).

All the experiments were repeated three times independently.

Table 1
Primer sequences used for gene expression analyses.

Gene name	Primer name	Sequence 5′ to 3′
F–box LRR (At1g66290)	LRR F	GGCTACTTGTTCCTGGAAGTG
	LRR R	AGTGATACGAACTCGGGCTTTG
F–box (At5g03100)	F-box F	TTTGGCTACTTGTTCCTGGAA
	F-box R	AGTGATACGAACTCGGGCT
CPK11(At2g30360)	CPK11 F	TTCTTACTCGTCGGGATTGG
	CPK11 R	CCCTCCATCTCAAACCCATA
CPK33 (At1g50700)	CPK33 F	TAGCCAAGAAATACGGATTGG
	CPK33 R	TCTTCGTACGGCTTCTCG
AtWRKY44 (At2g37260)	AtWRKY44 F	CAATGGGACAAGACCCTAACA
	AtWRKY44 R	GCTACTCTTGCAACCGGAAT
ZFBP68 (At5g66270)	ZFBP68 F	CCATGTCCAGTGCTCTCTCA
	ZFBP68 R	CAAGAGCTGCAAGGGAAAAG
LTP1 (At2g38540)	LTP1 F	AATCACATCGAACGCTGC
	LTP1 R	GCTCTAGCGGCACCTTGAAT
GSTPM24 (At4g02520)	GSTPM24 F	AGTGGCTTCAAAGCTTGCTTTT
	GSTPM24 R	TCGGTGAAGAGCTTCTTGGT
PR1 (At2g14610)	PR1 F	ACAACCAGGCACGAGGAG
	PR1 R	AAGTCACCGCTACCCCAG
PR4 (At3g04720)	PR4 F	CTAGCGGACCAGGGGAGAG
	PR4 R	GCAAGAAGCTTGACCACGAG
PDF1.2 (At5g44420)	PDF1.2 F	TTGCTTCCATCATCACCCTTA
	PDF1.2 R	TGCTGGGAAGACATAGTTGC
PLC1 (At5g58670)	PLC1 F	TCACTCGTGTCAATGTCGGTA
	PLC1 R	TCATACGTTTCATAATTTTGCTGA
PLDα (At3g15730)	PLDα F	GGACCAGATCCCGAATTCTT
	PLDα R	GGGCATCAACTTCATAGATGG
PLDδ (At4g35790)	PLDδ F	TGGGGTTTCTTTTGATCCTG
	PLDδ R	CACAGCATCATTATCCGATTTT
PDK1 (At5g04510)	PDK1 F	TGATCCAAACAAATTCCCTCA
	PDK1 R	AGAACCGTTGGATCTGTTGG
Ptp1 (At1g71860)	Ptp1 F	ACAGAGGATCAGCCCATGTC
	Ptp1 R	CAGACATATCGCCAGCAAGA
NPR1 (At1g64280)	NPR1 F	GGAAGAGCTTGTTAAAGAGATAATTG
	NPR1 R	GGATATTATTACATTCAACCGCCAT
Hsfa2 (At2g26150)	Hsfa2 F	GGAGGAAACGGAGGCTTACT
	Hsfa2 R	CGCTGCTTCCAAATTACCAT
Hsfa21 (At4g18880)	Hsfa21 F	CGATAAAGATAAAGCCGGAGTA
	Hsfa21 R	CAAGTGATAAATAAACAGCTGAACC
Oxi1 (At3g25250)	Oxi1 F	TGGTGATAAGGGAACCGATG
	Oxi1 R	TCAACCCCTTAACCCATTCC
Mpk3 (At3g45640)	Mpk3 F	GTGGCCAATACACGGATTTT
	Mpk3 R	CATCGCTACTAGCTCGTTCGT
Mpk6 (At2g43790)	Mpk6 F	GCGGCTGATACAGAGATGAC
	Mpk6 R	TCATAGCCGAACAAACGATG
WRKY25 (At2g30250)	WRKY25 F	TTCACCGACCTTCTTGGTTC
	WRKY25 R	TGTGAGGAGCTGAGAAGCAG
WRKY6 (At1g62300)	WRKY6 F	GCAGCGTTTTCTCAGTCACA
	WRKY6 R	TGTTTCCTATAACCCAGTTGGAA
WRKY33 (At2g38470)	WRKY33 F	AGCAAAGAGATGGAAAGGGGACAA
	WRKY33 R	TTGCGTTTGAAGGTTGTGTTG
Zat12 (At5g59820)	Zat12 F	GGAGCTTGGAAGAACAGTTTATTGA
	Zat12 R	CATAACAAATCTCCAATGCTACAA
Atrbohf (At1g64060)	Atrbohf F	CGTTGCATTAAAATAAATGTGGC
	Atrbohf R	GAACGTTGACGGAGAGAGAGA
Atrbohd (At5g47910)	Atrbohd F	GAGACGAGGCAATTCAAGTAACGA
	Atrbohd R	GCGAATCTTGCGTTTTTCTT
Apx1 (At1g07890)	Apx1 F	CGATTGCATTTGAACTTTGTCA
	Apx1 R	GAGGCATAAATATCGAGAGAAATTA
Cat3 (At1g20620)	Cat3 F	CCAAAAACTCATGTTTCCTTCC
	Cat3 R	TGATCCATTTCAGGATTCTCC
SOD (At1g12520)	SOD F	TGTGTGTGCGTTTTGTGTGT
	SOD F	TTAATGGGACAGGGGCATC
UBI 10 (At4g05320)	UBI10 F	AAAGAGATAACAGGAACGGAAACATAGT
	UBI10 R	GGCCTTGTATAATCCCTGATGAATAAG
PP2A (At1g13320)	PP2A F	TAACGTGGCCAAAATGATGC
	PP2A R	GTTCTCCACAACCGCTTGGT

2.10. ROS detection in LOS-treated plants

The presence of ROS was visualized by the specific probe 2′-7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA; Sigma Aldrich), which is rapidly oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of H_2O_2. Five-week-old plants were infiltrated with LOS and water (control) as described above; four inoculated and systemic leaves from five treated and control plants were harvested 4 and 24 h after treatment. Half number of leaves was incubated in a solution of 20 μM DCFH2-DA in 10 mM Tris-HCl (pH 7.4) for 45 min under dark. As a negative technical control the remaining half was incubated in 10 mM Tris-HCl (pH 7.4) only, under the same conditions.

After staining, the samples were washed three times in fresh buffer for 10 min to remove the excess of fluorophore and mounted on slides. Fluorescence was then observed under a LSM 710 confocal microscope (Carl Zeiss Microscopy GmbH, Germany) with Planneofluoar × 40/1.30 objective. Two laser excitations lines were used (i.e., 488 nm for probe detection and 563 nm for chlorophyll auto fluorescence). Data were processed using Image J software (http://rsbweb.nih.gov/ij/).

3. Results

3.1. LOS affinity capture experiments

The defense signaling network triggered by LOS from *Xcc* in Arabidopsis was investigated by an affinity capture procedure to identify LOS specific protein interactors that might elicit plant innate immune response. The LOS from *Xcc* was extracted and biotinylated by incubation with biotin-p-nitrophenyl-ester according to the procedure developed at our laboratory (Giangrande et al., 2013). The extent of modification was tested by SDS-PAGE analysis developed with both silver staining and streptavidin/HRP conjugate (data not shown). Biotinylated LOS was conjugated to avidin resin as described in Materials and methods section.

A total protein extract was prepared from Arabidopsis leaves and incubated with avidin beads. This pre-clearing procedure was introduced to decrease the number of possible non-specific interactions of plant proteins with the beads, thus avoiding false positives. Following incubation, the unbound fraction was collected and incubated overnight with the biotin-LOS beads. Both resin samples were extensively washed with the binding buffer and finally proteins retained by the beads were eluted as described in Materials and methods section.

3.2. Identification of LOS interacting proteins

Both the control and samples mixtures were reduced with DTT and cysteine residues were alkylated with iodoacetamide. Samples were then digested with trypsin and the resulting peptide mixtures directly analyzed by LC-MS/MS providing both the accurate molecular mass and sequence information on individual peptides. This information was used to search protein databases using an in house version of the Mascot software. Common proteins identified in both

the sample and the control mixtures were discarded and only those solely occurring in the sample were identified as putative LOS interactors, thus greatly decreasing the number of false positives. All the selected proteins showed Mascot scores greater than the significance threshold.

Eight proteins have been found that putatively interact with the LOS moiety. Among them are two members of the F-box protein family involved in pathogen recognition, namely F-box (At5g03100) and F box-LRR (At1g66290) as well as two protein kinases involved in cellular signal transduction pathways, CIPK11 (At2g30360) and CIPK33 (At1g50700). Moreover, two proteins responsive to biotic and abiotic stresses, i.e. non-specific lipid transfer proteins (NsLTP1-At2g38540) and glutathione S-transferase (GST PM24-At4g02520), the transcription factor AtWRKY44 (At2g37260) and the RNA binding protein ZFBP68 (At5g66270) were also detected.

3.3. Transcript profiling of genes coding LOS interacting proteins

In order to shed some light on defense signaling network triggered by *Xcc* LOS, the transcriptomic profile of the genes coding the identified proteins was investigated by real time PCR. Moreover, expression analysis was also performed on marker defense genes coding PR proteins, as PR1 (At2g14610), responsive to the SA pathway, and PR4 (At3g04720) and PDF1.2 (At5g44420), both responsive to the JA pathway. To this end, Arabidopsis leaves were infiltrated with *Xcc* LOS and sampled at different time points after treatment, as reported in Material and Methods section. In addition, systemic leaves were harvested at the same time points to monitor the defense response in tissues far from the infection site. Control leaves were infiltrated with water. Three biological replicates and three technical replicates of qPCR experiments were carried out.

The results shown in Fig. 1, representative of three independent gene expression studies, demonstrated that *Xcc* LOS weakly induced *F-box* gene expression in both infiltrated and systemic leaves collected at 4 and 8 h after treatment, whereas its level of expression was lower in infiltrated leaves collected 24 and 48 h after treatment; no significant change was observed in systemic samples harvested at 24 and 48 h with respect to the respective controls. Expression study carried out on the *F box-LRR* gene showed a discontinuous pattern of expression irrespectively to time of harvesting either in infiltrated or systemic leaves besides a strong induction detectable only in systemic leaves harvested 48 h upon treatment.

Weak activation of *CIPK33* and *CIPK11* gene expression with respect to the control was detectable at 8 h after treatment either in infiltrated or systemic samples. Noteworthy, strong *CIPK33* induction was detected in infiltrated and systemic leaves harvested 48 h upon treatment, whereas *CIPK11* level of homologous samples were lower than that at 8 h upon treatment. *Xcc* LOS caused increase in accumulation of transcripts for both transcription factors coding genes: in particular *ZFBP68* and *AtWRKY44* showed a very weak induction in both infiltrated and systemic leaves analyzed 8 h upon treatment, whereas the level of induction was higher in samples harvested 48 h upon treatment. As far as *GST PM24* gene is concerned, statistically significative high level of expression was detected in infiltrated leaves collected 4 h upon treatment and also in infiltrated and systemic samples collected later on at 8 h after treatment. However, light inhibition in both infiltrated and systemic samples was detected at 24 h, whereas a weak induction was detected in systemic samples collected at 48 h upon induction. *NsLTP1* gene was not influenced by the treatment in all samples analyzed at 4 and 8 h upon treatment with respect to the corresponding controls, whereas the level of induction at later time points were very low though statistically significative. Interestingly, we observed a strong inhibition of both *PDF 1.2* and *PR4* gene expression at all time point considered especially in infiltrated leaves, whereas a very strong accumulation of *PR1* gene was detected in all samples an at all time points considered.

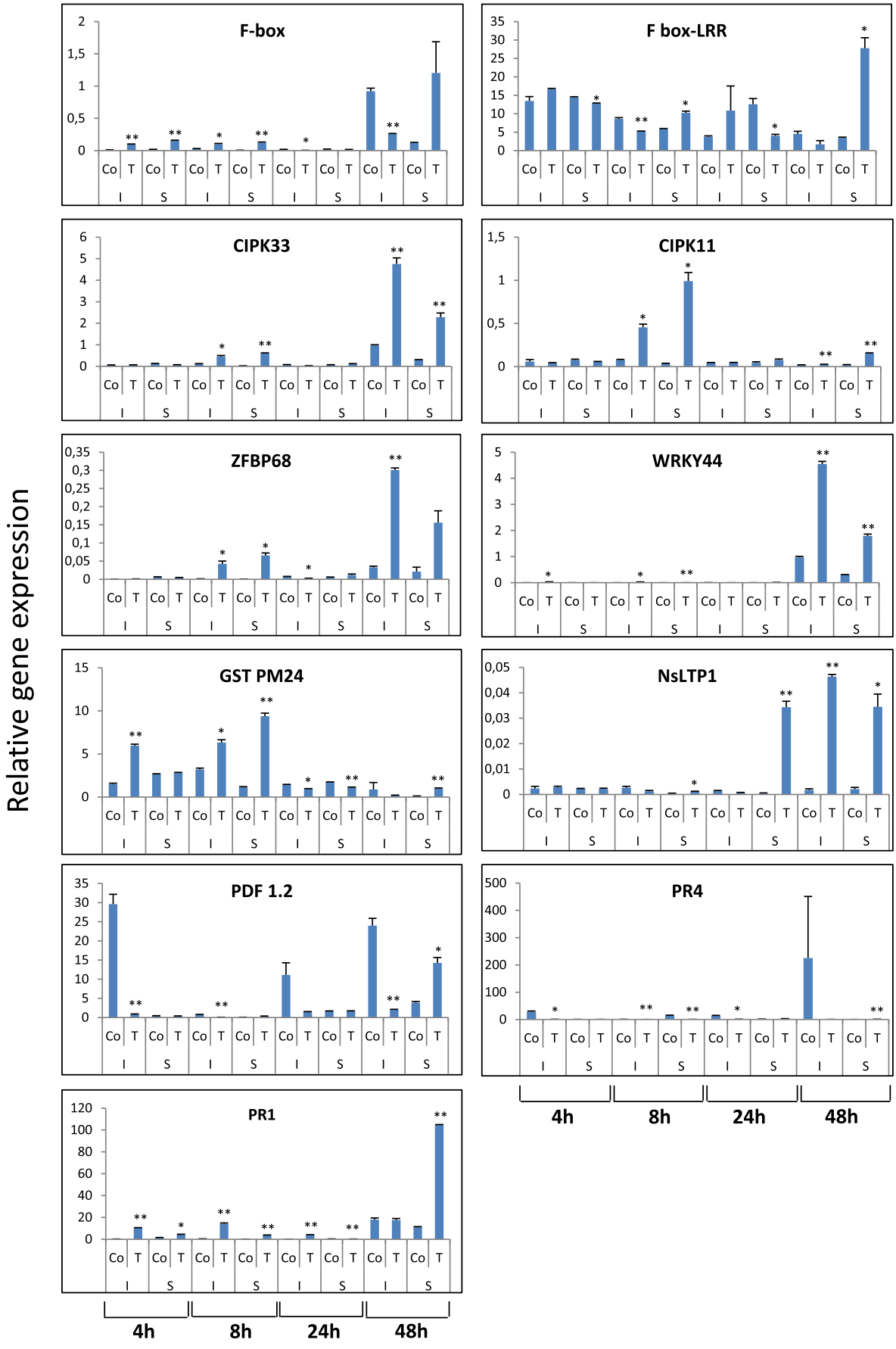
3.4. ROS detection in LOS-treated Arabidopsis plants

In order to highlight the presence of ROS in Arabidopsis plants treated with *Xcc* LOS, five-week-old Arabidopsis leaves were incubated with 2,7 DCFH2-DA to reveal the presence of H_2O_2 triggered by LOS treatment. This compound diffuses through the plasma membrane into the cytoplasm where is deacetylated by intracellular esterase and after oxidized by H_2O_2 producing the green fluorescent dye DCF. Systemic leaves of plants were also incubated with 2,7 DCFH2-DA in order to evaluate the presence of H_2O_2 in plant tissues far from the site of LOS infiltration. LOS-infiltrated and systemic leaves harvested 4 and 24 h after LOS infiltration were then immediately incubated with 2,7 DCFH2-DA. Leaves incubated with buffer only were used as a negative control. Fluorescence was analyzed with a confocal microscope using a 488 filter to evaluate the green fluorescence of the probe and a 563 nm filter (red) to detect the auto-fluorescence of the chlorophyll. The experiment was repeated three times independently and representative results are shown in Fig. 2. Panel A shows the results obtained at 4 h after LOS treatment. Control plants treated with buffer showed only the red fluorescence due to chlorophyll, while LOS-treated plants showed very strong green fluorescence due to 2,7 DCF highlighting the presence of high levels of H_2O_2. As expected, the same samples treated with buffer showed only red fluorescence due to the absence of 2,7 DCFH2-DA. Systemic leaves harvested from LOS-treated plants showed a less pronounced levels of green fluorescence, which is absent in systemic leaves harvested from control plants infiltrated with water. As above, samples treated with buffer showed the red fluorescence only. Panel B shows representative results obtained at 24 h after LOS treatment. Analogously to the above presented data, control plants treated with 2,7 DCFH2-DA and with buffer showed only the auto fluorescence of the chlorophyll whereas LOS-treated and systemic leaves from LOS-treated plants showed a less pronounced fluorescence in the green channel with respect to the results obtained at 4 h after LOS treatment.

3.5. Gene expression studies on markers of LOS-triggered oxidative burst

In order to highlight the ROS-signaling pathway in Arabidopsis, we performed gene expression studies on markers of oxidative stress on the same samples harvested for transcript profiling of genes coding LOS interacting proteins. Three biological replicates and three technical replicates of RT-PCR experiments have been carried out.

We selected several genes involved in the homeostasis of ROS with different function within plant cell. Among them, are three different isoforms of phospholipase, namely phospholipase C1 (PLC), phospholipase Dα (PLDα) and Dδ (PLDδ). Phospholipases are involved in the production of inositol triphosphate and diacylglycerol, the latter being the precursor of phosphatidic acid that in turn leads to the increasing of intracellular ROS (Testerink and Munnik, 2005). As shown in Fig. 3, *PLC* and *PLDδ* were induced both in infiltrated or systemic leaves harvested 48 h upon treatment, whereas *PLDα* was not influenced by treatment in all samples analyzed. The same expression pattern of *PLC* and *PLDδ* was found for the kinase *PDK1*, involved in ROS-mediated signal transduction pathway (Mittler et al., 2004; Matsui et al., 2010).


Relative gene expression
F-box
F box-LRR
CIPK33
CIPK11
ZFBP68
WRKY44
GST PM24
NsLTP1
PDF 1.2
PR4
PR1
Co T Co T Co T Co T Co T Co T Co T Co T
I S I S I S I S
4h 8h 24h 48h

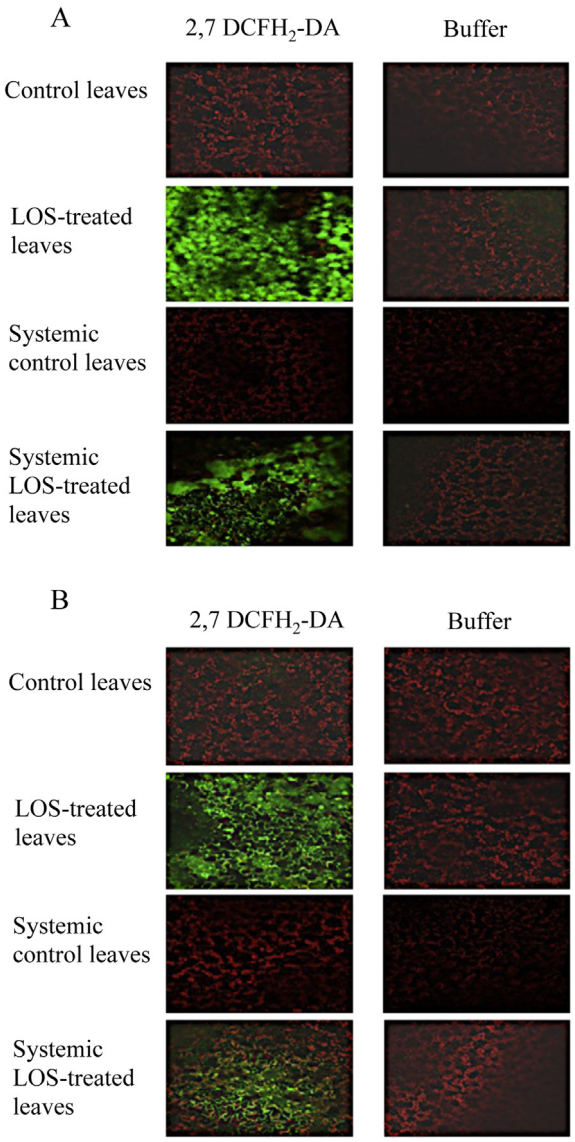


Fig. 2. Detection of H_2O_2 in Arabidopsis leaves using 2,7 $DCFH_2$-DA or buffer (negative technical control). Panel A: Detection of H_2O_2 4 h after LOS infiltration. Panel B: Detection of H_2O_2 24 h after LOS infiltration. Fluorescence was observed under a LSM 710 confocal microscope with Planneofluoar × 40/1.30 objective. Two laser excitations lines were used (i.e., 488 for probe detection and 563 nm for chlorophyll autofluorescence).

Several studies suggested the involvement of phosphatases in regulating ROS homeostasis (Bartels et al., 2009). In our studies, we selected the phosphatase PTP1 (Protein Tyrosin Phospatase1), whose expression is altered in response to several stress. In our experimental conditions, *PTP1* level was not influenced by LOS treatment. *NPR1* (inhibitor of PR1) involved in SAR onset was weakly induced both in infiltrated or systemic leaves harvested 4 and 8 h upon treatment; at later time points it was induced only in infiltrated leaves. We also investigated two transcription factors (TFs) susceptible to heat shock, namely *HSFA2* and *HSFA21*, induced in response to H_2O_2. *HSFA2* was induced by LOS treatment in systemic leaves after 4 h from infiltration and was repressed both in treated or systemic leaves harvested 24 and 48 h upon treatment, whereas *HSFA21* was repressed only in systemic leaves harvested 4 h upon treatment, being unaffected in all other samples.

It has been demonstrated that the kinases OXI1 as well as MAPK3 and MAPK6 play a central role in ROS-mediated signal transduction pathway (Matsui et al., 2010). In our study, *OXI1* was induced in both infiltrated or systemic leaves in all samples collected at early and late time points, whereas *MAPK6* was induced in infiltrated and systemic leaves only within 24 h. In our experimental conditions, *MAPK3* was totally unaffected.

In this study we have also analyzed some TFs known to be overexpressed during oxidative burst, namely WRKY25, WRKY6, WRKY33 and Zat12. As shown in Fig. 3, *WRKY25* and *WRKY33* were not sensitive to the treatment, whereas *WRKY6* was induced only in infiltrated leaves harvested at 4 and 8 h upon treatment. On the contrary, *ZAT12* was induced in infiltrated leaves at early and late time points selected, but in systemic leaves only at later time points.

Two more genes belonging to the family of NADPH oxidase, namely *AtrbohD* (Arabidopsis thaliana respiratory burst oxidase homolog D) and *AtrbohF* that are involved in the generation of oxidative burst (Ma et al., 2011), were investigated. In our experimental conditions, *AtrbohD* was induced in infiltrated leaves collected at all time points upon treatment, whereas it was induced in systemic leaves only in samples harvested 8 and 24 h upon treatment. On the contrary, *AtrbohF* expression was found to be insensitive in all conditions.

Finally, we analyzed some ROS scavenging/detoxifying enzymes such as ascorbate peroxidase (APX1, E.C. 1.11.1.11), catalase (CAT3, E.C. 1.11.1.6) and superoxide dismutase (SOD, E.C. 1.15.1.1). While *APX1* and *CAT3* seemed to be inhibited by the treatment with LOS, *SOD* was induced at the early time selected, but was insensitive at later time points. Ubiquitin (*UBI 10*) was used to normalize the quantity of RNA used for PCR.

4. Discussion

4.1. LOS-triggered innate immunity

It is well known that MAMPs/PAMPs trigger innate immunity in various vertebrate and invertebrate organisms acting as general elicitors of defense response. In particular, perception of PAMPs is frequently mediated by Toll-like receptors containing extracellular leucine-rich repeats (LRRs) in insect and vertebrates (O'Neill, 2002). Similar recognition system has been reported also in plants, highlighting remarkable conservation between distant organisms (Zipfel and Robatzek, 2010 and references therein). Like animals, plants have acquired the ability to recognize PAMPs that are characteristic of microbial organisms such as LPS, LOS, flagellin, which are not found in potential host plants.

LOS is the predominant PAMP in some strains of *Xcc*, the causative agent of black rot, able to infect both cruciferous crops as well as non-crop crucifers such as Arabidopsis. Here we report the

Fig. 1. qPCR analysis of genes coding for proteins identified by mass spectrometry. I: LOS-infiltrated leaves; S: systemic leaves; Co: control; T: LOS-treated plants. *F box-LRR* (At1g66290) and *Fbox* (At5g03100): F-box genes involved in pathogen recognition; *CIPK33* (At1g50700) and *CIPK11* (At2g30360): kinase-coding genes involved in cellular signal transduction pathways; *ZFBP68* (At5g662709): gene coding RNA binding protein; *WRKY44* (At2g37260): gene coding transcription factor belonging to the WRKY family; *GST PM24* (At4g02520): gene coding glutathione S-transferase, responsive to biotic and abiotic stresses; *NsLTP1* (At2g38540): gene coding Lipid Transfer Protein, responsive to biotic and abiotic stresses; *PDF 1.2* (At5G44420): plant defensin, responsive to jasmonic acid and ethylene signaling; *PR4* (At3G04720) and *PR1* (At2G14610), responsive to salicylic acid signaling. The transcript profiling is representative of three independent gene expression studies. Bars represent means ± SD. Asterisks indicate statistically significant differences compared to control plants (Student's *t* test, $^{*} = P < 0.05$; $^{**} = P < 0.01$).

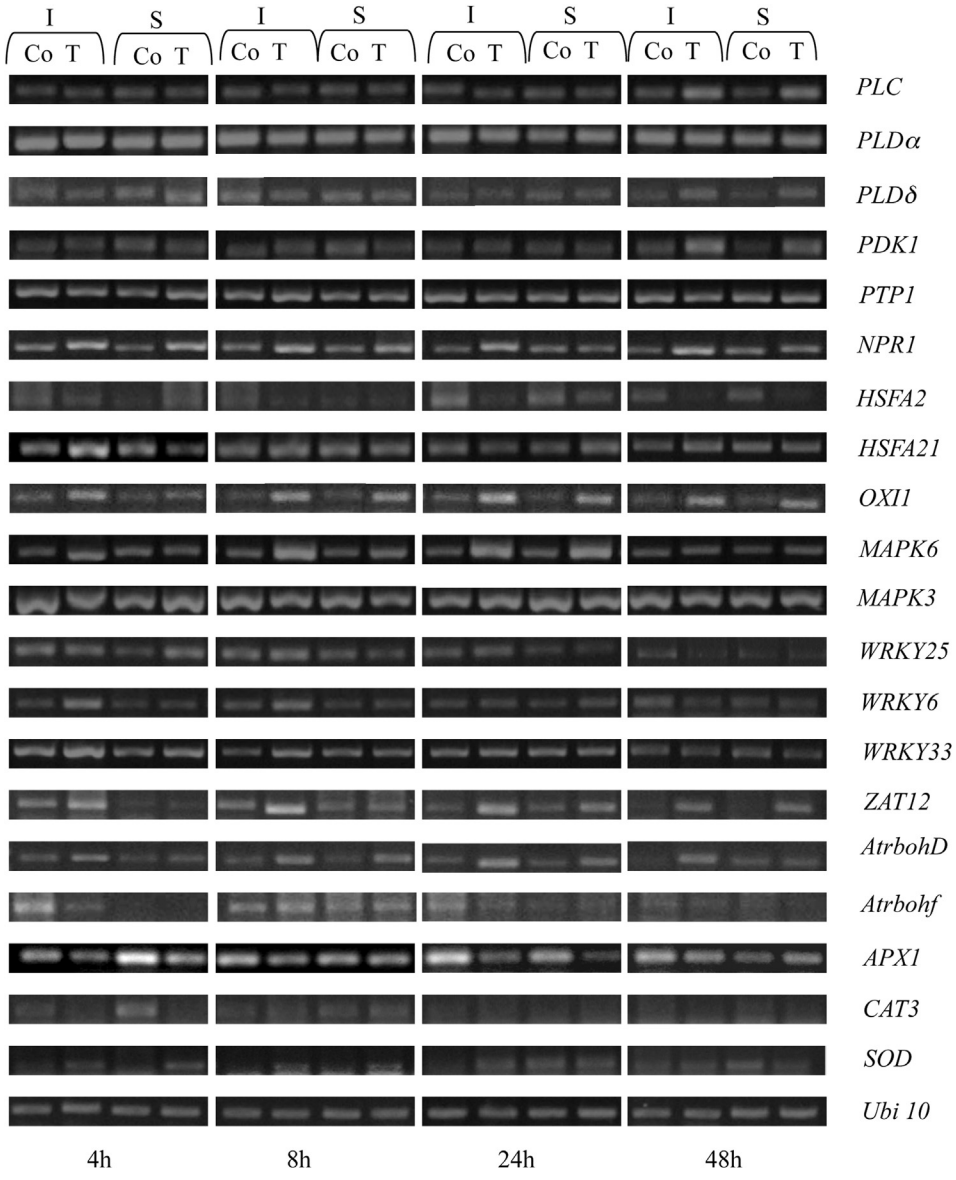


Fig. 3. Semi-quantitative RT-PCR analysis of marker genes of oxidative stress. I: LOS-infiltrated leaves; S: systemic leaves; Co: control; T: LOS-treated plants. *PLC* (At5g58670): Phospholipase C1; *PLDα* (At3g15730) and *PLDδ* (At4g35790): Phospholipase Dα and Dδ; *PDK1* (At5g04510): kinase; *PTP1* (At1g71860): phosphatase; *NPR1* (At1g64280): inhibitor of Pathogenesis Related 1; *HSFA2* (At2g26150) and *HSFA21* (At4g18880): transcription factors susceptible of heat shock; *OXI1* (At3g25250), *MAPK6* (At2g43790) and *MAPK3* (At3g45640): kinases; *WRKY25* (At2g30250), *WRKY6* (At1g62300), *WRKY33* (At1g62300) and *Zat12* (At5g59820): transcription factors; *AtrbohD* (At5g47910) and *AtrbohF* (At1g64060): NADPH oxidase involved in the generation of oxidative burst; *APX* (At1g07890) ascorbate peroxidase, *SOD* (At1g12520) superosside dismutase, *CAT3* (At1g20620) catalase3: ROS scavenging enzymes; *Ubi 10* (At4g05320): standard ubiquitin gene. The transcript profiling is representative of three independent gene expression studies.

identification of specific *Xcc* LOS protein interactors in Arabidopsis exploiting functional proteomic approach followed by mass spectrometry as well as transcriptomic analyses. By using "ad hoc" modified LOS bait, we designed affinity capture experiments by challenging the immobilized LOS with the entire protein extract from Arabidopsis leaves. Eight proteins specifically retained by the bait were identified. Some of these interactors could be assigned to putative signal transduction pathways triggered by treatment of Arabidopsis with the *Xcc* LOS. Among the putative interactors is Fbox-LRR, an F-box protein that could be involved in the first steps of elicitor recognition event. F-box proteins contain a conserved F-box domain (35-60 amino acids) that mediates protein-protein interactions in a variety of processes, such as polyubiquitination, transcription elongation, centromere binding, translation repression as well as disease resistance and hormone signaling (Vierstra, 2003). The C-terminal part of F-box proteins often contains LRRs and it has been shown to specifically bind to substrates (Kobe and Kajava, 2001). However, the majority of F-box proteins have unknown association motifs and the functions of most of these proteins have not been defined yet. Nevertheless, recent researches have shown a major involvement of F-box proteins in the proteasome-mediated degradation of proteins. Moreover, several reports on plant hormone signaling pathways showed that the ubiquitin proteasome system is a central regulatory mechanism in the signal transduction pathways of different plant hormones (Devoto et al., 2003). One of the best-characterized F-box protein in plants is TIR1 (Transport Inhibitor Response 1) that functions as an auxin receptor (Dharmasiri et al., 2005). Following auxin binding, TIR1 interacts with the repressor proteins Aux/IAA to promote their ubiquitination and degradation thereby releasing the auxin

response factors (ARFs) from their repressive effects. Other F-box-containing protein receptors are GID2 and SLY1, described in rice and Arabidopsis respectively, both involved in gibberellin signaling (Itoh et al., 2003) and the F-box protein COI1 involved in the JA signal response in Arabidopsis (Feys et al., 1994).

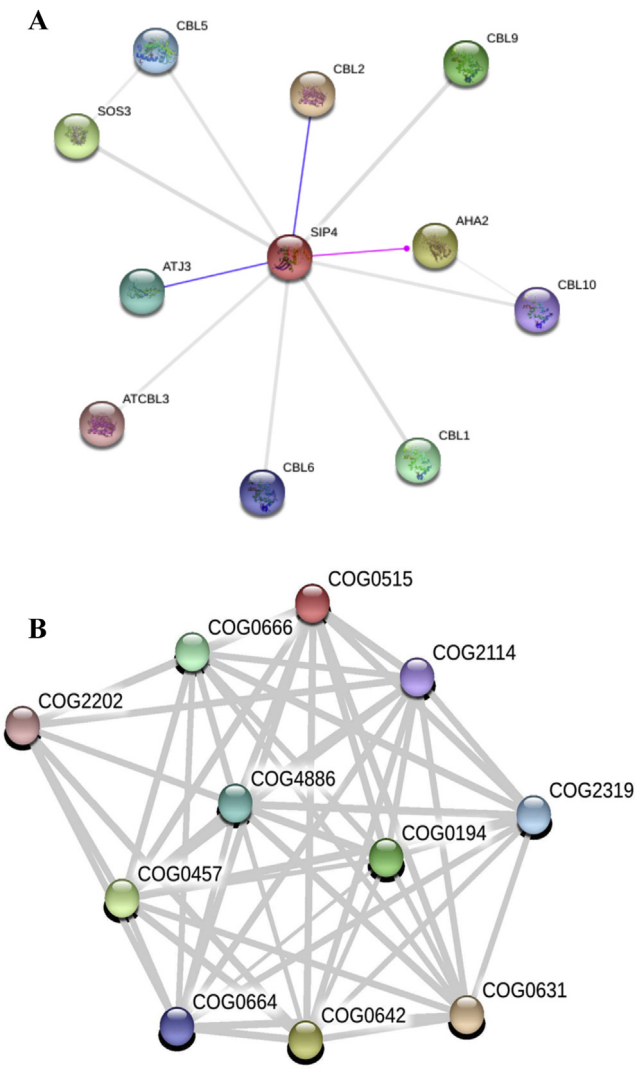


Fig. 4. A: Arabidopsis predicted functional partners of CIPK11 highlighted by the STRING program (http://string-db.org/). Kinase CIPK11 is termed SIP4 (SOS3-INTERACTING PROTEIN 4) by the program as it encodes a SOS2-like protein kinase that is a member of the CBL-interacting protein kinase family. Putative partners are: CBL2: Calcineurin B -like 2, calcium ion binding, member of AtCBLs (Calcineurin B-like Calcium Sensor Proteins); CBL1: Calcineurin B-like protein 1, calcium ion binding, member of AtCBLs; AHA2: ATPase/hydrogen-exporting ATPase; ATJ3: involved in protein binding, homologous to the co-chaperon DNAJ protein from *E. coli*; SOS3: Salt Overly Sensitive 3, calcium ion binding/calcium-dependent protein serine/threonine phosphatase; ATCBL3: Arabidopsis thaliana Calcineurin B-like 3, calcium ion binding, member of AtCBLs; CBL6: Calcineurin B-like protein 6, calcium ion binding, calcium sensor protein; CBL5: Calcineurin B-like protein 5, calcium ion binding, encodes calcineurin B-like protein 5; CBL9: Calcineurin B-like protein 9, calcium ion binding, member of AtCBLs; CBL10: Calcineurin B-like protein 10, calcium ion binding, member of AtCBLs. B: Orthologous predicted functional partners of CIPK11, termed COG0515 by the STRING program. Putative partners are: COG0631: Serine/threonine protein phosphatase; COG0642: Signal transduction histidine kinase; COG0457: FOG-TPR repeat; COG0194: Guanylate kinase; COG0666: FOG-Ankyrin repeat; COG4886: Leucine-rich repeat (LRR) protein; COG2319: FOG-WD40 repeat; COG0664:cAMP-binding proteins; COG2114: Adenylate cyclase, family 3 (some proteins contain HAMP domain); COG2202: FOG-PAS/PAC domain.

In analogy with TIR1, Fbox-LRR might then be involved in the recognition of *Xcc* LOS by activating a proteasome-mediate destruction of repressor proteins, which negatively regulate target genes participating to plant defense. Expression analysis of *Fbox-LRR* gene supports its active role in a MAMPs/PAMPs-mediated pathway as this gene is induced in systemic leaves harvested in the late stage after treatment with LOS.

Proteomic experiments identified the CIPK11 (At2g30360) kinase as a specific *Xcc* LOS interactor. Gene expression analysis demonstrated that CIPK11 was induced in infiltrated and systemic leaves 8 h after LOS treatment. CIPK11 belongs to the SOS2-like (Salt Overly Sensitive2) protein kinase, a member of the CBL (Calcineurin B-Like)-interacting protein kinase family (CIPK). These kinases regulate different aspects of salt tolerance by interacting with distinct CBL sensors (Beom-Gi et al., 2007) transducing the signal in a calcium-mediated pathway. In order to unravel the intricate pattern of interaction of CIPK11 in Arabidopsis, we used the freely available STRING program (http://string-db.org/). As shown in Fig. 4A, ten putative functional partners of CIPK11, SIP4 in the figure, were identified by the program, seven of which were CBL proteins. Besides CBL proteins, one cation-transporting ATPase (AHA2), one putative molecular chaperon homologous to the heat shock protein DnaJ from *Escherichia coli* (ATj3) and SOS3 (SALT OVERLY SENSITIVE 3), a calcium ion binding/calcium-dependent protein serine/threonine phosphatase were found as predictive interactors in Arabidopsis. We also asked to the program to find out all putative interactors of orthologous CIPK11 and the results are shown in Fig. 4B. Among the most significant predicted partners, the software indicated the leucine-rich repeat protein COG4886. This finding supports our experimental identification of the leucin-rich repeat protein Fbox-LRR in the LOS recognition complex. On this basis we can speculate that upon recognition between LOS and

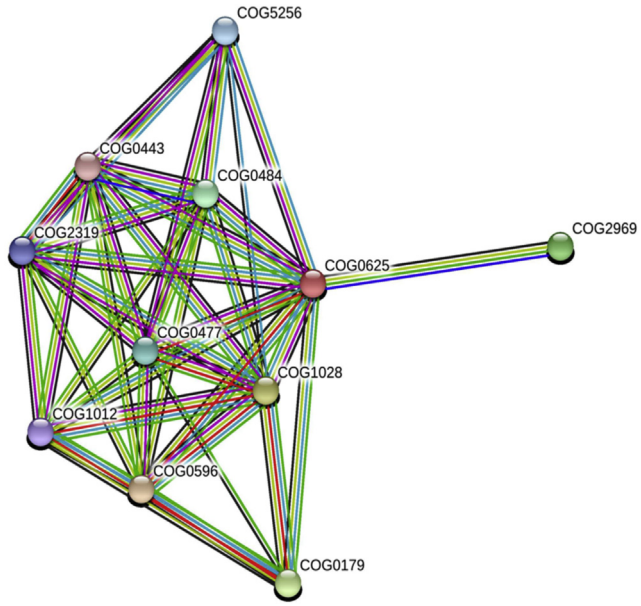


Fig. 5. Orthologous predicted functional partners of GST PM24 highlighted by the STRING program. GST PM24 is termed COG0625 by the program. Putative partners are: COG0596: predicted hydrolases or acyltransferases (alpha/beta hydrolase superfamily); COG1028: dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases); COG0179: 2-keto-4-pentenoate hydratase/2-oxohepta-3-ene-1,7-dioic acid hydratase (catechol pathway); COG2969: stringent starvation protein B; COG0484: DnaJ-class molecular chaperone with C-terminal Zn finger domain; COG0477: permeases of the major facilitator superfamily; COG5256: translation elongation factor EF-1alpha (GTPase); COG2319: FOG- WD40 repeat; COG1012: NAD-dependent aldehyde dehydrogenases; COG0443: molecular chaperone.

Fbox-LRR, a signal is transmitted to the CIPK11 protein, which in turn could phosphorylate target proteins. Other relevant predicted partners of orthologous CIPK11 are proteins with WD40 repeats (COG2319), belonging to the transducing protein family, which have been found in a number of eukaryotic species that cover a wide variety of functions including adaptor/regulatory modules in signal transduction.

Glutathione S-transferase (GST) PM24 (At4g02520) was also identified by mass spectrometry. GSTs are involved in many biological process as auxin-mediated signaling pathways, defense response to bacteria, toxin catabolic processes, and more in general in response to several biotic and abiotic stresses (Mullineaux and Rausch, 2005). Expression of *AtGST PM24* (also known as *AtGSTF2*) was found to be SA-dependent, showing a very early induction pattern as compared with the expression of the well-known SA marker PR1 (Lieberherr et al., 2003). When the STRING program was used to find putative partners of *AtGST PM24* orthologous, we found interaction with the DnaJ chaperone (COG0484) and the WD40-repeats containing protein (COG2319) (Fig. 5) that had previously been identified as putative CIPK11 interactors. Whether AtGST PM24 is a real functional partner of CIPK11 through its binding with DnaJ or WD40 proteins is still matter of study.

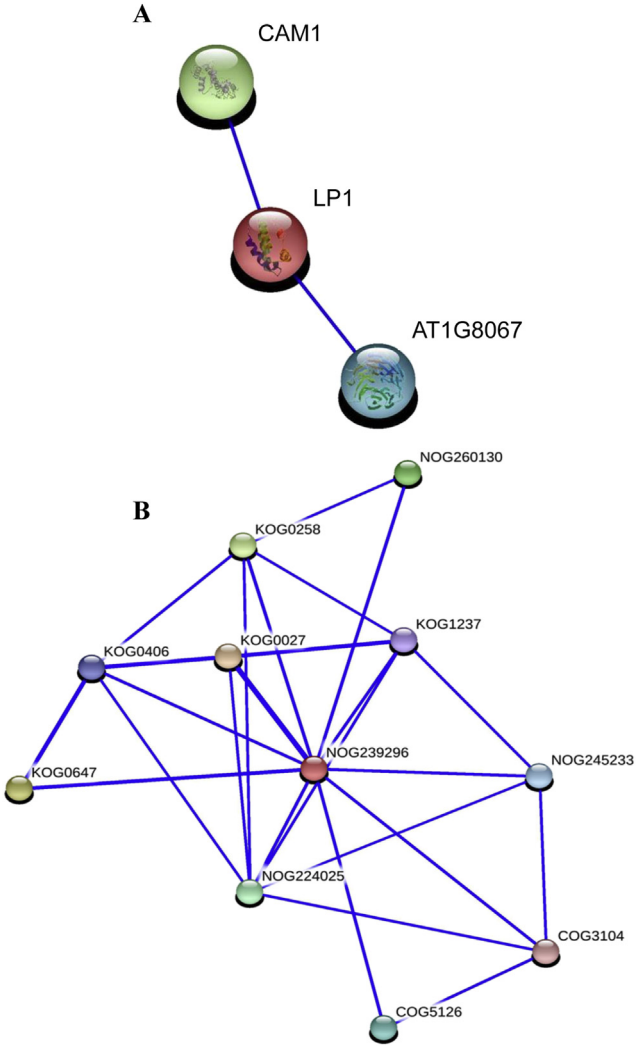


Fig. 6. A: Arabidopsis predicted functional partners of LTP highlighted by the STRING program LTP is termed LP1 by the program. Putative partner is CAM1: calmodulin 1, calcium ion binding. B: Orthologous predicted functional partners of LTP highlighted by the STRING program. LTP is termed NOG239296 by the program. Putative partners are: KOG0027: calmodulin and related proteins (EF-Hand superfamily); KOG0647: mRNA export protein (contains WD40 repeats); KOG0258: alanine aminotransferase; NOG260130: photosystem II protein; NOG224025: non supervised orthologous group; COG5126: Ca2+-binding protein (EF-Hand superfamily); NOG245233: nutrient reservoir protein; KOG0406: glutathione S-transferase; KOG1237: H+/oligopeptide symporter; COG3104: dipeptide/tripeptide permease.

In order to explain the presence of non-specific lipid transfer proteins (NsLTP1) between the putative interactors highlighted by the affinity procedure described in this study, we searched also for its putative partners using the STRING program. NsLTP1 (termed LP1 by the program) was found to interact with calmodulin 1 and a protein belonging to the transducing family protein containing WD-40 repeat (AT1G8067) (Fig. 6A). Moreover, besides other putative interactors, search for orthologous partners of NsLTP1 (termed NOG239296 by the STRING program) highlighted interaction with glutathione S-transferase (KOG0647) and an mRNA export protein containing WD40 repeats (Fig. 6B). On the basis of these evidences, we can speculate that NsLTP1 might be correlated to CIPK11 through its binding with WD40 proteins or through interaction with AtGST PM24, which in turn is able to recognize both WD40 proteins and DnaJ.

Finally, we investigated the expression profile of *WRKY44* that encodes for the transcription factor WRKY44 (At2g37260) identified in the affinity capture experiment. WRKY TFs recognize the W-box sequence, (T) (T)TGAC(C/T), and are involved in plant defense (Pandey and Somssich, 2009). *WRKY44* was found to be induced in both infiltrated and systemic leaves analyzed 48 h upon treatment. This result clearly indicates that following LOS challenge a signal transduction pathway is activated that uses *WRKY44* to modulate the expression of genes containing W-box in their promoters.

More studies are in progress to validate the hypothesized signal cascade through the use of specifically designed mutants. To date, just few reports are available on bibliographic databases about the phenotype of these mutant lines. Between the genes found to be putatively involved in LOS transduction pathway, mutant Arabidopsis lines have been produced of calcineurin B-like interacting protein kinase (CIPK) and WRKY transcription factors. CIPKs are found to be differentially regulated by a variety of conditions as demonstrated by their expression pattern under cold, drought, salt, ABA, low K^+ ion level and various developmental stages (D'Angelo et al., 2006; Tripathi et al., 2009). Studies based on mutant analysis under different stress conditions have clearly shown the involvement of different CBL-CIPK partners in sensing specific or multiple stress conditions by modulating different downstream targets such as potassium channels (AKT1), sodium efflux pumps (Na+/H + -antiporter) and transporters (SOS1, CHL1) (Lee et al., 2007; Xu et al., 2006). Interestingly, CDPK (calcium-dependent protein kinase) tobacco transgenic lines have shown that they are essential for mediating the Cf-9/Avr9-induced hypersensitive response in plants (Romeis et al., 2001). Difference between CDPKs and CIPKs is that the first directly interact with Ca^{2+}, whereas CIPKs are sensitive to Ca^{2+} stimulus by interaction with calcineurin B-like (CLB) proteins. Since CDPKs and CIPKs are often reported to be induced by the same stress conditions, we could hypothesize that also CIPKs can act downstream PAMPs recognition activating signal cascade, as described in present paper. Several mutant WRKY Arabidopsis lines have been also produced, demonstrating that WRKY factors are crucial regulators of the defense transcriptome and disease resistance in response to both biotic and abiotic stress (Li et al., 2006; Zheng et al., 2006).

Finally, it is well known that MAMPs/PAMPs response triggers activation of several intracellular signaling pathways characterized by the presence of common nodal regulators. In order to investigate whether LOS signaling involves SA and/or JA pathways, the expression profile of *PR1* as well as *PR4* and *PDF 1.2*, well known

marker genes of the two pathways respectively, was determined. The results clearly showed that *PR1* is induced after LOS treatment in both treated and systemic leaves, whereas both *PR4* and *PDF 1.2* are repressed in treated as well as systemic leaves, thus indicating that LOS treatment activates an SA-dependent signal transduction pathway.

4.2. LOS-induced oxidative pathway

Reactive oxygen species (ROS) play a central role in defense against several pathogens through a large network comprising more than 152 genes in Arabidopsis (Mittler et al., 2004). Among them are specific receptors that are still unknown at the present, different kinases and phosphatases and transcription factors, which sense redox changing within the cells (Mittler et al., 2004). It has been suggested that ROS species could activate at least three different pathways. One of them is activated following interaction with receptors and results in the direct activation of phospholipase C and D leading to the synthesis of phosphatidic acid (PA), which in turn activates different kinases that act in a signaling pathway including sequentially PDK1, OXI1 and MAPK3/6. Activation of MAP kinase signaling leads to induction or activation of different transcription factors that regulate the ROS-scavenging and ROS-producing pathways. The ROS-scavenging pathway leads to the synthesis of enzymes like SOD, CAT and APX, resulting in localized or general defense response. The ROS-producing pathway might be activated by low levels of ROS and results in enhanced ROS signals in specific cellular location via the activity of NADPH oxidases. The balance between ROS-scavenging and ROS-producing pathways will determine the intensity and localization of ROS signals in specific cellular locations or the entire cell. (Mittler et al., 2004). The second pathway involves the activation of a Ca2+ signaling, which leads to the direct activation of OXI1. The third pathway triggered by ROS leads to the inhibition of phosphatases that might interrupt the above-mentioned pathways by dephosphorylation of kinases.

In this paper, we report the activation of a ROS signaling mediated by *Xcc* LOS. We proved evidence that *PLC* and *PLDδ* are induced in Arabidopsis leaves treated with LOS, whereas *PLDα* are unaffected. Moreover, kinases like PDK1, OXI1 and MAPK6 were found to be overexpressed following LOS treatment both in infiltrated or systemic leaves. Analogously, *WRKY6* and *Zat12* were induced, probably acting in a ROS-scavenging pathway according to the enhanced expression of SOD. In our experimental condition, *AtrbohD* (NADPH oxidase) was found to be induced in either infiltrated or systemic leaves according to the activation of ROS-producing pathway. It is well known from the literature that phosphatase PTP1 is able to inactivate *in vitro* MAPK6 (Gupta and Luan, 2003). In this study, phosphatase *PTP1* was found to be unaffected by LOS treatment, suggesting that in these conditions both ROS-scavenging and ROS-producing pathways were active.

In order to confirm that treatment of Arabidopsis leaves with LOS is responsible for an increased production of ROS and to validate the results obtained with the transcriptomic analysis, we carried out the detection of H_2O_2 in Arabidopsis leaves following LOS treatment. Confocal microscopy confirmed the presence of high levels of this reactive oxygen species in both infiltrated or systemic leaves with respect to the control.

On the basis of the results presented in this paper, we can conclude that LOS treatment of Arabidopsis plants triggers several signal transduction pathways leading to plant defense. In particular, we can postulate that following LOS treatment in Arabidopsis a signal cascade pathway is activated that include at least the F box-LRR receptor, the CIPK11 kinase and the transcription factor WRKY44. Even though specific substrates of CIPK11 have not been identified, we believe that GST PM24 plays a role in triggering plant defense through the indirect interaction with CIPK11 mediated by DnaJ and/or WD40 repeats-containing proteins. To summarize, we proved evidence that LOS triggers a signal transduction pathway that uses nodal regulators that are in common with the SA-mediated pathway and also an oxidative burst leading to the activation of ROS-scavenging and ROS-producing pathways. Finally, the hypothesis that LOS might be able to stimulate SA production is strengthened from our results on HSFA2, APX1 and CAT3 repression, since previous study has already demonstrated a negative effect of SA on the expression of above genes (Chen et al., 2007).

Contribution

Experimental design, plant treatment and transcriptomic analysis (S.P.); purification of *X. campestris* LOS (A.M.); experimental design of the affinity procedure (A.A. and P.P.); execution of affinity procedure (C.G.); mass spectrometry analysis (A.A., P.P.); detection of H_2O_2 in Arabidopsis plants (S.P. and L.B.); putative interaction partners search (C. C., L.B. and C. C.); supervision of the experimental procedure and paper writing (C. Caruso).

Acknowledgments

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References

Bedini, E., De Castro, C., Erbs, G., Mangoni, L., Dow, J.M., Newman, M.-A., Parrilli, M., Unverzagt, C., 2005. Structure-dependent modulation of a pathogen response in plants by synthetic O-antigen polysaccharides. J. Am. Chem. Soc. 127, 2414–2416.

Bartels, S., Anderson, J.C., Gonzàles Besteiro, M.A., Carreri, A., Hirt, H., Buchala, A., Métraux, J.P., Peck, C.S., Ulm, R., 2009. MAP kinase phosphatase1 and protein tyrosine phosphatase1 are repressors of salicylic acid synthesis and SNC1-mediated responses in Arabidopsis. Plant Cell. 21, 2884–2897.

Chen, J., Zhu, C., Li, L., Sun, Z., Pan, X., 2007. Effects of exogenous salicylic acid on growth and H2O2-.metabolizing enzymes in rice seedling under lead stress. J. Environ. Sci. 19, 47–49.

D'Angelo, C., Weinl, S., Batistic, O., Pandey, G.K., Cheong, Y.H., Schültke, S., Albrecht, V., Ehlert, B., Schulz, B., Harter, K., Luan, S., Bock, R., Kudla, J., 2006. Alternative complex formation of the Ca-regulated protein kinase CIPK1 controls abscisic acid-dependent and independent stress responses in Arabidopsis. Plant J. 48, 857–872.

De Castro, C., Parrilli, M., Holst, O., Molinaro, A., 2010. Microbe-associated molecular patterns in innate immunity: extraction and chemical analysis of gram-negative bacterial lipopolysaccharides. Meth. Enzymol. 480, 89–115.

Desaki, Y., Miya, A., Venkatesh, B., Tsuyumu, S., Yamane, H., Kaku, H., Minami, E., Shibuya, N., 2006. Bacterial lipopolysaccharides induce defense responses associated with programmed cell death in rice cells. Plant Cell. Physiol. 47, 1530–1540.

Devoto, A., Muskett, P.R., Shirasu, K., 2003. Role of ubiquitination in the regulation of plant defence against pathogens. Curr. Opin. Plant Biol. 6, 307–311.

Dharmasiri, N., Dharmasiri, S., Estelle, M., 2005. The F-box protein TIR1 is an auxin receptor. Nature 435, 441–445.

Erbs, G., Newman, M., 2011. In: Knirel, Y.A., Valvano, M.A. (Eds.), Bacterial Lipopolysaccharides: Structure, Chemical Synthesis, Biogenesis and Interaction with Host Cells. Springer, pp. 417–433.

Feys, B., Benedetti, C.E., Penfold, C.N., Turner, J.G., 1994. *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell. 6, 751–759.

Galanos, C., Lüderitz, O., Westphal, O., 1969. A new method for the extraction of R lipopolysaccharides. Eur. J. Biochem. 9, 245–249.

Giangrande, C., Colarusso, L., Lanzetta, R., Molinaro, A., Pucci, P., Amoresano, A., 2013. Innate immunity probed by lipopolysaccharides affinity strategy and proteomics. Anal. Bioanal. Chem. 405 (2–3), 775–784.

Gupta, R., Luan, S., 2003. Redox control of protein tyrosine phosphatases and mitogen-activated protein kinases in plants. Plant Physiol. 132, 1149–1152.

Itoh, H., Matsuoka, M., Steber, C.M., 2003. A role for the ubiquitin-26S proteasome pathway in gibberellin signaling. Trends Plant Sci. 8, 135–142.

Kobe, B., Kajava, A.V., 2001. The leucine-rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. 11, 725–732.

Lee, S.C., Lan, W.Z., Kim, B.G., Li, L., Cheong, Y.H., Pandey, G.K., Lu, G., Buchanan, B.B., Luan, S., 2007. A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc. Natl. Acad. Sci. U S A 104, 15959–15964.

Li, J., Brader, G., Kariola, T., Tapio Palva, E., 2006. WRKY70 modulates the selection of signaling pathways in plant defense. Plant J. 46, 477–491.
Lieberherr, D., Wagner, U., Dubuis, P.-H., Metraux, J.-P., Mauch, F., 2003. The Rapid Induction of glutathione S-Transferases AtGSTF2 and AtGSTF6 by avirulent *Pseudomonas syringae* is the result of combined salicylic acid and ethylene signaling. Plant Cell. Physiol. 44 (7), 750–757.
Livaja, M., Zeidler, D., Von Rad, U., Durner, J., 2008. Transcriptional responses of *Arabidopsis* to the bacteria-derived PAMPs hairpin and lipopolysaccharide. Immunobiology 213 (3–4), 161–171.
Ma, L., Zhang, H., Sun, L., Jiao, Y., Zhang, G., Miao, C., Hao, F., 2011. NADPH oxidase AtrbohD and AtrbohF function in ROS-dependent regulation of Na^+/K^+ homeostasis in Arabidopsis under salt stress. J. Exp. Bot. 63, 305–317.
Matsui, H., Miyao, A., Takahashi, A., Hirochika, H., 2010. Pdk1 kinase regulates basal disease resistance through the OsOxi1–OsPti1a phosphorylation cascade in rice. Plant Cell. Physiol. 51, 2082–2091.
Mittler, R., Vanderauwera, S., Gollery, M., Van Breusegem, F., 2004. Reactive oxygen gene network of plants. Trends Plant Sci. 9, 490–498.
Mullineaux, P.M., Rausch, T., 2005. Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. Photosynth. Res. 86, 459–474.
O'Neill, L.A., 2002. Toll-like receptor signal transduction and the tailoring of innate immunity: a role for Mal? Trends Immunol. 23, 296–300.
Pandey, S.P., Somssich, I.E., 2009. The role of WRKY transcription factors in plant immunity. Plant Physiol. 150, 1648–1655.
Pieterse, C.M.J., Leon-Reyes, A., Van der Ent, S., Van Wees, S.C.M., 2009. Networking by small-molecule hormones in plant immunity. Nat. Chem. Biol. 5, 308–316.
Romeis, T., Ludwig, A.A., Martin, R., Jones, J.D.G., 2001. Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J. 20, 5556–5567.
Silipo, A., Molinaro, A., Sturiale, L., Dow, J.M., Erbs, G., Lanzetta, R., Newman, M.A., Parrilli, M., 2005. The elicitation of plant innate immunity by lipooligosaccharide of *Xanthomonas campestris*. J. Biol. Chem. 280, 33660–33668.
Silipo, A., Erbs, G., Shinya, T., Dow, J.M., Parrilli, M., Lanzetta, R., Shibuya, N., Newman, M.-A., Molinaro, A., 2010. Glyco-conjugates as elicitors or suppressors of plant innate immunity. Glycobiolgy 20, 406–419.
Testernik, C., Munnik, T., 2005. Phosphatidic acid: a multifunctional stress signaling lipid in plants. Trends Plant Sci. 10, 368–375.
Tripathi, V., Parasuraman, B., Laxmi, A., Chattopadhyay, D., 2009. CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plant. Plant J. 58, 778–790.
Vierstra, R.D., 2003. The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. Trends Plant Sci. 8, 135–142.
Xu, J., Li, H.,D., Chen, L.Q., Wang, Y., Liu, L.L., He, L., Wu, W.H., 2006. A protein kinase, interacting with two calcineurin B-like proteins, regulates K+ transporter AKT1 in Arabidopsis. Cell 125, 1347–1360.
Zipfel, C., Robatzek, S., 2010. Pathogen-associated molecular pattern-triggered immunity: Veni, Vidi...? Plant Physiol. 154, 551–554.
Zeidler, D., Zähringer, U., Gerber, I., Dubery, I., Hertung, T., Bors, W., Hutzler, P., Durner, J., 2004. Innate immunity in *Arabidopsis* lipopolysaccharides activate nitric oxide synthase NOS and induce defense genes. Proc. Natl. Acad. Sci. U S A 101, 15811–15816.
Zheng, Z., Qamar, S.A., Chen, Z., Mengiste, T., 2006. Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J. 48, 592–605.