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Abstract: Fusarium verticillioides causes ear rot in maize and contaminates the kernels with the fumonisin mycotoxins. It is known that plant lipoxygenase (LOX)-derived oxylipins regulate defence against pathogens and that the host-pathogen lipid cross-talk influence the pathogenesis. The expression profiles of fifteen genes of the LOX pathway were studied in kernels of resistant and susceptible maize lines, grown in field condition, at 3, 7 and 14 days post inoculation (dpi) with F. verticillioides. Plant defence responses were correlated with the pathogen growth, the expression profiles of fungal FUM genes for fumonisin biosynthesis and fumonisin content in the kernels. The resistant genotype limited fungal growth and fumonisin accumulation between 7 and 14 dpi. Pathogen growth became exponential in the susceptible line after 7 dpi, in correspondence with massive transcription of FUM genes and fumonisins augmented exponentially at 14 dpi. LOX pathway genes resulted strongly induced after pathogen inoculation in the resistant line at 3 and 7 dpi, whilst in the susceptible line the induction was reduced or delayed at 14 dpi. In addition, all genes resulted overexpressed before infection in kernels of the resistant genotype already at 3 dpi. The results suggest that resistance in maize may depend on an earlier activation of LOX genes and genes for jasmonic acid biosynthesis.

Piacenza, August31, 2015

DearProf.Christian Wilhelm

Editor-in-Chief

Journal of Plant Physiology

I am sending herewith the manuscript "Resistance to *Fusariumverticillioides* and fumonisin accumulation in maize inbred lines involves an earlier and enhanced expression of lipoxygenase (LOX) genes" by Maschiettoand co-workers,revised and modified according to the suggestions and comments of reviewers.

I hope that the manuscript will be accepted in this form.

Yours sincerely,

Alessandra Lanubile

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Resistance to *Fusariumverticillioides* and fumonisin accumulation in maize inbred lines involves an earlier and enhanced expression of lipoxygenase (LOX) genes

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Summary

Fusariumverticillioides causes ear rot in maize and contaminates the kernels with the fumonisin mycotoxins. It is known that plant lipoxygenase (LOX)-derived oxylipins regulate defence against pathogens and that the host-pathogen lipid cross-talk influence the pathogenesis. The expression profiles of fifteen genes of the LOX pathway were studied in kernels of resistant and susceptible maize lines, grown in field condition, at 3, 7 and 14 days post inoculation (dpi) with *F. verticillioides*.Plant defence responses were correlated with the pathogen growth, the expression profiles of fungal *FUM* genes for fumonisin biosynthesis and fumonisin content in the kernels. The resistant genotype limited fungal growth and fumonisin accumulation between 7 and 14 dpi. Pathogen growth became exponential in the susceptible line after 7 dpi, in correspondence with massive transcription of *FUM* genes andfumonisins augmented exponentially at 14 dpi. LOX pathway genes resulted strongly induced after pathogeninoculation in the resistant line at 3 and 7 dpi, whilst in the susceptible line the induction was reduced or delayed at 14 dpi. In addition, all genes resulted overexpressedbefore infection in kernels of the resistant genotype already at 3 dpi. The results suggest that resistance in maize may depend on an earlier activation of LOX genes and genes for jasmonic acid biosynthesis.

Keywords:lipoxygenase genes,pathogen resistance, *Fusariumverticillioides*,fumonisin,*Zea mays*.

***Detailed Response to Reviewers**

Answers:

Reviewer#1: The authors greatly appreciated the positive comment of the reviewer.

Reviewer#2:

- Title: As suggested, the title has been modified as follows: "Resistance to *Fusariumverticillioides* and fumonisin accumulation in maize inbred lines involves an earlier and enhanced expression of lipoxygenase (LOX) genes."
- Abstract: As suggested, the last sentence in the abstract on page 2 has been modified as follows: "The results suggest that resistance in maize may depend on an earlier activation of LOX genes and genes for jasmonic acid biosynthesis."
- Conclusions: As suggested, the last sentence in the abstract on page 19 has been modified as follows: "These results collectively suggest that resistance in maize may depend on an overexpression of LOX pathway genes and highlighted the central role of JA in *Fv* resistance."

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Summary

Fusarium verticillioides causes ear rot in maize and contaminates the kernels with the fumonisin mycotoxins. It is known thatplant lipoxygenase (LOX)-derived oxylipinsregulate defence against pathogens and that the host-pathogen lipid cross-talk influence the pathogenesis.The expression profiles of fifteen genes of the LOX pathwaywerestudied in kernels of resistant and susceptible maize lines, grown in field condition, at 3, 7 and 14 days post inoculation (dpi) with *F. verticillioides*.Plant defence responses were correlated with the pathogen growth, the expression profiles of fungal *FUM*genes for fumonisin biosynthesisand fumonisin content in the kernels.The resistant genotype limitedfungal growth and fumonisin accumulation between 7 and 14 dpi. Pathogen growth became exponential in the susceptible lineafter 7 dpi, in correspondence with massive transcription of *FUM* genes andfumonisins augmented exponentially at 14 dpi. LOX pathway genes resulted strongly induced after pathogeninoculation in the resistant line at 3 and 7 dpi, whilst in the susceptible line the induction wasreduced or delayed at 14 dpi. In addition, all genes resulted overexpressedbefore infection in kernels of the resistant genotype already at 3 dpi. The results suggest that resistance in maize may depend on an earlier activation of LOX genes and genes for jasmonic acid biosynthesis.

Keywords:lipoxygenase genes,pathogen resistance, *Fusariumverticillioides*,fumonisin,*Zea mays*.

Abbreviations: dpi, days post inoculation; ET, ethylene; FC, fold change; *Fv*,*Fusariumverticillioides*; GLV, green leaf volatile; JA, jasmonic acid; LOX, lipoxygenase; ppm, parts per million; PR, pathogenesis-related; R, resistant CO433 line; S, susceptible CO354 line; SA, salicylic acid; sd, standard deviation; *Zm*,*Zea mays*,*ZmACX*,*Zmacyl-CoA oxidase*;*ZmAOS*,*Zmallene oxide synthase*;*ZmHPL*,*Zmhydroperoxidelyase*;*ZmLBP*,*Zmlipid binding protein ZmLOX*,*Zm lipoxygenase*;*ZmOPC*,*ZmOPC-8:0 CoA ligase1*;*ZmOPR8*,*ZmOPDA reductase8*; *Zmplt1*, *Zm phospholipid transfer protein*.

1. Introduction

Worldwide infection of maize seeds with the fungus *Fusarium verticillioides*(*Fv*)causes ear rot andcontaminates the kernels with fumonisins of B series, which are consideredcarcinogenic mycotoxins(Logrieco et al., 2002).Fumonisins are able to disrupt the metabolism of sphingolipids, important signalling molecules in animal and plants (Williams et al., 2007). Several infection pathways have been identified including silk infection, insect injury and systemic transmission from seeds or roots to kernels (Munkvold, 2003a). *Fusarium* ear rot resistance is a complex polygenic trait largely unknown and most commercial maize hybrids are susceptible to the disease and mycotoxin accumulation (Munkvold, 2003b). The process of *Fv* infection and mycotoxin accumulation in maize is influenced by environmental conditions,but alsoby host resistance and biochemical composition of kernel, including moisture, development stage and lipid composition(Lanubile et al., 2011; 2014; Sagaram et al., 2006; Woloshuk and Shim, 2013). The content of linoleic acid, some sphingolipids and the oxylipin (9S,10E,12Z)-9-hydroxy-10,12-octadecadienoic acid (=9-HODE) resulted positively correlated with fumonisin accumulation into maize kernels infected with *Fv*(Dall'asta et al., 2015; Gao et al., 2007).

Oxylipins comprehend around 150 oxygenated polyunsaturated fatty acids and derivatives, which in plants act as molecular signals to regulate growth and development, senescence, sex determination of reproductive organs, defence against biotic and abiotic stress and programmed cell death(Christensen and Kolomiets, 2011). On the other side, fungal oxylipins are potent regulatorsof spore development and secondary metabolism, including biosynthesis of mycotoxins, and pathogenicity(Sagaram et al., 2006).Oxylipins derived from either plants or their pathogens can mediate a host-pathogen cross-talk and host oxylipins may be important for successful colonization by certain fungal pathogens (Gao et al., 2007).

Most of plant oxylipins are produced through the lipoxygenase (LOX) pathway. Its starting point is the incorporationof molecular oxygen into either 9-or 13-position of the carbon chain of C18 polyunsaturated fatty acids, generating 9- and 13-hydroperoxides, respectively. This reaction is

mediated by 9-or 13-LOXs that use as substrates the linoleic (18:2) and linolenic (18:3) acids, cleaved from cell membranes by diverse lipases (Nemchenko et al., 2006).

Products of the 13-LOX enzymatic activity can be utilized by at least seven multienzyme pathway branches:the best characterized are the allene oxide synthase (AOS) and the hydroperoxide lyase (HPL) pathways, both using the same 13-hydroperoxide substrate(Feussner and Wasternack, 2002).The AOSpathway produces jasmonic acid (JA), its precursor 12-oxo-phytodienoic acid (12-OPDA) and derivatives, collectively known as jasmonates. Jasmonates function as signals in the pathogen- and insect-induced pathways that regulate expression of defence-related genes (Turner et al., 2002). The best studied HPL-derived oxylipins arethe green leaf volatiles (GLVs) thatpossess antimicrobial properties (Prost et al., 2005) and act as signals inducing the expression of defensive genes (Bate and Rothstein, 1998) and regulating plant-plant communication after insect elicitation (Christensen et al., 2013; Engelberth et al., 2004).

Functional and physiological analyses of LOXs were firstly addressed to dicots and recent researches aimed to unravel the role of LOX in plant-pathogen cross-talk with the use of maize mutants (Christensen et al., 2013; 2014; Gao et al., 2007)and *Fv* mutants(Lanubile et al., 2013; Scala et al., 2014).Our study providesthe first insight of the overexpression of genes involved in the LOX pathwayin resistant (R) and susceptible (S) maize lines, tested in field conditionover a time course of 21 days after inoculation with*Fv*. Moreover, the plant defence responses were correlated with the differential growth of *Fv*, the expression profile of fungal genes for fumonisin biosynthesis and the fumonisin contents in theinoculated kernels. The LOX pathway genes could be used as candidates to select hybrids and sources of resistance to *Fusarium* ear rot.

2. Materials and methods

2.1 Maize inbreds, growth conditions, fungal strains and inoculation assays

The maize inbred lines CO433 (R) and CO354 (S) were used, respectively, as resistant and susceptible genotypes to *Fv* infection (Lanubile et al., 2010; 2011; Reid et al., 2009). Both lines

were developed by the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Canada (Reid et al., 2009), and were maintained by sibling at the Department of Sustainable Crop Production, UniversitàCattolica del SacroCuore, Piacenza, Italy. The experiment was conducted at the Gaita experimental farm set in Caleppio di Settala, Milan, Italy. The sowing was made on the 28th April, 2012. Experimental units were single-row plots of 4 m length, separated by a distance of 0,80 m. Five rows were sown for each maize genotype. Plots were overplanted and thinned at the three to four leaf stage to 20 plants. Standard cultural practices for growing maize were followed. Fertilizer rates were applied as follow: 250 kg/ha N, 100 kg/ha P_2O_5 and 80 kg/ha K_2O. Irrigation was applied by drip system in order to prevent water stress. Maize ears were hand-pollinated starting from the 29th June.

The*Fv*strain used for inoculation was the fumonisin producer ITEM 1744, supplied by the Institute of Sciences of Food Production, National Research Council, Bari, Italy. Cultures and conidia were prepared as described in Lanubile et al. (2015), obtaining a final concentration of spore suspension of 3.5 x 10^6 conidia/ml.

Maize ears were inoculated at 15 days after hand-pollination using a side-needle inoculator, already described in Lanubile et al. (2015). For the detection of fungal strain, gene expression analyses and fumonisin content, the inoculated and immediately adjacent kernels were collected at 3, 7, 14 and 21 days post inoculation (dpi). Control ears were mock-inoculated (inoculation with water) at the same inoculation time listed above and kernels were sampled in the mock-inoculation point and immediately adjacent kernels. Three biological replicates were prepared for each time point, where replicates derived from the pool of kernels coming from three different maize ears. The collected kernels were ground in liquid nitrogen with a pestle and mortar and used both for gene expression analyses and fumonisin quantification.

2.2 RNA isolation and real-time RT-PCR expression analysis

Total RNA extraction and purification were performed according to Lanubile et al. (2015).Real-time RT-PCR experiments were performed on kernels collected at 3, 7 and 14 dpi using the 2x

iQSYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and the CFX-96 device (Bio-Rad). One µgof total RNA was used for cDNA synthesis following the iScript cDNA synthesis kit protocol (Bio-Rad). 20 ng of single strand cDNA determined by fluorometric assay (Qubit, Invitrogen) were used for real-time RT-PCR. Relative quantitative analysis was performed under the following conditions: 95°C for 3 min and 40 cycles at 95°C 15 s, 57-63°C for 30 s. A melting curve analysis, ranging from 60 to 95°C with a 0.5°C increment for 5 s, was used to identify different amplicons, including non-specific products. Three technical replicates (within each biological replicate) were employed for each tested sample and template-free negative controls. Gene-specific primers were downloaded from literature or designed possibly within consecutive exons, separated by an intron, using Primer3 software (Table S1). A quality filter for amplification efficiency of housekeeping and target genes was set to include only values ranging from 90 to 110% and standard curve with R^2>0.98. Relative quantification of maize genes was normalized to the housekeeping gene *β-actin* (Table S1) and FC values in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The same approach was used for relative quantification of fungal *FUM* genes, normalized with the fungal housekeeping gene *β-tubulin2* (Table S1). To evaluate the difference in response to mock-inoculation of the two maize lines, the expression values (ΔCt) of mock R were normalized to the corresponding ΔCt of mock S and then FCs were calculated according to the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). The same approach was used for the comparison between inoculated kernels of the two maize genotypes. To quantify the fungal growth, the copy number of *β-tubulin2* transcript was detected using real-time RT-PCR in inoculated kernels collected at 3, 7 and 14 dpi. The real-time RT-PCR thermal cycling conditions were the same as reported above. The *β-tubulin2* number of copies is related to ng of cDNA obtained from kernel tissues and determined based on the equation of the linear regression according to the technical manual (Bio-Rad). Fungal cDNA (20 ng) was serially diluted [1:1, 1:5, $1:5^2$, $1:5^3$, $1:5^4$, $1:5^5$] in sterile water and 20 ng of each kernel cDNA sample was compared to the dilution standard curve to determine fungal cDNA copy number.

2.3 Sample preparation and LC-MS/MS conditions for fumonisin quantification

5 g of homogenised sample was weighed into 100 mL Erlenmeyer flasks. The samples were extracted with 50 mL of mixture of methanol/water (70/30, v/v) on a rotary shaker (GFL 3017; GFL, Burgwedel, Germany) for 1 hour. The extract was transferred into a 50 mL polypropylene tube and centrifuged at 5,000 *g* for 3 min (GS-6 centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). 1 mL of extract was transferred into a vial and injected.

Detection and quantification was performed with a QTrap 5500 LC-MS/MS system (AB Sciex, Foster City, CA, USA) equipped with a TurboV electrospray ionization (ESI) source and a 1290 series UHPLC system (Agilent Technologies, Waldbronn, Germany). Chromatographic separation was performed at 40^{o}C on an Acquity UPLC$^{®}$HSS T3, 100 mm × 2.1 mm i.d., 1.8 µm (Waters, Milford, MA, USA). Both eluents contained 0.1% of formic acid. Eluent A consisted of methanol/water (40/60, v/v) and eluent B of methanol/water (90/10, v/v). A gradient program was set up with 0–3 min linear increase from 30% of B to 90% of B, at 3 min jump to 100% B and held for another 2 min at 100% B, at 5.1 min back to initial conditions of 30% B. Equilibration of the column at the starting conditions was set up for 2 min. The flow rate was 0.3 mL/min. The injection volume was 3 µL.

ESI-MS/MS was performed in the multiple reaction monitoring (MRM) mode in positive polarity with the following settings: source temperature 550°C, curtain gas 10 psi (69 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 50 psi (345 kPa of zero grade air), ion source gas 2 (drying gas) 50 psi (345 kPa of zero grade air), ion spray voltage +4000 V, collision gas (nitrogen) high. For quantitation two selected reaction monitoring transitions per compound were acquired with a dwell time of 30 ms: the protonated adducts of the analytes (m/z 722.5 for FB_1, m/z 706.5 for FB_2 and FB_3) were chosen as precursors and the declustering potential (DP) was 116 V for FB_1, 126 V for FB_2 and FB_3. The following product ions were chosen as quantifier and qualifier, respectively: for FB_1 m/z 334.4 (collision energy (CE) of 57 V) and m/z 352.3 (CE 55 V), for FB_2 and FB_3 m/z 336.4 (CE 59 V) and m/z 318.4 (CE 51 V). The integrated peaks were quantified on the matrix

matched calibration curves prepared using blank sample extract. The results were corrected to recovery. The overall average recoveries of the method calculated across all validated levels for free fumonisins were 92% for FB_1, 85% for FB_2 and 92% for FB_3. The limits of quantification were 4 µg/kg, 6 µg/kg and 7 µg/kg for FB_1, FB_2 and FB_3, respectively.

2.4 Statistical analyses

For gene expression analyses, standard deviations of the means were calculated on three technical replicates of three biological replicates.

The observed means of FB_1, FB_2 and FB_3 concentrations at 3, 7, 14 and 21 dpi within each genotype were subjected to ANOVA ($P<0.05$), considering the sampling time point and treatment as fixed factors, in order to test the difference in fumonisin content between mock and inoculated kernels within each genotype.

Two-factor ANOVA ($P<0.05$) was performed on the observed means of FB_1, FB_2 and FB_3 and the total fumonisin content at 3, 7, 14 and 21 dpi of only inoculated kernels of both genotypes. Genotypes and sampling times were considered as fixed factors to test their significances and the significance of their interaction.

3. Results and Discussion

3.1 Evaluation of fungal growth, expression of FUM genes and fumonisin content

The R and S maize genotypes showed contrasting resistances to *Fv* and several other fungal pathogens (Reid et al., 2009). The defence responses of theS genotype to the same pathogen were assayed in previous works at early stage after inoculation, performing microarray and RNA-Seq analyses for gene expression profilesand through enzymatic assays (Lanubile et al., 2010; 2012a; b; 2013; 2014).In this study, a differentresistant line and a longer time course, with sampling at 3, 7, 14 and 21days post inoculation (dpi), were considered.

The growth of *Fv* was assayed in the inoculated maize kernels by absolute quantification of the *β-tubulin2* transcript through real-time RT-PCR (Table 1). The RNA resulted strongly degraded at last time of sampling due to the advanced fungal infection and then it was not suitable for further fungal and plant gene expression analyses. The R genotype showed a reduced growth of the pathogen in comparison to Sin all sampled times. Moreover, the divergence of pathogen growth in the two genotypes increased over the time course: *β-tubulin2* copy number was 1.4, 1.7 and 1.8 times greater in the S genotype at 3, 7 and 14 dpi, respectively. The reduced fungal growth in R over the time coursewas in line with our previous findings in earlier stages of infection(Lanubile et al., 2010; 2012a; b; 2013; 2014).

Most field isolates of *Fv*produce predominantlyfour B-series fumonisins, calledFB_1, FB_2, FB_3, and FB_4(Butchko et al., 2006). These mycotoxins are synthesized through theactivity of enzymes encoded by the fumonisin biosynthetic(*FUM*) gene cluster, consisting in 15 co-regulatedgenes designated *FUM1-FUM3*, *FUM6-FUM8, FUM10,FUM11,* and *FUM13-FUM19*(Proctor et al., 2003). Furthermore, the *FUM21*geneencoding a zinc cluster transcription factor, regulates the expression of almost all *FUM* genes(Brown et al., 2007).The expression of selected crucial*FUM* genes (*FUM1*, *FUM2*, *FUM8* and *FUM21*) was determined at 3, 7 and 14 dpi. The fold changes (FC) of inoculated kernels over mockkernels are reported in figure 1A-D. The four genes presented a similar trend: gene expression increased rapidly in R at 7 dpi and then it decreased to initial values. On the other side, gene induction was less marked in Sat 3 and 7 dpi, but increased exponentially at 14 dpi. In this way, up-regulation resulted stronger at 3 and 7 dpi in R line in comparison to S, but it reached huge values in the latter at 14 dpi, in line with the fungal growth assay (Table 1).

FB_1, FB_2 and FB_3 contents were measured in mock and inoculated kernels at the four time points of the experiment.The levels of fumonisinswere either far below or very close to limit of quantification (LOQ) in the mock samples of both genotypes. Therefore, only the contents determined in inoculated kernels are reported in figure 1E. Significant differences (P<0.001) inFB_1, FB_2 and

FB_3contents were found between the inoculated kernelsand the corresponding mock samplesat 7,14 and 21 dpi in R genotypeand at 14 and 21 dpi in S genotype. A significant (P<0.001) difference was detected in each genotype between time of sampling, treatment and their interaction. Moreover, considering only the inoculated kernels, genotypes accumulated significantly (P<0.001) different levels for each fumonisin and their total content at 14 and 21 dpi (Fig. 1E). This result corresponded to the exponential expression of the *FUM* genes and the augmented growth of the pathogen in the S line at 14 dpi. The most concentrated fumonisin was FB_1, whose content ranged from 0.5 ppm (3 dpi) to 74.7 ppm (21 dpi) in the Rkernels and from 0.5 ppm (3 dpi) to 495.8 ppm (21 dpi) in the Skernels. As for fungal growth, fumonisin contents increased faster in S genotype, with a ratiofor FB_1between genotypes (S/R) of 1.1 (3 dpi), 0.7 (7 dpi), 4.4 (14 dpi) and 6.6 (21 dpi). Similar ratios were obtained also for FB_2 and FB_3.

These results collectively demonstratedthe resistance of the R line in comparison to S, as highlighted by a limited growth of the pathogen, alow fumonisin content and reduced transcription of genes for fumonisin biosynthesis.These findings are noteworthy because *Fv* development, *FUM* gene expression and fumonisin content were monitored for the first time*in planta*, under field conditions, and in relation to the host resistance.

3.2 Regulation of the expression of maize 9-LOX genes

Thirteen different maize LOXs with several functions, localization and regulation within the plant were reported (Yan et al., 2012). Metabolism of 9-hydroperoxy fatty acids generates a distinct group of oxylipins, but structurally related to 13-LOX oxylipins, whose role is less known (Howe and Schilmiller, 2002). However several studies demonstrated their hormone-like signalling activities in plant growth and development and defence against pests and pathogens (Christensen and Kolomiets, 2011; Gao et al., 2008a). In this workthe expression levels of selected 9-LOX genes, *ZmLOX3, ZmLOX4, ZmLOX5* and *ZmLOX12* were measured by real-time RT-PCR. In order to establish the strength of induced defence responses determined by wounding (mock-inoculation) in the two maize genotypes, apart from *Fv* inoculation, the FCs were calculated as transcript content of

mock R over mock S kernels (Fig. 2).A similar approach was used to evaluate the response to pathogen inoculation, with the comparison of the inoculated samples (inoculated R/ inoculated S), reported in figure S1. In these cases, FC>1 means the overexpression in the R line, whilst FC<1 means the overexpression in S.It is evident that at 3 and 7 dpi all 9-LOXs, excluding *ZmLOX5*, were overexpressed in the mockR in comparison to S line (Fig. 2). This indicated that R was capable to activate a rapid gene transcription in response to wounding. On the other side, the S line responded to woundingonly subsequently, at 14 dpi, with a moderate overexpression of *ZmLOX3*, *ZmLOX5* and *ZmLOX12* (Fig. 2). The reduced readiness of the S line in facing the attack of *Fv*and other mycotoxigenic fungi was demonstrated to be due to a lack in constitutive defences at the transcription and enzymatic activity levels, including PR genes, reactive oxygen species-scavenging and other genes of the secondary metabolism (Lanubile et al., 2012a; b; 2014; 2015), and, according to this study, to genes of the LOX pathway.

The expression profiles of the 9-LOXsare reported in figure 3A-D, as FCs of inoculated kernels over mock kernels. Expression of *ZmLOX3*, *ZmLOX4*, *ZmLOX5* and *ZmLOX12*was greater in the R line at 7 dpi, with FCs of 4.4, 11.3, 39.9 and 54.9, respectively and then strongly decreased at 14 dpi. In the S line lower FCs were measured at 7 dpi for all 9-LOXs, followed by a moderate decrement in induction that carriedhigher FCs at 14 dpi in comparison to R.The highest expression values of the 9-LOXs at 7 dpi in inoculated R in comparison to S and the opposite expression profile at 14 dpi areevident in figure S1.

ZmLOX3 occurs in developing embryos(Gao et al., 2007) and is further up-regulated in susceptible maize kernels during *Fv*infection under laboratory condition(Wilson et al., 2001). This study demonstrated that *ZmLOX3* resulted up-regulated in both genotypes after infection also in the field and the unusual lowest response of the R line at 3 and 14 dpi (Fig. 3A) is in line with the knowledge that oxylipins specifically mediated by *ZmLOX3* are required for *Fv* pathogenesis, including disease development and production of spores and mycotoxins, but not vegetative growth(Gao et al., 2007). Maize *lox3* mutant have shown that the 9-LOX pathway controls plant germination, root growth

and senescence (Gao et al., 2008a) and contributes to maize resistance to *Aspergillus spp.*(Gao et al., 2009) and susceptibility to several fungal pathogens, including *Fv*(Gao et al., 2007). In the latterpathosystems9-LOX-derived oxylipinsseem to mimic the action of the pathogen-synthesized oxylipins (e.g. psi factors) by inducing production of conidia and mycotoxins (Gao et al., 2007).

ZmLOX4 and *ZmLOX5* are segmentally duplicated 9-LOX genes that have distinct organ-specific and stress induced expression patterns (De La Fuente et al., 2013).*ZmLOX4*transcript wasdetected mainly in the roots and the shoot apical meristem, while *ZmLOX5* was expressed predominantly in above ground organs, especially the silks (Park et al., 2010). Since*ZmLOX4* was only induced in leaves by JA and not by wounding (Park et al., 2010), it may function in JA-mediated pathways elicited by pathogens and not by leaf herbivores(De La Fuente et al., 2013), supporting the existence of separate wound- and pathogen-induced JA-dependent pathways (Dombrecht et al., 2007; Lorenzo et al., 2004). Moreover *ZmLOX4*, resulted strongly up-regulated in maize kernels under field condition at 4 dpi with *A. flavus*(Dolezal et al., 2014). Consistentlywith these findings,*ZmLOX4* resulted responsive to *Fv* infection in both maize genotypes, showingthe highest FCs associated to the R genotype at 7 dpi andconstant and reduced expression in S during the time course (Fig. 3B). Anyway, *ZmLOX4* was detected at different expression levels in the two genotypes after mock-inoculation (Fig. 2), suggesting that also wounding could lead to itsregulation in the ear tissue. Additional expression profiles of unchallenged ears could clarify the role of this gene in maize resistance.

ZmLOX5 was induced in leaves by JA, salicylic acid (SA), insect herbivory and by wounding (Park et al., 2010). Due to its localization in silks, it was supposed that*ZmLOX5*affects aflatoxin resistance (De La Fuente et al., 2013). This gene could be considerably involved also in *Fv* resistance since its expression resulted similar in mock kernels of both genotypes (Fig. 2), but at 7 dpi the inductionwas quadrupled in R inoculated kernelsin comparison to the S line (Fig. S1).

The monocot-specific 9-LOX *ZmLOX12* was recently discovered to accumulate predominantly in mesocotyls after *Fv* infection(Christensen et al., 2014). *Lox12* mutant was more susceptible to *Fv*

colonization of mesocotyls, stalks and kernels and fumonisin contamination of the kernels, showing diminished levels of jasmonatesand expression of genes of the JA-biosynthetic pathway(Christensen et al., 2014). Our study demonstrated the central roleof *ZmLOX12* in maize resistance towards this pathogen in the ear grown in field condition, since this genewas induced up to 6.6 times more in theR genotype at 7 dpiin comparison to S, whereas its induction decreased during the time course in S (Fig. 3D).

3.3 Regulation of the expression of maize 13-LOX genesand ZmLOX6

13-LOXs produce (9Z,11E,15Z)-(13S)-hydroperoxyoctadeca-9,11,15-trienoate (=13S-HPOTE), which is the substrate for both GLVs andjasmonates.Among the 13-LOXs, the expression profilesof *ZmLOX8, ZmLOX10* and *ZmLOX11* were analysed (Fig. 3E-G).In maize, GLV and JA pathway are physically separated from each other and then do not compete for substrate, as evinced by the localization of JA-producingLOX8 to chloroplasts (Acosta et al., 2009) and GLV-producing LOX10 to organelles distinct from chloroplasts (Christensen et al., 2013). So far,*ZmLOX10* is the unique 13-LOX isoform responsible for generating 13S-HPOTE for the GLV biosynthesis pathway, (Christensen et al., 2013).

ZmLOX8, ZmLOX10 and *ZmLOX11* resulted overexpressed in mock R kernels at 3 dpi, whilst only *ZmLOX10* kept the overexpression also at 7 and 14 dpi (Fig. 2).*ZmLOX8* was strongly induced at 7 dpi in R, showing a FC value of 20.5 compared to 4.3 of the S genotype (Fig. 3E). A similar pattern was observed for *ZmLOX10* and *ZmLOX11*, which were strongly up-regulated at 7 dpi in both genotypes (Fig. 3F,G). The paralog *ZmLOX10* and *ZmLOX11*are expressed differentially in leaves and silks of unchallenged plants, respectively (Nemchenko et al., 2006). *ZmLOX10* transcripts increased transiently by wounding, chilling, treatments with JA, SA and abscissic acid (ABA) and inoculation with an avirulent fungal strain of *Cochlioboluscarbonum*(Nemchenko et al., 2006).The lowest FC of *ZmLOX10* at 7 dpi in the R line in comparison to S (35.5 vs 235.1, respectively) (Fig. 3F) could be explained by its greater expression already in mock R kernels (about 14 times higher at 7 dpi) (Fig. 2). *ZmLOX10* was induced by wounding, especially in R and the expression

augmented after pathogen inoculation. The involvement of *ZmLOX10* in direct and indirect herbivore defence responses was demonstrated by *lox10* mutant seedlings, impaired in both GLV and JA production and suggestinga cross-talk signalling between *ZmLOX10*-mediated GLV and *ZmLOX8*-mediated JA branches (Christensen et al., 2013). The strong up-regulation inRkernels of *ZmLOX8*at 7 dpi and the increased expression of *ZmLOX10*after mock-inoculationmight suggest that JA and GLV branches contributedequally in the early activation of ear defence mechanism, the former addressed to pathogen resistance and the latter to a more generic wound response.

A putative role in osmotic stress response was hypothesized for *ZmLOX11*, induced in leaves only by ABA (Nemchenko et al., 2006). In line with these findings, wounding (mock-inoculation) did not issue a significant difference in response among the two genotypes. However, in contrast with the down-regulationin maize kernels at 4 dpi with *A. flavus*(Dolezal et al., 2014), *ZmLOX11* was similarly induced in both genotypes by *Fv* inoculation, and the FCs at 7 dpi reached 71.2 in the R genotype and 48.6 in S (Fig. 3G).

This study included also theevaluation of expression pattern of the plastidial*ZmLOX6*,whichrepresents a novel LOX subfamily, distinct from 9- and 13-LOXs previously characterized, withthe capability of metabolize 13-LOX-derived fatty acid hydroperoxides(Gao et al., 2008b).*ZmLOX6* was subjected to a moderate up-regulation in both genotypes at 3 and 7 dpi (Fig. 3H), and its induction resulted almost 8 times greater ininoculated Sat 14 dpi in comparison to R (Fig. S1). Since this gene was not responsive to wounding or insects, but was induced by JA and by*C.carbonum* during compatible interactions, it may contribute to susceptibility to this pathogen(Gao et al., 2008b) and also to *Fv*, as evinced by our results.

3.4 Regulation of genes for the biosynthesis ofgreen leaf volatiles and jasmonates

The AOS branch of the 13-LOX pathway leads to the jasmonate family of compounds including JA/Methyl JA and their metabolic precursor 12-OPDA (Feussner and Wasternack, 2002). JA production begins in the chloroplast with the conversion of 13S-HPOTE to allene oxide by AOS, followed by the formation of 12-OPDA by allene oxide cyclase,the conversion to oxophytoenoic

acid in the peroxisomes by an OPDA reductase (OPR), and endswith three beta-oxidation steps (Christensen and Kolomiets, 2011). The genes involved in synthesis of jasmonates, namely*ZmAOS*, *ZmOPR8, OPC-8:0 CoA ligase1* (*ZmOPCL*) and *acyl-CoA oxidase* (*ZmACX*), were overexpressed in R mock kernelsat 3 and 7 dpi, especially *ZmAOS* (Fig. 2). All four genesresulted generally induced in both genotypes at 3 and 7 dpi (Fig. 4A-D), but fungal inoculation induced higher gene expression in R line at 7 dpi in comparison to S (Fig. S1). A similar expression trend occurred for*ZmAOS* and *ZmOPR8*:FCs were 24.8 and 38.7 for *ZmAOS*, and 8.3 and 9.0 for*ZmOPR8*in S and R lines, respectively (Fig. 4A,B). *ZmOPCL* and *ZmACX* FC values decreased in the S line over the time course, while in R reached the maximum at 7 dpi (FC of 4.1 and 17.7, respectively; Fig. 4C,D). The multiple role of JA was investigated in maize mutants of *OPR7* and *OPR8* genes: the double mutant showed a dramatic reduction of JA, strong developmental defects, delayed leaf senescence showing reduced ethylene (ET) and ABA levels, and increased susceptibility to insects and pathogens (Yan et al., 2012), including *Fv*(Christensen et al., 2014).

The strong defence role of JA and ET signalling pathway towards *Fv*has been demonstrated(Christensen et al., 2014; Lanubile et al., 2014). It is known that plant resistance to biotrophic/hemibiotrophic pathogens activated by gene for gene recognition is controlled largely by SA-mediated signalling pathways, while resistance to necrotrophic pathogens is mediated, antagonistically, by the JA and ET signalling pathways (Glazebrook, 2005).It was proventhat wheat defence against the hemibiotrophic*F. graminearum* is sequentially regulated by SA and JA during the early and later stages of infection, respectively (Ameye et al., 2015; Ding et al., 2011). Wheat cultivars at 32 and 72 hours post inoculation revealed the existence of a biphasic strategy against *F. graminearum*: a JA- and ET-mediated defence mechanismwas directed against fungal growth and sporulation and induced the transcription in the resistant cultivar of a set of genes encoding antimicrobial peptides,PR proteins as lipid transfer proteins, defensins and thioninsand the second mechanism was specifically directed towards fungal mycotoxins and proteases(Gottwald et al., 2012).On the contrary, SA-related genes were not significantly induced in resistant and susceptible

maize genotypes at 3 dpi with *Fv*, but the activation of typical JA- and ET-responsive defence genes and transcription factors, such as chitinases, LOXs, PR10 and ACC oxidase, was observed with an enhanced induction in a resistant line (Lanubile et al., 2014). It is possible that the biotrophic*Fv* phase triggering SA-related defences occurred earlier than 3 dpi and then it was not detected. New studies covering earlier sampling times are required to solve the heterogeneity in behaviour of *Fusarium* spp. Nevertheless, our study demonstrated that an effective resistance to *Fv*in the long-term needed the transcription of genes of the JA-biosynthetic pathway and LOX genes, including *ZmLOX8* which is so far the isoform responsible for JA production (Acosta et al., 2009).The R line showed a prompt activation of these genes immediately after wounding derived by the mock-inoculation, suggesting the initial overlap of JA- and wound-mediated defence responses.

Lipid Transfer Protein (LTPs) bind to and transfer lipids from membrane and have shown multiple roles in defence against pathogens, in cuticle synthesis and as modulators of plant growth and development(Yeats and Rose, 2008) and have been linked to *F. graminearum* resistance in wheat (Schweiger et al., 2013; Sun et al., 2008; Xiao et al., 2013). In our study, the expression profiles of two LTPs, namely *phospholipase transfer protein homolog1* (*Zmplt1*) and *lipid binding protein* (*ZmLBP*) were analysed. Both genes were overexpressed in the S line before fungal inoculation, except for *Zmplt1* at 3 dpi (Fig. 2).*Zmplt1* was subjected to a modest induction in both genotypes, showing decreasing values over the time course for the S genotype (FC of 3.4 at 3 dpi and 1.3 at14 dpi) and a higher expressionin R line only at 7 dpi (FC of 3.0) (Fig. 4E). The highest FC for the S line at 3 dpi was consistent with transcriptomic analysis, since this line showed at this time point expression values for this gene superior to 1000 FPKM (Fragments Per Kilobase of exon model per Million mapped reads) (Lanubile et al., 2014).*ZmLBP*resulted strongly induced in R at 7 dpi, showing a FC of 34.3 and only 2.73 in the S line (Fig. 4F) and an overexpression 8 times greater in R inoculated kernels (Fig. S1). The same gene was strongly down-regulated after infection at 3 dpi in both genotypes (Lanubile et al., 2014), although in the presented study a not significant variation was detected among inoculated and mock samples for both genotypes. The analysis has to be

extended to the expression profiles of other genes of the LTP family before their selection as candidate genes for *Fv* resistance in maize.

The HPL branch uses 13-HPOTE as substrate to produce GLVs, a number of volatile C6-aldehydes and alcohols (Gao et al., 2008a). GLVs have been suggested to be essential in the activation of wound-related pathways, some of which are JA independent (Bate and Rothstein, 1998), although a molecular cross-talk for a co-operation in defence signalling between these two 13-LOX branches was proven (Halitschke et al., 2004). In the present study, the*ZmHPL* gene for the synthesis of GLVs resulted strongly up-regulated at 7dpi inR line, whilst its induction decreased over the time course in S(Fig. 4G). As for genes involved in biosynthesis of jasmonates, *ZmHPL* resultedmore expressed in mock-kernels of R line at 3 dpi (Fig. 2) and in R inoculated kernels at 7 dpi (Fig. S1).GLV role in priming, which refers to a sensitization of plants to respond faster and/or more strongly to future herbivore insect attack, was demonstrated in maize(Engelberth et al., 2004), but recently also in wheat againstfungal pathogens(Ameye et al., 2015). Primed wheat plants with the GLV Z-3-hexenyl acetate (Z-3-HAC) showed reduced fungal spread in subsequent *F. graminearum*infections, although accumulated higher levels of mycotoxins(Ameye et al., 2015). Moreover Z-3-HAC was responsible for the transition from SA- toJA-dependent defences, occurring at 2 dpi(Ameye et al., 2015). This study showed that wounding (mock-inoculation) triggered genes for GLV production, including *ZmLOX10* with an enhanced response in R. The connection between *Fv* inoculation and the activation of these genes in relation to host resistance should be further determined, although it is evident that the pathogen augmented the induction of plant responses at 7 dpi.

3.5 Conclusions

This work investigatedthe expression pattern of fifteenmaize genes includingLOXsand genes forJA and GLV biosynthesis in kernels of two commercial maize lines,tested in field condition,showing contrasting resistance to *Fv*.In addition, the expression levels of fungal *FUM* genes and the quantification of fungal growth were correlated to the amount of fumonisins. The long-

timecourseconsidered allowed to monitor the early and late plant responses in parallel with the progression of pathogen infection.The pathogen growth resulted greater in the S genotype in all time points. Its growth became exponential after 7 dpi, in correspondence with massive transcription of *FUM* genes. Interestingly, at 7 dpi expression of several *FUM* genes appeared more abundant in R genotype, but it went back to basal levels at 14 dpi. In parallel, fumonisin levels increased exponentially at 14 dpi in kernels of the S line, whereas a reduced contamination was determined in R. It is evident that plant resistance was mounted between 7 and 14 dpi and the R genotype was more efficient in limiting fungal growth and fumonisin accumulation. Genes belonging to LOX pathway resulted strongly induced after *Fv* inoculation in the R line at 3 and 7 dpi, whilst inS the induction was reduced or delayed at 14 dpi.In addition, all genes weremore induced in mock R kernels already at 3 dpi, suggesting that this line activated earlier and more efficiently the transcription of defence responses. Moreover, since a significant difference in gene induction between the two maize lines occurred at 7 dpi in inoculated kernels and not in mock samples, it could be hypothesized that in the R genotype a separate wounding-independent response to *Fv* could ariseat this time point. Finally, among 9-LOX genes,*ZmLOX5* and *ZmLOX12* resulted strongly up-regulated after pathogen inoculation at 7 dpi in the R line and their expression values were not significantly different among genotypes in mock kernels, suggesting that these two genes are related to resistance and exclusively responsive to pathogen inoculation.These results collectively suggest that resistance in maize may depend on an overexpression of LOX pathway genes and highlighted the central role of JA in *Fv* resistance. Thehormonal and oxylipin profiles will be considered in further analyses to better explain*Fv*resistance in maize. Moreover our current work includesthe use of LOX pathway genes as candidate genes in QTL mapping and their use in breeding programs for increasing maize resistance against *Fusarium* ear rot.

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APPENDIX A. SUPPLEMENTARY DATA

Fig. S1 *Real-time RT-PCR analyses in Fv inoculated kernels.*FC ofexpression between inoculated kernels of R and S lines of *ZmLOX*, *ZmAOS*, *ZmOPR8*, *ZmOPCL*, *ZmACX*, *Zmplt1*, *ZmLBP*and*ZmHPL*at 3, 7 and 14 dpi. Vertical bars indicate ± sd.

Tab. S1 Sequence of primers used for real time RT-PCR analyses.

TABLE

Tab. 1 Transcript copy number (± sd) of the *Fvβ-tubulin2* gene inR and S inoculated kernelsat 3, 7 and 14 dpi.

Genotype	3 dpi	7 dpi	14 dpi
S	2700 ± 344	3045 ± 974	5529 ± 848
R	1999 ± 407	1841 ± 462	3085 ± 378

FIGURE LEGENDS

Fig. 1*Evaluation of host resistance to Fv.***(A)** FC ofexpression of*FUM1*, **(B)***FUM2*, **(C)***FUM8*, **(D)***FUM21* genes,between *Fv*inoculated and mock kernels at 3, 7 and 14 dpi in R and S lines. Vertical bars indicate ± sd. **(E)**Contents of fumonisin B_1, fumonisin B_2 and fumonisin B_3 in *Fv*inoculated kernels of R and S genotypes at 3, 7, 14 and 21 dpi.*** indicate significant differences (P<0.001) among genotypes for the total fumonisin content and for each detected fumonisin; ns indicate not significant differences, according to ANOVA.

Fig. 2 *Real-time RT-PCR analyses in mock-inoculated kernels.*FC ofexpression between mock kernels of R and S lines of *ZmLOX*, *ZmAOS*,*ZmOPR8*, *ZmOPCL*, *ZmACX*, *Zmplt1*,*ZmLBP*and*ZmHPL*, at 3, 7 and 14 dpi. Vertical bars indicate ± sd.

Fig. 3 *Real-time RT-PCR analyses of LOX genes.*FC ofexpression between *Fv*inoculated and mock kernels of R and S lines at 3, 7 and 14 dpi of **(A)** *ZmLOX3*,**(B)***ZmLOX4*, **(C)***ZmLOX5*,

(D)*ZmLOX12*, **(E)***ZmLOX8*, **(F)***ZmLOX10*, **(G)***ZmLOX11* and **(H)***ZmLOX6*. Vertical bars indicate ±sd.

Fig. 4 *Real-time RT-PCR analyses of genes of JA and GLV biosynthesis*.FC ofexpression between *Fv*inoculated and mock kernels of R and S lines at 3, 7 and 14dpi of **(A)** *ZmAOS*, **(B)***ZmOPR8*, **(C)***ZmOPCL*, **(D)***ZmACX*, **(E)***Zmplt1*,**(F)***ZmLBP*and **(G)***ZmHPL*. Vertical bars indicate ± sd.

Table 1

Tab. 1 Transcript copy number (± standard deviations) of the *Fusarium verticillioides β-tubulin2* gene in inoculated kernels of CO433 (R) and CO354 (S) lines at 3, 7 and 14 days post inoculation (dpi).

Genotype	3 dpi	7 dpi	14 dpi
S	2700 ± 344	3045 ± 974	5529 ± 848
R	1999 ± 407	1841 ± 462	3085 ± 378

Figure 1

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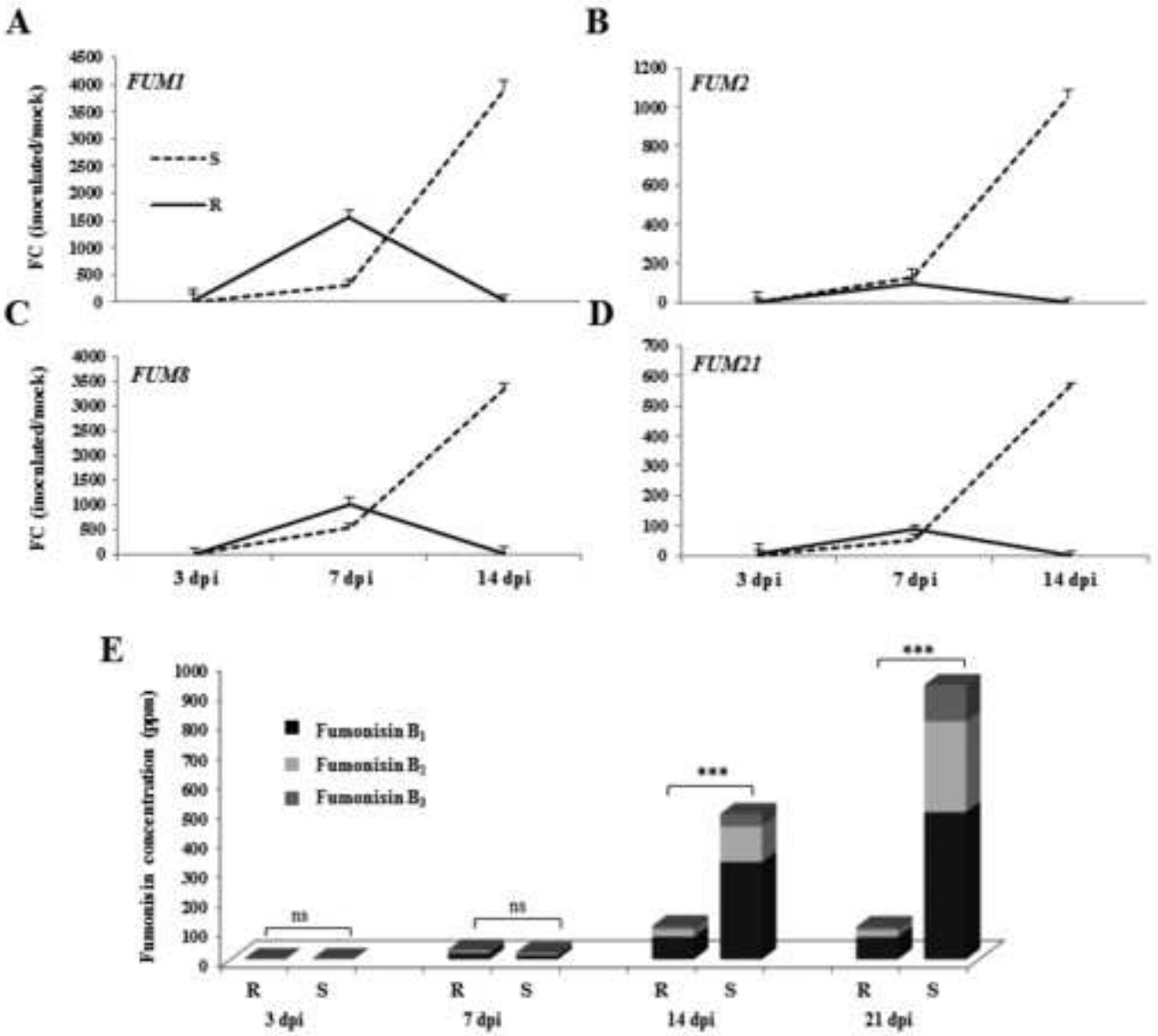

Figure 2

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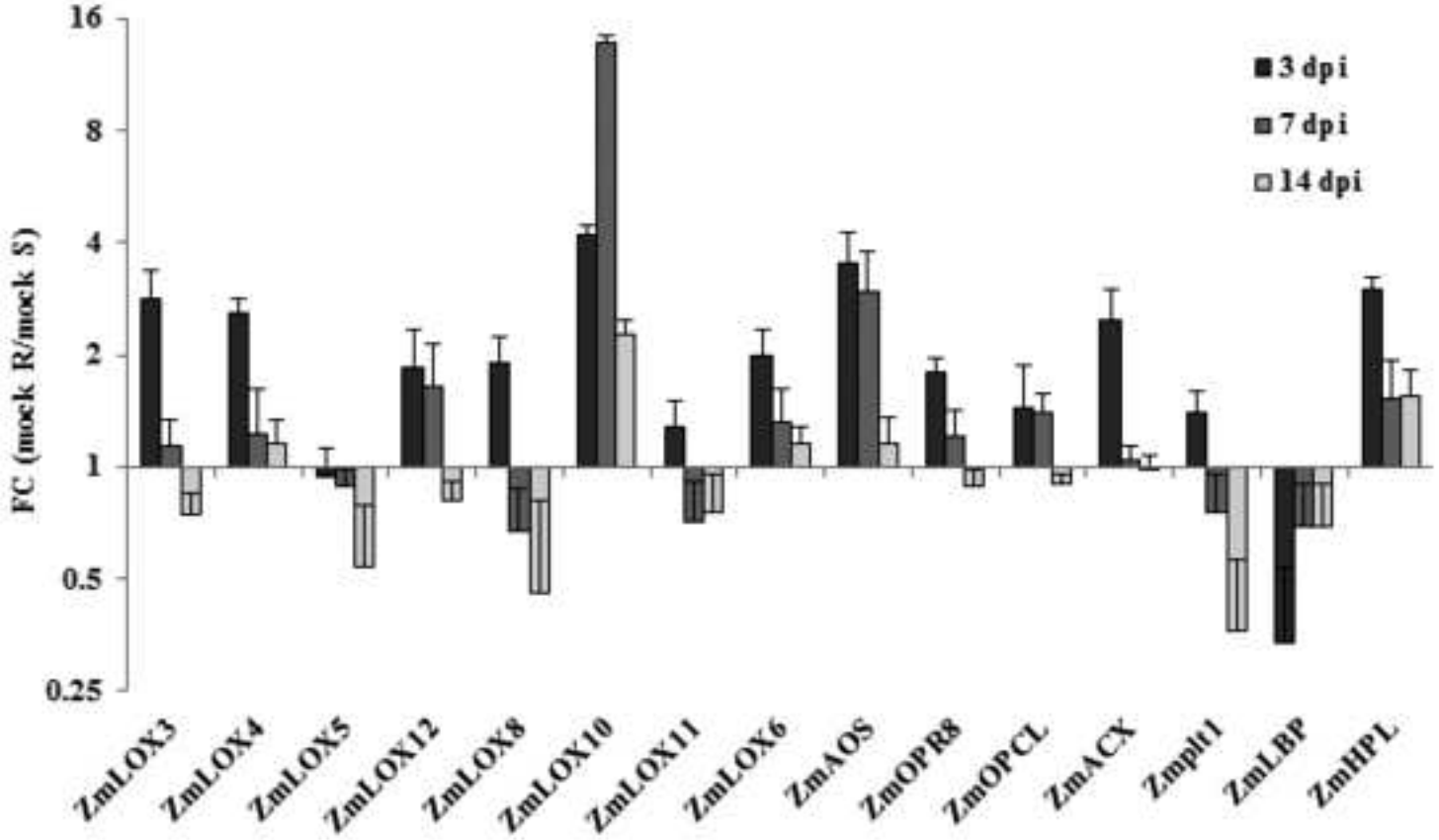

Figure 3

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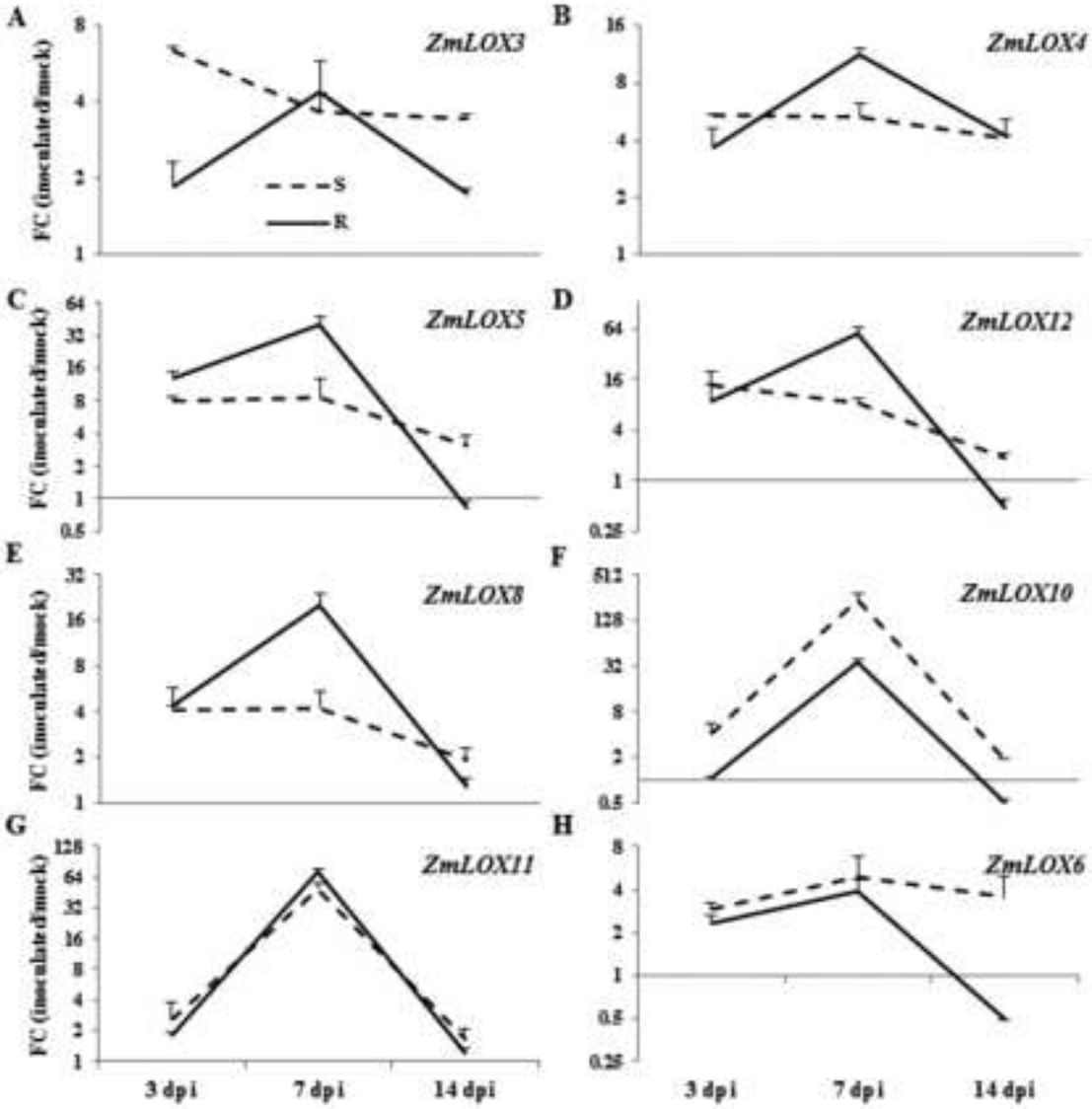

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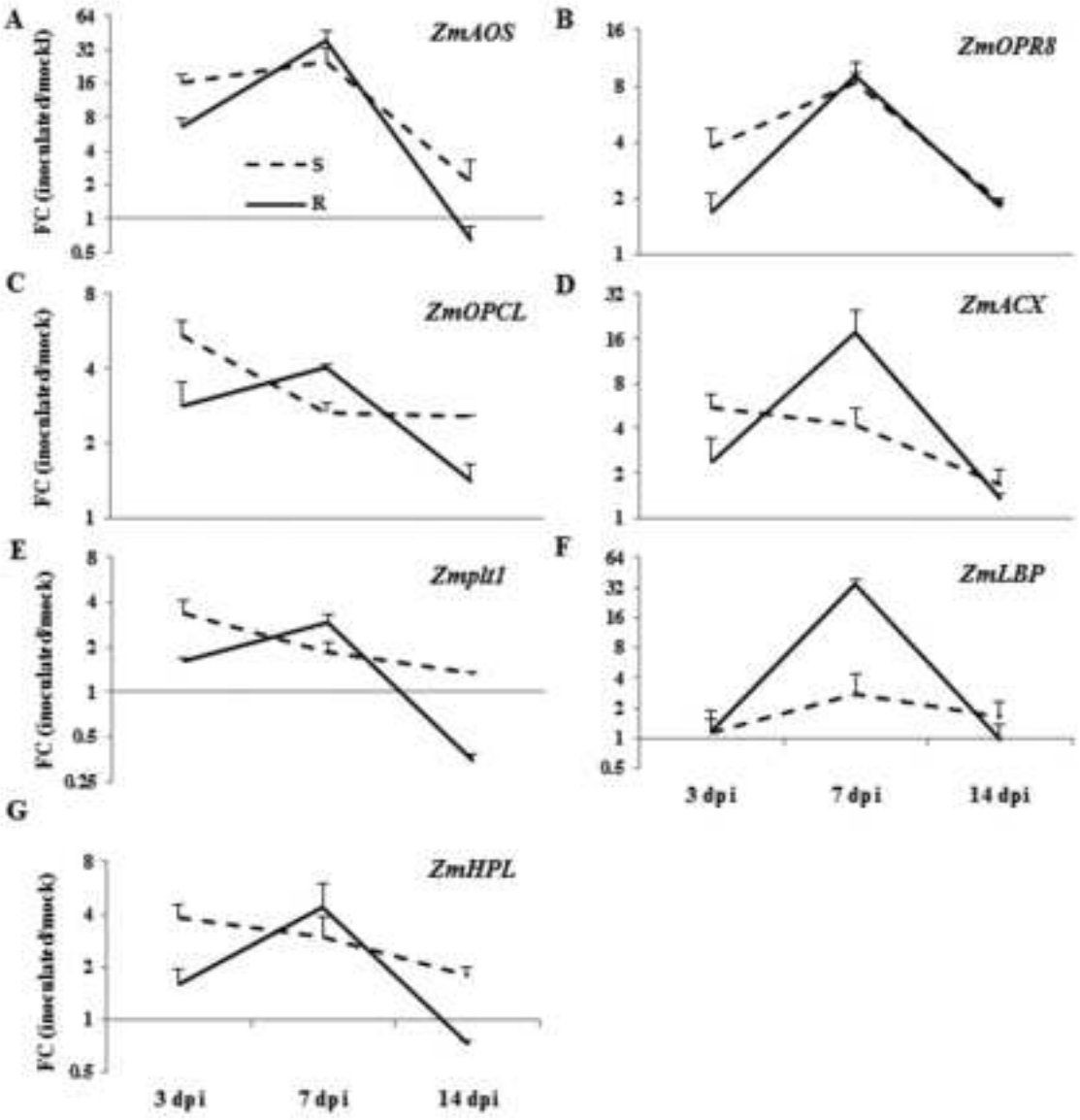

Figure S1

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Table S1

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