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Upregulation of gray mold-induced plant genes in uninfected rachis tissue

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ABSTRACT

Molecular detection of infections is normally performed by searching for the DNA of the pathogen in infected tissues. We consider the possibility of detecting an ongoing infection by analyzing the systemic response. The genes coding for FAD-binding domain-containing protein and two Cytochrome P450s were upregulated in visually healthy tissue of pathogen-inoculated leaves at 24 and 48 hours post infection, both during tip and side leaflet inoculations. The upregulation of these genes was statistically not different between rachis and inoculation spot. The results suggest the possibility to sense B. cinerea single leaflet infections by measuring plant gene expression in rachis-derived samples.

Abbreviations: qPCR: quantitative real time PCR; LD: leaf disk; IS: infection spot; POI: point of interest other than IS; hpi: hours post inoculation; DEG: differentially expressed gene

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Introduction

Leaf diseases cause high losses in most crops. Without applying fungicides protectively, the early detection of diseases is a prerequisite for their control. In this case 'early' is meant in good time for curative treatments which is usually in a pre-symptomatic state. Curative control of diseases could help reduce the amount of fungicides applied in agriculture. An in-depth understanding of the interaction of plants with their compatible pathogens can help to develop sophisticated methods for pre-symptomatic disease detection. Attack by a compatible plant–pathogen may lead in the plant to thousands of differentially expressed genes (DEGs) within hours and days from the infection (Boller and Felix 2009). In tomato leaves, approximately 4700 DEGs were identified at 24 h post infection (hpi) comparing *Botrytis cinerea*- with mock-infected tissue (Rezzonico et al. 2017). Twenty-four hpi is earlier than visual symptom development for this experimental set up. A systemic expression of some of these genes induced by *B. cinerea* infection could help identify the disease before symptom development even when sampling visually healthy tissue. This could become a valuable control method, for example, to deliver healthy tomato seedlings. The spatial regulation of DEG measured between infected and non-infected leaves of the same plant was reported earlier in the literature. Some 825 DEGs were identified in close vicinity (6–12 mm) of the infection site in *B. cinerea*-inoculated lettuce leaves (Mulema and Denby 2012). However, in non-inoculated leaves of the same lettuce, no DEG was determined to be induced by *B. cinerea* inoculation among the set of genes that was strongly upregulated in inoculated leaves (Cremer et al. 2013). In other plant–pathogen systems, other spatially differentiated expression patterns were found. For instance, an increased chitinase and phenylalanine ammonia-lyase (PAL) mRNA expression in non-infected leaf tissue as well as a systemic induction of 1,3-beta-glucanase mRNA were measured in potato plants infected with *Phytophtora infestans* at 35 and 96 hpi, respectively (Schröder et al. 1992). Maldonado-Bonilla et al. (2008) found an upregulation of sesquiterpene phytoalexin biosynthesis enzyme-coding genes both in inoculated ('local') and non-inoculated ('systemic') leaves of the same pepper and tobacco plants. These controversial results and the involvement of different genes do not allow a direct development of a *B. cinerea* disease detection method with a sampling of non-infected tissue.

In our study, we selected four genes out of the 25 strongest early upregulated genes during the *B.*

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cinerea-tomato interaction (Rezzonico et al. 2017). They belong to the genes that code for *F-box-like* proteins, the *cytochrome P450*, and FAD-binding domain proteins. The *F-box* genes were demonstrated to be involved in several regulatory function cascades such as protein–protein interaction and cell cycle regulation (Craig and Tyers 1999) as well as in pathogen-mediated cell death (van den Burg et al. 2008). The enzyme protein superfamily of cytochrome P450 are involved in biotic and abiotic stress response. For instance, they play important role in phytoalexin biosynthesis, hormone metabolism, and biosynthesis of terpenoids, alkaloids, and cyanogenic glucosides (Xu et al. 2015). The flavin adenine dinucleotide (FAD)-binding domain-containing proteins are involved in diverse processes including electron transfer to redox centers, and activation of oxygen for oxidation and hydroxylation (Fraaije and Mattevi 2000).

In two different experiments, we investigated the spatial expression of these DEGs in tomato compound leaves induced by *B. cinerea* drop infections at the tip leaflet or at a side leaflet in susceptible tomato leaf tissue challenged for 24 h with *B. cinerea*. For these four genes, we report on the systemic expression at 24 and 48 hpi within one tomato compound leaf infected with a single drop of *B. cinerea* suspension or mock inoculum.

Materials and methods

Plant material

Tomato plants (*Solanum lycopersicum*, Heinz 1706 cultivar) were grown in standard soil (Floragard Bio, HaGaFe GmbH, Saterland, Germany, with 20% perlite, Ricoter, Aarberg, Switzerland) in a semi-regulated greenhouse with open windows. The temperature was set to 20–26 °C with maxima during sunny summer days of up to 40 °C. On cloudy days, artificial light was used to achieve minimal constant lighting of 80 kW per square meter for 16 h per day. Once a week, cuttings were produced from tomato 'mother' plants that were treated weekly with sulfur (Stulln WG, Andermatt Biocontrol, Grossdietwil, Switzerland). The cuttings were then placed in approximately 100% rel. humidity for one week to develop roots. Thereafter, they were acclimatized to the same greenhouse conditions mentioned above. Young and fully unfolded leaves were harvested from two-week-old cuttings for inoculation trials. Six compound leaves were placed in miniature grow boxes (30 × 60 cm and 14 cm in height with a clear plastic cover; Landi, Dozigen, Switzerland) on paper towels wetted with distilled sterile water. Separate boxes were used for pathogen and mock inoculation.

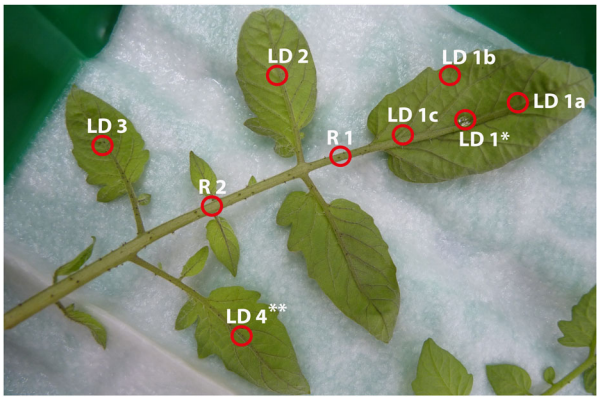

Figure 1. Spatial distribution of sampling sites on tomato leaflet of leaf disks (LD) and rachis samples (R). Sampling locations used in 'experiment A' with inoculation at center of tip leaflet LD1*: LD1*, LD1a, LD1b, LD1c, LD2, LD3, R1, and R2. Sampling locations of 'experiment B' with inoculation at center of side leaflet LD4**: LD1, LD2, LD3, LD4**, R1, and R2.

Inoculum preparation, inoculation, and sampling for relative mRNA expression analysis

B. cinerea strain T4, which was kindly provided by Philippe Nicot, INRA Centre de Recherche PACA, Montfavet, France, was grown on 15 g/l malt agar (Fluka, Sigma-Aldrich, Buchs, Switzerland) plates for 3–8 weeks. Spores were harvested with 20 ml half-strength grape juice (Farmer, Landi, Dotzingen, Switzerland, 1:1 diluted with tab water) and diluted to 1.2×10^6 spores per ml. The spore suspension was used directly for inoculation with a single 10-µl-drop placed on the adaxial surface of either the primary leaflet (indicated as LD1*) or the side leaflet (LD4**) (Figure 1). The inoculated leaves were stored in the dark at 18 °C. Mock inoculations were performed under the same conditions using half-strength grape juice for inoculation.

Sampling: Two experimental series were conducted: In experiment A, the tip leaflet was inoculated in its center; in experiment B, a side leaflet was inoculated (Figure 1). Experiments A and B were independently repeated three times. Six leaves were inoculated per experimental replicate and inoculum: samples of twice two leaves were pooled for further analysis and as backup, the third pair was kept for infection control by observing the sporulation after four days after infection. The backup was used only in case of samples being lost, for example, during RNA extraction. Leaf disks (LD) of the inoculation site (LD1* and LD4**) and additional sites (experiment A: LD1a, LD1b, LD1c, LD2, LD3, R1, and R2; experiment B: LD1, LD2, LD3, R1, and R2) were cut at 24 hours post inoculation (hpi; experiments A and B) and 48 hpi (experiment B) with a 5 mm-diameter cork borer. The remaining inoculum was removed from the LDs with a paper towel. LDs of two different compound

Table 1. Primer pairs used in this study.

Gene ID/primer name	Protein name	Primer sequence forward	Primer sequence reverse	Efficiency[a]	Reference
Solyc02g080840.1.1	F-box-like	CGCGATCAAGGAAGCAGTTG	TACTGGTAGCTGCAATGCCA	97	Rezzonico et al. (2017)
Solyc03g112040.1.1	Cytochrome P450	GGCTGCTGGAACTCAAGTCT	GACAACCTCTTCTGCCTGCA	97	Rezzonico et al. (2017)
Solyc06g065060.1.1	FAD-binding domain-containing protein	ACTTTGCAGGGTTCCCAACA	TCCATCGAGACCTCCCTCTG	98	Rezzonico et al. (2017)
Solyc10g078230.1.1	Cytochrome P450	TGGGAAGGATTTGATATGGGCC	ACATTGCCAGGCTTAGTACAGT	117	Rezzonico et al. (2017)
LSm7	U6 snRNA-associated Sm-like protein LSm7	GGTGGAAGACAAGTGGTTG GAACAC	CGTCTGGCTGAACAAAAGG ATTGG	78a	Müller et al. (2015)
SlCBL1	Calcineurin B-like protein	CCATCCAAATGCTCCGATCGATGA	TGCCTCTCAATGAAGCCTTGTTGC	89a	Pombo et al. (2014)

[a]Measured under our laboratory conditions (Rezzonico et al. 2018).

leaves were pooled, and shock-frozen in liquid nitrogen. Samples were stored at −80 °C until further processing.

RNA extraction and quantitative real time PCR

Total RNA was extracted using NucleoSpin® RNA Plant (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions, including DNase treatment. RNA quality and quantity were estimated using the micro volume spectrophotometer (Q3000 UV quawell, Quawell Technology, San Jose, CA, USA). Total RNA was transcribed into first strand cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA).

Four upregulated genes from a previous RNA-seq study were selected (Rezzonico et al. 2018). For each of these genes, primers sets were designed (Table 1) using Primer3 software (Untergasser et al. 2012). Primer pairs LSM7 and SlCBL1 that amplify constitutively expressed genes encoding an U6 snRNA-associated Sm-like protein LSm7 and a Calcineurin B-like protein, respectively, were used as reference genes (Pombo et al. 2014; Müller et al. 2015; Rezzonico et al. 2018). The qPCR was conducted with 0.2 µM forward and reverse primers and 1 µl template (24 ng/µl) in 10 µl reactions on a LifeCycler480 (Roche, Basel, Switzerland) with a Fast EvaGreen® qPCR Master Mix (Biotium, Haywardm, CA, USA). For all primer pairs used in this study, the fast amplification protocol suggested by the master-mix provider was used, consisting of initial denaturation at 95 °C for 2′, followed by 40 cycles at 95 °C for 5″, and 60 °C for 30″. A melting curve analysis was performed from 60 to 100 °C in 0.1 °C steps at the end of the run. All samples were analyzed in duplicates. Primer efficiency and stable expression of reference genes were evaluated previously (Rezzonico et al. 2017, 2018). Relative expression levels of pathogen-infected vs. mock-infected samples were analyzed based on the $\Delta\Delta C(t)$-method (Livak and Schmittgen 2001) with software GenEx v6 (bioMCC, Freising, Germany).

The relative expression values of the non-infected samples should be analyzed in relation to the inoculated sample, allowing to measure the difference of gene expression. This comprised a multilayer relative analysis consisting of several relation and normalization steps (Figure 2): First, the qPCR-derived threshold cycle values (*Ct*-values) of target genes were efficiency-corrected, duplicates averaged and normalized to the *Ct*-values of reference genes LSM7 and SlCBL1. Then the values obtained with the *B. cinerea*-inoculated leaves were normalized with the corresponding reference mock-infected samples. The resulting values at the positions of interest (POI, sampling positions other than inoculation spot) were normalized to the inoculation spot (IS). To display and test with Tukey's multiple comparisons of means for significant differences, data were $\log_2$-transformed ($\log_2$-transformed 'fold-change' (logFC ($\log_2$)). Thereafter, IS has by definition a value of zero and POIs are displayed as negative values because differential expression in the POIs was weaker than at the IS.

Results

Spatial expression depends on inoculation site

Tip leaflet inoculation

Tomato (*S. lycopersicum* cv. Heinz 1706) compound leaves were inoculated (as indicated with a*) at the center of the tip leaflet (LD1*, Figure 1, experiment A). Four samples were taken at different positions on the tip leaflet (LD1*, LD1a, LD1b, and LD1c), two on the center of different side leaflets (LD2 & LD3) and two along the leaf rachis (R1 & R2). At 24 hpi expression levels of genes were highest (approximately 31–50 log2-fold change, Supplemental Figure 1, as described in Rezzonico et al. (2017)) at the inoculation spot (IS) LD1* compared to the other sampled locations for *F-box-like* (02g080840.1.1), two *Cytochrome P450*s (10g078230.1.1 and 03g112040.1.1), and *FAD-binding domain-containing protein* (06g065060.1.1) (raw *Ct*-values: Supplemental Table 1). A qualitative

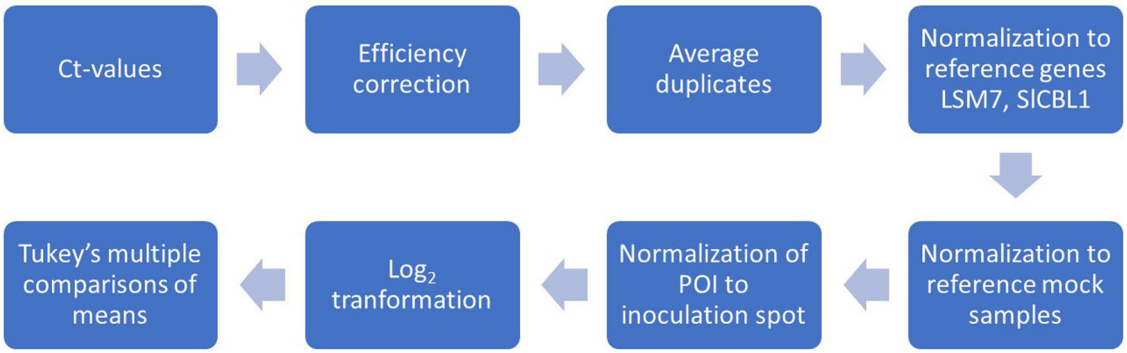

Figure 2. Schematic illustration of multilayer relative analysis using the $\Delta\Delta C(t)$-method (Livak and Schmittgen 2001) with software GenEx v6 (bioMCC, Freising, Germany). POI, point of interest, that is, sampling positions other than inoculation spot.

comparison of the differences of the efficiency- and reference gene-corrected *Ct*-values demonstrate the strong upregulation in IS (Supplemental Figure 2(A)).

Comparing the relative gene expression (log2-fold change) at 24 hpi of LD1a-c, LD2-LD3, R1 and R2 to LD1* using a Tukey multiple comparison of means, demonstrated no significant differences in upregulation for *FAD-binding domain-containing protein* (06g065060.1.1) and *Cytochrome P450* (03g112040.1.1) among all sampling sites throughout the compound leaf (Figure 3). A statistically lower upregulation compared to IS was found at certain sampling positions for genes *F-box-like* (02g080840.1.1, LD1* differing from LD1b and LD3), and *Cytochrome P450* (10g078230.1.1, LD1* differing from LD2 and LD3) (Figure 3).

Side leaflet inoculation

In a second experiment (experiment B), we inoculated the side leaflet at position LD4** (Figure 1) and sampled at 24 and 48 hpi. As in experiment A, the differences of corrected *Ct*-values of mock-minus pathogen-infected samples show highest values in the IS (LD4**, Supplemental Figure 2(B and C)). At 24 hpi no significant difference in expression between the sampling locations in the rachis (R1 and R2) and the IS (LD4**) was found for the two *Cytochrome P450*s (10g078230.1.1 and 03g112040.1.1) and *FAD-binding domain-containing protein* (06g065060.1.1). At 24 hpi, expression of all four target genes inside leaflet LD4** was significantly different (i.e. higher) with respect to the leaflets on the other side of the rachis (sampled at LD2 and LD3; Figure 4).

At 48 hpi, the target genes were expressed in all sampling locations. No significant difference between the sampling sites could be found at 48 hpi for all four tested genes (Figure 4B). Cytochrome P450 (03g112040.1.1) was expressed with a relatively low but comparable intensity across the whole leaf. However, the standard deviation calculated for the differences of corrected *Ct*-values of mock- minus pathogen-infected samples were higher compared to 24 hpi (Supplemental Figure 2(B and C)).

Discussion

The presented results indicate a leaf-wide gene regulation caused by fungal infection in tissue away from the infected location at 24 and 48 hpi. Comparing diseased vs. mock-infected compound leaves, the expression of *Cytochrome P450* (03g112040.1.1) and *FAD-binding domain-containing protein* (06g065060.1.1) was found not to differ significantly over the whole compound leaf when the tip leaflet was inoculated (experiment A). However, the expression in the inoculation spot was stronger when comparing the *Ct* value differences (Supplemental Figure 2). Other works described the upregulation of mRNAs of several genes like (1) Phenylalanine ammonia-lyase (PAL), (2) 4-coumarate:CoA ligase (4CL), (3) S-adenosyl-L-methionine:bergaptol *O*-methyltransferase (BMT), and (4) pathogenesis related protein (PR1) only in infected tissue of the non-host parsley, inoculated with the pathogen *Phytophthora magasperma* f. sp. *Glycinea* (Schmelzer et al. 1989). In contrast, the *Arabidopsis thaliana* promoter of gene *DJ1E* was reported to be similarly active in the whole *A. thaliana* leaf after drop infection with *B. cinerea* (Lehmeyer et al. 2016), which is in agreement with our results. In our study, other genes such as the *F-box-like*, gene *07g056210.2.1* and *Cytochrome P450* (10g078230.1.1) differed in two out of eight sampling locations when inoculation occurred in the tip leaflet. This means the most sampling locations selected for this study were regulated similarly to the inoculation site and hence a *B. cinerea* infection could be sensed also in healthy tissue of infected leaves. Interestingly, the two cytochromes P450 were not equally regulated, what may be explained by the potentially different functions of the cytochromes belonging to the protein super family (Xu et al. 2015). Our results suggest that the mRNA of *Cytochrome P450* (10g078230.1.1) and *FAD-binding*

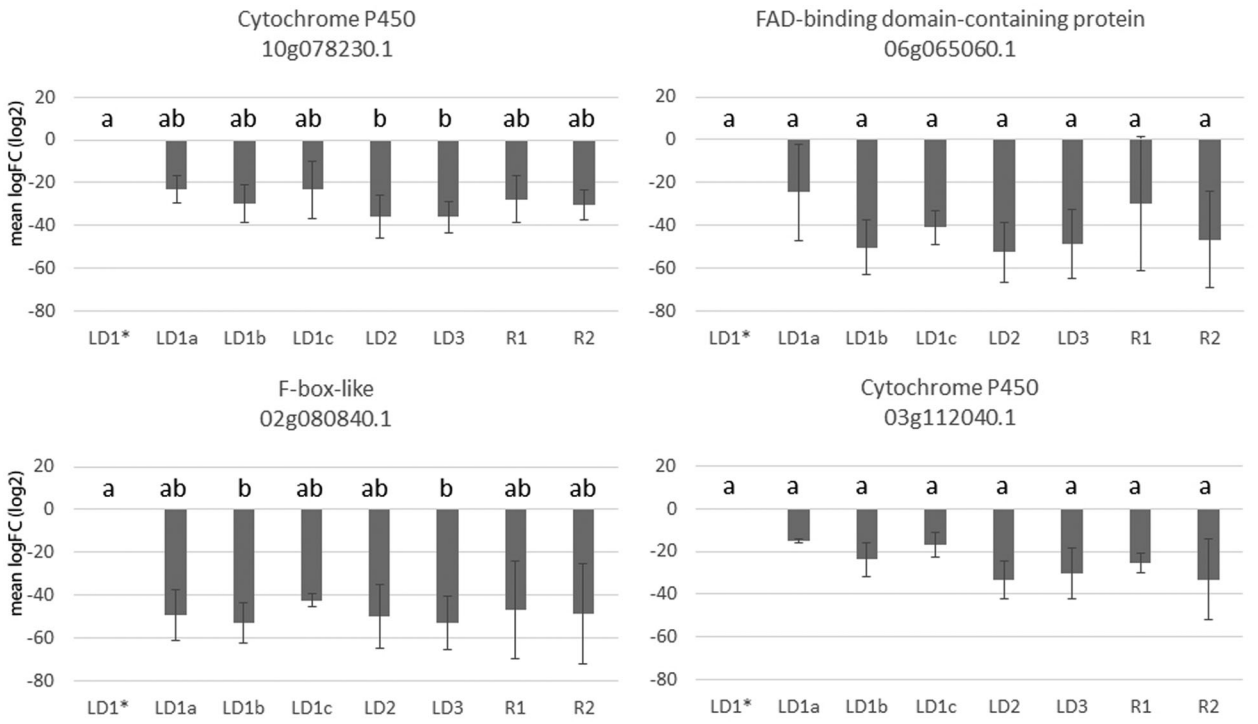

Figure 3. Gene expression (mean logFC (log_2)) normalized to the inoculation site LD1* on tip leaflet of the compound leaf at 24 hpi. The gene expression upregulation at the inoculation site was strongest and therefore the normalized values of samples at other sampling points are negative. LD1*, LD1a–c, LD2, LD4, and R1 and R2 are leaf disks and rachis pieces, respectively, collected according to Figure 1. qPCR-derived expression *Ct*-values were normalized to reference gene primer sets LSM7 and SlCBL1, compared between *B. cinerea*- and mock-inoculated samples and averaged of three experiment repetitions. Error bars represent standard deviations. Letters indicate statistical significance computed with Tukey's multiple comparisons of means calculated for each gene separately, $p = .05$.

domain-containing protein are expressed with the same relative expression level along the main vein in rachis and the tip leaf of the compound leaf when inoculated at the tip leaflet. When infecting a lateral leaflet of the compound leaf, the two *Cytochrome P450*s (10g078230.1.1 and 03g112040.1.1) and the *FAD-binding domain-containing protein* (06g065060.1.1) were expressed in a comparable manner in rachis samples and at the infection site for both sampling times 24 and 48 hpi. Considering the difference of corrected *Ct*-values, the strongest upregulation was always found at the infection points. Why the genes are expressed differently as a function of the inoculation site, cannot be answered by our data.

Since all four genes described as upregulated in the infected tissue were also upregulated, even if to a lesser extent, in healthy tissues, a combination of such genes (i.e. genes upregulated in rachis plus reference genes) could be used in a multiplexed qPCR assay for detection of *B. cinerea* infections in asymptomatic leaf tissue. However, establishing such an assay would need from further experiments with larger number of samples. Compared to classical microbial disease detection and isolation techniques using selective growth medium, this would provide a fast indication of diseased leaf samples, for instance, during the production of tomato seedlings. However, if this kind of gene regulation is specific to *B. cinerea* and the influence of other biotic and abiotic stresses needs further evaluation. The presented results suggest sampling of rachis tissue because the genes of the two *Cytochromes P450*s (10g078230.1.1 and 03g112040.1.1), and the *FAD-binding domain-containing protein* (06g065060.1.1) were analogously regulated in rachis samples with respect to the infection site – independently of infection location. Whether this is true in whole plants will be tested in future works. The promoters of such genes could be of further use to engineer plants that provide a visually detectable signal as shown in grapevine with the nondestructive quantification of gene expression (Li et al. 2011).

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Disclosure statement

No potential conflict of interest was reported by the authors.

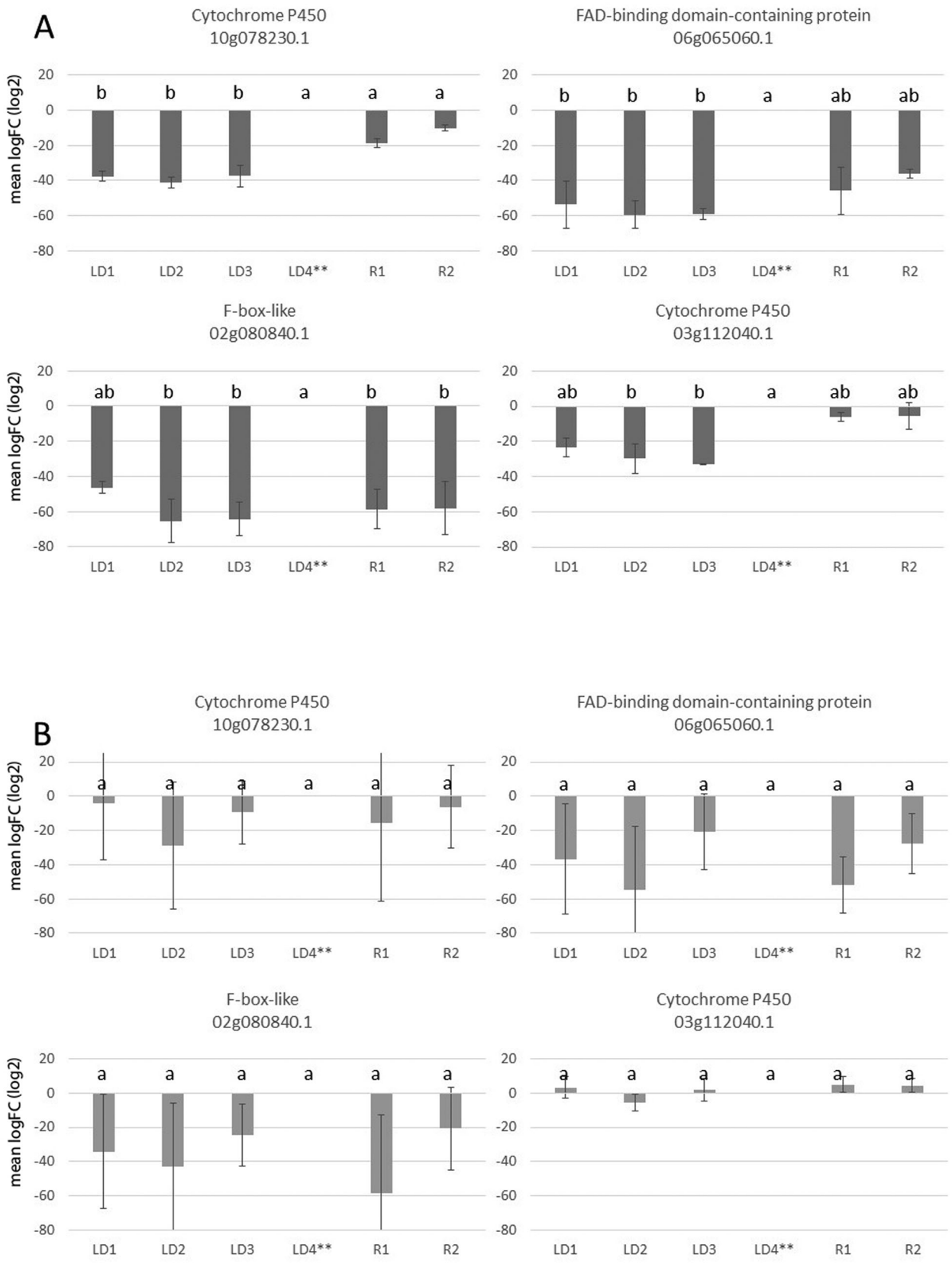

Figure 4. Gene expression (mean logFC (log_2)) normalized to inoculation site on side leaflet LD4 of the compound leaf at A: 24 hpi and B: 48 hpi. The gene expression upregulation in inoculation site was strongest and therefore the normalized values at other sampling points are negative. LD1–4 and R1 and R2 are leaf disks and rachis pieces collected according to Figure 1. qPCR-derived expression *Ct*-values were normalized to reference gene primer sets LSM7 and SlCBL1, compared between *B. cinerea*- and mock-inoculated samples and averaged of three experiment repetitions. Error bars represent standard deviations. Letters indicate statistical significance computed with Tukey's multiple comparisons of means calculated for each gene separately, $p = .05$.

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