

RESEARCH ARTICLE

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Lyso-phosphatidylethanolamine primes the plant immune system and promotes basal resistance against hemibiotrophic pathogens

Ronny Völz^{1*} , Ju-Young Park², William Harris², Sungkee Hwang³ and Yong-Hwan Lee^{1,2,4,5*}

Abstract

Background: Lyso-phosphatidylethanolamine (LPE) is a natural phospholipid that functions in the early stages of plant senescence. Plant innate immunity and early leaf senescence share molecular components. To reveal conserved mechanisms that link-up both processes, we tried to unravel to what extent LPE coordinates defense response and by what mode of action.

Result: We found that LPE-treatment induces signaling and biosynthesis gene expression of the defensive hormone salicylic acid (SA). However, jasmonic acid and ethylene triggered gene induction levels are indistinguishable from the control. In accordance with gene induction for SA, oxidative stress, and reactive oxygen species (ROS) production, we detected raised in-situ hydrogen peroxide levels following LPE-application. Yet, ROS-burst assays of LPE-pretreated plants revealed a reduced release of ROS after PAMP-administration suggesting that LPE interferes with an oxidative burst. Our data refer to a priming effect of LPE on SA/ROS-associated genomic loci that encode pivotal factors in early senescence and considerably improve plant basal immunity. Thus, we challenged *Arabidopsis thaliana* with the hemibiotrophic pathogen *Pseudomonas syringae*. Consistently, we found an increased resistance in the LPE-pretreated *Arabidopsis* plants compared to the mock-pretreated control.

Conclusions: Our results underscore a beneficial effect of LPE on plant innate immunity against hemibiotrophs. Given the resistance-promoting effect of exogenously applied LPE, this bio-agent bears the potential of being applied as a valuable tool for the genetic activation of defense-associated traits.

Keywords: Lyso-phosphatidylethanolamine, Plant immunity, Salicylic acid, ROS, *Pseudomonas syringae*

Background

Phospholipids possess a wide spectrum of biological functions, including the storage of energy, contribution to signaling transduction, and the structural integrity of cell membranes. Notably, phospholipids regulate plant-microbe interactions by stimulating the defense signaling

system. Many different phospholipids can be cleaved by specific phospholipases to generate second messengers [1–4]. Phospholipase C and phospholipase D are the key enzymes involved in the generation of phospholipid-derived second messengers, phosphatidic acid, diacylglycerol, and inositol 1,4,5-trisphosphate [5]. Phospholipase A2 hydrolyzes the structural phospholipid, phosphatidylethanolamine and generates lyso-phosphatidylethanolamine (LPE) *in planta* as a minor component of cell membranes [6, 7].

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LPE was reported to stimulate mitogen-activated protein (MAP)-kinase signaling cascades [8] and to promote the activation of phenylalanine ammonia-lyase and extracellular acid invertase [9], which are considered to be important metabolic enzymes and pathogenesis-related proteins [10, 11]. In tomatoes, LPE accumulates in wounded leaves and is systemically enriched in non-wounded leaves of injured plants [4]. Nowadays, LPE is administered on a wide range of crops, such as green pepper, sweet cherry, strawberries, and tomatoes [12–14]. Exogenously applied LPE delays early senescence while simultaneously accelerating fruit ripening and increasing the half shelf-life of crop plants [12, 15, 16]. LPE's influence depends on the stage of fruits' ripening. LPE stimulates ripening in mature fruits; however, LPE interferes with ethylene-production in ripening fruits resulting in an improved structural strength, which consequently extends the shelf life. After 5 days of LPE-treatment, fruits possessed a significantly lower production of ethylene and a diminished level of electrolyte leakage than the controls. The specific role of LPE in aging and senescence might be explained by the inhibitory effect of LPE on phospholipase D, an enhancer of senescence progression [15, 17, 18].

Plant immunity and especially pathogen recognition can be classified into at least two distinct branches. Firstly, the perception of characteristic pathogen-associated molecular components/pattern (PAMP) by the plasma membrane receptor complex FLAGELLIN-INSENSITIVE2 / BRI1-ASSOCIATED RECEPTOR KINASE activates a MAP-kinase signaling cascade that links up-stream signals to downstream targets by phosphorylation and refers to the PAMP-triggered immunity (PTI). FLAGELLIN22 (flg22), a 22 amino acid peptide from within the bacterial flagellin protein, is a crucial PAMP that triggers PTI in plants [19, 20]. Secondly, the perception of bacterial effectors by cytosolic plant receptors triggers a robust defense response, called effector-triggered immunity (ETI) [21, 22]. Plant defense against fungi and other microbial pathogens relies on the induction of both local and systemic resistance [23].

The three phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are the classical immunity signaling agents, that orchestrate defense processes depending on the kind of biotic threat. The local and systemic acquired resistance (SAR) depends on SA action [24, 25]. SA-biosynthesis and signaling are induced after direct exposure to a wide range of biotrophic and hemibiotrophic pathogens, like *Pseudomonas syringae* (PstDC3000). Pathogen-induced SA biosynthesis mainly takes place in chloroplasts catalyzed by *ISOCHORISMATE SYNTHASE 1* (*ICS1*) [26, 27]. Several transcription factors (TFs) have been reported to be directly recruited to the *ICS1* promoter in order to activate its

expression. Of these, SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60 g are essential for *ICS1* induction and SA accumulation after pathogen perception [28, 29]. The WRKY family members WRKY28 and WRKY46 have been shown to promote the induction of *ICS1* as well, partly in complex with SARD1 [30, 31]. ENHANCED-DISEASE SUSCEPTIBILITY (EDS) 1 and PHYTOALEXIN-DEFICIENT (PAD) 4 contribute to SA synthesis in guard cells [32], form heterodimers, and are regulated by the positive feedback of SA which in turn potentiates SA action. Moreover, the SA-efflux-transporter EDS5, which is localized in the chloroplast envelope, contributes to intracellular SA-homeostasis [33–35]. A hub in SA-signaling is formed by NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES (NPR) 1 and its counteracting paralogs NPR3 and NPR4. These factors act as SA receptors [36, 37], whose function is mediated by members of the TGA family [38, 39] and by the NIM1-INTERACTING (NIMIN) proteins [40]. However, NPR3/4 exert distinct roles in the transcriptional regulation of SA-inducible genes [41] by mediating the action of the transcriptional regulator NPR1 which is considered the 'master regulator' of the SAR responses [41, 42]. A feed-forward loop between SA and reactive oxygen species (ROS) production, such as hydrogen peroxide (H₂O₂) in the defense response, was reported, and ROS signals are involved both upstream and downstream of SA signaling in response to stress [43]. The majority of intracellular H₂O₂ is produced from molecular oxygen by a stepwise reaction via a superoxide anion intermediate which undergoes enzymatic reduction to H₂O₂ [44]. In this context, H₂O₂ originating in chloroplasts and peroxisomes triggers SA biosynthesis. H₂O₂ is essential for the main outputs of the multifaceted defense response comprising transcriptional reprogramming, cell death, and stomatal closure [43]. By contrast, JA and ET signaling promote immunity against necrotrophs such as the fungal pathogen *Alternaria brassicicola* [45–47], *Botrytis cinerea* [48] or *Cochliobolus miyabeanus* [49]. Moreover, a large number of studies have revealed that the SA signaling pathway prevalently acts antagonistically to ET/JA-mediated signaling [50, 51] in plant defense response.

We analyzed the effect of LPE on plant immunity. LPE-pretreated plants express molecular markers for SA-biosynthesis and signaling. Moreover, in-situ ROS-determination of LPE-treated plants revealed the accumulation of H₂O₂, which correlates with the induced expression of oxidative stress markers. We found that LPE-pretreated *Arabidopsis* plants are more resistant to PstDC3000, indicating LPE as a plant defense-promoting factor.

Results

LPE-treatment induces the expression of SA-signaling and biosynthesis genes

To evaluate the impact of LPE on plant gene expression, we analyzed the induction of defense genes and immunity markers following LPE-application. We found that several components determining SA-biosynthesis were differentially expressed after LPE-treatment. *ICS1*, which encodes a protein that catalyzes the rate-limiting step in the SA-metabolism for plant defense [26, 27] was more strongly expressed after LPE-treatment than in the mock-treated control (Fig. 1a). In addition, we observed elevated transcript levels of *WRKY46*, which encodes a protein that contributes to *ICS1* expression [31] (Fig. 1a). Moreover, the number of transcripts of *EDS1*, *EDS5*, and *PAD4* increased in LPE-treated plants (Fig. 1a). The hub in SA-reception and signaling is formed by *NPR1* and its counteracting paralogs *NPR3* and *NPR4*. Interestingly, *NPR1* (Fig. 1b), encoding the SA-receptor [36, 52], and the expression of the SA-receptor components, *NIM1-INTERACTING (NIMIN) 1* (Fig. 1b), *NIMIN2* (Fig. 1b) [39, 53] and *WRKY38* (Fig. 1b), were increased following LPE-treatment. However, the expression of *NPR3* and *NPR4* (Fig. 1b), was indistinguishable from the mock-treated control. The transcriptional induction of the PAMP [54] and SA-response marker [55, 56] *FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)* was more strongly induced following LPE-administration (Fig. 1b). This result suggests that LPE activates the immune MAP-kinase cascade thereby initiating plant immunity.

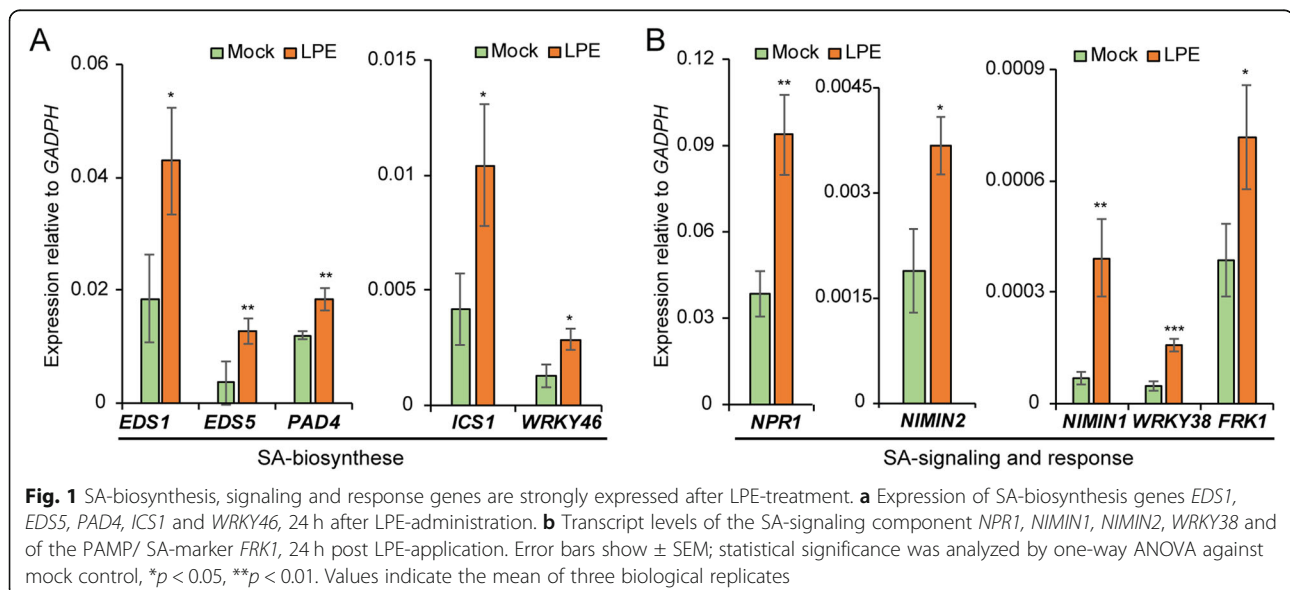
Importantly, key-marker genes for JA/ET-biosynthesis and signaling were found at mock treatment levels. These genes include *OXOPHYTODIENOATE REDUCTASE*

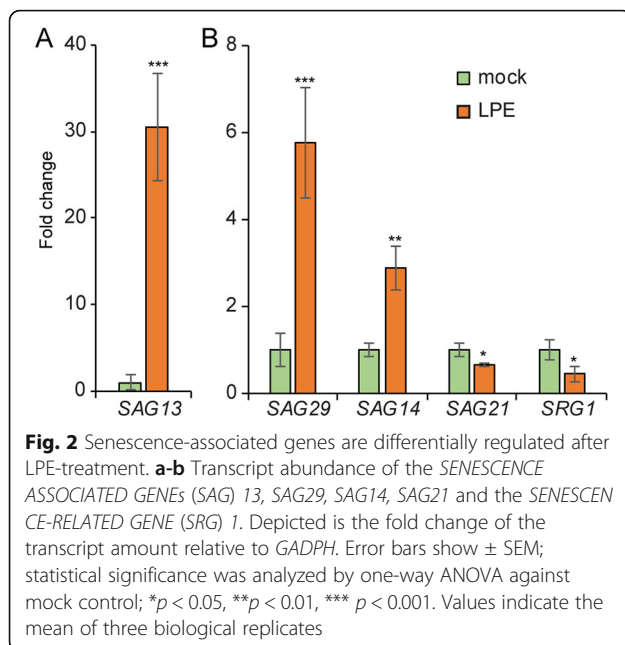
(*OPR3*), whose encoded protein catalyzes the rate-limiting step in the JA-biosynthesis, the JA-response gene *VEGETATIVE STORAGE PROTEIN 2 (VSP2)*, and the key-target factors of the synergistic JA/ET-response *PLANT DEFENSIN 1.2A (PDF1.2)*, *ETHYLENE RESPONSE FACTOR 1 (ERF1)* and *OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59 (ORA59)* (Fig. S1) [57–59]. The regular expression of these pivotal factors mediating JA/ET action suggests that LPE does not modulate JA/ET-dependent defense before pathogen-perception.

Altogether, in solely LPE-treated plants, the differential expression of SA-metabolic and SA/PAMP signaling genes, suggests a degree of plant genome priming that supports defense-associated processes and mechanisms.

Senescence-associated genes are differentially expressed

Changes in SA-induced and age-dependent gene expression show a high degree of overlap in genome-wide transcriptomic data sets, that demonstrate the dual role of SA in leaf senescence and plant immunity [60]. Therefore, we analyzed whether the LPE-induced up-regulation of SA-metabolic genes corresponds with the differential expression of genes involved in senescence and aging. *SENESCENCE ASSOCIATED GENE (SAG) 13* was shown to be induced by ROS and to be involved in immunity against necrotrophic and biotrophic pathogens [61]. *SAG29* belongs to the SWEET sucrose efflux transporter family and is strongly expressed in young inflorescent buds but the expression declines in senescent leaves [62]. The transcript abundance of *SAG13* and *SAG29* is strongly increased after LPE-treatment (Fig. 2). Likewise, *SAG14*, a blue copper-binding protein [63], was upregulated (Fig. 2). Yet, *SAG21* [63], involved in oxidative stress tolerance, and the *SENESCENCE-*





RELATED GENE (SRG) 1 [64] exhibit a reduced transcript quantity following LPE-treatment (Fig. 2). In summary, the differential expression of SAGs is in accordance with previous findings concerning the inhibitory effect of LPE on fruit ripening and senescence progression.

LPE enhances the hydrogen-peroxide accumulation

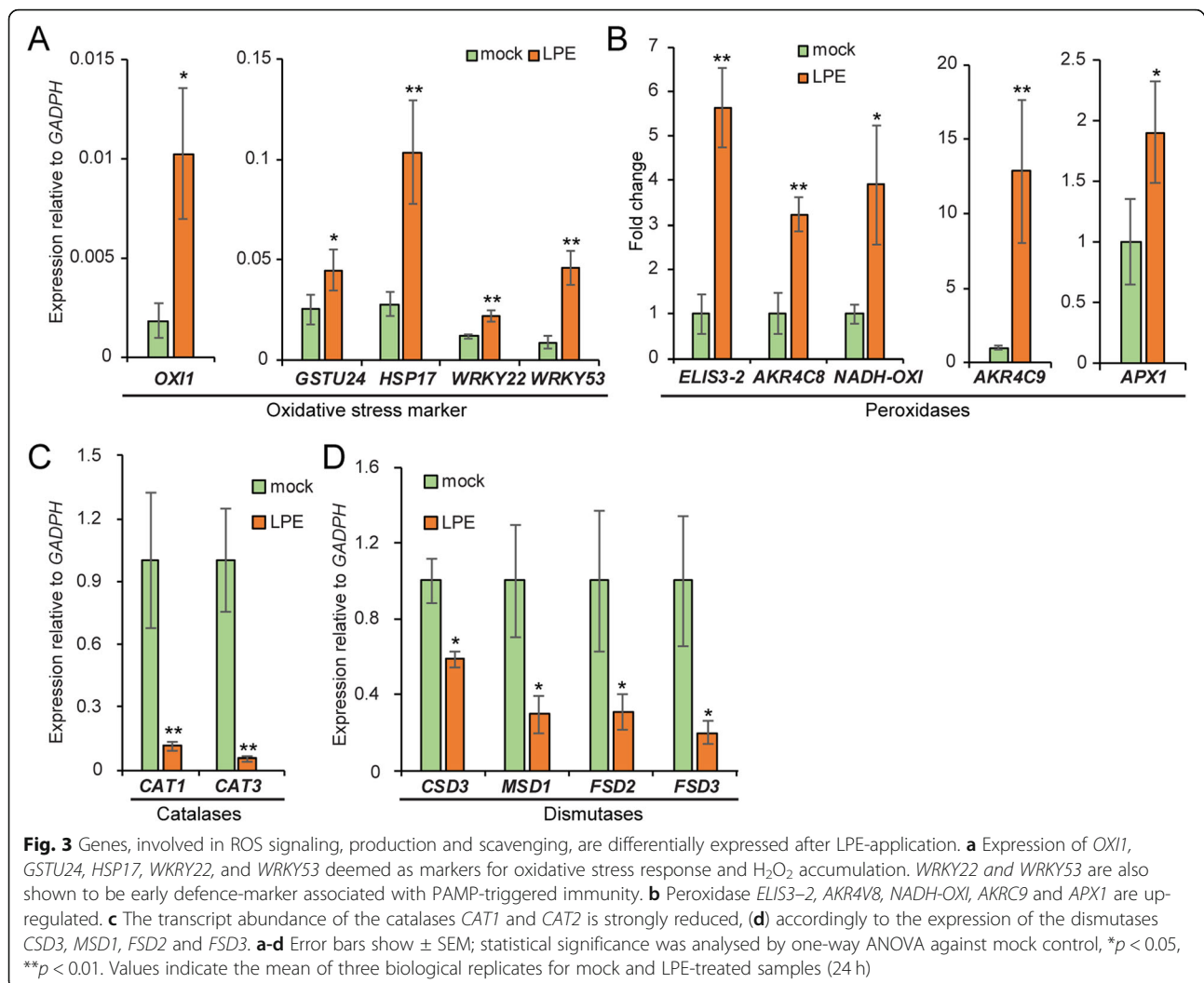
SA plays a critical role in transcriptional reprogramming, cell-death, and systemic-acquired resistance during defense response. Several lines of evidence indicate that the non-radical ROS hydrogen peroxide H_2O_2 , originating in chloroplasts and peroxisomes, triggers SA-biosynthesis. Furthermore, SA also promotes ROS production during the early-stages of defense response [43].

We analyzed whether the induction of key genes involved in oxidative signaling and response to intracellular, cytosolic H_2O_2 accumulation correlates with our findings. Thus, we determined the induction of the H_2O_2 -inducible genes *Arabidopsis thaliana* *GLUTATHIONE S-TRANSFERASE 24* (*GSTU24*) [65, 66], involved in the glutathione-related signaling during enhanced H_2O_2 metabolism. Besides, we analyzed the small heat shock gene *HSP20-LIKE CHAPERONES SUPERFAMILY PROTEIN* (*HSP17.6A*) [67] and the *OXIDATIVE SIGNAL-INDUCIBLE 1* (*OX11*) that encodes a serine/threonine kinase induced in response to a wide range of H_2O_2 -generating stimuli [68]. Compared to the mock-treated control all three key genes are significantly upregulated after LPE-treatment (Fig. 3a). Likewise, *WRKY22*, an early-immunity marker [54] previously shown to have an

elevated expression in response to H_2O_2 [69], and its H_2O_2 -inducible upstream regulator *WRKY53* [70], are more highly expressed 24 h after LPE-application (Fig. 3a). In summary, our expression data suggests that LPE primes the plant genome and promotes genes involved in oxidative stress and H_2O_2 accumulation.

Plants have a complex antioxidant system for maintaining the homeostasis of ROS. In general, this system can be divided into enzymatic and non-enzymatic ROS-scavenging mechanisms [71]. Thus, we determined the expression levels of genes involved in the metabolism and catabolism of different sorts of ROS. Three types of antioxidative enzymes, superoxide dismutases (SOD), catalases (CAT) and peroxidases, play a major role in keeping superoxide radicals and H_2O_2 at steady-state levels under non-stress conditions [72]. Oxidoreductases, known formerly as oxidases, reductases, dehydrogenases and peroxidases, predominately generate ROS in various cell compartments. The oxidoreductase encoding genes *CINNAMYL-ALCOHOL DEHYDROGENASE B2* (*ELIS3-2*) [73], two *CHLOROPLASTIC ALDO-KETO REDUCTASEs* (*AKR4C8* and *AKR4C9*) [74], a *NADH:UBIQUINONE/PLASTOQUINONE OXIDOREDUCTASE* (*NADH-OXI*) and *ASCORBATE PEROXIDASE 1* (*APX1*) [75] exhibited higher transcript levels after LPE-treatment (Fig. 3b). By contrast, *CAT1* and *CAT3*, which encode proteins that degrade H_2O_2 [76], are strongly downregulated (Fig. 3c). SODs are a diverse set of enzymes that generate H_2O_2 thereby containing either iron (Fe), manganese (Mn), or copper/zinc (Cu/Zn), and are present in the cytosol, chloroplasts, mitochondria and peroxisomes [77, 78]. We found a reduced expression of SODs (*CSD3*, *MSD1*, *FSD2*, *FSD3*) after LPE-treatment (Fig. 3d). In summary, genes that contribute to the antioxidant system for maintaining the homeostasis of ROS are differentially regulated after LPE-application thereby suggesting an altered ROS titer.

Therefore, we raised the question of whether the differential expression of these genes corresponds to deviating in situ ROS levels. To assess the accumulation of the intracellular ROS H_2O_2 24 h after LPE-application, we carried out an in situ 3,3'-diaminobenzidine (DAB) staining of mock and LPE-treated plants (Fig. 4a). In the untreated control without applied DAB, the staining value is approximately 165 arbitrary units and was set as the default unstained level. After DAB staining, mock-treated plants showed a weak staining intensity of 125 compared to strong staining of 100 in LPE-treated plants. This staining difference indicates that LPE triggers H_2O_2 accumulation *in planta*. To validate these results, we quantitatively determined H_2O_2 concentrations based on the spectrophotometric analysis of potassium iodide oxidation [79]. The absorbance at different wavelengths between 280 and 420 nm was consistently



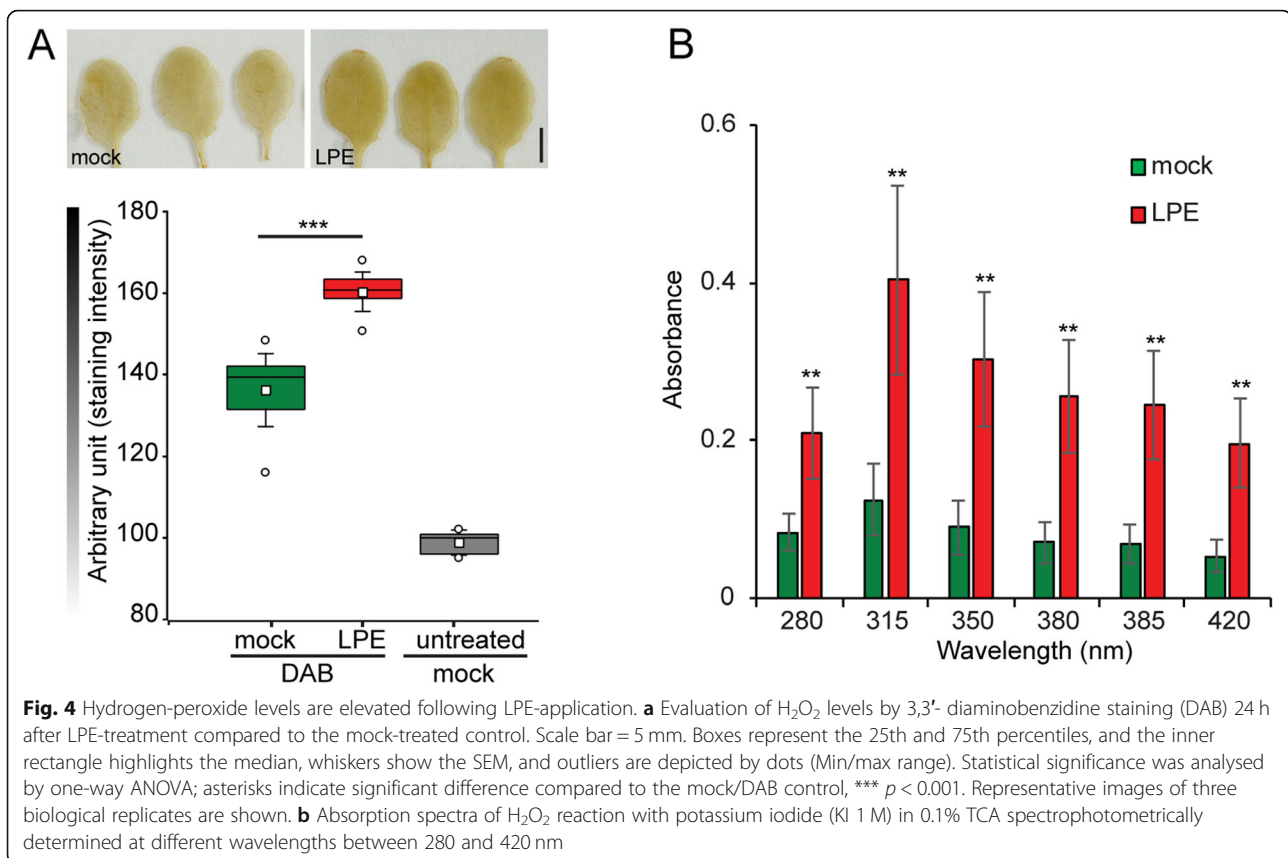
elevated compared to the mock-treated controls, demonstrating higher ROS levels (Fig. 4b). Consequently, our results indicate that LPE changes the transcriptomic profile of antioxidant genes thereby influencing ROS homeostasis.

LPE interferes with the oxidative burst following PAMP perception

Furthermore, we analyzed whether LPE triggers the ROS-burst, one of the first defense reactions in plants after the perception of pathogen-associated molecular pattern (PAMP) [20]. In the ROS-burst assay, as a positive control, we applied the PAMP flg22 (100 nm), which was demonstrated to massively increase the ROS-burst [20]. However, the applied LPE (50 mg/l) did not trigger ROS-burst and the outcome was indistinguishable from the mock-treated control (Fig. 5a). Subsequently, we examined whether LPE pretreated plants show a difference in the ROS-burst after flg22 perception. Interestingly,

the ROS-burst was consistently diminished in 3 biological replicates after flg22 application compared to the mock-pretreated control (Fig. 5b). This finding suggests that LPE interferes with the ROS-burst machinery after PAMP perception.

The predominant source of apoplastic H_2O_2 is catalyzed by apoplastic peroxidases and NADPH oxidases also referred to as respiratory burst oxidases (RBOHs) [80]. RBOH-D and RBOH-F were found to be required for a full oxidative burst in response to avirulent strains of *PstDC3000* [81]. We found the main members *AtRBOH-C*, *AtRBOH-D*, and *AtRBOH-F* being downregulated after LPE-treatment (Fig. 5c). This downregulation aligns with the attenuated ROS-burst observed after LPE + flg22-treatment compared to the mock+flg22 control. Taken together, our results show that LPE enhances the H_2O_2 accumulation over 24 h, which correlates with the expression of markers for oxidative signaling and elevated H_2O_2 levels. Yet, the



burst of H_2O_2 and further members of the ROS family, like singlet oxygen and superoxide anions, is diminished by LPE after PAMP perception. This finding identifies LPE as a negative regulator of apoplastic ROS production and burst.

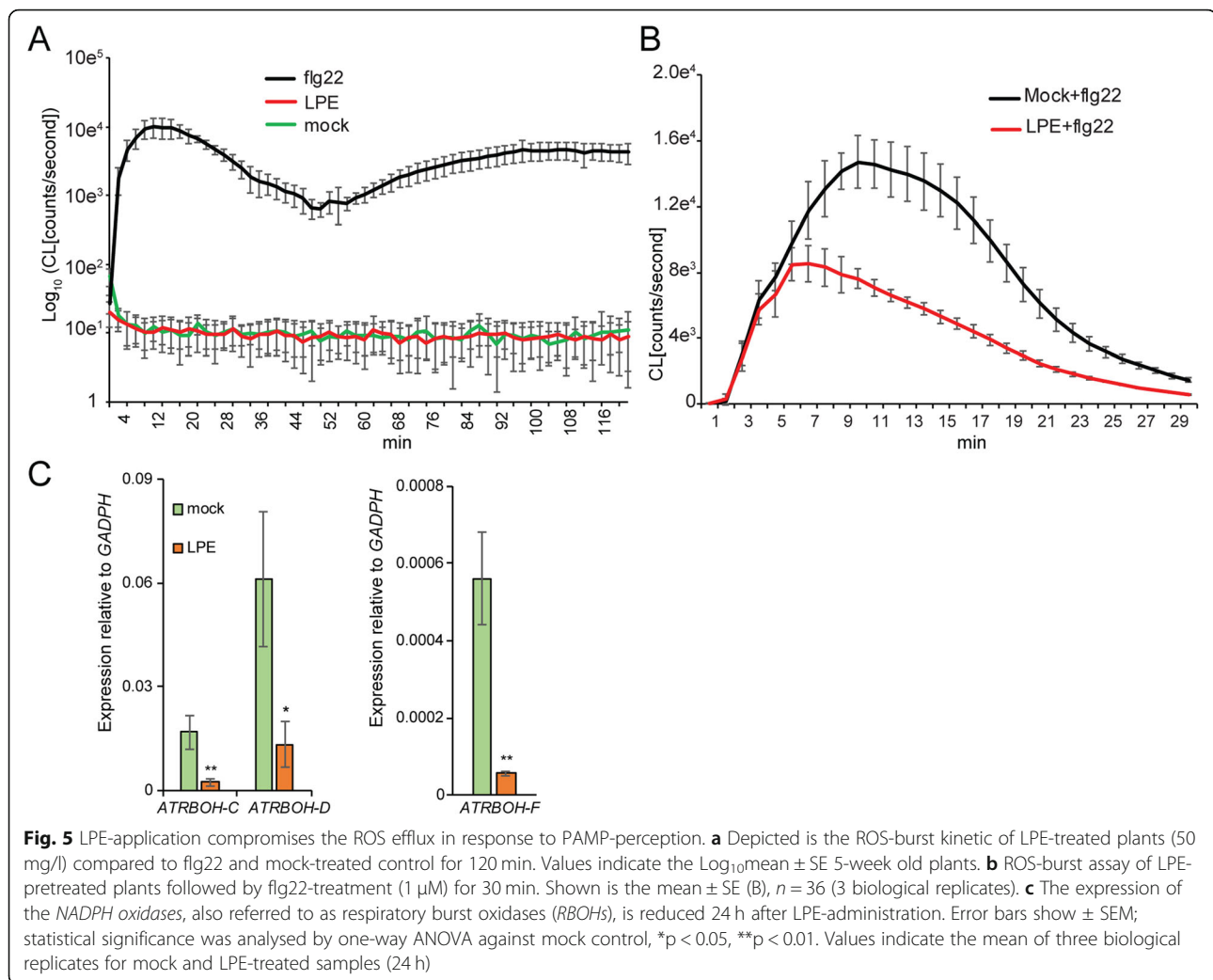
LPE-application increases the resistance of *Arabidopsis thaliana* against *Pseudomonas syringae*

To study the effect of LPE on plant immunity, we treated four to five-week-old *A. thaliana* plants (ecotype Columbia) with LPE, 24 h before inoculation (Fig. 6b, c) and subsequently challenged these plants with *PstDC3000*. Two hours after spray inoculation, the bacterial proliferation levels in LPE-pretreated plants corresponded to those in the control plants indicating that stomatal immunity, and hence, bacterial leaf-accessibility was not affected by the applied LPE. However, the bacteria proliferation was consistently diminished from 48 to 154 hpi in LPE-pretreated plants compared to the mock-pretreated control, thereby indicating a reduced susceptibility of *Arabidopsis* after LPE-application. In summary, these results demonstrate that administered LPE increases resistance against the hemibiotrophic pathogens *PstDC3000*.

Discussion

We discovered that the phospholipid LPE, a regulator of early senescence in plants, contributes to plant innate immunity. LPE-pretreated plants exhibit enhanced resistance against *PstDC3000*.

ROS can be produced in different organelles in response to a broad range of specific environmental conditions [82]. In this regard, ROS accumulation enables the activation of a multitude of signaling cascades, which results either in acclimation or in cell death depending on the biotic/abiotic stress. Whether ROS would serve as signaling molecules or could cause oxidative damage to the tissues depends on the delicate equilibrium between ROS production, and their scavenging. In mitochondria and chloroplasts, generated ROS is an unavoidable by-product of bioenergetic processes. Within chloroplasts, triplet state excited chlorophylls, and the electron transfer chain are major sites of ROS production [83]. We found that LPE-application enhances the level of hydrogen peroxide, which goes along with the differential expression of cytosolic markers for oxidative stress, H_2O_2 production and scavenging, as well as senescence-associated genes. The dismutation of two molecules of H_2O_2 into water and oxygen is catalyzed by CATs. We found that *CAT1* and *CAT3* were downregulated after



LPE treatment which may explain the increase in H_2O_2 levels. SODs, which generate H_2O_2 , showed a reduced expression that might be caused by a negative feedback loop on *SOD* expression at high H_2O_2 levels.

Following the downregulation of plasma membrane-localized respiratory burst oxidase genes, which lead to the catalysis of apoplastic H_2O_2 , the ROS-burst was diminished after PAMP perception in LPE-pretreated plants. This result refers to a reduced apoplastic H_2O_2 titer. However, we found markers for cytosolic H_2O_2 accumulation strongly expressed in agreement with high observed H_2O_2 measurements. Eventually, our findings support the notion that LPE triggers the increase of cytosolically-localized H_2O_2 , but diminishes the amount of apoplastic ROS. The obtained results suggest that the LPE-mediated increase of intracellular H_2O_2 levels favors plant immunity over the release of ROS as an immediate defense response.

Differentially regulated genes (DEGs) after LPE-treatment were pooled and used to generate a functional

protein interaction network by the use of STRING [84]. The network revealed that the DEGs can be grouped in functional clusters that are interconnected (Fig. 6a). This result shows that LPE exerts transcriptional regulation on molecular components that concomitantly contribute to ROS homeostasis and senescence.

The early immunity markers *WRKY22* and *FRK1* are induced by the PAMP-triggered MAP-kinase signaling cascade (MEKK1, MKK4/MKK5, and MPK3/MPK6) [54] that function downstream of the flagellin receptor FLS2, a leucine-rich-repeat (LRR) receptor kinase. The expression of these marker genes indicates the activation of the MAP-kinase cascade, which results in the promotion of the SA signaling cascade and the accumulation of reactive oxygen species [55]. Eventually, the activation of this MAP-kinase cascade confers resistance to bacterial pathogens [85, 86], which is in accordance with our findings.

Damage-associated molecular patterns (DAMPs) are tissue-derived alarm signals that trigger cellular signaling cascades which prevalently initiate defense responses.

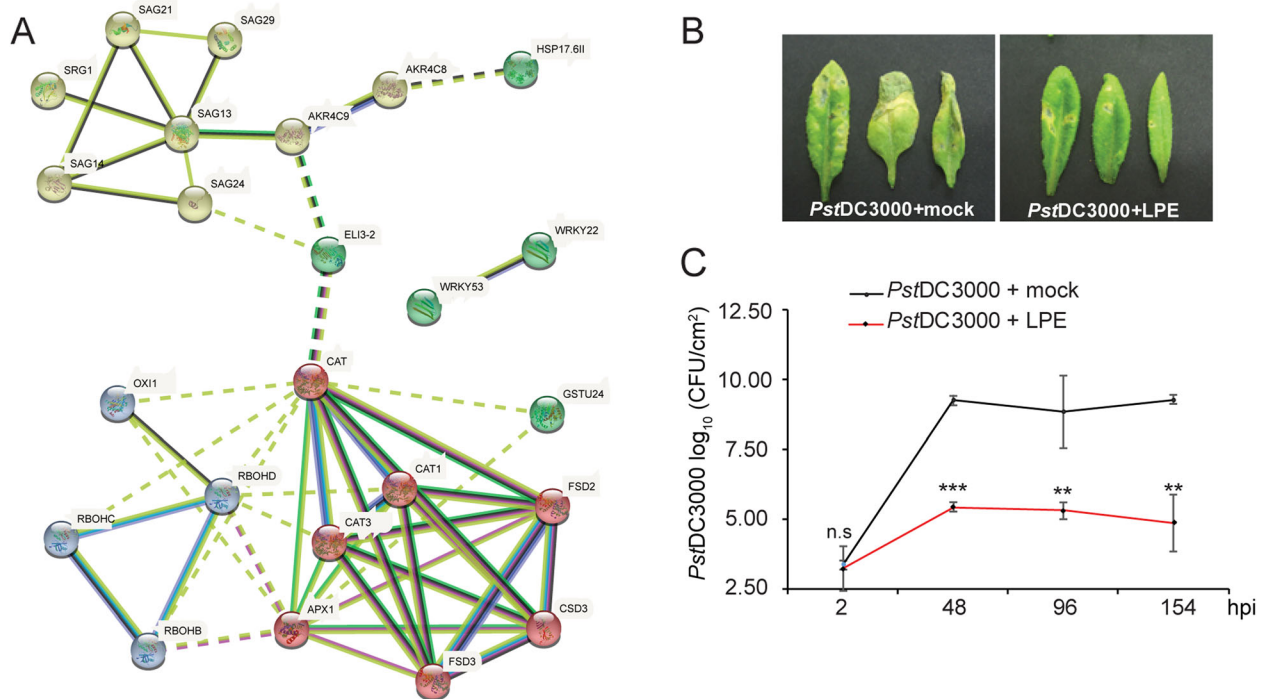


Fig. 6 Exogenously applied LPE increases plant resistance to *Pseudomonas syringae*. **a** Differentially regulated genes after LPE-treatment were pooled and used to generate a functional protein interaction network by the use of STRING (version 10.0) within the Cytoscape environment. Interconnected clusters contain targets involved in senescence (yellow), ROS metabolism (blue, red) and MAP kinase signaling (green). **b** Overview of four-week-old WT plants, after mock- and LPE-pretreatment, followed by *Pst*DC3000 spray-inoculation. **c** WT (Col) plant, were challenged with *Pst*DC3000 after LPE- or mock-pretreatment. Three biological replicates of ten plants ($n = 30$) were spray-inoculated with a bacterial suspension of $OD_{600} = 0.2$, the density of colony-forming units (CFU) was analyzed 2, 48, 96 and 154 h post-inoculation (hpi). Statistical significance was analyzed by one-way ANOVA, asterisks indicate significant difference, n.s., non-significant; ** $p \leq 0.01$; *** $p \leq 0.001$. Error bars show $\pm$ SEM

DAMPs are deemed as any molecules that are usually not exposed to cells under non-stress conditions, such as cell wall components, nucleic acid fragments, peptides, extracellular ATP, and further components [87]. Importantly, DAMPs are derived from the injured organism in which the response cascade will be initiated. Thus, LPE, as a general plant cell-membrane component, can be taken into account as a DAMP that triggers defense-associated signaling pathways.

Conclusion

Taken all together, our results indicate that LPE acts as an immunity-promoting agent that activates a wide-range of defense-related-traits associated with SA-metabolism and H_2O_2 turnover. This outcome suggests that SA/ROS homeostasis is a crucial element for the LPE-enhanced plant immunity against hemibiotrophs. These findings open up the possibility to apply LPE in farming, not only to delay senescence of crops but also to improve their resistance to biotic threats under inhospitable conditions, and further work on that aspect is warranted.

Methods

Plant materials

Seeds of *Arabidopsis thaliana* ecotype Col-0 were obtained from the European Arabidopsis Stock Center (N1093). *Arabidopsis* plants were grown in a growth chamber (Percival) at 22 °C, 70% relative humidity with 16 h illumination.

Pathogen inoculation

Four- to five-week-old *A. thaliana* plants, grown in soil and bottom-irrigated, were sprayed with either 250 ppm Tween 80 (mock-solution) or 50 ppm LPE (50 mg/l) [16] in 250 ppm Tween 80 solution 24 h before pathogen inoculation. Afterward, plants were covered by a hood and kept in the growth chamber at 22 °C. *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 strains were grown for 24 h at 28 °C on King Agar B media plates (Sigma 60, 786) with 50 mg/l rifampicin. *Pst* DC3000 were regained from plates in 10 mM $MgCl_2$. Plants were spray-inoculated with *Pst*DC3000 at $OD_{600} = 0.2$ in 10 mM $MgCl_2$ containing 0.04% Silwet L-77 and sampled 2 h, 48 h, 96 h and 154 after inoculation. To determine the

level of colonization (colony-forming units (CFU)), a total of 30 plants (3 biological replicates) were sampled by taking 3 leaf discs per plant and the bacterial titers of the plant samples were determined by conducting a dilution series at 28 °C on King Agar B plates with 50 mg/l rifampicin [88].

RNA extraction and qRT-PCR

RT-PCR was performed to analyze expression levels of individual marker genes. Plants were grown on plates [half-strength Murashige and Skoog (MS, Sigma M6899), 0.5% sucrose (sigma S5016), 1% agar (sigma A1296), and 0.5% MES (sigma M8250), pH adjusted to 5.7 with KOH] at 23 °C in long-day conditions. Total RNA from LPE (50 mg/l) and mock-treated 14-days old seedlings was extracted using the easy-spin™ Total RNA Extraction Kit (iNtRON BIOTECHNOLOGY).

An amount of 5 µg of total RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The RT-qPCR analysis was performed by the use of an Applied Biosystems 7500 Real-Time PCR system™ (Applied Biosystems, Foster City, CA). An SYBR green PCR master mix (Applied Biosystems, Warrington, UK) was used. Data generated from duplicates of at least three biological replicates (biorep) were averaged. The relative increase or decrease of mRNA abundance between samples was determined by comparing the threshold cycle values. Oligo-nucleotides used to determine the transcript levels can be found in Table S1.

Histochemical staining and ROS-burst assay

In situ detection of H₂O₂ was performed from LPE (50 mg/l) and mock-treated 4 weeks old plants by the use of 3,3'-diaminobenzidine (DAB) histochemical staining, as previously described [89]. Experiments were repeated three times with similar outcomes and shown is a representative result. Detection of ROS-burst after LPE, mock and flg22 (100 nM)-treatment was carried out as previously described [90]. In brief, ROS-burst was measured using a luminal-based assay. Leaf discs (1/4 in. in diameter) of LPE and mock-pretreated plants were incubated overnight in a white 96-well plate (Costar, Fisher Scientific) containing sterile water. After 24 h, the water was replaced by 100 µl of the elicitation solution (34 µg/ml luminol, 20 µg/ml horseradish peroxidase and 1 mM flg22). Luminescence was measured using the GLOMAX 96 MICROPLATE LUMINOMETER, and signal integration time was 0.5 s. Data of three bioreps (consisting of 12 technical replicates per biorep) were acquired, and the average value is presented.

Spectrophotometrical analysis of H₂O₂ levels

The measurement was carried out by following the instructions of Nounjan et al., 2012 [79] with some

modifications. In brief, 14 day-old Arabidopsis seedlings (50 mg) were homogenized and subsequently taken up in 1.25 ml of solution containing 1.0 ml trichloroacetic acid (TCA) (0.1% w:v) and 0.25 ml potassium phosphate buffer (10 mM). The suspension was centrifuged (12,000 rpm, 15 min, 4°C). Each time 500 µl of the supernatant were added to either 200 µl potassium-jodide (1 M) or to 200 µl water, respectively. Subsequently, 200 µl of each tube were placed in UV-microplate wells and left at room temperature for 1 h. Samples and blanks were analysed in triplicates. The oxidation of potassium iodide was determined by the Epoch-spectrophotometer from Bio-Tek at 280, 315, 350, 380, 385 and 420 nm.

Statistical analysis

Statistical significance was calculated based on one-way ANOVA with Tukey post-test. Different letters above bars indicate significant differences, $p < 0.05$. Samples sharing letters are not significantly different. Asterisks indicate significant differences n.s., non-significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12896-020-00661-8>.

Additional file 1: Figure S1. Genes, involved in JA/ET metabolism and SA signalling repression, are not differentially expressed after LPE-treatment.

Additional file 2: Table S1. Oligo-nucleotides used to determine the transcript levels.

Abbreviations

LPE : Lysophosphatidylethanolamine; SA: Salicylic acid; ET: Ethylene; JA: Jasmonic acid; ROS: Reactive oxygen species; H₂O₂: Hydrogen peroxide; MAP: Mitogen-activated protein; flg22: Flagellin22; PAMP: Pathogen-associated molecular pattern; PTI: PAMP-triggered immunity; ETI: Effector-triggered immunity; DEGs: Differentially regulated genes; ICS1: ISOCORISMATE SYNTHASE 1; SARD1: SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1; EDS : ENHANCED-DISEASE SUSCEPTIBILITY; PAD: PHYTOALEXIN-DEFICIENT; NPR: NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES; NIMIN : NIM1-INTERACTING; FRK1 : FLAGELLIN22-INDUCED RECEPTOR-LIKE KINASE 1; OPR3: Oxophytodienoate reductase 3; VSP2: VEGETATIVE STORAGE PROTEIN 2; PDF1.2: Plant defensin 1.2A; ERF1: Ethylene response factor 1; ORA59: OCTADECANOID-RESPONSIVE ARABIDOPSIS 59; GSTU24: Arabidopsis thaliana GLUTATHIONE S-TRANSFERASE 24; HSP17.6A : HEAT SHOCK PROTEIN20-LIKE CHAPERONES SUPERFAMILY PROTEIN; OX1: OXIDATIVE SIGNAL-INDUCIBLE 1; SAG: SENESCENCE-ASSOCIATED GENE; SRT: SENESCENCE-RELATED GENE; ELIS3-2: ELICITOR-ACTIVATED GENE 3-2; AKR: ALDO-KETO REDUCTASE; NADH-OX: NADH:UBIQUINONE/PLASTOQUINONE OXIDOREDUCTASE; APX1: ASCORBATE PEROXIDASE 1; CAT: CATALASE; CSD: COPPER/ZINC SUPEROXIDE DISMUTASE 1; MSD: MANGANESE SUPEROXIDE DISMUTASE 1; FSD: IRON SUPEROXIDE DISMUTASE 1; ATRBOH: RESPIRATORY BURST OXIDASE HOMOLOG; DAB: 3,3'-diaminobenzidine; PstDC3000 : *Pseudomonas syringae* pv. tomato; Col-0: Columbia; biorep: biological replicate

Acknowledgements

Not Applicable

Authors' contributions

RV, JYP, and YHL designed, performed, and analyzed the experiments. SH and WH analyzed data. RV wrote the original draft. YHL and WH edited the manuscript. The authors read and approved the final manuscript.

Funding

This work was financially supported by the National Research Foundation of Korea (NRF-2017R1A2A1A17069504, NRF-2015M3A9B8028679, and NRF-2018R1A5A1023599), grants were received by Yong-Hwan Lee. The funding body was not involved in the design of the studies, collection, analysis, and interpretation of the data; nor in the writing of the manuscript.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Nothing to declare.

Consent for publication

not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 18 February 2020 Accepted: 8 December 2020

Published online: 03 February 2021

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