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Gene Expression and Metabolomics Profiling of the Common Wheat Obtaining Leaf Rust Resistance by Salicylic or Jasmonic Acid through a Novel Detached Leaf Rust Assay

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Abstract: Wheat leaf rust caused by *Puccinia triticina* is a destructive fungal disease causing considerable grain yield loss. In this study, we developed a novel assay to test the rust resistance of detached wheat leaves on defined media with retarded senescence. We observed that salicylic and jasmonic acid confer leaf rust resistance to a susceptible *Keumkang* wheat (*Triticum aestivium* L.). Transcription analysis revealed that *atchi8* was highly expressed with an increased chitinase activity in the salicylic acid-treated leaves, while expression of *PR-9*, *atpodL*, and *PR-5* increased in the jasmonic acid-treated leaves. Additionally, the metabolic profile suggested that the phenylalanine pathway might link flavonoid production to leaf rust resistance in the salicylic acid-treated leaves, while the alanine, aspartate, and glutamate metabolism might control the production of other amino acids to enhance pathogen stress response in the jasmonic acid-treated leaves. Finally, all identified genes and metabolites could be potential targets for screening chemical compounds for leaf rust resistance. Future studies on the underlying mechanisms of leaf rust resistance obtained by exogenous treatment of salicylic and jasmonic acids remain necessary.

Keywords: common wheat; *Triticum aestivum* L.; leaf rust; *Puccinia triticina*; biotic stress; salicylic acid; jasmonic acid; plant metabolomics

1. Introduction

Common wheat (*Triticum aestivum* L.) is one of the most important cereal crops worldwide, and its production must increase to ensure a sustainable development goal that aims to end poverty and protect the planet [1,2]. However, wheat rusts, including leaf, stem, and stripe rust, are economically devastating fungal pathogens that have been causing serious reductions in wheat productivity worldwide [3–6]. In particular, leaf rust occurs more regularly worldwide than stem rust or stripe rust.

Thus, controlling the leaf rust disease to prevent a serious yield loss in wheat production has become an urgent matter [7].

A basal defense system using phytohormonal regulation has evolved in plants against different pathogens and stresses; salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA) play crucial roles in plant growth regulation and responses to biotic and abiotic stresses [8,9]. Among them, SA and JA participate in the regulation of various physiological processes as an endogenous growth regulator and the defense response to fungal biotrophic pathogens. For example, exogenous treatment with SA and JA induce chitinase homologs during a fungal pathogen defense response in pine, while SA confers resistance to the biotrophic rust pathogen, *Puccinia substriata*, by expressing certain genes involved in oxidative stress and pathogenesis-related genes in pearl millet [10,11]. In addition, SA increases the expression of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, and catalase in wheat seedlings as a defense to pathogens [12]. SA and JA induce expression of pathogenesis-related (PR) proteins and thus increase resistance against a wide range of biotic stress in cereal crops, including wheat [13,14]. In particular, β-1,3-glucanase and thaumatin-like proteins that are induced by SA and JA enhance resistance to *Stagonospora nodorum*, which is a major pathogen of wheat and related cereals. By increasing PR protein expression, SA increases the resistance of broad bean leaves to the rust fungus *Uromyces fabae* [15] and protects wheat from Fusarium head blight [16]. Although there is substantial evidence that SA is involved in the defense mechanism against fungal pathogens, particularly in rust disease, its role in wheat rust is still poorly understood [17–19].

Here, we first developed a detached leaf assay to conveniently and quickly evaluate leaf rust infection on wheat leaves. Then, we examined the effect of exogenously applied phytohormones on the resistance of common wheat to leaf rust using the assay systems at the physiological, transcriptomic, and metabolomic levels. We further investigated the underlying mechanism of leaf rust resistance in common wheat obtained by treatment with SA.

2. Materials and Methods

2.1. Plant Material

Ten ecotypes of *Aegilops tauchii* (RDA-GenBank Information Center) and 40 *Triticum aestivum* L. Korean wheat cultivars (Korea Research Institute of Chemical Technology) were used in this study. Wheat seeds were sterilized with 5% NaOCl for 7 min and washed 5 times with distilled water. The seeds were then stored at 4 °C for germination. After 2 days of incubation, the seeds were sown in autoclaved soil. The seedlings were grown at 25 °C for 14 days under daylight conditions in the greenhouse.

2.2. Senescence Retardant Chemicals and Preparation of the Medium

Senescence retardant chemicals at different concentrations and combinations were compared with 1.2% phytoagar (PHYTO AGAR, Duchefa Biochemie, Haarlem, The Netherlands) media using a detached leaf assay (Table 1). Three types of 1.2% phytoagar media were prepared and autoclaved. The media was cooled, and approximately 25 mL of media was dispensed into each Petri dish (90 mm × 15 mm). SA, JA, ABA, and chitosan were applied to the media for the leaf rust resistance study. SA (100 μM or 500 μM), JA (50 μM or 100 μM), ABA (50 μM or 100 μM), or chitosan (2 μM) was diluted with ethanol or acetic acid. Then, it was added to the autoclaved BAP (6-Benzylaminopurine) medium at 50 to 60 °C before gelling.

Table 1. Compositions of the defined medium.

Media	Components	Amount
Control	Phytoagar	12 g/L
	Sucrose	20 g/L
	Murashige and skoog	2 g/L
	Streptomycin	100 µg/L
BK medium (Benzimidazole + kinetin)	Phytoagar	12 g/L
	Sucrose	20 g/L
	Murashige and skoog	2 g/L
	Benzimidazole	30 mg/L
	Kinetin	10 mg/L
	Streptomycin	100 µg/L
BAP medium (6-Benzylaminopurine)	Phytoagar	12 g/L
	Sucrose	20 g/L
	Murashige and skoog	2 g/L
	6-Benzylaminopurine	100 mg/L
	Streptomycin	100 µg/L

2.3. Detached Leaf Assay Preparation

Sterilized aluminum foils (60 mm × 60 mm) were cut and placed in the middle of the medium. Sections (5 cm) were detached from the midpoint of each 1st leaf fragment using flame sterilized scissors, and the cut ends of the detached leaves disinfected with a solution of 2% NaOCl. The cut ends of the detached leaves were subsequently rinsed in distilled water, placed in the lower part of the leaf surface on the medium, and the rest was placed at an angle on the sterilized foil. The experiment was conducted in triplicate with each setup placed in a chamber at 25 °C under daylight conditions.

2.4. Chlorophyll Assay

A total of 50 mg of leaf fragments was collected 7 days after excision using a modified detached leaf assay. The leaf material was ground with a mortar and pestle in liquid nitrogen. We used 80% acetone to extract the chlorophyll from the samples. Chlorophyll extraction was performed overnight at 4 °C in the dark. The concentrations of chlorophyll a, chlorophyll b, and total chlorophyll were measured using a SPARK spectrophotometer at 663 and 645 nm (TECAN, Meilen, Zurich, Switzerland) and calculated using the following equations:

$$\text{Chlorophyll a} = (12.7 \times A663 - 2.69 \times A645) \times V/1000 \times W \quad (1)$$

$$\text{Chlorophyll b} = (12.7 \times A663 - 2.69 \times A645) \times V/1000 \times W \quad (2)$$

$$\text{Total Chlorophyll (a + b)} = (12.7 \times A663 - 2.69 \times A645) \times V/1000 \times W \quad (3)$$

In the equations, V is the extract volume (mL), and W is fresh leaf weight (g). The blank consisted of 80% acetone [20,21].

2.5. Leaf Rust Inoculation

Puccinia triticina strain *HSZ0742* (NCBI Gene Bank DQ41710.1) was supplied by the Korea Research Institute of Chemical Technology. The inoculum was prepared by diluting the uredospores of *P. triticina* (750 mg/L) in distilled water containing 120 µL/L of Tween 20 (DaejungChem, Siheung, Korea). After inoculation, the infected wheat was incubated at 100% relative humidity for 24 h at 20 °C in the dark after excision with a modified detached leaf assay or in a pot without being cut for whole seedling assay. After dark incubation, the phenotypic infection type was evaluated 10 days post-inoculation under normal growing conditions [22]. The evaluation of seedling response varied from 0 to 4, where 0 indicated no visible symptoms, 1 = minute uredinia surrounded by mostly necrotic tissue, 2 = small- to

medium-sized uredinia surrounded by chlorotic and necrotic tissue or both, 3 = large uredinia without surrounding chlorosis, and 4 = large uredinia without chlorosis or necrosis.

2.6. Fluorescence Microscopy

The infected *Keumkang* wheat leaves treated with SA (100 μM or 500 μM), JA (50 μM or 100 μM), and ABA (50 μM or 100 μM) 10 days after inoculation of the leaf rust were stained with lectin from *Triticum vulgaris* (Sigma-Aldrich, St. Louis, MO, USA) [23]. Microscopy images of leaf fragments were captured using a fluorescence microscope (DMi8, LEICA, Wetzlar, Hesse, Germany) with a GFP filter (GFP-EN, LEICA, Wetzlar, Hesse, Germany). For the microscope image, the detached leaf samples were collected and submerged in 1 M KOH for 48 h. After washing 3 times with 50 mM Tris-HCl for 10 min, 1 mg/mL WGA-FITC diluted with 50 mM Tris-HCl was added to leaf samples and incubated for 1 h at 25 °C.

2.7. Real-Time Quantitative Reverse Transcription PCR Analysis for Gene Expression

Leaf samples (50 mg) treated with SA, JA, and ABA by using the detached leaf assay at 0, 6, and 24 h post-inoculation were ground to a powder in liquid nitrogen with a mortar and pestle. Total RNA was isolated with TRIzol solution (Ambion, Life Technologies, Carlsbad, CA, USA), and template cDNAs were synthesized using the RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) [24,25]. PCR amplification of the cDNA was conducted with BrightGreen 2X qPCR MasterMix (abm, Richmond, Canada) using a thermocycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Forward and reverse primers (10μM) diluted to working concentration. The reaction mixture in thermal cycler for 40 cycles of the amplification, which includes enzyme activation (95 °C for 10 min), denaturation (95 °C for 15 s), annealing/extension (60 °C for 60 s). Plant actin eu97 was used as the internal control. The primer sequences used for qRT-PCR analysis are provided in Table S1.

2.8. Chitinase Assay

Leaf fragments (50 mg) treated with the detached leaf assay were collected at 0, 24, and 48 h post-infection and ground into a powder with a mortar and pestle. A total of 700 μL PRO-PREP protein extraction solution (iNtRON Biotechnology, Seoul, Korea) was added to the samples and incubated on ice for 30 min after vigorous vortexing. Subsequently, the extracts were centrifuged at 13,000 rpm for 15 min at 4 °C. The collected supernatant was used for the chitinase assay. The 3,5-dinitrosalicylic acid (DNS) method was used to measure chitinase activity after inoculation [26–28]. The reaction mixture included 50 μL crude protein extract, 40 μL 1.6% (*w*/*v*) colloidal chitin, and 80 μL 0.2 M sodium phosphate buffer (pH 6.5). The mixture was incubated at 37 °C for 1 h. The reaction was stopped by adding 40 μL of 1% NaOH followed by boiling for 5 min. After centrifugation at 4000 rpm for 5 min at 4 °C, the supernatant was mixed with 1% DNS solution at a ratio of 1:1 and boiled for 5 min. After cooling for 10 min, the absorbance was measured using a spectrophotometer at 540 nm (TECAN, Meilen, Zurich, Switzerland).

2.9. Peroxidase Activity

For the peroxidase (POD) activity assay, extracted enzymes were collected at 0, 24, and 48 h post-infection. The reaction mixture consisted of 20 μL crude enzyme extract, 930 μL 25 mM guaiacol (2-methoxyphenol), and 50 μL 2% H_2O_2. The mixture was incubated at 25 °C for 2 min. After a fivefold dilution, the absorbance was measured using the SPARK spectrophotometer at 470 nm (TECAN, Meilen, Zurich, Switzerland) [29].

2.10. Gas Chromatography–Mass Spectrometry for Metabolite Analysis

The collected leaf fragments (50 mg) at 0 and 7 days after inoculation with SA, JA, and ABA using a modified detached leaf assay were ground into a powder with a mortar and pestle and stored at −80 °C until metabolite extraction [30]. Each sample was extracted with 1 mL of 80% methanol using a vortex for 1 min, followed by 10 min of sonication at 4 °C. Subsequently, the extracts were centrifuged at 13,000 rpm for 10 min at 4 °C, and 500 µL of the supernatant was filtered through 0.2 µm PTFE syringe filters. The filtered supernatants were completely dried using a freeze dryer. The dried samples were then derivatized. First, 25 µL of MOX solution (10 mg of methoxyamine, 1 mL of pyridine) was added to each sample and vortexed for 15 s. The reaction mixture was incubated at 37 °C for 30 min after centrifugation for 10 min at 10,000 rpm. Following this, silylation was performed by adding 125 µL of *N*,*O*-bis(trimethylsilyl)acetamide to the reaction mixture, followed by incubation at 60 °C for 20 min. GC-MS/MS analysis was then carried out using an Agilent 7890A/7000 GC triple quadrupole mass spectrometer system (Agilent Technologies, Palo Alto, CA, USA). The samples were injected in the splitless mode with an Agilent 7683A Injector, and the temperature was set to 250 °C. The GC was equipped with an Agilent J&W HP-5ms UI 15 m × 0.25 mm × 0.25 µm (P/N 19091S-431UI) capillary column. Helium was used as the carrier gas with a constant column flow rate of 1 mL min^{-1}. The oven temperature of the GC column was programmed to rise from 120 °C to 280 °C at 15 °C min^{-1} (hold 3 min). The total run time for the analysis was 18 min, and a solvent delay time of 3 min was used at the start of the run. Mass spectrometry was performed in the electron ionization mode at 70 eV; full scan. The software Agilent MassHunter Data Acquisition Software (Ver. B.04.00, Agilent Technologies, Palo Alto, CA, USA) was used for data acquisition. Quantitative analysis (version B.07.01 SP1/Build 7.1.524.1 unknowns analysis, Agilent Technologies, Palo Alto, CA, USA) was used for the hypothesis test. MetaboAnalyst was used for pathway analysis.

3. Results and Discussion

3.1. Development of Defined Media for Detached Leaves with Retarded Senescence

There have been efforts to inoculate wheat leaf rust on a detached leaf grown on an artificial medium for rapid and convenient evaluation of the resistance of various wheat cultivars to leaf rust infection. However, leaf senescence progresses rapidly, making it difficult to evaluate the phenotype of leaf rust infection on a detached leaf. To address this issue, we modified the medium previously used in the detached leaf rust assay to further retard senescence [31]. First, 14-day-old seedling leaves were cut from common wheat (Keumkang) and then grown on the three types of media for 7 days: control medium, BK medium containing kinetin and benzimidazole, and BAP medium containing 6-benzylaminopurine (Table 1). Particularly, the BK medium was previously reported to retard senescence in detached leaves of common wheat [32]. Interestingly, the BAP medium was most effective in retarding the senescence of common wheat leaves. The progression of senescence in the BK medium seems to be more than that in the BAP medium due to the appearance of a few yellow spots and thus the BK medium was less effective in retarding the senescence than the BAP medium (Figure 1a). Chlorosis is caused by a lack of chlorophyll and leaves the leaf tissue yellow. Following this, chlorophyll a, chlorophyll b, and total chlorophyll content were analyzed to quantify the degree of senescence. Consistent with the morphological phenotype shown in Figure 1a, the concentrations of all chlorophyll contents were much higher in leaves grown on BAP medium than those grown on the control and BK media, whereas leaves grown on the BK medium had slightly higher concentrations of chlorophyll a than those grown on the control medium (Figure 1b). Taken together, the results indicate that BAP medium was the most effective retardant medium for wheat leaves in delaying senescence and chlorosis progression. The BK medium was also effective in delaying senescence of wheat leaves as previously reported; however, it might be less suitable for evaluating leaf rust resistance of wheat varieties using the detached leaf rust assay as some degree of senescence was observed in the wheat

leaves grown on the BK medium for 7 days, and the leaf rust infection phenotype becomes evident 10 days after infection.

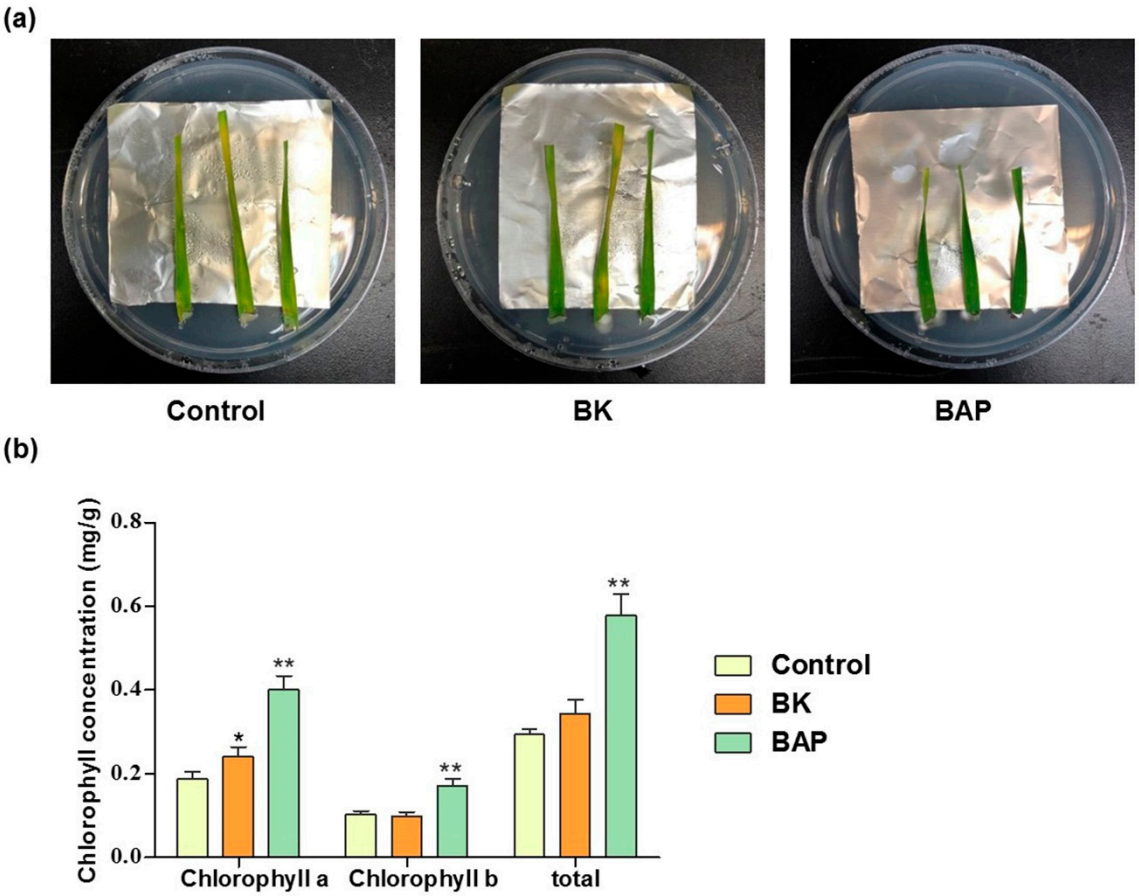

Figure 1. Comparison of senescence of detached *Keumkang* wheat leaves grown on defined medium containing chemical retardants, the BK (Benzimidazole + kinetin) or the BAP (6-Benzylaminopurine) medium. (**a**) Morphologies and (**b**) chlorophyll contents in detached leaves of 14-days-old seedlings of the common wheat grown on the three types of media for 7 days. BK contains kinetin and benzimidazole, and BAP contains 6-benzylaminopurine. Asterisks indicate significant differences compared to control in each type. Error bars represent standard deviation (n = 3). Data were statistically analyzed by a Student's t-test (*: $p < 0.05$, **: $p < 0.01$).

3.2. Evaluating Leaf Rust Resistance of Wheat Varieties Using the Detached Leaf Rust Assay in a Controlled Environment

The infection phenotype of leaf rust was examined 10 days after the inoculation of the urediniospores of Puccinia triticina on a detached leaf grown on each defined medium (control, BK, and BAP). As expected, wide yellow spots and curl morphology were observed in the leaves grown on the control and BK media, whereas it was not observed in the leaves grown on BAP medium. Accordingly, the leaf rust phenotype was more clearly observed in the leaves grown on the BAP medium (Figure 2a).

Rust resistance assessment is typically carried out using adult plants in the field or whole seedling in a controlled greenhouse. We sought to determine whether the whole seedling assay using intact leaves in a greenhouse and the detached leaf rust assay using cut leaves grown on the BAP medium presented the same pathophenotype. For this, we performed the whole seedling assay and the detached leaf rust assay with 50 wheat cultivars, including *Triticum aestivum* L. and Aegilops tauschii. For the inoculation of urediniospores of P. triticina, 14-day-old wheat cultivars were used for both assays and then evaluated based on a 0–4 scale [33]. Among 40 common wheat (*Triticum aestivum* L.) cultivars,

Jonong and Ol were resistant to leaf rust infection (0–1 scales for DLA and WSA), while others were susceptible to leaf rust infection (2–4 scales for DLA and WSA). On the other hand, among 10 ancient wheat (Aegilops tauschii) cultivars, 6 cultivars, 16, 79TK057-324, PI 349037, PI 431603, PI 603221, PI 603224, were resistant to leaf rust infection (0–1 scales for DLA and WSA), while others were susceptible to leaf rust infection (2–4 scales for DLA and WSA). Currently, limited data are available for ancient wheat cultivars obtaining leaf rust resistance, and thus further study is required for identifying genes associated with leaf rust resistance to understand the molecular mechansim and compare with common wheat. Interestingly, the results showed that the whole seedling assay and the detached leaf rust assay were significantly correlated (Spearman, $r^2 = 0.97$), indicating that although both assays were performed under different conditions, the detached leaf rust assay can replace the whole seedling assay to evaluate the pathophenotype of *P. triticina* on wheat leaves (Table 2 and Figure 2b).

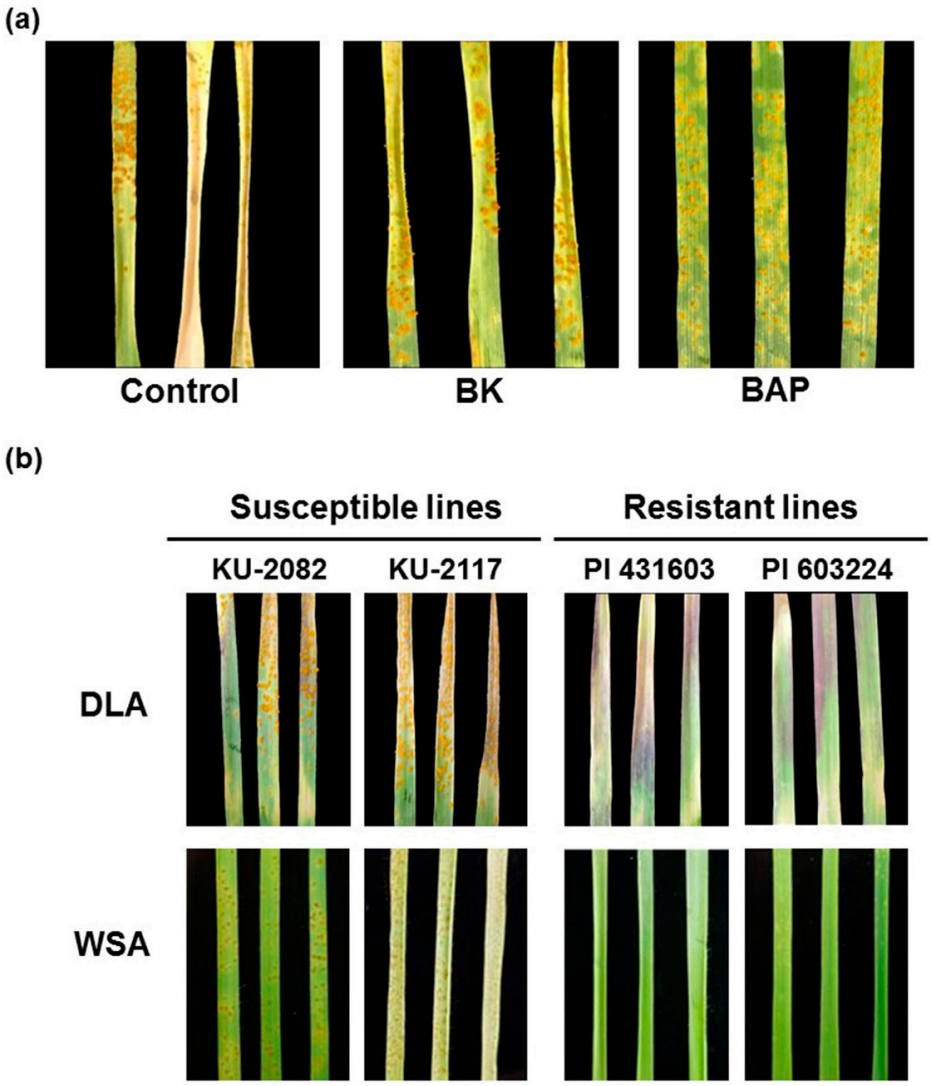

Figure 2. Pathophenotype of leaf rust on detached and undetached wheat leaves. Detached and undetached leaves of 14-days-old seedlings were grown for 10 days after inoculation with leaf rust for the DLA (detached leaf rust assay) and the WSA (whole seedling assay), respectively. (**a**) The DLA using the control, BK, or BAP medium was performed for comparing morphological variations of *Keumkang* wheat leaves. (**b**) The DLA using the BAP medium or the WSA was performed for comparing morphological variations of *Aegilops tauschii* in the greenhouse. *Keumkang* wheat is known to be susceptible to leaf rust. Among all *Aegilops tauschii* used, *KU-2082* and *KU-2117* are known to be susceptible, and *PI 431603* and *PI 603224* are known to be resistant to leaf rust.

Table 2. Correlation of the phenotypes of various wheat cultivars inoculated with leaf rust between the DLA and the WSA.

Phenotypic Reaction *					
Accession Name (*Triticum aestivum* L.)	**DLA**	**WSA**	**Accession Name (*Triticum aestivum* L.)**	**DLA**	**WSA**
Alchan	3	3	*Jojoong*	3	3
Anbaek	3	3	*Jokyung*	3	3
Baekchal	3	3	*Jonong*	1	1
Baekjoong	2	2	*Jopum*	3	3
Baek-kang	2	2	*Keumkang*	3	3
Chung-kye	3	3	*Milseoung*	2	2
Dabun	3	3	*Namhae*	3	3
Dahong	3	3	*Ol*	1	1
Dajung	3	3	*Olgeru*	2	2
Eunpa	2	2	*SaeKeumKang*	3	3
Geuru	3	3	*Saeol*	3	3
Gobun	3	3	*Seodun*	2	2
Goso	3	3	*Shinmichal*	3	3
Hanbaek	3	3	*Shinmichal No.1*	2	2
HoJoong	2	2	*Suan*	3	3
Jeokjoong	3	3	*Sukang*	3	3
Jinpum	3	3	*Taejoong*	2	2
Joa	3	3	*Tapdong*	2	2
Jo-eun	2	2	*Uri*	2	2
Johan	2	2	*Younbaeck*	2	2
Accession Name (*Aegilops tauschii*)	**DLA**	**WSA**	**Accession Name (*Aegilops tauschii*)**	**DLA**	**WSA**
16	0	1	*PI 330489*	3	3
79TK057-324	1	1	*PI 349037*	1	1
KU-2019	3	4	*PI 431603*	0	0
KU-2082	3	4	*PI 603221*	1	0
KU-2117	3	3	*PI 603224*	0	0

* Phenotypic performance after leaf rust infection: 0 = no visible symptoms, 1 = minute uredinia surrounded by mostly necrotic tissue, 2 = small- to medium-sized uredinia surrounded by chlorotic and necrotic tissue or both, 3 = large uredinia without surrounding chlorosis, and 4 = large uredinia without chlorosis or necrosis.

The correlation between DLA (detached leaf rust assay) and WSA (whole seedling assay) was significant at the 0.01 level (Spearman, $r^2 = 0.97$)

3.3. Salicylic Acid and Jasmonic Acid Confer Leaf Rust Resistance to Common Wheat

It has been shown that many plants are tolerant to abiotic and biotic stress by treating phytohormones such as SA, JA, and ABA [34–37]. However, their function is poorly understood for leaf rust disease in wheat varieties [38–40]. In this regard, SA, JA, and ABA were applied to the detached leaf rust assay to examine their role in the resistance of common wheat to leaf rust disease. BAP media containing SA (100 μM and 500 μM), JA (50 μM and 100 μM), or ABA (50 μM and 100 μM) were used to cultivate *Keumkang* wheat leaves inoculated with *P. triticina*. Particularly, *Keumkang* wheat is a highly sensitive common wheat to leaf rust and thus a proper model to screen compounds conferring leaf rust resistance. Interestingly, leaves grown on media containing either SA or JA had dramatically increased resistance to leaf rust, whereas the leaves grown in media containing ABA did not (Figure 3a). In particular, no urediniospores were observed in the leaves grown on media containing 100 μM or 500 μM SA. When SA at concentration lower than 50 μM was added to the BAP medium, leaf rust progression was also not observed (data are not shown). However, we observed some urediniospores on the surface of the leaves grown on the 50 μM of JA. Following this, fluorescent-labeled lectin staining was performed to observe leaf rust progression clearly. Consistently, much of the fluorescence signal

was observed in the leaves grown on the control medium and ABA-containing medium but was lower in the leaves grown on the JA-containing medium, and none in the leaves grown on the SA-containing medium (Figure 3b). Consequently, the results indicated that SA is the most effective phytohormone to prevent the progression of leaf rust in common wheat. Additionally, JA is also effective, but not as much as SA.

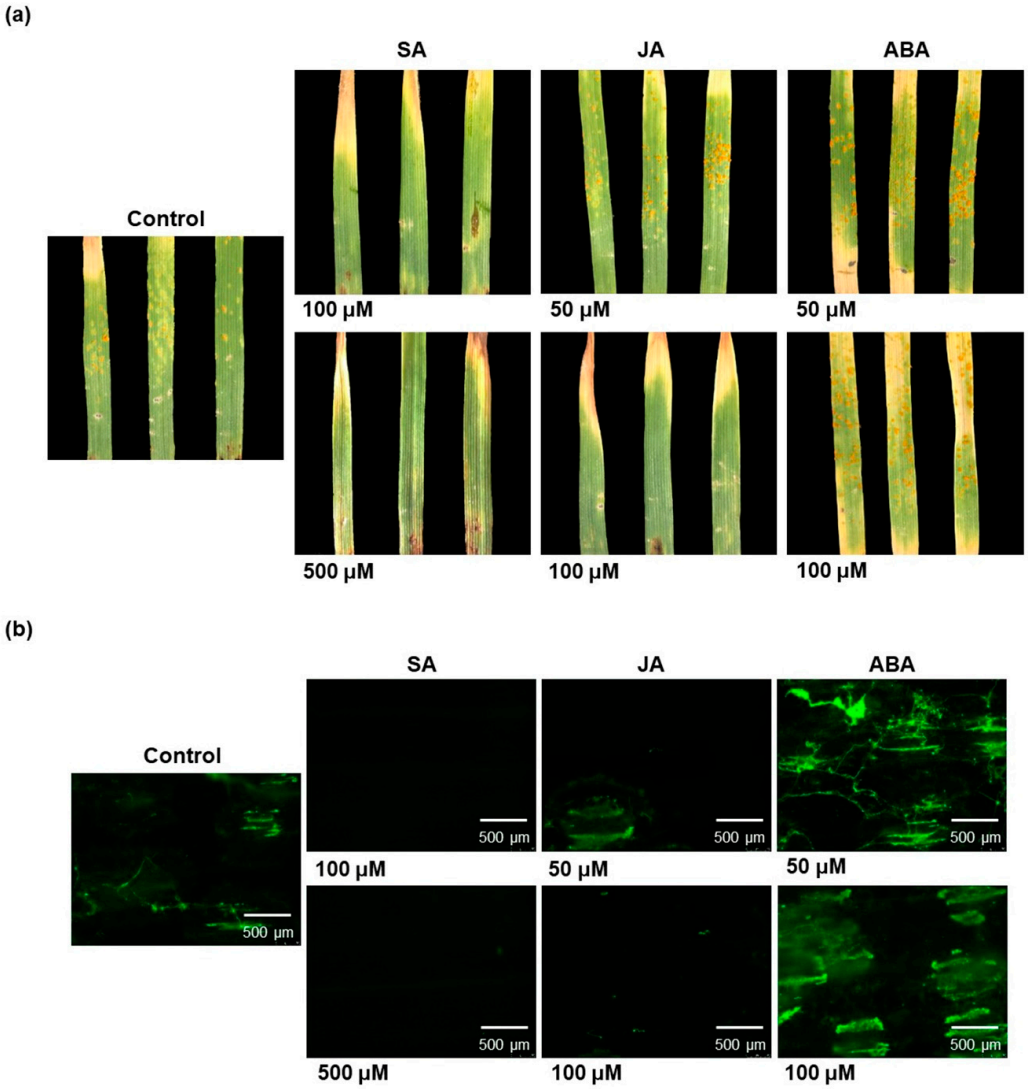

Figure 3. Effect of salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA) on *Keumkang* wheat leaf rust resistance. (**a**) Morphologies and (**b**) fluorescence microscopy images were compared among the detached *Keumkang* wheat leaves of 14-days-old seedlings grown on the BAP media containing SA, JA, or ABA for 10 days after inoculation with leaf rust. The evaluation was done by staining with a lectin from *Triticum vulgaris*.

3.4. Salicylic Acid Increases the Expression and Activity of Chitinase in Wheat Leaves Inoculated with Leaf Rust

To understand the potential mechanisms of leaf rust resistance to wheat, gene expression in *Keumkang* wheat leaves treated with or without 100 μM of SA, JA, or ABA after inoculation with leaf rust was analyzed using qRT-PCR. In particular, eight genes that were shown to be highly expressed in the leaf rust-resistant wheat [41] were analyzed at 0, 6, and 24 h after treatment with the phytohormones (Figure 4). Among the eight genes, expression of *atchi8* dramatically increased at 24 h in SA-treated leaves (~5000-fold), but not in untreated leaves or JA- or ABA-treated leaves. Moreover, expression of *PR-9* and *atpodL* was much higher at 6 and 24 h in JA-treated leaves than in leaves of other treatments.

In addition, expression of *PR-5* increased at 24 h in JA-treated leaves (~180-fold) but not in untreated leaves or SA- or ABA-treated leaves. Consequently, this result suggests that SA and JA might have different mechanisms responsible for leaf rust resistance to common wheat. For example, *PR-9* and *atpodL* encode pathogen-related proteins that have peroxidase activity. It was previously reported that rust-resistant wheat has increased antioxidant activity via increased peroxidase activity [42,43]. Therefore, JA might confer resistance to leaf rust to common wheat by upregulating the expression of peroxidases and subsequently increasing antioxidant activity. Moreover, *atchi8* is a chitinase-related gene with the highest increase in the leaf rust-resistant strain of *Triticum aestivum* L. when inoculated with leaf rust [44]. Hence, we surmise that SA may increase chitinase activity, thereby conferring leaf rust resistance to common wheat.

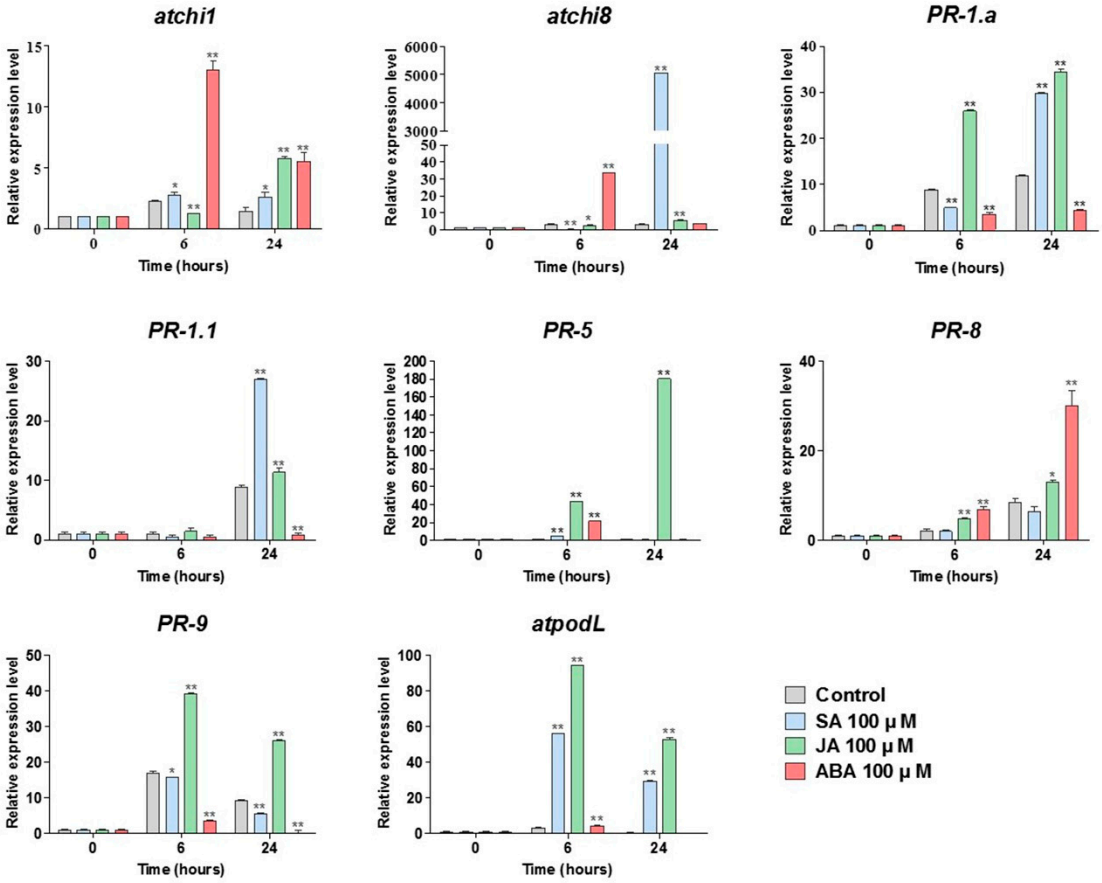

Figure 4. Transcription analysis of eight genes in detached *Keumkang* wheat leaves treated with 100 μM SA, JA, or ABA. mRNA expressions of *atchi1*, *atchi8*, *PR-1.a*, *PR-1.1*, *PR-5*, *PR-8*, *PR-9*, and *atpodL* in detached *Keumkang* wheat leaves of 14-days-old seedlings grown on the BAP media containing SA, JA, or ABA after inoculation with leaf rust were analyzed at 0, 6, and 24 h using quantitative RT-PCR. Control used vehicle ethanol without SA, JA, and ABA. Asterisks indicate significant differences compared to control each time. Data were statistically analyzed by a Student's t-test (*: $p < 0.05$ and **: $p < 0.01$).

Along with the expression results, chitinase and peroxidase activities were measured in the wheat leaves treated with 100 μM of SA or 100 ppm chitosan after inoculation with leaf rust (Figure 5). Chitosan is known to increase the expression of chitinase and peroxidase as an antifungal elicitor. When compared with the control, which was untreated after inoculation with leaf rust, chitinase activity in the SA-treated leaves was much higher at 24 and 48 h than that in the control and the chitosan-treated leaves, whereas peroxidase activity in the SA-treated leaves was similar to that of the control.

Consequently, this result supports the idea that exogenous SA treatment further increases chitinase activity in wheat leaves inoculated with leaf rust.

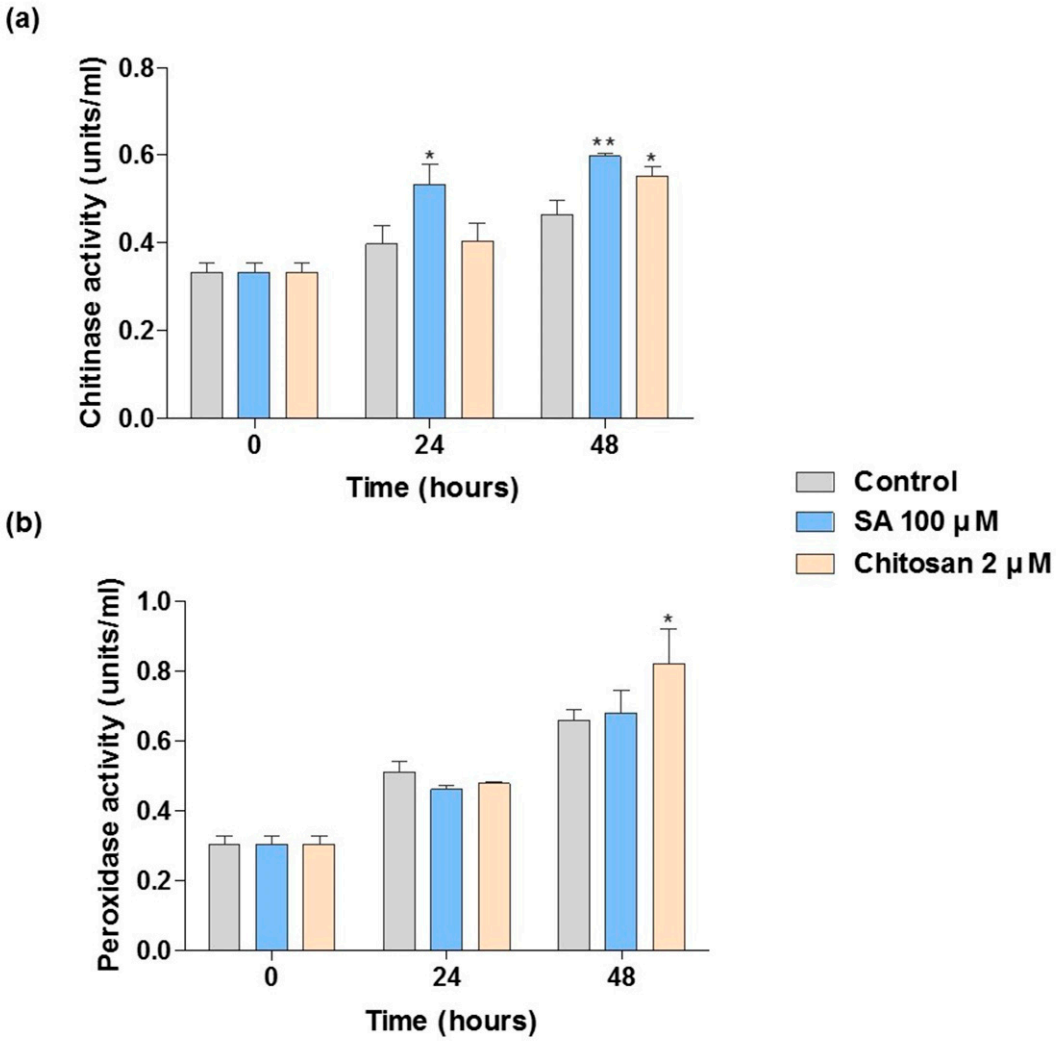

Figure 5. Chitinase and peroxidase activity of detached *Keumkang* wheat leaves treated with SA or chitosan. (**a**) Chitinase and (**b**) peroxidase activity in detached *Keumkang* wheat leaves of 14-days-old seedlings grown on the BAP media containing SA or chitosan after inoculation with leaf rust were measured at 0, 24, and 48 h. Error bars represent standard deviation (n = 3). Asterisks indicate significant differences compared to control each time. Data were statistically analyzed by a Student's t-test (*: $p < 0.05$, **: $p < 0.01$).

3.5. Exogenous SA Application Alters Metabolite Profiles Related to the Defense Biosynthesis Pathway

The biochemical mechanisms by which wheat metabolism is associated with leaf rust resistance are not yet widely understood [45,46]. Therefore, identifying the metabolites associated with pathophysiology under leaf rust stress provides a novel approach to understanding the underlying mechanism of leaf rust resistance [47]. To address this question, we performed untargeted metabolomics using GC-MS in *Keumkang* wheat leaves treated with or without 100 µM of SA, JA, or ABA after inoculation with leaf rust. Using the platform, approximately 200 metabolites were detected in each of the samples. Among them, approximately 45 metabolites were selected as promising biomarkers that represent significantly different levels between the control group and the SA, JA, or ABA-treated group; they were present in the heatmap and were used for the pathway analysis and the diagnosis of principal component analysis (PCA) (Figures 6 and 7 and Figures S1 and S2).

In the pathway analysis, representation of all metabolic pathways displayed according to their significance or pathway impact. The graph presents a view of all the matched pathways arranged by −log10(*p*) values (pathway enrichment analysis) on the y-axis, and the pathway impact values (pathway topology analysis) on the x-axis. The SA-treated group showed that the phenylalanine pathway was highly scored in both the pathway impact and the −log10(*p*) value; the pathway impact alternatively represented that the number of score or circle size was evaluated based on the core metabolites, and the −log10(*p*) value alternatively represented that the number of score or color was evaluated based on the number of metabolites involved in the pathway [48]. In addition, aminoacyl-tRNA synthesis and the glucosinolate biosynthesis pathway were highly scored in the −log10(*p*) value. It has been reported that exogenous SA treatment increases flavonoid biosynthesis as a secondary metabolite [49,50]. Many flavonoids are synthesized through the phenylpropanoid pathway, where phenylalanine is used to produce 4-coumaroyl-Co, which becomes the backbone of flavonoids. Flavonoid accumulation plays a crucial role in defending pathogen infection, and thus upregulation of the phenylalanine pathway by SA might contribute to increasing leaf rust resistance of common wheat. Additionally, glucosinolate is also known to play a role in protecting many plants from pathogens [51]. Therefore, the glucosinolate biosynthesis pathway may also be involved in increasing leaf rust resistance.

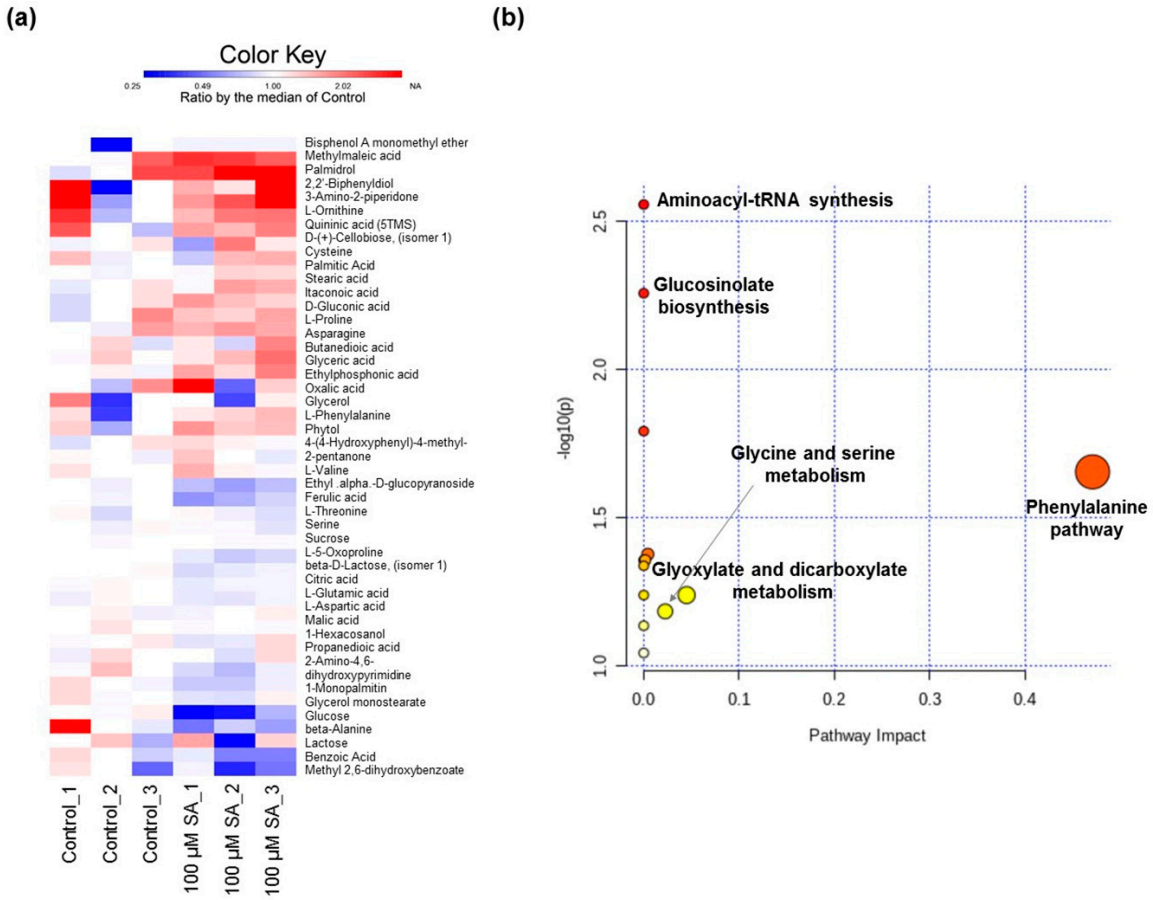

Figure 6. Metabolomics analysis of detached *Keumkang* wheat leaves treated with SA. (**a**) Heatmap and (**b**) pathway analyses of metabolites identified from the detached *Keumkang* wheat leaves of 14-days-old seedlings grown on the BAP media containing SA or vehicle ethanol (control) for 7 days after inoculation with leaf rust using a detached leaf assay. In (**a**), the color range is from red to blue, representing from high to low expression, respectively. In (**b**), the pathway impact is represented alternatively as the score in the x-axis or circle size, and the −log10(*p*) value is represented alternatively as the number of scores in the y-axis or color. The color range is from red to yellow, representing from high to low −log10(*p*) value, respectively.

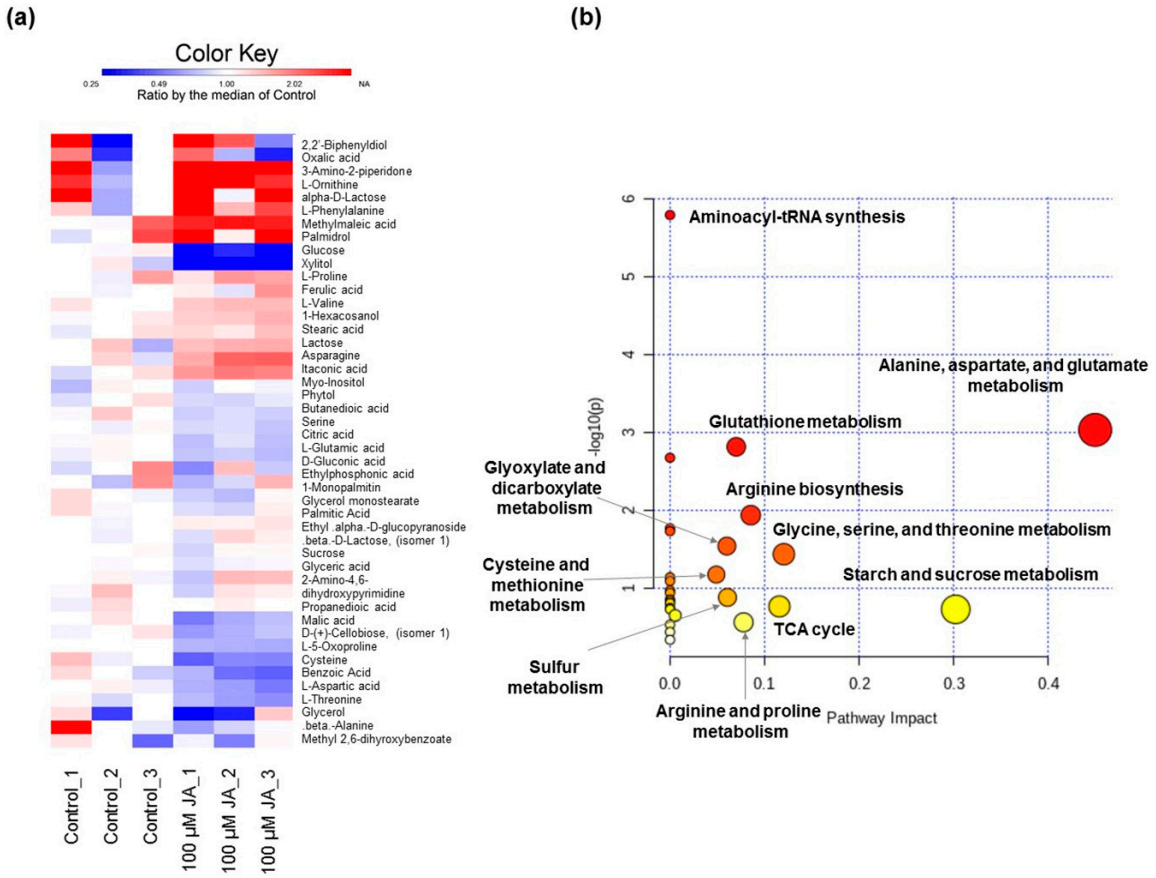

Figure 7. Metabolomics analysis of detached *Keumkang* wheat leaves treated with JA. (**a**) Heatmap and (**b**) pathway analysis of metabolites identified from the detached *Keumkang* wheat leaves of 14-days-old seedlings grown on the BAP media containing JA or vehicle ethanol (control) for 7 days after inoculation with leaf rust using a detached leaf assay. In (**a**), the color range is from red to blue, representing from high to low expression, respectively. In (**b**), the pathway impact is represented alternatively as the number of scores in the x-axis or circle size, and the −log10(*p*) value is represented alternatively as the number of scores in the y-axis or color. The color range is from red to yellow, representing from high to low −log10(*p*) value, respectively.

In contrast, the JA-treated group showed that the alanine, aspartate, and glutamate metabolism were highly scored in both the pathway impact and the −log10(*p*) value, while the starch and sucrose metabolism was highly scored in the pathway impact and the aminoacyl-tRNA synthesis, glutathione metabolism, arginine biosynthesis, and glycine, serine, and threonine metabolism were highly scored in the −log10(*p*) value. Interestingly, the metabolic pathways identified in the JA-treated group involved amino acid metabolism, including alanine, aspartate, and glutamate metabolism, which play a crucial role in plant stress response [52]. In particular, glutamate participates not only in amino acid metabolism via α-amino group transfer but also in upregulating the activities of β-1,3-glucanase, polyphenol oxidase, peroxidase, and chitinase in response to pathogen stress [53,54]. The starch and sucrose metabolism participates in protecting plants from pathogens by activating plant immune responses [55]. All considered, the metabolomics analysis showed differential accumulation of metabolites between the two groups treated with SA or JA under post-infection conditions. Both of these phytohormones are effective in increasing the leaf rust resistance of common wheat. However, they may utilize different metabolic pathways to confer leaf rust resistance to common wheat; SA may enhance accumulation of secondary metabolites such as flavonoids via the phenylalanine pathway, while JA might enhance the accumulation of primary metabolites such as glutamate, glucose, sucrose, and other amino acids.

4. Conclusions

In conclusion, we developed an advanced method for evaluating leaf rust resistance of detached wheat leaves grown on defined media with retardant senescence. A convenient and well-controlled environment is provided for this method. Using this method, we demonstrated that SA and JA confer leaf rust resistance to common wheat. Regarding the mechanism underlying this resistance mediated by exogenous SA or JA treatment, transcription analysis evidence showed that expression of chitinase-related genes increases in SA-treated wheat leaves, while expression of the peroxidase-related genes increases in the JA-treated wheat leaves. Furthermore, metabolomics analysis suggests that the phenylalanine pathway might contribute to conferring leaf rust resistance to common wheat by increasing the secondary metabolites including flavonoids in the SA-treated wheat leaves, while the alanine, aspartate, and glutamate metabolisms might contribute to conferring leaf rust resistance of common wheat by increasing the levels of primary metabolites, including glutamate and other amino acids after JA treatment. Finally, exogenous SA or JA treatment would be useful for defending wheat leaves against leaf rust to improve crop production. Moreover, the identified metabolites and pathways would be a target to screen chemical compounds that provide leaf rust resistance and to understand further the underlying mechanism of leaf rust resistance at the molecular level.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/11/1668/s1, Figure S1: Metabolomics analysis of detached wheat leaves treated with ABA. (**a**) Heatmap and (**b**) pathway analysis of metabolites identified from the detached leaves (*Keumkang* wheat, susceptible) of 14-days-old seedlings of the common wheat grown on the BAP media containing ABA or vehicle ethanol (control) for 7 days after inoculation with leaf rust using a detached leaf assay. In (**a**), the color range is from red to blue, representing from high to low expression, respectively. In (**b**), the pathway impact is represented alternatively as the number of score in the x-axis or circle size, and the −log10 (*p*) value is represented alternatively as the number of score in the y-axis or color. The color range is from red to yellow, representing from high to low −log10 (*p*) value, respectively; Figure S2: Principal components analysis (PCA) of metabolites identified from the detached leaves (*Keumkang* wheat, susceptible) of 14-days-old seedlings of the common wheat grown on the BAP media containing SA, JA, or ABA for 7 days after inoculation with leaf rust, Table S1: List of primers used in the study.

Author Contributions: Conceptualization, M.K., A.L. and B.C.L.; formal analysis, M.K. and A.L.; investigation, M.K., Y.J.R., H.M.L., Y.J. and H.C.; methodology, Y.J.R., D.W.C. and M.C.; project administration, B.C.L., H.L. and N.C.; supervision, B.C.L., C.C., S.-i.E.; validation, H.M.L., Y.J. and H.C.; visualization, D.W.C. and M.C.; writing—original draft, M.K. and B.C.L.; writing—review and editing, all authors. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. The authors have filed a patent application relating to the technology.

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