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Characterization of two leaf rust-resistant *Aegilops tauschii* accessions for the synthetic wheat development

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Abstract

Aegilops tauschii (*Ae. tauschii*) is a diploid ($2n=2x=14$) wild grass species, which has been reported as the progenitor of hexaploid wheat (*Triticum aestivum*) with D-genome. In this study, 68 *Ae. tauschii* accessions with diverse geographical backgrounds were investigated for their resistance to infection by the leaf rust fungi *Puccinia triticina*. Two *Ae. tauschii* accessions that exhibited hyper-resistance to leaf rust at both seedling and adult stages were identified. Utilizing two susceptible *Ae. tauschii* ecotypes and keumkang, a common Korean wheat cultivar known to be susceptible to leaf rust, as the negative control, further investigations were conducted for understanding the mechanism underlying immunity to leaf rust disease of these two resistant accessions. Resistant accessions displayed the increased β -1,3-glucanase activity to prevent fungal penetration and the better peroxidase activity to cope with leaf rust-induced oxidative stress. Moreover, transcriptional analyses reveal the important role of the LRR receptor-like serine/threonine-protein kinase FLS2 (*Irr*) to the disease resistance of the two ecotypes. *Ae. tauschii* is a remarkable genetic source, especially for abiotic and biotic stress resistance genes, as the plant is known for its wide-ranging geographical habitat and adaptability to different environments. This, combined with the fact that *Ae. tauschii* and wheat share a close evolutionary relationship, is indicative of the immense benefit of using *Ae. tauschii* as a material for improving the quality of synthetic wheat. Our aim was to identify and evaluate the strongest *Ae. tauschii* contenders for breeding leaf rust-resistant synthetic wheat.

Keywords: *Aegilops tauschii*, β -1,3-Glucanase, 3-Glucanase, Leaf rust resistance, *Puccinia triticina*, Synthetic wheat

Introduction

Leaf rust (LR) disease, which is caused by the rust fungi *Puccinia triticina* (*P. triticina*), is the most destructive pathogenic disease of cereal crops [39]. *P. triticina* has one of the most complicated life cycle of all fungi [38, 62] and is an obligatory biotrophic pathogen that parasitizes wheat [31, 52, 64]. Rust fungi usually have five spore forms; among these, the urediospore is the most

important asexual form [52]. Urediospore infection of LR has three phases: (i) penetration, (ii) parasitic growth, and (iii) sporulation. In the parasitic phase, after the pathogen has successfully penetrated plant tissues, the rust growth rapidly utilizes the resources of the host plant, leading to major yield loss of the infected crop [62]. In the sporulation phase, the rust spores burst out from the host plants and are disseminated to new hosts via wind and rain. A study showed that a single rust uredium generates up to 600 urediospores per day [19], which is approximately equal to 10^{12} – 10^{13} urediospores on 1 ha of infected plant field per day [18]. Furthermore, spores disseminated by the wind can travel large distances, spreading the rust infection across countries and even continents [12, 19,

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32, 46, 62]. The massive range and scale of the rust disease indicates that the chance of wheat being infected by this pathogen will always be high. Therefore, increasing the rust resistance of cereal food crops such as bread wheat is undoubtedly important.

Plants have highly evolved and complex mechanisms of recognizing and responding to pathogenic threats. Chitinase, β -1,3-glucanase, and other pathogenesis-related (PR) proteins play major roles in defense against fungal attack. PR proteins constitute a group that is induced in plant cells suffering from pathogenic attack. Among these, β -1,3-glucanase and chitinase are two key factors involved in plant anti-fungal counter measures [9, 22, 53]. A portion of the cell wall of various pathogenic fungi consists of β -1,3-, -1,6-glucanase and chitin; therefore, many plant chitinases and β -1,3-glucanases can inhibit fungal growth, possibly by dissolving the tip of the fungal germ tubes and hyphae [41]. The combination of these two enzymes possesses stronger anti-fungal activity for a broader range of fungi than the individual enzymes [41]. Other PR proteins are known for their function in acquired resistance, contributing toward long-term resistance based on acquired memory [34, 59, 60].

Bread wheat (*Triticum aestivum*) is a primary cereal crop and constitutes a large portion of the modern diet in many countries [20, 66]. As a hexaploid ($6n=6x=42$) species, wheat has an exceptionally large and complex genome, containing more transposable elements than any other plants [17, 68]. This has impeded wheat genomic research compared to research on any crop with smaller genomes, such as rice and sorghum. *Aegilops tauschii* (*Ae. tauschii*) is a diploid wild grass species, which was reported to be the progenitor of the D-genome in wheat [30, 39, 42]. As *Ae. tauschii* is an important genetic resource for wheat, genomic studies on *Ae. tauschii* contribute significantly in understanding the large and complex genomes of wheat species. *Ae. tauschii* has the most diverse geographical habitat among *Aegilops* species, and thus can adapt extensively to various environmental conditions [28, 39]. *Ae. tauschii*, the progenitor of D-genome in wheat, is a good source of rust resistance genes that can be transferred to common wheat via homologous recombination using synthetic wheat as a bridge [11]. *Ae. tauschii* is reported to harbor resistant alleles of seedling LR-resistant genes *LR21* (1D), *LR32* (3D), *LR39* (2D), and *LR42* (1D), and the adult plant LR-resistant gene *LR22a* (5D) [39]. Although genetic manipulations can be used to rapidly modify food crops, the consumption of genetically modified organisms (GMOs) is controversial because of safety issues. We strongly believe that the selection of remarkable *Ae. tauschii* accessions as materials for synthetic wheat breeding is a more authentic approach in producing LR-tolerant bread wheat. In this

study, 68 *Ae. tauschii* accessions with diverse geographical backgrounds were selected and their growth performance was closely monitored after infection with the LR fungi. The two most remarkably resistant accessions were selected for further characterizations. These resistant ecotypes are invaluable genetic resources for developing the LR-tolerant synthetic hexaploid wheat via breeding in the future.

Materials and methods

Plant and pathogen materials

Sixty-eight ecotypes of *Ae. tauschii* were obtained from the RDA-Genebank Information Center. The *P. triticina* strain was provided by the Korea Research Institute of Chemical Technology. Amplification of urediospores for LR resistance screening was performed using the Korean wheat cultivar, keumkang.

LR inoculation conditions

The seeds were sterilized with NaOCl for 15 min and rinsed several times with autoclaved distilled water. The seeds were then stored at 4 °C for 3 days and then grown for 14 days after sowing in soil. The inoculum was prepared by diluting the urediospores of *P. triticina* in distilled water containing Tween 20 (120 μ L/L) (Sigma-Aldrich, P5927). The seedling leaves were sprayed evenly with the inoculum using an airbrush and incubated in the dark at 100% relative humidity for 24 h at 20 °C, the seedling then were grow normally in the growth room (23 °C, 16:8 h light/dark photoperiod). The infection type was evaluated 10 days post-inoculation. All experiments were performed in triplicate.

LR resistance at adult plant stage was screened on the field. As a spreader, a susceptible cultivar, keumkang, was placed between the *Ae. tauschii* cultivars. The infection type was evaluated 10 days post-inoculation.

Microscopy

The experiment was conducted as previously [6, 57]. Leaf samples were stained with wheat germ agglutinin conjugated to fluorescein isothiocyanate (WGA-FITC, Sigma-Aldrich) 7 days after leaf rust inoculation. Microscopic pictures of the leaf samples were obtained using the fluorescence upright microscope Axio Imager M1 (Carl-Zeiss) with a GFP fluorescence filter.

Rust biomass assay

The experiment was conducted as described by Ayliffe et al. [6]. Leaf samples (100 mg each) were collected 14 days after LR inoculation, washed with 50 mM Tris-HCl pH 7.5, and homogenized using sonication. WGA-FITC (Sigma-Aldrich) was added to each homogenate and the solutions were incubated for 1 h at

room temperature. The homogenates were washed again before being transferred to a 96-well microplate. The fluorescence values of each sample was measured using the multi-detection microplate reader Sense (Hidex) at 485 nm excitation and 535 nm emission, with 1.0 s measurement time [57].

Preparation of crude enzyme extracts

For extracting crude enzyme from the seedlings, 40 mg leaf samples were ground in liquid nitrogen. Pro-prep protein extraction solution was added and vortexed vigorously. The samples were centrifuged at 13,000 rpm for 5 min at 4 °C after incubation for 30 min on ice. The supernatant was used for the following enzymatic assays [1].

Chitinase assay

Chitinase activity after inoculation was measured using a chitinase assay kit (CS0980, Sigma-Aldrich). Changes in chitinase activity after treatment with elicitors were detected using the 3-, 5-dinitrosalicylic acid (DNS) method [44]. The reaction mixture consisted of 80 µL 0.2 M sodium phosphate buffer (pH 6.5), 50 µL crude enzyme extract, and 40 µL 1.6% (w/v) colloidal chitin. The reaction mixture was incubated at 37 °C for 1 h and stopped by adding 1% NaOH and boiling for 5 min. After centrifugation at 4000 rpm for 5 min at 4 °C, the supernatant and 1% of DNS were mixed in the ratio of 1:1 and boiled for 5 min. After cooling the tubes for 10 min, the absorbance was measured using the multi-detection microplate reader Sense (Hidex) at 540 nm. A standard curve was constructed using *N*-acetyl-D-glucosamine. One unit is defined as the amount of enzyme required to produce 1 µmol *N*-acetyl-D-glucosamine per minute under the conditions described [35].

β-1,3-Glucanase assay

β-1,3-Glucanase activity was measured using the DNS method. The reaction mixture consisted of 80 µL 0.2 M sodium phosphate buffer (pH 6.5), 20 µL crude enzyme extract, and 40 µL 1% (w/v) laminarin. The reaction mixture was incubated at 37 °C for 1 h and halted by adding 1% NaOH and boiling for 5 min. After centrifugation at 4000 rpm for 5 min at 4 °C, the supernatant and 1% DNS were mixed in 1:1 ratio and boiled for 5 min. After cooling the tubes for 10 min, the absorbance was measured using the multi-detection microplate reader Sense (Hidex) at 540 nm. A standard curve was constructed using glucose. One unit is defined as the amount of enzyme required to produce 1 µmol glucose per minute under the conditions described [10, 44].

Peroxidase (POD) activity assay

The reaction mixture consisted of 940 µL 25 mM guaiacol (2-methoxyphenol), 10 µL crude enzyme extract, and 50 µL 2% H₂O₂. The reaction mixture was incubated at room temperature for 2 min. After fivefold dilution, the absorbance was measured using the multi-detection microplate reader Sense (Hidex) at 470 nm. One unit of peroxidase is defined as the amount of enzyme required to convert guaiacol to tetraguaiacol in 1 min under the conditions described [10].

RNA sequencing and quantitative reverse transcription (qRT)-PCR

One hundred milligram fresh leaves of each sample were collected and ground in liquid nitrogen. RNA was extracted from the ground samples using the TRIzol method and was reverse synthesized to cDNA using the Thermo Scientific RevertAid first strand cDNA synthesis kit [36, 48]. qRT-PCR analyses were conducted using a Bio-Rad Thermocycler. All analyses were performed in independent triplicate. The primers used are listed in Additional file 1: Table S1.

Control and LR-inoculated samples of KU-2084 were prepared for RNA sequencing analysis as previously described [51].

Each experiment was performed in independent triplicate. Error bars indicate standard deviation and asterisks show the significant differences between control and LR-infected sample means (*P<0.05, **P<0.01, ***P<0.001). Statistical data was computed using one-way ANOVA of the Statistical Analysis System (SAS 9.4) program.

Results

Evaluation and classification of *Ae. tauschii* accession with respect to resistance to LR infection

The growth performances of 68 *Ae. tauschii* accessions of diverse geographical origins were assessed under LR inoculation conditions (Table 1). Seedlings and adult plant reactions of *Ae. tauschii* accessions to LR infection were assessed independently. Each *Ae. tauschii* accession was categorized to a certain infection type (IF) based on the severity of the rust system present on the leaf surface. IF 0 = no visible symptoms, 0+ = hypersensitive flecks, 1 = minute uredinia surrounded by mainly necrotic tissue, 2 = small to medium-sized uredinia surrounded by chlorotic and/or necrotic tissue, 3 = large uredinia without surrounding chlorosis, 4 = large uredinia without chlorosis or necrosis (Table 1, Fig. 1) [26]. Our results did not show any correlation between the reactions of *Ae. tauschii* to LR in the seedling and adult stages. Furthermore, the

Table 1 Origins and leaf rust resistance profiles of 68 *Ae.tauschii* specimens used in this study

Accession Name	Origin	SR	AR	Accession Name	Origin	SR	AR	Accession Name	Origin	SR	AR
16	Russia	1	2	82-Ae 3	China	2	2	KU-2117	Iran	3	0
337	Afghanistan	2	4	84TK501-003	Turkey	2	3	KU-2126	Iran	4	0
2001	Pakistan	2	2	84TK501-009	Turkey	2	0	PI 330489	–	3	3
2016	Pakistan	2	–	84TK501-010	Turkey	3	2	PI 349037	Azerbaijan	1	1
2036	Afghanistan	2	3	84TK501-012	Turkey	2	2	PI 431602	Turkmenistan	3	2
2095	Afghanistan	2	4	84TK530-002	Turkey	2	2	PI 431603	Azerbaijan	0	1
2111	Iran	2	0	84TK534-004	Turkey	2	2	PI 452130	China	2	3
2112	Iran	4	1	84TK572-004.00	Turkey	2	0	PI 560534	Turkey	4	0
2128	Iran	2	4	84TK573.1-001	Turkey	2	1	PI 560535	Turkey	2	–
2140	Iran	2	1	Ae 45	–	3	–	PI 560536	Turkey	2	2
2144	Iran	1	1	Ae 46	China	2	0	PI 560538	Turkey	4	3
2146	Iran	3	3	D.I.V. 16185	–	2	0	PI 560755	Turkey	2	1
2147	Iran	0	0	KU-2001	Pakistan	2	1	PI 574467	Russia	2	0
2168	Iran	2	3	KU-2019	Afghanistan	4	0	PI 574468	Armenia	3	1
2170	Iran	2	3	KU-20-2	–	2	0	PI 603221	Western Asia	0	1
2051-2	Afghanistan	2	2	KU-2059	Afghanistan	2	3	PI 603224	Russia	0	0
79TK057-318	Turkey	2	3	KU2069	Iran	3	0	PI 603225	Turkmenistan	0	2
79TK057-322-1	Turkey	2	2	KU-2073	Iran	2	0	PI 603230	Azerbaijan	4	2
79TK057-324	Turkey	1	3	KU-2074	Iran	1	0	PI 603233	Azerbaijan	2	1
79TK075-400	Turkey	2	3	KU-2075	Iran	4	2	PI 603246	Portugal	3	0
79TK075-405	Turkey	3	3	KU-2082	Iran	4	0	PI 603252	Iran	2	2
79TK091-455-2	Turkey	2	3	KU-2083	Iran	2	2	PI 603255	Armenia	2	2
79TK093-471	Turkey	2	1	KU-2084	Iran	0	0				

SR seeding reaction, APR adult plant reaction

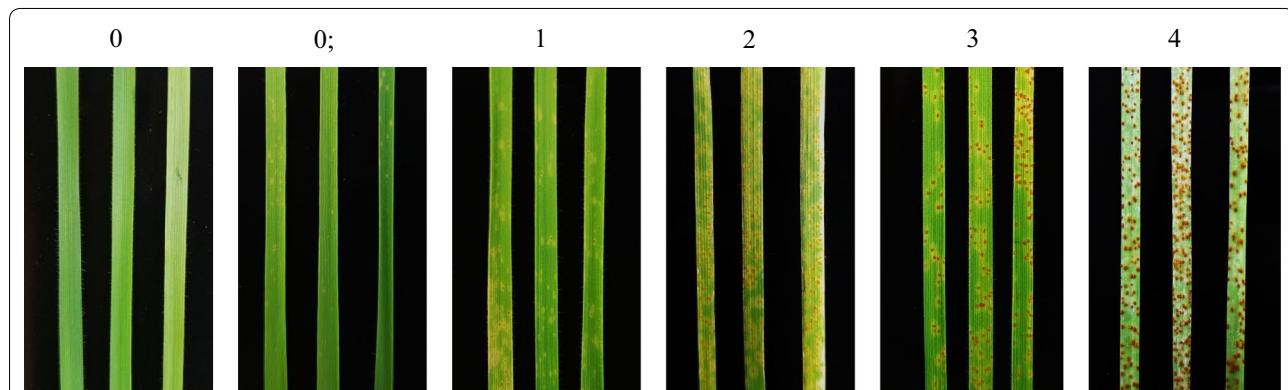


Fig. 1 Infection types of wheat leaf rust in *Ae. tauschii*. Infection types: 0 = no visible symptoms, 0+ = hypersensitive flecks, 1 = minute uredinia surrounded by mainly necrotic tissue, 2 = small to medium-sized uredinia surrounded by chlorotic and/or necrotic tissue, 3 = large uredinia without surrounding chlorosis or necrosis, 4 = large uredinia with surrounding chlorosis or necrosis

connection between geographical backgrounds and LR resistance was negligible, and accessions of the same origin reacted varyingly to rust infection (Table 1).

Two accessions with outstanding resistance to LR were identified. KU-2084, which showed no visible rust symptoms in either stage, and 2147, which showed only

hypersensitive flecks at the adult stage, were selected for further investigation. 79TK075-405 and PI560538, two ecotypes with severe LR symptoms were used as negative controls to complement the study on the two resistant ecotypes. Since our objective was to evaluate *Ae. tauschii* accessions as genetic materials for synthetic

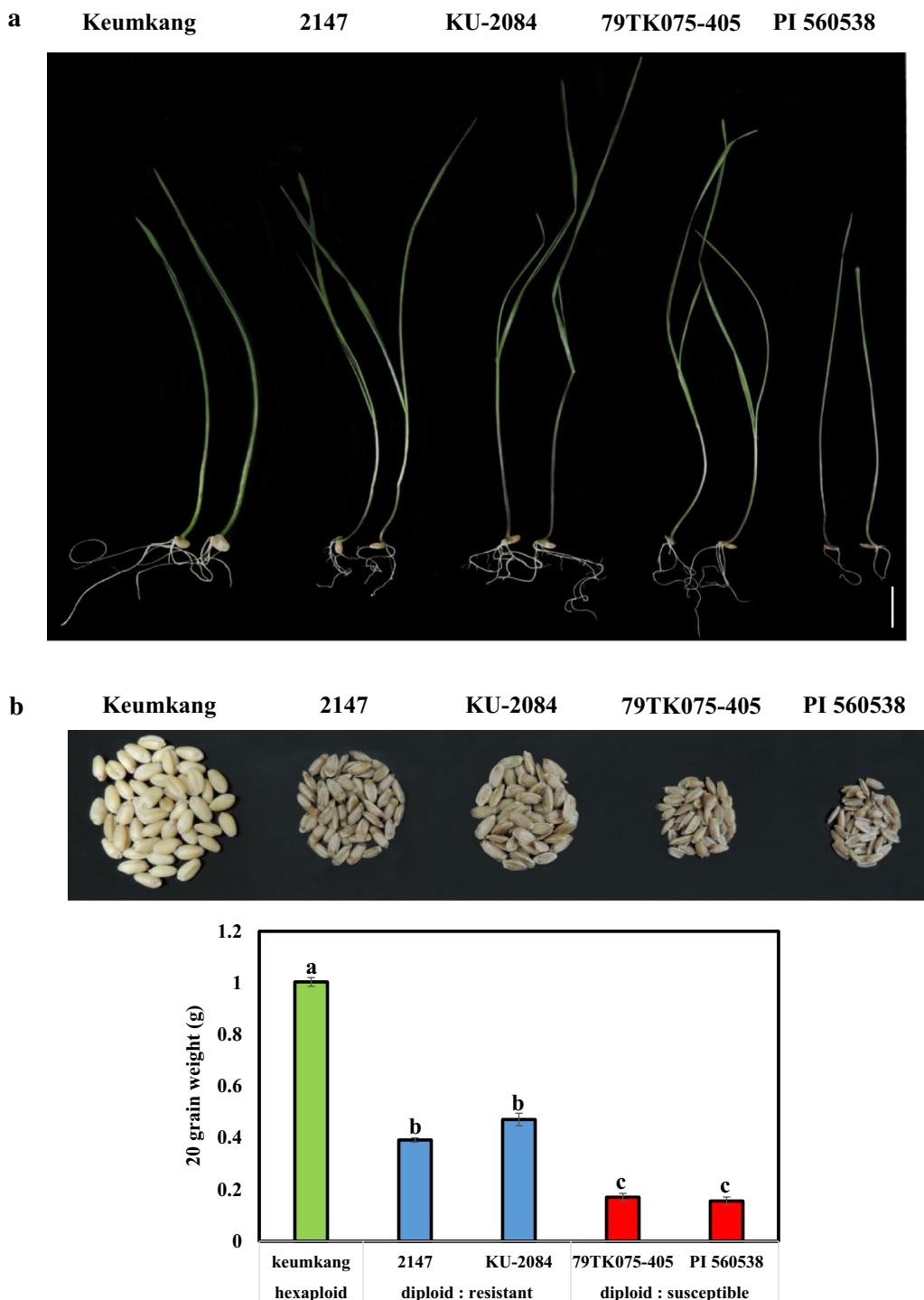


Fig. 2 Growth performance under normal condition. **a** Growth performance of keumkang, KU-2084, 2147, 79TK075-405, and PI560538 under normal conditions. Photographs of seedlings were obtained 14 days after germination. **b** Size and weight differences between keumkang, KU-2084, 2147, 79TK075-405, and PI560538 under normal conditions. Twenty kernels of keumkang and the *Ae. tauschii* lines were used for weight measurement

wheat breeding, we also included keumkang for the investigation.

Growth performance under normal conditions

Under normal (infection-free) conditions, no distinct morphological trait was detected in any of the four *Ae. tauschii* accessions (Fig. 2a). Figure 2b shows the seed sizes and measured grain weights (per 20 seeds) of keumkang and the four *Ae. tauschii* ecotypes under normal condition. The differences in seed size were evident; the kernels of 79TK075-405 and PI560538 were noticeably smaller than those of the two resistant ecotypes. Measured grain weight revealed approximately 40% heavier seed weight in the resistant accessions than in the susceptible accessions. However, this large difference in seed size and weight was not related to the LR resistance proficiency. Comparison of grain weight among different IF accessions indicated no correlation between grain weight and LR-resistant status (Additional file 1: Figure S2). As previous studies have not indicated any relationship between seed weight/size and LR resistance in *Ae. tauschii*, the differences in this case are possibly related to variations in adaptation to dissimilar environmental conditions (Table 1).

LR resistance of KU-2084 and 2147

The LR fungi, *P. triticina*, was introduced to 2-week-old seedlings of keumkang, KU2084, 2147, 79TK075-405, and PI560538 via spraying. Photographs of seedling leaves were obtained 10 days after the inoculation. As can be seen in Fig. 3, keumkang, 79TK075-405, and PI560538 exhibited severe rust urediospore development under identical LR treatment. On the contrary, 2147 and KU-2084 did not display any signs of rust sporulation on

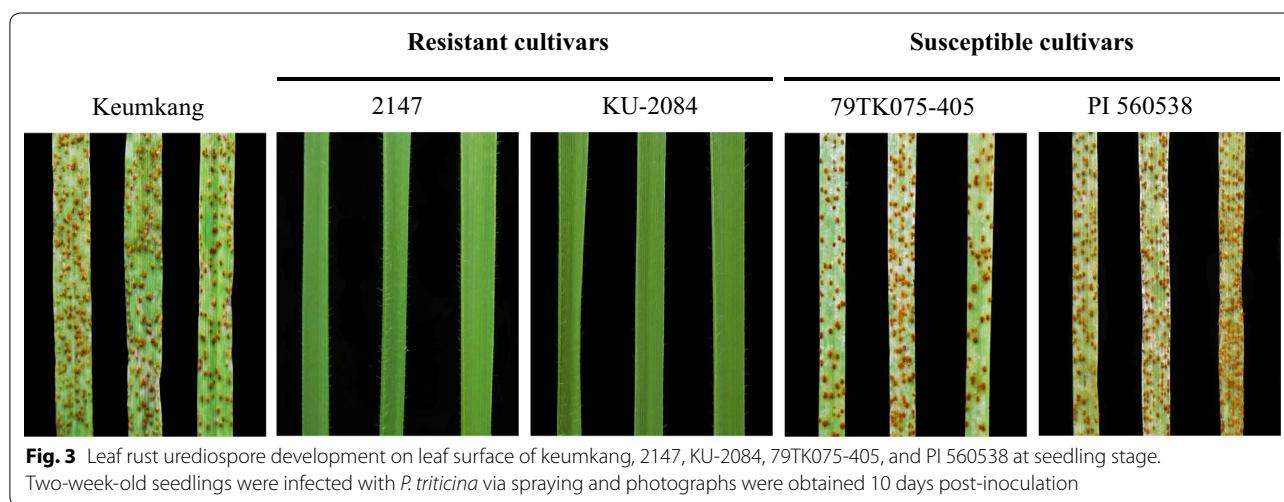
the leaf surface. Keumkang was classified as IF 3 in the seedling stage.

Microscopic analysis utilizing WGA-FITC, which binds to the fungal cell wall, demonstrated high density of fungal cells on the leaf surface of keumkang, 79TK075-405, and PI560538 [6, 57]. Conversely, fungal growth on the infection sites of 2147 and KU-2084 was prohibited (Fig. 4a). Rust biomass quantification on leaf surface indicated 75–80% reduction in rust colonization in 2147 and KU-2084 compared to that in keumkang and the two susceptible lines (Fig. 4b).

Plant defense against rust infection in 2147 and KU-2084

Leaf samples of 2-week-old keumkang and the four *Ae. tauschii* accessions were collected 6 h and 24 h after inoculation with *P. triticina*; the control sample was not treated with LR. Enzymatic activity of chitinase, β -1,3-glucanase, and POD were assayed using these samples. β -1,3-Glucanase enzymatic activity of 2147 increased slightly at 6 h and significantly at 24 h post-inoculation, whereas that of Ku-2084 showed a sudden increase at 24 h (Fig. 5b). Keumkang, 79TK075-405, and PI560538 displayed no real increase in β -1,3-glucanase activity level in response to rust infection.

The chitinase activity level of keumkang and KU-2084 were reduced 24 h post-inoculation, whereas no alteration in activity was observed in the three other *Ae. tauschii* accessions 6 h or 24 h post-infection (Fig. 5a). POD activity increased significantly in all *Ae. tauschii* accessions in response to rust infection after 24 h, with notable increase at 6 h post-inoculation in only the 2147 and 79TK075-405 accessions (Fig. 5c). In contrast, the POD activity of keumkang was unchanged. This implied that all four *Ae. tauschii* accessions reacted more rapidly



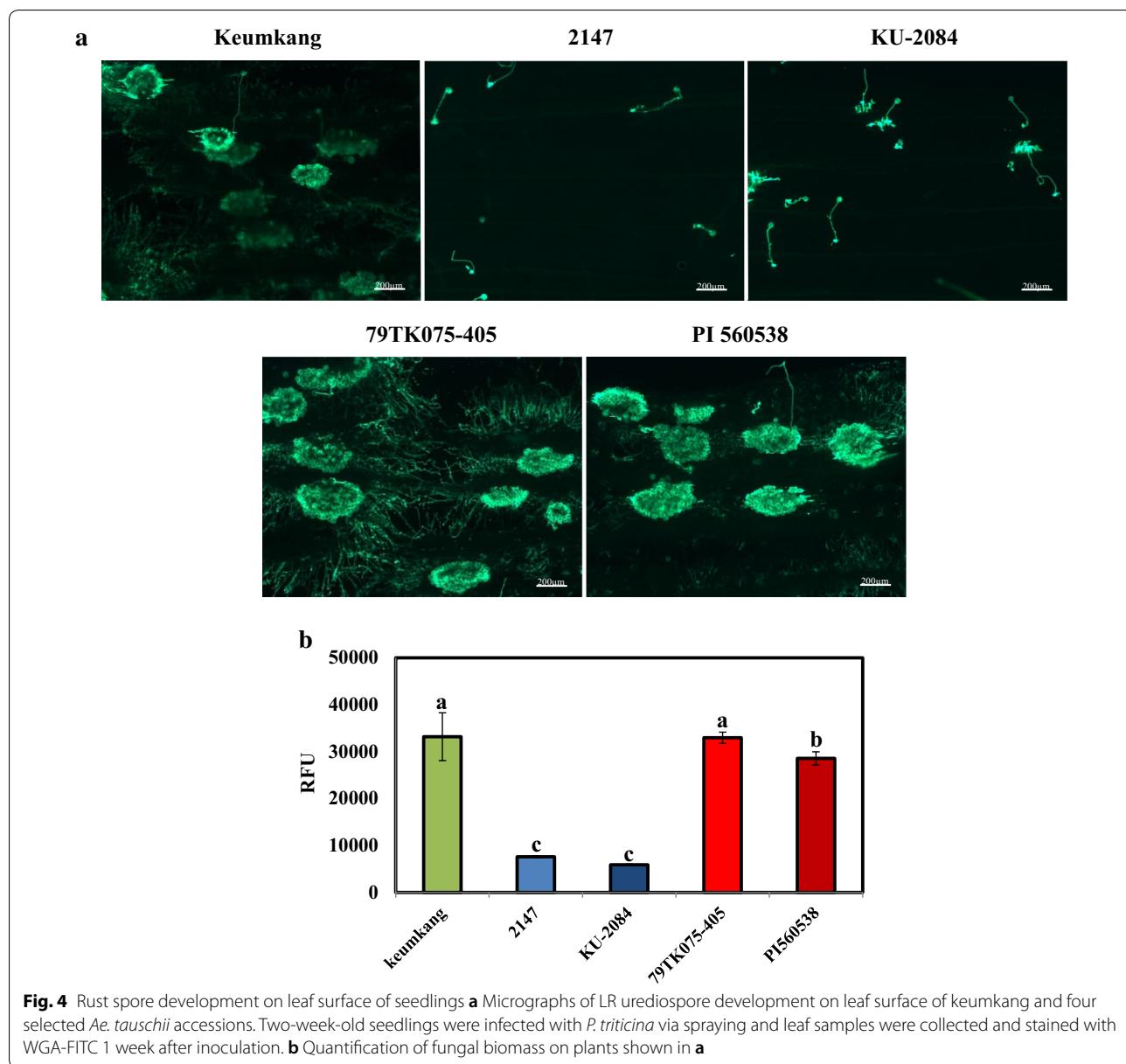


Fig. 4 Rust spore development on leaf surface of seedlings **a** Micrographs of LR urediospore development on leaf surface of keumkang and four selected *Ae. tauschii* accessions. Two-week-old seedlings were infected with *P. triticina* via spraying and leaf samples were collected and stained with WGA-FITC 1 week after inoculation. **b** Quantification of fungal biomass on plants shown in **a**

to fungal infection-induced oxidative stress than the wheat cultivar keumkang.

Expression of genes involved in pathogenic response in *Ae. tauschii*

To delineate the mechanism underlying the hyper-resistance of 2147 and KU-2084 to LR, RNA sequencing analysis was performed on control and LR-inoculated samples of KU-2084. Several genes, the expression levels of which were altered in response to LR infection, were identified (Table 2). In agreement with the measured POD activity shown in Fig. 5c, peroxidase 2-like (*atpod2*), peroxidase 1-like (*atpod1*), and peroxidase like (*atpodL*) were

highly upregulated in the LR treatment group compared to that in the control. Surprisingly, no β -1,3-glucanase-related gene was detected in the analysis. Higher expression of chitinase 8-like (*atchi8*), chitinase 1-like (*atchi1*), WRKY transcription factors 24, 60, 70, 72, leaf rust 10 disease resistance locus receptor protein kinase (*lr10r*), LRR receptor-like serine/threonine-protein kinase (*lrr receptor*) and ABC transporter 50 (*ABCG50*) were also observed. Rust infection also repressed the transcription of many chlorophyll a-b binding proteins and altered host plant secondary metabolism as shown by alterations in many genes associated with metabolic biosynthesis (Table 2).

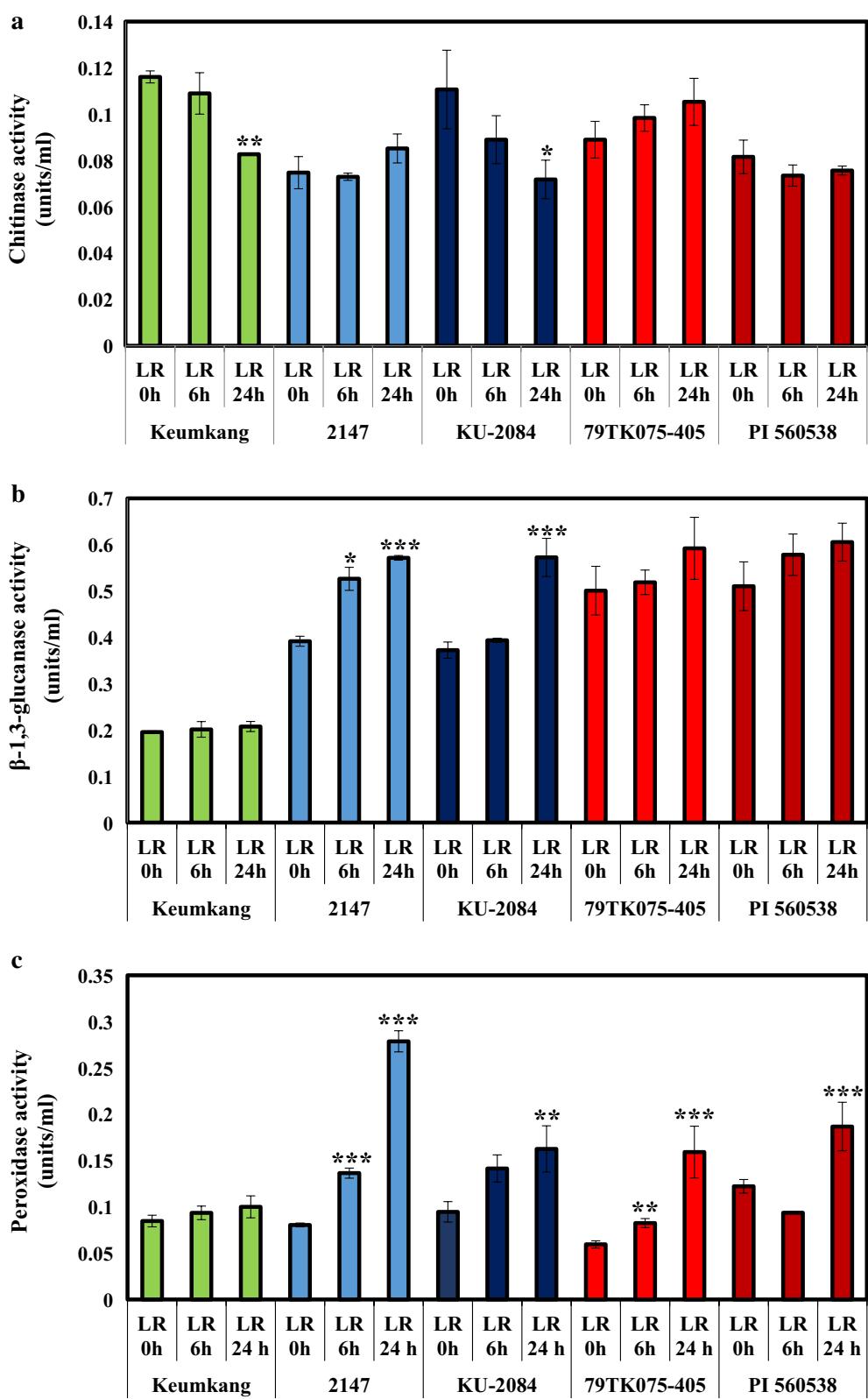
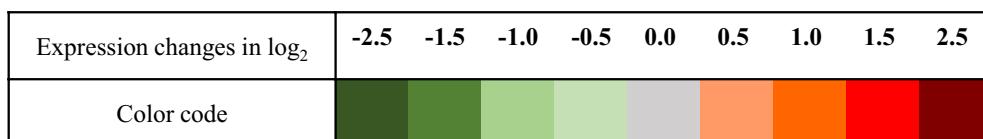


Fig. 5 Quantitative enzymatic activity assays under leaf rust treatment of keumkang and the four selected *Ae. tauschii* accessions. **a** Chitinase activity assay, **b** glucanase activity assay, and **c** peroxidase activity assay

Table 2 RNA sequencing analysis of control and leaf rust-treated samples of KU-2084

Gene ID	Description	Control vs LR treated
AET1Gv20521700	Aegilops tauschii subsp. tauschii chitinase 8-like	2.40236654
AET2Gv20218300	Aegilops tauschii subsp. tauschii peroxidase 2-like	2.19622896
AET2Gv20218200	Aegilops tauschii subsp. tauschii peroxidase-like	1.65561393
AET2Gv21079000	Aegilops tauschii subsp. tauschii probable WRKY transcription factor 70	1.565072528
AET7Gv20872800	Aegilops tauschii subsp. tauschii chitinase 1-like	1.35955613
AET3Gv20097200	Aegilops tauschii subsp. tauschii LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like 2.4	1.35802721
AET7Gv20224500	Aegilops tauschii subsp. tauschii ABC transporter G family member 50	1.15725761
AET7Gv20864500	Aegilops tauschii subsp. tauschii peroxidase 1-like	1.306333745
AET1Gv20977100	Aegilops tauschii subsp. tauschii probable WRKY transcription factor 72	1.404705995
AET4Gv20024800	Aegilops tauschii subsp. tauschii WRKY transcription factor WRKY24-like	1.324644015
AET5Gv20460100	Aegilops tauschii subsp. tauschii WRKY transcription factor WRKY62-like	1.065861576
AET6Gv20027800	Aegilops tauschii subsp. tauschii LRR receptor-like serine/threonine-protein kinase FLS2	1.30147096
AET1Gv20996400	Aegilops tauschii subsp. tauschii chlorophyll a-b binding protein of LHCII type III, chloroplastic	-1.64107854 3
AET5Gv20117400	Aegilops tauschii subsp. tauschii protein NRT1/PTR FAMILY 2.11-like	-1.45981367 1
AET7Gv20563000	Aegilops tauschii subsp. tauschii chlorophyll a-b binding protein 1B-21, chloroplastic	-1.39524811 4
AET5Gv20519300	Aegilops tauschii subsp. tauschii protein TIFY	-2.46091560 2
AET7Gv20671300	Aegilops tauschii subsp. tauschii chlorophyll a-b binding protein of LHCII type 1-like	-2.06844528 2
AET5Gv20809800	Aegilops tauschii subsp. tauschii chlorophyll a-b binding protein of LHCII type 1-like	-2.16033212 1
AET4Gv20506100	Aegilops tauschii subsp. tauschii probable 2-oxoglutarate-dependent dioxygenase At5g05600	-2.12886622 6
AET3Gv20669000	Aegilops tauschii subsp. tauschii anthocyanidin 5,3-O-glucosyltransferase-like	-1.97442017 1



Furthermore, qRT-PCR analysis of *atpod1*, *atpod2*, *atpodL*, *atchi1*, *atchi8*, *ABCG50*, *lr10r* and *lrr receptor* genes was performed on keumkang and the four *Ae. tauschii* accessions (Fig. 6). The expression levels of the three pod-related genes, *atpod1*, *atpod2*, and *atpodL*, were low under non-infected conditions, but were significantly induced by LR in keumkang and all four *Ae. tauschii* lines. Induction of pod-related gene expression

was distinct between the two resistant ecotypes and between keumkang, the susceptible ecotypes, and resistant lines. The chitinase-related genes *atchi1* and *atchi8* were highly upregulated in response to LR treatment in keumkang and the four *Ae. tauschii* accessions both 6 h and 24 h post-inoculation. KU-2084 displayed high fold induction in *atchi8* and *atchi1* expression 6 h post-inoculation, whereas 2147 showed the highest expression of

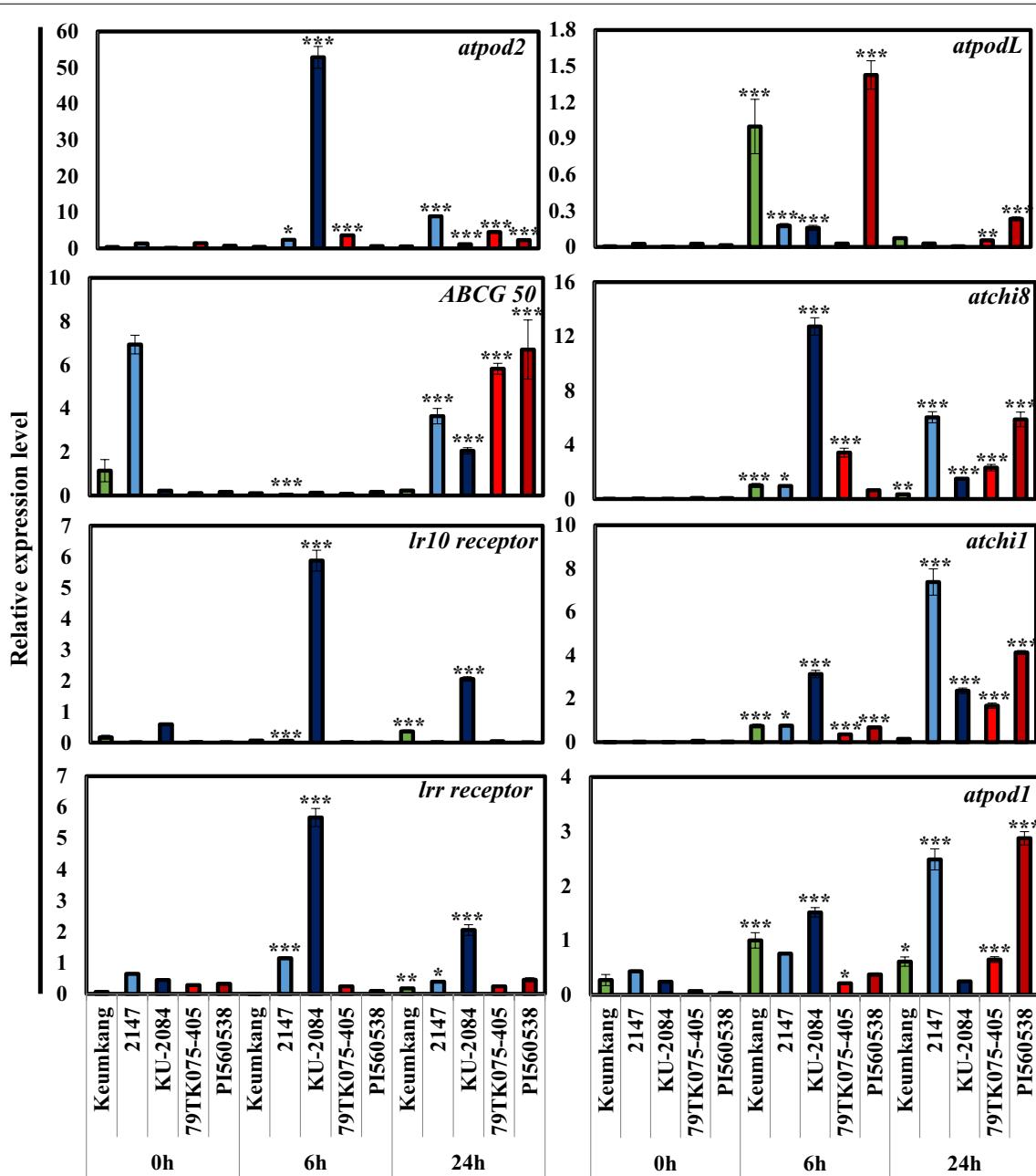


Fig. 6 qRT-PCR analysis of *atpod1*, *atpod2*, *atpodL*, *atchi1*, *atchi8*, *ABCG50*, *lr10r*, and *lrr receptor* in 0 h, 6 h, and 24 h post-inoculation samples each of keumkang, 2147, KU-2084, 79TK075-405, and PI 560538

atchi1 and *atchi8* among all tested samples 24 h after the treatment. Interestingly, under LR infection, transcriptional induction of the *lrr receptor* was only observed in the two resistant *Ae. tauschii* accessions 6 h and 24 h after the inoculation. Upregulation of *lr10 receptor* occurred in only KU-2084 line among the 4 tested *Ae. tauschii* accessions. Transcription of *ABCG50* was induced by LR infection 24 h after inoculation in KU-2084, 79TK075-405, and PI560538, whereas that of 2147 was significantly repressed by LR treatment at both 6 h and 24 h post-inoculation. These results implied that albeit identical geographical backgrounds, the hyper-resistances of KU-2084 and 2147 to LR are originated from different underlying mechanisms.

Discussion

Aegilops tauschii is a diploid wild grass species and is reportedly a progenitor of hexaploid wheat with D-genome [30, 39, 42]. *Ae. tauschii* is well-known for its adaptability to diverse environmental conditions as it has wide geographical distribution. Hence, *Ae. tauschii* can be a good source of genes conferring resistance to biotic and abiotic stresses for synthetic wheat. Synthetic hexaploid wheat technique was derived from mimicking the origin of natural hexaploid wheat in order to artificially recombine favorable traits of the wild progenitors (AB and D) [14, 45, 67]. The genetic material responsible for these favorable traits can be transferred to common wheat via homologous recombination using synthetic wheat as a bridge [11]. In addition, due to high level of genetic polymorphism between synthetic hexaploid wheat and common wheat, the former has been used as a good gene mapping tool [11]. Therefore, selection of good genetic resources in *Ae. tauschii* for generating synthetic hexaploid wheat is the important first step in both breeding of common wheat with desired properties and in advancing genetic studies in wheat.

LR, caused by *P. triticina*, is infamous for destroying cereal crops. Parasitic growth of *P. triticina* on wheat starves the host plant and can lead up to 30% loss in yield [54]. Furthermore, the rust pathogen is also known for its high adaptability to diverse climate condition, rapid growth, and ability to spread to large distances [12, 16, 19, 32, 46, 62], explaining why LR disease is a global threat to all crop plants on earth. Thus reinforcement of LR resistance in wheat is crucial for both maintaining agricultural values of the crop plant and controlling the spread of the disease [5, 28]. In this study, the growth performances of 68 *Ae. tauschii* accessions with various origins were monitored after LR inoculation. These accessions were then classified into five IF groups (Fig. 1) [26]. The KU-2084 and 2147 *Ae. tauschii* ecotypes displayed remarkable immunity to LR disease in both seedling and adult stages

(Table 1). Closer evaluation revealed that after LR infection, KU-2084 and 2147 did not develop any rust spots, whereas keumkang and two susceptible ecotypes showed severe LR symptoms (Fig. 3). Microscopic observation and rust biomass indicated complete inhibition of fungal cell growth on the leaf surface of KU-2084 and 2147.

PR proteins constitute a plant protein family, which is induced by pathogenic intrusion and is highly specific for pathogen infection [9]. They are a part of the induced systemic resistance (ISR) activated by a series of complex defense responsive signals triggered by pathogen-associated molecular pattern (PAMP) receptors upon recognition of pathogenic attack [27, 47, 60, 61]. Chitinase and β -1,3-glucanase are two PR members that play key roles in plant defense against fungal infection [9, 22, 53]. The cell wall of pathogenic fungi usually consists of β -1,3-, -1,6-glucanase and chitin; hence, many plant chitinases and β -1,3-glucanases produced as part of ISR can suppress fungal growth or block fungal infection by dissolving the tip of germ tubes and hyphae [41]. The combination of these two enzymes reportedly exhibits stronger anti-fungal activity against a broad range of fungi than the individual enzymes [41]. In the present study, we observed significantly higher β -1,3-glucanase activity in the two resistant *Ae. tauschii* accessions, KU-2084 and 2147, compared to that in keumkang and the two susceptible lines (Fig. 5b) at as early as 6 h and 24 h post-inoculation. Nonetheless, during our 24 h LR treatment, no noticeable difference in chitinase enzymatic activity was observed between resistant and susceptible lines. Previous studies on wheat resistance to LR revealed that the intracellular activities of chitinase and β -1,3-glucanase differed with respect to response to early infection at the seedling stage. β -1,3-Glucanase activity increased immediately on the 1st day after inoculation, whereas chitinase activity either remained unaltered or reduced slightly 48 h post-inoculation, and then increased dramatically thereafter [2, 3, 43]. This implied that in our study, chitinase may contribute to LR resistance of the two resistant *Ae. tauschii* lines, KU-2084 and 2147, later during the infection. However, β -1,3-glucanase activity appeared to be the primary factor contributing to LR resistance in KU-2084 and 2147 during early infection. Increased β -1,3-glucanase activity in KU-2084 (24 h) and 247 (6 h and 24 h) (Fig. 5) completely inhibited rust infection and growth (Figs. 3 and 4), possibly by dissolving the fungal penetration tools such as fungal germ tubes and hyphae. [41]. The gradual increase in POD activity in all four *Ae. tauschii* lines (Fig. 4) and the significant upregulation of *atpod1*, *atpod2*, and *atpodL* (Fig. 6) 6 h and 24 h post-inoculation indicated that the resistant and susceptible lines combatted LR-induced oxidative stress in identical manner.

The results of RNA sequencing further confirmed our hypothesis that chitinase contributed in the later stage of LR resistance of KU-2084 and 2147 (Table 2). The chitinase-related genes were highly upregulated by LR infection (Table 2 and Fig. 6). Interestingly, no β -1,3-glucanase-related gene expression was detected in our analysis. One of the reasons for this could be that these genes were triggered by infection earlier than the time of sample collection. In rice, the transcription of photosynthetic genes, such as that encoding ribulose 1,5-diphosphate carboxylase, and chlorophyll a/b-binding genes was suppressed in both the susceptible and resistant cultivars under fungal infection; in barley, LR infection reduced the total nitrogen and chlorophyll content [13, 24, 40]. These results corroborated our observation that transcription of many chlorophyll a–b binding genes and *NRT1.1* was repressed in LR-infected *Ae. tauschii* (Table 2). This indicated that the detrimental effects of *P. triticina* on *Ae. tauschii* and crop plants such as rice and barley were similar. WRKY transcription factors are involved in numerous plant physiological processes, including hormone response, abiotic and biotic stress response, and defense [23, 37, 49, 65]. In the present study, LR treatment induced the expression of four WRKY transcription factors in KU-2084, namely WRKY24 (homolog of TaWRKY41), WRKY62 (homolog of Tawrky15), WRKY70 (homolog of TaWRKY 45), and WRKY72 (homolog of TaWRKY19). As TaWRKY45 reportedly enhances transgenic wheat resistance to *Fusarium* head blight [23] and several WRKY members are involved in PAMP recognition of pathogen threat in plants [8, 37], the upregulation of these WRKY genes in infected KU-2084 may represent a mechanism of either plant-pathogenic response or pathogenic defense.

The qRT-PCR analysis revealed that the *Ae. tauschii* LRR receptor-like serine/threonine-protein kinase FLS2 (*lrr receptor*) was the only gene that exclusively induced in resistant *Ae. tauschii* accessions under LR treatment. LRR receptor-like serine/threonine-protein kinase FLS2 is a member of receptor-like kinase (RLK) family, which was reportedly functioned in either cell growth and development or as pattern recognition receptors (PRRs) in PAMP [7]. According to [7], LRR receptor-like serine/threonine-protein kinase FLS2 was classified as transmembrane receptor kinase (TMRK) with specified function of disease resistance. In *Arabidopsis thaliana*, LRR receptor-like serine/threonine-protein kinase FLS2 operates as PPR that determines the specific perception of flagellin (flg22), a potent elicitor of the defense response to pathogenic threat [7, 21]. Induction of *lrr receptor* transcript in response to LR measured at 6 h post inoculation in KU-2084 and 2147 hinted that recognition of LR infection via the elicitor flagellin and the downstream

signaling pathway activated by the receptor could be the major contributors to the hyper-resistance to the disease of the 2 ecotypes. The *Ae. tauschii LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR-LIKE PROTEIN KINASE-like 2.4 (lr10r)* gene was significantly induced only in KU-2084 after LR treatment (Fig. 6). The wheat receptor-kinase-like gene *TaWAKL4/Stb6* is the closest homolog of *lr10r* of *Ae. tauschii* with 97% similarity in sequence identity and 61% query cover. Previous studies indicate that *TaWAKL4/Stb6* plays an important role in resistance to the fungal disease septoria tritici blotch (STB) in many cereal plants [4, 15, 56]. *TaWAKL4/Stb6* can confer pathogen resistance without triggering hypersensitive response in plants [55]. A study found that *TaWAKL4/Stb6* encoded receptor kinase (WAK)-like protein can detect presence of fungal apoplastic elicitors to trigger downstream defense mechanisms [56]. As shown in the increase of *lr10r* transcript only in KU-2084 and not 2147 under LR condition, it suggests that there are distinct downstream signaling pathways activated after LR infection contributing to the hyper-resistance phenotype of each ecotype. However, more studies are required to clarify these speculations.

ABC transporters are one of the largest protein families. In plants, ABC transporters play important roles in many essential processes such as organ growth, plant nutrition, plant development, response to abiotic stresses, pathogen resistance, and plant's ability to interact with the environment [29, 63]. The ABC subfamily G (ABCG) transporter is the largest ABC subfamily [29]. Genome-wide transcription profiling of ABCG transporters in *Arabidopsis* revealed that 50% of these transporters are induced by jasmonic acid (JA) and/or salicylic acid (SA), two well-known phytohormones involved in plant biotic stress response. This implied that several ABCGs participate in plant pathogen defense and/or pathogen response. Studies indicated that the anti-fungal activities of ABC transporters can be because of anti-pathogenic plant secondary metabolites such as phenolics, terpenoids and their derivatives, alkaloids, glucosinolates, and cyanogenic glycosides, the biosynthesis of which is mediated by induced ABC transporters [29, 50]. *NpPDR1*, an ABCG protein, is reportedly involved in plant immunity against the necrotrophic fungi *Botrytis cinerea* in *Nicotiana plumbaginifolia* [25]. *AtABCG36/AtPDR8/PEN3* is required for powdery mildew resistance in *A. thaliana*. A gene encoding a full-sized ABCG transporter was identified as the responsible gene for the resistance allele of the multi-pathogen resistance gene *LR34* [33, 57, 58]. These evidences suggested that *ABCG50* might contribute to LR tolerance in *Ae. tauschii*.

In conclusion, we identified two LR hyper-resistant *Ae. tauschii* ecotypes, KU-2084 and 2147. Further

investigations revealed that chitinase activity contributes considerably to the immunity of the two accessions against LR. These two *Ae. tauschii* ecotypes are valuable genetic sources of anti-LR that can be transferred to bread wheat via selective breeding. We strongly believe that breeding using carefully selected materials such as these two *Ae. tauschii* lines is an authentic approach for creating new and better wheat cultivars.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s13765-020-00496-z>.

Additional file 1: Figure S1. Sequence of *Puccinia triticina* strain HSZ0742. The strain was identified by comparing sequence similarity using NCBI/BLAST (*Puccinia triticina* strain HSZ0742 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; 28S ribosomal RNA gene, partial sequence). **Figure S2.** Weight comparison of 20 kernels by infection types. **Figure S3.** PCR results of leaf rust markers. Marker band size: csLV34: 150 bp; Xgwm539: 157 bp; Xgdm 35: 189 bp; Xcf4: 262 bp. **Figure S4.** Visualization of RNA-sequencing results of control and LR-infected KU-2084 samples. **Table S1.** Primers used in this study.

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Authors' contributions

AL, CST and HJL developed ideas and designed experiments. AL, CST, WJL, DP and IC carried out experiments. MK and HL provided microbial source and analytical tools. CC provided plant materials. DP and IC did RNA sequencing analysis. CST, AL and HJL wrote the manuscript, NC, SWH and HJL revised the manuscript. All authors read and approved the manuscript.

Competing interests

All authors declare that there is no conflict of interests.

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