

Article

Development and Characterization of Synthetic Hexaploid Wheat for Improving the Resistance of Common Wheat to Leaf Rust and Heat Stress

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Abstract: Synthetic hexaploid wheat (SHW) is a valuable resource for breeding because it possesses more desirable traits, such as better yield and abiotic and biotic stress tolerance than common wheat. In this study, our group developed a SHW line, named ‘SynDT’, which has markedly better characteristics than Korean bread wheat ‘Keumkang’. The SynDT line is thermotolerant as it rapidly expresses heat shock proteins under heat stress. In addition, this line exhibits resistance to leaf rust by inducing the expression of antifungal enzymes, mainly chitinase, along with the rapid and high expression of pathogen-related genes. Moreover, it possesses the favorable traits of its parent wheat lines *Triticum durum* #24 and *Aegilops tauschii* #52. Therefore, the SynDT wheat line can be used as a breeding material for improving local common wheat cultivars.



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Keywords: synthetic wheat; heat stress; leaf rust

1. Introduction

Hexaploid common wheat or bread wheat (*Triticum aestivum* L.; hexaploid genome = AABBDD) is a crucial cereal crop worldwide. A third of the global population depends on this crop, and the rise in global population has led to an increased demand for bread wheat. However, sustainable wheat production is threatened as wheat productivity has not improved significantly over time [1]. This challenge can be explained by the narrow genetic diversity of bread wheat, which is the result of a bottleneck event during its evolution, as only a few accessions of the donor species were involved in its origin and evolution [2]. Synthetic hexaploid wheat (SHW) has been artificially developed to address this issue. To mimic the natural hybridization of bread wheat, tetraploid *Triticum turgidum* L. wheat ($2n = 4x = 28$, AABB) and diploid *Aegilops tauschii* Coss. ($2n = 2x = 14$, DD) were selectively crossed to pass the elite genes of desirable traits to their progenies [2]. Consequently, SHW is a valuable source of genetic variability, with favorable traits, such as improved yield and abiotic and biotic stress tolerance, which are the primary targets of modern breeding strategies [3]. A previous study on genetic diversity in SHW has reported that this breed has higher levels of diverse amplified fragment length polymorphisms (39%) than common bread wheat (12–21%) [4]. To date, China is one of the successful countries to have managed to completely utilize SHW as a genetic resource. The well-known representative SHW line, “Chuanmai 42”, has a high average yield, and its synthetic derivative lines (SDLs) are commonly used in wheat breeding [5].

Leaf rust (LR) is a common disease in wheat caused by the fungi *Puccinia triticina* [6]. The fungi usually attack the leaf blades and cause a decrease in kernel weights and numbers

per head. LR disease in the early stage of growth results in 30% yield loss [7]. SHW is a genetically variable source of LR resistance. “Syn137” (68.111/RGB-U//Ward/3/A. *tauschii* [WX629]) is resistant to LR at the seedling stage owing to the presence of a dominant LR resistance gene on chromosome 5D [8]. Moreover, “Synthetic 45” is resistant to 20 pathotypes of LR at the seedling stage. Genetic mapping has shown that the resistant gene, *LrSyn45*, is located on chromosome 1D [9]. A novel LR-resistant gene, *Lr32*, has also been identified in synthetic wheat [5]. Therefore, developing LR-resistant SHW is one of the main strategies for crop improvement.

Climate change has caused an increase in global temperature, adversely affecting plant growth. In plants, heat stress (HS) is known to cause irreversible damage when the temperature exceeds threshold levels [10]. HS negatively affects all stages of plant development [11]. Additionally, wheat is sensitive to HS. It is estimated that an increase in temperature of 1 °C causes a 6% reduction in wheat production [12]. Thus, developing SHW resistant to HS is essential. Advanced wheat lines derived from SHW have shown 13% higher yield under HS conditions than their parent lines [13]. Multiple synthetic derivatives have also shown high tolerance to HS [14].

Despite the outstanding tolerance of SHW to environmental stresses, it still processes undesirable agronomic traits, making it unsuitable for commercial usage [9]. Thus, these desirable traits of SHW are usually transferred to domesticated bread wheat to generate SDLs for the improvement of local common wheat cultivars. Many SDLs that have been registered worldwide show higher genetic diversity than their parent lines [9]. In this study, for common wheat cultivar improvement, SHW lines that can confer resistance to HS and/or LR were developed. These lines can be used as breeding materials for improving local cultivars. Our findings showed that, in comparison to Keumkang, a bread wheat cultivar that is widely grown in Korea, the SHW line developed in the present study (named SynDT) was tolerant to HS and LR. In the future, this SHW line can be crossed with local bread wheat cultivar for improving stress tolerance.

2. Materials and Methods

2.1. Plant Materials

The SHW lines, SynDT #1 and #2, are the progeny of artificial crossing between *T. durum* #24 (*T. turgidum* LEUCURUM 33) and *Ae. tauschii* #52 (*Ae. tauschii* TA 1616). Both *T. durum* #24 and *Ae. tauschii* #52 were obtained from the International Maize and Wheat Improvement Center (CIMMYT) (Table S3). The embryo was rescued, and the plantlets were grown for several weeks in half-strength MS media before it was transferred to soil. Before the heading stage, the chromosomes were doubled by colchicine treatment (solution concentration: 0.5 mg/mL); next, the plants were grown until the grains were set. The collected grains were checked and amplified. Keumkang was used for comparison with SHW.

2.2. Confirmation of D Genome in SynDT

For crossing, *T. durum* were chosen as receivers and *Ae. tauschii* as donors. Therefore, to verify the success of pollination by confirming the presence of the D genome on the synthetic wheat line, which is inherited from *Ae. tauschii*, a SCAR-marker, SCS253, was utilized. This marker is linked to *Lr19* gene and on 7D chromosomes. The PCR condition, which is described by Huseynova et al. (2013) [15], is used to get the SCS253 bands in SynDT line and its parent wheats.

2.3. LR Infection

The *P. triticina* strain, HSZ0742 (NCBI Gene Bank DQ41710.1), was provided by the Korea Research Institute of Chemical Technology.

The wheat grains were sown in soil for eight days (23 °C, 16 h light/8 h dark) before infection with *P. triticina*. Three pots per wheat line were planted for each replicate. The

plants were infected as described in a previous study [16]. The phenotype images were acquired at 8 days postinoculation (dpi).

2.4. HS Treatment

The grains were sown in soil for six days (23 °C, 16 h light/8 h dark) before being exposed to HS. Three pots per wheat line (20 grains in each pot) were planted for each replicate. At day 6, the wheat was exposed to 45 °C temperature for 8 h and then returned to normal conditions (23 °C, 16 h light/8 h dark). At day 7 or 8, HS exposure, as performed on day 6, was repeated. Images were acquired after 4 days of recovery.

2.5. Microscopy and Rust Biomass Assay

At 8 dpi, LR samples from 5 leaves were collected for either microscopy or rust biomass assays. This was performed according to the detailed protocol described by Truong et al. (2019) [16].

2.6. Protein Extraction and Enzyme Assays: Chitinase, β -1,3-Glucanase, Peroxidase Activity Assays

Fifty milligrams of LR samples from 3 leaves were collected at 0, 6, and 24 h post-inoculation (hpi). Total protein was extracted using PRO-PREP™ protein extraction solution (iNtRON Biotechnology). The extracted solution was utilized for three enzyme assays: chitinase, β -1,3-glucanase, and peroxidase assays. These assays were performed based on a previously reported protocol [16].

2.7. Real Time RT-PCR Analysis

For HS, 6-day-old seedlings were exposed to a temperature of 45 °C for 0, 4, and 8 h. In contrast, 8-day-old seedlings were infected with *P. triticina* spores at 0, 6, and 24 h. The samples from 3 leaves were collected and ground in liquid nitrogen. Total RNA was extracted using TRIzol (Ambion, Life Technologies, Carlsbad, CA, USA) solution. After incubation, the samples were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was transferred to new Eppendorf following by adding 200 μ L chloroform, mixing and incubating for 3 min at room temperature. The samples were then centrifuged, and the supernatant was added isopropanol, inverting, and standing on ice for 10 min. After centrifugation, the supernatant was discarded, washed with 70% ethanol and the pellets were dried for an hour. After that, DEPC distilled water was added and incubated in 55 °C for 10 min. The quality of RNA is checked by agarose gel running. From that, cDNA is synthesized by using the Thermo Scientific RevertAid first strand cDNA synthesis kit [17]. The difference in transcript levels was determined by qRT-PCR analysis (CFX connect Real-Time PCR, Bio Rad, Hercules, California 94404, USA). The internal control for normalization was Plant actin *eu97*. The primers used in this study are listed in Table S1. The qRT-PCR data were analyzed by Bio-Rad CFX Manager 3.1 software.

2.8. Data Analysis

For robust results, each experiment was repeated at least twice. One set of representative data was chosen for analysis. The standard deviation (SD) value was calculated from three replicates of each treatment. Statistical Analysis System (SAS) was used to perform one-way analysis of variance (ANOVA) and Tukey's test ($\alpha = 0.05$). Among lines within a treatment condition, different letters (a, b, or c) indicate significant different based on one-way ANOVA ($p < 0.05$).

3. Results and Discussion

3.1. Process of Generating Synthetic Wheats and Their Morphology

In this study, more than 50 lines each of *T. durum* and *Ae. tauschii* cultivars were obtained from the CIMMYT. Preliminary screening was done to select the cultivars with at least mild tolerance to HS or LR (data not shown). Both *T. durum* and *Ae. tauschii* were

planted in the field for grain amplification and crossing. For crossing, we chose *T. durum* as receivers and *Ae. tauschii* as donors. Before the grain-filling stages, we performed emasculation. The anthers inside each spikelet from *T. durum* were removed and covered with polythene bags. Three days after emasculation, the pollens of *Ae. tauschii* were transferred to the emasculated *T. durum*. After three weeks, we collected the synthetic grains without endosperms. The embryos of synthetic grains were rescued and placed on MS media containing NAA hormone until they were large and strong enough to grow in soil (Figure 1A). At this stage, the synthetic plants were haploid; when the plants attained maturity, we treated them with colchicine to double their chromosome number and let them grow until the kernels were set. Figure 1B illustrates the process of colchicine treatment: removing the plants from the soil, leaving small leaves, washing off the soil in roots, immersing the plant roots in colchicine solution and aeration for 6 h, rinsing with water, and replanting the treated plants back to the soil. After colchicine treatment, the spikes, and kernels of the synthetic plants were successfully set (Figure 1C). To verify our synthetic wheats, we checked the presence of the D genome from *Ae. tauschii*, using the SCS253 marker. Through the preliminary PCR experiments, we found that this marker is only shown bands in the *Ae. Tauschii* but not *T. durum* lines, which is a specific marker to check the presence of D genome in hexaploid wheat. As shown in Figure 1D, *T. durum* #24, which contains the A and B genomes, did not have any band of SCS253 marker. Only *A. tauschii* #52 and SynDT #1 and #2 containing the D genome showed bands at 737 bp. The confirmed synthetic wheat plants were planted in the field for grain amplification.

In this study, the responses of our synthetic wheat (SynDT) to LR and HS were compared to those of Keumkang. In addition, we also examined *T. durum* #24 and *Ae. tauschii* #52, which are the parents of SynDT. Before studying their response to LR and HS, we also observed that the studied wheat varieties had different grain morphology and weight. SynDT had large grains, with long and thin shape. Compared to Keumkang, *T. durum* #24 is longer in length but similar in width. Keumkang has short and round shapes, whereas those of *Ae. tauschii* #52 were the smallest (Figure 2A,B). Furthermore, there is a correlation between grain size and weight. The order of weights of 100 grains/line is SynDT > Keumkang > *T. durum* #24 > *Ae. tauschii* #52 (Figure 2C, Table S4). The size of wheat grains is one of the most crucial agronomic traits because of its effect on grain weight, milling quality, and market price [18]. In addition, larger grains may have favorable traits of increased yield and seedling vigor [19]. Taken together, our synthetic wheat lines have larger and heavier grains, which is a promising trait for breeding value.

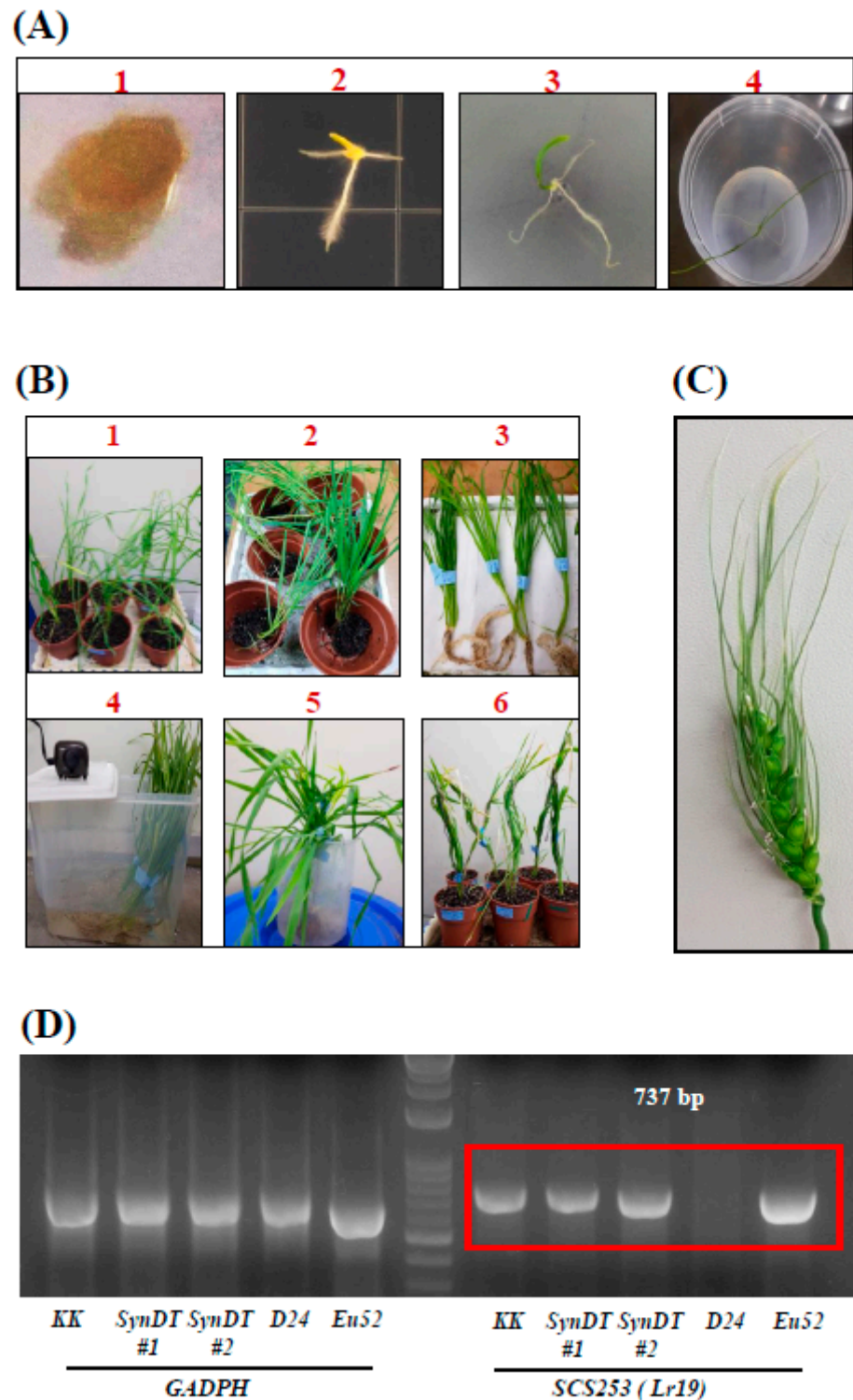


Figure 1. Procedure for generation and confirmation of synthetic wheat lines. **(A)** Rescue and growth of embryo of synthetic grains. (1) The embryo of synthetic grains. (2) One-week-old synthetic plants. (3) Two-week-old synthetic plants. (4) One-month old synthetic plants. **(B)** Procedure of colchicine treatment. (1) The ready and mature plants for colchicine treatment. (2) The soil from the root sites was removed, along with small, weak leaves. (3) The roots were washed with water and allowed to dry for 30 min. (4) The plants were immersed in colchicine solution and aerated for 6 h. (5) The plants were rinsed for 30 min with water. (6) The treated plants were re-planted inside the pots. **(C)** Spike of SynDT. **(D)** Confirmation of D genome in SynDT using a SCS253 marker.

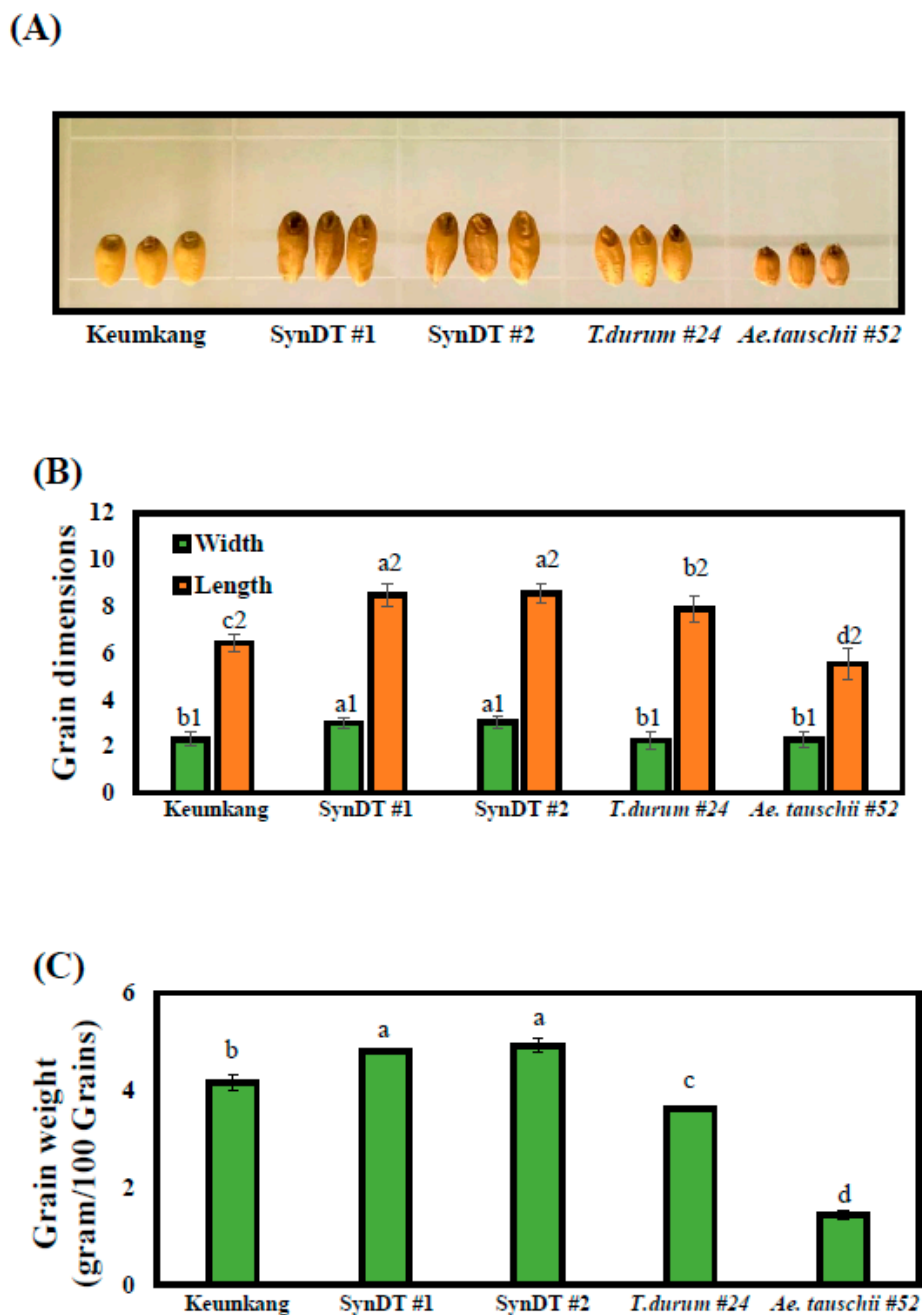


Figure 2. Grain morphology of all studied wheat cultivars. (A) Pictures of grain morphology of Keumkang, SynDT #1 and #2, *T. durum* #24, and *Ae. tauschii* #52. (B) Grain dimensions of Keumkang, SynDT #1 and #2, *T. durum* #24, and *Ae. tauschii* #52. (C) Weight of 100 grains of Keumkang, SynDT #1 and #2, *T. durum* #24, and *Ae. tauschii* #52. Standard deviation value was calculated from three replicates in each treatment. One-way ANOVA and Tukey's test ($\alpha = 0.05$) were performed using Statistical Analysis System (SAS). Different letters (a, b, or c) indicate significant differences ($p < 0.05$).

3.2. Tolerance of SynDT to HS Due to the Induction of HSPs

First, we examined the response of our synthetic wheat to HS. The 6-day-old wheat seedlings were subjected to extreme HS at 45 °C for 1, 2, or 3 consecutive days for 8 h per day. After 4 days of recovery, we observed that SynDT lines grew better than *Keumkang*, with full second leaves emerging from the stem. Most of the *Keumkang* plants were severely affected by HS, with their second leaves being damaged and growing abnormally (Figure 3A,B). Therefore, based on the growth statuses of the second leaves, we measured

the recovery rates of the studied wheat lines, and found that SynDT lines have higher recovery rates than Keumkang (Figure 3C, Table S5). Keumkang was included in this study for good comparison [16] because, in a previous study, Keumkang had the highest tolerance to HS among the common Korean wheat cultivars. Additionally, we checked the response of the parent wheat, *T. durum* #24 and *Ae. tauschii* #52, to HS and found that *Ae. tauschii* #52 is more tolerant to HS than Keumkang and *T. durum* #24 (Figure S1). Therefore, we believe that the HS tolerance of SynDT lines is mainly inherited from *Ae. tauschii* #52. In addition, some studies have revealed that the D genome from *Ae. tauschii* is a valuable resource for the variability in improved agronomic traits [20,21].

At the molecular level, the response to HS is mainly characterized by the expression of HSPs. The increased accumulation of HSPs might contribute to thermotolerance [22]. Under stress conditions, HSPs play a role in protein stabilization and refolding [23]. Therefore, we examined the transcript levels of *HSP17.3* (NCBI X58279.1), *HSP70.1* (NCBI KJ027551.1), and *HSP101b* (NCBI AF097363) (Tables S2 and S6). *HSP17.3* is a small HSP (sHSP) that binds to non-native proteins to prevent thermal aggregation [23]. The transcript levels of *HSP17.3* were quickly induced in response to HS (Figure 4A). At the 4 h time point, HS significantly increased the expression levels of *HSP17.3* in SynDT #1 compared to that in Keumkang (106-fold); *HSP17.3* expression in *T. durum* #24 was also upregulated. At 8 h, the transcript levels of *HSP17.3* were reduced in most wheat lines except in Keumkang. In a previous study, the expression of *HSP17* was also induced in response to HS in “HD2329” and “C-306” wheat cultivars [24]. *HSP70* belongs to one of the main families of HSPs and functions as a chaperone in protein refolding and prevents aggregation [22]. *HSP70* is also involved in the response to various environmental stress factors, such as heat, cold, oxidative stress, heavy metals, and pathogens [25]. In our study, HS exposure also induced the expression of *HSP70.1*, particularly in SynDT #1 at 4 h (Figure 4B). At 8 h of HS, the expression levels of *HSP70.1* showed no difference between Keumkang and SynDT #1. *HSP70.1* expression in the parent *T. durum* #24 was maintained at 4 and 8 h of HS exposure. Similarly, SynDT #1 showed significantly higher *HSP101b* expression than Keumkang at 4 h (262-fold). There was no difference in the transcript levels of *HSP101b* after 8 h of HS. Moreover, the expression of *HSP101b* in *T. durum* #24 was higher at 8 h than at 4 h. In contrast, *Ae. tauschii* #52 had higher transcript levels of *HSP101b* at 4 h compared to that at 8 h. *HSP101* is known to reactivate aggregated proteins [26]. In maize, the accumulation of *HSP101* protein was increased in response to HS [27]. Thus, SynDT showed higher expression levels of HSPs than Keumkang, especially at 4 h, indicating that the former responded to HS more quickly than Keumkang to protect the plants from damage. Moreover, in the parent lines, *HSP17.3* and *HSP70.1* were mainly expressed in *T. durum* #24, while *HSP101b* was expressed in both.

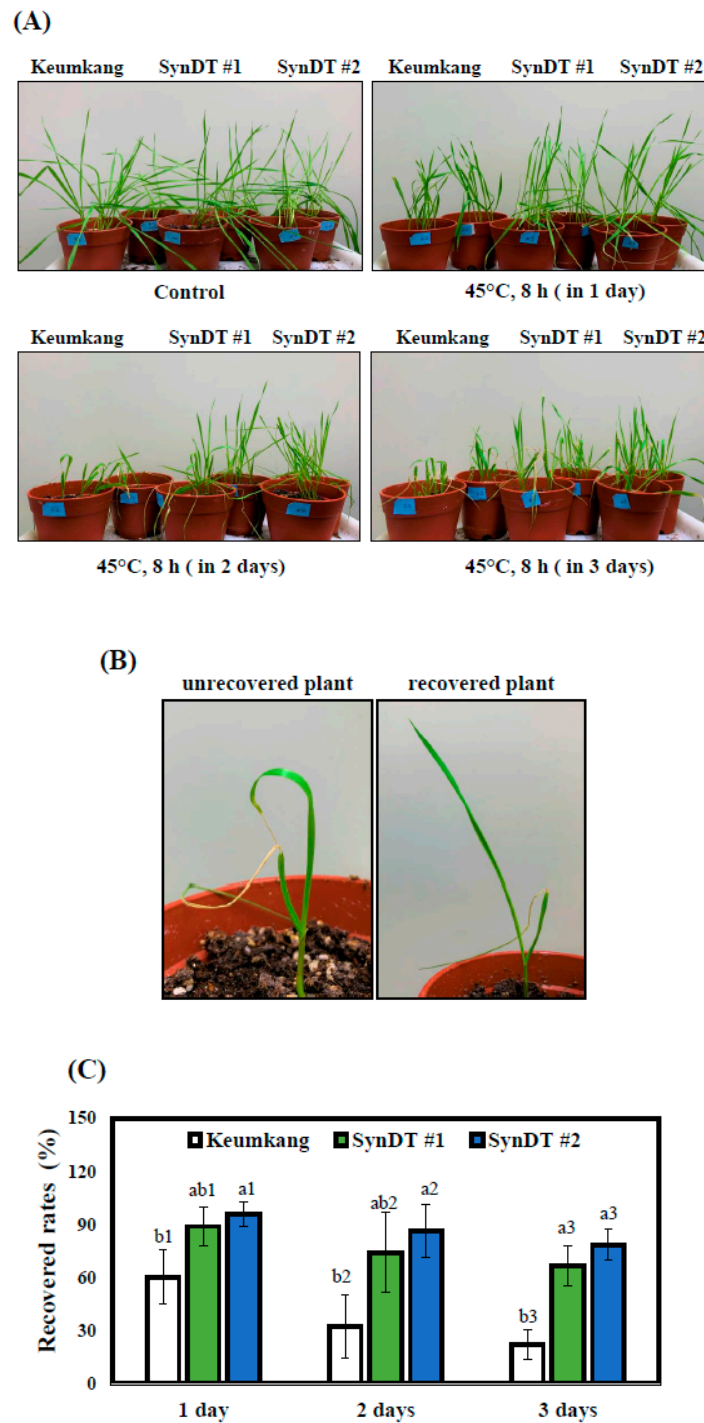


Figure 3. Growth performance of Keumkang and SynDT #1 and #2 in response to HS. (A) The phenotype of Keumkang, SynDT #1, and SynDT #2 after 4 days of recovery from HS at 45 °C for 8 h/day. (B) The criteria to distinguish between unrecovered and recovered plants from HS. (C) The recovery rates of Keumkang, SynDT #1, and SynDT #2. Six-day-old wheat seedlings were exposed to HS at 45 °C for 8 h for 1, 2, or 3 consecutive days. The pictures and quantification data were obtained 4 days after HS treatment. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. One-way ANOVA and Tukey's test ($\alpha = 0.05$) were performed using SAS. Different letters (a, b, or c) indicate significant differences ($p < 0.05$).

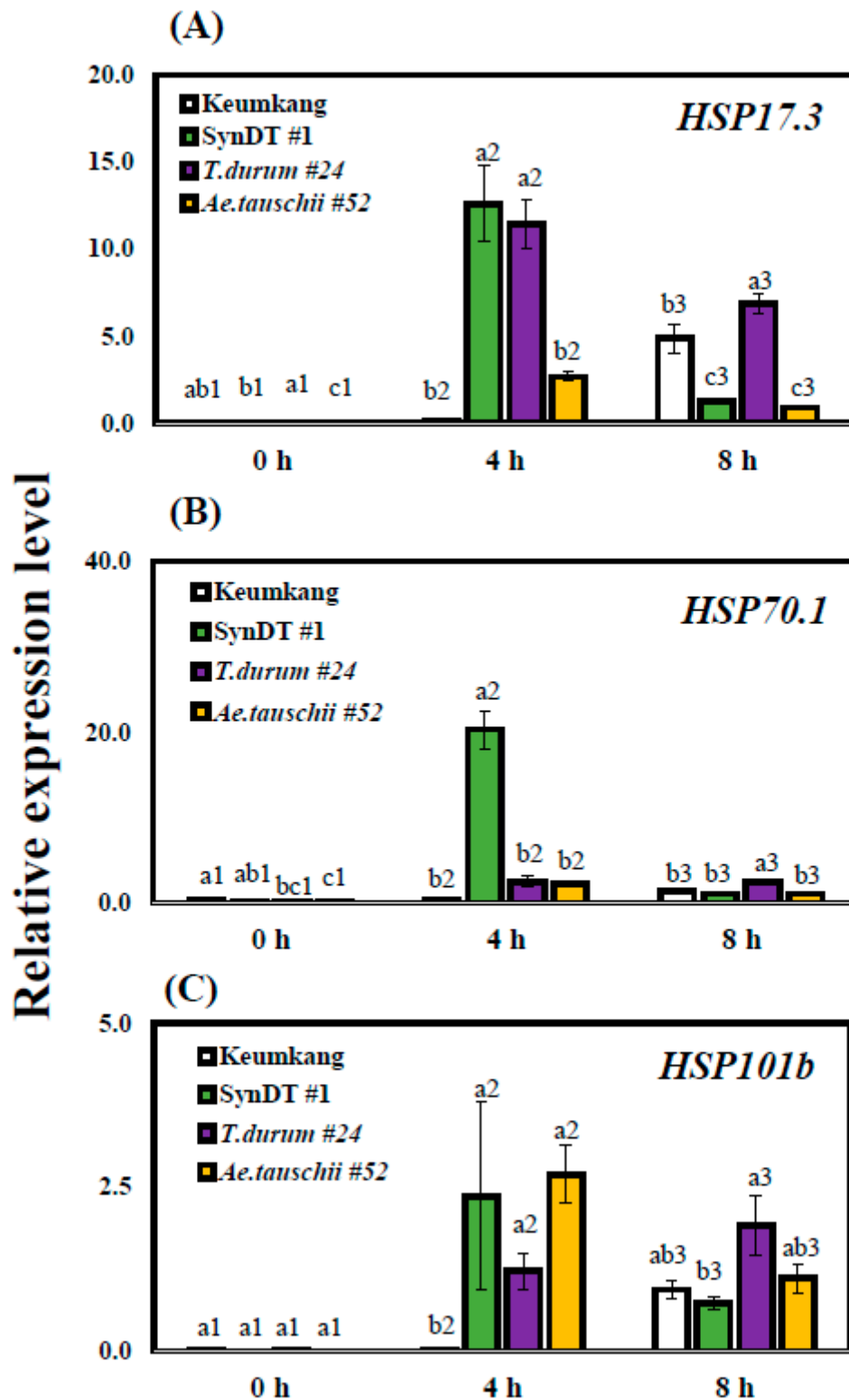


Figure 4. Transcript levels of *HSP* genes in response to HS. (A) *HSP17.3*. (B) *HSP70.1*. (C) *HSP101b*. Six-day-old wheat seedlings were exposed to HS at 45 °C for 4 and 8 h. The samples were collected for RNA extraction and cDNA synthesis. *Actin97* was used as the internal control. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. One-way ANOVA and Tukey's test ($\alpha = 0.05$) were performed using SAS. Different letters (a, b, or c) indicate significant differences ($p < 0.05$).

3.3. Resistance of SynDT Wheat Cultivar to LR by the Induction of Chitinase and PR Genes

All 8-day-old wheat lines were infected with *P. triticina*. After 10 dpi, urediniospores were observed on the leaf surface of Keumkang, whereas no spores were detected on those of SynDT #1 and #2 (Figure 5A). Microscopy revealed that a mass of hyphae had developed from the infected sites in Keumkang, but not in the synthetic wheats (Figure 5B). In addition, the results of rust biomass were consistent with those observed using microscopy (Figure 5C, Table S7). We also checked the response of *T. durum* #24 and *Ae. tauschii* #52 to LR and found that *T. durum* #24 was mildly sensitive to LR, whereas *Ae. tauschii* #52 was resistant (Figure S2). Therefore, we believe that our synthetic wheats may have inherited the LR-resistant phenotype from *Ae. tauschii* #52.

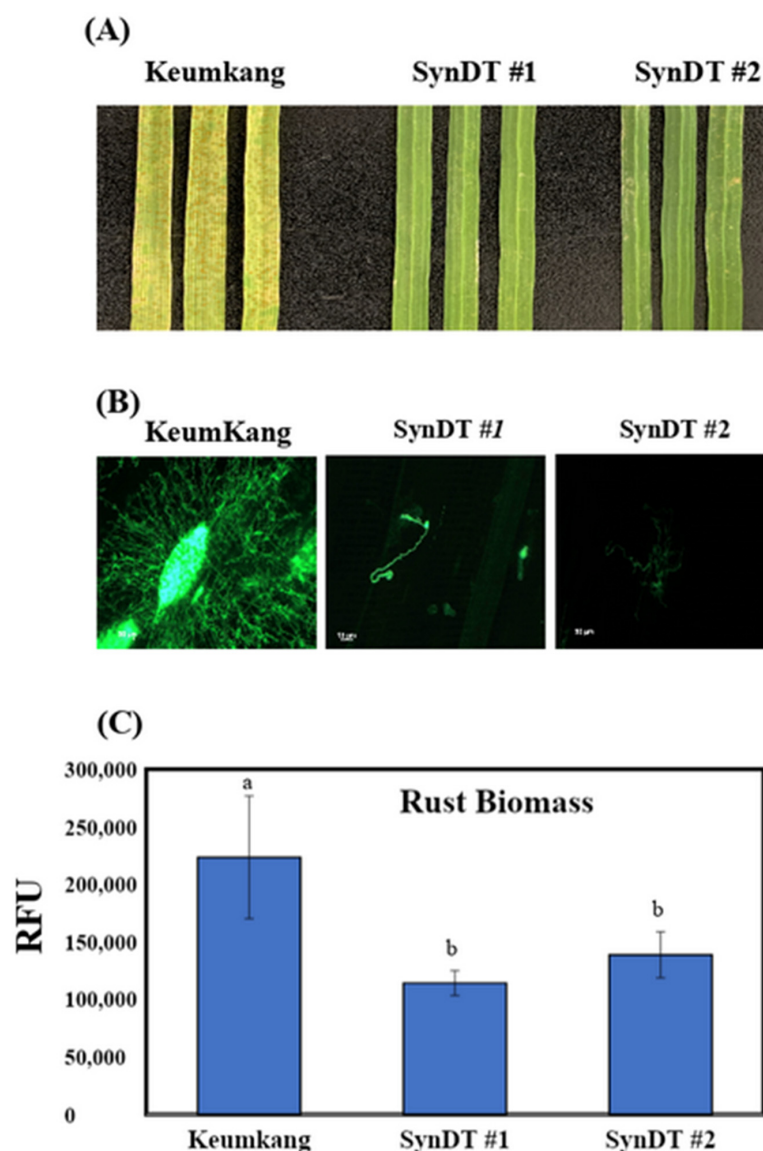


Figure 5. Growth performance of Keumkang and SynDT #1 and #2 in response to LR infection. (A) The phenotype of Keumkang, SynDT #1, and SynDT #2 in response to LR. (B) The development of hyphae under the microscope. (C) The quantification of rust biomass. Eight-day-old seedlings were exposed to LR. After 10 days, a picture was taken, and samples were collected for observations under a microscope or to quantify LR. The experiments were conducted at least twice to verify that the results were consistent. The standard deviation value was calculated from three replicates in each treatment. One-way ANOVA and Tukey's test ($\alpha = 0.05$) were performed using SAS. Different letters (a, b, or c) indicate significant differences ($p < 0.05$).

Plants have multiple mechanisms to defend themselves from pathogenic infections. Among these, the accumulation of antifungal proteins is vital in inhibiting fungal growth. Chitinase, β -1,3-glucanase, and peroxidase are common antifungal proteins that induce LR. The first component of fungi in contact with plants is its cell wall, of which chitin is one of the fundamental components. Chitinase can hydrolyze the substrate, chitin, into N-acetylglucosamine oligomers [28,29]. We determined the chitinase levels of 8-day-old infected seedlings. Both Keumkang and SynDT cultivars showed a gradual reduction in chitinase levels at the time of LR exposure. However, at 6 and 24 hpi, the chitinase activity of the SynDT cultivar was significantly higher than that of Keumkang (1.6-fold and 1.4-fold, respectively) (Figure 6A, Table S8). Furthermore, the transcript levels of *CHI8*, a chitinase-encoding gene, were upregulated when Keumkang and SynDT lines were infected with LR, and SynDT showed the highest expression at 6 hpi (Figure 6B, Table S8). There was no difference in the transcript levels of *CHI8* at 24 hpi, indicating that *CHI8* is an early responsive gene during infection, especially in the SynDT cultivar. We also checked the transcript levels of the PR genes involved in systemic acquired resistance (SAR) [30]. Among the PR genes, *PR4* encodes the chitin-binding protein [31]. We found that the expression levels of *PR4* were upregulated in response to LR (Figure 6C, Table S8). At 6 hpi, the SynDT line had significantly higher *PR4* levels than Keumkang. The transcript levels of *PR4* in SynDT substantially increased at 24 hpi. Between parent wheats, *T. durum* #24 showed a greater induction of *PR4* transcript levels compared to that of *Ae. tauschii* #52 at 6 and 24 hpi.

β -1,3-Glucan, a substrate of β -1,3-glucanase, is another important component of the fungal cell wall. In combination with chitinase, β -1,3-glucanase catalyzes the hydrolytic cleavage of β -1,3/1,6-glucans in the cell wall [32,33]. In our study, in response to LR, the glucanase levels of Keumkang and SynDT were similar at 6 hpi but decreased in the SynDT line at 24 hpi (Figure 7A, Table S9). Moreover, the *PR2* gene, which encodes proteins with β -endoglucanase activity, was more induced in SynDT at 6 and 24 hpi, compared to Keumkang. *PR2* was also highly expressed in *Ae. tauschii* #52 (Figure 7B, Table S9). Numerous studies have shown the upregulation of PR genes in plants in response to pathogenic infections [34–36]. Both *PR2* and *PR4* are considered important proteins that can be targeted in the fungal cell wall, thereby leading to fungal death. The coaction of PR genes can increase the resistance to pathogenic attacks in plants [37]. Therefore, the upregulation of *PR2* and *PR4* in the SynDT line can partially explain its resistant phenotype. β -1,3-glucanase and chitinase belong to the *PR2* and *PR4* families, respectively. Moreover, it is known that chitinase, along with β -1,3-glucanase, can inhibit the hyphal growth of many pathogenic species [38]. Our results reconfirmed that chitinase is the primary enzyme responsible for LR resistance in the SynDT wheat cultivar.

We further checked peroxidase activity and observed that the levels of peroxidase increased gradually in response to LR in both Keumkang and SynDT, but it was slightly higher in the latter (Figure 8, Table S10). The enzyme peroxidase is crucial for the synthesis of lignin, leading to cell wall lignification, which serves as a physical barrier against infections [39]. In plants, protective levels vary, which are determined by specific enzyme activity, enzyme concentration and localization inside cells, host–pathogen interactions, and fungal characteristics [39]. Therefore, the study on defensive responses is complicated. In our study, the combination of different antifungal proteins, especially chitinase activity, could be involved in the cascade to prevent LR in SynDT cultivar.

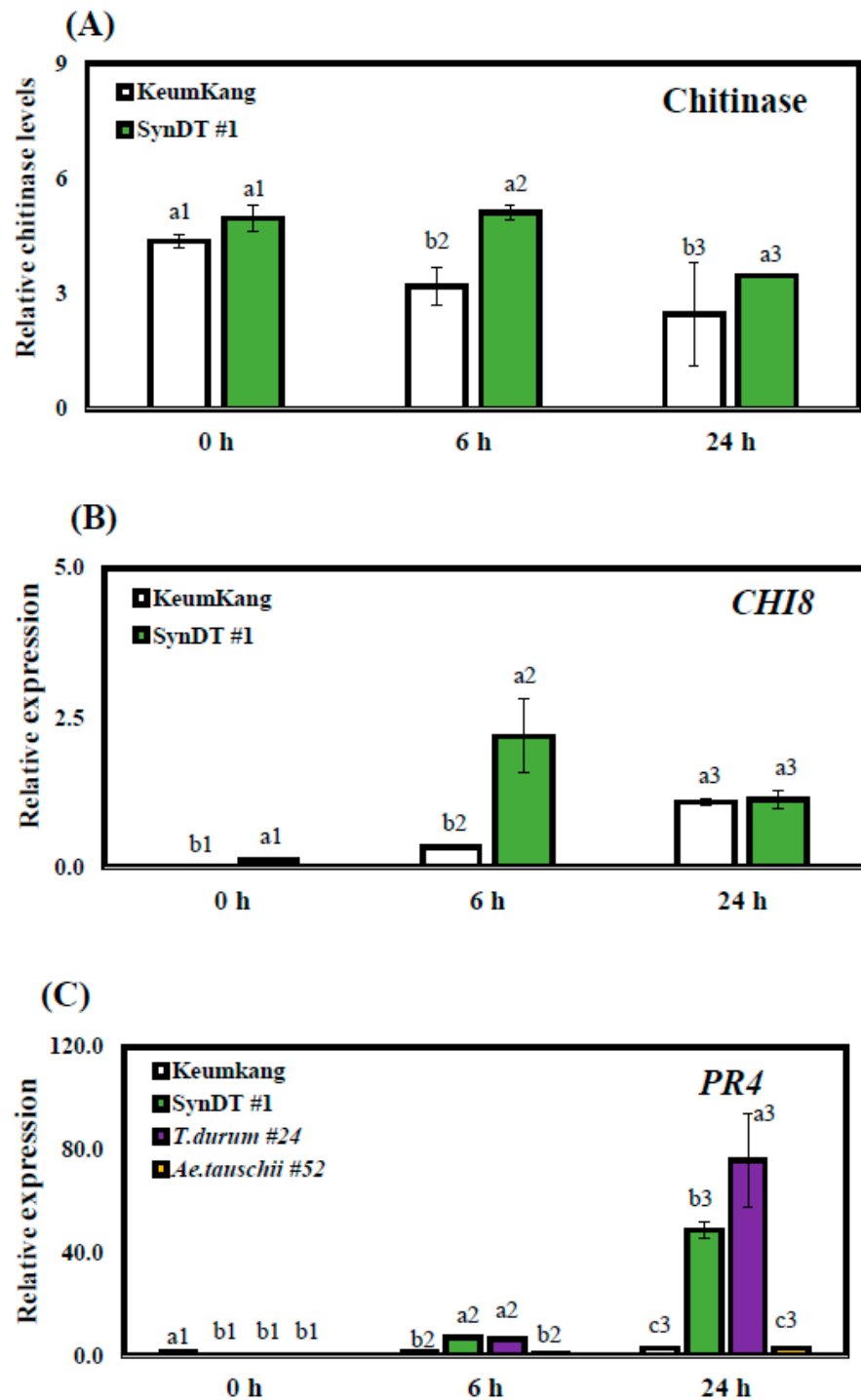


Figure 6. Chitinase levels in response to LR infection. (A) The chitinase levels of KeumKang and SynDT #1 in response to LR at 0, 6, and 24 hpi. (B) The transcript levels of *CHI8*, chitinase-encoding enzymes in KeumKang and SynDT #1, in response to LR at 0, 6, and 24 hpi. (C) The relative expression levels of *PR4* of KeumKang, SynDT #1, *T. durum* #24, and *Ae. tauschii* #52 in response to LR at 0, 6, and 24 hpi. Eight-day-old seedlings were exposed to LR. The samples were collected after 0, 6, and 24 hpi for enzymatic assay or to measure the transcript levels. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. One-way ANOVA and Tukey's test ($\alpha = 0.05$) were performed using SAS. Different letters (a, b, or c) indicate significant differences ($p < 0.05$).

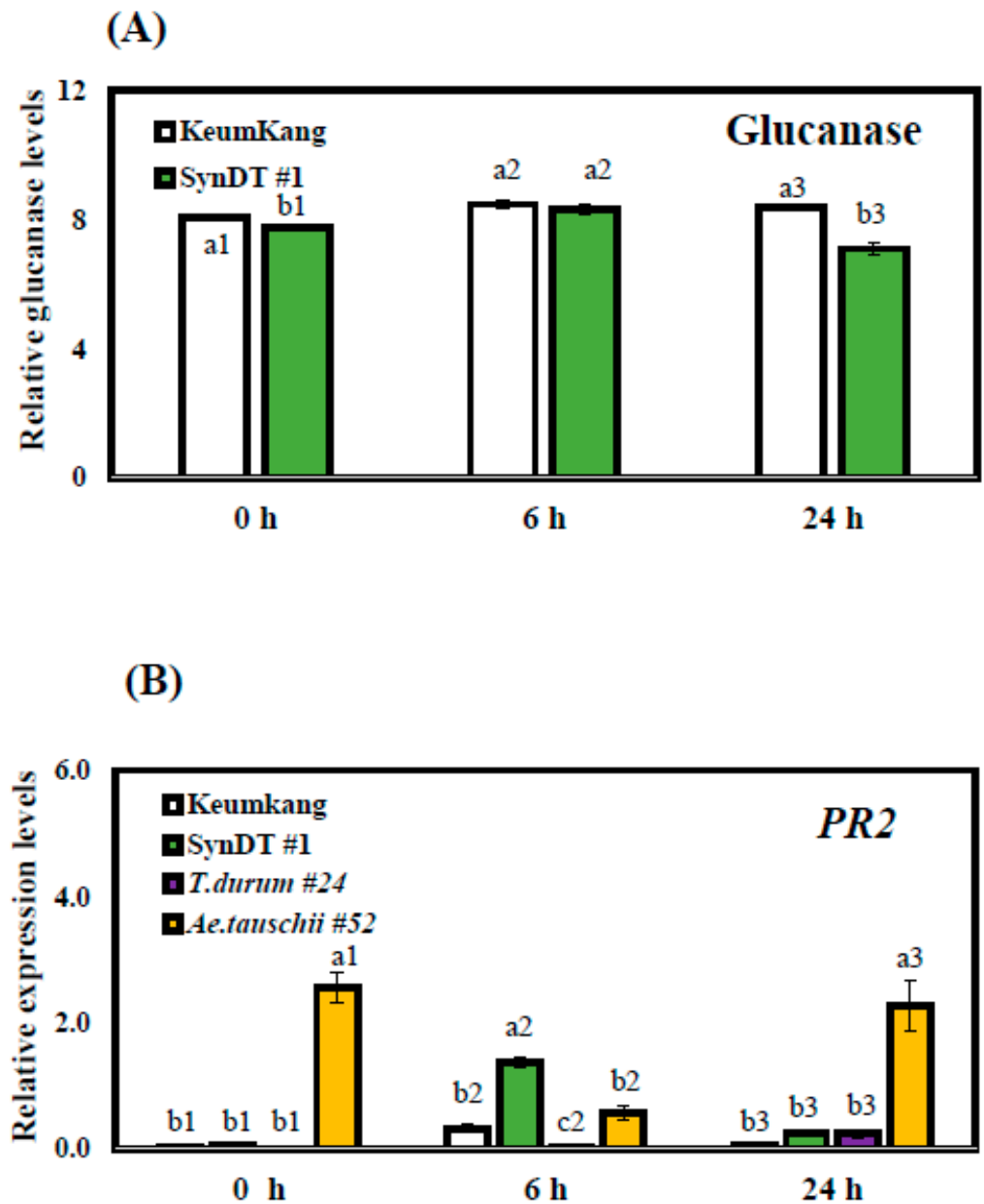


Figure 7. Glucanase levels in response to leaf rust (LR) infection. (A) Levels of glucanase in KeumKang and SynDT #1 in response to LR at 0, 6, and 24 hpi. (B) Relative expression levels of *PR2* in KeumKang, SynDT #1, *T. durum* #24, and *Ae. tauschii* #52 in response to LR at 0, 6, and 24 hpi. Eight-day-old seedlings were exposed to LR. Samples were collected after 0, 6, and 24 hpi for enzymatic assay or to measure transcript levels. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. One-way ANOVA and Tukey's test ($\alpha = 0.05$) were performed using SAS. Different letters (a, b, or c) indicate significant differences ($p < 0.05$).

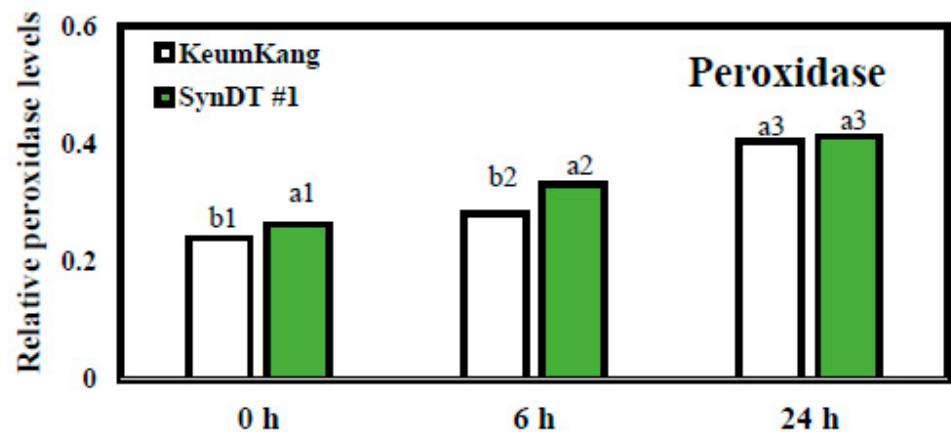


Figure 8. Levels of peroxidase in KeumKang and SynDT #1 in response to leaf rust (LR) at 0, 6, and 24 hpi. Eight-day-old seedlings were exposed to LR. Samples were collected after 0, 6, and 24 hpi for enzymatic assay. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. One-way ANOVA and Tukey's test ($\alpha = 0.05$) were performed using SAS. Different letters (a, b, or c) indicate significant differences ($p < 0.05$).

Lastly, *Lr34*, which encodes the ATP-binding cassette (ABC) transporter, is a well-known LR resistance gene in many wheat cultivars [40]. *Lr34* is located on chromosome 7DS and is one of the crucial resistance genes in wheat breeding because of its ability to inhibit multiple pathogenic species [39]. Our results showed that *Lr34* had significantly higher expression levels in SynDT than in KeumKang (14.1-fold), particularly at 6 hpi (Figure 9, Table S11). Therefore, the fast response of *Lr34* in SynDT may contribute to LR resistance.

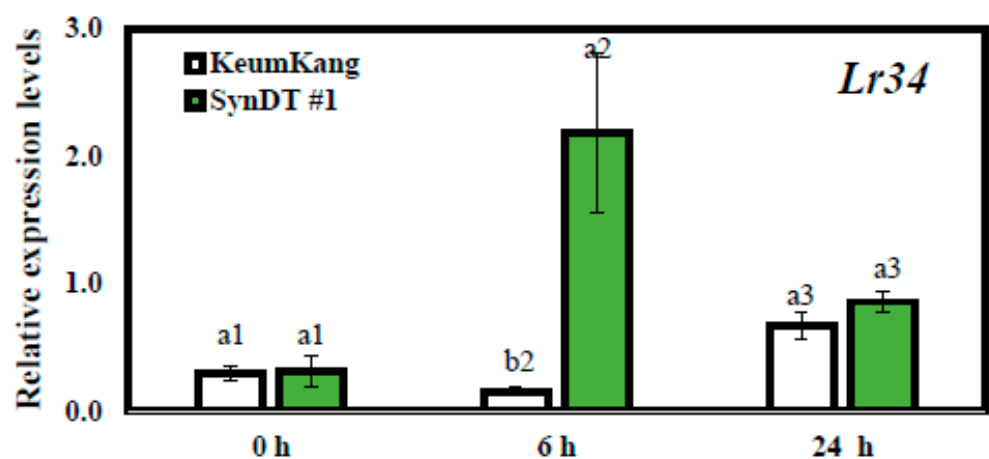


Figure 9. Relative transcript levels of *Lr34* gene in KeumKang, SynDT #1 in response to leaf rust (LR) at 0, 6, and 24 hpi. Eight-day-old seedlings were exposed to LR. Samples were collected after 0, 6, and 24 hpi for RNA extraction and cDNA synthesis. *Actin97* was used as internal control. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. One-way ANOVA and Tukey's test ($\alpha = 0.05$) were performed using SAS. Different letters (a, b, or c) indicate significant differences ($p < 0.05$).

4. Conclusions

Thus, in comparison to Keumkang, SynDT showed higher resistance to both HS and LR. SynDT is tolerant to HS, owing to the elite genes from its parents and fast-induced HSPs that protect cells from detrimental effects. SynDT also showed enhanced chitinase activity and high transcript levels of PR genes that help to cope with LR. Recently, the use of SHW in wheat breeding as a genetic resource has gained promising results for the improvement of common wheat worldwide. Furthermore, the possession of valuable genes in SynDT is critical in common wheat breeding for stress tolerance. In the future, SynDT can be used as a breeding material for local Korean bread wheat cultivars.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2073-4395/11/1/18/s1>, **Figure S1: Phenotype and recovery rates of Keumkang, *Triticum durum* #24, and *Aegilops tauschii* #52 in response to heat stress (HS).** (A) Growth performance of Keumkang, *T. durum* #24, and *Ae. tauschii* #52 after 4 days of recovery from HS. (B) Recovery rates of Keumkang, *T. durum* #24, and *Ae. tauschii* #52 in response to HS. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. One-way ANOVA and Tukey's test ($\alpha = 0.05$) were performed using SAS. Different letters (a, b, or c) indicate significant differences ($p < 0.05$). **Figure S2: Phenotype, microscopy, and rust biomass of Keumkang, *Triticum durum* #24, and *Aegilops tauschii* #52 in response to leaf rust (LR).** (A) Phenotype of Keumkang, *T. durum* #24, and *Ae. tauschii* #52 in response to LR. (B) Development of hyphae under the microscope in Keumkang, *T. durum* #24, and *Ae. tauschii* #52. (C) Quantification of the rust biomass of Keumkang, *T. durum* #24, and *Ae. tauschii* #52. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. One-way ANOVA and Tukey's test ($\alpha = 0.05$) were performed using SAS. Different letters (a, b, or c) indicate significant differences ($p < 0.05$). **Table S1: The lists of used primers in this study. Table S2: The lists of IWGCS RefSeqv1.1 gene name in this study.** The IWGCS RefSeqv1.1 gene name of three HSPs genes used in this study were searched by using the BLAST tool from Ensembl Plants (<http://plants.ensembl.org/index.html>). **Table S3: The accession number and sources of *T. durum* and *Ae. tauschii* in this study.** The accession number of *T. durum* #24, and *Ae. tauschii* #52 were used in this study and the resources were provided for purchase purpose. **Table S4: Grain morphology of all studied wheat cultivars in Figure 2.** Grain weight (in grams—g), grain width (in millimeter s—mm), and grain length (mm) of Keumkang, SynDT #1 and #2, *T. durum* #24, and *Ae. tauschii* #52. Standard deviation value was calculated from three replicates in each treatment. The data represent the mean of three technical replicates and standard deviation (mean \pm SD). **Table S5: The recovery rates of Keumkang, SynDT #1, and SynDT #2 in Figure 3C.** Six-day-old wheat seedlings were exposed to HS at 45 °C for 8 h for 1, 2, or 3 consecutive days. The percentages (%) of survival plants were obtained 4 days after HS treatment. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. The data represent the mean of three technical replicates and standard deviation (mean \pm SD). **Table S6: Transcript levels of HSP genes (*HSP17.3*, *HSP70.1*, *HSP101b*) in response to HS in Figure 4.** Six-day-old wheat seedlings were exposed to HS at 45 °C for 4 and 8 h. The samples were collected for RNA extraction and cDNA synthesis. *Actin97* was used as the internal control. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. The data represent the mean of three technical replicates and standard deviation (mean \pm SD). **Table S7: The rust biomass of Keumkang and SynDT #1 and #2 in response to LR infection in Figure 5C.** Eight-day-old seedlings were exposed to LR. After 10 days, a picture was taken, and samples were collected for observations under a microscope or to quantify LR. The experiments were conducted at least twice to verify that the results were consistent. The data represent the mean of three technical replicates and standard deviation (mean \pm SD). **Table S8: Chitinase levels, the transcript levels of *CHI8*, the relative expression levels of *PR4* and in response to LR infection at 0, 6, and 24 hpi in Figure 6.** Eight-day-old seedlings were exposed to LR. The samples were collected after 0, 6, and 24 hpi for enzymatic assay or to measure the transcript levels. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. The data represent the mean of three technical replicates and standard deviation

(mean \pm SD). **Table S9: Glucanase levels, relative expression levels of PR2 in response to LR infection at 0, 6, and 24 hpi in Figure 7.** Eight-day-old seedlings were exposed to LR. Samples were collected after 0, 6, and 24 hpi for enzymatic assay or to measure transcript levels. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. The data represent the mean of three technical replicates and standard deviation (mean \pm SD). **Table S10: Levels of peroxidase in Keumkang and SynDT #1 in response to LR at 0, 6, and 24 hpi in Figure 8.** Eight-day-old seedlings were exposed to LR. Samples were collected after 0, 6, and 24 hpi for enzymatic assay. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. The data represent the mean of three technical replicates and standard deviation (mean \pm SD). **Table S11: Relative transcript levels of Lr34 gene in Keumkang, SynDT #1 in response to leaf rust (LR) at 0, 6, and 24 hpi in Figure 9.** Eight-day-old seedlings were exposed to LR. Samples were collected after 0, 6, and 24 hpi for RNA extraction and cDNA synthesis. *Actin97* was used as internal control. The experiments were conducted at least twice to verify that the results were consistent. Standard deviation value was calculated from three replicates in each treatment. The data represent the mean of three technical replicates and standard deviation (mean \pm SD).

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