Transgenic Expression of the Functional Fragment Hpa1₁₀₋₄₂ of the Harpin Protein Hpa1 Imparts Enhanced Resistance to Powdery Mildew in Wheat

Defu Wang, Yajun Wang, Maoqiang Fu, Shuyuan Mu, Bing Han, Hongtao Ji, Hongsheng Cai, Hansong Dong, and Chunling Zhang, National Ministry of Education Key Laboratory of Integrated Management of Crop Diseases and Insect Pests, Nanjing Agricultural University, Nanjing, 210095 China

Abstract

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Powdery mildew, one of devastating diseases of wheat worldwide, is caused by *Erysiphe graminis* f. sp. *tritici*, a fungal species with constant population changes, which often poses challenges in disease management with host resistance. Transgenic approaches that utilize broad-spectrum resistance may limit changes of pathogen populations and contribute to effective control of the disease. The harpin protein Hpa1, produced by the rice bacterial blight pathogen, can induce resistance to bacterial blight and blast in rice. The fragment comprising residues 10 through 42 of Hpa1, Hpa1₁₀₋₄₂, is reportedly three- to eight-fold more effective than the full-length protein. This study evaluated

the transgenic expression of the Hpa1₁₀₋₄₂ gene for resistance to powdery mildew in wheat caused by *E. graminis* f. sp. *tritici*. Nine Hpa1₁₀₋₄₂ transgenic wheat lines were generated. The genomic integration of Hpa1₁₀₋₄₂ was confirmed, and expression of the transgene was detected at different levels in the individual transgenic lines. Following inoculation with the *E. graminis* f. sp. *tritici* isolate Egt15 in the greenhouse, five transgenic lines had significantly higher levels of resistance to powdery mildew compared with nontransformed plants. Thus, transgenic expression of Hpa1₁₀₋₄₂ conferred resistance to one isolate of *E. graminis* f. sp. *tritici* in wheat in the greenhouse.

Powdery mildew is one of the most devastating diseases of wheat in many areas with a cool or maritime climate, and it causes extensive yield losses worldwide (41). The pathogen is an ascomycete fungus classified in the species *Blumeria graminis* (DC.) Speer (gametophyte generation) or a conidia-producing filamentous fungus designated as *Erysiphe graminis* f. sp. *tritici* É.J. Marchal (asexual generation). This fungus constantly experiences population changes, which can limit effectiveness of resistance that is deployed in traditional breeding approaches, especially when resistance (*R*) genes, such as *Pm* alleles from wild emmer wheat (4,8,46,47), are used in breeding programs. Thus, it is practically difficult to control powdery mildew through traditional breeding measures. Instead, transgenic approaches that utilize broad-spectrum resistance may limit changes of pathogen populations and contribute to effective control of the disease. For instance, the expression of defense response genes, such as barley genes encoding chitinase (7,34) and β -1, 3-glucanase (6), has been shown to confer enhanced resistance against powdery mildew in transgenic wheat lines.

Effective resistance could be also triggered by certain broad-spectrum defense elicitors, such as harpins produced by gram-negative plant-pathogenic bacteria. Harpins are multifunctional proteins that induce plant growth and defense responses (2,23,50) and, therefore, have a great potential for practical use in crops. In this regard, well-characterized harpins include HrpN_{Ea} identified in *Erwinia amylovora*, a bacterial pathogen that causes fire blight disease in rosaceous plants (25,50), and Hpa1 produced by *Xanthomonas oryzae* (Ishiyama) Swings et al., an important bacterial

pathogen of rice (24,56). Transgenic expression of the HrpN_{Ea} or Hpa1 gene in plants improved the resistance response to diseases caused by several different types of pathogens (21,22,35,37, 38,43,45). For example, the HrpN gene expression in transgenic tobacco plants represses the infection by *Tobacco mosaic virus* and alleviates the necrosis symptoms caused by the virus (35). The HrpN_{Ea}-expressing tobacco plants are also enhanced in resistance to the Granville wilt disease caused by the bacterium *Pseudomonas solanacearum* (Smith) Smith (35) and fungal diseases brown spot and gray mold, caused by *Alternaria alternata* (Fr.) Keissl. and *Botrytis cinerea* Pers., respectively (22,35,45). The HrpN_{Ea} expression in transgenic potato plants confers resistance to late blight disease caused by the oomycete *Phytophthora infestans* (Mont.) de Bary (25). Similarly, Hpa1-expressing transgenic rice plants had greater resistance to bacterial blight and to sheath spot caused by the fungi *Rhizoctonia oryzae* Ryker & Gooch (37,38). In addition, disease resistance can be also induced by the external application of Hpa1 to plants (10,40). In this case, plant growth was greater along with higher levels of disease resistance (10,11).

The effects of the Hpa1 protein in plants do not necessarily require the full length of the protein but, instead, certain regions of the total protein sequence are more effective (10,11). A fragment comprising residues 10 through 42, Hpa1₁₀₋₄₂, from the N-terminal region of Hpa1, has been isolated by prokaryotic expression (10,11) and analyzed for its effects in tobacco, tea, and rice. In tobacco, levels of Hpa1₁₀₋₄₂-induced expression of defense-related genes (*NPRI* and *PR1*) and growth-promoting genes (*ARF1* and *EXPI*) are approximately twice that induced by Hpa1 (10). In tea plants, Hpa1₁₀₋₄₂ is 1.3-fold more effective than Hpa1 in elevating the yield of the top three leaves used as drinking material (51). In rice, Hpa1₁₀₋₄₂ is 7.5 and 2.7 times stronger than Hpa1 in eliciting resistance to bacterial blight (10) and rice blast (11), respectively. Meanwhile, the vegetative growth enhancement is 1.5-fold higher (10) and the grain yield increase is 2.0-fold more (11) in rice plants treated with Hpa1₁₀₋₄₂ in contrast to the full-length Hpa1.

These findings indicate that Hpa1₁₀₋₄₂ may have a practical value for disease control in agriculturally significant crops (10,11), such as common wheat (*Triticum aestivum* L.). Therefore, the objective of this study was to determine whether transgenic expression of Hpa1₁₀₋₄₂ in a background of a common wheat cultivar that is sus-

Corresponding author: Chunling Zhang, E-mail: zhangcl@njau.edu.cn

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ceptible to powdery mildew could enhance the resistance response to this disease.

Materials and Methods

Plant material. The transformation host plant, 'Yangmai16' (Y16), is a vernal wheat cultivar widely planted in the East China wheat-production area. Y16 has a moderate yield of grains with high flour qualities, resists several diseases (such as head scab, Rhizoctonia root rot, and sharp eyespot), and is susceptible to powdery mildew (<http://baike.so.com/doc/6292923.html>). Y16 seed used in the initial experiment were provided by Dr. Yong Zhang (Academy of Agricultural Sciences of Yangzhou City, Jiangsu, China). The *HpaI*₁₀₋₄₂-expressing transgenic Y16 (Y16:HpaI₁₀₋₄₂) lines were generated in this study and their T₃ progenies were used in all experiments stated below.

Plant growth conditions. Soil for these studies was a natural loam collected from a wheat field near Pailou Village, Xuanwu District, Nanjing City, Jiangsu Province. This was used directly (without sterilization). Plants grown to Feekes stage 11.4 (grains ready to harvest) were grown in large pots (35 cm tall, 30-cm top diameter, and 20-cm bottom diameter) filled with the natural loam. Plants grown to Feekes stage 4.0 (when leaf sheaths lengthen) were grown in the natural loam in small pots (12 cm tall, 10-cm top diameter, and 8-cm bottom diameter). In both cases, seed were sown and germinated in the soil in pots (8 seeds/pot) and subsequent plants were grown under controlled temperature (16 to 20°C in the first 15 days followed by 21 to 25°C) and natural light conditions in the greenhouse affiliated with the Nanjing Agricultural University and located at Pailou Village. Fertilization, irrigation, and other agronomic managements were performed regularly as in the field.

Generation of transgenic wheat lines. The plant binary vector pAHC25 (14) used in Y16 transformation contains the *uidA* gene encoding β -glucuronidase and the *bar* gene encoding phosphinothricin acetyltransferase (PAT) as selective markers. The vector also contains the maize *ubiquitin (Ubi)* gene promoter to control transgene transcription and the transcription terminator from the *nopaline synthase* gene of *Agrobacterium tumefaciens*. In addition, the pAHC25 vector possesses multiple cloning sites, including bases for *SmaI* and *SacI* restrictions. The *HpaI*₁₀₋₄₂ gene was cloned from *X. oryzae* pv. *oryzicola* DNA (10) by polymerase chain reaction (PCR) using specific primers (Supplementary Table S1) that contained *SmaI* and *SacI* recognition bases. The PCR product was confirmed by sequencing and inserted into pAHC25 by replacing the *uidA* gene, creating the recombinant pAHC25:*HpaI*₁₀₋₄₂ vector. The orientation and the integrity of *HpaI*₁₀₋₄₂ in the recombinant vector were confirmed by sequence analysis. Then, the recombinant pAHC25:*HpaI*₁₀₋₄₂ vector was introduced into Y16 by biolistic bombardment (12). Transformants were screened based on the PAT activity that confers resistance to the phosphinothricin-containing herbicide (17). Seeds were incubated at 24 to 26°C on Murashige and Skoog (MS) agar medium containing the herbicide product Bialaphos (Goldbio Diagnostics Trading Co. Ltd.) at 5 μ g/ml. Twenty days later, surviving seedlings were transplanted into pots and grown in the greenhouse. Molecular characterization of transgenic plants was performed by using regular protocols (54). The homozygosity of the transgene locus was confirmed by screening T₃ seed on Bialaphos-containing MS agar medium and PCR analyses of genomic DNAs from T₄ seedlings. If all 10 seeds of a transgenic line germinated and subsequent T₄ seedlings grew well on the medium, and the *HpaI*₁₀₋₄₂ insert was detected from the T₄ seedlings, the transgenic line was regarded as homozygous at the insertion locus. Homozygous T₃ seed of every transgenic line were used in this study.

PCR and PCR-Southern blot analyses. Genomic DNA was extracted from leaves of 15-day-old (approximate tillering Feekes stage 2) wheat plants grown in small pots and analyzed by PCR using primers specific to the *Ubi::HpaI*₁₀₋₄₂ gene fusion. The PCR reaction was performed in a 25- μ l solution consisting of 1 μ l (100 ng) of the genomic DNA, 4 μ l of dNTPs (TaKaRa Biotech. Co.

Ltd.) each at 2.5 mM, 12.5 μ l of 2 $\times$ GC buffer I (TaKaRa), 0.25 μ l of Taq polymerase (TaKaRa) at 5 units/ μ l, 1 μ l of 5'-3' primer (10 μ M), 1 μ l of 3'-5' primer (10 μ M), and 5.25 μ l of highly pure water. The PCR program was denaturation at 94°C for 5 min, 32 amplification cycles (30 s at 94°C, 30 s at 58°C, and 1 min at 72°C), and a final extension at 72°C for 10 min. The PCR product was resolved on a 1% (wt/vol) agarose gel and visualized by staining with ethidium bromide. PCR-Southern blotting analysis was performed by using the standard protocol (39) and the probe specific to the *Ubi::HpaI*₁₀₋₄₂ fusion gene. Prior to hybridization to the blot, the probe was labeled with digoxigenin by using the DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Diagnostics Trading Co. Ltd.).

Reverse-transcriptase PCR analyses. For use in gene expression analysis, total RNA was isolated from the top first and second expanded leaves of 15-day-old plants grown in small pots and analyzed by semiquantitative reverse-transcriptase (RT)-PCR and real-time RT-PCR protocols. Both protocols were performed to detect the expression of pathogenesis-related (*PR*) genes *PR-1* and *PR-5* by using specific primers and using the constitutively expressed *actin* gene as a reference. Genes were amplified for <26 cycles with a range of template concentration increases of 0.5 ng from 0 to 3.0 ng in 25- μ l reaction solutions to select desired concentrations. Reaction treatments, RT-PCR protocols, product cloning, and sequencing verification were performed as previously described (10,26). The 25- μ l reaction mixture contained 1 μ l of first-strand cDNA diluted 1:10, 2.5 μ M primer, and 1 $\times$ SYBR Premix Ex Taq (TaKaRa). All reactions were performed in triplicate with null-template controls in which cDNA was absent. Mean expression levels of tested genes were normalized to the null-template controls and quantified relative to the concentration of *actin* transcripts.

Isolate. *Erysiphe graminis* f. sp. *tritici* isolate Egt15 was originally identified in a leaf colony of Y16 grown in a field near Nanjing and provided by our colleague Dr. Zhengqiang Ma. Inoculum was maintained on Y16 seedlings grown in small pots in an environment-controlled chamber with 50 to 55% humidity, and a cycle of 12 h of light and 12 h of darkness, with light at 250 μ E/m²/s, 26 $\pm$ 1°C in light, and 22°C $\pm$ 1 in darkness. Egt15 conidia and conidiophores were transferred to fresh nursery seedlings every 2 weeks with a soft painting brush. Conidia newly produced until 10 days on the most recent nursery seedlings were used as inoculants.

Disease assays. Fifteen-day-old (approximate tillering Feekes stage 2) plants grown in small pots and 55-day-old (approximate last-leaf-visible Feekes stage 8) plants grown in large pots were inoculated with Egt15 conidia from nursery seedlings with an electric hairdryer (49). Even inoculum density was maximized by blowing conidia into a large (1 m tall, 70 cm long, and 60 cm wide) plastic inoculation box through a large (50 cm long and 10 cm in diameter) glass tube. The inoculated plants were kept under the inoculation box for 30 min before being moved to the greenhouse. Following inoculation, plants were divided into three experimental groups. In the first, the top first and second leaves were excised 5 days after inoculation and washed in 5 ml of pure water to make conidial suspensions. Conidia in a 5- μ l suspension were counted by microscopy, and the number of conidia per leaf was calculated. In the second experimental group, total RNA was isolated from the top first and second leaves of plants 5 days after inoculation and analyzed by real-time RT-PCR performed with primers specific to Egt15 18S ribosomal (r)RNA. Plants in the third group were assessed at 15 days after inoculation for severity of powdery mildew. The disease severity was evaluated by the criterion disease index, which was determined based on levels of plant infection (11,20). Infection levels were divided into 1 to 4 classes; namely, class 1 representing no visible infection and classes 2 to 4 referring to low, moderate, and high levels of infection, respectively, based on both the extent of leaf necrosis and the visible amount of fungal growth on leaf surfaces. Each infection class was arbitrarily given a representative number (RN): class 1, RN = 1; class 2, RN = 2; and class 3, RN = 3. The number

of plants (NP) in every infection class was recorded. Then, disease index was calculated using the formula $\text{disease index} = 100 \times \Sigma(\text{RN of a class} \times \text{NP in the class}) / (\text{RN of the greatest class} \times \text{total NP surveyed})$ (11). Therefore, the disease index criterion can reflect the variation in disease severity in large plant populations, and a high value of disease index indicates a high level of disease severity.

Plant resistance evaluation. Levels of resistance to the powdery mildew disease in Y16:Hpa1₁₀₋₄₂ lines in contrast to Y16 were evaluated by statistical analyses of the disease index data. If the disease index of a Y16:Hpa1₁₀₋₄₂ line was significantly lower than that of Y16, the transgenic line was considered more resistant.

Chemiluminescence. The chemiluminescence observation was performed on the top second leaves of 15-day-old plants grown in small pots for the accumulation of hydrogen peroxide (H₂O₂) and callose deposition. The growth of Egt15 on leaf surfaces was visualized by staining the leaves with lactophenol trypan blue staining solution, and leaf staining was performed by a previously described method (36). The accumulation of H₂O₂ in leaves was observed after leaf staining with the dye 3,3'-diaminobenzidine hydrochloride (DAB; 52). Leaf callose was detected by the aniline blue staining method (28). The stained leaves were observed by microscopy under visible light (for trypan blue and DAB staining) and UV light (for aniline blue staining), respectively.

Measurements of antioxidant enzyme activities. The top second leaves of 15-day-old plants were excised to isolate superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD). These enzymes were extracted with the extraction solution made of 50 mM phosphate buffer (pH 7.0), 10 mM reduced ascorbate, and 4.0% (wt/vol) polyvinylpyrrolidone. The top second leaves of plants grown in small pots were excised and homogenized with liquid nitrogen. Ground powder from a 0.2-g leaf sample was extracted with 5 ml of the extraction solution. The homogenate was centrifuged at 10,000 × g for 30 min at 4°C, and the supernatant was collected for enzyme activity assays. Activities of SOD, CAT and POD were measured as previously described by Gianopolitis and Ries (18), Aebi (1), and Chance and Maehly (9), respectively.

Statistical analysis. All experiments were carried out at least three times. The statistical significance of disease index differences among wheat lines (Y16 and Y16:Hpa1₁₀₋₄₂ lines) and the difference between Y16 and a Y16:Hpa1₁₀₋₄₂ line were analyzed by the analysis of variance (ANOVA) method along with one-tailed Fisher's least significant difference (LSD) tests, respectively. Similar analysis methods were also applied to defense response data. When data from every plant line complied with the formal distribution pattern, they were analyzed by ANOVA and LSD. All statistical analyses were performed with the IBM SPSS19.0 software package (44) developed by IBM Corporation (<http://www-01.ibm.com/software/analytics/spss/>).

Results

Characterization of transgenic wheat plants. Transformation of Y16 wheat with the recombinant pAHC25:Hpa1₁₀₋₄₂ vector (Fig. 1A) resulted in the generation of nine Hpa1₁₀₋₄₂-expressing transgenic Y16 (Y16:Hpa1₁₀₋₄₂) lines (Fig. 1B–D). All of the transgenic wheat seedlings grew normally to maturity and produced fertile seed under normal growth conditions in the greenhouse (Supplementary Table S2). PCR (Fig. 1B) and PCR-Southern blotting (Fig. 1C) analyses indicated that the transgene Hpa1₁₀₋₄₂ was present in the genome of every transgenic line but not in the nontransformed Y16 plant. The presence of the Hpa1₁₀₋₄₂ sequence in the genome of every transgenic line was also confirmed by sequencing and alignments of the Hpa1₁₀₋₄₂ and flanking sequences isolated from transgenic plants (Supplementary Figure S2). Subsequently, the semiquantitative RT-PCR data suggested that Hpa1₁₀₋₄₂ was substantially expressed in Y16:Hpa1₁₀₋₄₂ numbers 3, 9, 11, 39, 40, 41, and 44 plants, except for Y16:Hpa1₁₀₋₄₂ numbers 45 and 46, both of which had low levels of the Hpa1₁₀₋₄₂ transcript (Fig. 1D). This result was confirmed by real-time RT-PCR analyses, which indicated that levels of the Hpa1₁₀₋₄₂ expression were highest in the transgenic line Y16:Hpa1₁₀₋₄₂ number 3, lowest in Y16:Hpa1₁₀₋₄₂ number 46, and moderate in Y16:Hpa1₁₀₋₄₂ number 39 and the other Y16:Hpa1₁₀₋₄₂ lines (Fig. 1E).

Enhanced resistance to powdery mildew in seven of nine Y16:Hpa1₁₀₋₄₂ lines. Nine transformed Y16 (Y16:Hpa1₁₀₋₄₂) lines

Fig. 1. Molecular characterization of Hpa1₁₀₋₄₂ transgenic wheat lines. **A**, Part of the recombinant vector used in transformation of Y16 wheat. The restriction sites used in the construction are indicated, and elements used to control the transgene expression include UbiP (promoter of the ubiquitin-encoding gene), Tnos (terminator of the gene encoding nopaline synthase), and the Bar gene (encoding phosphinothricin acetyltransferase) as a selective marker. In addition, the "intron" devised to modulate the promoter activity is also shown to indicate the position of the Hpa1₁₀₋₄₂ insert in the recombinant vector. **B** and **C**, Polymerase chain reaction (PCR) and PCR-Southern analyses of the Hpa1₁₀₋₄₂ gene insert. The primer pair and the probe specific to an Hpa1₁₀₋₄₂-containing 788-bp region present in the recombinant vector were used in the PCR and Southern blotting analyses, respectively. The recombinant vector was used as a "positive control", and the "negative control" indicates non-template reaction performed along with the PCR amplification of the genomic DNA of the indicated wheat lines. **D**, Semiquantitative reverse-transcriptase (RT)-PCR analyses with Hpa1₁₀₋₄₂-specific primers and RNA isolated from the indicated wheat lines. The constitutively expressed actin gene was used as a reference. **E**, Real-time RT-PCR analyses of Hpa1₁₀₋₄₂ expression in transgenic Y16:Hpa1₁₀₋₄₂ lines compared with Y16. The Hpa1₁₀₋₄₂/actin transcript ratios were quantified as mean values ± standard deviation bars of results from three experiments (24 plants per experiment for every wheat line).

were compared with the nontransformed Y16 plant for resistance to *E. graminis* f. sp. *tritici* isolate Egt15. Resistance was evaluated by assessing Egt15 propagation and Egt15-caused powdery mildew in inoculated plants. Two of the low *Hpa1*₁₀₋₄₂-expressing lines (Y16:Hpa1₁₀₋₄₂ numbers 45 and 46) developed similar levels of infection following inoculation with *E. graminis* f. sp. *tritici* isolate Egt15 (Fig. 2A–C). In contrast, seven high *Hpa1*₁₀₋₄₂-expressing lines (Y16:Hpa1₁₀₋₄₂ numbers 3, 9, 11, 39, 40, 41, and 44) were more resistant than the nontransformed Y16 plant for colonization of the fungus and disease severity. Egt15 produced conidia on leaves of all plants in 5 days but the number of conidia was significantly ($P < 0.01$) smaller in seven of the nine transgenic lines compared with the nontransformed Y16 plant (Fig. 2A). By 15 days after inoculation, powdery mildew developed on leaves of all plants; however, the disease severity was significantly ($P < 0.01$) lower in seven of the transgenic lines than in the nontransformed Y16 plant (Fig. 2B). Mean powdery mildew disease index values in the seven transgenic lines were 21 to 84% of that in Y16 (Fig. 2C). Thus, *Hpa1*₁₀₋₄₂ reduced the colonization and overall inoculum production of *E. graminis* f. sp. *tritici* isolate Egt15 in the transgenic lines Y16:Hpa1₁₀₋₄₂ numbers 3, 9, 11, 39, 40, 41, and 44.

Consistent impediments of the pathogen growth in Y16:Hpa1₁₀₋₄₂ plants. To analyze whether reduction of the pathogen growth was a consistent characteristic of *Hpa1*₁₀₋₄₂-transformed plants, we measured Egt15 growth in the nontransformed Y16 plant and transformed lines Y16:Hpa1₁₀₋₄₂ numbers 3, 39, and 46, which acquired high, moderate, and low levels of resistance to powdery mildew (Fig. 2). When Egt15 was inoculated on 15-day-

old (approximate tillering Feekes stage 2) seedlings, the hyphal growth was observed on leaves of all plants at 5 days after inoculation (Fig. 3A). However, levels of the hyphal growth were significantly ($P < 0.01$) lower on leaves of Y16:Hpa1₁₀₋₄₂ numbers 3 and 39 compared with Y16 and Y16:Hpa1₁₀₋₄₂ number 46 plants (Fig. 3A). This difference was correlated with that of the growth of Egt15 in leaf tissue, as quantified by the expression of the fungal 18S rRNA, which was highly expressed in Y16 and Y16:Hpa1₁₀₋₄₂ number 46; however, the expression level was reduced ($P < 0.01$) in Y16:Hpa1₁₀₋₄₂ number 3 and 39 plants (Fig. 3B). Consistently, the number of conidia was significantly smaller ($P < 0.01$) on leaves of Y16:Hpa1₁₀₋₄₂ number 3 or 39 versus Y16 and Y16:Hpa1₁₀₋₄₂ number 46 (Fig. 3C). As a result, powdery mildew was evidently alleviated in Y16:Hpa1₁₀₋₄₂ numbers 3 and 39 compared with Y16 or Y16:Hpa1₁₀₋₄₂ number 46 as observed at 5 days (Fig. 3A) and 15 days (Fig. 3D) after inoculation. Similar results were obtained in the inoculation experiments performed on 55-day-old (approximate last-leaf-visible Feekes stage 8) plants. By 5 days after inoculation, Egt15 had a robust expression of its 18S rRNA (Fig. 3E) and produced a large number of conidia in Y16 or Y16:Hpa1₁₀₋₄₂ number 46 (Fig. 3F); however, both the expression of the fungal 18S rRNA and the number of conidia were decreased significantly ($P < 0.01$) in Y16:Hpa1₁₀₋₄₂ number 3 or 39 (Fig. 3E and F). By 15 days after inoculation (when plants were 70 days old, approximately at head-visible Feekes stage 10.1), the powdery mildew severity was much lower in Y16:Hpa1₁₀₋₄₂ number 3 or 39 than in Y16 or Y16:Hpa1₁₀₋₄₂ number 46 (Fig. 3G). The reduction in pathogen growth suggests that resistance to this pathogen was

Fig. 2. Comparison of nine transformed Y16 (Y16:Hpa1₁₀₋₄₂) lines and the nontransformed Y16 plant following inoculation with *Erysiphe graminis* f. sp. *tritici* isolate Egt15. **A–C.** Fifteen-day-old plants were inoculated with Egt15. **A**, Production of conidia on the top first and second leaves 5 days after inoculation. **B** and **C**, Powdery mildew disease severities surveyed 15 days after inoculation. **A** and **C**, Data shown are mean values $\pm$ standard deviation bars of results from three experiments (40 plants per experiment for every wheat line). Different letters on bar graphs indicate significant differences by Fisher's least significant difference test at $P < 0.01$.

enhanced in Y16:Hpa1₁₀₋₄₂ number 3 and 39 plants at both the young and adult stages. In both stages, the resistance level was higher in Y16:Hpa1₁₀₋₄₂ number 3 than in Y16:Hpa1₁₀₋₄₂ number 39 (Fig. 3).

Defense responses in Y16:Hpa1₁₀₋₄₂ plants. To gain information on the physiological and molecular bases of Hpa1₁₀₋₄₂-enhanced resistance, transgenic lines Y16:Hpa1₁₀₋₄₂ numbers 3, 39, and 46 were compared with the nontransformed Y16 plant in terms of defense responses. Several defense responses known to associate with plant disease resistance were analyzed in leaves of 15-day-old (approximate tillering Feekes stage 2) plants following inoculation with *E. graminis* f. sp. *tritici* isolate Egt15. First, H₂O₂ is an important type of reactive oxygen species (ROS) involved in an oxidative burst that is thought to be one of the key responses in plant disease resistance (3). Previously, it was reported that harpin-mediated disease resistance is accompanied by the production of H₂O₂, which is synthesized by infected plant tissues to inhibit pathogen development (19,36). H₂O₂ was produced at higher levels in Y16:Hpa1₁₀₋₄₂ number 3 or 39 than in the Y16 or Y16:Hpa1₁₀₋₄₂ number 46 (Fig. 4A). Second, callose deposition is responsible for sealing leaf-surface injuries and is often deposited in cells that activate defense responses (13). Callose deposition was greater in leaves of Y16:Hpa1₁₀₋₄₂ number 3 or 39 compared with Y16 or Y16:Hpa1₁₀₋₄₂ number 46 (Fig. 4B). Next, the levels of *PR-1* and *PR-5* transcripts were compared because *PR* genes are molecular markers of plant responses to pathogen attacks (42,48) and they have been reported previously to be involved in powdery mildew resistance of wheat (32). *PR-1* and *PR-5* behaved differently in Y16 and Y16:Hpa1₁₀₋₄₂ plants following inoculation with Egt15. Semiquantitative RT-PCR did not detect the expression of *PR-1*

and *PR-5* in Y16 and Y16:Hpa1₁₀₋₄₂ number 46 plants until 5 days after inoculation (Fig. 4C). However, the *PR* gene expression in inoculated Y16:Hpa1₁₀₋₄₂ numbers 3 and 39 plants was increased greatly over the constitutive expression found in both plants without inoculation (Fig. 4C). Based on real-time RT-PCR analyses, levels of *PR-1* and *PR-5* expression were significantly ($P < 0.01$) greater in Y16:Hpa1₁₀₋₄₂ numbers 3 and 39 plants than in the Y16 or Y16:Hpa1₁₀₋₄₂ number 46 plant following inoculation with Egt15 (Fig. 4D). Finally, we measured leaf content of SOD, CAT, and POD, three major antioxidant enzymes that are involved in ROS removal in plants (30,31). Higher antioxidant activities were observed in Y16:Hpa1₁₀₋₄₂ numbers 3 and 39 plants than in Y16 or Y16:Hpa1₁₀₋₄₂ number 46 plant in response to pathogen exposure (Fig. 5).

Discussion

Previous studies have shown that the expression of full-length harpin proteins induces defense responses and promotes growth and development in transgenic plants (29,35,55). This study is focused on the disease resistance role of Hpa1₁₀₋₄₂, a functional fragment isolated from the Hpa1 protein sequence (10,11,51) and expressed in wheat, one of the most agriculturally significant crops. The findings of this study demonstrate that transgenic expression of the *Hpa1*₁₀₋₄₂ gene in Y16 wheat results in a reduction of powdery mildew following inoculation with one isolate of the pathogen *E. graminis* f. sp. *tritici*. The Hpa1₁₀₋₄₂-enhanced resistance in Y16:Hpa1₁₀₋₄₂ lines can be effective in impeding the growth and propagation of the pathogen isolate and the subsequent development of the powdery mildew symptoms under greenhouse conditions.

Fig. 3. Comparison of the nontransformed Y16 plant and three transgenic Y16 (Y16:Hpa1₁₀₋₄₂) lines for the colonization, conidial production, and powdery mildew development following inoculation with *Erysiphe graminis* f. sp. *tritici* isolate Egt15. For assays of young seedlings, 15-day-old plants were inoculated. **A**, Five days later, the hyphal growth of Egt15 on the top second leaves were observed and the leaves were photographed; **B**, Egt15 18S rRNA transcript in the top first and second leaves was quantified in relative to the wheat *actin* gene and **C**, the number of conidia on the second leaves was also determined. **D**, By 15 days after inoculation, plants were photographed to show the powdery mildew symptoms. For assays of adult plants, 55-day-old plants were inoculated with Egt15. **E**, Five days later, Egt15 18S rRNA transcript was quantified in relative to the wheat *actin* gene and **F**, the number of conidia on the second leaves was determined. **G**, By 15 days after inoculation, plants were photographed to show the powdery mildew symptoms. **B**, **C**, **E**, and **F**, Data shown are mean values $\pm$ standard deviation bars of results from three experiments (40 plants per experiment for every wheat line), and different letters on bar graphs indicate significant differences (least significant difference test at $P < 0.01$).

It is interesting that the expression of *Hpa1*₁₀₋₄₂ confers resistance to powdery mildew in all but two of the transgenic wheat lines tested at the seedling stage. The *Hpa1*₁₀₋₄₂ expression in five transgenic wheat lines (Y16:Hpa1₁₀₋₄₂ numbers 3, 9, 11, 39, and 41) resulted in reduced disease development as compared with the nontransformed host Y16 plant. In Y16:Hpa1₁₀₋₄₂ number 3, 9, 11, 39, and 41 plants, resistance levels were consistent with levels of the transgene expression. This indicates that the resistance to powdery mildew was enhanced in those transgenic lines. In Y16:Hpa1₁₀₋₄₂ numbers 3 and 39, moreover, enhanced resistance was observed at both the young and adult stages. Nevertheless, the *Hpa1*₁₀₋₄₂ gene expression did not enhance powdery mildew resistance in Y16:Hpa1₁₀₋₄₂ number 45 and 46 plants. The difference in levels of resistance may be caused by different insertion points of the exogenous gene in the wheat genome but this assumption remains to be examined. In addition, enhanced powdery mildew resistance was correlated with upregulated *PR* gene expression in Y16:Hpa1₁₀₋₄₂ number 3, 9, 11, 39, and 41 plants. This result was consistent with previous studies, where the induction of disease resistance and defense responses is a conserved function of harpin proteins, either applied as a product to plants (10,11,40) or produced in transgenic plants (37,38,45).

In addition to *PR* gene expression, ROS is also a part of the plant defense response to pathogens. ROS plays a dual role in plants: at a low concentration, ROS acts as a signaling molecule to regulate the normal growth and development of plants but, at a high concentration, ROS can cause plant senescence or even programmed cell death (5,24). Plants protect themselves against ROS-induced cellular injury by employing an antioxidative defense system compris-

ing antioxidative enzymes such as SOD, CAT, and POD (16,31). In this study, the POD and SOD activities in the resistant transgenic wheat lines were higher than those in the nontransformed plant at every time point during the course of experiment, although they peaked at different times. One possible explanation is that different antioxidative enzymes in wheat play their respective roles at different developmental stages (33). However, the CAT activity differed, mainly because powdery mildew is a biotrophic fungal pathogen that colonizes living plant tissues and obtains nutrients from living host cells. The lower CAT activity, weaker ROS scavenging ability, and greater toxicity to cells contributed to reduced powdery mildew growth and reproduction (5,16). Moreover, the activities of CAT, SOD, and POD in the nontransformed plant also changed during the time course after inoculation. These analyses indicate that *Hpa1*₁₀₋₄₂ expression contributed to antioxidant enzyme activities on the basis of their induction by the fungal infection. Although the increase in enzyme activities was positively correlated with the powdery mildew resistance, this may only be a secondary response to elevated level of ROS production due to fungal infection and may not necessarily have a direct link to resistance (33).

The most sustainable strategy to protect plants from diseases is to breed resistant cultivars. In breeding programs, *R* genes have been extensively used because they protect plants very effectively against pathogen infection and are easy to select. In wheat, many *R* genes have been identified (4,8,27,47,53) but, to the best of our knowledge, only *Pm3* was overexpressed with enhanced powdery mildew resistance in a wheat cultivar (46,47). Additionally, *R* genes are often race specific and are overcome rapidly if they are present as a single resistance source in varieties that are widely

Fig. 4. Comparison of the nontransformed Y16 plant and three transformed Y16 (Y16:Hpa1₁₀₋₄₂) lines for defense responses following inoculation with *Erysiphe graminis* f. sp. *tritici* isolate Egt15. Fifteen-old-plants were inoculated. **A**, H₂O₂ production and **B**, callose deposition in the top second leaves at 2 and 4 days after inoculation. **C**, Semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) and **D**, real-time RT-PCR analyses of the expression of *PR-1* and *PR-5* genes in the top first and second leaves. **D**, Data shown in bar graphs are mean values ± standard deviation bars of results from three experiments (30 plants per experiment for every wheat line). Different letters on bar graphs indicate significant differences (least significant difference test at *P* < 0.01).

cultivated. Therefore, breeders are constantly challenged to broaden the resistance spectrum when breeding crop plants. Fortunately, with the aid of genetic engineering, numerous transgenic plants with enhanced disease resistance have been produced, including transgenic lines that are enhanced in powdery mildew resistance (6,7,34,47). In most cases, the resistance is due to the direct antifungal effect of the gene itself. However, the *Hpa1*₁₀₋₄₂ expression enhances powdery mildew resistance mainly through the activation of the defense responses, which are effective to limit infection by different pathogens, not specific to a particular pathogen (10,15,35,37). Therefore, the transgenic wheat lines presented here may provide a potential wheat germplasm source for enhanced resistance to powdery mildew and other pathogens.

However, it should be pointed out that results detailed in this study were obtained from the inoculation experiments using only one isolate of the pathogen under greenhouse conditions. At least two additional conditions should be considered in regard to the potential of agricultural use of *Hpa1*₁₀₋₄₂-expressing plants. First, in the future, it will be necessary to study whether the *Hpa1*₁₀₋₄₂ expression is effective to resist the pathogen isolates other than Egt15 or simultaneous infection by different isolates of the pathogen. Second, many experiments are required to evaluate the environmental fitness of *Hpa1*₁₀₋₄₂-expressing plants under natural field conditions.

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Literature Cited

- Aebi, H. 1984. Catalase in vitro. *Method Enzymol.* 105:121-126.
- Alfano, J. R., and Collmer, A. 2004. Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annu. Rev. Phytopathol.* 42:385-414.
- Alvarez, M. E., Pennell, R. L., Meijer, R. J., Ishikawa, A., Dixon, R. A., and Lamb, C. 1998. Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92:773-784.
- Ben-David, R., Xie, W. L., Peleg, Z., Saranga, Y., Dinor, A., and Fahima, T. 2010. Identification and mapping of *PmG16*, a powdery mildew resistance gene derived from wild emmer wheat. *Theor. Appl. Genet.* 121:499-510.
- Bhattacharjee, S. 2005. Reactive oxygen species and oxidative burst: roles in stress, senescence and signal transduction in plant. *Curr. Sci.* 89:1113-1121.
- Bieri, S., Potrykus, L., and Futterer, J. 2003. Effects of combined expression of antifungal barley seed proteins in transgenic wheat on powdery mildew infection. *Mol. Breed.* 11:37-48.
- Bliffeld, M., Mundy, J., Potrykus, L., and Futterer, J. 1999. Genetic engineering of wheat for increased resistance to powdery mildew disease. *Theor. Appl. Genet.* 98:1079-1086.
- Cao, A. H., Xing, L. P., Wang, X. Y., Yang, X. M., Wang, W., Sun, Y. L., Qian, C., Ni, J. L., Chen, Y. P., Liu, D. J., Wang, X., and Chen, P. D. 2011. Serine/ threonine kinase gene *StpK-V*, a key member of powdery mildew resistance gene *Pm21*, confers powdery mildew resistance in wheat. *Proc. Natl. Acad. Sci. USA* 108:7727-7732.
- Chance, B., and Maehly, A. C. 1955. Assay of catalase and peroxidase. *Method Enzymol.* 2:764-775.
- Chen, L., Qian, J., Qu, S. P., Long, J. Y., Yin, Q., Zhang, C. L., Wu, X. J., Sun, F., Wu, T. Q., Beer, S. V., and Dong, H. 2008. Identification of specific fragments of *HpaG*_{Xooc}, a harpin protein from *Xanthomonas oryzae* pv. *oryzicola*, that induce disease resistance and enhance growth in rice. *Phytopathology* 98:781-791.
- Chen, L., Zhang, S. J., Zhang, S. S., Qu, S. P., Ren, X. Y., Long, J. Y., Yin, Q., Qian, J., Sun, F., Zhang, C. L., Wang, L. X., Wu, X. J., Wu, T. Q., Zhang, Z. K., Cheng, Z. Q., Hayes, M., Beer, S. V., and Dong, H. 2008. A fragment of the *Xanthomonas oryzae* pv. *oryzicola* harpin *HpaG*_{Xooc} reduces disease and increases yield of rice in extensive grower plantings. *Phytopathology* 98:792-802.
- Chen, L., Zhang, Z. Y., Liang, H. X., Liu, H. X., Du, L. P., Xu, H. J., and Xin, Z. Y. 2008. Overexpression of *TiERF1* enhances resistance to sharp eyespot in transgenic wheat. *J. Exp. Bot.* 59:4195-4204.
- Chen, X. Y., and Kim, J. Y. 2009. Callose synthesis in higher plants. *Plant Signal. Behav.* 4:489-492.
- Christensen, A. H., and Quail, P. H. 1996. Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. *Transgenic Res.* 5:213-218.
- Dong, H. S., Delaney, T. P., Bauer, D. W., and Beer, S. V. 1999. Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. *Plant J.* 20:207-215.
- Foyer, C. H. 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* 17:1866-1875.
- Gao, C., Liu, J., and Nielsen, K. K. 2009. *Agrobacterium*-mediated transformation of meadow fescue (*Festuca pratensis* Huds.). *Plant Cell Rep.* 28:1431-1437.
- Giannopolitis, C. N., and Ries, S. K. 1977. Superoxide dismutase I. Occurrence in high plants. *Plant Physiol.* 59:309-314.
- Gopalan, S., Wei, W., and He, S. Y. 1996. *Hrp* gene-dependent induction of *hin1*: a plant gene activated rapidly by both harpins and the *avrPto* gene-mediated signal. *Plant J.* 10:591-600.
- Huang, X. Q., Wang, L. X., Xu, M. X., and Röder, M. S. 2003. Microsatellite mapping of the powdery mildew resistance gene *Pm5e* in common wheat (*Triticum aestivum* L. em. Thell.) *Theor. Appl. Genet.* 106:858-865.
- Huo, R., Wang, Y., Ma, L. L., Qiao, J. Q., Shao, M., and Gao, X. W. 2010. Assessment of inheritance pattern and agronomic performance of transgenic rapeseed having harpin_{Xooc}-encoding *hrf2* gene. *Transgenic Res.* 19:841-847.
- Jang, Y. S., Sohn, S. I., and Wang, M. H. 2006. The *hrpN* gene of *Erwinia amylovora* stimulates tobacco growth and enhances resistance to *Botrytis cinerea*. *Planta* 223:449-456.
- Kim, J. G., Park, B. K., Yoo, C. H., Jeon, E., Oh, J., and Hwang, I. 2003. Characterization of the *Xanthomonas axonopodis* pv. *glycines* *HpaG* pathogenicity island. *J. Bacteriol.* 185:3155-3166.
- Li, P., Lu, X. Z., Shao, M., Long, J. Y., and Wang, J. S. 2004. Genetic diversity of harpins from *Xanthomonas oryzae* and their activity to induce hypersensitive response and disease resistance in tobacco. *Sci. China C Life Sci.* 47:461-469.
- Li, R., and Fan, Y. 1999. Reduction of lesion growth rate of late blight plant

Fig. 5. Comparison of the nontransformed Y16 plant and three transformed Y16 (Y16:Hpa1₁₀₋₄₂) lines for three antioxidant enzymes. Fifteen-day-old plants were inoculated with Egt15 and enzyme activity in the top second leaves was analyzed in a time course assay. Data shown are mean values $\pm$ standard deviation bars of results from three experiments (30 plants per experiment for every wheat line).

- disease in transgenic potato expressing harpin protein. *Sci. China C Life Sci.* 42:96-101.
26. Liu, R. X., Chen, L., Jia, Z. H., Lü, B. B., and Dong, H. 2010. Transcription factor AtMYB44 regulates induced expression of the *ETHYLENE INSENSITIVE2* gene in *Arabidopsis* responding to a harpin protein. *Mol. Plant-Microbe Interact.* 24:377-389.
 27. Liu, Z. Y., Sun, Q. X., Ni, Z. F., Nevo, E., and Yang, T. M. 2002. Molecular characterization of a novel powdery mildew resistance gene *Pm30* in wheat originating from wild emmer. *Euphytica* 123:21-29.
 28. Lü, B. B., Sun, W. W., Zhang, S. P., Zhang, C. L., Qian, J., Wang, X. M., Gao, R., and Dong, H. 2011. HrpN_{EP}-induced deterrent effect on phloem feeding of the green peach aphid *Myzus persicae* requires *AtGSL5* and *AtMYB44* genes in *Arabidopsis thaliana*. *J. Biosci.* 36:127-137.
 29. Miao, W. G., Wang, X. B., Li, M., Song, C. F., Wang, Y., Hu, D. W., and Wang, J. S. 2010. Genetic transformation of cotton with a harpin-encoding gene *HpaX_{oo}* confers an enhanced defense response against different pathogens through a priming mechanism. *BMC Plant Biol.* 15:67-80.
 30. Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7:405-410.
 31. Mittler, R., Vanderauwera, S., Gollery, M., and Van Breusegem, F. 2004. Reactive oxygen gene network of plants. *Trends Plant Sci.* 9:490-498.
 32. Niu, J. S., Liu, R., and Zheng, L. 2007. Expression analysis of wheat *PR-1*, *PR-2*, *PR-5* activated by *Bgt* and SA, and powdery mildew resistance. *J. Triticeae Crops* 27:1132-1137.
 33. Niu, L. Y., Wang, H. S., and Shi, M. W. 2004. Changes of SOD and POD activities in wheat leaves infected by wheat powdery mildew and their relations to resistance. *J. Henan Vocation-Tech. Teachers Coll.* 4:5-8. (In Chinese with English abstract)
 34. Oldach, K. H., Becker, D., and Lörz, H. 2001. Heterologous expression of genes mediating enhanced fungal resistance in transgenic wheat. *Mol. Plant-Microbe Interact.* 14:832-838.
 35. Peng, J. L., Bao, Z. L., Ren, H. Y., Wang, J. S., and Dong, H. 2004a. Expression of harpin_{X_{oo}} in transgenic tobacco induces pathogen defense in the absence of hypersensitive response. *Phytopathology* 94:1048-1055.
 36. Peng, J. L., Dong, H., Dong, H. P., Delaney, T. P., Bonasera, B. M., and Beer, S. V. 2003. Harpin-elicited hypersensitive cell death and pathogen resistance requires the *NDR1* and *EDS1* genes. *Physiol. Mol. Plant Pathol.* 62:317-326.
 37. Ren, H. Y., Gu, G. Y., Long, J. Y., Qian, J., Wu, T. Q., Song, T., Zhang, S. J., Chen, Z. Y., and Dong, H. 2006. Combinative effects of a bacterial type-III effector and a biocontrol bacterium on rice growth and disease resistance. *J. Biosci.* 31:617-627.
 38. Ren, H. Y., Song, T., Wu, T. Q., Sun, L. J., Liu, Y. X., Yang, F. F., Chen, Z. Y., and Dong, H. 2006. Effects of a biocontrol bacterium on growth and defence of transgenic rice plants expressing a bacterial type-III effector. *Ann. Microbiol.* 56:281-287.
 39. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor Laboratory, NY.
 40. Sang, S. L., Li, X. J., Gao, R., You, Z. Z., Lu, B. B., Liu, P. Q., Ma, Q. X., and Dong, H. 2012. Apoplastic and cytoplasmic location of harpin protein Hpa1_{X_{oo}} plays different roles in H₂O₂ generation and pathogen resistance in *Arabidopsis*. *Plant Mol. Biol.* 79:375-391.
 41. Schulze-Lefert, P., and Vogel, J. 2000. Closing the ranks to attack by powdery mildew. *Trends Plant Sci.* 5:343-348.
 42. Shah, J. 2003. The salicylic acid loop in plant defense. *Curr. Opin. Plant Biol.* 6:365-371.
 43. Shao, M., Wang, J. S., Dean, R. A., Lin, Y. J., Gao, X. W., and Hu, S. J. 2008. Expression of a harpin-encoding gene in rice confers durable nonspecific resistance to *Magnaporthe grisea*. *Plant Biotechnol. J.* 6:73-81.
 44. Shi, L. W. 2012. SPSS19.0. Pages 109-143 in: *Statistical Analysis from Accidence to Conversance*. Tsinghua University Press, Beijing. (In Chinese)
 45. Sohn, S. I., Kim, Y. H., Kim, B. R., Lee, S. Y., Lim, C. K., Hur, J. H., and Lee, J. Y. 2007. Transgenic tobacco expressing the *hrpN_{EP}* gene from *Erwinia pyrifoliae* triggers defense responses against *Botrytis cinerea*. *Mol. Cell* 24:232-239.
 46. Susanne, B., Daniel, S., Carolina, D. Q., Gabriele, B., Gerhard, H., Francis, P., Pierre, B., Caroline, T., Christof, S., Michael, W., and Beat, K. 2012. Transgenic *Pm3* multilines of wheat show increased powdery mildew resistance in the field. *Plant Biotechnol. J.* 10:398-409.
 47. Susanne, B., Severine, H., Gerhard, H., Olena, K., Simone, V. B., Simon, L. Z., Bernhard, S., Michael, W., and Beat, K. 2011. Transgenic *Pm3b* wheat lines show resistance to powdery mildew in the field. *Plant Biotechnol. J.* 9:897-910.
 48. Thulke, O. U., and Conrath, U. 1998. Salicylic acid has a dual role in the activation of defense related genes in parsley. *Plant J.* 14:35-42.
 49. Wang, Y. P., Nishimura, M. T., Zhao, T., and Tang, D. Z. 2011. ATG2, an autophagy-related protein, negatively affects powdery mildew resistance and mildew-induced cell death in *Arabidopsis*. *Plant J.* 68:74-87.
 50. Wei, Z. M., Lacy, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 257:85-88.
 51. Wu, X. J., Wu, T. Q., Long, J. Y., Yin, Q., Zhang, Y., Chen, L., Liu, R. X., Gao, T. C., and Dong, H. 2007. Productivity and biochemical properties of green tea in response to full-length and functional fragments of HpaG_{X_{oo}}, a harpin protein from the bacterial rice leaf streak pathogen *Xanthomonas oryzae* pv. *oryzicola*. *J. Biosci.* 32:1119-1131.
 52. Xiao, S., Brown, S., Patrick, E., Brearley, C., and Turner, J. G. 2003. Enhanced transcription of the *Arabidopsis* disease resistance genes *RPW8.1* and *RPW8.2* via a salicylic acid-dependent amplification circuit is required for hypersensitive cell death. *Plant Cell* 15:33-45.
 53. Yahiaoui, N., Srichumpa, P., Dudler, R., and Keller, B. 2004. Genome analysis at different ploidy levels allows cloning of the powdery mildew resistance gene *Pm3b* from hexaploid wheat. *Plant J.* 37:528-538.
 54. Yang, M., Qin, B. P., Liu, C. L., Cai, H. S., Wang, Z. L., Liang, Y. C., and Yin, Y. P. 2013. The molecular identification of transgenic Hpa1₁₀₋₄₂ wheat and resistance evaluation on Fusarium Head Blight. *Sci. Agric. Sin.* 46:657-667. (In Chinese with English abstract)
 55. Zhang, L., Xiao, S. S., Li, W. Q., Feng, W., Li, J., Wu, Z. D., Gao, X. W., Liu, F. Q., and Shao, M. 2011. Overexpression of a Harpin-encoding gene *hrf1* in rice enhances drought tolerance. *J. Exp. Bot.* 62:4229-4238.
 56. Zhu, W. G., Magbanua, M. M., and White, F. F. 2000. Identification of two novel *HpaG*-associated genes in the *HpaG* gene cluster of *Xanthomonas oryzae* pv. *oryzae*. *J. Bacteriol.* 182:1844-1853.