Eur J Plant Pathol
DOI 10.1007/s10658-012-0048-8

Enhanced overall resistance to Fusarium seedling blight and Fusarium head blight in transgenic wheat by co-expression of anti-fungal peptides

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Accepted: 26 July 2012

Abstract Introduction of alien genes into wheat has been proposed as a strategy to breed cultivars with improved resistance to Fusarium seedling blight (FSB) and Fusarium head blight (FHB). In this study, we co-transformed different anti-fungal peptides (AFPs) into an elite wheat cultivar Yangmai11. We identified the genetically stable transgenic wheat lines carrying single or multiple genes by PCR, qRT-PCR and Southern blot analyses. Transgenic wheat lines 451 and 513 expressing two AFPs displayed a consistent, significantly improved overall resistance to FSB and FHB, whereas only FHB resistance was observed from other lines. Furthermore, crude proteins extracted from the lines 451 and 513 showed a clear inhibitory activity against *F. graminearum* in vitro. Taken together, it was essential to properly combine and express AFPs in transgenic wheat in order to obtain an improved overall resistance to *Fusarium* pathogens.

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Published online: 12 August 2012

Keywords Anti-fungal peptide · Fusarium head blight · Fusarium seedling blight · Overall resistance · Transgenic wheat

Introduction

Fusarium seedling blight (FSB) and Fusarium head blight (FHB), caused by *Fusarium* spp., are economically devastating diseases of wheat and other small grain cereal crops worldwide (Bai and Shaner 2004; Li et al. 2010a). FSB in wheat occurs at the germination stage, throughout the succeeding seedling development, and it provides inoculum for the subsequent epidemics of FHB that takes place in spikes with reddish and scabby symptoms. Both FSB and FHB pathogens produce *Fusarium* mycotoxins, which are toxic to domestic animals, humans and plants. Epidemics of FSB and FHB frequently occur in the middle and lower regions of the Yangtze River, and in Heilongjiang Province in northeastern China (Qu et al. 2008). It has been reported that the incidences of mycotoxicosis result from the consumption of *Fusarium* mycotoxin-contaminated wheat flour in China (Chen et al. 2003), which presents a serious threat to public health. Recently, the spread and severity of FSB and FHB to even wider regions in China have worsened due to global climate change (Liang et al. 2007). Since the mid-1990s, FHB has re-emerged as a serious problem for agriculture in Europe and North America (Parry et al. 1995; Windels 2000).

Endogenous expression of resistance genes is the most effective control strategy of fungal diseases to prevent infection in the field and during storage. However, germplasm with innate FSB and FHB resistance is inadequate in nature. Only a few wheat cultivars have a modest resistance to FHB, such as well-known Chinese local cultivars Sumai3 and Wangshuibai, while a high susceptibility to FSB is observed in these lines (Li et al. 2010a; Wu et al. 2005). Recent studies have shown that different quantitative trait loci (QTL) are attributed to FSB and FHB resistance (Li et al. 2010b; Tamburic-Ilincic et al. 2009). Moreover, transgenic wheat expressing an *Arabidopsis thaliana NPR1* gene showed an increased disease severity of FSB although an enhanced FHB resistance was obtained (Gao et al. 2012). Therefore, resistance or susceptibility of wheat to FSB and FHB in wheat deals with a complex regulation. Developing wheat varieties with an overall resistance to both FSB and FHB becomes a major challenge for effectively reducing yield loss and mycotoxin loads in agriculture products.

Although genetic engineering has been used to improve FHB resistance in wheat (Li et al. 2008, 2011), no study has reported an enhanced overall resistance to FSB and FHB, nor for expression *in planta* of anti-fungal proteins *Pep3* or *MsrA1* for improvement of resistance to Fusarium pathogens. Moreover, all the current transgenic elite wheat lines for improvement of resistance carry either herbicide or antibiotic resistance genes. In addition, no study has been carried out for segregation and heritability of transgenes derived from co-bombardment of multiple gene cassettes in transgenic progenies for hexaploid wheat. In our present study, we aimed to generate genetically stable transgenic wheat lines without using any sequences coding for herbicide or antibiotic resistance. Moreover, we planned to select the transgenic plants with improved resistance to both FSB and FHB, to characterize transgene integration and expression patterns after segregation, and to determine their anti-fungal activities against *Fusarium* pathogens with the resistant wheat-derived proteins.

Materials and methods

Gene constructs

Sequences coding for anti-fungal proteins, *MsrA1* and *Pep3*, were chemically synthesized (Genescript, Nanjing, China) according to their amino acid sequences (Cavallarin et al. 1998; Osusky et al. 2000). A *Chitinase* (*Chi*) gene (GenBank ID: M62904) was cloned from a barley cultivar Epi3233. *Rs-CWP2* sequence was released from the plasmid pTRAkc (Peschen et al. 2004), while *Pmi* (phosphomannose isomerase) gene was derived from a pBML-PMI vector and used as a selection marker (Zhang et al. 2006). The *Pmi* gene encodes a phosphomannose isomerase that can make use of mannose as a carbon source. *Pmi* and *Chi* were constructed into the expression cassette in a head-to-head manner (Fig. 1a), which were individually regulated by a maize ubiquitin promoter and a *nos* terminator from pAHC25 (Christensen and Quail 1996), respectively. The other three genes, *MsrA1*, *Pep3* and *Rs-CWP2*, were individually constructed into the expression cassette under the regulation of the same

promoter and terminator used above (Fig. 1b). All the expression cassettes were constructed into a pBluescript SK vector and contained a 150-bp flanking sequence at their both ends to protect the cassette from digestion or degradation. Each expression cassette was released by respective restriction enzymes *PvuII/KpnI*. Released cassettes were separated on agarose gels and purified with the AxyPrep gel purification kit (Axygen, USA).

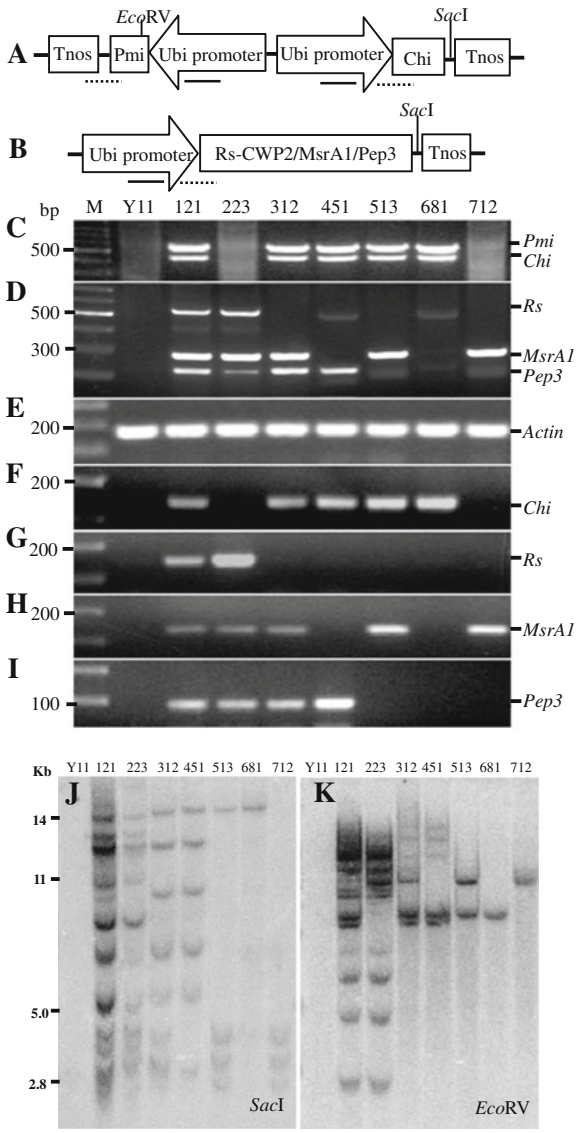

Fig. 1 Expression cassettes used for transformation (**a**, **b**) and identification of transgenic plants by PCR (**c**, **d**), RT-PCR (**e-i**) and Southern blots (**j**, **k**). Transgenic lines are indicated on the top of the panels (**c-k**), and genes are indicated in the cassettes or the right of the panel (**c-i**). Solid lines indicate the DNA fragment used as a probe in Southern blots; dotted lines indicate the fragments amplified by PCR

Plant materials and transformation

A commercial Chinese wheat cultivar (cv.) Yangmai11 (Y11) that is currently widely cultivated in the middle and lower regions of the Yangtze River, was grown in an experimental field in Wuhan, China. Spikes were collected on the 14th day after pollination. Immature embryos with a size of about 0.8–1.2 mm were isolated and placed on callus induction medium with the scutellum facing upwards. Embryonic calli were co-bombarded with all the four expression cassettes using equal amounts of DNA molecules (Li et al. 2008). Mannose (10 g/l, w/v) was used in the medium as selection agent. The callus induction, transformation and regeneration were carried out as previously described (Huang et al. 2012; Li et al. 2008). Briefly, immature embryos from wheat spikes collected approximately 14 days post-anthesis were bombarded with a biolistic gun, PDS-1000 He (Bio-Rad, USA) at a pressure of 1100 psi. The bombarded embryos were cultured in callus induction medium (MS with 2 mg/l 2, 4-D) in dark for 2 weeks. The calluses were grown on regeneration medium (MS with 0.5 μm IAA, 1 μm BA, 10 g/l mannose) under light for 14–20 day, and subcultured on the regeneration medium with 5 g/l mannose for a further 20 days. The plantlets generated were transferred to selection medium (1/2 MS with 0.5 μm NAA, 15 g/l mannose) for 14 days, and the surviving plantlets with strong roots and shoots were planted in pots filled with soil. Transgenic wheat plants after vernalization were grown in a growth chamber under conditions of 16/8 h day/night at 18–20 °C and harvested at physiologic maturity.

DNA extraction, PCR and Southern blot

Genomic DNA of wheat was extracted from young leaves of transgenic wheat of different generations and non-transgenic controls as previously described (Li et al. 2008). Two sets of primers (PmiF/NosR1, ChiF/NosR2; Table 1) were used in PCR amplification of *Pmi-Chi* cassette. For PCR amplification of *RS-CWP2*, *MsrA1* and *Pep3* genes, 0.75 μM 5' Co-primer UbiF (Table 1) based on the ubiquitin promoter and 0.25 μM of each gene-specific 3' primer (RsR, MR and PR, Table 1) were used. PCR reaction and

Table 1 Primers and sequences used for PCR, RT-PCR and qRT-PCR

Primer name	Annealing site	Sequence (5'–3')	DNA fragment (bp)
PmiF	*Phosphomannose isomerase* (*pmi*)	AGTGATGGCAAACTCCGATAACG	578
NosR1	*Nos* terminator	CTCATAAATAACGTCATGC	
ChiF	*Chitinase*	TCTCCCACAACTACAACTATGGACCTGC	410
NosR2	*Nos* terminator	CAAATGTTTGAACGATCGGGGAAAT	
UbiF	*Ubiquitin* promoter	GTTTCTTTTGTCGATGCTCACCC	
RsR	*Rs-CWP*$_2$	ACGAATTCCAACCCCTTGCCG	544
MR	*MsrA1*	AAGCTGGGAGTCCAGTGGTAAGC	281
PR	*Pep3*	CCTTGAGGATTTTCTTGAAGAGCTT	221
ActF1	*Actin*	GCTGTTCCAGCCATCTCATGT	156
ActR1	*Actin*	CGATCAGCAATTCCAGGAAAC	
ChiF1	*Chitinase*	CACGGACGCCACTGTGGGCT	124
ChiR1	*Chitinase*	TGCGGCCCGGTCAGCCCCTGAC	
CWP2F1	*Rs-CWP*$_2$	CGTCCTCGGTGTCAGCAAAC	145
CWP2R1	*Rs-CWP*$_2$	GGTCTGTTGGCGCTGTCATC	
MsrAF1	*MsrA1*	CTTCCTCCTCATCAGCGCCT	124
MsrAR1	*MsrA1*	TGGGAGTCCAGTGGTAAGCA	
PepF1	*Pep3*	CTTCCTCCTCATCAGCGCCT	122
PepR1	*Pep3*	CGAGTGCACCCAGATCCTCT	
UbiP5	*Ubiquitin* promoter	CATCTCTGTATATGCATCAG	495
UbiP6	*Ubiquitin* promoter	CGGTAGTTCTACTTCTGTTC	

Primers PmiF/NosR1 and ChiF/NosR2 were used to amplify *Pmi* and *Chi* genes, respectively. Primers UbiF, RsR, MR and PR were used to amplify *Rs-CWP2*, *MsrA1* and *Pep3* genes, respectively. Primers ActF1/R1, chiF1/R1, CWP2F1/R1, MsrA1F1/R1 and PepF1/R1 were used in RT-PCR and qRT-PCR to amplify *Actin*, *Chi*, *Rs-CWP2*, *MsrA1* and *Pep3*, respectively. The PCR products of UbiP5/6 were used as a probe in Southern blot

Southern blot analysis were carried out as previously described (Li et al. 2008). Briefly, genomic DNA from seven T4 transgenic lines and the non-transgenic Y11 was digested by two different restriction endonucleases, *Sac*I and *Eco*RV for Southern blots. The probe used in hybridization was amplified using P5/P6 primers (Fig. 1a and b; Table 1) and labeled with a-[^{32}P]-dCTP.

RNA extraction, RT-PCR and qRT-PCR

Total RNA was extracted from wheat spikelets and young leaves with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. In order to minimize the genomic DNA contamination, purified RNA was treated with RNase-free DNase I (Takara, Dalian, China) before final ethanol precipitation. Subsequently, 5 μg of total RNA was reversely transcribed into cDNA (Li et al. 2010a, b). Gene-specific primers were used for RT-PCR as previously described (Li et al. 2008, 2010a).

For quantitative real-time PCR (qRT-PCR), about 5 μg total RNA from wheat spikes (T4 and T5) or seedlings (T5) was used for reverse-transcription. qRT-PCRs were performed in a volume of 25 μL containing Sybr Green I PCR Master Mix (Toyobo; Osaka, Japan), 10 pmol each of forward and reverse gene-specific primers (Table 1), and 10 μl cDNA (1:100 dilution). Gene-specific primers were designed using Primer Premier5 software. Wheat actin gene was co-amplified as an internal control to normalize the total amounts of cDNAs present in each reaction and to eliminate the differences among the samples. PCR amplification was performed in an iQ5 Cycler (Bio-Rad, U.S.A.) under the following conditions: 95 °C for 4 min, followed by 40 cycles of 94 °C for 15 s, 62 °C for 20 s, 72 °C for 20 s. The plate read was at 72 °C for 30 s. A melting

curve was performed to determine the specificity of each PCR primer by incubating the reaction at 95 °C for 20 s, cooling to 55 °C for 10 s, and increasing to 95 °C at a rate of 0.5 °C/10 s. Quantifications of relative mRNA of target genes were determined using the ΔΔCT method as previously described (Schmittgen and Livak 2008):

$$\text{Relative expression level} = 2^{-(\text{CT of target gene} - \text{CT of } \beta - actin \text{ gene})}$$

Western blot

Total wheat crude proteins were extracted from 7-day-old seedlings (300 mg) with one volume of the protein extraction buffer (50 M Tris-HCl, pH 7.8; 1 mM EDTA; and 1 mM phenylmethansulphonyl fluoride). Total proteins were extracted from the ground leaf powders with the protein extraction buffer (50 M Tris-HCl, pH 7.8; 1 mM EDTA; and 1 mM phenylmethansulphonyl fluoride). After centrifugation of the extracts (12,000×g, 4 °C, 20 min), the supernatant was stored at 4 °C with the addition of 0.01 % (wt/vol) sodium azide for preservation. Wheat leaf protein extracts (20 μl) were then separated on 12 % sodium dodecylsulphate-polyacrylamide gels (SDS-PAGE) and transferred onto membrane (Li et al. 2008). Rabbit-derived polyclonal antisera against a peptide (SRAQFDRMLLHRNDGAC) of chitinase (Shin et al. 2008) were used as primary antibody (1:2000 dilution), and alkaline phosphotase-conjugated goat anti-rabbit antibody (1:5000 dilution) was used as secondary antibody (Neweast, Wuhan, China). Signals were analyzed and quantified using Quantity One 4.5.0 software (Bio-Rad, USA).

FHB resistance assay

A macroconidial suspension of a *F. graminearum* strain 5035 that had been isolated from a scabby wheat spike in Hubei, China (Zhang et al. 2007) was prepared as previously described (Wu et al. 2005) and used for the inoculation of spikes and seedlings throughout this study. Wheat plants were grown in either experimental fields (2 m in line length, 0.4 m between lines, and 30 plants per line) (T4) or plastic houses (T3, T4 and T5). Plastic houses (20 m×8 m×3 m) are the normal experimental fields that are covered with plastics. A total of 40 plants from each transgenic line or control were inoculated by single-floret injection (T3, T4 and T5) or spray inoculation (T5) at mid-flowering stage (Li et al. 2008). For single floret injection, one spike per plant was inoculated with a 10-μl droplet of the conidia (5×10^5 spores/ml) that was injected by a pipette tip to the central floret of one middle spikelet (Wu et al. 2005). The fungus-inoculated spikes were kept humid within the plastic bag for 3 days.

At the same stage as for the single-floret inoculation, the wheat spikes were sprayed once by a portable sprayer until the whole spikes were fully covered by the conidia suspension (5×10^4 spores/ml) in the plastic house. The sprayed plants were kept humid for 3 days. A FHB-resistant cv. Sumai3, a FHB-susceptible cv. Annong8455 and the non-transgenic Y11 were used as controls. Infected spikelets were visually scored at 21 day post inoculation (dpi), and disease severity was determined according to the percentage of infected spikelets.

FSB resistance assay

A total of 40 transgenic plants from T4 and T5 generations were inoculated at seedling stage with conidiospores as previously described (Wu et al. 2005; Li et al. 2010a). Briefly, the wheat seeds were soaked with 0.1 % $HgCl_2$ for 1 min followed by two rinses in sterilized distilled water. The sterilized seeds were incubated on moist filter paper in growth chamber at 20 °C for 3 day, and the top 2–3 mm of each seedling was cut-off. An aliquot of 3 μl of suspension (1×10^6 spores/ml) was added to the slant side of the cut seedling. The inoculated seedlings were maintained in a growth chamber at 25 °C with 95 % humidity and a 12 h photoperiod of light. A FSB-resistant cv. Annong8455, a FSB-susceptible cv. Sumai3 and the non-transgenic Y11 served as controls. The brown lesions of diseased seedlings were measured at 7 dpi, and the lesion length was determined as previously described (Li et al. 2010a).

Estimation of yield components

T4 transgenic plants and non-transgenic control Y11 inoculated by single-floret injection were harvested after maturation, and thousand kernel weights (TKW) were determined accordingly. The percentages of non-inoculated controls were referred to as the

relative thousand kernel weights (RTKW) as previously described (Li et al. 2008).

In vitro anti-fungal activity assay

An agar-gel diffusion inhibition assay was carried out in order to determine the in vitro anti-fungal activity for inhibition of mycelial growth of *F. graminearum*. A 5-mm diameter mycelial disk of the strain used for the inoculation described above was placed in the center of a PDA plate and incubated at 22 °C for 2 days. This mycelial disk was from a 3-day-old culture. At the 3rd day, a 50-μl aliquot of total wheat proteins from each transgenic line or the non-transgenic control Y11 that were prepared for western blots was applied to the wells in the PDA plate 1 cm away from the edge of the fungal colony, followed by a 1-day incubation. Inhibitory zone from different transgenic samples was visually compared with that from the non-transgenic Y11.

Statistical analysis

Data analysis was performed using SAS release 8 (Statistics Analysis System, SAS institute, Cary, NC, USA), and the Student *t* test was used for relevant data by SPSS software. P value of less than 0.05 was considered as statistically significant.

Results

Transformation and characterization of transgenic wheat plants

The wheat cv. Y11 was used for co-bombardment with four expression cassettes: *Pmi-Chi*, *Rs-CWP2*, *MsrA1* and *Pep3*. T0 transgenic plants were selected in the presence of mannose and identified by PCR. One transgenic plant containing all four cassettes and five intact gene coding sequences was selected for further analysis in the succeeding generations. After selfing of T0 plant, 11 identified T1 plants were obtained to produce T2 transgenic lines and finally seven lines were selected for subsequent characterization (Table 2). All T1 plants and 40 plants from each transgenic lines through T2 to T4 generations were identified by PCR (Fig. 1c and d) and RT-PCR (Fig. 1e-i). Table 2 shows that seven different T3 transgenic lines with various gene combinations were identified as follows: lines 121 (*Pmi*, *Chi, MsrA1*, *Rs-CWP2* and *Pep3*) and 312 (*Pmi, Chi, MsrA1* and *Pep3*) contained five and four genes, respectively; lines 223 (*Rs-CWP2*, *MsrA1* and *Pep3*), 451 (*Pmi*, *Chi* and *Pep3*) and 513 (*Pmi*, *Chi* and *MsrA1)* contained three genes, whereas line 681 (*Pmi* and *Chi*) contained two genes, and line 712 (*MsrA1*) contained only one gene. Among them, lines 223 and 712 did not contain *Pmi* gene. All individual expression cassettes, including *Pmi-Chi* and the associated regulatory sequences, were inherited as an intact unit in the transgenic lines (data not shown).

In our present study, we confirmed the transgene integration and investigated the integration patterns in T4 transgenic wheat lines by Southern blot. Figure 1a shows that *SacI* restriction site was present within each cassette, whereas *EcoRV* existed only within the *Pmi* sequence. As shown in Fig. 1j, more than 10 hybridization bands were detected from lines 121 and 223, followed by line 312 with seven bands. Moreover, six, four and three bands were detected from lines 451, 513 and 712, respectively, whereas only one band was detected from line 681. Figure 1k illustrates a simpler integration pattern. Therefore, all the seven transgenic lines indeed carried transgenes in their genomes, and each displayed a unique transgene integration pattern.

Expression analysis of transgenes by qRT-PCR and Western blot

Comparative qRT-PCR analyses revealed a consistent and comparable pattern for seven transgenic lines at seedlings (T5) and spikes (T4 and T5) in different generations, although there were variations among different genes and lines (Fig. 2a-c). *Chi* accumulated more transcripts than others in all five lines carrying *Chi* gene. The introduced barley chitinase sequence was amplified with primers ChiF1/ChiR1, not endogenous analogs from wheat (data not shown). Transgenic lines 121 and 223 showed more copies of transgenes in Southern blot (Fig. 1j and k) but displayed a relatively low mRNA expression. Therefore, the consistent expression patterns through different generations and developmental stages suggested genetic stability of transgenes in wheat and their constitutive regulation by ubiquitin promoter.

We detected one specific band from all samples using Western blot, probably due to a cross-reactivity with the antisera. Three transgenic lines (451, 513 and

Table 2 Percentage of infected spikelets and lesion length in T3 to T5 transgenic wheat and RTKW in T4 transgenic wheat as compared with non-transgenic control

Line	Genotype[a]	Single-floret injection (%)[b]				Spray floret (%)[b]	Seedling lesion (cm)[b]		RTKW (%)[b]
		T3 plastic house	T4 field	T4 plastic house	T5 plastic house	T5 plastic house	T4	T5	T4 field
121	Pm,R,M,P	17.64±3.10	28.66±2.89***	16.76±0.60***	26.45±3.82	26.11±1.53*	0.96±0.09	1.08±0.09	55.57±1.83**
223	R,M,P	17.15±2.63	33.23±5.09***	11.56±1.13***	32.20±2.36	28.98±1.79*	0.90±0.11	1.03±0.07	48.88±2.21*
312	Pm,M,P	13.78±2.08*	22.92±2.46***	14.54±0.21***	18.89±2.71**	27.72±4.83*	1.01±0.07	0.98±0.05	62.82±3.15***
451	Pm,P	7.19±0.31*	16.13±3.93***	9.94±1.25***	16.09±1.27**	14.42±1.81***	0.58±0.04*	0.36±0.06*	89.85±4.32***
513	Pm,M	12.49±2.08*	15.70±3.65***	11.67±1.62***	15.62±1.31**	19.21±1.25***	0.63±0.04*	0.53±0.07*	74.37±6.26***
681	Pm	21.71±5.57	12.01±2.54***	19.21±1.25***	21.73±1.87*	27.78±3.17*	0.74±0.05	0.79±0.07	80.56±5.82***
712	M	15.70±2.08	22.23±4.64***	27.04±2.10	25.52±2.89	28.47±3.11*	0.97±0.08	0.88±0.05	51.83±4.31**
Y11		30.47±3.43	73.02±4.16	34.29±2.89	37.33±2.53	40.06±3.17	1.05±0.08	1.04±0.08	34.93±1.63
AN		69.02±1.43	93.59±4.07	89.03±6.84	83.15±7.52	79.17±4.27	1.52±0.07	1.46±0.07	-
S3		5.77±0.46	10.76±1.65	4.96±0.65	13.12±1.54	29.39±4.04	2.77±0.10	2.35±0.09	-

[a] Pm, R, M and P represent *Pmi-Chi*, *Rs-CWP2*, *MsrA1* and *Pep3* gene, respectively

[b] Data are an average ± standard error from 40 plants

Y11 non-transgenic Yangmai11; *AN* Annong8455; *S3* Sumai3; - not determined

*, **, ***: $P<0.05$, 0.01 and 0.001, respectively

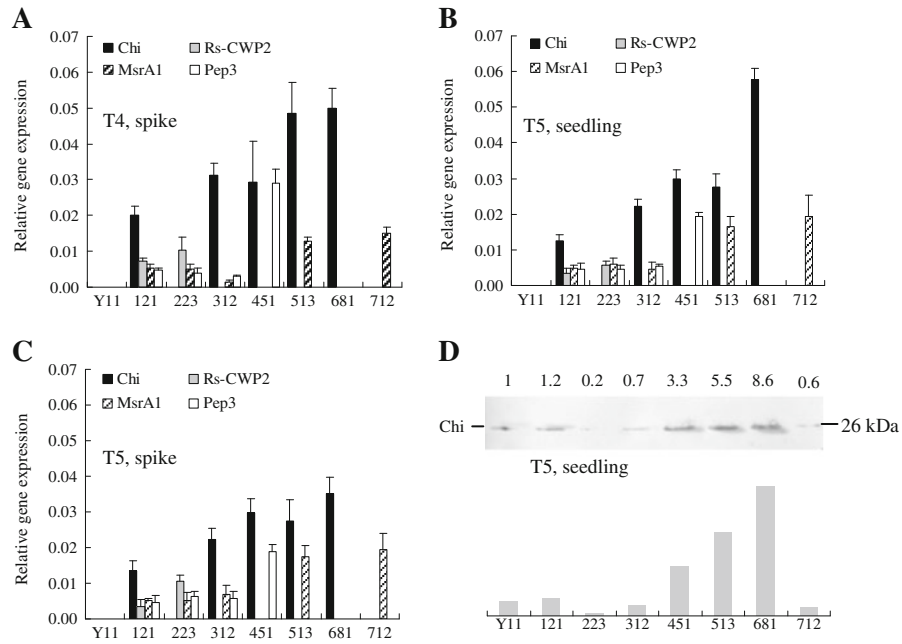

Fig. 2 qRT-PCR analyses of four genes in T4 and T5 generations of seven transgenic lines and non-transgenic control Y11 (**a**, **b** and **c**). Western blot of a chitinase (Chi) detected with poly-antisera and quantified with Bio-Rad imaging system (**d**). Amounts of transgene transcripts in qRT-PCRs were calculated relative to endogenous β-actin gene expression

681) showed a strong signal (Fig. 2d). These results suggested that transgene chitinase was profoundly accumulated at seedlings in these three wheat lines.

FHB resistance of transgenic wheat

In our present study, we assessed the FHB resistance of seven transgenic wheat lines of T3, T4 and T5 generations (Table 2). A significant difference was observed between some transgenic wheat lines and non-transgenic control Y11. In T3 generation, after the single-floret injection, line 451 had 7.2 % of infected spikelets, with a reduction of 76 % relative to the control Y11. Line 513 had 12.5 % of infected spikelets, with a reduction of 59 % relative to the control Y11. Furthermore, we assayed the plants of T4 generation in both plastic house and field. Our data showed that the percentage of infected spikelets from six transgenic wheat lines ranged from 12–33 % (field) and 10–19 % (plastic house), respectively. These rates were significantly reduced by 55–84 % and 44–71 % compared with their respective control Y11. The line 712 had a significantly lower infection rate than the control Y11 in the plastic house assay only. In T5 generation, significant reduction of infected spikelets in three transgenic lines (312, 451 and 513) was observed based on single-floret injection, with a reduction ranging from 49–58 % relative to the control Y11. In addition, a lower infection was observed in four lines (121, 223, 312 and 712) based on spray inoculation, and a much significantly lower infection was observed from two lines (451 and 513), with a reduction ranging from 53–64 % relative to the control Y11. Figure 3a and b demonstrate the representative wheat plants at 21 dpi. The single floret infection revealed *Fusarium* spp. spreading (i.e., a type II resistance), whereas the spray inoculation assessed infection incidence (i.e., a type I resistance) (Li et al. 2008). Therefore, expression of anti-fungal peptides (AFPs) in elite wheat cultivar could enhance both type I and type II FHB resistances.

FSB resistance of transgenic wheat

In order to investigate whether FSB resistance was also improved in transgenic wheat plants with enhanced FHB resistance, we assayed all the transgenic wheat lines for their FSB response (Table 2). In T4 generation, the lesion length of lines 451 and 513 was 0.58 and 0.63 cm, respectively, which was significantly reduced by 45 % and 40 % compared with the control Y11 (1.05 cm). In T5 generation, the lesion length of lines 451 and 513 was 0.36 and 0.53 cm, respectively, which was significantly reduced by 65 % and 49 % compared with the control Y11 (1.04 cm). Figure 3c shows the examples at 7 dpi. These results

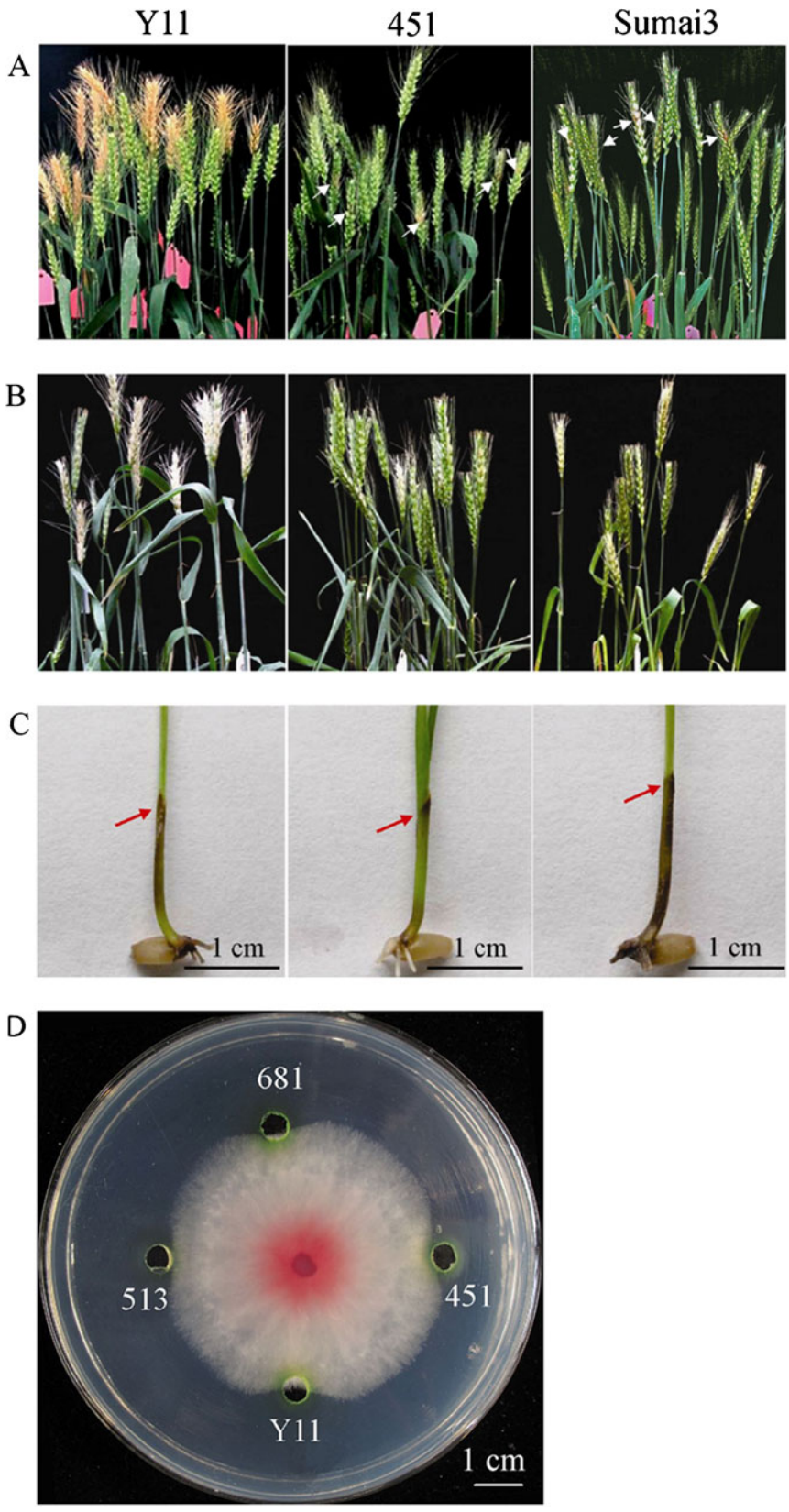

Fig. 3 Phenotype of representative plants after resistance assays (**a**, **b** and **c**) and anti-fungal activity assay in vitro (**d**). **a** 21 day after single-floret injection; **b** 21 d after spray inoculation; **c** 7 day after seedling inoculation; **d** 1 day after application of crude proteins. Transgenic lines and cultivars are indicated on the top of panel or in petri dish. Arrows indicate inoculated spikelet (**a**) or various lengths of lesions (**c**)

indicated that the transgenic wheat lines 451 and 513 were also FSB-resistant in addition to FHB resistance.

Yield analysis of transgenic wheat

In our present study, we also measured the RTKW of all seven T4 transgenic wheat lines in field FHB assay and non-transgenic control Y11 at physiological maturity after inoculation with single-floret injection (Table 2). Results revealed a very significant difference between the transgenic wheat and the control Y11. RTKW of the transgenic lines ranged from 48.9 to 89.8, whereas it was 34.9 for the non-transgenic control Y11. Therefore, the TKW of the transgenic wheat lines was significantly increased by 40 % to 157 % compared to the control Y11.

In vitro anti-fungal activity against Fusarium pathogen

In order to address more detailed information on mode of resistance in the transgenic wheat plants, we extracted the total crude proteins from wheat seedlings and analyzed their anti-fungal activities against *F. graminearum* in vitro. Figure 3d shows an example that a clear inhibitory zone was observed from the proteins extracted from two transgenic wheat lines 451 and 513, exhibiting significant FSB and FHB resistances in different generations, whereas the proteins from the remaining transgenic lines or the non-transgenic control Y11 did not show discernible inhibitory zone, in which mycelia overlaid part of the wells for the Y11 and transgenic wheat line 681. These results suggested the expression of AFPs in transgenic wheat resulted in a reduced fungal spreading in wheat spikes and seedlings through the *in planta* restriction of fungal growth.

Discussion

It becomes a major challenge to integrate both FSB and FHB resistances into one elite wheat cultivar since these two resistances are regulated by independent genetic loci and only a limited amount of resistant germplasm exists in nature (Li et al. 2010b; Tamburic-Ilincic et al. 2009). In the present study, we aimed to investigate whether transgenic wheat could have an improved overall resistance to *Fusarium* pathogens at seedlings and spikes. In order to comparatively identify the wheat lines resistant to both FSB and FHB, we co-transformed different genes into an elite wheat cultivar and selected the genetically stable transgenic wheat lines from the transgenic progenies.

It is essential to properly combine a number of different transgenes in transgenic wheat in order to enhance overall disease resistance. Table 2 shows that two transgenic lines 451 and 513 exhibited a consistent resistance to FSB and FHB throughout various generations. Each of these two lines carried two genes: line 451 carried *Chi* and *Pep3*, while line 513 carried *Chi* and *MsrA1*. qRT-PCR and Western blot analyses revealed that the transgenes were highly expressed in these lines (Fig. 2a-d). Although carried more genes, transgenic lines 121, 223 and 312 only showed an enhanced FHB resistance in some generations, whereas no FSB resistance was observed (Figs. 1 and 2). We found that the expression of transgenes was relatively low in these three lines (121, 223 and 312). This could be associated with the high copy numbers of transgenes, which might affect the expression of each other. Furthermore, transgenic lines 681 and 712 carried a single AFP and showed an enhanced resistance only to FHB. Therefore, a single AFP might not be sufficient to confer a resistance to FSB although it had a high expression at both the mRNA and protein levels (line 681, Fig. 2).

The significantly enhanced overall resistance might be attributed to efficient inhibition of fungal growth by synergistic action of different AFPs. Figure 2d shows that a high level of Chi protein was detected from three transgenic wheat lines 451, 513 and 681 by Western blot, among which line 681 exhibited the highest expression. However, only the proteins expressed in the lines 451 (*Chi* and *Pep3*) and 513 (*Chi* and *MsrA1*) showed a clear inhibitory activity against *F. graminearum* in vitro (Fig. 3d) as well as a consistent resistance to *Fusarium* pathogens throughout various generations and developmental stages (Table 2). *Chi* encodes a barley chitinase, and a chitinase-mediated breakdown of fungal chitin has been observed in vivo (Benhamou and Theriault 1992). Chitin is a major structural component of the cell wall of *F. graminearum,* and it has been proposed as a target for controlling the pathogen (Xu et al. 2010). *Pep3* from immune haemolymph of the *Cecropia* moth interferes with phospholipids on the fungal cell membrane, affecting channel formation or membrane disruption (Cavallarin et al. 1998). Derived from an N terminus-modified cecropin-melittin cathionic peptide chimera, the positively charged *MsrA1* is

thought to interact with negatively charged phospholipids of fungal membranes by depolarization (Osusky et al. 2000). In our present study, the observed resistances *in planta* and anti-fungal activity in vitro in the lines 451 and 513 could be caused by a combined effect of chitin breakdown together with the interference of fungal membrane. Therefore, selection of two different AFPs with a complementary, synergistic activity towards *Fusarium* pathogens could facilitate breeding of wheat cultivars with an improved overall resistance to FSB and FHB. *In planta* assay appeared to be a pivotal step for this selection.

FSB response appeared to be a more stringent reaction than the FHB assay when challenged with *F. graminearum*. Only two transgenic lines (451 and 513) demonstrated a FSB resistance, and their FHB resistance was consistent in all assays. At the seedling stages of wheat, young tissues may favor fast invasion and spread by the fungus. Therefore, we evaluated FSB severity at 7 dpi; for such a short period, only proteins with a strong anti-fungal activity were able to efficiently restrict the fungal spreading. However, glumes of wheat spikes are largely lignified, leading to a requirement of more time and energy for the fungus to invade the tissues. Therefore, FHB symptom was assayed at 21 dpi. Under such circumstance, the response of wheat to FHB might differ from FSB. These results implied that FSB assay alone at the seedlings was able to effectively identify transgenic plants with an overall resistance to both FSB and FHB.

The current study described an efficient selection of genetically stable transgenic wheat lines expressing different AFPs. The selected transgenic wheat with proper combination of transgenes exhibited a significantly enhanced resistance to both FSB and FHB. These results suggested that co-expression of different AFPs provided a promising alternative to improve an overall resistance to *Fusarium* pathogen in wheat through genetic engineering.

Acknowledgments This work was supported by the National Basic Research Program of China (2009CB118806), the Ministry of Agriculture of China (2008ZX08002-001, 2009ZX08002-001B).

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