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ORIGINAL PAPER

GmPGIP3 enhanced resistance to both take-all and common root rot diseases in transgenic wheat

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Abstract Take-all (caused by the fungal pathogen *Gaeumannomyces graminis* var. *tritici*, *Ggt*) and common root rot (caused by *Bipolaris sorokiniana*) are devastating root diseases of wheat (*Triticum aestivum* L.). Development of resistant wheat cultivars has been a challenge since no resistant wheat accession is available. GmPGIP3, one member of polygalacturonase-inhibiting protein (PGIP) family in soybean (*Glycine max*), exhibited inhibition activity against fungal endopolygalacturonases (PGs) in vitro. In this study, the *GmPGIP3* transgenic wheat plants were generated and used to assess the effectiveness of GmPGIP3 in protecting wheat from the infection of *Ggt* and *B. sorokiniana*. Four independent transgenic lines were identified by genomic PCR, Southern blot, and reverse transcription PCR (RT-PCR). The introduced *GmPGIP3* was integrated into the genomes of these transgenic lines and could be expressed. The expressing GmPGIP3 protein in these transgenic wheat lines could inhibit the PGs produced by *Ggt* and *B. sorokiniana*. The disease response assessments postinoculation showed that the *GmPGIP3*-expressing transgenic wheat lines displayed significantly enhanced resistance to both take-all and common root rot diseases caused by the infection of *Ggt* and *B. sorokiniana*. These data suggested that *GmPGIP3* is an attractive gene resource in improving resistance to both take-all and common root rot diseases in wheat.

Keywords Wheat · Polygalacturonase-inhibiting protein · *GmPGIP3* · *Gaeumannomyces graminis* var. *tritici* · *Bipolaris sorokiniana*

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important food crops in the world. It provides ~20 % of the calories consumed by humankind (Fu et al. 2009). Take-all disease, caused by the necrotrophic fungus *Gaeumannomyces graminis* var. *tritici* (*Ggt*), is one of the most destructive root diseases of wheat worldwide (Gutteridge et al. 2003; Daval et al. 2011). The symptoms of take-all include black lesions on the roots and based stems, plant stunting, premature ripening, and white heads even empty spikes, leading to severely yield losses of wheat (Gutteridge et al. 2003; Daval et al. 2011). Common root rot is primarily caused by the soil-borne fungus *Bipolaris sorokiniana* and impacts the wheat production in the many areas of the world (Kumar et al. 2002). Take-all and common root rot diseases occur simultaneously to the wheat plants in some fields (Shivanna et al. 1996).

In plant–pathogen interactions, plants have evolved a multilayered immunity system to counter infection of microbial pathogens. The plant cell wall represents the first line of defense for plant cells against pathogen infection. To gain access to the plant tissue, most of fungal pathogens secret a variety of enzymes that degrade the plant cell wall. Necrotrophic pathogens kill host plant cells via secreting abundant hydrolytic enzymes and feed on the dead tissues (Laluk and Mengiste 2010). Endopolygalacturonase (PG; EC

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3.2.1.15) is one of secreted enzymes, which cleave the α-1,4 linkages between D-galacturonic acid residues in homogalacturonan and cause cell separation and maceration of host plant tissues (Cantu et al. 2008). The significance of PG in pathogen infection and colonization has been demonstrated for many fungal pathogens, such as *B. sorokiniana* (Clay et al. 1997), *Botrytis cenerea* (ten Have et al. 1998), *Alternaria citri* (Isshiki et al. 2001), *Sclerotinia sclerotiorum* (Zuppini et al. 2005), and *Claviceps purpurea* (Oeser et al. 2002), and for bacteria pathogens including *Agrobacterium tumefaciens* (Rodriguez-Palenzuela et al. 1991) and *Ralstonia solanacearum* (Huang and Allen 2000).

Many plants possess the polygalacturonase-inhibiting proteins (PGIPs) with extracellular leucine-rich repeat motif, belonging to the cell wall glycoproteins (Jones and Jones 1997; D'Ovidio et al. 2006). Some PGIPs are capable of inhibiting the pathogen PG activity and protecting the host tissues from degradation. Moreover, the interaction between plant PGIPs and pathogen PGs favors accumulation of oligogalacturonides, which elicit a wide range of defense responses (Cervone et al. 1997; Ridley et al. 2001; D'Ovidio et al. 2006). In the defense responses, plants produce various isoforms of PGIPs that display differential recognition specificity and inhibition efficiency to PGs (De Lorenzo et al. 2001). Many *Pgip* genes have been cloned and characterized from various plants. The effectiveness of some PGIPs in limiting pathogens infection has been verified in *Arabidopsis* (Ferrari et al. 2003), tomato (Powell et al. 2000), tobacco (Joubert et al. 2006), and grape (Aguero et al. 2005). The ectopic expression of bean *PvPGIP2* improves resistance to *B. sorokiniana* and *Fusarium graminearum* in transgenic wheat (Janni et al. 2008; Ferrari et al. 2012). In the recent study, four soybean *Pgip* genes were cloned (D'Ovidio et al. 2006). Only GmPGIP3 showed broad inhibition spectrum to PGs from eight different fungi in vitro, with the highest inhibition activity against PGs from *F. graminearum* (D'Ovidio et al. 2006). However, it is not clear whether the expression of *GmPGIP3* in plants can enhance resistance to fungal pathogens.

In this study, we generated *GmPGIP3* transgenic wheat lines and further investigated if the ectopic expression of *GmPGIP3* improves resistance to the infection of *Ggt* and *B. sorokiniana* in wheat. The results showed that *GmPGIP3*-expressing transgenic wheat lines displayed significantly enhanced resistance to the infection of the two pathogens.

Materials and methods

Plant and fungal materials

The wheat cultivar (cv.) Yangmai 18 was used as the recipient of *GmPGIP3* transformation. Yangmai 18, provided by Professor Boqiao Zhang in Yangzhou Agricultural Institute, is a Chinese spring wheat variety with susceptibility to both *Ggt* and *B. sorokiniana*. Soybean cv. Zhonghuang 13, provided by Prof. Lianzheng Wang in Institute of Crop Science, CAAS, was used to clone *GmPGIP3* sequence.

The fungal pathogen *Ggt* XNQS-2 was isolated, identified, and provided by Dr. Yang Wang, College of Plant Protection, Northwest A&F University, China. The fungal pathogen *B. sorokiniana* ACC30209 was purchased from the Agricultural Microbial Culture Collection, CAAS, China.

DNA and RNA extraction and the first-strand cDNA synthesis

The genomic DNA was extracted from leaves of wheat plants using the CTAB method (Saghai-Maroof et al. 1984). Total RNA was extracted from wheat roots or soybean leaves using TRIzol reagents (Invitrogen) and subjected to the RNase-free DNase I for removing the genomic DNA. Two micrograms of total RNA was used to synthesize the first-strand complementary DNA (cDNA) for each sample using M-MLV reverse transcriptase (TaKaRa, Dalian, China).

Construction *GmPGIP3*-expressing transformation vector

The *GmPGIP3* sequence containing complete open reading frame (ORF) was cloned from cDNA of soybean Zhonghuang 13 using primers GmPGIP3-OF (5′- CATT CATTCAAGATAGATGTCAAAGTT-3′) and GmPGIP3-OR (5′-TTAAGTGCATGGT GGAAGAGGAG-3′), which were designed based on the *GmPGIP3* sequence (GenBank accession no. AJ972662). The ORF was subcloned into *Sma* I and *Sac* I sites of the monocot expression vector pAHC25 (Christensen and Quail 1996), resulting in the transformation vector pA25-GmPGIP3. In the *GmPGIP3* expressing cassette of pA25-GmPGIP3 vector, *GmPGIP3* transcription was controlled by the maize *ubiquitin* promoter and the terminator of nopaline synthase (*Tnos*) gene (Fig. 1a).

Wheat transformation by biolistic bombardment

The transformation of pA25-GmPGIP3 into wheat cv. Yangmai 18 was performed using biolistic bombardment following the procedure described by Xu et al. (2001) and Chen et al. (2008). Here, a biolistic gun, PDS-1000 He (Bio-Rad, USA), was used. A total of 1200 immature embryos of Yangmai 18 were bombarded with 1-μm gold particles coated with pA25-GmPGIP3 DNA under a pressure of 1100 psi.

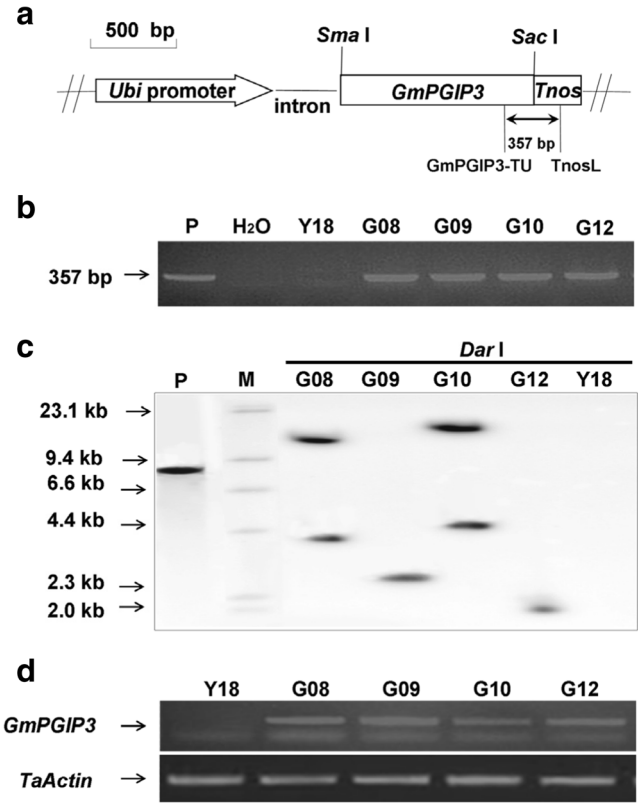

Fig. 1 *GmPGIP3* transformation vector and molecular characterization of transgenic wheat. **a** Schema of the transformation vector pA25-GmPGIP3. *Ubi promoter*, maize ubiquitin promoter; *intron*, the first intron from the ubiquitin promoter; and *Tnos*, terminator of the *Agrobacterium tumefaciens* nopaline synthase gene. The *arrow* indicates the region amplified in the PCR assays and used for the Southern blot probe. **b** PCR pattern of the *GmPGIP3* transgenic lines in T_3 generation using the primers specific to *GmPGIP3-Tnos* expressing cassette. *Lane P*, pA25-GmPGIP3 plasmid as the positive control of PCR assay; *lane* H_2O, template is H_2O; *lane Y18*, nontransformed Yangmai 18 as the negative control; and *lanes G08*, *G09*, *G10*, and *G12*, *GmPGIP3* transgenic lines. **c** Southern blot assay. The *Dra* I-digested genomic DNAs from transgenic lines G08, G09, G10, G12, and nontransformed Yangmai 18 were hybridized with the fragment specific for *GmPGIP3*. *Lane P*, pA25-GmPGIP3 vector DNA; *lane M*, λDNA/*Hin*d III markers. **d** RT-PCR analysis on transcription of *GmPGIP3* in the four transgenic lines. Housekeeping gene *TaActin* in wheat as internal reference is used to normalize the initial DNA contents among samples. *Lane Y18*, nontransformed Yangmai 18; *lanes G08*, *G09*, *G10*, and *G12*, *GmPGIP3* transgenic lines

PCR and Southern blot analyses on *GmPGIP3* transgenic wheat plants

The presence of *GmPGIP3* in transgenic wheat plants in T_0–T_3 generations was detected by PCR analysis of leaf genomic DNA using primers GmPGIP3-TU (GGACGC TACCTCAGGGACTTAC, locating in *GmPIGP3* sequence) and TnosL (ATGTATAATTGCGGGACTCTAA T, locating in the *Tnos* sequence of pA25-GmPGIP3). The PCR reaction was performed in a total volume of 20 μl containing 1× *Taq* buffer, 1.5 mM Mg^{2+}, 0.05 mM each dNTP, 0.4 mM each primer, 1 unit *Taq* polymerase (TaKaRa, Dalian, China), and 50 ng template DNA. The PCR cycle was performed with an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 45 s and a final extension at 72 °C for 10 min. The amplified products were resolved on a 1.5 % agarose gel and visualized by ethidium bromide staining. The nontransformed Yangmai 18 and pA25-GmPGIP3 were separately used as negative control and positive control.

Southern blotting was performed following a modified protocols described by Sharp et al. (1989) and Zhang et al. (2012). Genomic DNAs (~20 μg each) of four transgenic wheat lines and nontransformed Yangmai 18 were digested by the restriction enzyme *Dra* I and then separated on 0.8 % agarose gel, transferred to a Hybond N^+ nylon membrane (GE Amersham). The amplification fragments specific to transgenic *GmPGIP3* was labeled by α-^{32}P-dCTP and then used as probe. The membrane containing the DNAs was hybridized with the probe at 65 °C for 20 h.

Transcription analysis of *GmPGIP3* in transgenic wheat

RT-PCR was used to analyze *GmPGIP3* transcription in transgenic wheat plants. The primers specific to *GmPGIP3*, GmPGIP3-QF (CCTAATCGGTCAAATCCCCT), and GmPGIP3-QR (GACAAGTCCACGAACGCCA) were used for the evaluation of transcriptional level of *GmPGIP3* in transgenic wheat lines. The amplification of wheat *Actin* gene was used to normalize initial cDNA contents among samples using the specific primers including TaActin-F (CACTGGAA TGGTCAAGGCTG) and TaActin-R (CTCCATGTCATCCC AGTTG).

Extraction of *Ggt* and *B. sorokiniana* PGs and expressing GmPGIP3 protein

The fungal mycelium culture and PG extraction of *Ggt* and *B. sorokiniana* were performed following the method described by Janni et al. (2008) for *B. sorokiniana* PGs. The crude protein extracts containing GmPGIP3 were extracted from leaves of *GmPGIP3* transgenic plants as described by D'Ovidio et al. (2004a). Enzymatic activity of crude PGs from *Ggt* and *B. sorokiniana* was assayed following the protocol described by Taylor and Secor (1988) and Janni et al. (2008). The agarose plates containing PG reaction system were incubated at 30 °C for 18 h. Each reaction system contains 5 μl endo-PGs plus 25-μl 20-mM Na acetate solution (pH 4.7).

Assay of inhibitory activity of expressing GmPGIP3 protein

Inhibitory activity of the expressing GmPGIP3 protein extracted from the transgenic wheat plants was tested using agarose diffusion assay according to the modified method described by Taylor and Secor (1988) and Janni et al. (2008). Total volume of each reaction system was adjusted to 30 μl using 20 mM Na acetate (pH 4.7). Each reaction system contains 5 μl endo-PGs and 20 μg crude protein extract from transgenic wheat plants or nontransformed Yangmai 18. The agarose plates containing inhibitory activity reactions were incubated at 30 °C for 18 h.

Responses of transgenic wheat plants to *Ggt* and *B. sorokiniana*

At 4 weeks postinoculation with *Ggt* (Liu et al. 2013), take-all responses of *GmPGIP3* transgenic wheat and nontransformed Yangmai 18 plants were assessed. The average take-all index (TAI) for each line was calculated following Bithell et al. (2011). To further investigate the take-all resistance to these transgenic wheat lines, quantitative reverse transcription PCR (qRT-PCR) was used to assay the relative abundance of *Ggt* in transgenic wheat plants based on *Ggt* 18S ribosomal RNA (rRNA) in reference to wheat 18S rRNA as described by Liu et al. (2013).

Following inoculation with *B. sorokiniana* mycelia as described by Dong et al. (2010), common root rot responses of the transgenic and nontransformed wheat lines were evaluated. The average disease index of common root rot for each line was scored at plant maturity according to Dong et al. (2010). The disease assessments were performed in T_1–T_3 transgenic plants. In each disease assessment, at least 20 plants per line were tested.

Results

Generation and molecular characterization of *GmPGIP3* transgenic wheat

To assess the effectiveness of *GmPGIP3* in improving wheat resistance to the infection of *Ggt* and *B. sorokiniana*, transgenic wheat plants expressing *GmPGIP3* were generated by bombarding pA25-GmPGIP3 into the immature embryos of wheat cv. Yangmai 18. The presence of *GmPGIP3* in transgenic wheat plants was detected by the desired fragment (357 bp) of genomic PCR with *GmPGIP3* transgene-specific primers. Genomic PCR assays showed that the expected band of *GmPGIP3* transgene was present in four transgenic wheat lines (G08, G09, G10, and G12) from T_0 to T_3 generations, but not in nontransformed Yangmai 18 (Fig. 1b). The results indicated that the introduced *GmPGIP3* was inheritable in these four transgenic lines and that the transformation efficiency was 0.33 %. Using the probe derived from the *GmPGIP3* transgene-specific amplified fragment, Southern blot assay showed that the *GmPGIP3* expressing cassette was integrated into the genomes of the four transgenic lines with one to two copies (Fig. 1c). The hybridization patterns in the four transgenic lines were different, indicating that these transgenic lines were derived from independent transformation events.

Expression and inhibitory activity of GmPGIP3 in transgenic wheat plants

RT-PCR assays were used to analyze the transcription of the introduced *GmPGIP3* in these four transgenic wheat lines (G08, G09, G10, and G12). The results showed that the transcription of *GmPGIP3* in these transgenic lines was

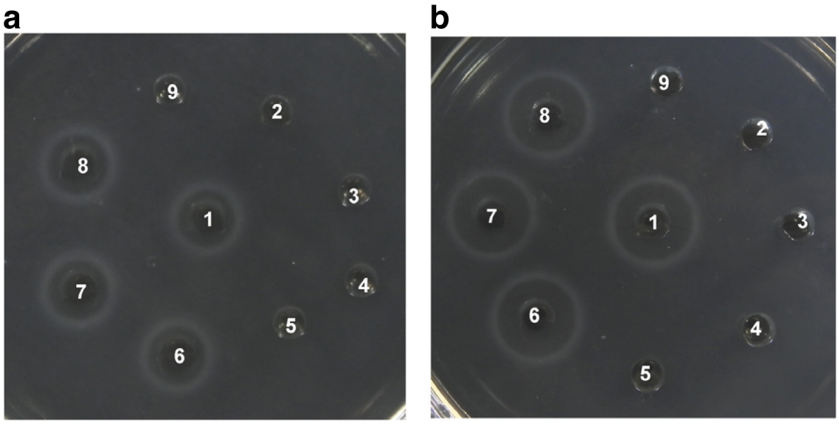

Fig. 2 Agarose diffusion assay of crude protein extracts from leaves of *GmPGIP3* transgenic wheat lines and nontransformed Yangmai 18. All incubation mixtures (except for 8) contain 5 μl endo-PGs produced by *Gaeumannomyces graminis* var. *tritici*, *Ggt*) (**a**) and *Bipolaris sorokiniana* (**b**) in liquid culture. *1*, crude endo-PGs; *2–6*, crude endo-PGs plus crude protein extract (20 μg) from transgenic wheat lines G08, G09, G10, G12, and nontransformed Yangmai 18, respectively; *7*, crude endo-PGs plus boiled crude protein extract from the transgenic wheat line G08; *8*, pectolyase Y-23 (0.5 μg); and *9*, boiled crude endo-PGs

detected with higher levels compared with nontransformed Yangmai 18 (Fig. 1d).

The inhibitory activity of transgenically expressing GmPGIP3 protein was detected by a semiquantitative agarose diffusion assay using the endo-PG activity produced in liquid culture from *Ggt* and *B. sorokiniana*. The results showed that endo-PGs from *Ggt* or *B. sorokiniana* had the PG enzymatic activity (Fig. 2a, b). The crude protein extracts from these four transgenic wheat lines (G08, G09, G10, and G12) significantly inhibited the activity of endo-PGs from *Ggt* and *B. sorokiniana*, as indicated by the lack of the halo. In contrast, crude protein extract from nontransformed Yangmai 18 did not have any effect on the activity of endo-PGs from *Ggt* and *B. sorokiniana*. The assays of the boiled extracts did not exhibit any activity, excluding the possibility of a nonproteinaceous inhibitor (Fig. 2a, b). These results revealed that the transformed GmPGIP3 proteins were expressed in these four transgenic wheat lines and possessed inhibitory activity against endo-PGs from *Ggt* and *B. sorokiniana*.

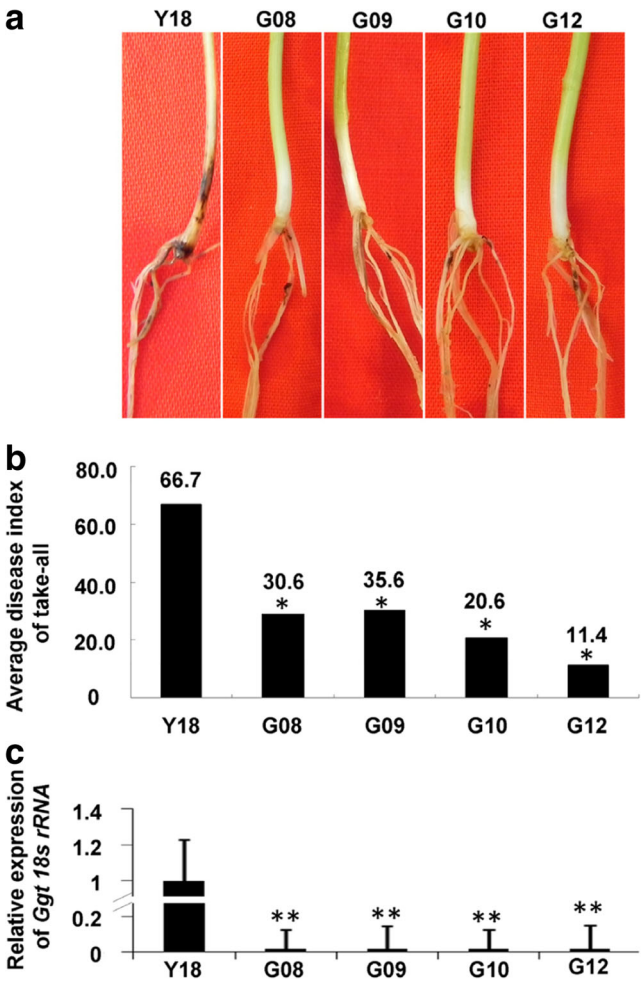

Fig. 3 Take-all responses of *GmPGIP3* transgenic and nontransformed wheat lines. **a** The typical take-all phenotypes on the roots of nontransformed Yangmai 18 (Y18) and four transgenic lines (G08, G09, G10, and G12). **b** Average disease index of take-all in nontransformed Yangmai 18 and these transgenic lines (*asterisks* indicate $P<0.05$). **c** *Ggt* biomass in the roots of nontransformed Yangmai 18 and these transgenic lines were analyzed using quantitative RT-PCR. The relative transcript levels of *Ggt* 18S rRNA in transgenic lines were relative to those in nontransformed Yangmai 18. Three biological replicates of each line were averaged and statistically treated using *t* test (*double asterisks* indicate $P<0.01$). *Bars* indicate the standard error of the mean

Expression of *GmPGIP3* improved resistance to both take-all and common root rot in transgenic wheat

To investigate if expression of *GmPGIP3* enhances resistance to both soil-borne diseases in transgenic wheat, the responses to take-all and common root rot were assessed in the four transgenic wheat lines and nontransformed Yangmai 18 following inoculation with the fungal pathogens *Ggt* and *B. sorokiniana*. The results showed that the *GmPGIP3*-expressing transgenic lines exhibited a significant reduction of take-all symptoms compared with nontransformed Yangmai 18 (Fig. 3a). The average TAIs of these four transgenic wheat lines were ranged from 11.44 to 35.56 depending on the different lines, whereas that of Yangmai 18 was 66.67 (Fig. 3b). Additionally, *Ggt* biomass was significantly decreased in these transgenic lines compared with that in nontransformed Yangmai 18, indicating that the expression of *GmPGIP3* markedly improved wheat resistance to take-all caused by *Ggt* infection (Fig. 3c). Moreover, the assessment

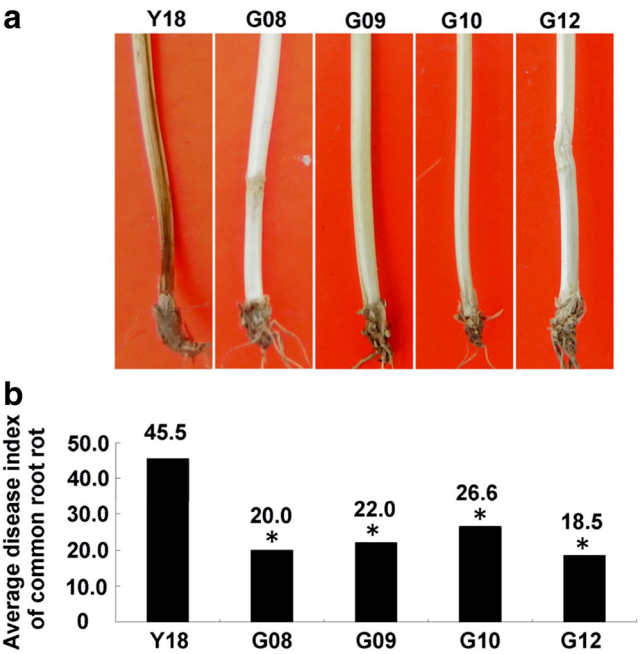

Fig. 4 Common root rot responses of *GmPGIP3* transgenic wheat and nontransformed Yangmai 18 lines. **a** The typical common root rot phenotypes on the roots and stem base of nontransformed Yangmai 18 (Y18) and the four transgenic lines (G08, G09, G10, and G12). **b** Average disease index of common root rot in nontransformed Yangmai 18 and these transgenic lines (*asterisks* indicate $P<0.05$)

on common root rot responses showed that these four *GmPGIP3*-expressing transgenic lines exhibited a significant reduction of the disease symptoms compared with nontransformed Yangmai 18 (Fig. 4a). The average disease index in transgenic lines were ranged from 18.5 to 26.6, whereas that in nontransformed Yangmai 18 was 45.5, suggesting that the ectopic expression of *GmPGIP3* in transgenic wheat enhanced resistance to common root rot caused by *B. sorokiniana* infection (Fig. 4b).

Discussion

More than 30 members of *PGIP* family were isolated from various plant species (D'Ovidio et al. 2004b; 2006; Ferrari et al. 2003; Janni et al. 2013; Li et al. 2003; Lu et al. 2012; Wang et al. 2013). The effectiveness of some members in increasing disease resistance has been verified in *Arabidopsis*, potato, tobacco, grape, and wheat (Aguero et al. 2005; Ferrari et al. 2003; Janni et al. 2008; 2013; Joubert et al. 2006; Powell et al. 2000). The bean PvPGIP2 is one of most effective inhibitors so far characterized (D'Ovidio et al. 2004a; 2006; Janni et al. 2008, 2011). Recently, *GmPGIP3*, one of *PGIP* family members in soybean, was isolated and studied (D'Ovidio et al. 2006). Sequence comparisons revealed that the deduced amino acid sequence of GmPGIP3 contains the key residues that are crucial for inhibition activity in PvPGIP2. Assays on inhibiting capacity to PGs revealed that the spectrum of inhibition of GmPGIP3 against eight different fungal pathogens was similar to that of bean PvPGIP2 (D'Ovidio et al. 2006). Interestingly, inhibitory activity of GmPGIP3 to PGs from fungal *Botrytis aclada* was three times more than that of PvPGIP2 (D'Ovidio et al. 2006). The accumulating knowledge on GmPGIP3 promotes us to investigate if ectopic expression of GmPGIP3 in wheat can improve resistance to the important fungal pathogens *Ggt* and *B. sorokiniana*.

In this study, we generated the *GmPGIP3* transgenic wheat plants by biolistic bombardment. Genomic PCR and Southern blot analyses revealed that the introduced *GmPGIP3* expressing cassette was integrated into the genomes of transgenic wheat lines and could be inherited from T_0 to T_3 generations. RT-PCR assay indicated that, compared with nontransformed Yangmai 18, *GmPGIP3* was expressed with higher levels in four transgenic wheat lines, including G08, G09, G10, and G12. The resistance test of these transgenic lines showed that the ectopic expression of *GmPGIP3* in transgenic wheat significantly improved resistance to both take-all and common root rot diseases. The inhibitory activity assays to endo-PGs showed that the transgenically expressing GmPGIP3 protein from the four transgenic wheat lines was capable of inhibiting the activity of endo-PGs from *Ggt* and *B. sorokiniana*. These data suggested that the expressing GmPGIP3 protein inhibited the activity of PGs produced by the fungal pathogens *Ggt* and *B. sorokiniana* and, in turn, might suppress the further infection and colonization of the two fungal pathogens, leading to the *GmPGIP3*-expressing transgenic lines that displayed significantly enhanced resistance to both take-all and common root rot diseases caused by the infection of the two pathogens. The transgenic wheat lines generated in this study may provide potential wheat germplasms for enhancing resistance to both take-all and common root rot diseases.

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