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The Expression of a Bean PGIP in Transgenic Wheat Confers Increased Resistance to the Fungal Pathogen *Bipolaris sorokiniana*

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A possible strategy to control plant pathogens is the improvement of natural plant defense mechanisms against the tools that pathogens commonly use to penetrate and colonize the host tissue. One of these mechanisms is represented by the host plant's ability to inhibit the pathogen's capacity to degrade plant cell wall polysaccharides. Polygalacturonase-inhibiting proteins (PGIP) are plant defense cell wall glycoproteins that inhibit the activity of fungal endopolygalacturonases (endo-PGs). To assess the effectiveness of these proteins in protecting wheat from fungal pathogens, we produced a number of transgenic wheat lines expressing a bean PGIP (PvPGIP2) having a wide spectrum of specificities against fungal PGs. Three independent transgenic lines were characterized in detail, including determination of the levels of PvPGIP2 accumulation and its subcellular localization and inhibitory activity. Results show that the transgene-encoded protein is correctly secreted into the apoplast, maintains its characteristic recognition specificities, and endows the transgenic wheat with new PG recognition capabilities. As a consequence, transgenic wheat tissue showed increased resistance to digestion by the PG of *Fusarium moniliforme*. These new properties also were confirmed at the plant level during interactions with the fungal pathogen *Bipolaris sorokiniana*. All three lines showed significant reductions in symptom progression (46 to 50%) through the leaves following infection with this pathogen. Our results illustrate the feasibility of improving wheat's defenses against pathogens by expression of proteins with new capabilities to counteract those produced by the pathogens.

In several plant–pathogen interactions, the plant cell wall represents the main barrier to the colonization of host plant tissue. To overcome this obstacle, most fungal pathogens produce a variety of enzymes that degrade the host plant cell wall. Endopolygalacturonase (endo-PG; EC 3.2.1.15) is one of the first enzymes secreted during the infection process and it cleaves the α-(1$\rightarrow$4) linkages between D-galacturonic acid residues in homogalacturonan, causing cell separation and maceration of host tissue. The importance of PG in pathogenesis has been demonstrated for the fungi *Botrytis cinerea* (ten Have et al. 1998) and *Alternaria citri* (Isshiki et al. 2001), and for the bacteria *Agrobacterium tumefaciens* (Rodriguez-Palenzuela et al. 1991) and *Ralstonia solanacearum* (Huang and Allen 2000).

Many plants possess a cell wall glycoprotein, the polygalacturonase inhibiting protein (PGIP), which is able to inhibit fungal endo-PGs. The interaction between fungal PGs and plant PGIP favors the accumulation of oligogalacturonides (OGs), which elicit a wide range of defense responses (Cervone et al. 1997; Ridley et al. 2001). PGIP belong to the subclass of proteins containing leucine-rich repeats (LRRs) of the extracytoplasmic type (Jones and Jones 1997). They typically contain 10 imperfect LRRs of 24 residues each, which are organized to form two β-sheets, one of which (sheet B1) occupies the concave inner side of the molecule and contains residues crucial for endo-PG recognition (Di Matteo et al. 2003; D'Ovidio et al. 2004; Leckie et al. 1999). Biochemical and molecular analyses reveal both the existence of a small PGIP-encoding gene family in several plant species and that members of the same PGIP family can possess distinct PG-inhibiting activities (De Lorenzo et al. 2001). These analyses also indicate that the bean PvPGIP2 is one of the most effective inhibitors of fungal PGs (D'Ovidio et al. 2004). The effectiveness of PGIP in limiting host tissue colonization has been shown in tomato, *Arabidopsis*, tobacco, and grape. In these dicot plant species, the overexpression of PGIP reduces the symptoms caused by the infection by *B. cinerea* (Aguero et al. 2005; Ferrari et al. 2003; Joubert et al. 2006; Manfredini et al. 2006; Powell et al. 2000).

The demonstration that PG is a pathogenicity factor for *Claviceps purpurea* during the infection of rye (Oeser et al. 2002) has reinforced interest in understanding the role of PGIP in monocots. Clay and associates (1997) demonstrated the involvement in leaf penetration of the endo-PG produced in vitro by the cereal pathogen *Bipolaris sorokiniana* (Sacc.) Shoemaker (teleomorph *Cochliobolus sativus*), the causal agent of foliar spot blotch, root rot, and black point of grains (Kumar et al. 2002). This PG is inhibited by both the bean and the wheat PGIP (Clay et al. 1997; Kemp et al. 2003); therefore, the overexpression of PGIP in plant tissue may be a useful tool to counteract this pathogen.

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In order to verify whether the PGIP may protect wheat tissue from PG degradation and colonization by fungal pathogens, we have produced 33 transgenic wheat lines that accumulate the wide-spectrum and very effective PG-inhibitor PvPGIP2 (D'Ovidio et al. 2004) from bean, and analyzed for both their ability to decrease the release of uronides upon PG treatment and their response to *B. sorokiniana*, the causal agent of foliar spot blotch, which causes significant yield losses (Kumar et al. 2002).

RESULTS

Transformation and selection of transgenic plants.

In all, 3,200 *Triticum aestivum* cv. Bobwhite immature embryos were co-transformed in five separate experiments using pUBISR2 (Fig. 1A) and *bar* selectable marker plasmids. Thirty-three T_0 independent transgenic lines resistant to bialaphos and containing the *Pvpgip2* gene were obtained, for a co-transformation efficiency of 1%. The presence of the *Pvpgip2* transgene was verified by polymerase chain reaction (PCR) analysis of genomic DNA from regenerated plants, using the primers 615F/853R to produce an amplicon of 239 bp (data not shown). This PCR assay also was used to follow the inheritance of *Pvpgip2* and to identify progeny that were homozygous for the transgene. No significant differences in morphology and growth were observed between transgenic lines, the corresponding "null" genotype that had lost the transgene by segregation (null segregant), and untransformed plants (*T. aestivum* cv. Bobwhite).

Genomic DNA blots of some of these lines were performed using the complete coding region of *Pvpgip2* as probe, and revealed that multiple copies of pUBISR2 had integrated into the wheat genome of each transgenic line. Cleavage with *Bam*HI produced the expected 1-kb fragment containing the complete coding region of *Pvpgip2* (Fig. 1B), whereas digestion with *Sph*I, which cuts once within the pUBISR2, produced a main hybridizing fragment of 5.7 kb (Fig. 1C), the size of the entire construct. This last result suggests the integration of multiple copies of the constructs in head-to-tail array into the genome of the transgenic lines. We also observed the presence of additional faintly hybridizing fragments that most likely represent different insertion sites, or rearranged copies of pUBISR2, as well as the terminal fragment of the array. As expected, no hybridization signals were present in genomic DNA of nontransformed and null segregant lines. A typical result is shown in Figure 1, which includes the three lines, named J82-23a, J82-200 and MJ7-4a, used for subsequent infection experiments.

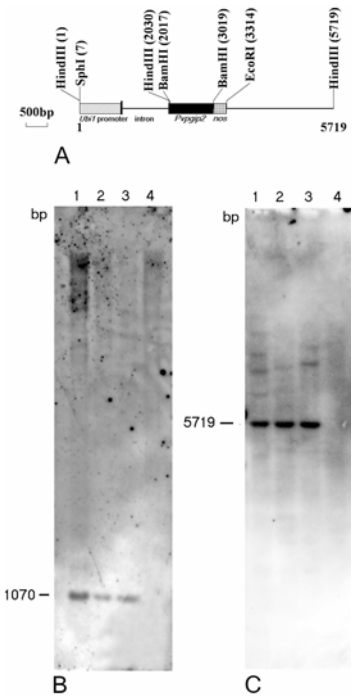

Fig. 1. Schematic representation of the pUBISR2 construct and Southern blots of regenerated transgenic lines. **A,** The pUBISR2 construct was prepared by cloning the bean *Pvpgip2* into the *Bam*HI site of of pAHC17 under control of the maize *Ubiquitin1* promoter and NOS terminator. Genomic DNA of T3 transgenic lines (8 μg) was digested with **B,** *Bam*HI or **C,** *Sph*I and probed with digoxigenin-labeled coding region of *Pvpgip2*. Line 1, J82-23a; line 2, J82-200; line 3, MJ7-4; and line 4, *Triticum aestivum* cv. Bobwhite (nontransgenic control).

Accumulation, activity, and apoplastic localization of PvPGIP2 in transgenic wheat lines.

Leaves from plants harboring the *Pvpgip2* transgene were subjected to crude protein extraction to measure accumulation levels and inhibitory activity of the transgenically expressed PvPGIP2. Western blotting analyses of transgenic and control plants, performed using an antibody raised against the bean PGIP, detected PvPGIP2 only in leaf extracts of transgenic plants. A clear immunodecoration signal, corresponding in size to purified PvPGIP2 used as control, was detected in all regenerated transgenic plants, whereas no signal was detected in crude protein extracts prepared from the corresponding null segregants and untransformed plants. An example of these results is reported in Figure 2A, which includes the three lines used for the infection experiments.

Because the pUBISR2 construct contains the entire coding sequence of *Pvpgip2*, including the signal peptide that targets the encoded protein to the apoplast, we used Western blots to assay extracellular fluids from hypocotyls for the presence of PvPGIP2. Fluid showed no activity of G6PDH, a cytosolic marker, indicating no detectable contamination by intracellular components. Antibodies raised against bean PGIP detected a band with the expected size of PvPGIP2 in apoplastic fluids of transgenic plants (Fig. 2B), confirming that the transgene-encoded protein is secreted in wheat.

The activity of the transgenically expressed PvPGIP2 was tested against a *Fusarium moniliforme* endopolygalacturonase (FmPG) because this enzyme is inhibited by PvPGIP2 (Leckie et al. 1999) but is resistant to the inhibition by the endogenous wheat PGIP (Fig. 3). The analysis performed by a semi-quantitative agarose diffusion assay showed that the crude protein extract of all transgenic lines expressing PvPGIP2 inhibited FmPG, as indicated by the lack of the halo. In contrast, the crude protein extract from the null segregants and untransformed plants did not affect the activity of the enzyme (Fig. 3). Protein extracts that had been boiled exhibited no inhibitory activity in this assay, excluding the possibility of a nonproteinaceous inhibitor (Fig. 3).

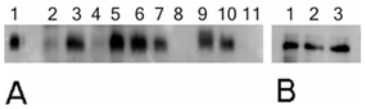

Fig. 2. Western blot of proteins from wheat leaves of regenerated transgenic lines, performed using an antibody raised against the bean polygalacturonase-inhibiting protein (PGIP). **A,** Lane 1, purified PvPGIP2 (50 ng); lanes 2 through 11, crude protein extracts (5 μg) from T1 wheat leaves; lane 2, MJ5-1; lane 3, J82-23; lane 4, MJ1-22c; lane 5, J82-23a; lane 6, J82-200; lane 7, MJ4-16; lane 8, J82-23a null segregant; lane 9, J82-159; lane 10, MJ7-4a; and lane 11, untransformed *Triticum aestivum* cv. Bobwhite. **B,** Extracellular fluids (1 μg) recovered by vacuum infiltration from hypocotyls of transgenic wheat lines: lane 1, J82-23a; lane 2, J82-200; and lane 3, MJ7-4a.

Based on these results, three homozygous transgenic lines were selected for subsequent characterization. Crude protein extracts from leaves of lines J82-23a, J82-200, and MJ7-4a and their corresponding null segregants were subjected to more accurate quantitative and tissue-specific inhibitor activity. The amount of crude protein extract from leaves of these lines needed to inhibit by 50% the FmPG activity—quantified as the increase in reducing end-groups over time—was found to be 300, 400, and 450 ng of crude protein extracts of J82-23a, J82-200, and MJ7-4a, respectively. PvPGIP2 inhibitory activity also was found in extracts from root, crown, stem, spikes, florets, ovaries, and anthers. The widespread distribution is expected because the *Pvpgip2* transgenes are under control of the constitutive Ubiquitin promoter. We also demonstrated that PvPGIP2 activity was stably transmitted to progeny over four generations (data not shown).

Transgenic wheat tissues show increased resistance to PG activity.

To test whether accumulation of PvPGIP2 in wheat resulted in increases in resistance to tissue degradation by the action of fungal PGs, we performed a PG activity assay on sliced wheat stems of transgenics J82-23a, J82-200, and MJ7-4a and their corresponding null segregant lines, following the procedure reported by Favaron and associates (1997). After 10 h of incubation with FmPG or water, the amounts of uronides released into the solution were measured. Tissues from null segregant lines released twice the number of uronides when incubated with FmPG, compared with incubation with buffer (Fig. 4). Tissues from the transgenic plants containing PvPGIP2 released only 17 or 18% more uronides when incubated with FmPG compared with buffer (Fig. 4). In addition, the nontransgenic stems released slightly more uronides in water compared with the transgenic plants. Taken together, these results show that PvPGIP2 accumulated in the transgenic tissue is active in inhibiting FmPG and possibly also interacts with the pectin of wheat tissue.

PG activity of *B. sorokiniana.*

It has been reported that *B. sorokiniana* produced endo-PG activity that is inhibited by PGIP (Clay et al. 1997; Kemp et al. 2003). However, before performing our infection experiments, we tested whether our strain of *B. sorokiniana* produced PG activity and whether this activity was inhibited by purified PvPGIP2 or crude protein extracts from wheat. We examined the production of PG during both the infection of wheat plants at the first leaf emerged stage (Zadoks stage 11, the growth stage used for infection experiments) and in culture conditions. Assays performed with crude protein extracts of infected tissue did not show significant PG activity, likely due to the presence of the endogenous PGIP. Instead, polygalacturonic acid (PGA)-agarose gel overlay performed after isoelectrofocusing separation of protein extracts showed that *B. sorokiniana* produced several PG isoforms during leaf wheat infection. The PG pattern was similar to that obtained by growing the fungus in liquid culture, with at least three corresponding isoforms (Fig. 5). Inhibition was measured by agarose diffusion assays using the crude PG activity produced in liquid culture by *B. sorokiniana*. This activity was completely inhibited by using 50 ng of the purified PvPGIP2 and 3 μg of crude protein extracts from each of the three transgenic lines. In contrast, inhibition obtained with crude protein extracts from null segregant lines or untransformed plants never exceeded 20%, even when 30 μg of crude protein extracts were used (Fig. 6).

Altogether, these results confirm that our strain of *B. sorokiniana* produces PG activity that is inhibited by both purified

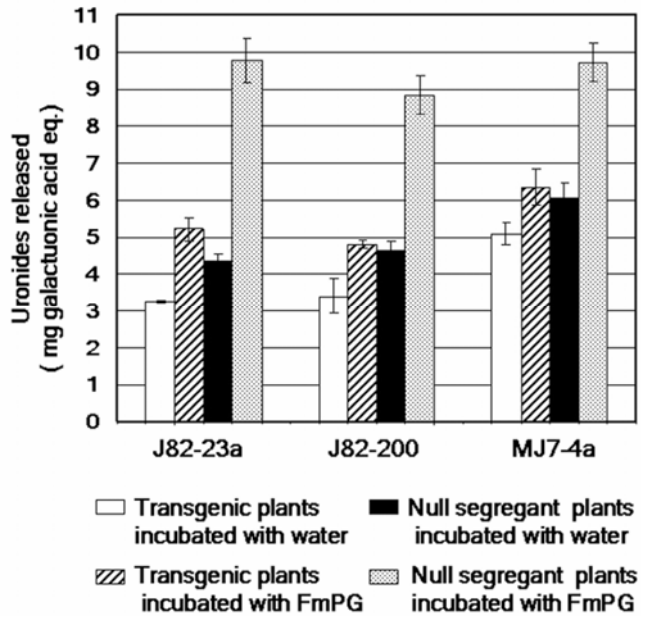

Fig. 4. Uronides released by 2-mm-thick wheat slices of transgenic (white and hatched bars) and corresponding null segregant lines (black and gray bars) in either water (white and black bars) or purified polygalacturonase (PG) from *Fusarium moniliforme* (FmPG) (hatched and gray bars) after 10 h of incubation. Each data point is the mean of three replicates. Bars indicate standard error.

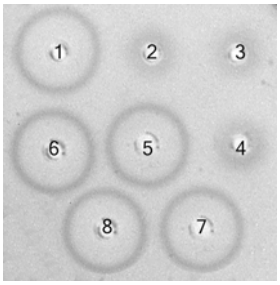

Fig. 3. Agarose diffusion assay of crude protein extract of leaves from transgenic and null segregant plants. The assay was performed using 0.0011 reducing units of *Fusarium moniliforme* endopolygalacturonase (FmPG). 1, Purified FmPG. Purified FmPG plus crude protein extract (1 μg) from 2, J82-23a; 3, J82-200; 4, MJ7-4a; 5, null segregant of J82-23a; 6, null segregant of J82-200; 7, null segregant of MJ7-4a; and 8, boiled J82-23a. The lack of the halo indicates the inhibition of FmPG activity.

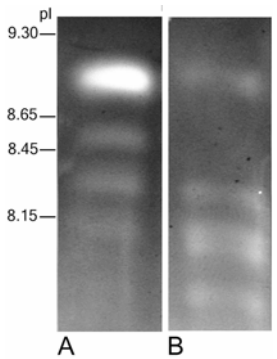

Fig. 5. Polygalacturonic acid-agarose gel overlay assay of polygalacturonases (PGs) of *Bipolaris sorokiniana* obtained from **A,** 3-day-old liquid culture and **B,** wheat tissue harvested 3 days after fungal inoculation. The polyacrylamide gel was loaded with 0.06 reducing units of PG activity from liquid culture or with fresh homogenized infected tissue; pI standards are shown on the left.

PvPGIP2 and crude wheat protein extract. Moreover, this PG activity is much more strongly inhibited by the crude protein extract of the transgenic lines than by that of the corresponding null segregant lines.

Transgenic wheat lines show increased resistance to *B. sorokiniana* infection.

Three independent greenhouse tests for resistance to *B. sorokiniana* were performed on the first emerged leaf of J82-23a, J82-200, and MJ7-4a transgenic and corresponding null segregant lines, using 15 plants for each experiment. Disease symptoms usually appeared 48 h post infection (hpi) and, at 96 hpi, lesions were well formed but not confluent in the transgenic plants, whereas they were confluent in the majority of the null segregant plants. In order to facilitate the analysis of single lesions, we chose the time point at 72 hpi for collecting the data. Disease symptoms were evaluated as the ratio between leaf area showing symptoms and the total leaf area, expressed as percentage. Statistical analysis of the data demonstrated that all lines had a significant reduction in disease symptoms compared with the corresponding null segregant lines (Table 1; Fig. 7). In particular, the J82-23a and J82-200 lines showed a statistically significant reduction in symptom severity manifested in both the mean value calculated for each individual test ($P \leq 0.05$) and the combined data ($P \leq 0.01$); whereas, for line MJ7-4a, the reduction in symptom severity was statistically significant in tests 2 and 3 ($P \leq 0.05$) and in the combined test ($P \leq 0.01$) (Table 1; Fig. 7). By considering the combined results, the transgenic lines J82-23a, J82-200, and MJ7-4a showed symptom reductions of 50, 50, and 46%, respectively (Fig. 7).

In addition, we analyzed the number of lesions and the average area per lesion. Analysis of variance of the combined data tests showed that the transgenic lines have a significant reduction of both the number of infection sites ($P \leq 0.01$) and the average area per lesion ($P \leq 0.05$) (Table 2). In particular, the transgenic lines J82-23a, J82-200, and MJ7-4a showed a reduction in the number of lesions of 35, 26, and 19%, respectively, compared with the null segregant lines. The same transgenic lines showed a reduction in the average lesion area of 56, 53, and 43%, respectively.

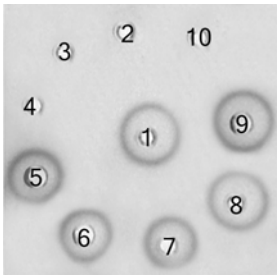

Fig. 6. Agarose diffusion assay of crude protein extract of leaves from transgenic and null segregant plants. The assay was performed using 0.0032 reducing units of crude polygalacturonase (PG) activity produced in liquid culture by *Bipolaris sorokiniana* (BsPG). 1, crude BsPG. Crude BsPG plus crude protein extract (3 µg) from 2, J82-23a; 3, J82-200; 4, MJ7-4a; and 5, boiled J82-23a. Crude protein extract (30 µg) from 6, null segregant of J82-23a; 7, null segregant of J82-200; 8, null segregant of MJ7-4a; and 9, *Triticum aestivum* cv. Bobwhite (nontransgenic control). 10, Purified PvPGIP2 (50 ng). The lack of the halo indicates the inhibition of BsPG activity.

DISCUSSION

Accumulation of the bean protein PvPGIP2 endows transgenic wheat lines with new PG-inhibiting capabilities, and such lines have reduced disease symptoms when challenged with the necrotrophic fungus *B. sorokiniana*. The demonstration that the

Table 2. Mean values and standard errors of infected sites and average area per lesion of transgenic plants (T) and null segregant (NS) lines inoculated with *Bipolaris sorokiniana* macroconidia[a]

Lines	No. of infection sites[b]	Average area per lesion (mm^2)[c]
J82-23a T	22.7 ± 2.3	1.26 ± 0.008
J82-23a NS	35.1 ± 3.7	2.87 ± 0.020
J82-200 T	17.0 ± 2.1	0.70 ± 0.004
J82-200 NS	22.9 ± 2.3	1.50 ± 0.011
MJ7-4a T	20.7 ± 2.2	0.93 ± 0.040
MJ7-4a NS	25.5 ± 1.8	1.63 ± 0.009

[a] Data represent the means of 45 replicates.
[b] Means are significantly different at $P \leq 0.01$.
[c] Means are significantly different at $P \leq 0.05$.

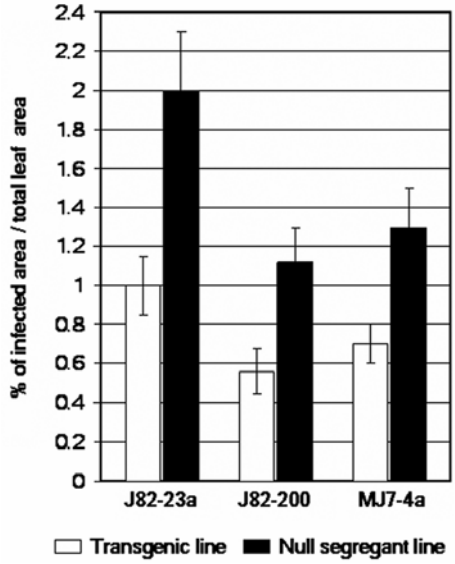

Fig. 7. Symptom spread 72 h after infection with *Bipolaris sorokiniana* for J82-23a, J82-200, and MJ7-4a transgenic (open bars) and corresponding null segregant (filled bars) lines. Bars show averages and standard errors of the area of lesions divided by the total leaf area (cm^2) for 15 plants of each genotype in three independent tests.

Table 1. Mean values and standard errors of symptomatic leaf area of transgenic plants (T) and null segregant (NS) lines inoculated with *Bipolaris sorokiniana* macroconidia[a]

Lines	Test 1	Test 2	Test 3	Combined data tests 1–3[b]
J82-23a T	0.84 ± 0.14*	1.68 ± 0.40*	0.65 ± 0.10*	1.00 ± 0.15**
J82-23a NS	1.43 ± 0.27	3.27 ± 0.78	1.15 ± 0.30	2.00 ± 0.30
J82-200 T	0.99 ± 0.30*	0.47 ± 0.11*	0.28 ± 0.05*	0.56 ± 0.12**
J82-200 NS	2.30 ± 0.48	0.85 ± 0.15	0.50 ± 0.09	1.12 ± 0.18
MJ7-4a T	0.58 ± 0.12[ns]	0.33 ± 0.16*	1.18 ± 0.21*	0.70 ± 0.10**
MJ7-4a NS	0.74 ± 0.27	0.61 ± 0.09	2.41 ± 0.48	1.30 ± 0.20

[a] Symptom severity (lesion expansion area cm^2/total leaf area cm^2) × 100. Data represent the means of 15 replicates; * and ** = means significantly different at $P \leq 0.05$ and 0.01, respectively, and ns = not significantly different.
[b] Values represent the means of the combined data of three tests.

size of fungal lesions are reduced in transgenic tissues provides an indirect evidence that pathogen PGs are important during the infection of *B. sorokiniana* on wheat tissues and that PGIP can regulate the fungal PG activity during the infection process. The effectiveness of PGIP in reducing pathogen symptoms has been reported already in transgenic dicot plant species infected with the necrotrophic fungus *Botrytis cinerea* (Aguero et al. 2005; Ferrari et al. 2003; Joubert et al. 2006; Manfredini et al. 2006; Powell et al. 2000). Here, we provide the first demonstration, to our knowledge, that the presence of a new PGIP can also protect wheat, a monocot plant species possessing a low cell wall pectin content, against fungal colonization. The wheat lines J82-23a, J82-200, and MJ7-4a showed comparable levels of protection of approximately 46 to 50% that are similar to those reported for transgenic tobacco plants expressing *Vitis vinifera* PGIP1 (VvPGIP1) and infected with *B. cinerea* (Joubert et al. 2006). Moreover, the significantly reduced number of lesions and average area per lesion suggest that PGIP might interfere with the pathogen growth during both the initial phase of infection and in the subsequent tissue colonization.

The expression of *Pvpgip2* was under control of the maize Ubiquitin promoter to allow the accumulation of the inhibitor in all tissues. Consequently, PvPGIP2 was easily detected in all tissues analyzed. The protein also was correctly targeted to the apoplast, indicating that its signal peptide, derived from the dicot species *Phaseolus vulgaris*, is correctly recognized in the wheat tissue.

To demonstrate the occurrence of novel PGIP activities in the transgenic wheat tissue, we took advantage of the lack of endogenous wheat PGIP activity against the PG of *F. moniliforme*. The activity assays were particularly useful as preliminary tests on the transgenic wheat tissue before challenging the transgenic lines with pathogens. The significantly increased resistance of the transgenic tissues to FmPG degradation was a consequence of the improved PGIP activity in the transgenic lines J-82-23a, J82-200, and MJ7-4a, in comparison with the corresponding null segregant lines, and provides important evidence for a direct role of PvPGIP2 in limiting tissue degradation by fungal PGs.

Moreover, the reduced release of uronides in the water-treated transgenic plants may be due to the interaction of PvPGIP2 and pectin components and supports the hypothesis that PvPGIP2 interacts with the pectin component of the cell wall (Spadoni et al. 2006) and contributes to the hypothesis that the specific interaction of PGIP with polygalacturonic acid may be strategic to protect pectins from the degrading activity of PGs (Spadoni et al. 2006). However, the possibility that the transgenically expressed PGIP can induce changes in cell wall architecture that make it less accessible to PG activity cannot be ruled out.

The ability of wheat PGIP to inhibit an endo-PG isoform produced in culture conditions by *B. sorokiniana* was reported previously by Kemps and associates (2003). We extended this observation by showing that PvPGIP2 also inhibits the PG activity produced in vitro by *Bipolaris sorokiniana*. Although the expression pattern of PG isoforms in some fungi can vary, depending on culture conditions or host tissue (ten Have et al. 2001), we showed that the most abundant *B. sorokiniana* PG isoform produced in culture conditions and inhibited by PvPGIP2 also was secreted in vivo on infected wheat tissue. As a consequence of the combined presence of both endogenous PGIP and PvPGIP2, the inhibitory activity of crude protein extracts from leaves of the transgenic wheat lines was more that 40 times greater than that of the null segregant plants. This increased PGIP activity could be responsible for the significant levels of protection that the transgenic plants showed when challenged with *B. sorokiniana.*

The occurrence of PG isoforms not inhibited by PGIP or the action of other cell-wall-degrading enzymes can explain the incomplete protection of the transgenic tissue. *B. sorokiniana* produces different PG isoforms during the infection of wheat tissue; however, apart from the effectiveness against the endo-PG expressed in vitro and in vivo, we do not know the inhibitory efficiency of PvPGIP2 or of the endogenous PGIP on the different PG isoforms of *B. sorokiniana* produced in vivo. Several other factors could explain the incomplete protection, including an inadequate accumulation of PvPGIP2 in the apoplast of the transgenic tissue or the loss of effectiveness of the inhibitor during different phases of infection. For example, it has been reported that a PG isoform of *Sclerotinia sclerotiorum* can escape PGIP inhibition at pH 3.6, most probably because the PGIP is inactive at this pH (Favaron et al. 2004).

In conclusion, the effectiveness of PGIP to protect wheat is important evidence of the wide range of effectiveness of this inhibitor and an additional demonstration that the improvement of pre-existing defense mechanisms is a feasible strategy for crop protection.

MATERIALS AND METHODS

Generation and selection of transgenic plants.

To assemble the monocot expression vector pUBISR2, the coding region of *Pvpgip2* (Lekie et al. 1999), including the N-terminal signal sequence, was inserted into the *Bam*HI site of pAHC17 under control of the maize *Ubiquitin1* promoter and napoline synthase terminator (Christensen and Quail 1996). The *Bam*HI sites flanking the complete coding region of *Pvpgip2* were generated by PCR amplification using the forward and reverse primers (PvPGIP2BF) 5′-CAGGATCCATGT CCTCAAGCTTAAGCAT-3′ and (PvPGIP2BR) 5′-TTGGATC CTTAAGTGCAGGCAGGAAGA-3′, respectively. PCR reactions were performed in a reaction volume of 50 µl with 10 ng of plasmid DNA (pPvpgip2), 2.5 units of FastStart High Fidelity PCR system (Roche Diagnostics, Monza, Italy), 1× *Taq* PCR buffer, 50 ng of each of the two primers, and 100 µM each deoxyribonucleotide. Amplification conditions were 1 cycle at 95°C for 2 min; 30 cycles at 95°C for 30 s, 60 °°C for 1 min, and 72°C for 1 min; and a final step at 72°C for 5 min. After *Bam*HI digestion, the amplicon was inserted into the *Bam*HI site of pAHC17 generating a 5,719-bp plasmid, pUBISR2. The correct coding sequence of *Pvpgip2* and the insertion sites were confirmed by nucleic acid sequencing.

For the transformation experiments, the plasmid pAHC20 (Christensen and Quail 1996), carrying the *bar* gene that confers resistance to the bialaphos herbicide, was co-bombarded with pUBSR2 in a 1:3 molar ratio. Constructs were introduced into immature embryos of *T. aestivum* cv. Bobwhite excised from plants in Zadoks stage 72, using a Model PDS-1000/He Biolistic particle delivery system (Bio-Rad, Hercules, CA, U.S.A.) and the protocols described in Okubara and associates (2002).

The presence of *Pvpgip2* in bialaphos-resistant plants and their progeny was verified by PCR using total DNA obtained from leaf sections of mature plants (Tai and Tanksley 1991). Control DNAs included untransformed *T. aestivum* cv. Bobwhite (negative) and pUBISR2 (positive). DNA was amplified using oligonucleotides designed for the specific sequence of *Pvpgip2*, having the following sequences: (615F) 5′-CCTCAC CGGGAAGATTCCG-3′ and (853R) 5′-TTAGCTGCGTCAGT CCCTGC-3′. Amplifications were carried out according with the procedures specified for Ready Mix RedTaq polymerase (Sigma-Aldrich, Milan, Italy) at an annealing temperature of 60°C.

To identify homozygotes, a minimum of eight progeny were sampled from each of two successive generations and tested

for amplification of *Pvpgip2*. Homozygotes from a number of lines were identified and three of the lines, J82-23a, J82-200, and MJ7-4a, were characterized further.

Southern blot analysis.

Genomic DNA was extracted from 0.1 or 5 g of green material following the procedure reported by Tai and Tanksley (1991) or D'Ovidio and associates (1992), respectively.

Genomic DNA (8 to 10 μg) was cleaved with *Sph*I or *Bam*HI restriction enzyme and separated on 1.2% agarose gel, transferred to a positively charged nylon membrane (Roche Diagnostics) as described by Sambrook and associates (1989), and hybridized with the coding region of *Pvpgip2* labeled with digoxigenin (digoxigenin-11-uridine-5′-triphosphate; Roche Diagnostics) following the procedure reported by D'Ovidio and Anderson (1994).

Fungal PGs, PGIP extraction, and enzymatic assays.

Fungal growth and endo-PG preparations were performed as previously described by Caprari and associates (1996) for *F. moniliforme* strain FC10. *B. sorokiniana* strain DSMZ 62608 was grown at 20°C on potato dextrose agar (PDA) (Sigma-Aldrich) and crude PG preparation was performed as follows. Five mycelium discs (4 mm in diameter) of *B. sorokiniana* were taken from the edge of actively growing colonies (4 days old) and placed in 250-ml Erlenmeyer flasks containing 100 ml of liquid culture medium (0.5% [wt/vol] $NaNO_3$, 0.1% [wt/vol] K_2HPO_4, 0.05% [wt/vol] $MgSO_3$, ferric ammonium citrate at 1 mg liter^{-1}, thiamine at 0.1 mg liter^{-1}, biotin at 0.01 mg liter^{-1}, and 1% citrus pectin [wt/vol]). After 3 days of culture in a rotary shaker at 110 rpm and 23°C, the contents of several flasks were pooled, filtered through Whatman (Brentford, U.K.) GF/A paper, dialyzed overnight at 4°C against 0.1 M sodium acetate buffer (pH 4.0), and concentrated 25-fold by a Minitan system (Millipore, Vimondrone, Milan, Italy) equipped with a polysulfonic membrane (PTGC, cut-off = 10 kDA). Activity and isoform patterns of crude PGs were determined as reported below.

Crude protein extracts containing PGIP activity were obtained from leaves of transgenic plants as previously described (D'Ovidio et al. 2004). Extracellular fluid was extracted from hypocotyls (Zadoks stage 47) by vacuum-infiltration with acetate buffer as described by Salvi and associates (1990). The extracellular fluid had negligible glucose-6-phosphate dehydrogenase activity, verifying that it was free of cytoplasmic contamination. Protein concentrations were determined with the "Coomassie plus-the better Bradford" assay kit (Pierce, Rockford, IL, U.S.A.). Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting were performed as previously described (Desiderio et al. 1997). Polyclonal antibodies raised in rabbit against PGIP purified from *P. vulgaris* pods were used for immunoblotting experiments. Purified PvPGIP2 was obtained as reported by D'Ovidio and associates (2004).

Enzymatic activity of PGs and inhibitory activity of PGIP were evaluated using both an agarose diffusion assay (Taylor and Secor 1988) in the presence of 20 mM Na acetate, pH 4.7, and by measuring the release of reducing end-groups from Na polygalacturonate (Milner and Avigad 1967). In the latter method, the incubation mixture contained 200 μl of 0.5% PGA from orange (Sigma-Aldrich) in 100 mM sodium acetate, pH 4.7, and bovine serum albumen at 0.1 mg ml^{-1}. After addition of the enzyme or crude protein extract, the mixture was incubated at 30°C for 1 h. PG activity was expressed as reducing units (RU). One RU was defined as the amount of enzyme required to release reducing groups at 1 μmol min^{-1} using D-galacturonic acid as standard.

The same mixture containing 0.0011 RU was used to assess the inhibitory activity of PvPGIP2 or crude protein extracts. One unit of PGIP activity was defined as the amount of inhibitor required to reduce PG activity by 50%.

Gel detection of PG activity of *B. sorokiniana*.

Infected leaf tissue was obtained as described below. At 72 h after inoculation, leaf segments displaying disease symptoms were cut with a razor blade, collected, frozen in liquid nitrogen, and stored at –80°C.

Detection of PG activity from infected leaves was performed after extracting leaf segments from 10 wheat leaves collected as reported above. Tissue was ground in 500 μl of water in a mortar with pestle, and approximately 50 μl of the extract together with tissue debris were placed directly upon the surface of 0.8-mm-thick polyacrylamide (PAA) gel containing 3.3% (vol/vol) carrier ampholytes (Amersham Biosciences, Uppsala, Sweden) covering the pH range 6 to 10.5. PG isoforms were detected by the agarose overlay technique (PGA-agarose gel overlay) described by Ried and Collmer (1985), blotting the gels for 45 min. Crude PG from liquid culture was also assayed with the same method.

PG activity on wheat tissue.

The PG activity of *F. moniliforme* on wheat tissue was determined by using a slightly modified procedure previously reported by Favaron and associates (1997). Slices (2 mm thick) were cut transversely from the middle region of stems of transgenic and null segregant plants. Slices were washed repeatedly with distilled water for 20 min, then briefly dried on filter paper and immersed in 2.0-ml conical Eppendorf vials (three slices per vial, approximately 30 mg fresh weight) containing 0.25 ml of 30 mM sodium acetate buffer (pH 4.6) and 0.5 RU of PG from *F. moniliforme*. After 10 h of incubation at 30°C on a platform shaker (100 strokes min^{-1}), 80 μl of reaction mixture was taken from each sample and the uronides released were measured by the m-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973) using D-galacturonic acid as a standard. Controls were performed by adding water in place of the enzyme. Experiments were carried out in triplicate.

Greenhouse testing.

Seed were surface sterilized by immersion in sodium hypochlorite (0.5% vol/vol) for 30 min, and then rinsed thoroughly in sterile water. Plants were grown in single pots at 18 to 23°C with a 14-h light period. Macroconidia were produced by culturing *B. sorokiniana* strain 62608 DSMZ on petri dishes containing PDA medium. Macroconidia were harvested by washing the culture surface with 3 ml of sterile water, and conidia concentration was estimated by a Thoma chamber (Tecnochimica Moderna, Monterotondo, Rome, Italy), adjusting the concentration to 3×10^5 conidia/ml. Tween 20 was added to a final concentration of 0.05%.

The upper surface of each leaf of T4 transgenic and control wheat plants in the first-leaf-emerged stage (Zadoks stage 11) was inoculated with 20 μl of conidia suspension. Plots were covered with plastic film and sprayed with water for the duration of the experiment in order to maintain approximately 100% relative humidity. Lesion numbers and sizes were determined 72 hpi by scanning the leaf surface and calculating the square centimeters of the infected area by using the Adobe Photoshop program (Microsoft, Segrate, Italy). The average area per lesion was calculated as weighted arithmetic mean. Data were analyzed statistically applying the student's *t* test and analysis of variance with general linear model (GLM) using Systat 12 (Systat Software Inc., CA, U.S.A.).

Each experiment included 15 plants of each of three transformed and corresponding null segregant lines. Three independent replicated experiments were performed.

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