Suppression of *Fusarium oxysporum* with recombinant polygalacturonase inhibiting proteins (BvPGIPs) extracted from sugar beet roots

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Abstract

Soil-borne fungus *Fusarium oxysporum* f. sp. *betae* (Fob) is the causative agent of Fusarium yellows in sugar beet. Leaf interveinal yellowing and root vascular discoloration significantly reduce root yield as well as sucrose content and juice purity. Fob, like other fungal pathogens, initiates disease development by secreting polygalacturonase (PG) enzymes to break down plant cell walls during early stages of infection. To protect themselves, plants produce polygalacturonase-inhibiting proteins (PGIPs). In our study of sugar beet root defense responses, several PGIP genes (*BvPGIPs*) were identified. To determine if *BvPGIPs* inhibit Fob PGs, genes *BvPGIP1*, *BvPGIP2* and *Bv(FC607)PGIP1* were fused with the CaMV 35S promoter and each was expressed individually in sugar beet hairy roots. We demonstrate that all three recombinant BvPGIP proteins inhibited Fob and *F. oxysporum* f. sp. *gladioli* (Fog) PGs. A comparable level of BvPGIP activity was observed against Fob PGs, while BvPGIP2 showed higher activity against Fog PGs. Similar results were obtained when recombinant PGIPs were used to bioassay effects on Fob and Fog spore germination and hyphal growth. This is a first report that documents *F. oxysporum* inhibition by overexpressing *BvPGIPs* that may lead to improved Fusarium yellows resistance in sugar beet.

Keywords PGIP · Sugar beet root · Fusarium yellows · *Fusarium oxysporum*

Abbreviations

PGIP	Polygalacturonase-inhibiting protein
PG	Polygalacturonase
Fob	<i>Fusarium oxysporum</i> f. sp. <i>betae</i>
Fog	<i>Fusarium oxysporum</i> f. sp. <i>gladioli</i>
<i>BvPGIP</i>	Sugar beet PGIP gene
CaMV 35S	Cauliflower mosaic virus promotor
LRR	Leucine-rich repeat
<i>hptII</i>	Hygromycin phosphotransferase II
Hyg	Hygromycin
kb	Kilobase
PDA	Potato dextrose agar

PDB	Potato dextrose broth
NBT/BCIP	Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate
AP	Alkaline phosphatase
kDa	Kilodalton
PVDF	Polyvinylidene difluoride

Introduction

The soil-borne fungus *Fusarium oxysporum* can cause vascular wilt or root rot on a wide range of plants (Kroes et al. 1998; Olivain and Alabouvette 1999). *F. oxysporum* f. sp. *betae* (Fob) is the causative agent of Fusarium yellows disease in sugar beet (*Beta vulgaris* L.). Fusarium yellows symptoms are characterized by interveinal yellowing on the leaves and vascular discoloration of the root (Hanson and Jacobsen 2009; Harveson 2009). Fusarium yellows significantly reduces root yield as well as sucrose content and juice purity. The disease has been prevalent in western United States for many years and current control measures rely on genetic resistance (Hanson and Jacobsen 2009; Windels et al. 2005). However, resistant cultivars when grown

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in different areas of the country did not provide consistent control to different Fob isolates found in those areas (Hanson et al. 2009, 2018).

In recent years, identifying and applying defense genes for molecular plant breeding has become an effective and environmentally safer approach for improving plant disease resistance. Polygalacturonase-inhibiting proteins (PGIPs) are plant cell wall, leucine-rich repeat (LRR) proteins that have proven to be effective in inhibiting fungal disease development (Kalunke et al. 2015). Using PGIPs for crop protection is considered environmentally safe since they are naturally present in most plants. The role of PGIPs in plant defense has been well documented in heterologous plants but is limited in homologous plants (Gomathi and Gnanamanickam 2004; Kalunke et al. 2015). In rice, over-expression of the endogenous rice *OsPGIP1* gene enhanced resistance to sheath blight (Wang et al. 2015; Chen et al. 2016).

Like other plant PGIPs, the sugar beet PGIPs (BvPGIPs) are encoded by gene families and at least two *BvPGIPs* were isolated from each of the breeding lines F1016, F1010 and FC607 (Li and Smigocki 2016). Over-expressing *BvPGIPs* in *Nicotiana* plants increased resistance to fungal pathogens *Rhizoctonia solani*, *F. solani* and *Botrytis cinerea* (Li and Smigocki 2018). The objective of this study was to determine the role of BvPGIPs in resistance to sugar beet pathogens, such as Fob. Since there is still no reliable, rapid method for whole plant regeneration of sugar beet plants from transgenic in vitro cultures, we employed the sugar beet hairy root system as a rapid approach (Smigocki et al. 2009) to obtain transformed sugar beet tissues that express the introduced *BvPGIPs*. Three highly homologous *BvPGIP* genes, *BvPGIP1* from breeding line F1016, *BvPGIP2* from F1010 and F1016 and *Bv(FC607)PGIP1* from FC607 (Li and Smigocki 2016, 2018), were engineered for over-expression in F1010 roots with no reported resistance to Fob. Inhibitory activities of the recombinant BvPGIPs were tested against fungal PGs purified from isolates of Fob and *F. oxysporum* f. sp. *gladioli* (Fog), a soil-borne fungus that causes Corm rot and yellows in gladiolus (Partridge 2003). The inhibition of Fob and Fog spore germination and hyphal growth by BvPGIPs was also analyzed.

Sugar beet germplasm F1010 (Cambell 1990) was used in this study. F1010 seeds were germinated and grown in a growth chamber at 25 °C day and 18–20 °C night temperatures with a day length of 16 h and 8 h dark photoperiod. Two Fob isolates, F19 and Fob220a, that are highly virulent on sugar beet, and Fog, not a pathogen of sugar beet, were used in this study (Partridge 2003; Hanson and Hill 2004; Windels et al. 2005; Hanson et al. 2009). All isolates were maintained on potato dextrose agar (PDA) (BD Difco™, USA).

The *BvPGIP1*, *BvPGIP2* and *Bv(FC607)PGIP1* genes were fused to the constitutive CaMV 35S promoter and each

construct was introduced into a plant transformation vector carrying the *hptII* selectable marker gene, as previously reported (Li and Smigocki 2018). The BvPGIP gene constructs were used to transform F1010 petioles using *Agrobacterium rhizogenes*-mediated transformation (Smigocki et al. 2009). Roots that regenerated on the petioles were excised and propagated as independent hairy root lines on half-strength B5 medium (PhytoTechnology Laboratories, Overland Park, KS) supplemented with 5 mg Hyg/l to select for hygromycin resistant transformed cells.

BvPGIP gene expression in transgenic hairy roots was analyzed by RT-PCR analysis using gene specific primers (Li and Smigocki 2016). Transcripts of the constitutively expressed actin gene were utilized as an internal control to normalize the RT-PCR results. Gene expression was quantified by densitometry with an AlphaImager HP (Alpha Innotech, San Leandro, CA). RT-PCR analyses were repeated two times with comparable results.

For Southern blot analysis, approximately 10 µg of genomic DNA was digested with restriction enzymes *XbaI/BamHI* or *NheI* (New England BioLabs Inc., Ipswich, MA) that have no recognition sites within the *BvPGIP* gene sequences, separated on agarose gels and transferred to nylon membranes. Full-length cDNA sequence of each gene was used as a probe. Hybridization signals were detected using alkaline phosphatase and DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Branchburg, NJ). Membranes were incubated in NBT/BCIP solution (nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt, 1:50 dilution) (Sigma, St. Louis, MO) for 2 h at 30 °C and photographed.

PGIP protein extracts were prepared from 10 g of hairy root tissues, homogenized in 100 ml of 20 mM Na-acetate buffer containing 1 M NaCl (pH 4.7), and purified with Amicon Ultra 15 filtration units (EMD Millipore, Billerica, USA) (Li and Smigocki 2016). Production of PG proteins in Fob and Fog strains was induced with 1% pectin (Berger et al. 2000). About 10⁵ spores harvested with sterile water were inoculated into a flask with 200 ml of potato dextrose broth (PDB), supplemented with 1% pectin. After a 3-day culture in a rotary shaker at 150 rpm, mycelia were removed by centrifugation at 10,000×g for 10 min. The supernatant was purified and concentrated using Amicon filtration units as described above. Protein concentration was determined using the Bradford method and ND-8000 spectrophotometer (NanoDrop Technologies Inc., DE, USA).

For western blot analysis, concentrated PGIP protein extracts (20 µg total) were electrophoresed on 12% Tris-Glycine mini gels (XCell SureLock Mini-Cell, Invitrogen, Carlsbad, CA). Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes using the XCell II Blot Module apparatus (Invitrogen) for 1 h 30 min. Membrane blotting and detection of primary antibody were

carried out using the WesternBreeze Chromogenic Immunodetection Kit (Invitrogen). Briefly, transferred membranes were incubated with rabbit polyclonal antibody against BvPGIP1 peptide (CVQITEAQDIYGIP) (GenScript Corporation, Piscataway, NJ) at 1:2000 dilution for 1 h and then incubated for 30 min with alkaline phosphatase conjugated secondary antibody (Goat Anti-Rabbit IgG). Alkaline phosphatase was detected with Chromogenic NBT/BCIP Substrate until purple bands developed on the membrane. The relative levels of the accumulated BvPGIP proteins were estimated by densitometry with an AlphaImager HP.

The inhibitory activity of the extracted BvPGIPs against fungal PGs was determined by agarose diffusion plate assay (Taylor and Secor 1988). BvPGIP activity was determined by calculating the reduction in size of the halo produced when one unit of PG was reacted with 15 µg of PGIPs isolated from the transgenic hairy lines as compared to the halo produced by the empty vector control line. One PG unit represents a halo of 5 mm radius produced after overnight incubation at 30 °C with 0.5% polygalacturonic acid substrate (Sigma, St. Louis, MO). Inactivated PGIPs (boiled at 100 °C for 5 min) mixed with F19 PG were included as a negative control. Each assay was conducted in triplicate and repeated two times. The size of halo is presented as mean $\pm$ standard deviation (SD) (Microsoft Excel program).

To test the inhibitory activity of recombinant BvPGIP on fungal spore germination, 10^3 spores harvested in sterile water were added to 15 µg of PGIP protein extract (20 µl total volume). The spore-PGIP mix was incubated at 30 °C in the dark for 0, 3, 24 and 48 h and then plated on PDA media for 24 h. Spore germination frequency from each incubation time point was photographed with a Zeiss SterEO Discovery microscope ($\times 20$) (Zeiss, SterEO Discovery, San Diego, CA). Spores that were treated with PGIP extracts for 24 h were used to determine PGIP activity on hyphal growth at 48 h after plating spores on PDA media. The recombinant BvPGIP activity was determined by the percentage of reduction of colony size relative to fungal growth of spores treated with PGIP extracts from hairy roots transformed with the empty vector that represents the background level of PGIP activity. Each experiment was repeated two times. Size of colony is presented as mean $\pm$ SD (Microsoft Excel program).

In this study, three genes that encode BvPGIP1, BvPGIP2 and Bv(FC607)PGIP1 were fused to the constitutive CaMV 35S promoter and were over-expressed separately in hairy roots of the sugar beet breeding line, F1010. At least three independently derived hairy root lines were regenerated from each BvPGIP transformed F1010 petioles. Southern blot analysis of BvPGIP1 (3-1 and 3-3), BvPGIP2 (7-2 and 7-3) and Bv(FC607)PGIP1 (1-3 and 1-5) hairy root lines revealed that 1–2 copies of each BvPGIP gene were integrated into F1010 genome (Fig. 1a). Since the sequences

of BvPGIP1, BvPGIP2 and Bv(FC607)PGIP1 are 97–98% identical, cross-hybridization is highly likely between the introduced transgene and the endogenous BvPGIP2 gene found in the F1010 background (Li and Smigocki 2016). The single hybridization signal generated with the *NheI* restriction enzyme in line BvPGIP1 3-1 likely represents two similar or identical size bands since two distinct size bands were detected when the DNA was digested with *XbaI/BamHI* restriction enzymes. A single hybridization band was detected in all empty vector control lines (C).

RT-PCR analysis revealed elevated BvPGIP transcript levels in all transgenic BvPGIP1, BvPGIP2 and Bv(FC607)PGIP1 lines (Fig. 1b). Relatively higher BvPGIP2 transcript levels were observed in lines 7-2 and 7-3 as compared to the control (C) that, as expected, showed low endogenous transcript levels in the F1010 background. No PGIP transcripts or very weak bands were detected in the BvPGIP1 or Bv(FC607)PGIP1 controls.

Western blot analysis using BvPGIP specific polyclonal antibody confirmed the presence of the recombinant PGIP proteins in transgenic hairy root lines (Fig. 1c). Proteins of about 42 kDa cross-reacted with the BvPGIP antibody in all samples tested, with consistently higher levels of PGIP proteins observed in the transgenic BvPGIP lines as compared to the controls. The two weak bands in the BvPGIP1 and Bv(FC607)PGIP1 control lines could be non-specific because these two genes were highly homologous with the endogenous BvPGIP2 gene in the controls. A weaker protein band corresponding to 36–40 kDa was observed in BvPGIP1 lines 3-1 and 3-3 and may represent a modified or partially degraded form of the 42 kDa PGIP protein, or some non-specific bands.

The activity of the recombinant BvPGIP proteins for Fob and Fog PGs was demonstrated using agarose diffusion assays (Fig. 2a, b). Protein extracts prepared from BvPGIP1 (3-1, 3-3), BvPGIP2 (7-2, 7-3) and Bv(FC607)PGIP1 (1-3, 1-5) lines inhibited the Fob and Fog PGs. Highest PGIP activity was observed against Fog PGs (45–87% reduction of halo) followed by Fob isolate F19 at 31–46% and Fob220a at 28–42% reduction, as compared to the activities of the endogenous BvPGIP2 in the empty vector control line (Fig. 2b). The higher activity against Fog PGs may be associated with the fact that Fog is not a pathogen of sugar beet and thus would be less likely to develop resistance to sugar beet PGIPs. BvPGIP1, BvPGIP2 and Bv(FC607)PGIP1 activities against Fob F19 or Fob220a PGs were similar, i.e. a reduction of the halo by 28–46% (Fig. 2b). However, BvPGIP2 protein extracts (line 7-2, 7-3) showed higher activity (80–87% reduction of halo) for Fog PGs than the BvPGIP1 and Bv(FC607)PGIP1 extracts (45–55% reduction). No inhibition of F19 PGs was observed when PGIP extracts were heat inactivated prior to mixing with the PGs (last row; Fig. 2a).

Fig. 1 *BvPGIP* gene expression in transformed sugar beet hairy roots. **a** Southern blot analysis of independently derived hairy root lines of *BvPGIP1* (3-1, 3-3), *BvPGIP2* (7-2, 7-3) and *Bv(FC607)PGIP1* (1-3, 1-5). Genomic DNA (10 µg) was digested with three restriction enzymes (*Xba*I, *Bam*HI and *Nhe*I) that do not cut within *BvPGIPs* and probed with the full-length cDNA fragment of each gene. C represents the control hairy roots transformed with the empty vector that has no *PGIP* gene inserted in the vector. **b** *BvPGIP* transcription in hairy root lines. RT-PCR was performed with total RNA using gene-specific primers that amplify about 1.0 kb of the coding region of each transgene. The sugar beet *actin* gene was used as an internal control. **c** Detection of recombinant *BvPGIP* proteins in hairy root lines. Western blot analysis was performed by separating *PGIP* proteins (20 µg) on 12% Tris–Glycine gels that were then transferred to PVDF membranes. Membranes were incubated with anti-*BvPGIP1* antibody followed by alkaline phosphatase (AP) conjugated secondary antibody (Goat anti-Rabbit IgG)

Recombinant *BvPGIP* protein extracts were also tested for their effect on fungal spore germination. When *BvPGIP1* 3-1 protein extract was mixed with *Fog* spores, a large reduction in the number of germinated spores was observed at 3 and 24 h after incubation (Fig. 3a, a2 and a3, respectively). An almost complete inhibition of spore germination was observed after 48 h (Fig. 3a, a4). Replacing *PGIP* extract with water or heat denatured protein extract had no effect on *Fog* spore germination (data not shown; Fig. 3a, a5, respectively).

Viability of *Fob* and *Fog* spores was determined after they were treated with each of the three *PGIP* extracts for 24 h. A significant reduction of fungal growth was observed for all spores treated with the *PGIP* extracts (Fig. 3b, c). *Fog* colony sizes were reduced by 40–80% when they were treated with one of the three extracts, followed by 40–62% reduction of

F19 and 27–41% reduction of *Fob220* colonies. As observed with the *Fog* *PG* extracts (Fig. 2), the *Fog* isolate was most strongly inhibited by the *BvPGIP2* extract showing a 70–80% colony size reduction, followed by *BvPGIP1* with 55–60% and *Bv(FC607)PGIP1* with 40–44% reduction. The vector control extracts showed a 17, 24, and 33% reduced colony sizes for F19, *Fob220a* and *Fog*, respectively as compared to *PG* control with no *PGIP* extract added.

Because *BvPGIP2* is the endogenous gene found in the F1010 genome that was used for the transformation experiments and as the vector control, we conclude that over-expressing *BvPGIP2* in F1010 hairy root lines 7-2 and 7-3 did not trigger the onset of gene co-suppression which would lead to reduced *BvPGIP2* gene activity. We speculate that the enhanced activity of the *BvPGIP2* protein extracts against *Fog* *PGs* may be due in part to the eight amino acid

Fig. 2 BvPGIP inhibition of fungal PGs. **a** Agarose diffusion assays showing PGIP inhibition of fungal PGs. PGIP extracts (15 μ g) from *BvPGIP1* (3-1, 3-3), *BvPGIP2* (7-2, 7-3) and *Bv(FC607)PGIP1* (1-3, 1-5) lines were each mixed with one unit of fungal PGs from isolates Fob F19, Fob220a and Fog. PG only represents one unit of PG that produces a halo of 5 mm radius after overnight incubation at 30 °C.

Heat inactivated PGIP extracts (100 °C for 5 min) were mixed with F19 PG as negative controls. **b** Comparison of halo sizes that represent the inhibitory activity of BvPGIPs against PGs of F19, Fob220a and Fog. Smaller halo indicates higher PGIP activity. Values are given as mean $\pm$ standard deviation (SD)

substitutions found between BvPGIP1 and BvPGIP2 (Li and Smigocki 2016). Of the eight amino acids, four (T/A, G/R, M/I, N/D) are located within the xxLxLxx motif, where the PGIP-PG recognition occurs (Desiderio et al. 1997). Therefore, the stronger recognition capacity between BvPGIP2 and Fog PGs most likely may involve one or more of these four amino acids.

In conclusion, this is a first report documenting the inhibitory activity of sugar beet PGIPs on Fog and Fob pathogens, causative agents of Corm rot and yellows in gladiolus and

Fusarium yellows in sugar beet, respectively. The recombinant BvPGIP1, BvPGIP2 and Bv(FC607)PGIP1 proteins over-expressed in sugar beet hairy roots inhibited Fog and Fob PGs, spore germination and hyphal growth, showing highest activity against Fog PGs. All three BvPGIP extracts showed similar level of activity against Fob F19 and Fob220a, while BvPGIP2 showed higher inhibition of Fog. Reports of PGIP inhibition of *F. oxysporum* PGs are limited. Only one study has shown that the activity of PGs isolated from *F. oxysporum* f.sp. *lycopersici* was partially inhibited

Fig. 3 BvPGIP inhibition of fungal growth. **a** Inhibition of spore germination. a1 No PGIP extract added to Fog spores. a2–a4 Fog spores were mixed with *BvPGIP1* 3-1 PGIP extract and incubated at 30 °C in the dark for 3 h, 24 h, and 48 h, respectively. a5 Fog spores were incubated with heat inactivated PGIP extracts for 24 h. Photos were taken with a Zeiss SteREO Discovery microscope ($\times 20$) at 24 h after plating the PGIP-spore mix on PDA media. Bar=1 mm. **b** Hyphal growth from F19, Fob220a and Fog spores treated with *BvPGIP1*

(3-1, 3-3), *BvPGIP2* (7-2, 7-3) and *Bv(FC607)PGIP1* (1-3, 1-5) extracts. No PGIP refers to spores not treated with recombinant PGIP extract. Heat inactivated PGIP extracts were mixed with F19 spores as negative controls. **c** Comparison of colony sizes that represent the inhibitory activities of recombinant PGIPs against growth of F19, Fob220a and Fog. Smaller size of colony indicates higher PGIP activity. Values are given as mean $\pm$ standard deviation (SD)

by the bean PvPGIP1, however, PvPGIP1 over-expression in tomato did not increase fungal resistance (Desiderio et al. 1997). Our findings demonstrate a relatively broad inhibitory capacity of recombinant sugar beet BvPGIPs against three different *F. oxysporum* isolates using transgenic sugar beet root lines. In addition, this study provides the basis for gaining a better understanding of the molecular mechanisms of sugar beet and *F. oxysporum* interaction and opens new strategies for controlling fungal diseases in sugar beet and other crops.

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Author contributions HL and ACS designed the experiments; HL performed the experiments, analyzed the data and wrote the manuscript; ACS conceived and directed the research, analyzed and interpreted the data and wrote and edited the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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