TÜBİTAK

Turkish Journal of Agriculture and Forestry

http://journals.tubitak.gov.tr/agriculture/

Research Article

Turk J Agric For
(2015) 39: 429-438

doi:10.3906/tar-1406-19

Generation of transgenic sugar beet (*Beta vulgarism* L.) overexpressing the polygalacturonase inhibiting protein 1 of *Phaseolus vulgaris* (PvPGIP1) through *Agrobacterium*-mediated transformation

Reza MOHAMMADZADEH[1,2], Mostafa MOTALLEBI[2,*], Mohammadreza ZAMANI[2], Zahra MOGHADDASSI JAHROMI[2], Peyman NOROUZI[3], Manuel BENEDETTI[4], Gilulia DE LORENZO[4]

[1]Department of Cell and Molecular Biology, Faculty of Science, Maragheh University, Maragheh, Iran

[2]National Institute of Genetic Engineering and Biotechnology, Tehran, Iran

[3]Sugar Beet Seed Institute, Karaj Iran

[4]Pasteur Fondazione Cenci Bolognetti Institute, "C. Darwin" Department of Biology and Biotechnology, Sapienza University of Rome, Rome, Italy

Received: 03.06.2014 • **Accepted:** 14.03.2015 • **Published Online:** 12.06.2015 • **Printed:** 30.06.2015

Abstract: Numerous diseases caused by fungal pathogens influence the annual production of sugar beet. In order to obtain a plant resistant to fungi, genetic transformation has been applied to the sugar beet. To invade a plant tissue, phytopathogenic fungi produce several cell wall-degrading enzymes (CWDEs); polygalacturonases (PGs) are pathogenicity factors produced at the earlier stages of a fungal infection that depolymerize the homogalacturonan. One of the strategies used by plants to limit the degradation of the cell wall polysaccharides by fungal CWDEs is the production of proteinaceous inhibitors. Against fungal, microbial, and insect PGs, plants produce cell wall-associated polygalacturonase-inhibiting proteins (PGIPs). The overexpression of PGIPs improves the resistance to fungal and bacterial necrotrophs in different plants. In this research, the gene encoding the PGIP1 fused downstream of the leader sequence for secretion in the extracellular environment was isolated from *Phaseolus vulgaris* and cloned into the expression vector pBI121 for the *Agrobacterium*-mediated transformation of sugar beet. Modified transformation protocol and selection strategies were developed. In comparison with the preexisting methods, the transformation efficiency was increased and different cultivars were transformed, highlighting the general effectiveness of the method applied. The presence of the transgene and the activity of PvPGIP1 were confirmed by PCR and agarose diffusion assay analyses, respectively, and the present and copy number of the transgene in the T0 plants' genome were demonstrated by Southern blot.

Key words: Plant transformation, *Phaseolus vulgaris*, phytopathogenic fungi, polygalacturonase, polygalacturonase-inhibiting proteins

1. Introduction

Crop losses caused by plant pathogens have reached 42% of total crop losses by all factors worldwide and $26 billion annually is spent on pest management (Oerke et al., 1994). Sugar beet (*Beta vulgaris* L.) is an economically important crop that provides more than 20% of the world's sugar supply (Joersbo, 2007); many significant agronomic problems of sugar beet, including susceptibility to phytopathogenic fungi, have not been solved yet by breeding (Bosemark, 1993; Cook, 1993). The three major fungal diseases of sugar beet are crown and rot root caused by *Rhizoctonia solani* K., root rot caused by *Aphanomyces cochlioides* D., and root diseases caused by *Fusarium oxysporum*. These diseases often occur synchronously in the same field (Harveson et al., 1994; Harveson and Rush, 1995a, 1995b), which notably increases the yield loss. Among these pathogens, *Fusarium oxysporum* is the least characterized in Iran (Zamani et al., 2004) and has been identified as the cause of 27.8% of sugar beet root rot (Mahmodi and Soltani, 2006). Fusarium wilt or Fusarium yellows of sugar beet is also caused by *Fusarium oxysporum*. f. sp. *betae* (Fob) (Hill et al., 2011) and causes significant reduction in sugar concentration, root yield, and juice purity (Hanson et al., 2009). Disease control is presently accomplished using integrated approaches, like cultural measures, resistant varieties, and fungicides. Among the possible biotechnological strategies, the introduction of genes encoding antifungal proteins is a useful starting point to obtain sugar beet resistant to pathogens.

* Correspondence: motalebi@nigeb.ac.ir

Although considerable progress has been made during last decade in the introduction of foreign genes into crops, sugar beet is still considered a plant recalcitrant to genetic transformation. *Agrobacterium tumefaciens*-mediated transformation (Lindsey and Gallois, 1990; D'Halluin et al., 1992a, 1992b; Krens et al., 1996; Snyder et al., 1999; Hisano et al., 2004), particle bombardment-mediated transformation (Snyder et al., 1999), and protoplast-based transformation of sugar beet (Hall et al., 1996) have already been described in the past. In general, *Agrobacterium*-mediated transformation is simpler, more efficient, and less expensive compared to other systems and also results in a low copy number of insertions. Sugar beet is highly susceptible in vitro to *A. tumefaciens* transformation (Krens et al., 1988; Lindsey and Gallois, 1990; D'Halluin et al., 1992b; Jacq et al., 1993; Zakharchenko et al., 2000) and susceptibility can be improved by preculturing explants before inoculation (Krens et al., 1996). Attempts have been made to develop transgenic sugar beet plants resistant to fungi; for example, a chitinase gene from pumpkin was transferred into sugar beet and suppression of disease symptoms caused by *R. solani* was detected in the transgenic plants (Hashimoto and Shimamoto, 2001; Gurel et al., 2008). To invade plant tissue, phytopathogenic fungi produce several cell wall-degrading enzymes (CWDEs). Polygalacturonases (PGs) are pathogenicity factors produced at the earlier stages of a fungal infection that depolymerize the major component of pectin, homogalacturonan (Lionetti et al., 2010). One of the strategies used by plants to limit the degradation of the cell wall polysaccharides by fungal CWDEs is the production of proteinaceous inhibitors (D'Ovidio et al., 2004; Ferrari et al., 2012). Against fungal, microbial, and insect PGs, plants produce cell wall-associated polygalacturonase-inhibiting proteins (PGIPs) (Spadoni et al., 2006) The overexpression of PGIPs improves the resistance to fungal and bacterial necrotrophs in different plants (Aguero et al., 2005; Ferrari et al., 2012). PGIPs found in the cell wall of many plants counteract fungal PGs by forming specific complexes with them (Torki et al., 2000; De Lorenzo et al., 2001; Protsenko et al., 2010; Benedetti et al., 2011), blocking their activity and favoring the accumulation of partially digested fragments of polygalacturonic acid, the oligogalacturonides, that induce the plant defense responses (Cervone et al., 1990; De Lorenzo et al., 2001; Martin et al., 2003). PGIPs are extracellular proteins belonging to the family of the leucine-rich repeat proteins (Mattei et al., 2001).

A single PGIP can recognize a broad range of fungal PGs and by limiting PG activity can prevent cell wall degradation and restrict fungal growth and colonization (Federici et al., 2006; Casasoli et al., 2009; Ferrari et al., 2011). For example, PvPGIP1 is able to recognize and inhibit several PGs produced by different phytopathogenic fungi such as *Aspergillus niger*, *Colletothricum acutatum*, *Staenocarpella maydis*, and *Botrytis cinerea* (D'Ovidio et al., 2004). Numerous studies have shown that PGIP reduces the susceptibility to fungal attack in different transgenic plants like tobacco, pear, apple, tomato, *Arabidopsis*, wheat, and grapevine (Benito et al., 1998; Powell et al., 2000; Atkinson et al., 2002; Faize et al., 2003; Ferrari et al., 2003; Tamura et al., 2004; Aguero et al., 2005; Manfredini et al., 2005; Joubert et al., 2006, 2007; Kortekamp, 2006; Oelofse et al., 2006; Gregori et al., 2008; Janni et al., 2008). In this study, the *Pgip1* gene of *P. vulgaris* (*Pvpgip1*), encoding one of the PG inhibitors thus far characterized (De Lorenzo et al., 2001; Benedetti et al., 2011), was transformed into sugar beet using an *Agrobacterium*-mediated genetic transformation.

2. Materials and methods

2.1. *Agrobacterium* strain and plasmids

The binary vector designed as pBIAH17 was used in the transformation experiment. pBIAH17 was generated from pBI121 through replacement of the GUS coding sequence with the *Pgip1* gene (Accession number: AY508111) of *P. vulgaris* (cultivar Naz Red Bean). Prior to cloning into the pBI121, a wild-type leader sequence for secretion was fused to the upstream of the *Pgip1* coding sequence such that the *Pgip1* gene product was secreted into the apoplastic space. The T-DNA of pBIAH17 retains the neomycin phosphotransferase (*nptII*) selection gene of pBI121 for plant selection. The T-DNA map, including the *nptII* and *Pvpgip1* (replaced with GUS int) expression cassettes, is shown in Figure 1. The freeze-thaw method (Sambrook and Russell, 2001) was used to mobilize

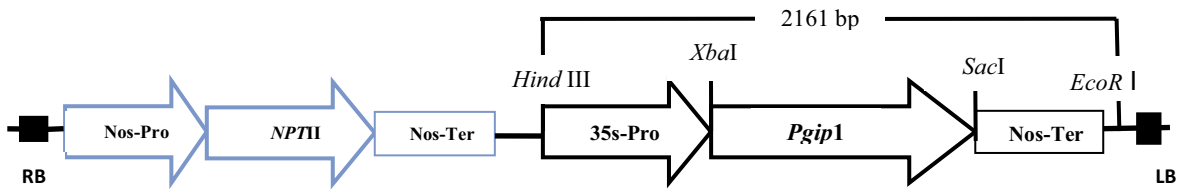

Figure 1. The T-DNA in the pBIAH17 plasmid used for transformation. pBIAH17 is a pBI121-derived plasmid containing the cassette for expression of the *Pvpgip1* gene under the control of the 35S-promoter and NOS-terminator in the pBI121 vector. Abbreviations: RB, right border; LB, left border.

pBIAH17 into *Agrobacterium tumefaciens* (GV3101). GV3101:pBIAB17 was cultured for 2 days at 28 °C on a rotary shaker at 180 rpm in liquid LB medium containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin (Sambrook et al., 1989; Norouzi et al., 2005) until an OD600 nm of 0.6–0.7 was reached. Bacterial cultures were centrifuged at 3500 rpm at 4 °C for 10 min and pellets were resuspended in bacterial-inducing medium (0.5X MSB (Murashige and Skoog, 1962 Basic) medium). After addition of 200 µM acetosyringone, bacteria were further cultivated at 28 °C to an OD600 nm of 1. The culture was diluted with liquid MS medium before cocultivation with plant explants to obtain a final OD600 nm of about 0.3 (Chilton, 1974; Mishutkina et al., 2010).

2.2. Plant materials and tissue culture conditions

Seeds of sugar beet cultivars SBSI-01 and SBSI-02 (provided by the Sugar Beet Seed Institute, Karaj, Iran) were scarified by immersion in concentrated sulfuric acid for 60 min and washed under running tap water. The seeds were then surface-sterilized using ethanol (70% w/v) for 5 min, rinsed 3 times in sterile distilled water, and immersed in 10% sodium hypochlorite plus Tween 20 (1 drop/100 cm^3 solution) for 10 min. Subsequently, the seeds were washed 3 times with sterile distilled water. After 3 rinses with sterile distilled water, seeds were placed onto petri dishes containing MSB medium containing 8 g L^{-1} agar and 0.5 mg L^{-1} 2,3,5-triiodobenzoic acid (TIBA). Seeds were left to germinate at 22–25 °C under a 16-h photoperiod. After 7–10 days of germination, the shoot apices of the germinates were excised (Figure 2a) and transferred onto shoot-inducing medium I [MSB, 1 mg L^{-1} N6-benzyl adenine (BA), 0.1 mg L^{-1} α-naphthalene acetic acid (NAA), and 0.5 mg L^{-1} TIBA] (Figure 2b). Two weeks later, shoots were transferred to shoot-inducing medium II [MSB, 0.5 mg L^{-1} BA and 0.1 mg L^{-1} indole-3-butyric acids (IBA)] for optimal shoot development (Figure 2c). For induction of shoot regeneration from butts (around the main vein of the leaves), leaf blades were cut from the shoots and placed on shoot-inducing medium II (Figure 2d). The shoots regenerated from the veins of the leaf blades were cut and the remainder of the leaf blades, carrying the shoot bases, were used as explants for transformation.

All tissue culture dishes were incubated in a growth chamber at 20 ± 2 °C and 70% humidity under a 16/8-h (light/dark) photoperiod with light provided by high pressure metal halide lamps (60 μm^{-2} s^{-1}) (Jafari et al., 2009). The MSB medium containing MS salts (Murashige and Skoog, 1962) and B5 vitamins (Gamborg, 1970) was used as basal medium. The pH of all media was adjusted to 5.8, except for the bacterial-induction medium (pH 5.5). MSB nutrient medium contained 30 g L^{-1} sucrose and 8 g L^{-1} agar.

2.3. Transformation procedure

Sugar beets of genotypes SBSI-01 and SBSI-02 were transformed by pBIAH17 containing a *Pvpgip1* gene and an *nptII* gene. Plants were regenerated and transformed as described by Norouzi et al. (2005) and Mohammadzadeh et al. (2012).

2.4. PCR analysis

In order to detect the presence of the *Pvpgip1* gene by PCR analysis, genomic DNA was extracted from the plant leaves using a CTAB method adapted from Dellaporta et al. (1983) and Doyle and Doyle (1990). Plant leaves were ground in liquid nitrogen and incubated in a lysis buffer, containing 50 mM Tris-HCl (pH 8.0),150 mM NaCl, and 100 mM ethylene diamine tetraacetic acid (EDTA), and 20% (w/v) sodium dodecyl sulfate (SDS), 5 M NaCl, and 20% CTAB (w/v). DNA was cleaned with an equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1) and precipitated using –20 °C isopropanol (1:1). Precipitated DNA was washed with 70% ethanol, dried, and suspended in 50 µL of water. PCR primers specific to the *Pgip1* gene, 5′-GCTCTAGAATGTCCTCAAG CTTAAGCAT-3′ (Fw) and 5′-GCACGAGCTCTTAAGTGCAGGCAGGAAG-3′ (Rw), were used, and the expected size of the amplified fragment was 1029 bp. Taq DNA polymerase was used in a standard 34-cycle reaction with annealing temperature of 54 °C. The plasmid pBIAH17 and the genomic DNA extracted from a nontransgenic plant were used as templates in the positive and negative controls, respectively.

2.5. Southern analysis

For Southern analysis, genomic DNAs were isolated from T0 plant leaves based on the protocol of the CTAB extraction method adapted from Doyle and Doyle (1990) and Dellaporta et al. (1983) with the addition of DNase-free RNase A treatment (Sigma, 0.5 mg L^{-1}, 37 °C, 10 min). Twenty-five micrograms of genomic DNA from each sample was digested with *Eco*R1 and *Eco*R1/*Hind*III. Both enzymes were cut once in the transgene cassette and the *Eco*R1/*Hind*III digestion released a 2161-bp diagnostic fragment. Plasmid DNA equivalent to one copy of the *Pvpgip*1 gene and nontransgenic plant DNA were used respectively as positive and negative controls. These were subjected to electrophoresis in a 0.8% agarose gel. The fractionated DNA was transferred to a positively charged nylon membrane (Roche Applied Science, Germany) by capillary transfer method and fixed on the membrane (Sambrook and Russell, 2001). A 1002-bp PCR-amplified fragment corresponding to the coding sequence of the *Pvpgip1* gene was used as a probe. The fragment was labeled with DIG-dUTP using the PCR DIG Probe Synthesis Kit (Roche Applied Science). Hybridization, high stringency washes, and detection were performed according to the instruction manual of the DIG DNA labeling and detection kit.

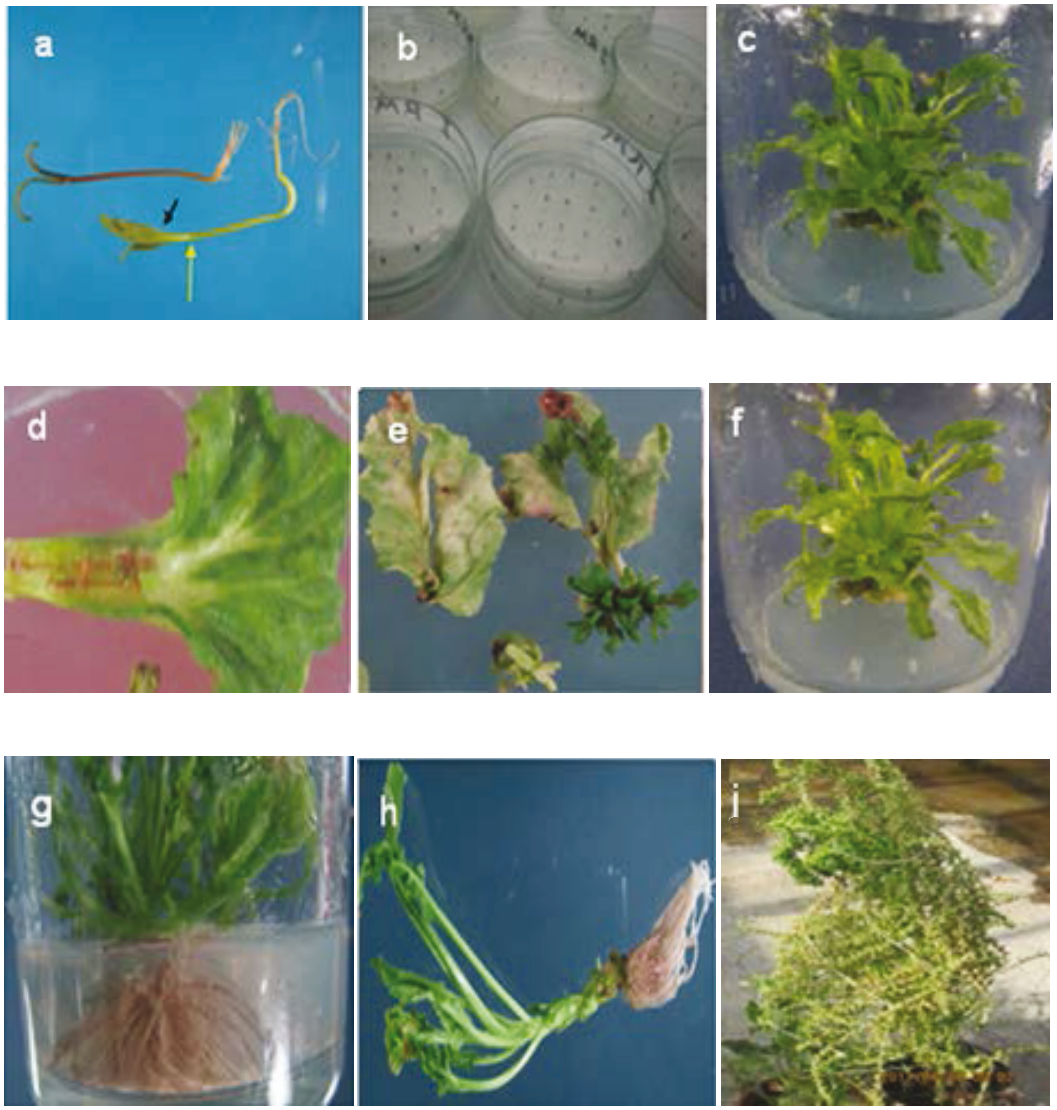

Figure 2. The process of transformation and regeneration of sugar beet plants: **(a)** the cutting of shoot apices; **(b)** shoot apices on shoot-inducing medium I; **(c)** shoots obtained from shoot apices; **(d)** leaves from shoots shown in (c) were used for transformation on shoot-inducing medium II; **(e)** chlorotic shoots and green kanamycin-resistant shoots formed on selection medium; **(f)** regenerated shoots transferred to growth medium and putative transgenic shoots propagated on shoot-propagation medium for preparation of clones, with many shoots formed around the shoot base; **(g, h)** putative transgenic plant with induced roots on root-inducing medium; **(i)** regenerated plant transplanted to a pot and acclimated to nonaseptic environment to obtained seeds.

2.6. Preparation of the crude-PG extract from *Colletotrichum lindemuthianum*

An isolate of *C. lindemuthianum* (Sacc. & Mgn.) Bri et Cav., race β, was grown for 20 days on potato dextrose agar (Oxoid, Italy) at 24 °C under constant light. Mycelium of *C. lindemuthianum* (1 cm^2) was harvested, used to inoculate Pectic Zymogram medium [$(NH_4)_2SO_4$ 2.64 g L^{-1}, KH_2PO_4 0.34 g L^{-1}, $MgSO_4.7H_20$ 0.14 g L^{-1}, pH 4.5], and supplemented with 1% apple pectin. Culture was incubated in a rotary shaker at 120 rpm and 21 °C for 12 days, and the filtrate was used for the PG activity assay.

2.7. Determination of the polygalacturonase inhibitory activity by agarose diffusion assay

Frozen leaves of the transgenic plants positive to PCR were subjected to protein extraction. Tissue was homogenized in liquid nitrogen and resuspended in 20 mM Na-acetate buffer (pH 4.6) containing 1 M NaCl. Homogenates were incubated under gentle shaking for 1 h at 4 °C and

centrifuged for 10 min at 10,000 × *g* and supernatants were transferred to fresh tubes. The protein content was determined against BSA according to the Bradford assay (Bradford 1976), and 30 µg of crude protein extract was assayed for inhibitory activity against a crude preparation of the endo-polygalacturonases produced by *Colletotrichum lindemuthianum* (ClPG).

The inhibitory effect of the PvPGIP1 against the ClPG activity was measured using an agarose diffusion assay (Taylor and Secor, 1988). The crude ClPG preparation and/or the plant protein extracts were added to the wells of 0.8% agarose plates containing 100 mM sodium acetate, pH 4.6, and 0.5% citrus pectin (Sigma P 3850). Plates were incubated for 16 h at 27 °C, and the halo caused by the enzyme activity was visualized after 1 min of treatment with 6 N HCl. Inhibitory activity was expressed in percentage as described by Ferrari et al. (2003).

2.8. Statistical analysis

The experiments were based on a completely randomized design with three replications per treatment. The data collected were subjected to analysis of variance test with SPSS software. The means were compared using Duncan's multiple range tests.

3. Results and discussion

3.1. *Agrobacterium*-mediated transformation of sugar beet plants

Leaves of regenerated plants were used as explants for transformation experiments. A total of 1000 explants of SBSI-01 and SBSI-02 (500 explants for each cultivar) were cocultivated with *Agrobacterium* strain GV3101 in the five transformation experiments reported here (Table 1). During the selection period on kanamycin (50 mg L^{-1}), the majority of explants gradually turned brown, whereas some green kanamycin-resistant sugar beet shoots were observed after 4 weeks (Figure 2e). The green shoots were subjected to selection with higher levels (100 mg L^{-l}) of kanamycin. The resistant plants were then subjected to a regeneration process (Figure 2f). In transformation experiments, 20% and 23.9% of plants showed resistance to the kanamycin selection for SBSI-01 and SBSI-02, respectively (Table 1). A total of 39 resistant green plants for both cultivars were positive to the PCR, confirming the presence of the transgene (Table 1). No albino plants were observed in the experiments. All the plants developed a functional root system in the selective rooting medium and survived transplantation (Figures 2g and 2h). The regenerated plant transplanted to a pot and acclimated to non-aseptic environment to obtained seeds (Figure 2i). Transgenic plants obtained from *Agrobacterium*-mediated transformation had simpler hybridization patterns and were estimated to have 1 to 3 transgene copies. In comparison with the previous systems reported for sugar beet transformation (Joersbo et al., 1998; Ivic-Haymes and Smigocki, 2005), our method was characterized by higher transformation efficiency, a lower transgene copy number in plants, and a shorter period to recover transgenic plants. Furthermore, low transgene copies reduce the possibility of gene silencing and increase the stability of the transgene (Iglesias et al., 1997; Li, 2008).

Agrobacterium-mediated transformation of sugar beet is often genotype-dependent (D'Halluin et al., 1992a), and in order to evaluate if the protocol was suitable for other cultivars, we transformed plants belonging to the cultivars SBSI-01 and SBSI-02. Among 1000 pieces of infected explants, 20% and 23.9% developed green shoots under 100 mg dm^{-3} kanamycin selection, and 33% and 34.5% of green shoots were PCR-positive for SBSI-01 and SBSI-02, respectively, showing high transformation efficiency and confirming the general effectiveness of the method.

Table 1. Transformation efficiency of tissue-cultured leaf explants of two sugar beet genotypes using the pBIAB17 plasmid carrying the *Pvpgip*1 gene.

Genotype	No. of explants	No. of regenerated shoots from explants at 50 mg/L kanamycin[a]	No. of green shoots at 100 mg kanamycin[b]	No. of PCR-positive plants[c]	No. of PCR-positive plants expressing PvPGIP1	Transformation efficiency[d]
SBSI-01	500	300 (60%)	60 (20%)	20 (33%)	12	4%
SBSI-02	500	230 (64%)	55 (23.9%)	19 (34.5%)	8	3.8%

[a] In parentheses, the number (×100) of regenerated shoots from explants incubated in the presence of 50 mg/L kanamycin / number of explants.

[b] In parentheses, the number (×100) of green shoots obtained at 100 mg/L kanamycin / number of regenerated shoots obtained at 50 mg/L kanamycin.

[c] In parentheses, the number (×100) of PCR-positive plants / number of green shoots obtained at 100 mg/L kanamycin.

[d] The number (×100) of PCR-positive plants / number of explants.

3.2. Detection of the inhibition capability of the transgenic sugar beet plants harboring the *Pvpgip1* gene against ClPG

PGIPs from *Phaseolus vulgaris* inhibit several PGs including the ones produced by *C. lindemuthianum* and *C. acutatum* (De Lorenzo et al., 2003; D'Ovidio et al., 2004). The inhibitory activities of the protein extract from wild-type (untransformed) and transgenic sugar beet plants were assayed by agarose diffusion assay against a crude preparation of PG from *C. lindemuthianum* (ClPG). Endogenous inhibitory activity against ClPG was not detected in the wild-type sugar beet while inhibition was found in several transgenic plants (Figure 3). The crude protein extracts of different plants inhibited ClPG to different extents, suggesting that the levels of the inhibitor varied in the different transgenic plants (Table 2). The boiled protein extracts did not show any inhibitory activity, confirming that the inhibition was due to proteins (data not shown). Different expression patterns of the *Pvpgip*1 gene occurred in 12 and 8 of the PCR-positive plants of cultivars SBSI-01 and SBSI-02, respectively (Table 2). Plants expressing the antifungal genes have shown increased resistance but not total resistance to the pathogens. This may be due to the variation in expression of the transgene, which is determined mainly by the site of insertion or promoter strength (Zhu et al., 1994). De Bolle (2003) also demonstrated a high variation of transgenic expression in *Arabidopsis thaliana*. The reduction in *Rhizoctonia solani* disease incidence ranged from 25% to 60% for the chitinase transgene-expressing plants (Howie et al., 1994). The inhibitory effect of PGIP1 against fungal PG activity was measured using an agarose diffusion assay (Taylor

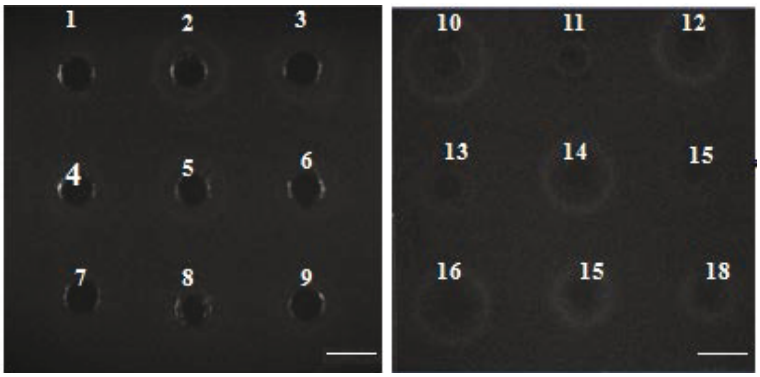

Figure 3. PGIP1 activity in a subset of transgenic sugar beet plants using a semiquantitative agarose diffusion assay. Halo indicates PG activity; the radius of the halo is related to the residual activity detected. Reduction in the radius or total disappearance of the halo means partial or total inhibition, respectively. ClPG plus 30 µg of protein extracts from different transgenic sugar beet plants. Samples 1 (T_0-017′), 7 (T_0-083), 8 (T_0-130), 9 (T_0-025′), 11 (T_0-144′), 13 (T_0-022′), 15 (T_0-063), 17 (T_0-061), and 18 (T_0-04) exhibited high inhibitory activity, while samples 14 (T_0 030) and 16 (T_0-12) did not show any inhibitory activity. 2 and 10: ClPG alone, 3 and 12: ClPG vs. 30 µg of untransformed sugar beet protein extract. Bar indicates 10 mm.

Table 2. Inhibition of PGIP1 activity in transgenic sugar beet plants harboring the *Pvpgip1* gene.

Transgenic plants of cultivar SBSI 01 (positive PCR)	Inhibition (%)[a]	Transgenic plants of cultivar SBSI 02 (positive PCR)	Inhibition (%)[a]
Control (wild type)	0	Control (wild type)	0
T0-017′, T0-015, T0-083, T0-114′, T0-066′, T0-04, T-034	75	T0-130, T0-025′, T0-063, T0-061′	75
T0-066, T0-036, T0-02′	50	T0-088, T0-065	50
T0-090, T0-068	25	T0-022′, T0-064	25

[a] Inhibitory activity was determined by agarose plate assay using a crude preparation of ClPG and 30 µg of protein extract from untransformed and transgenic plants. Twenty out of 39 independent transgenic plants showed expression of PvPGIP1.

and Secor, 1988) (Figure 3). Seven out of 20 PCR-positive plants showed high levels of expression of PGIP (≥75% inhibition) and 5 plants showed 25%–50% inhibition in the SBSI-01 cultivar (Figure 4; Table 2). In the SBSI-02 cultivar, 4 out of 19 PCR-positive plants demonstrated 75% and 4 plants demonstrated 25%–50% inhibition (Figure 4; Table 2). The interactive effect between PvPGIP1 and ClPG was found to be statistically nonsignificant for the remaining transgenic plants of both cultivars.

3.3. Southern blot analysis of transgenic sugar beet plants

Southern blot analysis was performed on the 6 putative T0 transgenic plants (5 plants showing 75% and 1 showing 25% inhibition) in order to confirm the transgenic nature of these plants (Figure 5). A 1002-bp amplified fragment of the *Pvpgip*1 gene was used as a probe. The various sizes of the restricted transgene bands among the analyzed plants indicated stable integration of the transgenes at different loci in the sugar beet genome. Plant genomic DNA was digested with the *Eco*RI enzyme by the presence of only one *Eco*RI restriction site between the right and left borders of T-DNA (Figure 1). The genomic DNA from each transgenic plant was digested with *Eco*RI/*Hind*III. Both enzymes were cut once in the transgenic cassette and released a 2161-bp diagnostic fragment (Figure 5). The *Pvpgip1* transgene copy number was detected as one copy in lines 083 and 22′; two copies in lines 017′, 114′, and 130; and three copies in line 063 (Figure 5). The single or multicopy insertions of the transgene were observed in both cultivars, indicating that the copy number of the transgene is genotype-independent. No hybridization signal occurred in the nontransgenic control plant (Figure 5).

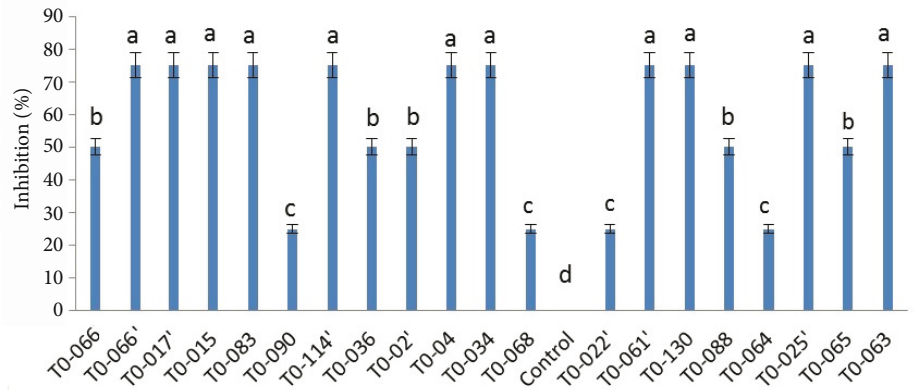

Figure 4. Data were obtained as the mean of 3 replications. Different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range tests. Vertical lines represent standard errors.

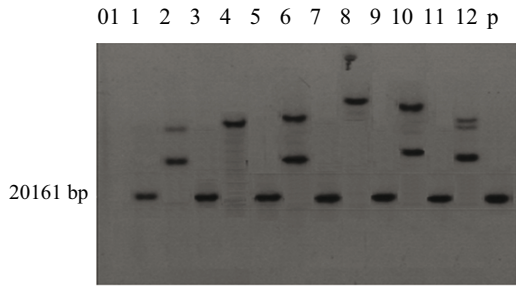

Figure 5. Southern blot analysis of transgenic sugar beet plants expressing PvPGIP1. Cultivar SBSI-01: Genomic DNA of transgenic plants expressing PvPGIP1 was digested with *Eco*R1/*Hind*III (017′, lane 1), *Eco*R1 (017′, lane 2), *Eco*R1/*Hind*III (083, lane 3), *Eco*R1 (083, lane 4), *Eco*R1/*Hind*III (114′, lane 5), and *Eco*R1 (114′, lane 6). Cultivar SBSI-02: Genomic DNA was digested with *Eco*R1/*Hind*III (022′, lane 7), *Eco*R1 (022′, lane 8), *Eco*R1/*Hind*III (130, lane 9), *Eco*R1 (130, lane 10), *Eco*R1/*Hind*III (063, lane 11), and *Eco*R1 (063, lane 12). Lane 01 represents the untransformed plant (negative control). The arrow indicates pBIAH17 digested with EcoR1/HindIII (shown in Figure 1), used as a positive control (lane p).

In conclusion, based on the results presented in this study, the *Pgip*1 gene from *Ph. vulgaris* is an efficient polygalacturonase inhibitor and it will be useful to improve sugar beet fungal resistance.

Acknowledgment
This research was funded by a grant (No. 353) from the National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.

References

Aguero CB, Uratsu SL, Greve C, Powell ALT, Labavitch JM, Meredith CP, Dandekar AM (2005). Evaluation of tolerance to Pierce's disease and botrytis in transgenic plants of Vitis vinifera L. expressing the pear PGIP gene. Mol Plant Pathol 6: 43–51.

Atkinson RG, Schroder R, Hallett IC, Cohen D, MacRae EA (2002). Over expression of polygalacturonase in transgenic apple trees leads to a range of novel phenotypes involving changes in cell adhesion. J Plant Physiol 129: 122–133.

Benedetti M, Leggio C, Federici L, De Lorenzo G, Pavel NV, Cervone F (2012). Structural resolution of the complex between a fungal polygalacturonase and a plant polygalacturonase-inhibiting protein by small angle X-ray scattering. Plant Physiol 157: 599–607.

Benito EP, ten Have A, van't Klooster JW, van Kan JAL (1998). Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*. Eur J Plant Pathol 104: 207–220.

Bosemark NO (1993). Genetics and breeding. In: Cooke DA, Scott RK, editors. The Sugar Beet Crop: Science into Practice. London, UK: Chapman & Hall, pp. 37–66.

Casasoli M, Federici L, Spinelli F, Di Matteo A, Vella N, Scaloni F, Fernandez-Recio J, Cervone F, De Lorenzo G (2009). Integration of evolutionary and desolvation energy analysis identifies functional sites in a plant immunity protein. P Natl Acad Sci USA 106: 7666–7671.

Cervone F, De Lorenzo G, Pressey R, Darvill AG, Albersheim P (1990). Can *Phaseolus* PGIP inhibit pectic enzymes from microbes and plants? Photochemistry 29: 447–449.

Cooke DA (1993). Pests. In: Cooke DA, Scott RK, editors. The Sugar Beet Crop: Science into Practice. London, UK: Chapman & Hall, pp. 428–483.

D'Halluin K, Bossut M, Bonne E, Mazur B, Leemans J, Botterman J (1992a). Transformation of sugar-beet (*Beta vulgaris* L.) and evaluation of herbicide resistance in transgenic plants. Bio-Technol 10: 309–314.

D'Halluin K, De Block M, Denecke J, Janssen J, Leeman J, Raynaert A (1992b). The bar gene as selectable and screenable marker in plant engineering. Methods Enzyme 216: 415e26.

Dellaporta SL, Wood J, Hicks JB (1983). A plant DNA minipreparation: Version II. Plant Mol Biol Rep 1: 19–20.

De Lorenzo G, D'Ovidio R, Cervone F (2003). The role of polygalacturonase-inhibiting proteins (PGIP) in defence against pathogenic fungi. Ann Rev Phytopathol 39: 313–335.

D'Ovidio R, Raiola A, Capodicasa C, Devoto A, Pontiggia D, Roberti S, Galletti R, Conti E, O'Sullivan D, De Lorenzo G (2004). Characterization of the complex locus of bean encoding polygalacturonase-inhibiting proteins reveals sub-functionalization for defense against fungi and insects. Plant Physiol 135: 2424–2435.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12: 13–15.

Faize M, Sugiyama T, Faize L, Ishii H (2003). Polygalacturonase-inhibiting protein (PGIP) from Japanese pear: possible involvement in resistance against scab. Physiol Mol Plant Pathol 63: 319–327.

Federici L, Di Matteo A, Fernandez Recio J, Tsernoglou D, Cervone F (2006). Polygalacturonase inhibiting proteins: players in plant innate immunity? Trends Plant Sci 11: 65–70.

Ferrari S, Sella L, Janni M, De Lorenzo G, Favaron F, D'Ovidio R (2012). Transgenic expression of polygalacturonase-inhibiting proteins in *Arabidopsis* and wheat increases resistance to the flower pathogen *Fusarium graminearum*. Plant Biol 14: 31–38.

Ferrari S, Vairo D, Ausubel FM, Cervone F, De Lorenzo G (2003). Tandemly duplicated Arabidopsis genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. Plant Cell 15: 93–106.

Gregori R, Mari M, Bertolini P, Sanudo Barajas JA, Tian JB, Labavitch JM (2008). Reduction of *Colletotrichum acutatum* infection by a polygalacturonase inhibitor protein extracted from apple. Postharvest Biol Technol 48: 309–313.

Gurel E, Gurel S, Lemaux PG (2008). Biotechnology applications for sugar beet. CRC Cr Rev Plant Sci 27: 108–140.

Hadfield KA, Bennett AB (1998). Polygalacturonases: many genes in search of a function. Plant Physiol 117: 337–343.

Hall RD, Riksen-Gruinsma T, Weyens GJ, Rosquin IJ, Denys PN, Evans IJ, Lathouwers JE, Lefebvre MP, Dunwell JM, Tunen AV et al. (1996). A high efficiency technique for the generation of transgenic sugar beet from stomatal guard cells. Nat Biotechnol 14: 1133–1138.

Harveson RM, Rush CM (1995a). Evaluation of genetic variability among *Fusarium oxysporum* f. sp. *betae* isolates by vegetative compatibility. Phytopathology 85: 11–17.

Harveson RM, Rush CM (1995b). Studies of vegetative compatibility among isolates of *Fusarium oxysporum* f. sp. *betae* causing different disease symptoms. J Sugar Beet Res 32: 142.

Harveson RM, Rush CM (1997). Genetic variation among *Fusarium oxysporum* isolates from sugar beet as determined by vegetative compatibility. Plant Dis 81: 85–89.

Hashimoto R, Shimamoto Y (2001). Transgenic sugar beet plants harboring a pumpkin chitinase gene demonstrating improved resistance to *Rhizoctonia solani*. Proc Jpn Soc Sugar Beet Technol 43: 24–28.

Hill AL, Reeves RL, Larson RL, Fenwick AL, Hanson LE, Panella L (2011). Genetic variability among isolates of *Fusarium oxysporum* from sugar beet. Plant Pathol 60: 496–505.

Hisano H, Kimoto Y, Hayakawa H, Takeichi J, Domae T, Hashimoto R (2004). High frequency *Agrobacterium* mediated transformation and plant regeneration via direct shoot formation from leaf explants in *Beta vulgaris* and *Beta maritima*. Plant Cell Rep 22: 910–918.

Iglesias VA, Moscone EA, Papp I, Neuhuber F, Michalowski S, Phelan T, Spiker S, Matzke M, Matzke AJM (1997). Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. Plant Cell Rep 9: 1251–1264.

Ivic-Haymes SD, Smigocki AC (2005). Biolistic transformation of highly regenerative sugar beet (*Beta vulgaris* L.) leaves. Plant Cell Rep 23: 699–704.

Jacq B, Lesobre O, Sangwan RS, Sangwan BS (1993). Factors influencing T-DNA transfer in Agrobacterium-mediated transformation of sugar beet. Plant Cell Rep 12: 621–624.

Jafari M, Norouzi P, Malbobi MA, Ghareyazei B, Valizadeh M, Mohammadi SA, Mousavi A (2009). Enhanced resistance to a lepidopteran pest in transgenic sugar beet plants expressing synthetic *cry1Ab* gene. Euphytica 165: 333–344.

Janni M, Sella L, Favaron F, Blechl AE, De Lorenzo G, D'Ovidio R (2008). The expression of a bean PGIP in transgenic wheat confers increased resistance to the fungal pathogen *Bipolaris sorokiniana*. MPMI 21: 171–177.

Joersbo M (2007). Sugar beet. In: Pua EC, Davey MR. Biotechnology in Agriculture and Forestry, Vol. 59. Berlin, Germany: Springer, pp. 355–379.

Joersbo M, Donaldson I, Kreiberg J, Petersen SG, Brunstedt J, Okkels FT (1998). Analysis of mannose selection used for transformation of sugar beet. Mol Breeding 4: 111–117.

Joubert DA, Kars I, Wagemakers L, Bergmann C, Kemp G, Vivier MA, van Kan JAL (2007). A polygalacturonase-inhibiting protein from grapevine reduces the symptoms of the endopolygalacturonase BcPG2 from *Botrytis cinerea* in *Nicotiana benthamiana* leaves without any evidence for in vitro interaction. Mol Plant Microbe In 20: 392–402.

Joubert DA, Slaughter AR, Kemp G, Becker JVW, Krooshoof GH, Bergmann C, Benen J, Pretorius IS, Vivier MA (2006). The grapevine polygalacturonase inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. Transgenic Res 15: 687–702.

Kortekamp A (2006). Expression analysis of defence related genes in grapevine leaves after inoculation with a host and a non-host pathogen. Plant Physiol Biochem 44: 58–67.

Krens FA, Trifonova A, Keizer LCP, Hall RD (1996).The effect of exogenously applied phytohormones on gene transfer efficiency in sugar beet (*Beta vulgaris* L.). Plant Sci 116: 97–106.

Krens FA, Zijistra CJ, Molen W, Jamar D, Huizing HJ (1988). Transformation and regeneration in sugar beet induced by 'shooter' mutants of *Agrobacterium tumefaciens*. Euphytica 5: 185–194.

Leckie F, Mattei B, Capodicasa C, Hemmings A, Nuss L, Aracri B, De Lorenzo G, Cervone F (1999). The specificity of polygalacturonase-inhibiting protein (PGIP): A single amino acid substitution in the solvent-exposed β-strand/β-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. EMBO J 18: 2352–2363.

Li J, Brunner AM, Meilan R, Strauss SH (2008). Stability of transgenes in trees: expression of two reporter genes in poplar over three field seasons. Tree Physiol 29: 299–312.

Lindsey K, Gallois P (1990). Transformation of sugarbeet (*Beta vulgaris* L.) by *Agrobacterium tumefaciens*. J Exp Bot 41: 529–536.

Lionetti V, Francocci F, Ferrari S, Volpi C, Bellincampi D, Galletti R, D'Ovidio R, De Lorenzo G, Cervone F (2010). Engineering the cell wall by reducing de-methyl-esterified homogalacturonan improves saccharification of plant tissues for bioconversion. P Natl Acad Sci USA 107: 616–621.

Mahmodi SB, Soltani J (2006). Rot root in Iran. Central Study and Research Industrial Sugar Beet of Iran Journal 178: 14–18 (in Persian with abstract in English).

Manfredini C, Sicilia F, Ferrari S, Pontiggia D, Salvi G, Caprari C, Lorito M, De Lorenzo G (2005). Polygalacturonase-inhibiting protein 2 of *Phaseolus vulgaris* inhibits BcPG1, a polygalacturonase of *Botrytis cinerea* important for pathogenicity, and protects transgenic plants from infection. Physiol Mol Plant P 67:108–115.

Martin GB, Bogdanove AJ, Sessa G (2003). Understanding the functions of plant disease resistance proteins. Annu Rev Plant Biol 54: 23–61.

Mattei B, Bernalda M, Federici L, Roepstorff P, Cervone F, Boffi A (2001). Secondary structure and post-translational modifications of the leucine-rich repeat protein PGIP (polygalacturonase-inhibiting protein) from *Phaseolus vulgaris*. Biochemistry 40: 569–576.

Mohammadzadeh R, Zamani MR, Motallebi M, Norouzi P, Jourabchi E, Benedetti M, De Lorenzo G (2012). *Agrobacterium tumefaciens*-mediated introduction of polygalacturonase inhibiting protein 2 gene from *Phaseolus vulgaris* (*Pv*PGIP2) into sugar beet (*Beta vulgaris* L.). AJCS 6: 1290–1297.

Oelofse D, Dubery IA, Meyer R, Arendse MS, Gazendam I, Berger DK (2006). Apple polygalacturonase inhibiting protein1 expressed in transgenic tobacco inhibits polygalacturonases from fungal pathogens of apple and the anthracnose pathogen of lupins. Phytochemistry 67: 255–263.

Powell AL, van Kan J, ten Have A, Visser J, Greve LC, Bennett AB, Labavitch JM (2000). Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol Plant Microbe In 13: 942–950.

Protsenko MA, Bulantseva EA, Korableva NP (2010). Polygalacturonase inhibiting proteins in plant fleshy fruits during their ripening and infections. Russ J Plant Physl+ 57: 356–362.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Sambrook J, Russell DW (2001). Molecular Cloning: A Laboratory Manual. 3rd ed. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Snyder GW, Ingersoll JC, Smigocki AC, Owens LD (1999). Introduction of pathogen defence genes and a cytokinin biosynthesis gene into sugar beet (*Beta vulgaris* L.) by *Agrobacterium* or particle bombardment. Plant Cell Rep 18: 829–834.

Tamura M, Gaob M, Tao R, Labavitch JM, Dandekar AM (2004). Transformation of persimmon with a pear fruit polygalacturonase inhibiting protein (PGIP) gene. Sci Hort 103: 19–30.

Torki M, Mandaron P, Mache R, Falconet D (2000). Characterization of a ubiquitous expressed gene family encoding polygalacturonase in *Arabidopsis thaliana*. Gene 242: 427–436.

Zakharchenko NS, Kalyaeva MA, Bur'yanov YI (2000). The method for genetic transformation of different sugar beet varieties. J Plant Physiol 47: 70–75.

Zamani MR, Motallebi M, Rostamian A (2004). Characterization of Iranian isolates of *Fusarium oxysporum* on the basis of RAPD analysis, virulence, and vegetative compatibility. Phytopathology 152: 499–503.