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Agrobacterium tumefaciens-mediated introduction of polygalacturonase inhibiting protein 2 gene (*PvPGIP2*) from *Phaseolus vulgaris* into sugar beet (*Beta vulgaris* L.)

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Abstract

Sugar beet (*Beta vulgaris* L.) is an important industrial crop, the yield of which is strongly affected by numerous diseases caused by fungal pathogens. To the aim of developing transgenic plants resistant to fungi, two transgenic diploid sugar beet genotypes expressing the gene encoding the polygalacturonase inhibiting protein 2 of *Phaseolus vulgaris* (PvPGIP2) were generated by *Agrobacterium tumefaciens*-mediated transformation. PGIPs are plant cell wall leucine-rich repeat (LRR) proteins that bind to and inhibit fungal polygalacturonase (PG), thus slowing down the plant cell wall degradation and limiting fungal colonization of the plant tissues. Leaf blade explants carrying the bases of regenerated shoots, a highly regenerative tissue, were used for transformation. PCR screening using specific primers showed the presence of the transgene in more than 40% of the regenerated kanamycin-resistant plants. A transformation rate of 4.4–4.2% (depending on the genotype) was achieved as revealed by agarose diffusion assay of the PvPGIP2 activity in the crude protein extracts of shoot tissues. The intact integration of the transgene cassette into the genome was confirmed by Southern blot analysis. The inhibitory activity against *Fusarium phyllophilum* polygalacturonase (FpPG) was found at various levels in several transgenic plants. No alterations of growth and development of the transgenic plants were observed.

Keywords: Phytopathogenic fungi, *Phaseolus vulgaris, Agrobacterium tumefaciens*, polygalacturonase, plant defence, plant transformation.
Abbreviation: AnPG-*Aspergillus niger* polygalacturonase; BA-N6-benzylAdenine; FpPG-*Fusarium phyllophilum* polygalacturonase; IBA-indole-3-butyric Acid; LRR-leucine-rich repeat; NAA-α-naphthaleneacetic acid; PGIP-polygalacturonase inhibiting protein; RIM-Root-inducing medium; SBSI-Sugar beet seed institute; SGM-Shoot growth medium; TIBA-2, 3, 5-triiodobenzoic acid.

Introduction

Sugar beet (*Beta vulgaris* L.) is an industrial crop that provides approximately 20% of the world's sugar and is becoming important for production of biofuel, alternative to fossil fuels (Joersbo, 2007). However, for the past 20 years, sugar yields have been steadily declining due to diseases caused by soilborne pathogens. The three major fungal diseases of sugar beet that often occur in the same fields are caused by *Rhizoctonia solani* Kühn, *Aphanomyces cochlioides* Drechs and by *Fusarium oxysporum* (Harveson and Rush, 1997). Fusarium wilt or Fusarium yellows of sugar beet is caused by *Fusarium oxysporum*. f. sp. *betae* (Fob) that can cause significant reduction in sugar concentration, root yield and juice purity (Hanson et al., 2009; Hill et al., 2011). *Fusarium oxysporum* is the less characterized sugar beet pathogen in Iran although *Fusarium* has been identified as the cause of the 27.8% sugar beet root rot (Mahmodi and soltani, 2006). Control of these diseases is presently accomplished using integrated approaches, like cultural measures, searching for resistant varieties and treatments with fungicides. Among the possible biotechnological strategies, there is the introduction of genes encoding antifungal proteins. 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 (Krens et al., 1996; Hisano et al., 2004), particle bombardment–mediated transformation (Snyder et al., 1999) and protoplast-based transformation of sugar beet (Hall et al., 1996) have been described. In general, *Agrobacterium* mediated transformation is simpler, more efficient and less expensive, compared to other systems. Sugar beet is highly susceptible *in vitro* to *A. tumefaciens* (Zakharchenko et al., 2000). However susceptibility is genotype-dependent (Jacq et al., 1993) and can be improved by pre-culturing 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 to sugar beet and suppression of disease symptoms caused by *R. solani* was seen in some transgenic plants (Hashimoto and Shimamoto, 2001). To penetrate plant cell walls and to release carbohydrates to be used as nutrients, pathogens secrete cell wall-degrading enzymes such as pectinases that cause the fragmentation and solubilization of pectic polymers (De Lorenzo et al., 2001). A critical role is played by endopolygalacturonases (PGs) that cleave the internal bonds of homogalacturonan, the constituent of the ''smooth region''

of pectin. The complete hydrolysis of homogalacturonan by fungal PGs can be hampered by polygalacturonase-inhibiting proteins (PGIPs), localized in the cell wall of many plants (Benedetti et al., 2011). PGIPs form specific complexes with PGs and modulate their activity, slowing down cell wall deconstruction and favouring the release of elicitor-active oligogalacturonides (Federici et al., 2006; Casasoli et al., 2009). PGIPs belong to the large family of the leucine-rich repeat (LRR) proteins (De Lorenzo et al., 1994). Several studies have shown that PGIP reduces the susceptibility to fungal attack in different transgenic plants like tobacco, pear, apple, tomato, Arabidopsis, wheat and grapevine (Powell et al., 2000; Ferrari et al., 2003; Tamura et al., 2004; Joubert et al., 2006; Oelofse et al., 2006; Janni et al., 2008; Manfredini et al., 2005; Ferrari et al., 2012). On the other hand, plants transformed with an antisense *pgip* gene are more susceptible to fungal diseases (Ferrari et al., 2006). In this study we performed an *Agrobacterium*-mediated genetic transformation of sugar beet using the *pgip2* gene of *P. vulgaris* (*Pvpgip2*), encoding one of the most efficient PG inhibitor so far characterized (Leckie et al., 1999; D'Ovidio et al., 2004; Casasoli et al., 2009).

Results and Discussion

Agrobacterium-mediated transformation of sugar beet genotypes SBSI-01 and SBSI-02

The aim of this work was to obtain transgenic sugar beet plants harboring the *Pvpgip2* gene of *P. vulgaris* cultivar Naz Red Bean, which encodes a protein identical to PVPGIP2 from the cultivar Pinto (Leckie et al., 1999; D'Ovidio et al., 2004; Casasoli et al., 2009), in the attempt of improving tolerance to fungal diseases. As a first step, we identified parameters for efficient *Agrobacterium*-mediated transformation of the diploid sugar beet genotypes SBSI-01 and SBSI-02. To this purpose we used the pBIAH23 plasmid, a pBI121 derivative containing the kanamycin resistance gene *(nptII)* as a selective gene and the *Pvpgip2* gene cloned downstream of the CaMV 35S promoter, replacing the GUS coding sequence.

It is known that the time of pre- and co-cultivation of the explants with *A. tumefaciens*, the concentration of acetosyringone and the virulence of *Aagrobacterial* strains affect the efficiency of transformation (Chumakov, 2001; Jacq et al., 1993; Hood et al., 1993). A pre-cultivation of the explants of 30-40 min before transformation, followed by a co-cultivation period of 4-6 min with *Aagrobacteria* in liquid medium and a cultivation of 4 days in solid medium was reported to allow efficient transformation. We used a combination of the methods described by Hisano et al (2004) and Norouzi et al (2005) with some modifications to optimize T-DNA delivery and regeneration. Parameters assessed were *Agrobacterium* density, salt composition, concentration of antibiotics and phytohormones (data not shown), as outlined by Jacq et al (1993) and Opabode (2006). The selective agent used in our experiments was kanamycin, due to the presence of the *nptII* gene in the pBIAH23 plasmid used for transformation. The use of an optimal concentration of selective agent, which would avoid the recovery of pseudo-transformants and would not negatively affect morphogenesis, is a crucial for obtaining transgenic sugar beet plants. Kanamycin is usually used at concentrations from 70 to 150 mgl^{-1} depending on the type of explants and genotype of sugar beet (Gurel et al., 2008). We determined survival of explants at different kanamycin concentrations (30, 50, 75, 100, and 150 mgl^{-1}) and found that a concentration of 75-100 mgl^{-1} over a selection period of 40 days is suitable for selections, because, in our experimental conditions, it determines 100% death of the control (non-transgenic) shoots (Fig. 2f). On the other hand, concentrations of kanamycin higher than 100 mgl^{-1} inhibited chlorophyll synthesis even in transgenic shoots (Norouzi et al., 2005) and repressed subsequent regeneration of transgenic plants (Ivic-Haymes and Smigocki, 2005). Except for the early stages, kanamycin was used at a concentration of 100 mgl^{-1} for selection of transformants. Useful features of *Agrobacterium*-mediated transformation include transfer of relatively large segments of DNA with defined ends and with minimal rearrangement as well as integration of small numbers of gene copies into plant chromosomes and a high quality and fertility of the transgenic plants. Although sugar beet is susceptible to *Agrobacterium* infection (Krens et al., 1988), efficiency of regeneration is low and genotype-dependent (D'Halluin et al., 1992). Direct organogenesis in sugar beet is less genotype-dependent (Jacq et al., 1993; Toldi et al., 1996). Direct shoot regeneration has been obtained from various explants including petiole and leaf (Detrez et al., 1988; Freytag et al., 1988; Krens and Jamar, 1989) as well as shoot base explants (Hisano et al., 2004). Transformation frequency is dependent on the explant type, genotype and selection conditions (Zakharchenko et al., 2000; Hisano et al., 2004; Bekheet and Solliman, 2007; Norouzi et al., 2005). We obtained transgenic shoots from leaf blade and regenerated shoot-base tissues co-cultivated with *Agrobacterium* strain GV3101 harbouring the pBIAH23 plasmid (Fig. 1). For each cultivar, the experiment was carried out with a total of 500 explants. The number of regenerated shoots obtained at 50 mgl^{-1} kanamycin was 240 and 260 for cultivars SBSI-01 and SBSI-02, respectively. Shoots were transferred to a medium containing 100 mgl^{-1} kamamycin and only 50 green shoots were obtained for each cultivar (Table 1). PCR analysis confirmed the presence of the *Pvpgip2* gene in 22 and 21 kanamycin-resistant plants of cultivars SBSI-01 and SBSI-02, respectively (Table. 1). Successful introduction of the *Pvpgip2* gene had therefore taken place in 4.4% and 4.2% of the analysed kanamycin-resistant plants of genotype SBSI-01 and SBSI-02, respectively.

Characterization of the transgenic sugar beet plants harbouring the Pvpgip2 gene

Before testing the sugar beet transgenic lines, the inhibitory activity of the protein extracts of wild type (untransformed) sugar beet plants was assayed by agarose diffusion assay against *Fusarium phyllophilum* PG (FpPG), which is inhibited by PvPGIP2 (Leckie et al., 1999; D'Ovidio et al., 2004). It is worth noting that the FpPG employed in this screening shows an aminoacid identity greater than 98% with the polygalacturonases produced by *Fusarium oxysporum*. Crude protein extracts prepared from frozen leaves of the 43 independent transgenic plants (T_0) containing the *Pvpgip2* gene were analyzed. Inhibitory activity against FpPG was found at various levels in several transgenic plants (Fig. 3). Expression of the *Pvpgip2* gene occurred in 8 and 7 of the analysed kanamycin-resistant and PCR positive plants of cultivars SBSI-01 and SBSI-02, respectively (Table. 1). De Bolle et al (2003) also demonstrated a high variation of transgenic expression in *Arabidopsis thaliana*. Four out of 22 transgenic plants showed high level of expression of the PGIP (≥75% inhibition) and 4 plants showed 25-50% inhibition in SBSI-01 cultivar (Fig.4). Also, in SBSI-02 cultivar it was shown that, three out of 21 transgenic plants

Table 1. Transformation efficiency of tissue-cultured leaf explants of two sugar beet genotypes using the pBIAH23 plasmid carrying the *Pvpgip2* 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 expression PvPGIP2	Transformation efficiency [d]
SBSI 01	500	240 (%48)	50(%21)	22(%44)	8	%4.4
SBSI 02	500	260(%52)	50(%19)	21(%42)	7	%4.2

[a] In parenthesis, the number (x100) of regenerated shoots from explants incubated in the presence of 50 mg/l kanamycin / number of explants, [b] In parenthesis, the number (x 100) of green shoots obtained at 100 mg/l kanamycin / number of regenerated shoots obtained 50 mg/l kanamycin, [c] In parenthesis, the number (x 100) of PCR –positive plants / number of green shoots obtained at 100 mg/l kanamycin, [d] The number (x 100) of PCR – positive plants / number of explants

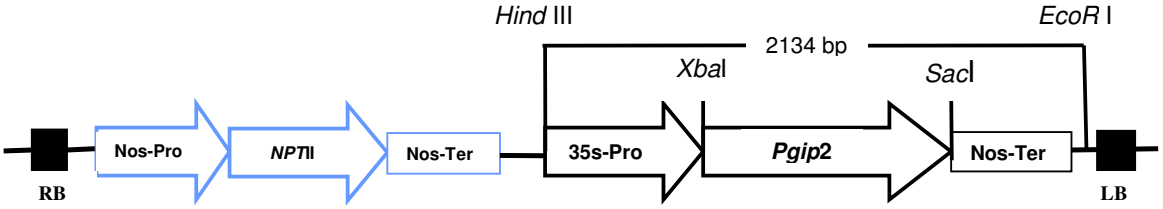

Fig 1. The T-DNA in the pBIAH23 plasmid used for transformation. pBIAH23 is a pBI121-derived plasmid containing the cassette for expression of the *Pvpgip2* gene under the control of 35S-promoter and NOS-terminator in the pBI121 vector from which the GUS gene was excised. Abbreviations: RB, right border; LB, left border.

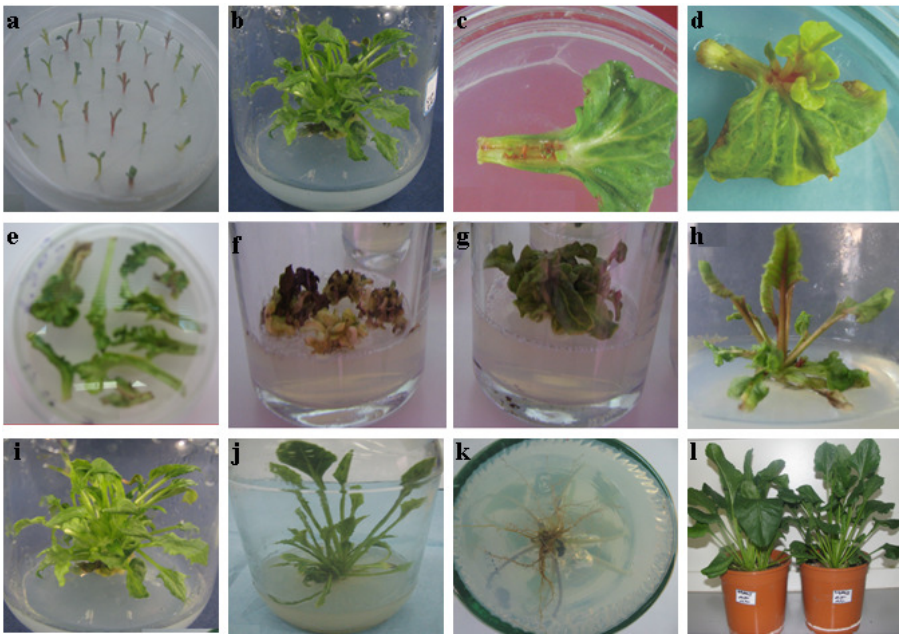

Fig 2. The process of transformation and regeneration of sugar beet plants. (a) Shoot apices on shoot-inducing medium I; (b) Shoots obtained from shoot apices; (c, d) Leafs from shoots shown in b were used for transformation on shoot-inducing medium II; (e) Co-cultivation of explants with *Agrobacterium*; (f,g) Chlorotic shoots and green kanamycin-resistant shoots formed on selection medium; (h) Regenerated shoots transferred to growth medium; (i) Putative transgenic shoots propagated on shoot-propagation medium for preparation of clones. Many shoots formed around the shoot base; (j,k) Putative transgenic plant with induced roots on root-inducing medium; (l) The regenerated plant transplanted to a pot and acclimated to non-aseptic environment.

demonstrated 75% inhibition and four plants showed 50% inhibition (Fig.4). The interactive effect between PvPGIP2 and FpPG was found to be statistically non-significant for the remaining of transgenic plants in both cultivars (SBSI-01 and SBSI-02). The wild type sugar beet extracts did not display any inhibitory activity against FpPG (Fig. 3 and 4). Many factors such as transgene location and copy number can contribute to variation in transgene expression (Matzke ans Matzke, 1998; Iyer et al, 2000; Matzke et al, 2000). The presence of the *PvPGIP2* gene in these plants was confirmed by Southern analysis. The different sizes of the transgene bands obtained from the digestion with the restriction enzymes indicated stable integration of the transgenes at different loci in the sugar beet genome (Fig. 5). The transgenic plants had simple hybridization patterns and were estimated to harbor 1 or 2 transgene copies. No alterations of growth and development were observed in the transgenic plants. In conclusion, based on the results presented in this study indicating that the *pgip*2 gene from *Ph. vulgaris* is an efficient polygalacturonase inhibitor, and it will be useful to improve sugar beet fungal resistance.

Materials and methods

Plant material and in vitro culture

Diploid sugar beet genotypes SBSI-01 and SBSI-02 were provided by the Sugar Beet Seed Institute, Karaj, Iran. These genotypes have a high potential yield. Seeds were scarified in concentrated H_2SO_4 for 60 min and washed three times with sterile deionized water for remove H_2SO_4. Seeds were then surface-sterilized for 1 min in 70% (v/v) ethanol and for 20 min in 5% (w/v) sodium hypochlorite. Sterilized seeds were immediately washed with sterile distilled water three times. 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 bacterial-induction medium (pH 5.5). MSB nutrient medium contained 30 gl^{-1} sucrose and 8 mgl^{-1} agar. Sterile seeds were placed onto petri dish containing MSB medium containing 8 mgl^{-1} agar and 0.5 mgl^{-1} of 2, 3, 5-triiodobenzoic acid (TIBA) and grown in the dark at 22–25°C. Seeds were germinated at 23°C –25°C under a 16 h photoperiod. After the seeds germinated, shoot-apex explants were excised from one-week-old seedling and were placed onto the shoot-inducing medium I [MSB, 1 mgl^{-1} N6-BenzylAdenine (BA), 0.1 mgl^{-1} of α-naphthaleneacetic acid (NAA) and 0.5 mgl^{-1} 2,.3,5-triiodobenzoic acid (TIBA)] (Fig. 2a). Two weeks later, shoots were transferred to the shoot-inducing medium II [MSB, 0.5 mg/l BA and 0.1 mgl^{-1} Indole-3-Butyric Acid (IBA)] for optimal shoot development (Fig. 2b). For induction of shoot regeneration from butts, leaf blades were cut from the shoots and placed on shoot-inducing medium II to induce regeneration of shoots around the main vein of the leaves (Fig.2c, d). 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^{-}) (Jafari *et al*., 2009)

Agrobacterium strain and plasmids

The binary vector pBI121 was used to obtain the plasmid pBIAH23, containing the *P. vulgaris pgip2* gene within the T-DNA region (Fig. 1). A *Xba*I-*Sac*I DNA fragment containing the *pgip2* gene of *P. vulgaris* cultivar Naz Red Bean (Accession number: DQ105560) was excised from the pAH21 plasmid, a pUC18 derivative, and cloned into the pBI121 vector upstream of the *Nos* terminator and downstream of the CaMV 35S promoter, replacing the GUS coding sequence. The pBIAH23 plasmid was introduced into *Agrobacterium tumefaciens* (GV3101) using the freeze-thaw method (Sambrook and Russell 2001). Transformed bacteria were grown in LB medium (Sambrook and Russell, 2001) containing 50 mgl^{-1} rifampicin and 50 mgl^{-1} kanamycin for 48 h at 28°C on a rotary shaker (200 rpm) and then used for genetic transformation.

Transformation and plant regeneration

Agrobacterium GV3101 cells harbouring the pBIAH23 construct were grown for 2 days at 28°C on a rotary shaker at 180 rpm in liquid LB medium containing 50 mgl^{-1} kanamycin, 50 mgl^{-1} rifampicin and Gentamicin 50 mgl^{-1} until an $OD_{600\ nm}$ of 0.6 - 0.7 was reached (Norouzi et al., 2005). Bacterial cultures were centrifuged at 3,500 rpm at 4 °C for 10 min and pellets were resuspended in bacterial-inducing medium (0.5X MSB medium). After addition of 200 µM of acetosyringone, bacteria were further cultivated at 28°C to an $OD_{600\ nm}$ of 1. The culture was diluted with liquid MS medium before co-cultivation with plant explants to obtain a final $OD_{600\ nm}$ of about 0.3 (Mishutkina et al., 2010; Chilton, 1974). The explants obtained as described above were immersed in the *Agrobacterium* suspension for 5 min, transferred on sterile filter paper and then onto a co-cultivation medium (0.5X MSB containing 100 µM acetosyringone) for 3 days (Fig. 2e).

For determination of optimal concentration of selective agent, explants, after co-cultivation, were placed onto shoot-induction medium I plates (20 explants per plate) containing different concentrations of kanamycin (30, 50, 70, 100, and 150 mgl^{-1}). Concentrations of 70-100 mgl^{-1} of kanamycin were found to be optimal. We therefore chose the following selection scheme: After a 3-day co-cultivation (Fig. 2e), explants were washed for 1 h under shaking with half-strength MSB containing 500 mgl^{-1} cefotaxime. Explants were then placed on selection medium containing MSB, 0.25 mgl^{-1} BA, 0.1 mgl^{-1} IBA, 50 mgl^{-1} kanamycin and 250 mgl^{-1} cefotaxime. After 2 weeks, explants carrying regenerated shoots were transferred onto fresh selection medium containing 100 mgl^{-1} kanamycin and sub-cultured every 2 weeks (Fig. 2f,g). When green shoots were large enough, they were transferred to shoot growth medium (SGM), composed of MSB medium supplemented with 30 gl^{-1} sucrose, 8 gl^{-1} agar, 0.25 mgl^{-1} BA, 0.1 mgl^{-1} IBA and 250 mgl^{-1} cefotaxime with no kanamycin, placed in a growth room and sub-cultured every 2 weeks (Fig. 2gh). Shoots longer than 60 mm were placed on root-inducing medium (RIM) which was half -strength MSB containing 30 gl^{-1} sucrose, 1.5 mgl^{-1} NAA, 1.5 mgl^{-1} IBA and 8 gl^{-1} agar (Fig. 2i). As soon as the root size reached approximately 5 mm, the regenerated plants were transferred onto the same medium containing no hormones to avoid malformation of the roots (Fig. 2jk). Plants were transferred into pots when roots looked branched and strong enough. After 2–4 weeks of growth in shade and high humidity conditions under plastic, plants were transferred to a regular growth chamber (Fig. 2l). Acclimated plants were vernalized for 2 months in a cold house at 4°C and then transplanted into the greenhouse for production of bolting shoots.

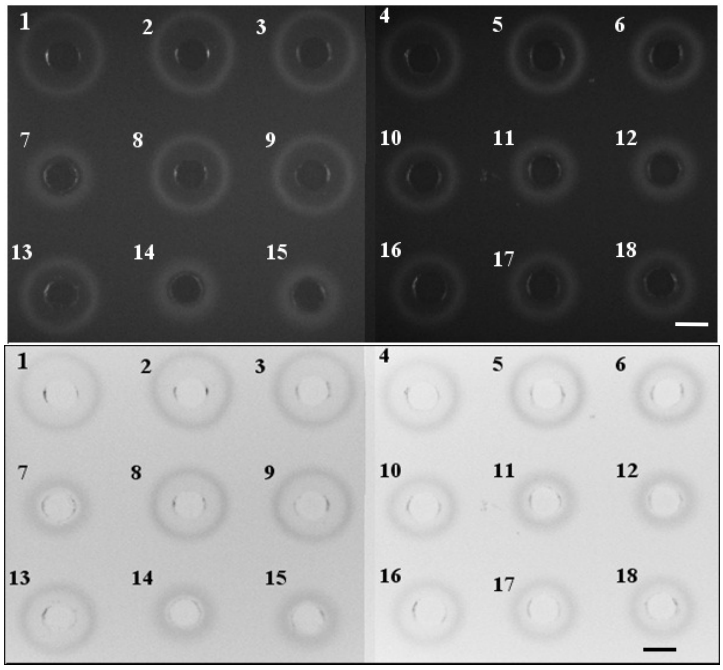

Fig 3. PGIP activity in a subset of the transgenic sugar beet plants analysed using a semi-quantitative agarose diffusion assay. 1**,** FpPG alone (control). 2**,** FpPG plus 30 µg of protein extract from untransformed sugar beet that do not display any inhibitory activity against FpPGs. 3-18, FpPG plus 30 µg of protein extracts from different transgenic sugar beet plants. Samples 7 (T_0-125), 11 (T_0-021), 12 (T_0-oII), 14 (T_0-036), 15 (T_0-119) exhibit high inhibitory activity, whilst samples 3 (T_0-027), 8 (T_0-0II), 9 (T_0-115) and 13 (T_0-116) do not show any inhibitory activity. Bottom, the experiment is shown also in the inverted color version. Bar indicates 10mm.

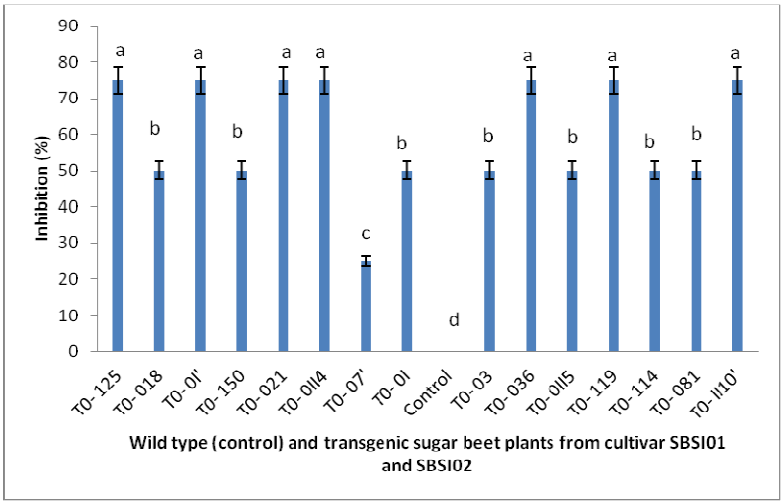

Fig 4. The data were obtained as a mean of 3 replications. The different letters denote a statistically significant difference at $P \leq 0.05$, as determined by Duncan's multiple range tests. Vertical lines represent standard errors.

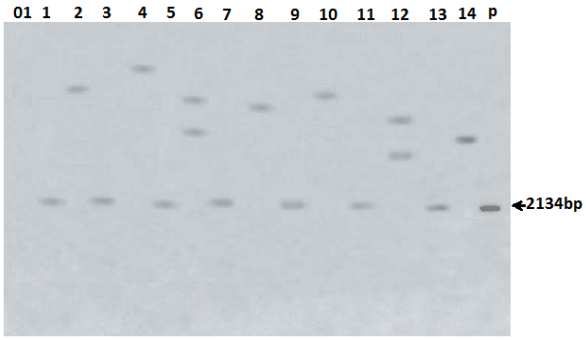

Fig 5. Southern blot analysis of transgenic sugar beet plants expressing PvPGIP2. Cultivar SBSI-01: Genomic DNA of transgenic plants expressing PvPGIP2 was digested with *EcoR1/HindIII* (T_0-125, lane 1), *EcoR1* (T_0-125, lane 2), *EcoR1/HindIII* (T_0-021, lane 3), *EcoR1* (T_0-021, lane 4), *EcoR1/HindIII* (T_0-0II4, lane 5), *EcoR1* (T_0-0II4, lane 6), *EcoR1/HindIII* (T_0-018, lane 7), *EcoR1* (T_0-018, lane 8). Cultivar SBSI-02: Genomic DNA was digested EcoR1*/HindIII* (T_0-036, lane 9), *EcoR1* (T_0-036, lane 10), *EcoR1/HindIII* (T_0-119, lane 11), *EcoR*1 (T_0-119, lane 12), *EcoR1/HindIII* (T_0-II10′, lane 13), *EcoR*1 (T_0-II10′, lane 14). Lane 01 represents the untransformed plant (negative control). The arrow indicates pBIAH23 digested with *EcoR1/HindIII* (shown in Fig.1), used as a positive control (lane P).

DNA extraction and PCR analysis

Genomic plant DNA was isolated by using a CTAB extraction method adapted from Doyle and Doyle (1990) and Dellaporta et al (1983). Tissue was ground in liquid nitrogen and incubated in a lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 100 mM ethylene diaminetetraacetic acid (EDTA) and 20% (w⁄v) sodium dodecyl sulphate (SDS), 5 M NaCl and CTAB 20% (w⁄v). DNA was extracted with an equal volume of phenol:chloroform: 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 water. Evidence for the presence of the transgene was provided by PCR amplification. The PCR primers, specific to the *pgip2* gene, were 2RB1 (5′-GCTCTAGAATGTCCTCAAGCTTAAGCAT-3′) and 2RB2 (5′-GCACGAGCTCTTAAGTGCAGGCAGGAAG-3′) to amplify the transgene (1002 bp). The PCR reaction conditions were as follows: initial denaturation for 5 min at 94°C, followed by 30 cycles consisted of denaturation for 30 min at 95°C, annealing for 45 min at 58°C, extension for 1 min at 72°C, and a further extension step for 10 min at 72°C. PCR products were separated by electrophoresis on a 1.0% agarose gel and visualized by ethidium bromide staining.

Southern blot hybridization

Southern blot analysis of PGIP2-overexpressing sugar beet plants was performed upon digestion of genomic DNA (25 µg) with *EcoR1* and *EcoR1/HindIII*. Both enzymes cut once in the transgene cassette and the *EcoR1/HindIII* digestion releases a 2134 bp diagnostic fragment. Digested DNA was separated by gel electrophoresis on a 0.8% agarose gel. The DNA was blotted onto 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 *Pvpgip2* gene was used as a probe. The fragment was labeled with DIG-dUTP using PCR DIG Probe Synthesis Kit (Roche Applied Science, Germany). Hybridization, high stringency washes and detection were performed according to the instruction manual of the DIG DNA labeling and detection kit.

PGIP 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, 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). The crude protein extracts were assayed for inhibitory activity against endo-polygalacturonases produced by *Fusarium phyllophilum* (FpPG). FpPG was expressed in *Saccharomyces cerevisiae* and prepared as described previously (Caprari et al., 1996). This PG had been indicated in the past as secreted by *Fusarium moniliforme* strain FC-10, which has been recently reclassified as *Fusarium phyllophilum* (Mariotti et al., 2008). The inhibitory effect of the PGIP2 against the fungal PG activity was measured using an agarose diffusion assay (Taylor and Secor, 1988). PGs and/or crude 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 P3850). Plates were incubated for 16 h at 27°C, and the halo caused by the enzyme activity was visualised after a 5 min treatment with 6 N HCl. Inhibitory activity was expressed as the ratio (in percentage) between the radius of the halo observed in the wells containing PG plus the plant protein extract and the halo observed with PG alone (0.5 cm, external to the inoculation well) (Ferrari et al., 2003).

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 by SPSS software. The means were compared using Duncan's multiple range tests.

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