

Transgenic Potato Plants Expressing Soybean β -1,3-Endoglucanase Gene Exhibit an Increased Resistance to *Phytophthora infestans*

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Z. Naturforsch. **53c**, 1012–1016 (1998); received May 11/June 5, 1998

Agrobacterium tumefaciens, Phytoalexins, Phytopathogen, *Solanum tuberosum*, Transgenic plants

Soybean β -1,3-endoglucanase represents a model system for studies on early plant responses to infection by fungal pathogens, and it has been implicated in the release of elicitors from fungal cell walls. In the present study, potato plants were transformed with the soybean β -1,3-endoglucanase cDNA via *Agrobacterium* delivery system. The transfer of the gene into potato genome was confirmed by (i) PCR amplification, (ii) Northern blot analyses, and (iii) an increase in the activity of β -1,3-endoglucanase in transgenic plants. The transformation resulted in an increased resistance of selected transgenic plants to infection by *Phytophthora infestans*, an important pathogen.

Introduction

Pathogenesis-related proteins have been found in all plant-pathogen interactions analysed so far. One of the most extensively studied groups of these proteins belongs to hydrolases, namely β -1,3-endoglucanases and chitinases (Kauffmann *et al.*, 1987; Linthorst, 1991; Beerhues and Kombrink, 1994). They are all characterised by low molecular weight, selective solubility at low pH, and resistance to proteolytic digestion. These proteins are believed to be key host components involved in the earliest plant-pathogen interactions, inducing cell responses (Takeuchi *et al.*, 1990; Linthorst, 1991).

Interactions between soybean and fungal pathogen *Phytophthora megasperma* f.sp. *glycinea* have been extensively studied (Keen and Yoshikawa, 1983; Takeuchi *et al.*, 1990; Umemoto *et al.*, 1997). It has been found that soybean tissues contain a factor, identified as β -1,3-endoglucanase, capable of releasing soluble and highly active elicitor

molecules from mycelial walls of *P. megasperma*; the released elicitors are probably responsible for initiation of phytoalexin accumulation in soybean plants infected with the fungus, triggering signalling events that are necessary for the onset of the defence response (Keen and Yoshikawa, 1983). A protein receptor for elicitors released by the soybean endoglucanase has recently been identified and characterised (Umemoto *et al.*, 1997). Transgenic tobacco plants expressing a cDNA for soybean β -1,3-endoglucanase exhibited high level of resistance to fungal disease (*P. parasitica* var. *nicotiana*) (Yoshikawa *et al.*, 1993), underlying the importance and uniqueness of the soybean glucanase. These tobacco plants were the first transgenics resistant to an Oomycete.

We have previously studied responses of potato cell suspensions to *P. infestans* infection (Awan *et al.*, 1997). The fungus causes the late blight diseases of potato and tomato, resulting in massive losses of revenue for these crops (Judelson, 1997). With this background, we were interested in the use of potato as a transgenic system to study responses and resistance to the pathogen. Potato is highly susceptible to a wide variety of viral, bacterial and fungal diseases, and attempts at improving its pathogen-resistance qualities have had high priority for potato research (Vayda and Belknap, 1992). In the present study, we transformed potato plants with soybean

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0939–5075/98/1100–1012 \$ 06.00 © 1998 Verlag der Zeitschrift für Naturforschung, Tübingen · www.znaturforsch.com. D



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β -1,3-endoglucanase gene, and studied preliminary aspects of the effects of the transformation on potato resistance to *P. infestans*.

Materials and Methods

Plasmid construction and potato transformation

The expression cassette containing soybean β -1,3-endoglucanase cDNA sequence (provided by Prof. M. Yoshikawa, Hokkaido Univ., Sapporo, Japan), under the control of cauliflower mosaic virus (CaMV) 35S promoter, was cloned as *Eco*RI-*Hin*III fragments into the binary vector pGA482 (Fig. 1). The vector contained also neomycin phosphotransferase II (NPT-II) as selection marker. The construct was mobilised into *Agrobacterium tumefaciens* strain that was cultivated at 28 °C in liquid or agar-solidified medium containing kanamycin (50 mg l⁻¹) and rifampicin (100 mg l⁻¹).

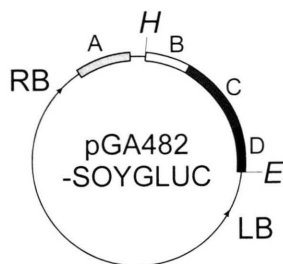


Fig. 1. Schematic representation of pGA482 vector carrying the soybean β -1,3-endoglucanase coding sequence. The construct contained : A) nopaline synthase promoter linked with NPT-II gene; B) 35S promoter; C) soybean β -1,3-endoglucanase coding sequence; and D) 35S polyA. Abbreviations used were: H – *Hind*III; E – *Eco*RI; RB, LB – right and left T-DNA borders, respectively.

Transformation of potato (Bzura cultivar) leaf fragments was performed as described elsewhere (Borkowska *et al.*, 1994), but omitting the feeder layer step and acetosyringone treatment, and replacing carbenicillin with cefotaxime. The *A. tumefaciens*-infected cut leaves regenerated shoots after 6–8 week-long cultivation on the medium containing kanamycin. The shoots which survived the selection pressure of kanamycin and formed roots were subcultured every fourth week. The control and transformed plants, ca. 3–4 cm tall, were transferred from *in vitro* to the growth chamber conditions and grown for 4–5 weeks, until they were ca. 15–20 cm tall. These plants were

used for resistance studies on *P. infestans* infection (see below).

PCR analysis

Total DNA was isolated from 100 mg fresh or liquid N₂-frozen leaves of control and transformed potato plants grown *in vitro* for four weeks. We used both old and young leaves for this procedure. The isolation protocol was generally as described by Fulton *et al.* (1995). The presence of soybean β -1,3-endoglucanase gene in the transformed potato plants was verified by PCR analysis, following standard procedures (Sambrook *et al.*, 1989). Nucleotide primers for amplification of a ca. 1.1 kb portion of DNA encoding soybean glucanase were: 5'-CCTCAACCTTCTTTCTTCTTA-3' and 5'-GTTCTATACCTGCCTCTTCTA-3'. The primers were based on cDNA sequence encoding the soybean protein (Takeuchi *et al.*, 1990), and they were designed to amplify the DNA of soybean but not potato-own glucanase (Beerhues and Kombrink, 1994). The PCR reactions were carried out for 30 cycles using thermocycler (Gene Amp PCR System 2400, Perkin Elmer), and the PCR products were analysed by electrophoresis on a 0.8% (w/v) agarose gel.

RNA preparation and RNA gel blots

Total RNA was extracted from potato leaves, according to protocol of Das *et al.* (1990), and run on glyoxal-agarose gels (Sambrook *et al.*, 1989). The RNA was transferred overnight onto Nylon membrane (Boehringer Mannheim GmbH) according to manufacturer's specifications. Blots were hybridised and processed according to the protocol of Church and Gilbert (1984), using random primed soybean β -1,3-endoglucanase cDNA (Takeuchi *et al.*, 1990) as a probe.

β -1,3-Glucanase activity in transgenic plants

The β -1,3-glucanase activity was assayed by measuring the release of reducing sugars from reduced laminarin (Kombrink and Hahlbrock, 1986), essentially as described by Awan *et al.* (1997). Activity of the enzyme was calculated in katal, *i.e.* moles of product released per second. Protein was determined by the method of Lowry *et al.* (1951).

Fungal growth and infection assay

P. infestans (MP 245, complex race), obtained from the Department of Genetics, Potato Research Institute, Młochów, was maintained on cholesterol-supplemented rye agar medium, as described by Awan *et al.* (1997). The spore suspension was obtained from 18 days old culture on rye agar medium by flooding with sterile distilled water, and sporangial titre was adjusted by using haemocytometer. The detached leaflets obtained from middle part of the control and transformed plants, grown in pots for five weeks in the growth chamber, were inoculated with a single droplet of 30 μ l spore suspension containing 100 sporangia. The controls were treated with distilled water. The inoculated leaflets (50 leaflets per treatment), put on moist filter paper, were covered with transparent foil to maintain the high humidity and were incubated in the dark for 4 h at 10 °C to induce the release of zoospores from sporangia. Then the samples were incubated in the dark for 24 h at 20 °C to facilitate the zoospore germination. Subsequently, leaflets were incubated under the regime of growth chamber. The development of disease symptoms was observed daily until the 7th day after inoculation. Five leaflets of equal size were selected randomly from each treatment and washed in 5 ml of distilled water. In the collected water wash, the sporangial titre was determined by counting in haemocytometer.

Results and Discussion

A construct, containing cDNAs for soybean β -1,3-endoglucanase and a kanamycin-selection marker (Fig. 1), was transferred *via* the *Agrobacterium*-mediated transformation of cut leaves into potato genome. The presence of the soybean gene in potato transformants, regenerated on a kanamycin-containing medium, was detected by PCR. Reactions containing nucleotide primers for the soybean gene gave positive results in the presence of DNA isolated from leaves of transformed, but not wild type, plants (Fig. 2). The size of PCR products was found to be about 1100 bp, which was consistent with positioning of the primers used for amplification in relation to cDNA for soybean β -1,3-endoglucanase (Takeuchi *et al.*, 1990). By Northern blot hybridisation, the soybean β -1,3-endoglucanase probe has been shown to hybridise to

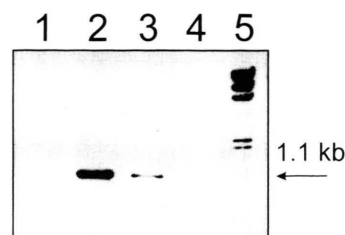


Fig. 2. Agarose gel electrophoresis of PCR products, corresponding to soybean β -1,3-endoglucanase, obtained through amplification of DNA isolated from control and transformed potato plants. Lanes from left to right are: 1 – untransformed Bzura; 2, 3 – transformed Bzura lines (no. 32 and 56); 4 – chemical control (all the chemicals used in the reaction except the DNA template); 5 – size marker (λ DNA/*Hind*III).

a single mRNA species of 1.3 kb in leaf tissue in selected (Bzura no. 32 and 56) transgenic lines (Fig. 3). This was consistent with the size of mRNA for the soybean enzyme (Takeuchi *et al.*, 1990; Yoshikawa *et al.*, 1993). Endogenous expression of potato own β -1,3-endoglucanase genes was not detected under the same conditions using the soybean cDNA, as evidenced by the lack of any bands for untransformed plants (Fig. 3). It is unknown at present how stable is the insertion of the soybean glucanase gene in the transformants. We normally propagate potato plants by vegetative growth (from tubers) rather than from seeds, and the Mendelian inheritance of the transferred gene in consecutive generations has not been tested yet.

The level of β -1,3-endoglucanase activity was checked in leaves of both control and transformed potato plants (Fig. 4A). Examination of individual transgenic plants showed an increased β -1,3-en-

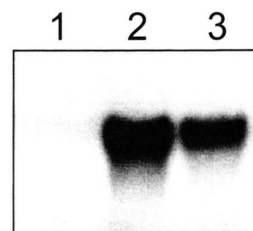


Fig. 3. Detection of the soybean β -1,3-endoglucanase transcript in transformed potato plants. Northern blot analysis was performed on 10 μ g RNA isolated from leaves of untransformed plants (lane 1) and transformed Bzura lines no. 32 and 56 (lanes 2 and 3, respectively). A cDNA for soybean β -1,3-endoglucanase (Takeuchi *et al.*, 1990) was used as a hybridisation probe.

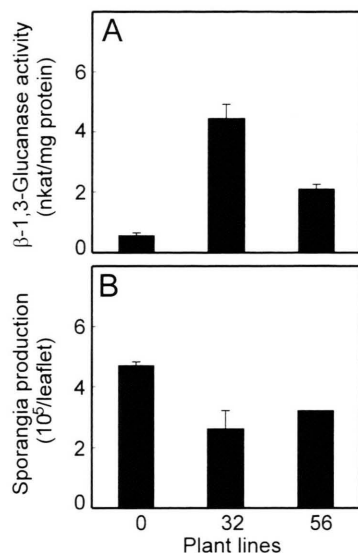


Fig. 4. The β -1,3-endoglucanase activity (A) and the production of sporangia by *P. infestans* mycelia (B) in the leaves of control (0) and transformed potato plant lines of Bzura (no. 32, 56). Results are means of three independent determinations; bars indicate standard error.

doglucanase activity in two selected lines (no. 32 and 56): the activity there was four- and eight-fold higher than in control (untransformed) plants. In comparison, selected tobacco plants transformed with soybean β -1,3-endoglucanase showed an up to four-fold increase in activity when compared to the level found in control plants (Yoshikawa *et al.*, 1993). Several other transformed potato plants that were analysed in the present study had no significant increase of β -1,3-endoglucanase activity. Thus, the presence of the soybean gene was not necessarily leading to higher levels of endoglucanase activity in transgenic potatoes, possibly reflecting constraints imposed by different positioning of the transferred gene in the genome of different potato lines. A similar phenomenon was reported for tobacco plants expressing soybean β -1,3-endoglucanase, where some of the transgenics did not produce the foreign protein (Yoshikawa *et al.*, 1993).

Transgenic potato Bzura lines no. 32 and 56 were subsequently used for studies on disease resistance to *P. infestans* treatment (Fig. 4B). Leaflets of both control (untransformed) as well as transgenic lines developed necrotic spots following inoculation with sporangia of *P. infestans*. The de-

velopment of necrosis was observed for up to four days after inoculation, while mycelia developed mainly after five days of treatment. After seven days of inoculation, the ratio of leaflets with mycelia was significantly lower in transgenic plants than in untransformed plants. The production of sporangia per leaflet was also lower in the former plants (Fig. 4B). For both Bzura lines 32 and 56, there was a correlation between β -1,3-endoglucanase activity (Fig. 4A) and the resistance to the fungus, measured as reduction in sporangia production (Fig. 4B). On the other hand, both control plants and those transgenics that did not show increased glucanase activity were uniformly highly susceptible to the fungus (Fig. 4, data not shown). When compared to wild-type plants, the transgenic Bzura lines no. 32 and 56 did not differ in the shape and size of leaves, but had slightly reduced growth rate (data not shown).

Unlike tobacco plants expressing soybean β -1,3-endoglucanase (Yoshikawa *et al.*, 1993), the potato transgenics were not fully immune to the pathogen attack and some necrosis was evident even in Bzura line no. 32 which had eight-fold higher activity of the glucanase when compared to control plants. Thus, high expression of the soybean gene was not sufficient for full resistance to the pathogen. This may underline some differences in mechanisms of resistance to the *Phytophthora* infection by tobacco in relation to potato plants. Further studies are necessary to elucidate whether the potato-expressed soybean glucanase is indeed involved in the elicitor-releasing process, as shown for the soybean/*Phytophthora* system (Keen and Yoshikawa, 1983; Anzai *et al.*, 1989; Yoshikawa *et al.*, 1993).

As chemical reagents for pathogen resistance control are removed from the market due to their toxicity, transgenic potatoes engineered to express resistance factors that occur naturally in other plant species represent logical and environment-friendly alternative (Vayda and Belknap, 1992). Unlike several other foreign genes used to obtain transgenic plants resistant to viral (Honda and Brar, 1989) and bacterial (Anzai *et al.*, 1989) diseases, the soybean β -1,3-endoglucanase cDNA used by Yoshikawa *et al.* (1993) and in the present study shows a relatively high homology (*ca.* 60% at nucleotide level) to cDNAs for tobacco (Takeuchi *et al.*, 1990) and potato glucanases

(Beerhues and Kombrink, 1994). Despite that, soybean β -1,3-endoglucanase conferred a degree of resistance to fungi infection of transgenic potato when compared to wild-type plants (Fig. 4B). Thus, structural differences between potato and soybean β -1,3-endoglucanases are sufficient for developing resistance to the pathogen. The same consideration applies to the soybean *versus* tobacco endoglucanases, based on work with transgenic tobacco plants (Yoshikawa *et al.*, 1993). Given the resurgence in the world-wide impact of the late blight disease caused by *P. infestans* (Judelsson, 1997), the present studies may represent a

promising step forward in attempts to fight off this disease in potato.

Acknowledgments

The authors are grateful to Prof. M. Yoshikawa for the gift of soybean β -1,3-endoglucanase expression cassette, and to Prof. H. Zarzycka for *Phytophthora* fungi. This research was supported, in part, by KBN grant 0416/P2/93/04 to K. K. M.F. M. A. was financially supported by a Ph.D. fellowship from the Ministry of Education of Poland, and L. Y. was supported by the European Fellowship Found.

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