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Generation of white mold disease-resistant sunflower plants expressing human lysozyme gene

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Abstract

Sunflower plants were transformed *via* co-cultivation of previously bombarded hypocotyl explants with *Agrobacterium tumefaciens* harboring the plasmid pNGL that contains the human lysozyme gene. The transformed shoots were selected using kanamycin and regenerated plants were analyzed using histochemical β-glucuronidase assay. Southern, Western and Northern blot analyses indicated the transfer, expression and stable integration of the foreign DNA into the sunflower genome. Resistance against the phytopathogenic fungus *Sclerotinia sclerotiorum*, which causes white mold disease, was confirmed using a phytopathogenic test and microscopic observation of the infection process.

Additional key words: *Agrobacterium tumefaciens*, fungal disease, *Helianthus annuus*, *Sclerotinia sclerotiorum*, transgenic sunflower.

Introduction

Sunflower (*Helianthus annuus* L.) is a major crop worldwide for the production of edible oils. However, cultivated sunflower is susceptible to several economically important fungal diseases such as downy mildew (*Plasmopara halstedi*), rust (*Puccinia helianthi*), white mold (*Sclerotinia sclerotiorum*), verticillium wilt (*Verticillium dahliae*), gray mold rot (*Botrytis cinerea*), and powdery mildew (*Erysiphe cichoracearum*). The fungus *Sclerotinia* causes sunflower diseases that account for half of disease-related yield losses (Laferriere 1986).

Since cultivated sunflower has a very narrow genetic base, an increase of its genetic variability by sexual and somatic hybridization with wild *Helianthus* species could provide the necessary diversity for many important agronomic traits including disease and insect resistance, gain in productivity and cytoplasmic male sterility for hybrid seed production (Rieseberg *et al*. 1999). However, sexual incompatibility between sunflower and these species as well as the sterility of hybrids significantly limits the feasibility of sexual hybridization. In addition, successful application of somatic hybridization has been very limited as a result of the lack of a protoplast regeneration system for some cultivated and wild sunflower species, and the lack of genetic markers that could be used for the selection of hybrids (Krasnyanski and Menczel 1995). Thus, utilization of genetic transformation technology for the introduction of disease resistance genes into sunflower is of special interest for improving sunflower cultivars (Müller *et al*. 2001).

There have been attempts to use gene technology as an alternative method to protect plants from microbial diseases (Lamb *et al*. 1992). Various genes have been introduced into plants such as tabtoxin acetyltransferase gene (Anzai *et al*. 1989), the stilbene synthase gene (Hain *et al*. 1993), the ribosome-inactivation protein gene (Longemann *et al*. 1992), the glucose oxidase gene (Wu *et al*. 1995) and the enhanced resistance against microbial pathogens has been demonstrated. In addition, genes encoding hydrolytic enzymes such as chitinase and glucanase that can degrade fungal cell wall components have been used for the production of fungal disease-resistant plants (Nishizawa *et al*. 1999, Yamamato *et al*. 2000). Furthermore, the use of the human lysozyme gene has been proved to be a simple and effective approach for an engineered disease resistance against phytopathogenic bacteria and fungi derived from a single transgene (Nakajima *et al*. 1997).

This is the first report describing the expression of the human lysozyme gene in transgenic sunflower plants and the protection it confers against the phytopathogenic fungus *Sclerotinia sclerotiorum*.

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Materials and methods

Plants: Greenhouse-grown sunflower plants (*Helianthus annuus* L. cv. Maick) were used to obtain hypocotyl explants in transformation experiments. The conditions under which the plants were cultivated were: temperature at least 20 °C, 16-h photoperiod with irradiance of 70 μmol m^{-2} s^{-1} provided by mercury lamps.

Plasmid DNA: The plasmid pNGL (Nakajima *et al*. 1997) was used for transformation of *Agrobaterium tumefaciens* LBA4404. It contains the structural gene for neomycin phosphotransferase-II *npt II*, which encodes resistance to the antibiotic kanamycin sulphate, the β-glucuronidase *gus* reporter gene and the human lysozyme gene. The expression of the human lysozyme gene is under the control of the cauliflower mosaic virus (CaMV) 35S promoter and is terminated by the nopaline synthase *Nos* gene terminator.

Plant transformation: Seeds were collected 10 - 14 d after pollination as described by Müller *et al*. (2001). They were surface sterilized in 70 % (v/v) ethanol for 2 min, followed by incubation in 4 % (v/v) sodium hypochlorite for 20 min. Afterwards they were washed four times in sterile water. Excised immature zygotic embryos (4 - 6 mm) were cut once transversally below the cotyledons. The resulting explants (HE) consisting of hypocotyl and radicle were placed with the cut surface on MSB medium containing Murashige and Skoog's salts (Murashige and Skoog 1962) and vitamins, 0.1 mg dm^{-3} 6-benzylaminopurine (BAP), 30 g dm^{-3} sucrose and 0.6 % agar. The pH was adjusted to 5.8 using 1 M NaOH. Explants were first cultured in the dark at 25 °C (Jeannin *et al*. 1998, Müller *et al*. 2001).

One-day-old HEs were placed densely within the central area of a Petri plate containing MSB medium. The HEs were bombarded with gold particles prior to *Agrobacterium* infection to increase transformation efficiency (Bindney *et al*. 1992). Flight distance of the uncoated gold particles (diameter 1.6 μm) was adjusted to 6 cm, and bombardments were carried out as described by Sawahel (1997) using a *PDS 1000/He* microprojectile gun (*Bio-Rad*, Leeds, UK).

Cells from *Agrobacterium tumefaciens* strain LBA4404 transformed with the plasmid pNGL were collected and washed once in one volume MS medium. Cells were adjusted with MS medium to an absorbance A_{546} of 1.0 and acetosyringone was added to a final concentration of 200 μM. Immediately after bombardment, the explants were covered with 0.05 cm^3 of this *Agrobacterium* suspension and allowed to grow for 3 d in the dark.

Regeneration and selection of transgenic lines: 3 d after transformation, explants were placed on MSB medium containing 50 mg dm^{-3} kanamycin as a selective agent (Knittel *et al*. 1994) and 250 mg dm^{-3} cefotaxime. Adventitious shoots emerged in clusters within the following 10 d. These shoot clusters were then dissected, transferred to new MSB medium and cultured in light (16-h photoperiod, 200 μmol m^{-2} s^{-1}). One week later shoots were separated and transferred to MSBS medium containing MS salts and Gamborgs BS vitamins (Gamborg *et al*. 1968). The pH and agar content were adjusted as described for MSB medium. Additionally, 5 mg dm^{-3} indole-3-acetic acid (IAA) was added to the autoclaved medium after filter sterilization. These shoots were allowed to grow for 5 d on this medium and finally transferred to MSBS medium containing only 0.5 mg dm^{-3} IAA where they developed roots in about 10 - 14 d. Plantlets with roots of about 1 cm length were potted into soil and transferred to the greenhouse.

Southern blot analysis: Total leaf genomic DNA was isolated using the *Genome Clean Kit* (*AGS*, Jena, Germany). DNA (15 μg) was digested with 25 U of the appropriate restriction enzyme, separated on a 0.8 % (m/v) agarose gel and blotted to nylon membranes. A fragment of human lysozyme gene was labelled using the *RadPrime* DNA labeling kit (*Life Technologies*, Stuttgart, Germany) and used as a probe. Blots were hybridized overnight at 65 °C and washed four times with sodium buffer (6x SSC; 2 times 2x SSC; 0.1x SSC) with an additional 1.0 % (m/v) SDS at 65 °C. Signals were visualized using *Kodak X-omat* films.

Western blotting: Total soluble protein was obtained by homogenizing young leaves of the transgenic and control sunflower plants in extraction buffer (200 mM Tris-HCI, pH 6.8, 20 % glycerol, 10 % 2-mercaptoethanol). Ten μg of protein was separated by 10 % SDS-polyacrylamide gel electrophoresis (Laemmli 1970) and transferred to nitrocellulose membrane (*Schleicher & Schuell*, Dassel, Germany). Immuno-detection using anti-human lysozyme antiserum was performed using a *hyperfilm-ECL* (*Amersham*, Oxford, UK) as recommended by the manufacturer.

Northern blot analysis: Total RNA was extracted from 1 g of the transgenic and control sunflower leaves and the poly (A)+RNA was purified using *ISOGENTM* (*Nippon Gene*, Tokyo, Japan) and *OligotexTM-dT30* (*Takara*, Kyoto, Japan) respectively. Two μg poly(A)+RNA were resolved on denaturing agarose gel and blotted onto *Hybond-Nylon* membrane (*Amersham*, Tokyo, Japan) by the capillary transfer method. Northern hybridization was carried out with labelled probe of human lysozyme gene prepared with random primers (Nakajima *et al*. 1997). The hybridized membrane was analyzed using an Image Analyzer *BAS2000* (*Fuji Film*, Tokyo, Japan).

Fungus infection assay: Sunflower plants were grown in chambers maintained at 25 °C under a 16-h photoperiod.

Sclerotinia sclerotiorum was cultured on potato dextrose agar (PDA) medium at 25 °C for 4 d. For plant inoculation, the agar discs (5 mm diameter) were excised and adhered to the upper surface of one-month-old plant leaves. The inoculated plants were maintained at 25 °C and 95 % humidity (Terakawa *et al*. 1997).

Light microscopy of *S. sclerotiorum* infection process: Light microscopic observations were made of leaves of the white mold resistant transformants and of the non-transformant after inoculation with conidia of *S. sclerotiorum*. About 0.025 cm^3 of the conidial suspension (10^4 conidia cm^{-3}) was dropped onto the surface of each leaf. These sunflower plants were incubated under saturated humidity for 48 h and then kept in an isolated greenhouse at 25 °C. From these leaves; discs (diameter of 10 mm) were cut out, fixed and discoloured in FAA solution (formaline:ethanol:acetic acid, 1:1:1). After staining with methyl blue solution, the leaf discs were observed under light microscopy.

Generation of transgenic plants expressing human lysozyme gene: Sunflower was transformed *via* co-cultivation of previously bombarded hypocotyl explants with *Agrobacterium tumefaciens*. Twenty-three clones regenerated under selection by kanamycin were used to measure GUS activity using histochemical assay (Jefferson 1987), and 6 clones with relatively high GUS activity were selected for Southern blot analysis.

Results

Southern blot analysis (Fig. 1) was carried out using genomic DNA isolated from 2 independent transgenic lines, SH-1 and SH-2, of primary transformant plants (Ro). The probe for the human lysozyme gene was hybridised to genomic DNA of the two plants, which had been digested with *BamHI*, and for the two transgenic plants bands of expected size of about 600 bp were observed (Fig. 1). The probe was further hybridised to undigested DNA of the two transgenic lines. Hybridisation occurred only to the uncut high molecular mass chromosomal plant DNA, and not to smaller pieces of DNA, indicating that the expressed transgene had integrated into chromosomal DNA of the transgenic plants (Fig. 1). The profile of genomic DNA digested with *BamHl* showed a single band in all transgenic lines, suggesting that each transgenic line had a single integration site with one or more copies of the transgene and the line SH-1 has higher copy number of integrated lysozyme gene than the line SH-2. There was no hybridisation signal from the untransformed sunflower DNA (Fig. 1).

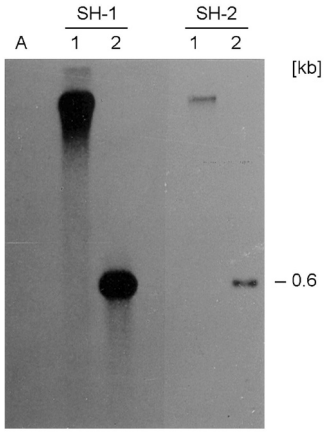

Fig. 1. Southern blot analysis of genomic DNA from 2 independent primary transgenic lines (SH-1 and SH-2) probed with the human lysozyme gene. *Lane A* - DNA isolated from untransformed plants, *lane 1* - uncut DNA from transformed plants, *lane 2* - *BamHI* digested DNA from transformed plants.

The regenerated transgenic plants displayed normal development in both the growth chamber and the greenhouse and were phenotypically indistinguishable from non-transgenic plants.

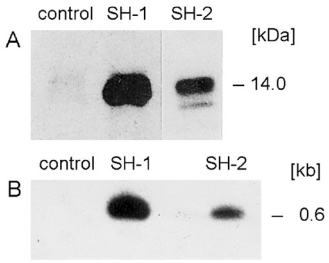

Fig. 2. Expression of the human lysozyme gene in control and transgenic sunflower plants. *A* - Western blot analysis of proteins extracted from 2 independent primary transgenic lines (SH-1 and SH-2). *B* - Northern blot analysis of poly(A)+ RNA isolated from 2 independent primary transgenic lines (SH-1 and SH-2).

In order to select elite candidates for the phyto-pathological test, the accumulation of human lysozyme gene was examined in these transformed clones. Leaf extracts prepared from the selected transformants were subjected to Western blot analysis with antiserum against human lysozyme (Fig. 2*A*). A specific positive band of approximately 14 kDa, which was consistent with the molecular mass of standard human lysozyme, was clearly detected in extracts from the two lines of the transformed clones SH-1 and SH-2. A positive signal was not observed in extracts from non-transgenic plant, even after a long incubation with the substrates for alkaline phosphatase. The accumulation of the synthesized human lysozyme in clone SH-1 was higher than that in the transgenic clone SH-2. In other transgenic clones, faint positive signals were observed.

The expression of the introduced human lysozyme

gene was also checked in these 2 clones by Northern blot analysis (Fig. 2*B*). Signals corresponding to approximately 0.6 kbp – that represent the correct size of the human lysozyme messenger RNA – were detected in these 2 clones but not in the non-transformed control. The expression of human lysozyme gene in SH-1 was remarkably higher than tkat in SH-2, which indicated a good correlation with the accumulation of 14 kDa proteins (Fig. 2*A*) and the high copy number of integrated lysozyme gene (Fig. 1).

Resistance against *Sclerotinia sclerotiorum* was examined for two selected clones (Table 1). The clones SH-1 and SH-2 showed a greatly reduced number of colonies and the efficiencies of colony formation were estimated to be 0.07 % and 0.82 %, respectively, which were lower than that of the non-transgenic control (2.65 %). In addition, SH-1 and SH-2 showed a drastic decrease in colony area (0.02 % and 0.05 %) compared with that of the non-transgenic control (9.67 %). A longer incubation of these plants led to an enlargement of colonies in both the non-transgenic and the transgenic plants SH-1 and SH-2, but a delayed development of symptoms in these transgenic plants was evident throughout the experiments. These results indicate that the expression of human lysozyme in plants enhances resistance to the *S. sclerotiorum*.

Table 1. Resistance of control and transgenic sunflower plants to *Sclerotinia sclerotiorum*. Colony formation is represented as the percentage of formed colonies to inoculated conidia. Colony area means percentage of area covered with colonies to total area of the leaf. Means ± SE of 3 independent experiments.

Plants	Colony formation [%]	Colony area [%]
Control	2.65 ± 1.31	9.67 ± 1.90
SH-1	0.82 ± 0.24	0.05 ± 0.01
SH-2	0.07 ± 0.03	0.02 ± 0.004

Under light microscopy, no distinct differences were found in conidial germination and mycelial growth between transformed and non-transformed plants at the early stage of infection. Three days after inoculation, however, the mycellial growth and formation of the haustoria were clearly inhibited on the leaf of the transgenic sunflower SH-1. The extension and development of hyphal branches were reduced to about 20 % of those of the non-transformed plants, while normal development of these structures was observed in non-transformant plants. In addition, hyphae from germinated conidia grew poorly and became malformed. Furthermore, the number of conidophores and secondary conidia drastically decreased in transgenic sunflower. Finally, the colonies were smaller in transgenic sunflower than in the non-transformed plants.

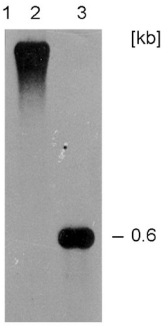

Fig. 3. Southern blot analysis of genomic DNA from R_1 plant obtained from the primary transgenic line SH-1. *Lane 1* - DNA isolated from an untransformed control, *lanes 2, 3* - uncut DNA and *BamHl* digested DNA from R_1 transformed plant.

These findings strongly suggested that the development of haustoria was remarkably disturbed in the epidermal cells of the transgenic sunflower expressing the human lysozyme gene.

In order to investigate the inheritance of resistance against *S. sclerotiorum*, the primary transgenic sunflower plants SH-1 were self pollinated and the progeny was examined for the presence of the human lysozyme gene by Southern hybridization and resistance against *S. sclerotiorum* by artificial infection. It was confirmed that 50 of 68 plants exhibited resistance against white mold. These progenies were found to harbour the human lysozyme gene (Fig. 3). The segregation of disease resistance among progenies was in accordance with the predicted Mendelian ratio of 3:1 (resistant:susceptible), as tested by χ^2 analysis ($\chi^2 = 0.078$, $P < 1$ %).

Discussion

Previous reports on transformation of sunflower plants (Everett *et al.* 1987, Schrammeijer *et al.* 1990, Knittel *et al.* 1994, Malone-Schoneberg *et al.* 1994, Burrus *et al.* 1996, Rao and Rohini 1999, Müller *et al.* 2001) described only the introduction of an antibiotic resistant gene and/or a β-glucuronidase or green fluorescent protein as marker genes. In this study, we have been successful in producing a novel transgenic sunflower plant that integrated a useful gene for improving characteristics useful in agriculture, which may make sunflower more profitable.

Transgenic sunflower plants harbouring the human lysozyme gene exhibited enhanced resistance against white mold. As the resistance was stably inherited in the progenies and since it is difficult to breed disease-resistant sunflower from interspecific hybrids, these transgenic sunflower plants are expected to serve as new breeding materials for disease resistance.

The human lysozyme cleaves the β-(1-4)-glycosidic bond of peptidoglycan in the bacterial cell wall and of

chitin in the fungal cell wall (Jolles and Jolles 1984). However, it is not clear whether the protection afforded by the human lysozyme transgene reflects a direct lytic effect killing the pathogen on contact, or perturbation of the growth of the pathogen, slowing it with respect to the activation of the endogenous pathogen-inducible defences. Previous reports suggested that a key aspect of the protection might be the engineered deployment of an effective defence mechanism prior to the activation of natural inducible defences (Jolles and Jolles 1984, Düring *et al*. 1993).

In conclusion, because of the wide spectrum of substrate specificity and high lytic activity of the human lysozyme, it might have some potential to protect plants from both bacterial and fungal disease (Nakajima *et al*. 1997). In addition, the introduction of the human lysozyme gene into sunflower plants represents a simple and effective approach for generating disease resistance against the phytopathogenic fungi *Sclerotinia sclerotiorum.*

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