Use of transgenic Oryzacystatin-I expressing plants enhances recombinant protein production

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Abstract

Plants are an effective and inexpensive host for the production of commercially interesting heterologous recombinant proteins. The *Escherichia coli*-derived glutathione reductase was transiently expressed as a recombinant model protein in the cytosol of tobacco plants using the technique of leaf agro-infiltration. Proteolytic cysteine protease activity progressively increased over time when glutathione reductase accumulated in leaves. Application of cysteine protease promoter-GUS fusions in transgenic tobacco identified a cysteine protease *Nt*CP2 expressed in mature leaves and being stress responsive to be expressed as a consequence of agro-infiltration. Transgenic tobacco plants constitutively expressing the rice cysteine protease inhibitor oryzacystatin-I had significantly lower cysteine protease activity when compared to non-transgenic tobacco plants. Lower cysteine protease activity in transgenic plants was directly related to higher glutathione reductase activity and also higher glutathione reductase amounts in transgenic plants. Overall, our work has demonstrated as a novel aspect that transgenic tobacco plants constitutively expressing an exogenous cysteine protease inhibitor have the potential for producing more recombinant protein very likely due to reduced activity of endogenous cysteine protease activity.

Keywords:

Cysteine protease; cysteine protease inhibitor; transient expression, tobacco agro-infiltration; glutathione reductase

Abbreviations:

OC-I, oryzacystatin-I; GR, glutathione reductase; GUS, β-glucuronidase; MUG, 4-methyl umbelliferyl glucoronide; MU, 4-methyl umbelliferone

Introduction

Plants are an effective and inexpensive host for the production of heterologous recombinant proteins [*31*]. In comparison to bacterial cells, plant cells are able to carry out post-translational modifications, correctly fold proteins and assemble multimeric proteins [*16*]. A major challenge is, however, ensuring stability of accumulated proteins for optimizing yield and protein quality [*15*]. Specifically proteases, naturally required for eliminating any mis-folded proteins and providing amino acids for protein production, severely affect protein production [*3, 14*, *23*, *39*].

Strategies to increase recombinant protein stability *in planta* might include using a plant host with reduced protease activity. In particular, co-expression of a protease inhibitor active against specific endogenous proteases in plants may limit proteolytic activity [*15*]. Kim *et al*. [*28*] reported that relative protease activity decreased in transgenic rice cells when the serine protease inhibitor II, active against chymotrypsin and trypsin, was expressed. However, research on the use of transgenic plants expressing a protease inhibitor as a production system for recombinant proteins is still very limited. Komarnytsky *et al*. [*29*] expressed and co-segregated a soybean Bowman–Birk trypsin inhibitor stabilizing recombinant antibodies secreted by transgenic tobacco roots. Rivard *et al*. [*38*] recently described a 'mouse trap' strategy including either a tomato cathepsin D inhibitor or bovine aprotinin, which are active against trypsin and chymotrypsin, for protein production using ribulose 1,5-bisphosphate carboxylase/oxygenase as a target enzyme. Both inhibitors provided protection during the protein recovery process. Cathepsin D inhibitor expression in the cytosol further increased total soluble protein amount in transgenic potato [*38*].

So far there is no detailed report about the use of transgenic plants expressing a cysteine protease inhibitor for recombinant protein production. In our group, ectopic expression of a

rice cysteine protease inhibitor (OC-I) in the cytosol of transgenic tobacco plants has been found decreasing endogenous cysteine protease activity and increasing the protein amount in leaves [*44*]. Using these transgenic plants, our group has also achieved protection of tobacco superoxide dismutase (SOD) as well as Rubisco from inactivation under stress [*12*, *36*]. However, any potential of these plants in recombinant protein production has so far not been evaluated.

To evaluate potential of these OC-I expressing tobacco plants for recombinant protein production, we transiently expressed in this study glutathione reductase (GR) derived from *E. coli* as a recombinant model protein. *Agrobacterium*-mediated transient expression of recombinant proteins is a useful technique for rapid accumulation of recombinant proteins in plants. Accumulation occurs just days after agro-infiltration without T-DNA integration into the plant genome [*17*, *26*, *41*]. Our aim was to test, as a novel aspect, if tobacco plants expressing OC-I have the potential for higher GR accumulation when compared to non-transgenic tobacco plants and if possible higher GR accumulation is related to decreased cysteine protease activity. We specifically used GR as a model enzyme due to its application in bio-research for determining the oxidized and reduced glutathione content of tissue extracts or of serum samples, easy measurement of activity and natural expression of the enzyme in the cytosol [*19*]. Results of our study show, as a novel aspect, that transgenic OC-I expressing tobacco plants have lower endogenous cysteine protease activity which was directly related to higher GR activity and amounts in agro-infiltrated leaves.

Materials and Methods

Plant material and growth

Seeds of non-transgenic tobacco (*Nicotiana tabacum* L. Cv. Samsun) and transgenic tobacco were germinated in plastic trays in commercially available germination soil. Transgenic tobacco lines used included line T4/5 expressing the oryzacystatin-I (OC-I) coding sequence only under the control of a constitutive CaMV 35S promoter sequence allowing expression of OC-I in the cytosol [*45*] without addition of any further signalling sequence. In addition, tobacco lines PNtCP1 and PNtCP2 were used carrying the coding sequence for β-glucuronidase (GUS) under the control of either the *Nt*CP1 promoter sequence derived from the senescence-induced tobacco cysteine protease *Nt*CP1 or the *Nt*CP2 promoter sequence derived from the tobacco cysteine protease N*t*CP2 expressed in mature leaves and being stress responsive [*4*]. Plant transformation was conducted using the protocol of Glick and Thompson [*22*].

Tobacco seedlings were grown at a 12/12 hours light/dark cycle with a day/night temperature of 26ºC/20ºC and 80% (v/v) relative humidity. Four weeks old plants were individually transferred into 25 cm diameter pots containing commercially available potting soil and then further grown for 12 weeks to obtain fully expanded leaves suitable for agro-infiltration. Plants were watered daily and fertilized once per week with a Murashige and Skoog (MS) solution [*35*].

Agro-infiltration

Agro-infiltration of leaves was carried out following the method described by d'Aoust *et al*. [*1*] using a syringe for infiltration of bacteria. In comparison to vacuum-based agro-infiltration, syringe-based agro-infiltration is fast and results in high-yield production of a recombinant protein particularly at the lab-scale. For infiltration, a 10 ml syringe (without a needle) was filled with an *Agrobacterium tumefaciens* (strain LBA4404) cell suspension (OD_{600} of 1). *Agrobacterium* cells harboured the pKG2 vector carrying the *E. coli* GR coding sequence (Fig. 1) [*19*] and were suspended in a 50 ml suspension solution (10 mM $MgCl_2$:$6H_2O$, 100 μM acetosyringone). The first fully-expanded leaf of four individual plants was infiltrated with *Agrobacterium* cells (LBA4404) harbouring plasmid pKG2 and all remaining non-infiltrated leaves were removed. The suspension was gently infiltrated with the syringe into the abaxial side of a fully expanded leaf after making a very small incision into the leaf with a blade to ease infiltration with bacterial cells. An even pressure was maintained during the infiltration to avoid any major wounding of the leaf tissue. Wetting of the leaf was observed as the suspension solution entered the tissue. Several points of infiltration were made in order to completely inoculate the leaf. As a control, leaves of plants were infiltrated with non-pKG2 carrying *Agrobacterium* cells. Plants were kept for 6 days in an environmentally controlled growth room for protein accumulation and they were watered daily. All leaves were harvested 6 days post-infiltration for analysis. For analysis of GUS activity in PNtCP1 and PNtCP2 tobacco plants, half of the infiltrated leaf was harvested at day 1 and the other half on day 6 for GUS analysis.

Leaf harvesting and protein content

Leaf material (100 mg) was homogenized in liquid nitrogen with a pestle and mortar with 20% PVPP (polyvinylpolypyrrolidone) added to the mixture. Soluble proteins were extracted by addition of 1 ml of a solution containing 50 mM Tris-HCl, pH 7.8, and 1 mM EDTA. The mixture was transferred to a 1.5 ml Eppendorf tube and then centrifuged at 13 000 rpm for 10 min at 4°C. The soluble protein containing supernatant was used for further analysis.

Total protein content from tobacco leaves extracts were determined using a commercially available protein assay (BioRad, UK), which is based on the Bradford method [*5*], using Bovine serum albumin (BSA, Sigma®-Aldrich, Germany) as a standard.

Glutathione reductase activity

Glutathione reductase (GR) activity was measured using a GSH recycling method where GSH, produced by GR and NADPH in the presence of oxidized glutathione, reacts with 5'-dithiobis (2-nitrobenzoic acid (DTNB, Ellman's reagent; Sigma-Aldrich, UK) to form the disulfide GSTNB. The produced yellow-colored compound 5-thio-2-nitrobenzoic acid (TNB) can be measured at 412 nm in a spectrophotometer [*18*]. For preparation of a GR standard curve for the GSH recycling method, purified GR from Baker's yeast (Sigma-Aldrich, UK) was freshly prepared to obtain a GR concentration of 20 units/ml where one GR unit produces 1 μmol $NADP^+$ from NADPH per min at pH 7.0 and 25ºC.

For GR activity determination in leaves, a leaf protein extract (20 μl corresponding to 25 μg of total soluble protein) was placed into a well (200 μl) of a micro-titre plate also containing 110 μl of 0.12 M NaH_2PO_4, pH 7.5, 6 mM EDTA, 10 μl of 12 mM DTNB, 10 μl of 10 mM NADPH, 40 μl dsH_2O and 10 μl of 4 mM GSSG (oxidised glutathione). The increase

in absorbance at 412 nm due to TNB formation was measured for 5 min at 25ºC using a spectrophotometer (Soft Max Pro, Molecular Device). GR activity of different samples (units/ml reaction mixture) was calculated by using the GR standard curve.

For *in vitro* testing of GR sensitivity to the model plant cysteine protease papain, which has cathepsin L- like activity, a solution of GR from Baker's yeast (Sigma-Aldrich, UK) was prepared by centrifugation of a $(NH_4)_2SO_4$-GR suspension at 13000 g at 4°C for 5 min. The supernatant was discarded and the GR-containing pellet was re-suspended in 500 µl re-suspension medium consisting of 0.12 M NaH_2PO_4 (pH 7.5) and 6 mM EDTA to obtain a GR concentration of 20 units/ml. In order to demonstrate GR degradation by papain, 10 µl of the GR solution (0.2 units) was incubated at 25°C for 5 min with 20 µl re-suspension medium containing either 1, 2.5, 5 or 10 µg papain before adding the mixture to 170 µl reaction mixture which was followed by measurement of GR activity using the DTNB method.

Protease activity

Total protease activity was measured according to the method of Michaud *et al*. [*34*] on a mildly denaturing 10% SDS polyacrylamide gel containing 1% gelatine but without treatment of samples with mercaptoethanol or boiling the samples before introducing onto the gel. After electrophoresis using 10 µl of total soluble protein (25 µg) for each sample, the gel was incubated in 2.5% Triton X-100 for 30 min at room temperature to re-nature the proteases. After 30 min, the gel was rinsed 3-times for 10 min with dH_2O to remove the Triton X-100 solution. The gel was then incubated overnight in a proteolytic buffer containing 0.1 M citrate phosphate buffer, pH 6.0, and 10 mM L-cysteine at 37°C for the developing of the protease reaction. The gel was subsequently transferred overnight to a staining solution (25% isopropanol, 10% acetic acid and 0.5% Coomassie Brilliant Blue; Thermo Scientific, US) at

room temperature and then de-stained for 2 hr with a de-staining solution (10% acetic acid, 40% dH_2O and 50% methanol). De-stained bands indicated the presence of protease activity.

For measurement of all cysteine proteases with cathepsin L-like activity in plant extracts, a fluorometric assay was conducted [*2*]. Protease activity was determined using the synthetic cathepsin L–like cysteine protease substrate Z-Phe-Arg-AMC (Sigma-Aldrich, Germany). For activity measurement, 10 µl of soluble protein (25 µg) was first mixed with 82 µl reaction buffer containing 50 mM sodium phosphate, pH 6.0, 1 mM L-cysteine and then 8 µl of a 100 µM Z-Phe-Arg-AMC solution dissolved in DMSO was added to start the reaction. For inhibition of protease activity, 10 µl of 100 µM E64 solution was added to the reaction mixture at the start of the reaction. The mixture was immediately placed into a well of a micro-titer plate and the increase in fluorescence due to released AMC was measured over 10 min using a Fluoroskan Ascent® fluorometer (Thermo Labsystems, US) at an excitation wavelength of 355 nm and emission wavelength of 460 nm.

GUS activity

Measurement of β-glucuronidase activity (GUS) was carried following the method described by Gallagher [*20*] using the fluorogenic GUS substrate 4-methyl umbelliferyl glucoronide (MUG). Fluorescence was measured due to the release of 4-methyl umbelliferone (MU) at an excitation of 355 nm and an emission of 460 nm using the Fluoroskan Ascent® fluorometer (Thermo Labsystems, US).

Immuno-blotting

Protein extracts (25 μg) were loaded onto a 10% SDS polyacrylamide gel and the gel was run for 3 hr at 100 V at room temperature. The separated proteins were transferred onto a nitrocellulose ImmunBlot^{TM} PVDF membrane (BioRad, UK) using a transfer buffer (25 mM of glycine, 192 mM Tris-base and 20% methanol, pH 8.2) on ice for 2 hr at 45 V and 200 mA. After 3 hr protein transfer, the membrane was incubated overnight on ice in a blocking solution containing 5% skimmed milk powder dissolved in TBST buffer (0.5 M Tris-HCl, 1 M NaCl and 0.1% Tween 20 at pH 7.6). The membrane was then incubated on ice for 16 hr with TBST buffer containing 5% skimmed milk powder and an antibody raised in a rabbit against either OC-I or GR. After overnight incubation at 4°C, the membrane was washed 3-times for 10 min with TBST buffer and a goat anti-rabbit antibody conjugated to alkaline phosphatase (Santa Cruz Biotechnology, USA) was added as a secondary antibody (1:2000 dilution) to the solution. The membrane was incubated with the antiserum for 1 hr at room temperature in TBST buffer containing 5% skimmed milk powder. Finally, the membrane was washed 3-times for 30 min at 10 min intervals in 30 ml of TBST buffer. The presence of either OC-I or GR on the membrane was detected using the alkaline phosphatase detection kit (BioRad, UK).

Data analysis

Data were analyzed using the SAS statistical package (SAS, 2002). All data were subjected to analysis of variance using the Proc ANOVA program. Means were compared using the F-test at $P = 0.05$ and the mean separation were performed using Duncan-Weller multiple range test.

Results and Discussion

We first measured expression of OC-I in transgenic OC-I tobacco plants to ensure that all plants used for agro-infiltration expressed OC-I. Expression of the inhibitor, with the correct size of about 12 kDa, was detected by immuno-detection with an antiserum raised against OC-I in all transgenic leaves used for the experiments (Fig. 2A). In agro-infiltrated leaves, OC-I expression was found over a 7 day time period with decreasing OC-I expression over time. However, OC-I expression was not detected in any non-transgenic control leaves (Fig. 2B).

Agro-infiltrated tobacco leaves could be distinguished from non-infiltrated leaves by the wet appearance of the leaf tissue and formation of infiltration lesions due to the very small incisions made by a blade to allow easier infiltration of leaf tissue with *Agrobacterium* cells using a syringe. Six days after inoculation harvested leaves showed localized chlorosis around infiltration points (Fig. 3). On nine days after inoculation, a yellow to brown leaf colour developed possibly due to the visible onset of leaf senescence (data not shown) suggesting a possible accumulation of phenolic compounds [*25*].

We subsequently analyzed proteolytic activities in both agro-infiltrated and non-infiltrated tobacco leaves of the two plant types, transgenic OC-I expressing and non-transgenic tobacco plants. Agro-infiltration of leaves caused a general increase in total proteolytic activity and this activity progressively increased over 9 days after infiltration. Increase in proteolytic activity was clearly visible on a gelatine-containing polyacrylamide gel by the appearance of more intense de-stained bands due to greater degradation of gelatine (Fig. 4). Inclusion of gelatine into gels has previously been shown to detect multiple proteolytic activities in crude plant extracts [*34*]. In our study, three bands of major proteolytic activity with different size were detected in extracts from infiltrated non-

transgenic and transgenic leaves (Fig. 4; P1, P2 and P3). A slight decrease in the intensity of bands was sometimes found in transgenic leaves (data not shown). Highest proteolytic activity was measured (9 days after infiltration) when leaves had a brown to yellow colour. Proteases are progressively expressed with the onset of leaf senescence causing degradation of proteinaceous cellular components with recycling to other organs within the plant [*11*].

We then determined activity of all cathepsin L-like cysteine proteases in these leaves using Z-Phe-Arg-AMC as a protease substrate [*2*, *27*]. Leaves from infiltrated or non-infiltrated transgenic plants had significantly ($P < 0.05$) lower cysteine protease activity when compared to infiltrated or non-infiltrated leaves of non-transgenic plants (Table 1). In comparison to non-infiltrated leaves, all leaves that had been agro-infiltrated had further significantly ($P < 0.05$) higher activity of cathepsin L-like cysteine proteases than non-infiltrated leaves (Table 1). Lower protease activity in these transgenic plants confirms results obtained with these OC-I expressing tobacco plants previously reported by Van der Vyver *et al*. [44] and Prins *et al*. [36, *45*]. Activity of cathepsin L-like cysteine proteases in leaves of all types of plants tested was further significantly lower ($P < 0.05$) when E-64 was added to the reaction mixture (Table 1). E-64 is an irreversible cysteine protease inhibitor forming a tight irreversible complex with cysteine proteases and acting as a pseudo-substrate by entering the active site of the target protease [*43*]. Although we cannot exclude that some lower inhibition by E64 found in transgenic leaves when compared to non-transgenic leaves might be due to production of E64 insensitive proteases which still can react with the protease substrate, detection of cysteine protease activity was almost at the limit after E64 addition and values might therefore not accurately reflect the difference in activity found. Higher cathepsin-L like cysteine protease activity found in infiltrated leaves than in non-infiltrated leaves might further be due to leaf wounding during the infiltration procedure [*44*]. Such cysteine protease

expression has been previously found after wounding [*30*], during leaf senescence [*4*] and also programmed cell death [*42*].

The influence of infiltration with the transgene was further tested in two tobacco lines expressing the GUS gene behind promoters for a senescence (NtCP1) and a stress (NtCP2) related cysteine protease [*4*]. GUS activity increased, although not significantly ($P > 0.05$) when a statistical analysis was carried out, in both non-infiltrated and infiltrated green leaves over 6 days when GUS activity under the control of the senescence-related *Nt*CP1 promoter sequence [*4*] was measured (Table 2). Also, GUS activity was higher at day 1 in agro-infiltrated leaves than in non-infiltrated leaves possibly indicating a response of *Nt*CP1 to the syringe-based agro-infiltration process (Table 2). GUS activity further increased ($P < 0.05$) over 6 days in both non-infiltrated and infiltrated leaves, when the GUS coding sequence was expressed under the control of the *Nt*CP2 promoter sequence active in mature leaves and responsive to stress [*4*]. This increase was 2-fold higher in agro-infiltrated leaves but only 1.28-fold higher in non-infiltrated leaves (Table 2). GUS activity was also higher at both day 1 and day 6 in agro-infiltrated leaves than in non-infiltrated leaves indicating a response of the *Nt*CP2 promoter to agro-infiltration and leaf wounding, which is known to induce cysteine protease expression [*30*], and leaf maturation. However, we currently cannot exclude the possibility that also the process of recombinant protein production itself induces cysteine protease expression. Since the *Nt*CP1 promoter was not greatly activated in our experiments, any severe senescence process had possibly not occurred after six days of GR accumulation.

To investigate if GR is sensitive to cysteine proteases with cathepsin L-like activity, we first tested *in vitro* the effect of purified papain, a model plant cysteine protease with cathepsin L-like activity, on GR activity. Treatment of GR with papain almost completely inactivated GR (Fig. 5). In a next step, we measured a possible difference in GR activity present in transgenic and non-transgenic tobacco leaves. A significantly ($P < 0.05$) higher GR

activity was found in agro-infiltrated leaves when compared to non-infiltrated leaves (Fig. 6A). Further, infiltrated leaves of transgenic OC-I plants had significantly higher ($P < 0.05$) GR activity than non-transgenic infiltrated leaves and infiltrated leaves of transgenic plants had also the highest GR activity of all plants tested.

We finally carried out an immuno-detection procedure using an antiserum, which was raised against *E. coli* GR, to determine GR accumulation in agro-infiltrated leaves. A band corresponding to a size between 45 and 50 kDa for GR [*40*] was detected in infiltrated leaves. Also, a band with stronger intensity, very likely due to a higher GR amount, was detected in agro-infiltrated transgenic OC-I leaves when compared to non-transgenic leaves indicating a protective effect of OC-I expression on GR accumulation (Fig. 6B). Since no signal peptide was attached to the GR sequence in addition to the CaMV 35S promoter sequence, GR has very likely not migrated out of the cytosol after its mRNA was translated [*3*]. Recombinant proteins often accumulate only at a very low amount in the cytosol despite that the transgene is efficiently transcribed and the protein is also stable in this cellular compartment [*8*, *10*, *13*, *33*, *37*]. Possible reasons are incorrect folding of proteins and the presence of the ubiquitin–proteasome proteolytic pathway for recognition and degradation of incorrectly folded proteins [23, *46*]. Future research has therefore to show if GR accumulation can be improved by expressing both OC-I and GR together in a different sub-cellular compartment that are more suitable locations for protein accumulation [9, *23*, *32*]. For example, production of the human growth hormone in the cytosol of *Nicotiana benthamiana* leaf cells resulted in about 0.01% hormone of total soluble protein whereas 10% of total soluble protein for the hormone was obtained when targeted to the apoplast [*21*]. A much higher accumulation of GR was also found when the transgene was expressed in the chloroplast instead of the cytosol [*19*]. Since OC-I expression has also been detected in the chloroplast of our OC-I tobacco plants [*36*], a

future study might therefore involve directing GR into the chloroplast of these transgenic plants to determine any protective effect by chloroplast-located OC-I.

From our experiments, we could further not exactly determine if OC-I acts either *in planta* or *ex planta*, or both, and where exactly OC-I protection of GR might have occurred *in planta*. Furthermore, we have also found in a previous study that the ectopic expression of OC-I in the cytosol of OC-I plants increases the total protein content of tobacco leaves by 40% [*45*]. Such higher protein content has also been found for transgenic potato leaves expressing a tomato cathepsin D inhibitor in the cytosol [*38*]. However, the exact reason for this higher protein production when an inhibitor is expressed is still unclear. Where inhibition of cysteine proteases by OC-I might have occurred is also unclear. Cysteine proteases are initially synthesized as inactive KDEL tailed-protease precursors by the endoplasmatic reticulum (ER) and form protease precursor vesicles before they are transported to vacuoles through a Golgi complex-independent route [6, *7*, *24*]. The question therefore remains, if cysteine proteases particularly sensitive to OC-I, are also located in the cytosol. Further, OC-I might also have acted *ex planta* after tissue harvesting and leaf homogenization during GR recovery by preventing extracted proteases from directly reacting with GR. This reaction could have been inhibited by co-produced OC-I [*38*]. A more detailed investigation is therefore required to exactly determine OC-I action on expressed proteins in our transgenic OC-I plants.

Conclusions

Major progress has been made to improve the yield of recombinant proteins in plant-based expression systems. Co-expression of a “companion” protease inhibitor has been recently proposed as a suitable strategy to protect plant-expressed recombinant proteins [*3*]. Our work

contributes to the utility of this strategy. The novel aspect of our work is that transgenic OC-I expressing plants with reduced cysteine protease activity increases accumulation and activity of a transiently expressed recombinant protein. Our study has further provided strong evidence that the use of OC-I expressing plants to control recombinant protein degradation is feasible. More research is, however, required to confirm whether findings with OC-I expressing tobacco plants may also hold true for other cysteine protease inhibitors, or combinations of inhibitors, and further for other transiently expressed proteins and engineered host plants. Future research might also focus on establishing which particular cysteine protease is involved in the degradation of a specific recombinant protein and to identify or design a specific inhibitor for such a protease.

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Table 1 Cathepsin L-like cysteine protease activity of leaf extracts without and with addition of the cysteine protease inhibitor E-64.

Line	Cysteine protease activity[b] Fluorescence units min^{-1} mg $protein^{-1}$	
	(-) E64	(+) E64
Non-transgenic		
Non-infiltrated	434±15	10±4
Infiltrated	665±14	12±6
OC-I transgenic[a]		
Non-infiltrated	216±6	24±2
Infiltrated	301±11	30±3

[a]OC-I transgenic tobacco plants are plants of line T4/5

[b]Data represent the mean ± SE of three different leaf samples

Table 2 GUS (β-glucuronidase) activity in non-infiltrated and agro-infiltrated leaves of transgenic tobacco lines PNtCP1 and PNtCP2.

	GUS activity	
Line	μg MU mg protein^{-1}min^{-1}	
	Non-infiltrated	Agro-infiltrated
PNtCP1		
Day 1	0.158±0.035	0.205±0.036
Day 6	0.246±0.060	0.238±0.037
Significance[b]	*ns*	*ns*
PNtCP2		
Day 1	0.099±0.010	0.112±0.006
Day 6	0.133±0.008	0.224±0.013
Significance[b]	*	*

[a]Data are the means ±SE of four independent leaf samples

[b]Significance determined via the Student's *t*-test within the column (ns - non significant; * $P < 0.05$)

Figure legends

Fig. 1. Gene-construct pKG2 used for agro-infiltration. LB, Left border; RB, right border; *nptII,* coding sequence of the neomycin phosphotransferase gene; P35S, CaMV 35S promoter; Pnos, promoter of the nopaline synthetase gene; T, CaMV terminator; Tnos, terminator of the nopaline synthetase gene.

Fig. 2. Immuno-blot analysis to detect OC-I expression in transgenic tobacco plants. (A) OC-I expression in the transgenic leaves used for the experiments. (B) Expression of OC-I at 1, 5 and 7 days after agro-infiltration in transgenic OC-I expressing leaves (TD1, TD5, TD7) and non-transgenic control leaves (CD1, CD5, CD7). A 1:10 000 dilution of the OC-I antiserum was used for hybridization. Positions of the 17 kDa molecular weight marker and of OC-I (about 12 kDa) are indicated.

Fig. 3. Three representative tobacco leaves 6 days post-infiltration with chlorotic lesions around points of infiltration with Agrobacterium cells.

Fig. 4. Activity gel analysis of changes in protease activity during senescence of agro-infiltrated non-transgenic tobacco leaves. Protein samples (25 μg) extracted from progressive stages of senescence induced by infiltration were resolved on a 10% mildly denaturing SDS-polyacrylamide gel containing 1% gelatine. Following re-naturation and protein staining, protease activities were detected as clear bands represented by P1, P2 and P3. Since samples were not treated with mercaptoethanol or boiled, the exact size of proteases could not be determined.

Fig. 5. Effect of different concentrations of the model plant cysteine protease papain on GR activity after incubation of papain with 0.2 units of GR from Baker's yeast. GR activity (0.2 units) without addition of papain was set as 100% GR activity and remaining GR activity was determined after incubation with different concentrations of papain.

Fig. 6. (A) GR activity in non-infiltrated (N-IN) and infiltrated (IN) non-transgenic (filled bars) and OC-I transgenic (open bars) leaves. TSP represents total soluble protein. For GR activity determination in different leaf extracts, 25 µg total soluble protein dissolved in 20 µl reaction buffer were assayed for determination of GR activity. Data shown represent the mean ± SEM from four different leaves. (B) Detection of transiently expressed *E. coli* GR in infiltrated and non-infiltrated tobacco leaves using immuno-blotting. Lane 1 represents transient GR expression in OC-I expressing transgenic tobacco leaves, lane 2 non-infiltrated transgenic leaves, lane 3 infiltrated non-transgenic leaves, lane 4 non-infiltrated non-transgenic leaves and lane 5 purified GR. For each sample, 25 µg of total soluble protein was loaded onto the SDS gel. For GR detection, a GR antiserum in a 1:1000 dilution was used and the position of detected GR is indicated.

Figure 1

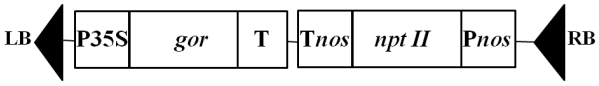


Figure 2

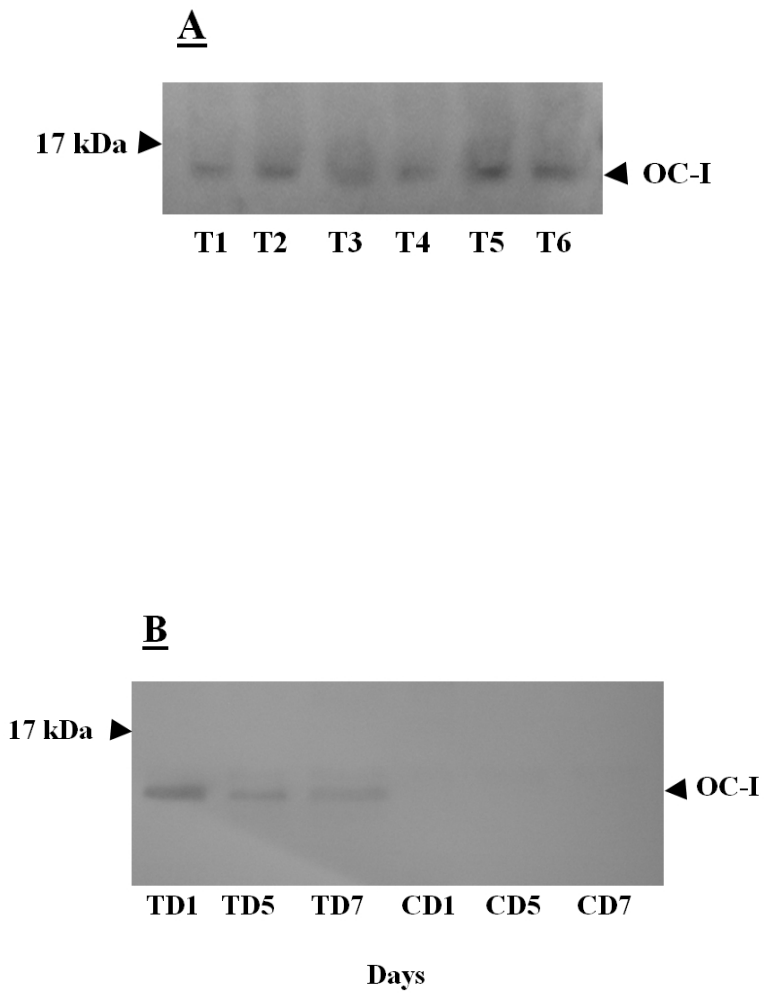

A
17 kDa
OC-I
T1 T2 T3 T4 T5 T6
B
17 kDa
OC-I
TD1 TD5 TD7 CD1 CD5 CD7
Days

Figure 3

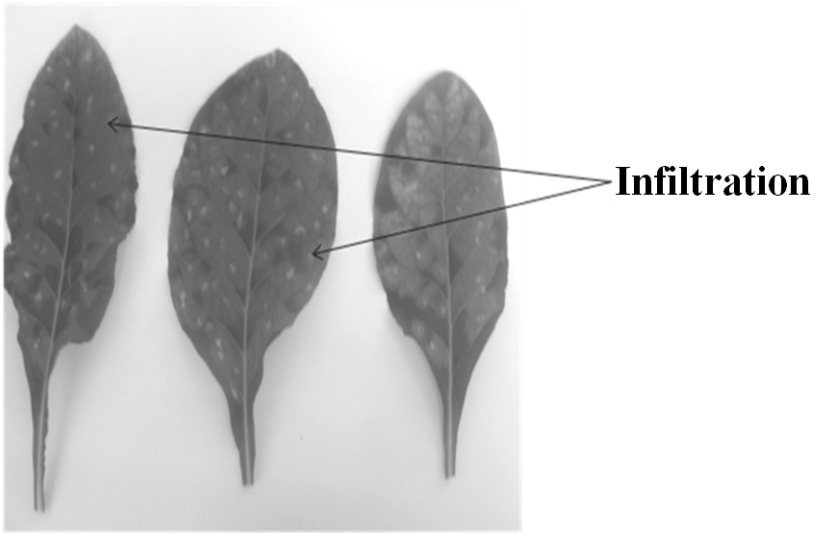


Figure 4

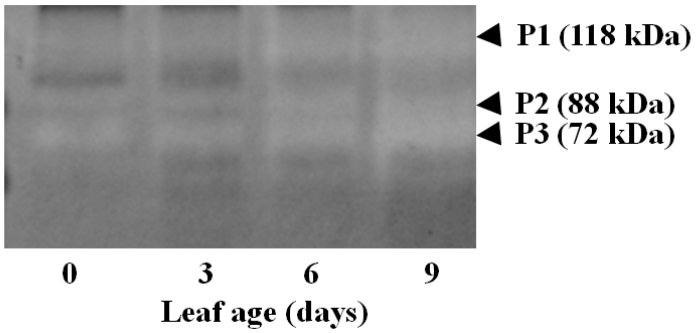


Figure 5

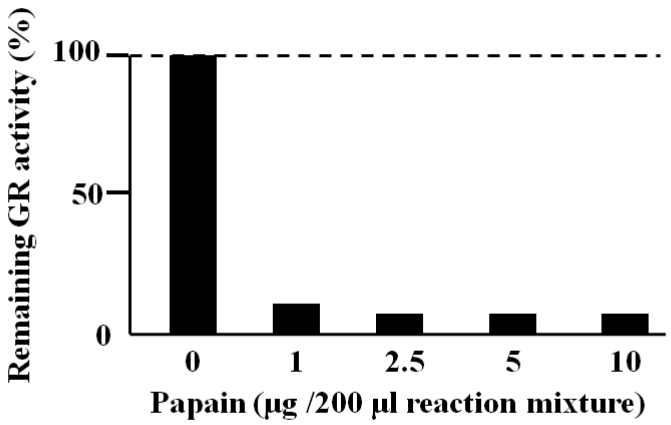
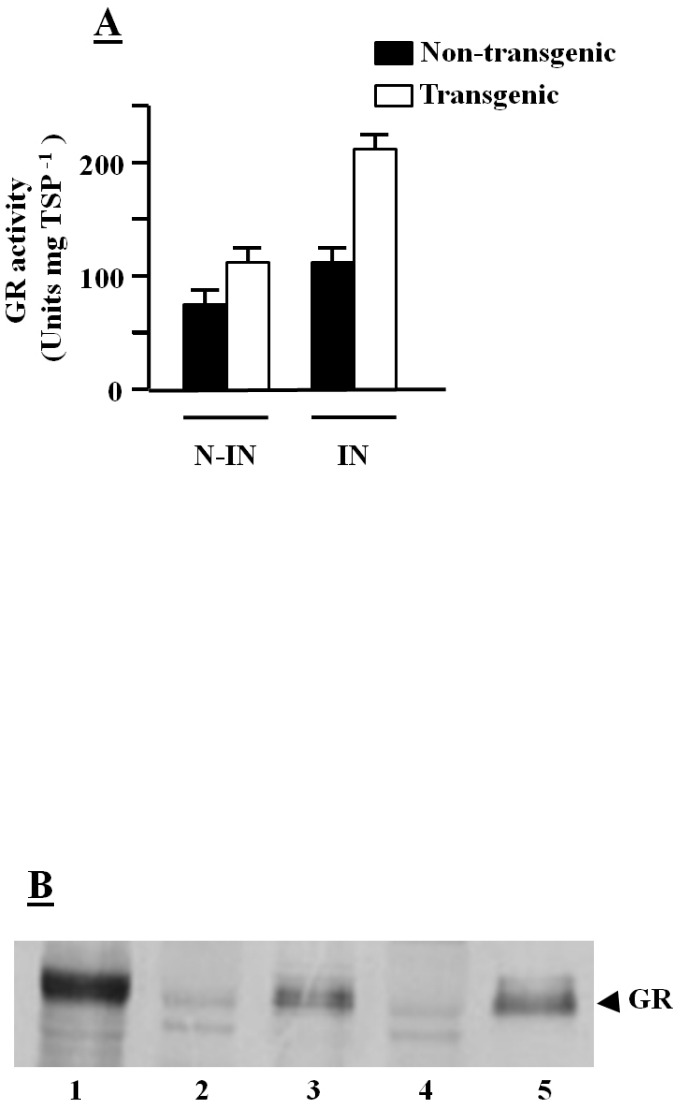

Remaining GR activity (%)
100
50
0
0
1
2.5
5
10
Papain (µg /200 µl reaction mixture)

Figure 6


A
Non-transgenic
Transgenic
GR activity
(Units mg TSP -1)
200
100
0
N-IN
IN
B
GR
1
2
3
4
5