Amino Acids (2009) 36:765–772
DOI 10.1007/s00726-008-0132-8

ORIGINAL ARTICLE

Molecular farming of human tissue transglutaminase in tobacco plants

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Received: 8 April 2008 / Accepted: 7 May 2008 / Published online: 2 July 2008

Abstract In this study we have utilized *Nicotiana tabacum* with a molecular farming purpose in attempt of producing transgenic plants expressing the human tissue transglutaminase (htTG). Three plant expression constructs were used enabling targeting and accumulation of the recombinant protein into the plant cell cytosol (*cyto*), the chloroplasts (*chl*) and the apoplastic space (*apo*). Analysis of transgenic T_0 plants revealed that recombinant htTG was detectable in all three transgenic lines and the accumulation levels were in a range of 18–75 μg/g of leaf material. In the T_1 generation, the recombinant htTG was still expressed at high level and a significant catalytic activity was detected into the leaf protein extracts. Southern blot analyses revealed that *apo* and *chl* plants of T_1 generation possess a high copy number of the recombinant htTG in their genome, while the *cyto* plants carry a single copy.

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Keywords Tissue transglutaminase · Bioreactor · Molecular farming · *Nicotiana tabacum*

Introduction

Transglutaminase (TG, E.C.2.3.2.13) family consists of transamidating enzymes (R-glutaminyl-peptide:amine γ-glutamyltransferase) that catalyze an acyl-transfer reaction between the γ-carboxyamide group of peptide-bound glutaminyl residues and ε-amino groups of endoprotein lysine as well as a variety of primary amines (Folk and Finlayson 1977).

After 50 years from its discovery, the human tissue transglutaminase (htTG) is still one of the most studied TG since it is related to different biological events and diseases (Griffin et al. 2002). Because of the type of reaction catalyzed, TGs can be considered an important way to manipulate proteins of different origins. Several proteins, useful for biotechnological applications in both food and medical fields, have been demonstrated to act as substrates of the tissue isoform of TGs. Furthermore, the identification of htTG as the major autoantigen in celiac disease (Dieterich et al. 1997) opened a new field of application for the diagnosis of this pathology. Thus a low cost production of this enzyme is required for its applications.

In the last decade, plants have been proposed in molecular farming strategies as an alternative eukaryotic system because of their capability in providing high biomass at low costs, thus representing an inexpensive source for the production of therapeutic and industrial biomolecules. Various plant species have been used as bioreactors to produce heterologous proteins at large scale (Giddings et al. 2000; Ma et al. 2003; Hellwig et al. 2004).

The aim of our project was to use *Nicotiana tabacum* suspension cells and plants as biofactory for the production of recombinant htTG. In a previous paper we have described the efficiency of tobacco Bright Yellow 2 (BY-2) cell cultures as bioreactor for the production of htTG and assessed the ability of recombinant enzyme to recognize autoantibodies in the serum of celiac patients (Sorrentino et al. 2005). In this study, we propose the whole transgenic plant as molecular farming system since it is well known that tobacco is a reliable plant and able to furnish high biomasses (Ma et al. 2003).

Nicotiana tabacum cv Petite Havana SR1 plant were transformed with three different plant expression constructs leading to targeting and accumulation of htTG into subcellular compartments of plant cells as the apoplastic space (*apo*), the cytosol (*cyto*) and the chloroplasts (*chl*) (Sorrentino et al. 2005). Transgenic T_0 and T_1 plants were produced and both Western and Southern analyses were performed in order to characterize such plants at molecular level. Furthermore, enzymatic activity and 2D-PAGE of the highest expressing plant were carried out.

Materials and methods

Plant expression constructs

The pTRAkc-htTG-*apo*, *cyto* and *chl* constructs, used for the experiments reported in this study, were previously prepared and tested as described in Sorrentino et al. (2005).

Tobacco transformation and cultivation

Nicotiana tabacum L. cv Petite Havana SR1 seeds were germinated under sterile conditions on MS-agar medium (4.3 g/l Murashige and Skoog basal salt with minimal organics [$MSMO^+$], 2% sucrose, 0.4 mg/l thiamin, 0.8% phyto-agar). Leaf material from 4-week-old sterile tobacco plants was used for whole plant transformation by co-cultivation with the transformed *Agrobacterium tumefaciens*. Briefly, leaf disc slices (approximate 1 cm^2) were incubated for 3 days in presence of recombinant agrobacteria on MS-agar medium at 22°C in dark, after which were transferred on modified MS-agar selective media (containing 100 mg/l kanamycin, 100 mg/l cefotaxime, and 1 mg/l BAP [6-benzylaminopurine]), and incubated at 22°C with a photoperiod of 16 h of light for the regeneration of *calli* and shoot germination.

Upon 4 weeks of incubation, the germinated shoots were picked and grown for rooting on MS-agar supplemented only with kanamycin and cefotaxime. The regenerated transgenic T_0 plants were maintained in square culturing vessels and subsequently transferred into soil for plant maturation, flowering, and seed collections. In order to obtain transgenic T_1 plants, seeds from htTG-producing T_0 plants were germinated onto MS-agar selective medium containing kanamycin. Kanamycin-resistant T_1 plants were selected and used for further analyses.

Segregation analysis

Segregation analysis was performed to evaluate the distribution of kanamycin resistance gene in the progeny T_1 of transformed T_0 plants. Hence, 123–188 seeds from T_0 plants were incubated onto MS-agar selective medium containing kanamycin. Resistant and susceptible (chlorotic) seedlings, as well as non germinating seeds were enumerated. The observed segregation frequency was calculated as the *ratio* between resistant and susceptible seeds while the expected segregation frequency was calculated as the *ratio* between the total number of analyzed seeds and the total number of genotypes, which for the segregation of a single copy of an hemizygote character are supposed to be 4 (in the *ratio* of 3:1). Student's χ^2-test was carried out and differences were considered to be significant at $P < 0.01$ for 1 *df*, as the segregant phenotypic classes are two.

Protein extraction and analysis

Leaf material from wild type (*wt*) or T_0 and T_1 transgenic tobacco plants was weighed and ground in mortar and pestle under liquid nitrogen. During grinding 2 vol. (w/v) of extraction buffer [200 mM Tris–HCl pH 7.5, 5 mM EDTA, 4 mM dithiotheitol (DTT), 5% PVPP (w/v)] were added to the leaf powder and then the mixture was poured in a test-tube. Leaf debris were removed by centrifugation at $20{,}000 \times g$ for 30 min at 4°C and plant extract supernatants, containing the total soluble proteins (TSP), were used for further analyses. TSP concentration was determined using a Bradford Reagent (Bradford 1976, Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblot analyses

Protein samples containing ~100 μg TSP were denatured at 95°C for 5 min in the presence of SDS-gel sample buffer (Laemmli 1970) and subsequently were separated on a 10% SDS-PAGE. The separated proteins were electrophoretically blotted at 250 mA for 60 min onto a nitrocellulose membrane (Hybond-C) by using a Trans-blot Cell (Bio-Rad Laboratories, Richmond, CA, USA). An amount of 200 ng of guinea pig tissue TG (gptTG) was used as a positive control. Western blot was carried out with mouse anti-tTG monoclonal antibody used at a 1:3000 dilution as the primary antibody (TG II Ab, Neomarkers, USA) which specifically recognizes the gptTG and the

htTG isoforms. The alkaline phosphatase-conjugated goat anti-mouse IgG (GAM^{PA}, Dianova, Germany) was used as the secondary antibody at a 1:5000 dilution. All antibody dilutions were made in TBS buffer (50 mM Tris, pH 7.5, 150 mM NaCl) supplemented with 2.5% (w/v) non-fat dry milk (Bio-Rad, USA). The immune complexes were detected using an NBT/BCIP (Bio-Rad) as chromogenic agent. The recombinant htTG yields were estimated using the FLUOR-S Multi Imager and Quantity One software ver 4.2.1 (Bio-Rad) using various concentrations of gptTG as standards. Estimated values were normalized for grams of fresh leaf material.

2D gel electrophoresis

Total soluble proteins from wild type and *chl*-8.2 (~270 µg) were added to 125 µl of sample rehydration buffer (Bio-Rad) [containing 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% biolyte 3/10 ampholyte, 0.001% bromophenol blue] and then loaded on 7-cm IPG strips (Bio-Rad), pH gradient 3–10. Isoelectric focusing (IEF) was performed at 20°C for 14 kVh on a Protean IEF Cell (Bio-Rad). Before IEF, the IPG strips were allowed to rehydrate for 12 h at 50 V. After IEF run, the IPG strips were incubated at room temperature for 10 min in 6 M urea, 2% SDS, 0.375 M Tris–HCl pH 8.8, 20% glycerol and 130 mM DTT. The second equilibration step was carried out for 10 min in the same solution, except that DTT was replaced by 135 mM of iodoacetamide. The second dimension (SDS-PAGE) was carried out on slab gels (5% stacking and 10% separating gels) and performed at constant current (200 V for 45 min). At the end the gels were Coomassie stained.

Enzyme activity assay

Enzymatic activity of plant-derived htTG was analyzed as previously described (Sorrentino et al. 2005). In particular, 10 µl of leaf extracts (~22 µg of TSP) were used as enzymatic source into 100 µl of assay mixtures. Reactions with *wt* protein samples and gptTG standard (1 µg) were also set up as controls. One enzyme unit was defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of Spd into DMC in 1 h under the described conditions.

Genomic DNA isolation

Genomic DNA from wild type and T_1 transgenic tobacco plants was isolated from leaf tissue by Doyle and Doyle (1987) modified protocol. In detail, ~1 g from young and green leaves was ground in mortar and pestle under liquid nitrogen. The leaf powder was resuspended in 3 ml of pre-heated at 60°C CTAB buffer (containing 0.1 M Tris–HCl pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 1% β-met and 2% CTAB) and then the mixture was poured in a 50-ml tube and incubated at 65°C for 50 min. Two extractions with 1 vol. of chloroform:isoamilic alcohol (24:1) were performed and then the DNA containing solution was incubated at 37°C for 15 min in presence of 10 µg/ml RNase A, in order to degrade the contaminating RNA. At this step, the DNA was precipitated by adding 2 vol. of 0.05 M Tris–HCl pH 8.0 containing 0.01 M EDTA and 1% CTAB, followed by centrifugation at 4,000×*g* for 10 min at 4°C. The pellet was resuspended in 600 µl of 1.5 M NaCl and then 2 vol. of 98% ethanol were added. The pellet of DNA was harvested again via centrifugation at 10,000×*g* for 15 min at 4°C, and then washed twice with cold 70% ethanol and finally resuspended in 50 µl of sterile H_2O. DNA concentration and purity were assessed by measuring the adsorbance values at 260 and 280 nm. The integrity of genomic DNA was evaluated by 1% agarose gel electrophoresis.

Southern blot analyses

A 10 µg aliquot of genomic DNA of each sample was subjected to Southern blot analysis. For this purpose, DNA was digested with 20 U of *Sca*I or *Eco*RI enzyme (Fermentas) in the appropriate buffer 1× in a final volume of 100 µl. The reactions were performed at 37°C over night under mineral oil to avoid the evaporation of samples. After digestion the DNA was precipitated by adding 0.1 vol. of 3 M sodium acetate (pH 5.2) and 2 vol. of absolute ethanol followed by centrifugation at 20,000×*g* for 30 min at 4°C. The pellet was washed with cold 70% ethanol and finally resuspended in 15 µl of sterile H_2O.

Digested DNA was separated by electrophoresis on 1% agarose gels and then capillary transferred onto nylon membrane (Hybond-N^+) with SSC 20× buffer (88.8 g/l sodium citrate, 175.3 g/l NaCl, pH 7.0) for 18 h. DNA was covalently bound to the membrane by UV-treatment for 50 s in UV-Cross-linker (Bio-Rad). Hybridizations were performed with digoxigenin (DIG) labeled probes (50 ng/ml), obtained by PCR amplification of specific htTG cDNA regions. Labeling of probes was performed by using the "PCR DIG Probe Synthesis kit" (Roche) with specific primer pairs in order to obtain the different S1, S2 and S3 probes, which are able to hybridize in different parts of htTG cDNA. All steps of incubations and treatments of agarose gels and membrane blots were performed following the standard procedures suggested by "The DIG system for filter hybridization" (Roche).

The hybridization bands were revealed with anti-DIG monoclonal antibody alkaline phosphatase-labeled and the CDP-STAR chemioluminescent substrate, both from "DIG

Luminescent Detection Kit" (Roche). The detection of specific hybridization signals was achieved by exposure of membrane blots to Kodak films for 30 min.

Results

Expression of recombinant htTG in T_0 and T_1 transgenic *Nicotiana tabacum* plants

Plant expression constructs (pTRAkc-htTG-*apo*, *cyto* and *chl*), obtained for the heterologous expression of htTG in three different plant cell compartments (Sorrentino et al. 2005), were used for the transformation of *N. tabacum* cv Petite Havana SR1 via co-cultivation with recombinant *A. tumefaciens* followed by selection for kanamycin resistance.

The T_0 transgenic tobacco plants, identified as *apo*, *cyto* and *chl*, were screened for the presence of recombinant htTG by immunoblotting analyses with monoclonal anti-tTG antibodies.

A protein band of expected size (77 kDa), corresponding to the recombinant htTG, was revealed in several transgenic plants for all three considered constructs (Fig. 1). However, not all the kanamycin resistant regenerated tobacco plants showed detectable recombinant htTG accumulation. In particular, the percentage of positive resulting plants was 54% for *apo* (12 out of 22 analyzed, Fig. 1a), 39% for *cyto* (7 out of 18, Fig. 1b) and 43% for *chl* (9 out of 21, Fig. 1c). The accumulation level of recombinant htTG in all cell compartments was approximately evaluated by densitometric analyses with Quantity One software (Bio-Rad). The obtained values revealed that the recombinant htTG accumulates at similar levels in the *cyto* and *chl* compartments, being 18–50 μg in *cyto* plants, and 25–50 μg in *chl* plants per gram of fresh leaf tissue. Highest accumulation levels were achieved in *apo* plants whose values were in a range of 18–75 μg/g of fresh leaf tissue.

T_0 plants showing highest expression levels were selected for self pollination to obtain transgenic lines then subjected to further molecular characterizations (Fig. 1, numbers marked with asterisk). For each construct, the highest expressing line was used to evaluate the accumulation levels of five kanamycin-resistant T_1 plants. In particular, T_1 seedlings from *apo*-5, *cyto*-11 and *chl*-8 lines were analyzed by western blot. The recombinant htTG was still expressed at consistent levels into the *apo* and *chl* transformed plant families (Fig. 2a, c), while weaker signals were detected in samples derived from plants with the cytoplasmic targeting of the recombinant protein (Fig. 2b). The highest accumulation value was registered for *chl*-8.2 plant (Fig. 2c, lane 2), thus this plant was further characterized.

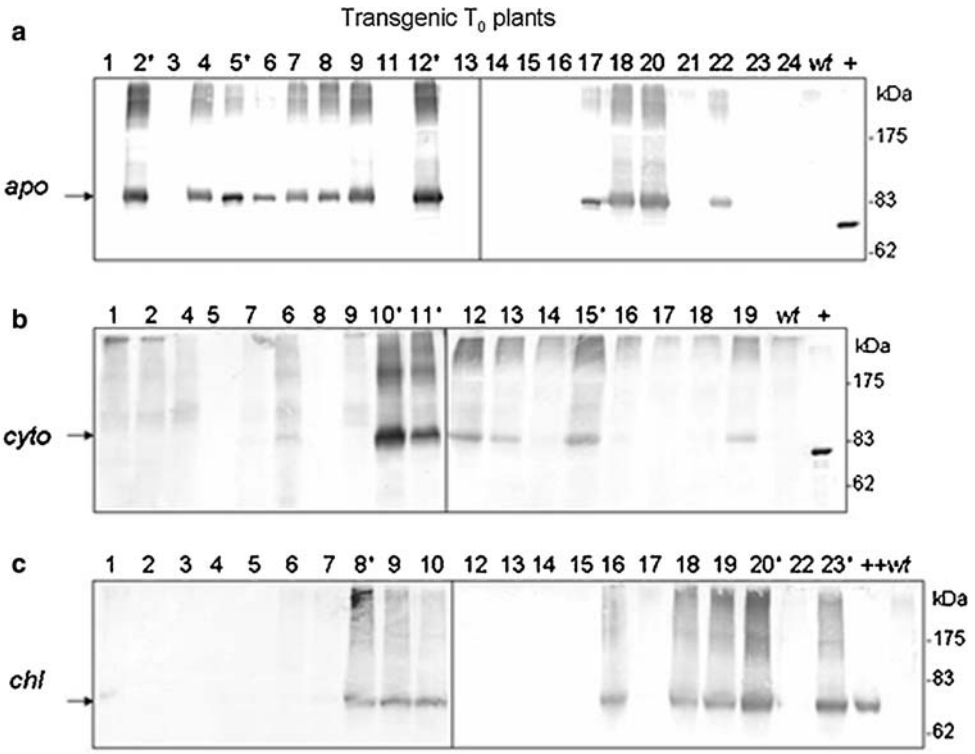

Fig. 1 Expression of recombinant htTG in *Nicotiana tabacum* T_0 plants. Immunoblot analyses of soluble proteins from T_0 plants transformed with pTRAkc-htTG derived constructs. *Numbers on lanes* identify selected transformed T_0 plants; *numbers marked with asterisk* refer to selected plants for further analyses. The *arrows* indicate the htTG size; "*wt*" protein extract from wild type plant. "+" 200 ng of gptTG (Sigma); "++" protein extract from *apo*-6 plant (panel **a**, *lane 6*)

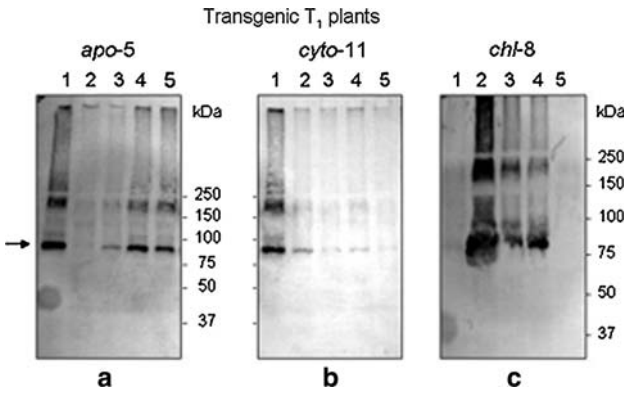

Fig. 2 Immunoblot analyses of transgenic T_1 plants. Immunoblot analyses of soluble proteins from T_1 plants transformed with pTRAkc-htTG derived constructs. *Numbers on lanes* identify selected transformed T_1 plants. The *arrow* indicates the htTG size

Biochemical characterization of transgenic *chl*-8.2 T_1 plant

Enzyme activity assay

The functionality of recombinant htTG produced in plant was evaluated by enzyme activity assay by using TSPs from leaf extracts as enzymatic source. Crude protein extract from *chl*-8.2 leaves was tested for the presence of enzymatic activity using a radiometric assay and the obtained enzymatic units were normalized for the milligrams of TSPs in order to calculate the specific activity. Compared to the *wt* TSPs, the *chl*-8.2 samples, containing the plant expressed recombinant htTG, exhibited a significant TG activity (Fig. 3b) with a calculated specific activity of ~26 U/mg of TSPs.

2D gel electrophoresis

Protein extracts from *wt* and chl-8.2 plants were analyzed via two-dimensional electrophoresis. For the first dimension, TSP (~270 μg) were focalized at their isoelectric point on 7-cm IPG strips pH 3–10; afterwards, proteins were separated on 10% SDS-PAGE for the second dimension.

As shown in Fig. 3c, in both analyzed plants proteomic pattern was essentially distributed between pH 4 and 8. However, a clear attenuation of protein bands, particularly at low molecular weights, occurred in *chl*-8.2, with a concurrent appearance at the top of the gel of new protein bands at higher mass, some of which were unable to penetrate the stacking gel (Fig. 3c, right).

Segregation in progeny

To confirm the transmission of the transgene to the next generation of transformed plants, nine T_0 plants were selected (Fig. 1, numbers marked with asterisk) and their T_1 seedlings were examined for kanamycin resistance. Four transgenic tobacco plant families showed the resistant to sensitive 3:1 mendelian segregation *ratio* (Table 1) expected for insertion of kanamycin resistance gene in a single locus. Assuming co-integration of htTG gene with the kanamycin resistance gene, the introduced htTG gene likely resides at the same locus.

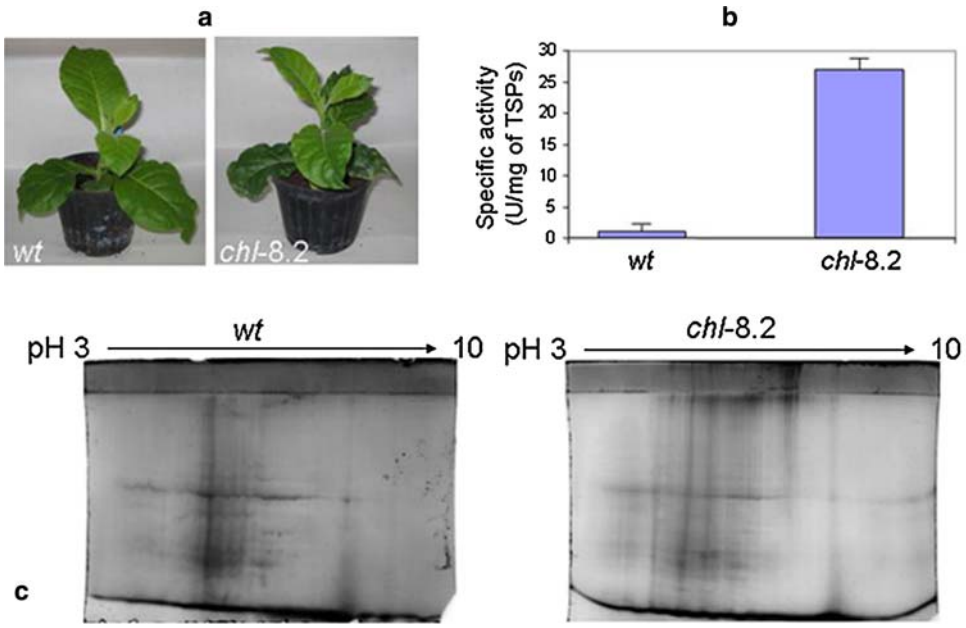

Fig. 3 Characterization of transgenic *chl*-8.2 plant compared to *wt* plant. Phenotypic aspect of the two plants (**a**); specific activity (**b**) and two-dimensional gel electrophoresis (**c**) of soluble proteins from leaf extracts; the first dimension was carried out by running 270 μg of proteins into 7-cm IPG-strip, pH 3–10, and second dimension was a 10% SDS-PAGE

Table 1 Segregation of kanamycin in T_1 progeny of transgenic plants

Transgenic line	Segregation (resistant/sensitive)	χ^2-Test (3:1)[a]
apo-2	76:58	37.92
apo-5	112:31	0.84[b]
apo-12	68:82	70.4
cyto-10	24:99	201.97
cyto-11	107:19	6.61[b]
cyto-15	163:25	13.72
chl-8	127:47	0.38[b]
chl-20	84:66	28.88
chl-23	98:26	1.075[b]

[a] The χ^2-values test the fit of counts of resistant/sensitive progeny to a 3:1 mendelian *ratio*

[b] Significant at $P < 0.01$ for 1 *df*

Molecular characterization of transgenic *apo*, *cyto* and *chl* T_1 plants

The copy number of htTG transgene in T_1 plants showing a segregation *ratio* of 3:1 was investigated by Southern blot analysis. Thus, total genomic DNA was isolated from five plants of each *apo*-5, *cyto*-11 and *chl*-8 lines, and then digested with *Sca*I or *Eco*RI. Both enzymes cut once in the expression cassette of pTRAkc constructs with the difference that *Sca*I cut in the htTG coding region and *Eco*RI cut at the 5'upstream the htTG sequence; neither enzyme has additional recognition sites elsewhere in the plasmids used for transformation. Therefore, in case of single or multiple interspersed integration events, probing with each of S1, S2 or S3 probes, we expected to find one band for a single copy, two bands for two copies and so on (Fig. 4a). Direct or inverted tandem copies integrations can also be distinguished by probing the DNA with S1 probe alone and with S1 + S2 or with S2 and S3, respectively (Fig. 4b, c).

For the transgenic lines analyzed in this detail, Southern blot results of *cyto*-11 plants demonstrated a unique hybridization band for all five analyzed genotypes (Fig. 5b), indicating a single integration event. Results obtained with *apo*-5 and *chl*-8 lines, instead, indicated multiple copy number for the transgene with a complex integration pattern (Fig. 5a, c). In particular, the DNA from *apo*-5 T_1 progeny showed six hybridization bands with S1, three bands with S3 (Fig. 5a), and at least seven bands with S1 + S2 probes (data not shown). These results are compatible with the presence of six copies of htTG transgene, integrated in three different inverted tandems. The *chl*-8 line showed the same five hybridization bands with S1 and S3 probe with identical electrophoretic profile for both probes (Fig. 5c). This atypical result was confirmed also with S1 + S2 hybridization (data not shown), suggesting that the insertion sites are linked, mapping on the same chromosome, although not in tandem among themselves.

It is worth noting that no hybridization signals were found in DNA from *wt* plants used as negative control (Fig. 5), indicating that probes were not able, as expected, to recognize any tobacco sequence in the employed stringency conditions.

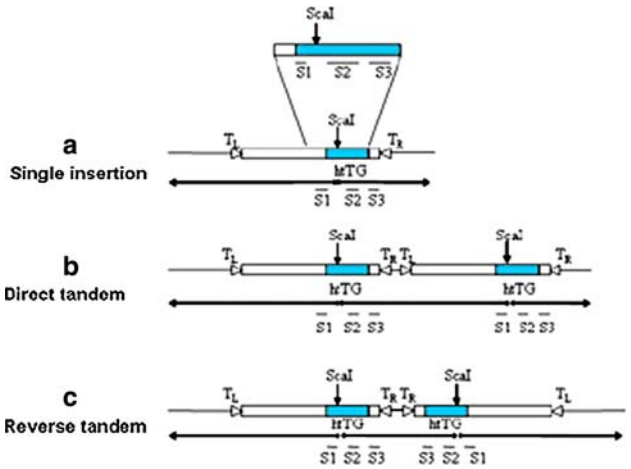

Fig. 4 Schematic representation of hybridization fragments expected in Southern blot analysis of *Sca*I digested genomic DNA from transgenic T_1 plants. Panel **a**, integration of single copy or multiple interspersed copies; hybridization with S1, S2, S3 probes → 1 copy = 1 band; 2 copies = 2 bands, and so on. Panel **b**, integration of two copies in direct tandem; hybridization with S1, S3 probes → 2 bands, with S1 + S2 probes → 3 bands. Panel **c**, integration of two copies in reverse tandem; hybridization with S1 probes → 2 bands, with S2, S3 probes → 1 band

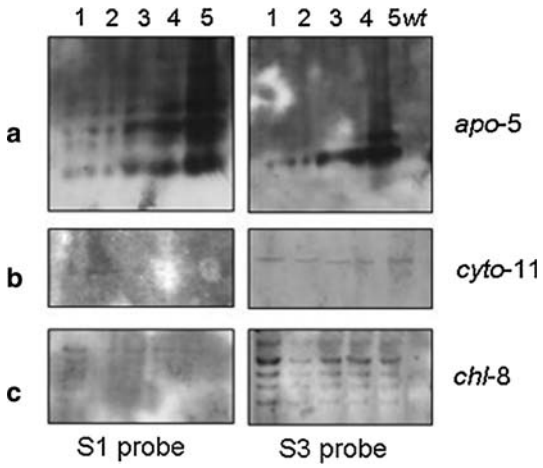

Fig. 5 Southern blot analysis of transgenic *apo*, *cyto* and *chl* T_1 plants. *Sca*I digested genomic DNA was hybridized with S1 and S3 probes. *Numbers on lanes* identify genotypes of T_1 plants; "*wt*", DNA from wild type plant used as negative control

Discussion

In this study, we have utilized *N. tabacum* cv Petite Havana SR1 with a molecular farming purpose in attempt of producing transgenic plants expressing the recombinant htTG. Three plant expression constructs were used enabling targeting and accumulation of the recombinant protein into the plant cell cytosol, in the chloroplasts and in the apoplastic space (Sorrentino et al. 2005).

Tobacco leaves were subjected to co-cultivation with recombinant *A. tumefaciens* and stably transformed plants were regenerated from transgenic *calli* growing on kanamycin selective substrates.

Immunoblot analyses of regenerated T_0 plants revealed that recombinant htTG was detectable in all three transgenic lines (Fig. 1) with a satisfying accumulation level ranging between 18 and 75 μg/g of leaf material. However, a higher percentage of expressing plants was achieved with *apo* plants (54%) followed by *chl* and *cyto* (43 and 39% respectively).

To be an efficient bioreactor a plant expression system must establish the heterologous character in the progeny and continue expressing it to high levels. Screening of T_1 generation showed that the recombinant htTG was still efficiently expressed in the majority of analyzed plant families (Fig. 2) with a particularly higher accumulation level in *chl*-8.2 than in other plants (Fig. 2c, lane 2). Thus, the *chl*-8.2 plant was further characterized and compared to a wild type plant. Despite the phenotypic aspect of the two plants, showing no substantial differences, (Fig. 3a), the *chl*-8.2 protein extracts displayed a consistent TG activity (Fig. 3b). The specific activity was calculated to be about 26 U/mg of TSPs, resulting ~2-fold higher than the value exhibited by the crude cell extract from BY-2-*chl*-targeted htTG (12.46 U/mg, Sorrentino et al. 2005). But then, the high molecular weight smear observed in most of the immunoblot analyzed samples (Figs. 1, 2) clearly indicates that the recombinant htTG contained in leaf extracts is endowed with enzymatic activity. In fact, crosslinking action upon itself and other endogenous proteins produces aggregates which are recognized by the specific anti-tTG antibody and absent in protein extracts from *wt* plants used as controls. Further evidences of a highly active recombinant htTG resulted from proteome analysis of the transgenic *chl*-8.2 plant. Once again, the crosslinking of endogenous leaf proteins was proved by the occurrence of new protein species of higher mass, some of which barely entered the separating gel, together with a concomitant decrease of the intensity of protein bands at lower molecular weight (Fig. 3c). This result is not surprising considering the wide substrate specificity of tissue isoform of TG, being able to modify a variety of proteins from plant origin (Griffin et al. 2002).

The reason why the percentage of regenerated *cyto* transformed T_0 plants was reduced (Fig. 1b) probably resides in the high functionality of recombinant htTG, while in the T_1 progeny, the accumulation level in the *cyto* compartment was the lowest detected (Fig. 2b). In fact, it is likely that the presence of highly active recombinant htTG could lead to the cross-linking of cytosolic proteins that become unable to accomplish their biological functions. Since the *cyto* construct gave the highest production of recombinant enzyme in infiltrated leaves (data not shown), it could be assumed that the regeneration of transgenic *cyto* plants from transformed *calli*, as well as the germination of transgenic *cyto* seeds could be prevented by the cross-linking activity of htTG. This hypothesis is confirmed by previously obtained results with BY-2 cells, where it was practically impossible to regenerate transgenic BY-2 *calli* transformed with the *cyto* construct (Sorrentino et al. 2005).

Moreover, evaluating the T_1 progeny of *cyto*-10 line, it comes out that the segregation *ratio* is almost inverted being the resistant seedlings about one-fourth of the sensitive ones (Table 1). This unexpected higher frequency of sensitive phenotype, reported less markedly also for other plant lines (see Table 1), suggests that the inability of growing on kanamycin substrates was due, most likely, to the toxicity of recombinant htTG rather than to the lack of marker gene. Thereby, presuming that a high copy number of transgene can lead to a high accumulation level of the recombinant enzyme, it is possible to argue that this may result lethal for the progeny of transgenic lines. For this reason, only transgenic lines showing a mendelian segregation *ratio* of 3:1 (Table 1) were selected for further characterizations.

According to the segregation analysis, Southern blot results revealed that *cyto*-11 plants possess a single copy of the htTG gene in their genomes (Fig. 5b), while, surprisingly, the *apo*-5 and *chl*-8 progenies carry a high copy number (Fig. 5a, c). These results reflect a complex integration pattern of transgenes which seem to be in linkage in the same locus at a no segregable distance, showing a segregation behavior compatible with mendelian *ratio* (Table 1).

A single copy of transgene justifies the lower expression level observed in *cyto*-11 plants (Fig. 2b), compared with the higher content of recombinant protein obtained in most of the *apo*-5 and *chl*-8 plants (Fig. 2a, c) that possessed multiple copies. The almost overall lack of signals noticed in *apo*-5.2, *chl*-8.1 and *chl*-8.5 plants (Fig. 2a, c) can be assigned to gene silencing that notoriously occur in transgenic plants (Yu and Kumar 2003). As reported by several authors, this phenomenon happens in presence of multiple copy of transgenes since plants are able to activate a mechanism of post-transcriptional gene silencing (PTGS)

that can be defined as a process leading to the degradation of homologous mRNAs (Kooter et al. 1999; Vaucheret et al. 2001 Matzhe et al. 2001; Waterhouse et al. 1998; Muskens et al. 2000; Wang and Waterhouse 2000). In fact, when multiple copies of a transgene are integrated as inverted-repeat insertions in the genome, RNA silencing of transgene may occur (Waterhouse et al. 1998; Muskens et al. 2000; Wang and Waterhouse 2000). A similar silencing mechanism can be hypothesized at least for *apo*-5.2 plant where three inverted-tandem insertions were found by Southern analyses, while additional investigations are required to better elucidate the events triggering the gene silencing in *chl*-8.1 and *chl*-8.5 plants. Nevertheless, PTGS can be overcome in the next generation of plants as RNA silencing does not affect the transcription of a gene locus, but only causes sequence-specific degradation of target mRNAs, which is not inherited in the progeny (Kooter et al. 1999).

In spite of silencing problems, the heterologous expression in the plant system can still be considered a suitable strategy for the scale up production of functional recombinant htTG. In fact, the enzyme is accumulated at appreciable levels in all three different compartments even though the chloroplast targeting would be preferable for the containment of cytotoxic activity.

The amount of recombinant enzyme expressed in the plant system was calculated to be 0.45–1.87% with respect to TSPs. As previously reported, also the BY-2 cells expression system allowed the accumulation of recombinant htTG in a range of 0.6–1.6% of TSPs (Sorrentino et al. 2005). Thus, whole plants as well as plant suspension cell cultures showed a comparable expression efficiency demonstrating their competitiveness towards other proposed bioreactors such as *Escherichia coli* (Shi et al. 2002) and baculovirus/insect cells (Osman et al. 2002). In conclusion, it can be assessed that both plant and cell expression systems are suitable bioreactors for htTG production.

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