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ORIGINAL ARTICLE

Transformation of *Solanum tuberosum* plastids allows high expression levels of β-glucuronidase both in leaves and microtubers developed in vitro

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Abstract Plastid genome transformation offers an attractive methodology for transgene expression in plants, but for potato, only expression of *gfp* transgene (besides the selective gene *aadA*) has been published. We report here successful expression of β-glucuronidase in transplastomic *Solanum tuberosum* (var. Desiree) plants, with accumulation levels for the recombinant protein of up to 41% of total soluble protein in mature leaves. To our knowledge, this is the highest expression level reported for a heterologous protein in *S. tuberosum*. Accumulation of the recombinant protein in soil-grown minitubers was very low, as described in previous reports. Interestingly, microtubers developed in vitro showed higher accumulation of β-glucuronidase. As light exposure during their development could be the trigger for this high accumulation, we analyzed the effect of light on β-glucuronidase accumulation in transplastomic tubers. Exposure to light for 8 days increased β-glucuronidase accumulation in soil-grown tubers, acting as a light-inducible expression system for recombinant protein accumulation in tuber plastids. In this paper we show that plastid transformation in potato allows the highest recombinant protein accumulation in foliar tissue described so far for this food crop. We also demonstrate that in tubers high accumulation is possible and depends on light exposure. Because tubers have many advantages as protein storage organs, these results could lead to new recombinant protein production schemes based on potato.

Keywords Potato · Tuber · Plastid transformation · Tobacco · Chloroplast · β-Glucuronidase

Abbreviations
TSP Total soluble proteins
GUS β-Glucuronidase

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Introduction

Potato [*Solanum tuberosum* and other cultivated tuber-bearing species (Hawkes 1990)] is, after cereals, the most important food crop worldwide (Caldiz 2007). Some traits that would be desirable for potato include increased yields (through disease resistance for example), modification of metabolic pathways, and production of new compounds (with the advantage of having a tuber as a storage organ). Nonetheless, breeding programs to generate improved

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cultivars have been limited by the tetraploid nature of cultivated potato and because many traits of agronomic interest are multigenic (Mullins et al. 2006). Transgenesis could circumvent many difficulties and limitations of conventional breeding programs.

Potato nuclear genome has been transformed with different transgenes (Mullins et al. 2006), either by direct (biolistic) or *Agrobacterium tumefaciens*-mediated transformation techniques, with varying results. Some observed limitations include low expression levels, triggering of post-transcriptional gene silencing, and the risk of horizontal transgene transfer to wild relative species.

Plastid genome (plastome) transformation is an attractive alternative methodology that allows higher levels of heterologous protein accumulation, with reported values of 51% and even 70% of total soluble protein (TSP) (Bock and Warzecha 2010; Lentz et al. 2010; Oey et al. 2009; Ruhlman et al. 2010) in *Nicotiana tabacum* plants. Advantages compared with the nuclear genome transformation include the absence of positional effects and the maternal inheritance of plastids in many of the cultivated species, reducing the risk of transgene transfer to compatible wild relatives (Daniell and Varma 1998).

Since 1990s, many species have been transformed at plastome level (Daniell 2006; Maliga and Bock 2011; Verma and Daniell 2007), but the methodology has been routinely used with *N. tabacum* only, probably due to tissue culture limitations (Maliga 2004; Ruhlman et al. 2010). In the case of potato, three publications showed stable plastid transformation. Sidorov et al. (1999) showed plastid transformation of the potato line FL1607, using vectors containing tobacco recombinogenic sequences to target integration of transgenes *aadA* and *gfp* between the genes *rbcL* and *accD*, or between the genes *trnV* and *rps7/3′*. They reported GFP expression levels of approximately 5 and 0.05% of TSP in leaves and microtubers, respectively, with low transformation efficiency when compared with tobacco (Sidorov et al. 1999). Nguyen et al. (2005) reported plastid transformation of the commercial potato cultivar Desiree. They tested vectors designed for tobacco plastid transformation that targeted the integration of *aadA* and *gfp* between the genes *rbcL* and *accD* or between *16S* rRNA and *orf70B*. In this report, the GFP expression levels were 0.0175 and 0.0035% of TSP in leaves and tubers, respectively, with lower transformation efficiency when compared with *N. tabacum*. More recently, Valkov et al. (2011) achieved 4% of TSP for GFP in leaves of transplastomic potato plants (Desiree) when the cassette containing *gfp* was targeted to the intergenic region between *rbcL* and *accD*, using potato recombinogenic sequences. In contrast, the best expression levels obtained in tubers were 0.02% TSP.

As summarized, only a few examples of potato plastid transformation have been published to date. In spite of improvements in the method, the levels of accumulation of heterologous proteins achieved in tubers are still insufficient for many biotechnological applications. In order to explore more alternatives for efficient plastid protein expression in potato, we used a plastid transformation vector designed to integrate the transgenes (*uidA* and *aadA*) into the *rrn* operon, between genes *16S* and *trnI*. With this vector we transformed the plastome of *N. tabacum* L. var. Petit Havana and *S. tuberosum* cv. Desiree. We achieved *β*-glucuronidase (GUS) accumulation levels up to 41% of TSP in mature leaves of transplastomic potato plants. To our knowledge, this is the highest reported value for potato plastid transformation. As expected, the levels of GUS expression in soil-grown minitubers were at least 10,000 times lower than the values observed in leaves. Nonetheless, the exposure of these soil-grown minitubers to light increased GUS accumulation levels. Interestingly, in microtubers developed in vitro, GUS accumulation was comparable to leaf values. Tubers are an attractive destination for protein accumulation since they are storage organs and heterologous proteins can remain stable for long periods of time (Artsaenko et al. 1998). These results open the opportunity for new schemes of heterologous proteins production in transplastomic potato plants.

Materials and methods

Transformation vectors

pBSW-utr was designed to express *β*-glucuronidase protein through plastid transformation in tobacco and potato plants. Briefly, *uidA* sequence was amplified by PCR with *Pfu* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) using DNA from pBI121 binary vector (Chen et al. 2003) as template. Two oligonucleotides were designed to include *Nde*I and *Xba*I restriction sites for subsequent cloning steps: gus5′(5′ CAGTCCCATATGTTACGTCCTG 3′) and gus3′(5′ GGAGAGTTCTAGATTCATTG 3′), respectively. Amplification product was sub cloned into pZeRO-2 (Invitrogen), and the fragment containing the *uidA* coding sequence was released by enzymatic restriction. Gel-purified *uidA* *Nde*I/*Xba*I fragment was sub-cloned into the chloroplast transformation vector pBSW-utr, previously digested with *Nde*I/*Xba*I to replace *hegf* sequence (Wirth et al. 2006). The final vector was designed pBSW-utrGUS (Fig. 1a), and the identity of *uidA* sequence was confirmed by sequencing and functional assay in *E. coli* (data not shown).

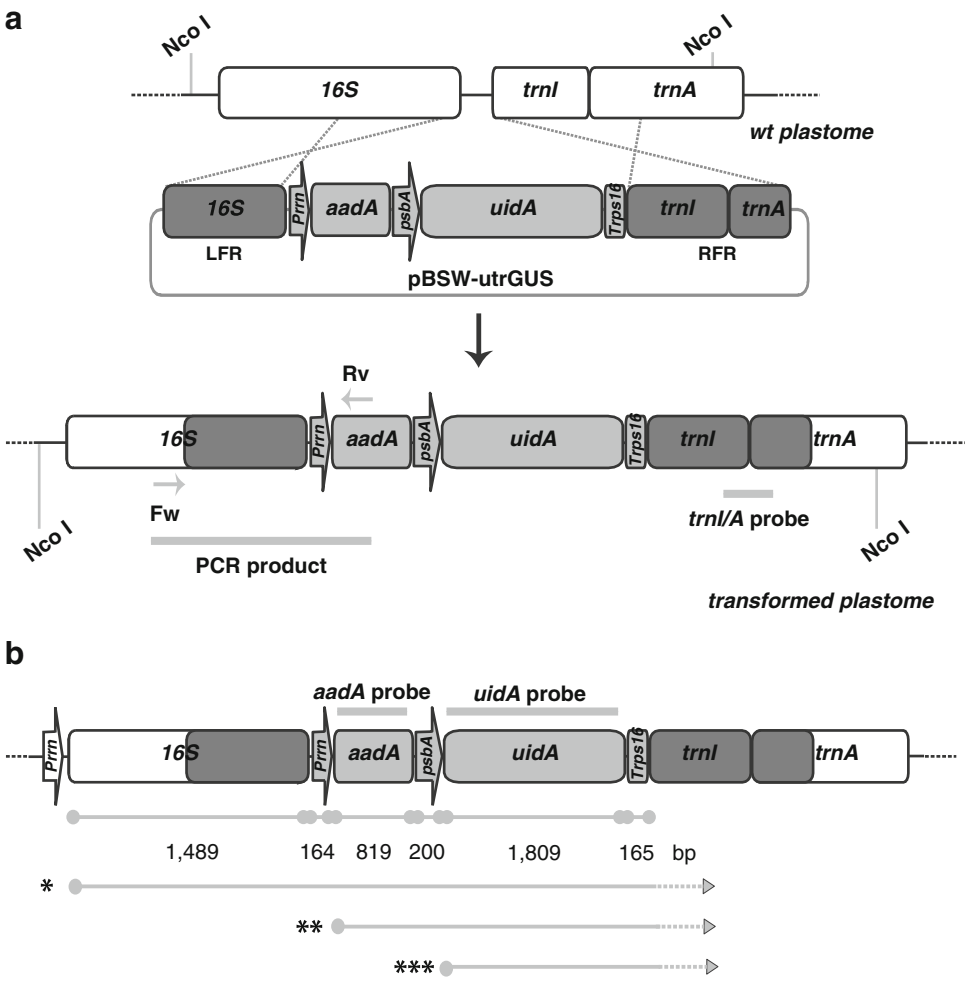

Fig. 1 Design of the plastid transformation vector pBSW-utrGUS. **a** The recombinant vector pBSW-utrGUS includes recombinogenic sequences from *N. tabacum* to target integration to the intergenic region between *16S* and *trnI*. The left flanking region (LFR 1,169 bp) includes the 3′ region of *rrn16*, and the right flanking region (RFR 1,015 bp) contains the full *trnI* and the 5′ region of *trnA*. Chloroplast physical map before and after insertion of the construct by homologous recombination is shown. *trnI/A*: probe used in Southern blot assays. *Arrows* indicate the location of the forward (Fw) and reverse (Rv) primers used for PCR analysis. **b** Diagram showing the mRNA transcripts expected from each promoter after the insertion of the transgenes into the plastid genome. *aadA* and *uidA*: probes used in northern blot assays

Tobacco plastid transformation

Plastid transformation in *N. tabacum* was carried out as previously described (Daniell 1997; Svab and Maliga 1993) using the PDS 1000/He biolistic particle delivery system (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, fully expanded leaves of in vitro cultured *N. tabacum* L. cv. Petite Havana plants (courtesy of Dr. Néstor Carrillo, Instituto de Biología Molecular y Celular de Rosario, Universidad Nacional de Rosario, Rosario, Argentina) were bombarded with 50 mg of 0.6-µm gold particles (Bio-Rad) coated with 10 µg of plasmid DNA using 1,100 psi rupture discs (Bio-Rad). Spectinomycin-resistant lines were selected on RMOP regeneration medium (Svab et al. 1990) containing 500 mg l^{-1} spectinomycin di-hydrochloride. Several independent transplastomic lines were subjected to additional rounds of regeneration on spectinomycin-containing regeneration medium to obtain homoplasmic lines. After root formation, plants were transferred to soil and grown under greenhouse conditions. In the greenhouse, natural light was supplemented 16 h per day by sodium lamps providing 100–300 µmol s^{-1} m^{-2}; the temperature was set at 26°C during day and 19°C in the night. The transplastomic nature of the regenerated plants was assessed by PCR amplification, using primers that anneal to the *16S* plastidic gene (upstream of the left flanking region or LFR) and to the *aadA* gene (Fw: 5′ GTATCTGGGGAATAAGCATCGG 3′ and Rv: 5′ CGAT GACGCCAACTACCTCTG 3′, respectively) (Fig. 1a). Therefore, a 1,450-bp specific fragment was amplified only in transplastomic plants.

Potato plastid transformation

Plastid transformation in *S. tuberosum* was carried out following the protocol described by Nguyen et al. (2005) with minor modifications. Briefly, young leaves from in vitro plants of *S. tuberosum* var. Desiree were excised, put with the abaxial side up onto sterile filter paper, in a petri dish containing CIM medium [MS salts (PhytoTechnology Laboratories, Overland Park, KS, USA) supplemented with glucose 1.6% (Sigma-Aldrich, St. Louis, MO, USA), 2,4-D 2 mg l^{-1} (Sigma-Aldrich), trans-zeatin riboside 0.9 mg l^{-1} (Sigma-Aldrich) and agar 0.8% (PhytoTechnology Laboratories)],

and kept in the dark. After 24 h, the leaves were bombarded using the PDS 1000/He biolistic particle delivery system (Bio-Rad) with similar parameters as those described for tobacco except that the distance between the sample and the macrocarrier was 6 cm. Bombarded leaves were cut into small pieces and put with the abaxial side down onto regeneration/selection media following the published protocol (Nguyen et al. 2005). Regenerated shoots were transferred to media containing MS salts and sucrose 2% supplemented with spectinomycin 400 mg l^{-1}. Internode explants were detached and subjected to a new round of regeneration according to published protocol (Beaujean et al. 1998), with a selection pressure of 400 mg l^{-1} spectinomycin. Once rooted, plants were transferred to soil and grown in the greenhouse. The transplastomic nature of the regenerated plants was assessed by PCR amplification in a similar way as described for tobacco.

Southern blot analysis

Integration and homoplasmy were assessed by Southern blot analysis. Total DNA was extracted from leaves as described previously (Dellaporta et al. 1983). The DNA (2 µg) was digested with *Nco*I (New England Biolabs, Beverly, MA, USA), electrophoresed in 0.8% agarose gels, and blotted onto HybondN+ Nylon membranes (Amersham Biosciences, Uppsala, Sweden). Specific DNA sequences were detected by hybridization with a ^{32}P-labeled *trnI/A* DNA probe, generated by random priming with a Prime-a-Gene kit (Promega, Madison, WI, USA). Pre-hybridization and hybridization were carried out at 65°C in Church's hybridization solution (Church and Gilbert 1984) for 2 and 16 h, respectively. After hybridization, membranes were washed for 15 min at room temperature in 2× SSC, 0.1% SDS, then for 15 min in the same solution at 65°C, and finally for 15 min in 0.2× SSC, 0.1% SDS at 65°C. The blot was exposed to a storage phosphor screen, which was analyzed using a Storm 840 PhosphorImager system (Amersham Biosciences).

Northern blot analysis

Total RNA was extracted from fully expanded young leaves using TRiZOL Reagent (Invitrogen). For tuber tissue, and extra step was included in order to reduce the sugar content. The aqueous phase obtained after the chloroform extraction was transferred to a new tube and 250 µl isopropanol plus 250 µl of a solution of sodium isocitrate 1.2 M/sodium chloride 0.8 M were added. The mix was incubated 30 min at 4°C, followed by a 10-min centrifugation at 13,400*g* (4°C).

The pellet was resuspended with 250 µl sodium acetate 3 M (pH 5.2). After centrifugation (10 min at 13,400*g*, 4°C), the pellet was washed with 750 µl 75% ethanol (Sintorgan, Argentina) and centrifuged 10 min at 13,400*g*, 4°C. Finally, the pellet was dried and resuspended in 50 µl of RNAse-free water. For northern blot assays, 3 µg of formaldehyde-denatured RNA were electrophoresed in a 1.5% agarose/formaldehyde gel and blotted onto HybondN+ Nylon membranes (Amersham Biosciences). Specific mRNA sequences were detected by hybridization with ^{32}P-labeled *uidA* or *aadA* DNA probes generated by random priming with a Prime-a-Gene kit (Promega). The blot was pre-hybridized, hybridized, and washed as described for Southern blot.

Protein extraction and analysis

Total soluble proteins were extracted as follows: 100 mg of tissue was processed with 400 µl (leaf tissue) or 100 µl (tuber tissue) of GUS extraction buffer (50 mM Na phosphate, pH 7, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, pH 8, 0.1% L-lauryl sarcosine, 0.1% Triton X-100). Homogenate was centrifuged for 30 min at 12,000*g* (4°C), and pellet was discarded. One aliquot of the supernatant was analyzed by the Bradford assay to determine TSP concentration (Bradford 1976). Remaining supernatant was mixed with 1 mM PMSF (Sigma-Aldrich), and used immediately for fluorometric assays (described below) or kept at 4°C for a few days or at −70°C for a few months, for further applications. For SDS-PAGE, the desired volume of this supernatant was mixed with Laemmli buffer (Laemmli 1970) and different quantities were loaded in polyacrylamide gels according to previous protocols (Sambrook et al. 1989). After electrophoresis, the gels were stained with Coomassie brilliant blue (CBB) or transferred onto nitrocellulose membrane for antibody detection. GUS protein was detected using a mouse antiserum specific for VP-βGUS (Lentz et al. 2010), and a second incubation step with alkaline phosphatase-linked rabbit anti-mouse antibody diluted 1:4,000 (Cell Signaling Technology, Beverly, MA, USA). After a final wash, phosphatase activity was determined by a chromogenic reaction using 5-bromo-4 chloro-3 indolyl phosphate and nitroblue tetrazolium (Sigma, St. Louis, MO, USA) as substrates. Quantification of GUS accumulation in the different leaves of transplastomic plants was done by comparing a dilution series of TSPs obtained from leaf samples of utrGUS-3 potato plants, with a dilution series of purified BSA. Known amounts of both BSA and leaf protein extracts were compared by SDS-PAGE and staining with CBB as described above. Stained gels were scanned and the protein band intensities of GUS and BSA were quantified using the ImageJ software (NIH, http://www.rsbweb.nih.gov/ij).

Detection of GUS activity in transplastomic plants

GUS enzymatic activity in transgenic plants was quantified using a fluorometric assay as previously described (Jefferson et al. 1987). Briefly, 25 mg of leaf tissue were ground in 125 µl of GUS extraction buffer (described in "Protein extraction" section). After centrifugation (30 min at 12,000*g*, 4°C), the supernatant containing the protein extract was diluted and 2 µl of this dilution was mixed with 498 µl of GUS assay buffer (1 mM of 4-methylumbelliferyl-*β*-D-glucuronide (Invitrogen) in GUS extraction buffer). The reaction was performed at 37°C. A previously characterized tobacco plant (*N. tabacum* cv. Xanthi) transformed with the *uidA* gene at the nuclear genome level obtained by the *Agrobacterium* method (nuGUS) was used as control. Protein extracts were diluted in order to achieve a confident fluorescence signal. A 100-µl aliquot was removed at different time points and added into 1.9 ml of stop buffer (0.1 M Na_2CO_3). GUS activity was determined with a DyNAQuant 200 fluorometer (Hoefer), using as standard 4-methyl-umbeliferone (4-MU). Total protein concentration of tissue homogenates was determined by the Bradford assay (Bradford 1976). The assay was repeated at least twice with each sample. Histochemical detection of GUS activity in plant samples was performed as previously described (Jefferson et al. 1987).

Light treatment of soil-grown potato tuber discs

Soil-grown minitubers (wt and utrGUS-4) were washed, peeled, and surface sterilized with 20% commercial bleach during 3 min. After washing in sterile water and removal of death tissue, tubers were cut into slices (2 mm width) and disposed into 1% agar plates, as previously described (Anstis and Northcote 1976; Virgin and Sundqvist 1992; Zhu et al. 1984). The discs were kept in the dark or illuminated by fluorescent tubes (70 µmol s^{-1} m^{-2}), under 16 h light/8 h dark photoperiod and 24°C temperature, for 8 days. Tuber discs from 0 h, 8 days dark and 8 days light treatments were processed with GUS extraction buffer and GUS enzymatic activity was measured as described above.

Results

Production of *N. tabacum* and *S. tuberosum* transplastomic plants

To obtain transplastomic tobacco and potato plants, we developed a vector named pBSW-utr (Lentz et al. 2010; Morgenfeld et al. 2009; Wirth et al. 2006). This vector contains *N. tabacum* sequences to target the integration of the desired genetic elements into the intergenic region between the genes *16S* and *trnI* (Fig. 1a), located in the Inverted Repeats (IRs) region of the plastome (Wakasugi et al. 1998). In pBSW-utr, the selector gene (*aadA*) and the gene of interest (*uidA*) can be transcribed as a policistronic RNA from the upstream *rrn* operon promoter. The *psbA* promoter and 5′UTR sequences included also direct transcription of *uidA*. The recombinogenic sequences used here show good nucleotide conservation (99% identity) between tobacco and potato, with only small insertions in the non-coding regions of the potato sequence. In addition, the regulatory elements included in our vector are also conserved between both species (100, 99 and 98% identity for *psbA* 5′UTR, *rrn* promoter and *rps*16 terminator sequences, respectively). Therefore, we used this vector without modifications to transform both tobacco and potato. We transformed leaf explants using the biolistic methodology, with 400 or 500 mg l^{-1} of spectinomycin for the selection of the transplastomic events in potato and tobacco, respectively. For *N. tabacum*, the transformation efficiency observed (data not shown) was similar to previous reports (Maliga 2004; Svab and Maliga 1993), with several shoots regenerated from each explant. In contrast, lower shoot regeneration efficiency was observed when *S. tuberosum* leaves were transformed; after the bombardment of 60 leaves, 22 calli developed on the abaxial side of the leaf, but only six regenerated into shoots (Fig. 2a–f). This observation points to a need for improvements in the regeneration protocol of leaf explants of *S. tuberosum* cv. Desiree, as recently shown (Valkov et al. 2011). We used *β*-glucuronidase activity assays to test for the transplastomic nature of the regenerated potato shoots and calli (Fig. 2g, h). Approximately 50% showed GUS activity, as evidenced by the quick coloration of the tissue and the buffer containing the substrate. The percentage of transplastomic potato shoots regenerated was in accordance with previous reports where integration was mediated by tobacco flanking regions (Nguyen et al. 2005; Valkov et al. 2011). The integration of transgenes into *N. tabacum* and *S. tuberosum* shoot plastomes was first confirmed by PCR (data not shown) using Fw and Rv primers indicated in Fig. 1a. Explants from PCR-positive shoots (lines utrGUS-3, 4 and 5) were subjected to new regeneration rounds under selective pressure, and mature plants from first and second regeneration rounds were then transfer to soil for further characterization. Tobacco transplastomic plants (*Nt*/utrGUS) were included for comparison.

Molecular characterization of transplastomic plants

The integration of the transgenes and analysis of homoplasmy were assessed by Southern blot (Fig. 3a). Total leaf DNA was extracted and fully digested with the restriction enzyme *Nco*I. Two *Nco*I recognition sites are located

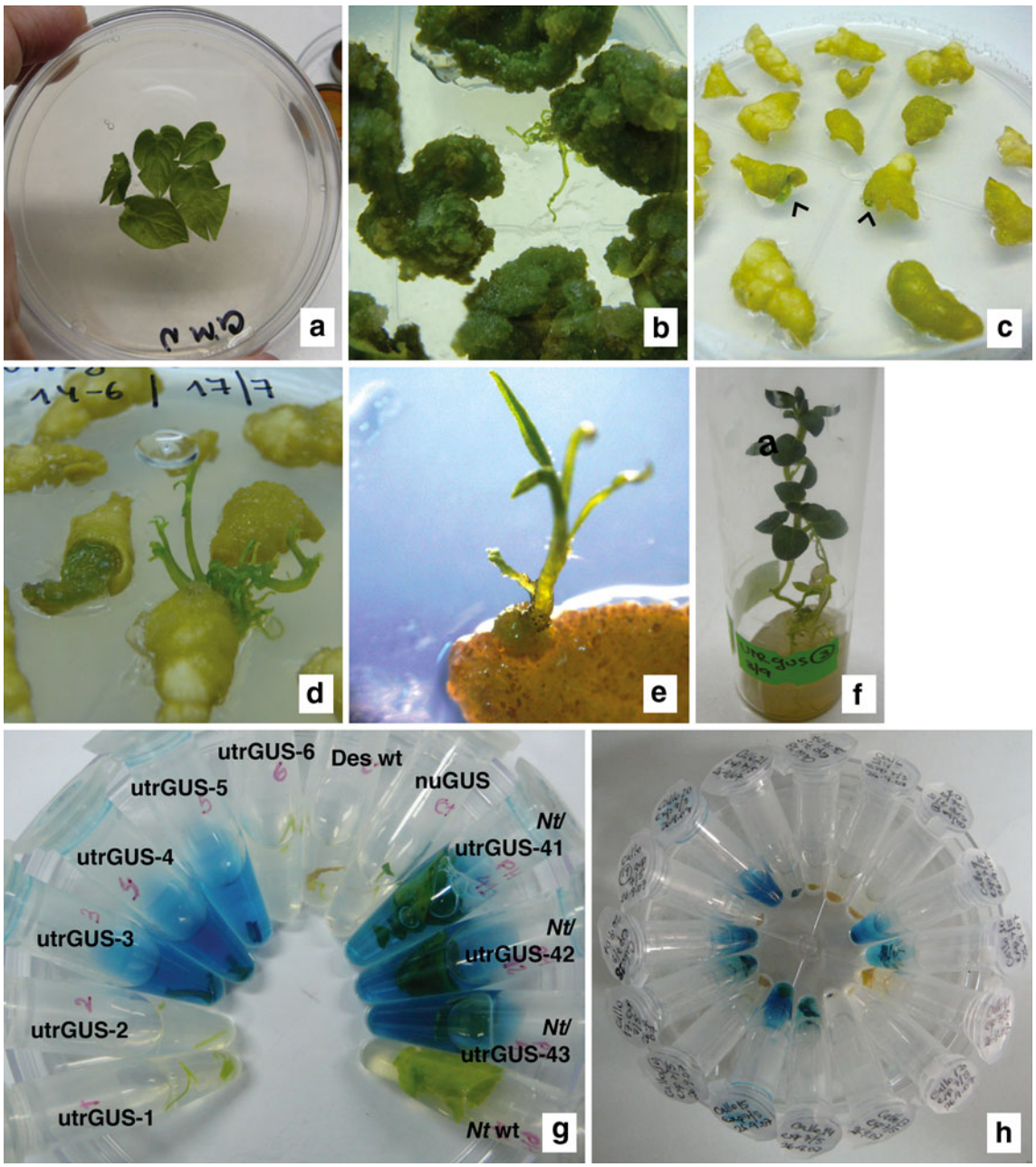

Fig. 2 Plastid transformation of *S. tuberosum* Desiree. Pictures (**a**–**f**) represent several stages in potato plastid transformation. **a** Leaves from in vitro plants used as explants for transformation; **b** regeneration control; **c**–**e** shoots regenerated 1 month after transferring the bombarded explants into Shoot Inducing Media with spectinomycin; **f** regenerated transplastomic shoot rooting in selective media. *β*-Glucuronidase activity in transplastomic potato shoots (**g**), tobacco leaves (**g**) and potato calli (**h**). *Arrowheads* in (**c**) indicate the regenerating shoots. utrGUS-1 to 6 are independent shoots regenerated from plastid transformation experiments in potato; *Nt*/utrGUS-41 to 43 indicate leaves from independent plants regenerated from plastid transformation experiments in tobacco. nuGUS: leaves from transgenic tobacco plant transformed with *uidA* at the nuclear genome level. All the transplastomic plants shown correspond to the first regeneration round

flanking the insertion site, creating fragments of different sizes for wt (6,461 bp for *S. tuberosum*) and transformed plastomes (9,672 bp for *S. tuberosum*) revealed with a probe homologous to *trnI*/*trnA* (Fig. 1a). We found that the initial transformation was sufficient to achieve homoplasmic state in transplastomic potato plants whereas three rounds of regeneration or passage to T1 generation were usually needed to achieve homoplasmy in tobacco (Maliga 2004). To evaluate the presence and relative abundance of the RNAs containing *uidA*, we performed northern blot analyses using total RNA extracted from tobacco and potato transplastomic leaves (Fig. 3b). In both species

uidA-RNA expression profile was similar, indicating that in potato leaves, the *uidA*-RNA transcribed from the *psbA* promoter is the most abundant, in accordance with previous reports for tobacco (Staub and Maliga 1993, 1994).

High β-glucuronidase accumulation in leaves of transplastomic potato and tobacco plants

We evaluated GUS accumulation in leaves of transplastomic plants by CBB-stained SDS-PAGE (Fig. 4a, b, d), fluorometric assays for GUS activity (Fig. 4c), and western blot (Fig. 4e) GUS protein was easily visualized after CBB staining, and in mature leaves (4th and 5th) its band intensity was similar to the band intensity of the large subunit of Rubisco (RbcL), the most abundant protein in the wild-type leaf (Fig. 4a, b). Moreover, protein extracts from older leaves showed enrichment in GUS protein, while RbcL protein started to decline. We selected the 5th leaf of utrGUS-3 potato plants to quantify GUS accumulation, as RbcL levels were still high in those leaves. After quantitation, values obtained for GUS protein accumulation were 41% of TSP (Fig. 4b). To our knowledge, this is the highest recombinant protein expression level reported for *S. tuberosum* plants. GUS accumulation values were similar for tobacco and potato and were around 4–5 magnitude orders above GUS expression levels measured in tobacco transgenic lines transformed at the nuclear genome level (nuGUS) (Fig. 4c). Expression of GUS in transplastomic potato leaves was not dependent upon growth conditions (in vitro or greenhouse) or the regeneration round (Fig. 4d, e). Additionally, potato plants sprouted from soil-grown minitubers or microtubers obtained from transplastomic plants showed the same levels of GUS expression as the original transplastomic lines (Fig. 4c–e), indicating the stability of the transgene trough the clonal propagation scheme usually applied for potato production.

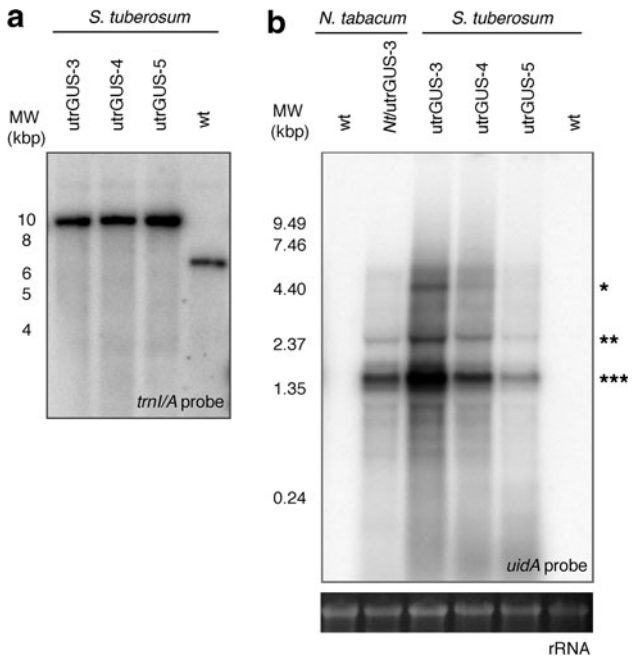

Fig. 3 Molecular characterization of transplastomic plants. Regenerated shoots were analyzed by Southern blot (**a**) and northern blot (**b**). **a** 2 µg of total DNA from potato (wt and transplastomic plants) was digested with *Nco*I, and electrophoresed in 0.8% agarose gel. After blotting, the membrane was hybridized with a *trn*I/*A* probe (wild-type plastome: 6,461 bp, transformed plastome: 9,672 bp; see Fig. 1a for details). **b** 3 µg of formaldehyde denatured RNA from potato and tobacco leaves (wt and transplastomic plants) was electrophoresed in 1.5% agarose/formaldehyde gel. After blotting, the membrane was hybridized with a *uidA* probe; *, ** and *** designate the different RNAs expected to be transcribed from three different promoters (see Fig. 1b for details). rRNA as observed under UV light was included as loading control

Low expression of *uidA* in soil-grown minitubers of transplastomic plants

To evaluate expression of *uidA* in tubers, we harvested soil-grown minitubers from transplastomic potato plants utrGUS-3, 4 and 5 and analyzed protein accumulation by SDS-PAGE (data not shown) and GUS activity fluorometric assays (Fig. 5a). GUS accumulation in soil-grown minitubers was lower than in leaves of potato and tobacco transplastomic plants, and similar to accumulation in transgenic tobacco plants transformed with *uidA* at the nuclear genome level. Despite GUS levels in the leaves of the three utrGUS lines were similar, GUS activity differed among their tubers, which also showed variations in their appearance (size and color, data not shown). We analyzed the expression of *uidA* at the RNA level by northern blot (Fig. 5b). As expected, profile of *uidA*-containing transcripts changed from leaves to soil-grown minitubers, with lower accumulation of RNAs transcribed from the three available promoters in tuber tissue, explaining at least partially the low accumulation of GUS protein (Fig. 5a). Also, a shift in the proportion of each transcript was observed, the one initiated from the vector promoter P*rrn* being the most abundant in soil-grown minitubers. We assume that this is due to *psbA* promoter and 5′UTR elements, responsible for *uidA* expression, being positively regulated by light (Staub and Maliga 1993, 1994).

High expression of *uidA* in microtubers of transplastomic plants developed in vitro

We evaluated expression of *uidA* in microtubers developed from transplastomic plants under in vitro conditions by GUS activity fluorometric assays (Fig. 6a) and CBB-stained SDS-PAGE (Fig. 6b). Interestingly, microtubers developed under in vitro conditions accumulated high

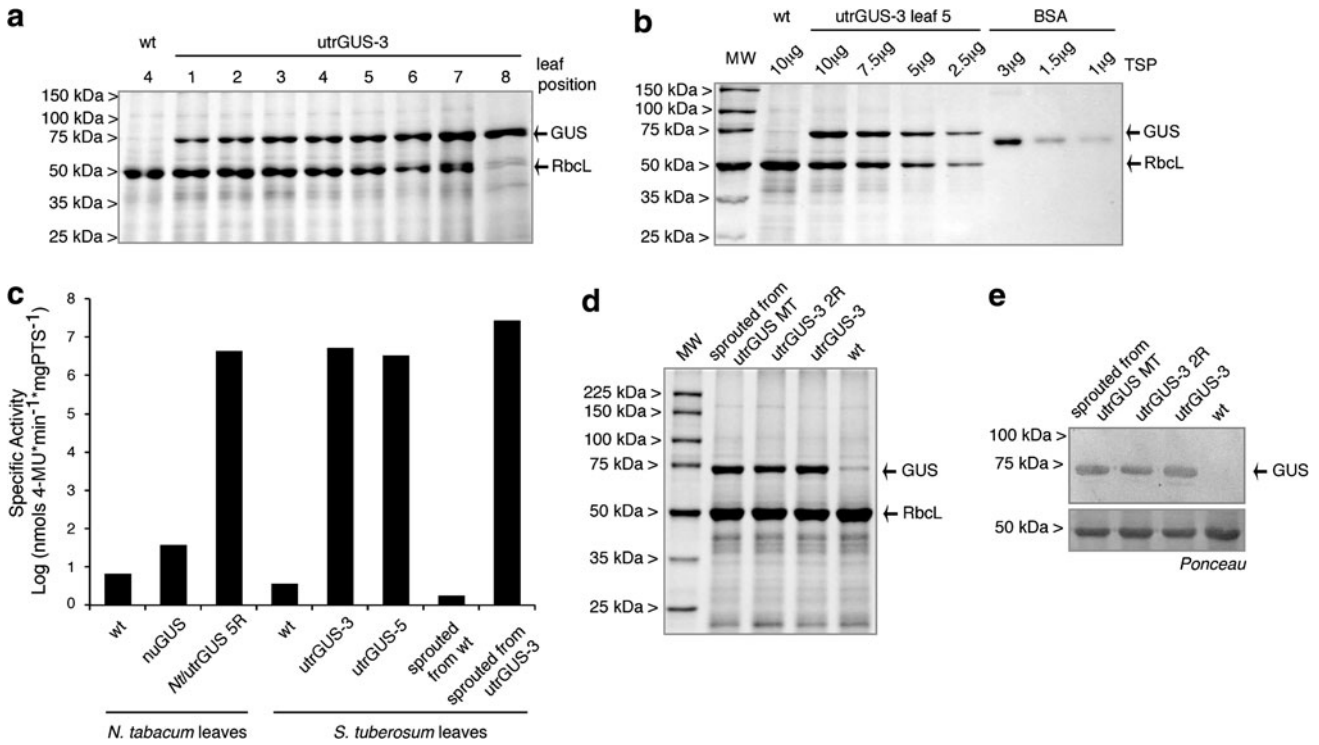

Fig. 4 Analysis of β-glucuronidase expression in leaves of transplastomic plants. **a** 10 µg of TSP from leaves of wt or utrGUS-3 potato plants were analyzed by 10% SDS-PAGE and CBB staining. Leaf position was established from young to older leaf, starting with the first expanded leaf as position 1. Relative positions for GUS and Rubisco large subunit (RbcL) are indicated. Sizes corresponding to Broad Range Protein Molecular Weigh Marker (Promega) were included. In **b**, the indicated amount of TSP extracted from wt or transplastomic leaves corresponding to position 5 (according to **a**) was loaded in each lane, including known amounts of bovine serum albumin (BSA) as standard. **c** β-Glucuronidase enzymatic activity determined by fluorometric assays using TSP from leaves of potato and tobacco transplastomic plants. Leaves from wt and nuclear transgenic plants (nuGUS) were included as controls. The common logarithm of Specific Activity was plotted. "Sprouted from" correspond to TSP from leaves of plants developed from soil-grown minitubers. **d–e** 15 µg of TSP from potato leaves from wt or utrGUS plants were analyzed by 10% SDS-PAGE and CBB staining (**d**) or western blot with anti-GUS antibodies (**e**). Sizes corresponding to Broad Range Protein Molecular Weigh Marker (Promega) were included. MT means "microtuber" (developed in vitro). (#)R designates the regeneration round of the plants from which leaves were taken. No number means that the plants were obtained from the initial transformation event

levels of GUS protein, with an average value of 9.8 ± 2.6% TSP.

The three different transcripts containing *uidA* (see Fig. 1b) accumulated with a similar profile between microtubers developed in vitro and potato leaves as indicated by northern blot (Figs. 3b, 6c). Transcripts driven by the *psbA* promoter were the most abundant (Fig. 6c).

These results suggest that high levels of recombinant protein accumulation can be reached in tuber tissue of transplastomic potato plants.

Increased expression of *uidA* in soil-grown minitubers of transplastomic plants after exposure to light

To determine if exposure to light could influence recombinant protein accumulation in soil-grown minitubers, we incubated wt and utrGUS-4 peeled minituber slices in a wet chamber with or without light exposure during 8 days. We selected this time point because glycoalkaloid accumulation exceeded the values considered safe for human consumption beyond 8 days' light exposure in Desiree tubers (Percival et al. 1996). GUS activity measured by fluorometric assay in the utrGUS-4 tuber slices exposed to light was in average 18 times higher compared with those kept in the dark (Fig. 6d). This result indicates that light-inducible expression of recombinant proteins can be achieved in tubers by the inclusion of light-responsive elements in the transformation vector (*psbA* promoter and 5UTR sequences).

Discussion

Yielding high levels of heterologous protein expression in transgenic plants is a critical problem in plant biotechnology. Plastid transformation is a very attractive methodology to overcome this problem (Bock 2001, 2007; Maliga 2004; Maliga and Bock 2011; Twyman et al. 2003).

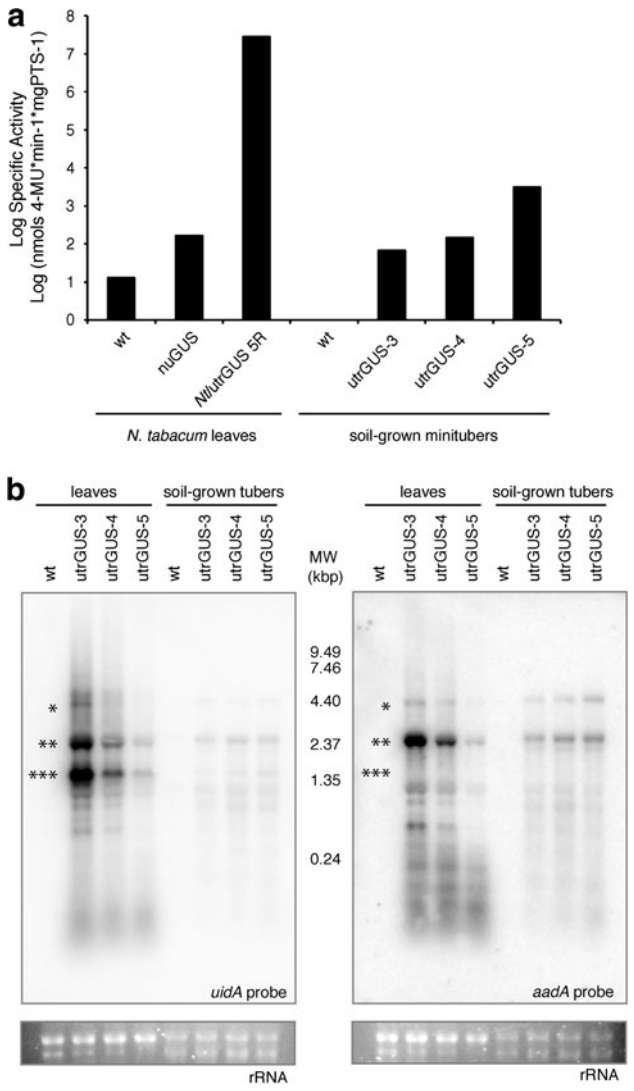

Fig. 5 Accumulation of β-glucuronidase in soil-grown minitubers of potato transplastomic plants. **a** β-Glucuronidase enzymatic activity was determined by fluorometric assays in TSP from tobacco leaves and potato soil-grown minituber samples (wt and transplastomic). The common logarithm of Specific Activity was plotted. **b** 3 μg of total RNA from potato leaves and soil-grown minitubers (from wt and transplastomic plants) was electrophoresed in 1.5% agarose/formaldehyde gel. After blotting, the membrane was hybridized with *uidA* or *aadA* probe; *, ** and *** designate the different RNAs expected to be transcribed from three different promoters (see Fig. 1b for details). rRNA as observed under UV light was included as loading control

Although several species were successfully transformed at the plastome level (Daniell 2006; Maliga and Bock 2011; Verma and Daniell 2007), most of the successful stories are related to tobacco transplastomic plants (Bock and Warzecha 2010; Maliga 2004).

Particularly for *S. tuberosum*, only two transgenes have been introduced into its plastome: *aadA* and *gfp*. The highest accumulation levels reported to date for GFP are 5% (leaves) and 0.05% (microtubers) of TSP in a non-commercial line (Sidorov et al. 1999), and 4% (leaves) and 0.02% (tubers) of TSP for Desiree (Valkov et al. 2011).

In this work we transformed the plastome of *S. tuberosum* cv. Desiree in order to evaluate expression levels of the reporter gene *uidA* in different potato tissues. We obtained stable homoplastic transplastomic plants, validating the intergenic region between *16S* and *trnI* genes as a suitable region for transgene integration in the potato plastome. Integration of foreign DNA into the plastome through homologous recombination does not require 100% similarity between the recombinogenic sequences included in the transformation vector and the target region in the plastome (Kavanagh et al. 1999). Nonetheless, utilization of endogenous regulatory elements and flanking sequences has a positive effect on transgene expression and transformation efficiency (Ruhlman et al. 2010; Valkov et al. 2011). Despite the heterologous origin of the recombinogenic sequences and regulatory elements included in the transformation vector, accumulation of GUS protein reached high levels in leaf tissue that were comparable to those obtained for tobacco, being up to 41% of TSP in mature leaves. To our knowledge, this is the highest expression level reported for a heterologous protein in *S. tuberosum.* In spite of the high expression level observed for GUS protein in leaves, plants were phenotypically normal, indicating that potato, like tobacco, can cope with the accumulation of high amounts of recombinant proteins in chloroplasts (Bally et al. 2009). However, GUS accumulation in soil-grown minitubers was much lower (four magnitude orders less than the values for the leaves of the same potato plant) and similar to values observed in Desiree transplastomic tubers (Nguyen et al. 2005) and in the leaves of nuclear transgenic tobacco plants. Even though *psbA* promoter and 5′UTR sequences included in the vector pBSW-utr do not seem to be the best choice for heterologous protein expression in amyloplasts, it has been demonstrated that *psbA*- and *rrn*-derived transcripts are the most abundant transcripts in these type of plastid (Brosch et al. 2007), although steady-state and polysome inclusion of other transcripts seem to be higher (Valkov et al. 2009). Northern blot analysis showed that in leaves of both potato and tobacco transplastomic plants *psbA* promoter and 5′UTR sequences allowed the highest transcript accumulation. When the RNA profile of soil-grown minitubers was analyzed, we observed that overall RNA accumulation was drastically reduced when compared with leaves, in agreement with previous observations (Brosch et al. 2007; Valkov et al. 2009). Moreover, RNAs transcribed from the *psbA* promoter accumulated to lower levels than those derived from the endogenous and introduced *rrn* promoters. These observations can be explained due to the presence of light-regulated elements in the *psbA* sequences included in pBSW-utr, allowing higher accumulation of transcripts

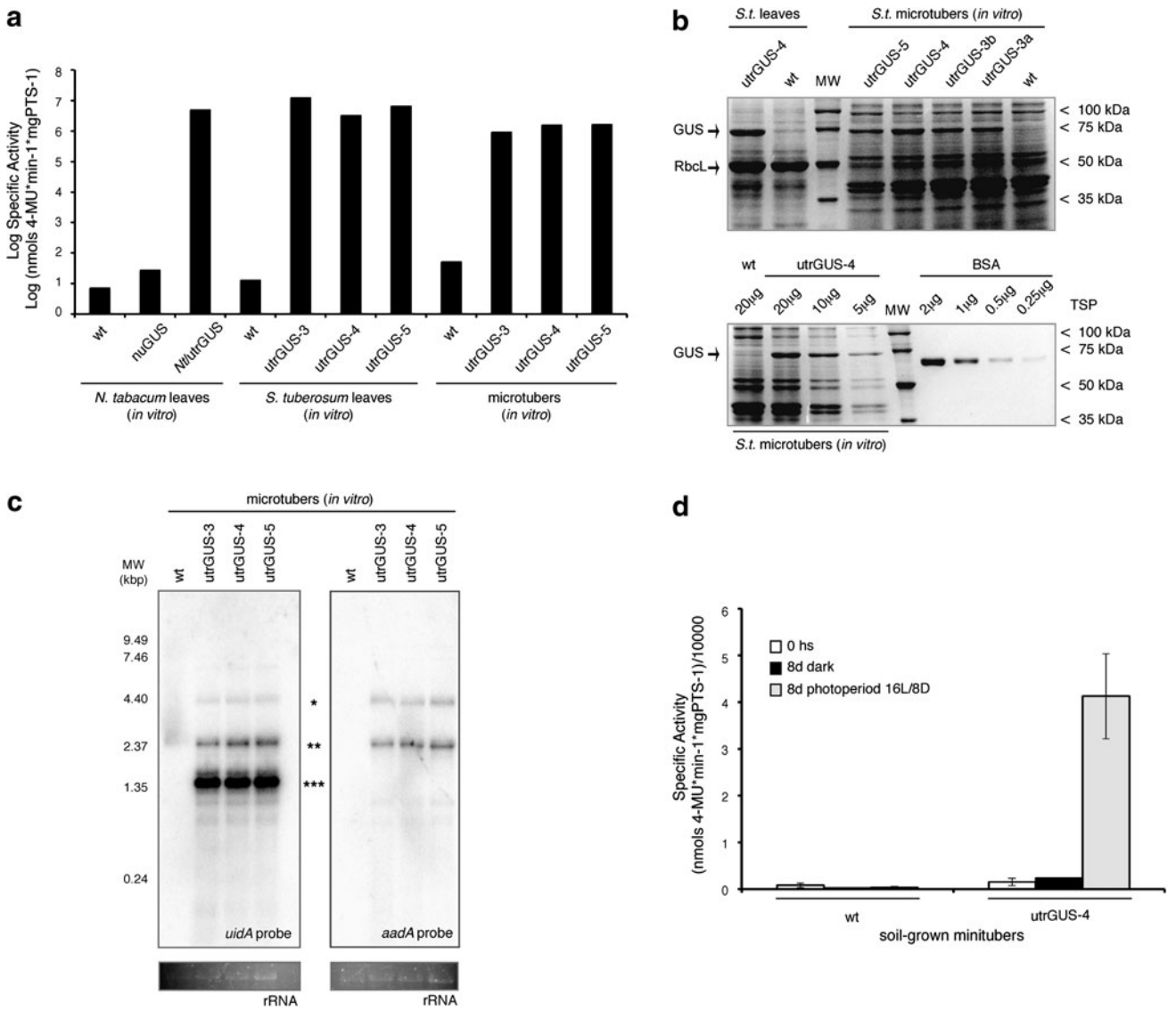

Fig. 6 Accumulation of β-glucuronidase in transplastomic microtubers (developed in vitro) or soil-grown minitubers exposed to light. **a** β-Glucuronidase enzymatic activity determined by fluorometric assays in TSP from leaves of wt and transplastomic tobacco and potato plants, and potato microtubers developed in vitro. The common logarithm of Specific Activity was plotted. **b** *Upper gel* 20 µg of TSP from microtubers developed in vitro of wt or utrGUS potato plants, and from potato leaves (utrGUS-4 or wt plants) were analyzed by 10% SDS-PAGE and CBB staining; *lower gel* the indicated amount of TSP extracted from wt or utrGUS-4 microtubers developed in vitro was loaded in each lane, including known amounts of BSA as standard. Relative positions for GUS and RbcL are indicated. Sizes corresponding to Broad Range Protein Molecular Weigh Marker were included. **c** 3 µg of total RNA from microtubers (from wt and transplastomic plants) was electrophoresed in 1.5% agarose/formaldehyde gel. After blotting, the membrane was hybridized with *uidA* or *aadA* probe; *, ** and *** designate the different RNAs expected to be transcribed from three different promoters (see Fig. 1b for details). rRNA as observed under UV light was included as loading control. **d** β-Glucuronidase enzymatic activity determined by fluorometric assays in TSP samples from soil-grown minituber slices (wt and utrGUS-4 transplastomic line) without treatment (0 h), after 8 days incubation in the dark, or after 8 days exposure to 16 h light/8 h dark photoperiod. *Bars* represent standard error of three biological replicates

in leaf chloroplasts but not in the amyloplasts of soil-grown tubers. Subtle differences observed among minitubers of utrGUS potato lines in terms of RNA transcribed from *psbA*-promoter and GUS protein levels could be related to differences in the developmental stage of the tubers. Brosch et al. (2007) reported the correlation between maturity of the potato tubers and changes in their transcript accumulation profile. When they analyzed the transcript profile of amyloplasts from older tubers, only transcripts derived from the *psbA* and the ribosomal operon (*rrn*) promoters were observed. It could be interesting to analyze in more detail how tuber maturation affects *uidA* transcripts profile and heterologous protein accumulation, to determine when the peak of transgene expression occurs.

Expression of *uidA* was also investigated in microtubers of transplastomic plants developed under in vitro conditions with normal photoperiod. Interestingly, GUS levels observed for these microtubers were around 10% of TSP, this value being much higher than the value observed for soil-grown minitubers. This observation could be the result of several processes, alone or in combination. The enrichment in the P*psbA*-derived RNA when compared with soil-grown minitubers could be indicative of a conversion of amyloplasts and/or leukoplasts into chloroplasts driven by the exposure to light, a phenomena already described in the literature (Anstis and Northcote 1976; Ljubicic et al. 1998). It was also demonstrated in tobacco leaf chloroplasts that *psbA* 5′UTR conferred light-inducible translation when included in heterologous transcripts (Eibl et al. 1999). As in vitro developed microtubers exhibit parameters characteristic of a physiologically older tuber (Stensballe et al. 2008), a different developmental state cannot be ruled out as at least partially responsible for the higher GUS accumulation observed in this kind of tuber. Since microtubers could be produced in fermentor like devices (Akita and Takayama 1994; Donnelly et al. 2003; Kämäräinen-Karppinen et al. 2010), an interesting approach for high-scale production of recombinant proteins arises.

According to recent publications, improvements of the transformation vector can increase further the accumulation of recombinant proteins in potato plastids. Among these, selecting endogenous sequences as components of the transformation vector can increase the recombinant protein expression levels (Ruhlman et al. 2010; Valkov et al. 2011). Moreover, particular arrangements of Shine–Dalgarno sequences (Drechsel and Bock 2011) and amino acid composition of the recombinant protein N-terminus (Apel et al. 2010; Gray et al. 2011) can also exert positive effects in recombinant protein accumulation. It would be interesting to see if by including these improvements in our transformation vector pBSW-utr we could increase even more recombinant protein expression in transplastomic potato plants.

In this work, we demonstrate that high levels of heterologous protein accumulation can be obtained in transplastomic potato plants without compromising plant development. Also, the high protein accumulation levels observed for microtubers developed in vitro, and its inducibility by light in soil-grown minitubers, opens the possibility for the development of new strategies for recombinant protein production in potato transplastomic plants.

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References

Akita M, Takayama S (1994) Induction and development of potato tubers in a jar fermentor. Plant Cell Tiss Organ Cult 36(2): 177–182. doi:10.1007/BF00037717

Anstis PJP, Northcote DH (1976) Development of chloroplasts from amyloplasts in potato tuber discs. New Phytol 72(3):449–463. doi:10.1111/j.1469-8137.1973.tb04394.x

Apel W, Schulze WX, Bock R (2010) Identification of protein stability determinants in chloroplasts. Plant J 63(4):636–650. doi:10.1111/j.1365-313X.2010.04268.x

Artsaenko O, Kettig B, Fiedler U, Conrad U, Düring K (1998) Potato tubers as a biofactory for recombinant antibodies. Mol Breed 4(4):313–319. doi:10.1023/A:1009676832273

Bally J, Nadai M, Vitel M, Rolland A, Dumain R, Dubald M (2009) Plant physiological adaptations to the massive foreign protein synthesis occurring in recombinant chloroplasts. Plant Physiol 150(3):1474–1481. doi:10.1104/pp.109.139816

Beaujean A, Sangwan RS, Lecardonnel A, Sangwan-Norreel BS (1998) Agrobacterium-mediated transformation of three economically important potato cultivars using sliced internodal explants: an efficient protocol of transformation. J Exp Bot 49(326):1589–1595. doi:10.1093/jxb/49.326.1589

Bock R (2001) Transgenic plastids in basic research and plant biotechnology. J Mol Biol 312(3):425–438. doi:10.1006/jmbi.2001.4960

Bock R (2007) Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering and molecular farming. Curr Opin Biotechnol 18(2):100–106. doi:10.1016/j.copbio.2006.12.001

Bock R, Warzecha H (2010) Solar-powered factories for new vaccines and antibiotics. Trends Biotechnol 28(5):246–252. doi:10.1016/j.tibtech.2010.01.006

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. doi:S0003269776699996

Brosch M, Krause K, Falk J, Krupinska K (2007) Analysis of gene expression in amyloplasts of potato tubers. Planta 227(1):91–99. doi:10.1007/s00425-007-0597-9

Caldiz DO (2007) Producción, cosecha y almacenamiento de papa en la Argentina. 2nd edn. McCain Argentina SA, Balcarce–BASF Argentina SA, Ciudad Autónoma de Buenos Aires, Argentina

Chen P-Y, Wang C-K, Soong S-C, To K-Y (2003) Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Mol Breed 11(4):287–293. doi:10.1023/a:1023475710642

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81(7):1991–1995

Daniell H (1997) Transformation and foreign gene expression in plants by microprojectile bombardment. Methods Mol Biol 62:463–489. doi:10.1385/0-89603-480-1:463

Daniell H (2006) Production of biopharmaceuticals and vaccines in plants via the chloroplast genome. Biotechnol J 1(10):1071–1079. doi:10.1002/biot.200600145

Daniell H, Varma S (1998) Chloroplast-transgenic plants: panacea—no! Gene containment—yes! Nat Biotechnol 16(7):602. doi:10.1038/nbt0798-602

Dellaporta S, Wood J, Hicks J (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1(4):19–21. doi:10.1007/BF02712670

Donnelly D, Coleman W, Coleman S (2003) Potato microtuber production and performance: a review. Am J Potato Res 80(2): 103–115. (doi:10.1007/BF02870209)

Drechsel O, Bock R (2011) Selection of Shine–Dalgarno sequences in plastids. Nucleic Acids Res 39(4):1427–1438. doi:10.1093/nar/gkq978

Eibl C, Zou Z, Beck A, Kim M, Mullet J, Koop H-U (1999) In vivo analysis of plastid *psbA*, *rbcL* and *rpl32* UTR elements by chloroplast transformation: tobacco plastid gene expression is controlled by modulation of transcript levels and translation efficiency. Plant J 19:333–345. doi:10.1046/j.1365-313X.1999.00543.x

Gray B, Yang H, Ahner B, Hanson M (2011) An efficient downstream box fusion allows high-level accumulation of active bacterial beta-glucosidase in tobacco chloroplasts. Plant Mol Biol 76(3):345–355. doi:10.1007/s11103-011-9743-7

Hawkes JH (1990) The potato: evolution, biodiversity and genetic resources. Belhaven Press, London

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6(13):3901–3907

Kämäräinen-Karppinen T, Virtanen E, Rokka VM, Pirttilä A (2010) Novel bioreactor technology for mass propagation of potato microtubers. Plant Cell Tiss Organ Cult 101(2):245–249. doi:10.1007/s11240-010-9679-7

Kavanagh TA, Thanh ND, Lao NT, McGrath N, Peter SO, Horvath EM, Dix PJ, Medgyesy P (1999) Homeologous plastid DNA transformation in tobacco is mediated by multiple recombination events. Genetics 152(3):1111–1122

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259): 680–685. doi:10.1038/227680a0

Lentz EM, Segretin ME, Morgenfeld MM, Wirth SA, Dus Santos MJ, Mozgovoj MV, Wigdorovitz A, Bravo-Almonacid FF (2010) High expression level of a foot and mouth disease virus epitope in tobacco transplastomic plants. Planta 231(2):387–395. doi:10.1007/s00425-009-1058-4

Ljubicic JM, Wrischer M, Ljubesic N (1998) Formation of the photosynthetic apparatus in plastids during greening of potato microtubers. Plant Physiol Biochem 36(10):747–752. doi:10.1016/S0981-9428(98)80025-9

Maliga P (2004) Plastid transformation in higher plants. Annu Rev Plant Biol 55:289–313. doi:10.1146/annurev.arplant.55.031903.141633

Maliga P, Bock R (2011) Plastid biotechnology: food, fuel and medicine for the 21st century. Plant Physiol. doi:10.1104/pp.110.170969

Morgenfeld M, Segretin ME, Wirth S, Lentz E, Zelada A, Mentaberry A, Gissmann L, Bravo-Almonacid F (2009) Potato virus X coat protein fusion to human papillomavirus 16 E7 oncoprotein enhance antigen stability and accumulation in tobacco chloroplast. Mol Biotechnol 43(3):243–249. doi:10.1007/s12033-009-9195-3

Mullins E, Milbourne D, Petti C, Doyle-Prestwich BM, Meade C (2006) Potato in the age of biotechnology. Trends Plant Sci 11(5):254–260. doi:10.1016/j.tplants.2006.03.002

Nguyen TT, Nugent G, Cardi T, Dix PJ (2005) Generation of homoplasmic plastid transformants of a commercial cultivar of potato (*Solanum tuberosum* L.). Plant Sci 168(6):1495–1500. doi:10.1016/j.plantsci.2005.01.023

Oey M, Lohse M, Kreikemeyer B, Bock R (2009) Exhaustion of the chloroplast protein synthesis capacity by massive expression of a highly stable protein antibiotic. Plant J 57(3):436–445. doi:10.1111/j.1365-313X.2008.03702.x

Percival G, Dixon GR, Sword A (1996) Glycoalkaloid concentration of potato tubers following exposure to daylight. J Sci Food Agric 71(1):59–63. doi:10.1002/(SICI)1097-0010(199605)71:1<59:AID-JSFA548>3.0.CO;2-1

Ruhlman T, Verma D, Samson N, Daniell H (2010) The role of heterologous chloroplast sequence elements in transgene integration and expression. Plant Physiol 152(4):2088–2104. doi:10.1104/pp.109.152017

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York

Sidorov VA, Kasten D, Pang SZ, Hajdukiewicz PT, Staub JM, Nehra NS (1999) Technical advance: stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. Plant J 19(2):209–216. doi:10.1046/j.1365-313X.1999.00508.x

Staub JM, Maliga P (1993) Accumulation of D1 polypeptide in tobacco plastids is regulated via the untranslated region of the *psbA* mRNA. EMBO J 12(2):601–606

Staub JM, Maliga P (1994) Translation of *psbA* mRNA is regulated by light via the 5′-untranslated region in tobacco plastids. Plant J 6(4):547–553

Stensballe A, Hald S, Bauw G, Blennow A, Welinder KG (2008) The amyloplast proteome of potato tuber. FEBS J 275(8):1723–1741. doi:10.1111/j.1742-4658.2008.06332.x

Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. Proc Natl Acad Sci USA 90(3):913–917. doi:10.1073/pnas.90.3.913

Svab Z, Hajdukiewicz P, Maliga P (1990) Stable transformation of plastids in higher plants. Proc Natl Acad Sci USA 87(21): 8526–8530. doi:10.1073/pnas.87.21.8526

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21(12):570–578. doi:10.1016/j.tibtech.2003.10.002

Valkov VT, Scotti N, Kahlau S, Maclean D, Grillo S, Gray JC, Bock R, Cardi T (2009) Genome-wide analysis of plastid gene expression in potato leaf chloroplasts and tuber amyloplasts: transcriptional and posttranscriptional control. Plant Physiol 150(4):2030–2044. doi:10.1104/pp.109.140483

Valkov VT, Gargano D, Manna C, Formisano G, Dix PJ, Gray JC, Scotti N, Cardi T (2011) High efficiency plastid transformation in potato and regulation of transgene expression in leaves and tubers by alternative 5′ and 3′ regulatory sequences. Transgenic Res 20(1):137–151. doi:10.1007/s11248-010-9402-9

Verma D, Daniell H (2007) Chloroplast vector systems for biotechnology applications. Plant Physiol 145(4):1129–1143. doi:10.1104/pp.107.106690

Virgin HI, Sundqvist C (1992) Pigment formation in potato tubers (*Solanum tuberosum*) exposed to light followed by darkness. Physiol Plant 86(4):587–592. doi:10.1111/j.1399-3054.1992.tb02174.x

Wakasugi T, Sugita M, Tsudzuki T, Sugiura M (1998) Updated gene map of tobacco chloroplast DNA. Plant Mol Biol Rep 16(3): 231–241. doi:10.1023/A:1007564209282

Wirth S, Segretin ME, Mentaberry A, Bravo-Almonacid F (2006) Accumulation of hEGF and hEGF-fusion proteins in chloroplast-transformed tobacco plants is higher in the dark than in the light. J Biotechnol 125(2):159–172. doi:10.1016/j.jbiotec.2006.02.012

Zhu YS, Merkle-Lehman DL, Kung SD (1984) Light-induced transformation of amyloplasts into chloroplasts in potato tubers. Plant Physiol 75(1):142–145. doi:10.1104/pp.75.1.142