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ORIGINAL PAPER

High efficiency plastid transformation in potato and regulation of transgene expression in leaves and tubers by alternative 5′ and 3′ regulatory sequences

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Abstract Transformation of potato plastids is limited by low transformation frequencies and low transgene expression in tubers. In order to improve the transformation efficiency, we modified the regeneration procedure and prepared novel vectors containing potato flanking sequences for transgene integration by homologous recombination in the Large Single Copy region of the plastome. Vector delivery was performed by the biolistic approach. By using the improved regeneration procedure and the potato flanking sequences, we regenerated about one shoot every bombardment. This efficiency corresponds to 15–18-fold improvement compared to previous results with potato and is comparable to that usually achieved with tobacco. Further, we tested five promoters and terminators, and four 5′-UTRs, to increase the expression of the *gfp* transgene in tubers. In leaves, accumulation of GFP to about 4% of total soluble protein (TSP) was obtained with the strong promoter of the *rrn* operon, a synthetic *rbcL*-derived 5′-UTR and the bacterial *rrnB* terminator. GFP protein was detected in tubers of plants transformed with only four constructs out of eleven. Best results (up to approximately 0.02% TSP) were achieved with the *rrn* promoter and *rbcL* 5′-UTR construct, described above, and another containing the same terminator, but with the promoter and 5′-UTR from the plastid *clpP* gene. The results obtained suggest the potential use of *clpP* as source of novel regulatory sequences in constructs aiming to express transgenes in amyloplasts and other non-green plastids. Furthermore, they represent a significant advancement of the plastid transformation technology in potato, of relevance to its implementation in potato breeding and biotechnology.

Keywords *Solanum tuberosum* · Homologous and homeologous recombination · Chloroplasts · Amyloplasts

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Introduction

The integration and the expression of transgenes in the plastome offers several potential advantages over the transformation of the nuclear genome in plants. These include the precise integration of transgenes in the host genome by homologous recombination, the high expression levels and the biological containment of transgenes and recombinant products, the cellular compartmentalization of compounds harmful to the plant, and the possibility of co-expressing several transgenes in prokaryotic-like operons. General concepts and applications of plastid transformation in basic research and biotechnology have been discussed in several recent reviews (e.g. Maliga 2004; Daniell et al. 2005; Bock 2007; Hasunuma et al. 2009).

In many cases, however, low transformation efficiencies have been reported for species other than tobacco (Sikdar et al. 1998; Sidorov et al. 1999; Ruf et al. 2001; Hou et al. 2003; Skarjinskaia et al. 2003; Zubko et al. 2004; Nguyen et al. 2005; Nugent et al. 2006; De Marchis et al. 2009). Low recovery of transplastomic shoots has been attributed to multiple reasons, such as a relatively inefficient homologous recombination system, non-optimal homology and length of flanking regions, the promoter used for the expression of the selectable marker gene, the kind of explant, or the regeneration protocol. An additional limitation related to the application of plastid transformation in breeding and biotechnology is the generally inefficient gene expression in non-green plastids, as a consequence of deficiencies in the expression machinery of non-green plastids compared to leaf chloroplasts (Brosch et al. 2007; Kahlau and Bock 2008; Valkov et al. 2009). Transgene expression with the plastid *rrn* and the bacterial *trc* promoters in potato tuber amyloplasts ranged from 1 to 20% of that in leaf chloroplasts, respectively, although GFP concentration attainable with the prokaryotic promoter was lower than with *rrn* one (0.004 vs. 0.05% of total soluble proteins), due to its low efficacy in both tissues (Sidorov et al. 1999; Nguyen et al. 2005). In transplastomic tomato plants, the fusion protein P24-Nef from HIV accumulated up to 40% TSP in leaves, but significantly less in green and ripe fruits (2.5% and nil, respectively) (Zhou et al. 2008). The relative expression level of transgenes in tomato fruit chromoplasts, however, was higher with other proteins (Ruf et al. 2001) and in any case sufficient to induce significant metabolic changes (Wurbs et al. 2007; Apel and Bock 2009). Very low levels of mRNA and GFP protein were detected in roots of transplastomic *Nicotiana benthamiana* plants (less than 2% of that in the leaves), albeit expression levels up to 40–75% of those in leaf chloroplasts could be achieved in petal leucoplasts of the same plants or in chromoplasts of carrot taproots (Kumar et al. 2004a; Davarpanah et al. 2009).

Despite recent progress, potato plastid transformation is still limited by low transformation frequencies and low transgene expression in tubers of transplastomic plants (Sidorov et al. 1999; Gargano et al. 2003; Nguyen et al. 2005; Gargano 2006). Plastid transformation is a long and complex process requiring organelle sorting out during repeated cell divisions in vitro, in order to achieve regeneration of homoplasmic transplastomic shoots (Bock 2001; Maliga 2004). It is therefore extremely important to devise an efficient selection and regeneration procedure. Furthermore, in all previous published reports, plastid transformation in potato was attempted with vectors designed for tobacco. However, since the complete potato ptDNA sequence became available (Gargano et al. 2005; Chung et al. 2006), vectors with homologous potato flanking sequences could be constructed and tested to examine the effect of increasing homology on plastid transformation efficiency.

Recent studies have reported the thorough characterization of gene expression in tuber amyloplasts (Brosch et al. 2007; Valkov et al. 2009), showing that gene expression in such organelles is generally impaired, with multi-step control occurring at transcriptional, post-transcriptional and translational levels. However, these studies have allowed the tentative identification of candidate regulatory sequences that potentially could improve transgene expression in amyloplasts and other non-green plastids. Overall, transcripts of plastid genes for the genetic system showed a smaller reduction in tubers, compared to leaves, than transcripts of photosynthesis-related genes (Valkov et al. 2009). These differences in transcript accumulation in tubers were mainly attributed to differential transcript stability (Brosch et al. 2007; Valkov et al. 2009). Although both the eubacterial-type (PEP) and phage-type (NEP) RNA polymerases were shown to be active in non-green

plastids (Brosch et al. 2007; Kahlau and Bock 2008; Valkov et al. 2009), genes with relatively higher levels of transcripts in tubers (e.g., *rrn*, *clpP, accD*, *ycf1*, *ycf2*) contain either NEP or multiple PEP and NEP promoters (Hajdukiewicz et al. 1997; Legen et al. 2002; Valkov et al. 2009), suggesting the involvement of NEP in tuber amyloplast gene expression. Indeed, recent data suggest that the two polymerases do not simply mediate gene class-specific transcription in different cells or plastid types, but differential processing, stability, and accumulation of the resulting transcripts and polypeptides (Legen et al. 2002; Cahoon et al. 2004; Zoschke et al. 2007) are involved in regulating gene expression. Alternative 5′- and 3′-UTRs of plastid genes can also play a significant role in transcript stability and translatability (Bock 2001; Maliga 2003).

Scant data are available on the use of alternative plastid 5′ and 3′ regulatory sequences in potato tubers (Sidorov et al. 1999; Nguyen et al. 2005). A full-length plastid *rrn* operon promoter, with both PEP and NEP consensus sequences, along with the ribosome-binding site of the phage T7 gene 10 leader and the 3′-UTR of the plastid gene encoding the ribosomal protein S16 were recently used to facilitate transgene expression in carrot chromoplasts (Kumar et al. 2004a). A similar approach was adopted by Wurbs et al. (2007), who used the *atpI* promoter containing both PEP and NEP signals, and the *rps16* 3′-UTR, to express carotenoid-related genes in tomato fruits. Nevertheless, the short *rrn* promoter, containing only the sequences for PEP recognition, was successfully used to express transgenes in fruit chromoplasts of transplastomic tomato, and in petal leucoplasts and root amyloplasts of tobacco and *Nicotiana benthamiana* (Khan and Maliga 1999; Ruf et al. 2001; Apel and Bock 2009; Davarpanah et al. 2009).

In this paper, we report the improvement of plastid transformation in potato to levels similar to those for tobacco, by optimizing the selection/regeneration procedure and by using homologous transformation vectors. Further, we tested several regulatory sequences for transgene expression in leaves and tubers, confirming general differences in expression patterns in the two organs. Although expression in tubers was generally low, the results obtained suggest the potential use of the plastid gene *clpP*, encoding the proteolytic subunit of the Clp ATP-dependent protease, as source of novel regulatory sequences to be used in constructs aimed at expression of transgenes in amyloplasts and other non-green plastids.

Materials and methods

Plastid transformation vectors

Plastid transformation vectors 1001, 1002, and 1003, contain a chimeric *gfp* gene under the control of *trc*, *rrn* and *psbA* promoters, respectively, and the *E. coli rrnB* terminator. They derive from pZS197 vector (Svab and Maliga 1993) and correspond to vectors described by Newell et al. (2003). The *aadA* selectable marker gene is under the control of the *rrn* promoter, the *rbcL* 5′-UTR and *psbA* 3′-UTR sequences. 1073, 1074, 1075 and 1076 vectors have *psbA* promoter and 5′-UTR as in the 1003 construct and different 3′-UTRs (*rbcL*, *psbA*, *petD*, *rpoA*, respectively) (Newell et al. 2003; Tangphatsornruang et al. 2003). Flanking and/or regulatory sequences driving the expression of *gfp* in the 1002 vector were replaced with sequences amplified from the potato plastome (DQ386163; Gargano et al. 2005), using *PfuUltra*® HF DNA polymerase (Stratagene, La Jolla, CA, USA), to produce pVL vectors.

The full-length potato *rrn* promoter sequence (P*rrnFL*; nucleotides 101600–101765), carrying both PEP and NEP transcription initiation sites, was amplified from potato genomic DNA using St-rrnFl_fd and St-rrnFl_rbs_rev primers (Supplementary Table S1) which introduced also a ribosome-binding site, digested with *Not*I and *Eco*RI and cloned in the 1002 vector (Newell et al. 2003) to produce pVL12. Tobacco flanking regions in pVL12 were replaced with the respective potato sequences as follows: after partial digestion, the *rbcL* flanking region between the *Sac*II and *Xba*I restriction sites (corresponding to nucleotides 57758–59202 of the tobacco plastid genome) was replaced by PCR-amplified *rbcL* flanking region from the potato plastid genome (nucleotides 56694–58164) using St-rbcL_fd and St-int_rev primers, generating the intermediate construct pVL4. The *gfp* and *aadA* expression cassettes and potato *rbcL* flanking region were excised from pVL4 as a *Sac*II/*Not*I fragment and cloned in pBluescript KS, producing the intermediate pVL5 vector. The a*ccD* flanking region (corresponding to nucleotides 58235–59506 of the potato plastid genome) was amplified with St-int_fd and St-accD_rev

primers, introducing *Not*I and *Eco*RV restriction sites, respectively. The PCR product was digested and cloned in *Not*I/*Hinc*II-restricted pVL5, producing the pVL13 vector carrying P*rrnFL-gfp* between *rbcL* and *accD* potato flanking regions. The pVL15 vector was produced by replacing the full-length *rrn* promoter (P*rrnFL*) in pVL13 with the short *rrn* promoter (containing only the PEP transcription initiation site) excised from the 1002 vector, using *Not*I and *Eco*RI restriction enzymes.

The potato *clpP* promoter region (nucleotides 73765–73945) was amplified with St-clpP_fd and St-clpP-rbs_rev primers, introducing a synthetic *rbcL* leader sequence, and cloned as a *Not*I/*Eco*RI fragment in 1002 and pVL13 to produce pVL2 and pVL14, respectively. The *clpP* promoter region including its 5′-UTR (P*clpP* 5′-UTR; nucleotides 73734–73945) was amplified using the St-clpP_fd and St-clpP_rev primers and cloned as above in 1002 and pVL13, producing pVL3 and pVL27, respectively.

All constructs used in this study were confirmed by sequencing. An overview of them is reported in Table 1.

Plant material

Potato plants (*Solanum tuberosum* L. *cv.* Désirée) were grown in vitro under controlled conditions (16 h light 40 μE m^{-2} s^{-1} and 8 h dark at 24°C) on MS medium with B5 vitamins (Duchefa, Haarlem, The Netherlands) and 30 g l^{-1} sucrose solidified with 0.8% (w/v) agar. Transplastomic plants were transferred to soil and maintained in a growth chamber (14 h light, 200 μE m^{-2} s^{-1}, at 25°C, and 10 h dark at 20°C) for tuberization. For all analyses, fully expanded leaves were collected from 4-week-old plants. Tubers were collected from 3-month-old plants. Three to four weeks

Table 1 GFP transcript and protein expression in leaves and tubers of potato transplastomic plants produced by plastid transformation with different constructs

Construct [a]	Source of *rbcL-accD* flanking regions[b]	Promoter (P)	5′ UTR (L)	Terminator (T)	Leaf		Tuber	
					Transcript[d]	Protein (%TSP)[e]	Transcript	Protein (%TSP)
1001	Tobacco	*trc*	*trc*	*rrnB*	1.0	0.066 ± 0.004	1.0	ND[g]
1002	Tobacco	*rrn*	Synthetic[c]	*rrnB*	–[f]	–	–	–
1003	Tobacco	*psbA*	*psbA*	*rrnB*	6.4 ± 2.7	0.058 ± 0.003	4.9 ± 0.2	ND
1073	Tobacco	*psbA*	*psbA*	*rbcL*	5.7 ± 0.4	0.098 ± 0.019	3.2 ± 0.4	ND
1074	Tobacco	*psbA*	*psbA*	*psbA*	6.2 ± 0.7	0.075 ± 0.015	1.3 ± 0.3	ND
1075	Tobacco	*psbA*	*psbA*	*petD*	4.7 ± 0.1	0.075 ± 0.015	2.2 ± 0.7	ND
1076	Tobacco	*psbA*	*psbA*	*rpoA*	4.6 ± 0.6	0.055 ± 0.015	0.7 ± 0.1	ND
pVL2	Tobacco	*clpP*	Synthetic	*rrnB*	–	–	–	–
pVL3	Tobacco	*clpP*	*clpP*	*rrnB*	–	–	–	–
pVL12	Tobacco	*rrnFL*	Synthetic	*rrnB*	1.5 ± 0.3	0.080 ± 0.020	3.3 ± 0.3	0.0013 ± 0.0003
pVL13	Potato	*rrnFL*	Synthetic	*rrnB*	2.2 ± 0.6	0.075 ± 0.015	3.3 ± 0.5	0.0013 ± 0.0001
pVL14	Potato	*clpP*	Synthetic	*rrnB*	ND	ND	0.2 ± 0.1	ND
pVL15	Potato	*rrn*	Synthetic	*rrnB*	17.3 ± 0.8	3.750 ± 0.750	8.0 ± 0.7	0.0148 ± 0.0019
pVL27	Potato	*clpP*	*clpP*	*rrnB*	0.6 ± 0.1	0.058 ± 0.005	0.2 ± 0.1	0.0175 ± 0.0014

[a] 1001–1003 (Newell et al. 2003); 1073–1076 (Tangphatsornruang et al. 2003); pVL2-pVL27, this manuscript. In all vectors, the selectable *aadA* marker gene with *rrn* promoter and *psbA* terminator was used

[b] Nucleotides 57758–60606 of the tobacco plastid genome (accession number Z00044) or 56694–59506 of the potato plastid genome (accession number DQ386163)

[c] Derived from *rbcL* gene (Svab and Maliga 1993)

[d] Transcript accumulation (means ± standard error) determined by northern blot hybridization and relative to construct 1001

[e] Protein accumulation (means ± standard error) determined by western blot analyses and presented as % of total soluble proteins

[f] – = Not available

[g] *ND* not detected

before tuber harvest, pots were covered with black tissue to prevent light influence on transgene expression in tubers developing near the soil surface.

Plastid transformation and regeneration

For biolistic bombardment, stock plants were grown in vitro on agar-solidified growth-regulator-free MS medium supplemented with B5 vitamins for 3–4 weeks, as described above. Two different protocols for plastid transformation were used. For protocol I, 2–5 leaves were placed on agar-solidified T1 callus induction medium containing MS salts with B5 vitamins, 3.0 mg l^{-1} zeatin riboside (ZR), 2.0 mg l^{-1} indole acetic acid (IAA), 1.0 mg l^{-1} gibberellic acid (GA_3), 16 g l^{-1} glucose (Nguyen et al. 2005) with 0.1 M sorbitol and 0.1 M mannitol as osmoticum. After incubation for 24 h in dark, leaves were bombarded with gold particles (0.6 μm diameter) coated with plasmid DNA using a PDS 1000/He Biolistic gene gun (BioRad, Hercules, CA, USA), 1,100 psi rupture disks (BioRad) and vacuum pressure of 28 Hg. Leaves were bombarded at a distance of 6 cm and incubated in dark conditions for 2 days. After this period, leaves were cut into small pieces (3 × 3 mm) and transferred to the same medium without osmoticum (i.e. sorbitol and mannitol), but supplemented with 400 mg l^{-1} spectinomycin and 250 mg l^{-1} cefotaxime. Leaf pieces were incubated under dim light (about 10 μE m^{-2} s^{-1}) in 16 h light, 8 h dark regime for 1 month.

Following protocol II, 24 h before bombardment, leaves were incubated in the dark on M6 medium (MS salts with B5 vitamins, 0.8 mg l^{-1} ZR, 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g l^{-1} sucrose) (Yadav and Sticklen 1995) with 0.1 M sorbitol and 0.1 M mannitol. After bombardment, leaves were cut into small pieces (3 × 3 mm) and incubated for 1 month on M6 medium (without sorbitol and mannitol) supplemented with 400 mg l^{-1} spectinomycin and 250 mg l^{-1} cefotaxime. Explants were then transferred to T1 medium (without sorbitol and mannitol) with the same antibiotics.

In both protocols I and II, leaf explants were transferred every 1–2 months to fresh selective T1 regeneration medium and incubated under the same conditions for a further 3–4 months. After this period, the first spectinomycin-resistant events were identified. Primary spectinomycin-resistant calli were excised and incubated for 1 month on DH medium, containing MS salts without NH_4NO_3, 0.268 g l^{-1} NH_4Cl, Nitsch vitamin mixture, 0.1 g l^{-1} casein hydrolysate, 0.08 g l^{-1} adenine hemisulfate, 2.5 g l^{-1} sucrose, 36.4 g l^{-1} mannitol, 2.5 mg l^{-1} zeatin, 0.1 mg l^{-1} IAA (modified from Haberlach et al. 1985), for further growth. Calli were transferred for regeneration on MON medium, containing MS salts with B5 vitamins, 30 g l^{-1} sucrose, 0.1 mg l^{-1} NAA, 5 mg l^{-1} ZR (Sidorov et al. 1999). Both DH and MON media were supplemented with 400 mg l^{-1} spectinomycin. Regenerated shoots were subcultured to growth regulator-free MS medium with 200 mg l^{-1} spectinomycin for root formation and finally transferred to soil. The pH of all media was adjusted to 5.8 prior to autoclaving.

PCR and Southern blot analyses

Total genomic DNA was extracted from 200 mg leaf material of putative transplastomic and control plants by a rapid miniprep procedure using sodium lauroyl sarcosine-based cell lysis (Pallotta et al. 2000). PCR analysis to verify the correct integration of the *gfp* gene in plants was performed using primers that anneal to the *gfp* gene (smGFP_rv) and to the native chloroplast gene (*accD*), and/or both primers homologous to the native chloroplast genes (*rbcL* and *accD*) (Supplementary Table S1).

RNase-treated DNA (2 μg) was digested overnight at 37°C with *Eco*RI/*Eco*RV or *Sac*II/*Eco*RV endonucleases, separated by electrophoresis in 0.9% (w/v) agarose gels and blotted onto Hybond N^+ membranes (GE Healthcare, Waukesha, WI, USA) by capillary transfer under alkaline conditions, according to the manufacturer's instructions. The hybridization probe was generated by PCR amplification of part of the plastid genome corresponding to *rbcL* and *accD* genes (nucleotides 56698–60237) using rbcL_fd and accD_rv primers (Supplementary Table S1). The PCR product was column purified and radiolabeled with [α-^{32}P]dCTP by random priming using the Prime-a-gene labelling system (Promega, Madison, WI, USA). Hybridizations were performed overnight at 65°C in Church buffer (Church and Gilbert 1984). After hybridization, the blot was washed first with 2× SSC for 5 min, followed by one wash with 2× SSC, 0.1% (w/v) SDS for 30 min and two washing steps

with 0.1× SSC, 0.1% SDS for 15 min each. The radioactivity was detected using a Typhoon 9200 Imager (GE Healthcare).

RNA isolation and northern blot analysis

Total potato RNA was extracted from 2 g tuber (2–3 cm length) or 0.5 g leaf tissue according to the phenol-based RNA protocol, described on the TIGR web site (http://www.jcvi.org/potato/sol_ma_protocols.shtml). After overnight precipitation with LiCl, purified RNA was recovered by centrifugation at 18,000*g* for 30 min at 4°C.

For northern blot analyses, 5 µg total RNA from leaves or 20 µg total RNA from tubers were separated by electrophoresis on 1.2% agarose/formaldehyde gels and blotted onto Hybond N^+ membranes (GE Healthcare). Part of the *gfp* gene was PCR amplified using GFP_fd and GFP_rv primers (Supplementary Table S1), column purified and used as a probe to investigate transcript accumulation in transplastomic plants. The probes for the *psbD* and 18S rRNA genes were PCR amplified from potato genomic DNA using psbD_fd/psbD_rv and 18S_fd/18S_rv primer pairs, respectively. [α-^{32}P]dCTP labelled probes were generated as described above. Hybridizations were carried out at 42°C following the membrane manufacturer's instructions (GE Healthcare).

After stripping the *gfp* probe, filters carrying leaf samples were hybridised with the *psbD* probe, while those with RNA from tubers were hybridized with the 18S rRNA probe. Hybridization signals were quantified with a Typhoon 9200 Imager (GE Healthcare) and normalized to *psbD* or 18S rRNA gene signals, respectively. At least two independent analyses of transcript accumulation per transplastomic line and per tissue were performed.

SDS–PAGE and immunoblot analysis

Total soluble proteins were extracted from 200 mg leaf, petal or tuber (2–3 cm size) tissues homogenized in 500 µl of buffer containing 100 mM Tris–Cl pH 7.8, 200 mM NaCl, 1 mM EDTA, 2% 2-mercaptoethanol, 0.2% Triton X-100 supplemented with 1 mM phenylmethylsulphonylfluoride (PMSF). After centrifugation at 11,000*g* for 10 min at 4°C, protein concentration was determined using Bradford Protein Assay Reagent Kit (Bio-Rad). Total protein (0.5–20 µg from leaves or 50 µg from tubers) was separated by SDS-12% PAGE and transferred onto nitrocellulose membrane (GE Healthcare). GFP protein was detected with rabbit anti-GFP antibodies (Invitrogen, Carlsvad, CA, USA) and the ECL Plus Western blotting Detection system (GE Healthcare) according to the manufacturer's instructions. Different amounts (50, 25, 10 and 2.5 ng) of purified recombinant GFP protein (rGFP, Clontech, Mountain View, CA, USA) were added to 20 µg protein from leaves or 50 µg protein from tubers of non-transformed Désirée plants and used as a standards. Two or three independent analyses of protein accumulation per transplastomic line and per tissue were performed.

Results

Construction of new vectors for plastid transformation

The transformation vectors used in this study are described in Table 1. Some of them were previously designed for plastid transformation of tobacco (Newell et al. 2003; Tangphatsornruang et al. 2003). They are based on the pZS197 vector (Svab and Maliga 1993) and contain a *gfp* gene with various 5′ and 3′ regulatory sequences. The pVL vector series were specifically constructed to improve either the integration frequency of transgenes in the potato plastome or their expression in tuber amyloplasts. The potato *rbcL-accD* sequences homologous to the tobacco sequences in pZS197 (Svab and Maliga 1993) were included in pVL13, pVL14, pVL15 and pVL27, whereas pVL2, pVL3 and pVL12 contained the original tobacco sequences. Additional modifications of the promoter and/or 5′-UTR sequences were introduced in the same constructs. pVL2, pVL3, pVL14 and pVL27 contained the *clpP* promoter; pVL12 and pVL13 contained a long version of the *rrn* promoter (*rrnFL*), including both PEP and NEP transcription initiation regions (Vera and Sugiura 1995), whereas pVL15 contained the "short" *rrn* promoter with only the PEP transcription initiation region. pVL2, pVL12, pVL13, pVL14, and pVL15 all contained a synthetic ribosome-binding site from the *rbcL* 5′-UTR (Svab and Maliga 1993), whereas the native *clpP* 5′-UTR was present in pVL3 and pVL27. All new vectors contained the *E. coli rrnB* terminator.

Overall, including the tobacco vectors described previously, 14 vectors were used in potato plastid tranformation experiments, allowing the comparison of tobacco and potato flanking regions, five promoters, four 5′-UTRs, and five 3′-UTR and terminator sequences.

Optimization of the plastid transformation protocol

The results of transformation experiments are summarized in Table 2. Seven vectors (1001–1003, 1073–1076, Table 1) were used in the first series of experiments following protocol I, based on single-step selection of green calli on T1 medium followed by shoot regeneration on DH/MON media. After ten bombardments of each vector, only five spectinomycin-resistant shoots were obtained: 1 with 1003, 3 with 1073 and 1 with 1075. Subsequent PCR analyses with internal/external and external/external primers, however, demonstrated that the *gfp* gene had been correctly integrated in the plastome in only one (20%) of these plants, transformed with the 1075 vector (data not shown). The same seven vectors plus pVL2, pVL3 and pVL12 were used for transformation following protocol II, consisting of a two-step selection procedure on M6/T1 media followed by shoot regeneration on DH/MON media. In total, 150 plates were bombarded. Ten or 15 bombardments per construct were generally performed, except for vectors 1002 and 1003 which were used for bombardment of 35 and 20 plates, respectively. Transformation with protocol II resulted in a total of 31 spectinomycin-resistant shoots (1–5 shoots per vector). Twenty-eight of these shoots were analyzed by PCR using internal/external and external/external primers and 15 (54%) demonstrated correct transgene integration after both analyses (data not shown). Transplastomic shoots were obtained with all vectors except pVL2 and pVL3. Finally, in order to compare the effect of the origin of the flanking regions on transformation efficiency, we performed other transformation experiment with the four constructs (pVL13, 14, 15, 27) containing the flanking sequences derived from potato. In total, 57 plates were bombarded (10–16 per construct) following the optimized selection/regeneration protocol II. These transformation experiments generated 69 spectinomycin-resistant shoots. PCR analyses of 36 putative transformants with *gfp*-specific primers and a primer pair flanking the transgene insertion site in the potato chloroplast genome confirmed the presence of the transgene in 31 lines (86%) (Table 2). Transplastomic shoots were recovered for all vectors, from a minimum of 2 with pVL27 to a maximum of 11 with pVL14. When the data in Table 2 were standardized to 100 bombardments, the number of transplastomic shoots obtained increased from 1.4 to 11.1 by adopting the two-step selection protocol II instead of the single-step protocol I, and from 11.1 to 104.2 using the potato flanking regions in the transformation vectors instead of the tobacco sequences.

Southern analysis (Fig. 1) confirmed the correct integration of the transgene in the plastome of transplastomic plants. Plants transformed with 1001, 1002, 1073, 1074, 1075 and 1076 constructs were analyzed by *Eco*RI/*Eco*RV digestion (Fig. 1b). *Eco*RV endonuclease did not cut inside the transformation vectors, but this site was recovered in the transplastomic plants due to the homologous recombination with the plastid genome. *Eco*RI endonuclease cut the plasmid vector between the promoter and *gfp* gene (Fig. 1a). Two bands were observed in transplastomic plants

Table 2 Efficiency of plastid transformation in potato using different regeneration protocols and flanking regions

Source of flanking regions	Regeneration protocol[a]	No. of bombarded plates	No. of Spec^R shoots	No. of shoots		No. of estimated transplastomic shoots/100 bombardments[b]
				Analyzed	PCR$^+$ (%)	
Tobacco	I.	70	5	5	1 (20)	1.4
Tobacco	II.	150	31	28	15 (54)	11.1
Potato	II.	57	69	36	31 (86)	104.2

[a] Transformation with: protocol I, one-step selection/regeneration procedure; protocol II, two-step selection/regeneration (see "Materials and methods" for details). For each protocol, 6–7 independent experiments were carried out using constructs reported in Table 1. Overall, 10–35 bombardments per construct were performed

[b] No. of Spec^R shoots × No. of PCR$^+$ shoots × No. of plates bombarded^{-1} × No. of shoots analyzed $^{-1}$ × 100

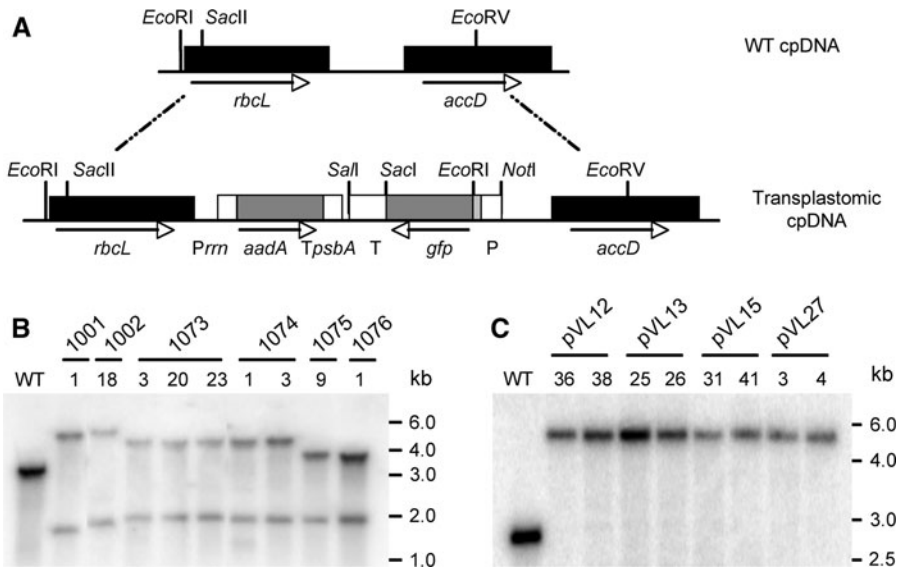

Fig. 1 Generation of transplastomic potato lines. **a** Maps of wild-type (Wt cpDNA) and transformed (transplastomic cpDNA) plastid genome regions involved in transgene integration. **b**, **c**, Southern blot analyses of independent transplastomic potato lines, obtained with ten different transformation constructs as indicated, and non-transformed Désirée plants (WT). Two µg of *Eco*RI/*Eco*RV (**b**) and *Sac*II/*Eco*RV (**c**) digested total DNA were probed with [α-32P]dCTP labelled *rbcL-accD* PCR amplified probe. See "Materials and methods" and Table 1 for vector details

(Fig. 1b). One, of approximately 1.5–1.9 kb, corresponded to the *Eco*RI-*Eco*RV fragment containing the *gfp* promoter and the *accD* flanking region, and the other, of 3.9–4.5 kb, included the *rbcL* flanking region, the *aadA* expression cassette and the remaining part of the *gfp* gene. The observed differences in the sizes of the fragments were due to the different sizes of the promoter and 3′-UTR/terminator sequences in the different constructs. Wild-type ptDNA produced only a single band of approximately 3.0 kb on digestion with *Eco*RI and *Eco*RV. This band was not detected in any transplastomic line shown (Fig. 1b), indicating that the plants were homoplasmic. Plants transformed with the pVL vectors were analyzed using *Sac*II and *Eco*RV restriction enzymes (Fig. 1c). All transplastomic plants analyzed showed the expected single band of about 5.9 kb, whereas in the non-transformed Désirée plants only the expected band of 2.8 kb was observed. Only two primary regenerants out of 24 analyzed displayed the wild type signal, indicating they were heteroplasmic (data not shown), whereas the majority (92%) of the plants were homoplasmic.

When transplastomic plants were transferred to a growth chamber, no differences in plant growth and tuber formation were detected among a majority of lines carrying different promoters, terminators or flanking regions. The only exceptions were lines 1002–18 and 1073–23; in both cases, plants were weak and displayed little or no tuber formation.

Transgene expression in leaves

Transgene transcript accumulation in potato leaves and tubers was investigated by northern blot hybridization. Transcripts were clearly detectable in both organs of all transplastomic lines (Figs. 2a, 3a), except in leaves transformed with pVL14 (P*clpP* with *rbcL* ribosome-binding site). When RNAs from tubers and leaves of the same plants were compared on the same filter, transcript accumulation was from 10- (pVL27 vector) to 40-fold (1003) lower in tubers than in leaves (data not shown).

The pattern of transcripts was similar in both tissues. All plants carrying the *gfp* gene with the *E. coli rrnB* terminator showed two bands of 1.0 and 1.2 kb, which are typical for this terminator, due to the presence of two sequences giving rise to stem-loop structures (Newell et al. 2003). Differences in the size and relative intensity of the bands were obtained from plants transformed with constructs containing plastid 3′-UTR sequences (Fig. 2a). In plants transformed with the 1073 construct, we

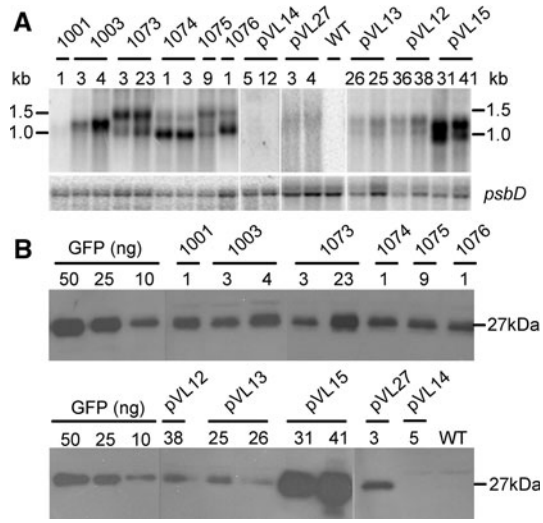

Fig. 2 Expression of *gfp* in leaves of transplastomic potato lines, obtained with eleven different transformation constructs as indicated, and non-transformed Désirée plants (WT). **a** Northern blot analysis of total RNA (5 μg) probed with [α-32P]dCTP labelled *gfp* probe. Hybridizations with *psbD* radiolabelled probe was used as loading control. **b** Western blot analysis of total soluble proteins (10 μg for pVL12, pVL13 and pVL15 vectors, and 20 μg for all other constructs) using anti-GFP antibodies. The recombinant GFP protein in 20 μg of WT protein from leaves was used as standard

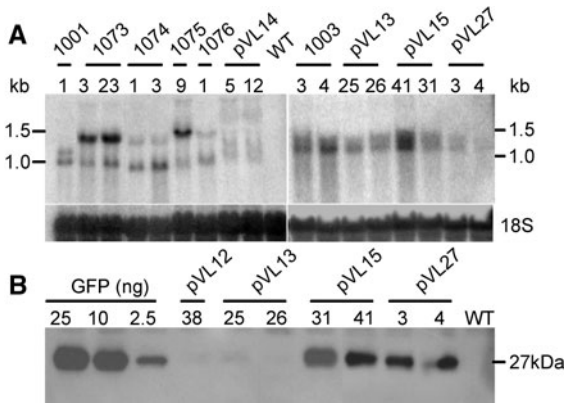

Fig. 3 Expression of *gfp* in tubers of transplastomic potato lines, obtained with ten different transformation constructs as indicated, and non-transformed Désirée plants (WT). **a** Northern blot analysis of total RNA (20 μg) probed with [α-32P]dCTP labelled *gfp* probe. Hybridizations with 18S radiolabelled probe was used as loading control. **b** Western blot analysis of total soluble proteins (50 μg) using anti-GFP antibodies. The recombinant GFP protein in 50 μg of WT protein from tubers was used as standard

observed a smaller band of about 0.97 kb and a longer, and more abundant, one of about 1.3 kb. For 1074, the predominant signal was the short band of about 0.95 kb, while a longer one of about 1.25 kb was barely detectable. For 1075, two bands of about 1.35 and 0.95 kb were evident. Finally, for 1076, a strong signal of about 1.0 kb was visible along with a weaker one corresponding to a longer (1.3 kb) transcript (Fig. 2a). These differences are most likely due to inefficient termination of transcription by plastid 3′-UTRs (Maliga 2003).

In leaves, the highest transcript accumulation levels were obtained with the pVL15 vector. The relative expression value for the pVL15 vector, compared to that obtained with the 1001 vector (=1), was 17.3 ± 0.8 (Fig. 2a; Table 1). In contrast, the lowest relative values (0–0.6×) were obtained with the constructs containing the *clpP* promoter and either the *rbcL*-derived or the *clpP* 5′-UTR (pVL14 and pVL27, respectively). Intermediate values were obtained from leaves with the constructs containing the *psbA* promoter (1003–1076, 6.4–4.6×), followed by those with the full-length P*rrn* (pVL12 and pVL13, 2.2–1.5×). No significant differences in transcript accumulation were detected between plants transformed with constructs differing only in the flanking regions (i.e. pVL12 and pVL13). All transplastomic lines, including those carrying pVL14, accumulated similar amounts of the *aadA* transcript (data not shown).

The accumulation of the GFP protein in chloroplasts and amyloplasts was investigated by immunoblot analyses. Except for plants transformed with pVL14, all other lines accumulated detectable amounts of GFP in leaves. The level of the recombinant protein, however, was significantly higher in plants transformed with the pVL15 vector (3.8 ± 0.8% TSP) than with the other constructs (Fig. 2b; Table 1). In plants transformed with the other constructs, the amount of GFP was generally low and ranged from 0.06 to 0.10% TSP, i.e. about 40–70-fold less than with pVL15. The results of western blot analysis were generally confirmed by measurements of GFP fluorescence in transplastomic plants (data not shown).

Transgene expression in tubers

Similar to leaves, the highest *gfp* transcript accumulation in tubers was obtained with the pVL15 vector containing the short *rrn* promoter (8× the expression level of the 1001 construct in the same tissues;

Fig. 3a and Table 1) and the lowest with pVL27 and pVL14 containing the *clpP* promoter (0.2×). Again, intermediate levels were obtained with other constructs, although, in comparison with leaves, wider differences (ranging from 4.9 to 0.7×) were detected among plants transformed with vectors containing the *psbA* promoter and 5′-UTR sequences, but different 3′-UTRs. Among these plants, the *rrnB* terminator gave the highest amount of *gfp* transcripts and the *rpoA* 3′-UTR the least. Slightly higher relative values than in leaves (3.3× vs. 1.5–2.2×) were shown by constructs containing the full-length *rrn* promoter (pVL12 and pVL13).

GFP accumulation was investigated in tubers about 2.5 cm in diameter collected from 3-month-old in vivo propagated plants. The recombinant protein was detected, at low levels, only in plants transformed with pVL27, pVL15, pVL12 and pVL13 (Fig. 3b; Table 1). With the first two vectors, containing either the *clpP* promoter and 5′-UTR, or the *rrn* promoter and the *rbcL* 5′-UTR, GFP accumulated to 0.015–0.018% TSP. With the other two vectors, GFP accumulation was about 10–15-fold lower (around 0.0013% TSP). GFP produced from the pVL15 and pVL27 constructs was still detectable in big (about 5 cm) and/or old (up to 1 year) tubers, at approximately 50% intensity of that detected in fresh, small (1–2 cm) tubers (data not shown).

In order to examine transgene expression in another tissue containing non-green plastids, the GFP content of petals was compared with leaves and tubers of plants transformed with the two best constructs (Supplementary Fig. S1). The two vectors pVL15 and pVL27 produced different relative amounts of GFP in the three tissues. pVL15 and pVL27 produced roughly the same amount of GFP in tubers, but the pVL15 directed about 10- and 50-fold higher GFP accumulation than pVL27 in petal leucoplasts and leaf chloroplasts, respectively. Parallel experiments on GFP accumulation in *E. coli* showed that, in contrast to pVL15, GFP protein failed to accumulate in pVL27-transformed cells (data not shown).

Discussion

In this study, we report the improvement of plastid transformation efficiency in potato. Previously, plastid transformation in this crop had been reported in the breeding line FL1607 (Sidorov et al. 1999), which is characterized by a high regeneration and transformation potential (Wenzler et al. 1989), and in the commercially important cultivar Désirée (Nguyen et al. 2005). In FL1607, a single-step regeneration procedure and several other tissue culture features were optimized, whereas a two-step procedure was adopted for Désirée. In both studies, however, only one transplastomic shoot per 15–18 bombardments was obtained with a progenitor vector of that used in the present study, and even lower transformation frequencies were obtained with other vectors (Sidorov et al. 1999; Nguyen et al. 2005).

Adopting the same genotype and basic regeneration protocol previously published (Nguyen et al. 2005), we approximately doubled the transformation efficiency, in comparison with previous results (Sidorov et al. 1999; Nguyen et al. 2005), by improving the procedure for the regeneration of transplastomic shoots from green spectinomycin-resistant calli, achieving about 11 shoots per 100 bombardments (one shoot every 9 bombardments) with vectors directing transgene integration in the *rbcL-accD* genomic region. Similar transformation frequencies were obtained with vectors for the *3′rps12-trnV* region (data not shown), representing in this case a more than three-fold improvement (Sidorov et al. 1999; Nguyen et al. 2005). A further dramatic increase of the transformation efficiency (equal to approximately one shoot per bombardment) was, however, obtained when tobacco flanking sequences in transformation vectors were replaced with homologous potato sequences. Results similar to those reported for potato in this study, and for tobacco elsewhere, have been obtained in carrot, cotton, soybean and lettuce, where, in biolistic approaches, the use of species-specific vectors allowed the improvement of plastid transformation frequencies up to 1–2 shoots per bombardment (Dufourmantel et al. 2004; Kumar et al. 2004a, b; Kanamoto et al. 2006). Conversely, the transformation efficiency in tobacco decreased by more than tenfold when flanking sequences derived from the petunia plastome were used for recombination (DeGray et al. 2001).

In contrast, no apparent reductions in transformation frequencies were observed in tobacco and tomato using homologous or homeologous *N. tabacum* and *S. nigrum* ptDNA sequences in transformation vectors (Kavanagh et al. 1999; Horváth et al. 2000; Nugent

et al. 2005; Nugent et al. 2006), or in transformations of *N. benthamiana* plastids with tobacco-derived vectors (Davarpanah et al. 2009), indicating that species-specific vectors are not always necessary. In the tobacco and tomato studies, it was demonstrated that integration of the cloned homeologous ptDNA sequences in the host plastome occurred by multiple recombination events in a similar region of the inverted repeats (IRs), that in comparison with "Single Copy" regions of the plastid genome, generally show a higher gene order conservation and a lower sequence divergence (Maier et al. 1995). The latter was estimated to be around 2.4% between tobacco and nightshade in the plastidial region used for transformation, and several homologous regions as short as 41–141 bp, or longer, were shown to be involved in multiple recombination events (Kavanagh et al. 1999). The comparable degree of recombination, resulting in similar transformation efficiency, attained in homologous and homeologous combinations, despite the sequence divergence between donor and recipient ptDNAs, was likely attributed to an active RecA-mediated, but also somehow suppressed mismatch, recombination/repair system in higher-plant plastids (Kavanagh et al. 1999).

In the *rbcL-accD* region used in the present study, we found 55 substitutions and 9 gaps (from 1 to 24 nucleotides) in 2867 nucleotides aligned between tobacco and potato, with an overall 95.6% identity. The expected efficiency of interspecific plastome recombination between tobacco and various other species was recently estimated, after alignment of the *rrn16-rrn23* internal transcribed spacer, considering the number of MEPS (Minimal Efficient Processing Segments), i.e. the number of blocks of sequences identical in the two partners and sufficiently large to allow the initiation of recombination by RecA (Vulić et al. 1997; McNutt et al. 2007). Based on a MEPS length in higher plant plastids equal to 45 bp, a 19% efficiency, in comparison with that achievable by homologous combinations, was estimated for divergence levels similar to that shown by the *rbcL-accD* regions in potato and tobacco (McNutt et al. 2007). Using the same procedure, in the latter region we could detect 23 fragments of identical sequence (from 45 to 237 bp long, average length = 92.7) between potato and tobacco, for a total of 1120 MEPS, a value approximately equal to 40% of that estimated using the homologous potato sequence. A similar reduction in efficiency should have been obtained comparing the homologous vs. the homeologous transformations, assuming conservation of cellular permissiveness towards homeologous recombination at different divergence levels (McNutt et al. 2007). Integration of transgene sequences in the recipient plastome has been recently shown to occur by a two-step process with recombination taking place at the ends of the targeting regions (Klaus et al. 2004; Sinagawa-Garcia et al. 2009). Hence, at least in a fraction of integration events, divergencies between tobacco and potato sequences could also lead to the formation of recombinant *rbcL* and/or *accD* coding sequences with lower functionality, likely affecting the regeneration of viable plants. In any case, the significant, and higher than expected, differences in transformation efficiency of the potato plastome using homologous potato and heterologous tobacco vectors indicate somehow the limits of nucleotide sequence heterogeneity, preventing the efficient integration of donor sequences and requiring the use of species-specific vectors for the *rbcL-accD* region. This further limits the adoption of this region for the plastid transformation of different species, considering that its use was already not possible in soybean and other leguminous crops, or in grasses, since the *rbcL-accD* gene order is either not conserved in the plastome of the former or the *accD* gene is absent in that of the latter (Maier et al. 1995; Dufourmantel et al. 2004).

Significantly greater similarity was found when the potato region investigated in this study was compared with other *Solanum* species, such as wild potato and tomato, or when other regions, previously employed in plastid transformation experiments (Maliga 2004), were scored for homology among Solanaceae species (data not shown), indicating the need for proper sequence analysis before attempting to use vectors developed in non-target species. The increasing number of plastome sequences released (http://www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=2759&type=4&name=Eukaryotae%20Organelles) should facilitate both such analyses and the development of species-specific vectors, if necessary. Anyway, the levels of sequence similarity between potato and tomato should allow in most cases the interchangeable use of vectors between these two important crops.

By contrast with tobacco, where 2–3 rounds of regeneration are usually needed to achieve homoplasmy (Bock 2001), almost all regenerated primary

transformants were homoplasmic in potato, probably as a consequence of the long cell growth phase in vitro before shoot regeneration. Only two regenerated lines out of 47 showed some growth abnormalities. These, however, were likely due to somaclonal variation, because other plants with the same expression cassettes were normal.

As far as the analysis of transgene expression in different plastid types was concerned, differences in *gfp* transcript accumulation in leaf chloroplasts were mainly due to the promoter used, suggesting that differences were likely due to promoter strength and resulting differential transcription rates. On the basis of results obtained, transplastomic plants could be ranked in three groups with decreasing expression levels: (1) those transformed with the pVL15 vector with the short *rrn* promoter and the synthetic 5′-UTR originally present in pZS197 (Svab and Maliga 1993); (2) those with the *psbA*-derived promoter and 5′-UTR sequences, and variable 3′-UTRs; (3) those with other vectors tested. The relative performances of the *rrn*, *psbA* and *trc* promoters largely confirm those previously shown in leaves of transplastomic tobacco plants transformed with the same vectors (Newell et al. 2003). In the present study, relatively low transcript accumulation in chloroplasts was obtained with the *clpP* promoter and, unexpectedly, with the full-length *rrn* promoter. However, since northern blot analyses revealed similar amounts of transcript when the *rrn, psbA* and *trc*-derived 5′ regulatory sequences drove the expression of an alternative gene (Birch-Machin et al. 2004), promoter performance and final transcript accumulation can vary significantly with the downstream sequence. In potato plants containing the same promoter/5′-UTR (*psbA*), the *rrnB* terminator gave the best results, confirming results obtained in tobacco, although variability among terminators was less evident than previously found (Tangphatsornruang et al. 2003).

GFP protein accumulation in chloroplasts was broadly similar to that achieved in tobacco with the same vectors (Newell et al. 2003), showing low amounts of protein in all transformed plants except those transformed with pVL15 (P*rrn*/rbcL 5′-UTR), which accumulated GFP to about 4% TSP, a value comparable with other transplastomic tobacco and potato plants (Sidorov et al. 1999; Reed et al. 2001; Newell et al. 2003). However, by comparing the accumulation of transcripts and proteins, a differential translatability of the different constructs could be seen. For example, compared to 1001-derived plants (P*trc*), pVL15 plants (P*rrn*) accumulated 17× and 57× more transcripts and proteins, respectively, while plants with the *psbA* promoter accumulated 5–6× more transcripts, without a notable impact on recombinant protein levels. An intermediate translatability in leaf chloroplasts was shown by pVL27 plants with the *clpP* 5′ regulatory sequences.

Overall, the vectors with the tobacco regulatory sequences used in this study gave comparable results in potato and tobacco leaves (Newell et al. 2003; Tangphatsornruang et al. 2003), confirming that regulatory sequences are sufficiently conserved at the structural and functional level to be used across species (Sidorov et al. 1999; Heifetz 2000; Kumar et al. 2004b; Kanamoto et al. 2006).

Previous profiling studies comparing transcript accumulation in potato leaf chloroplasts and tuber amyloplasts showed a generally low transcription rate of plastid genes and an increased transcript stability in amyloplasts (Brosch et al. 2007; Valkov et al. 2009). Interestingly, although, in the present study, transcript accumulation in tubers tended to match that in leaves ($r = 0.82$, $P < 0.01$), some differences were apparent between constructs with the same 5′ regulatory sequences (e.g. *psbA*), but different 3′-UTRs. This highlights the role played by the latter on transcript stability and, therefore, transcript accumulation in amyloplasts. Plants with the bacterial-derived *rrnB* terminator accumulated 5 and sevenfold more *gfp* transcripts than plants with *psbA* and *rpoA* 3′-UTRs, respectively, suggesting a positive effect of the *rrnB* terminator on mRNA stability.

In general, protein accumulation in amyloplasts was disappointingly low and was detectable with only a few constructs. Nevertheless, some useful observations could be made. Comparable protein accumulation was achieved in plants transformed with pVL15 (P*rrn*L*rbcL*) and pVL27 (P*clpP*L*clpP*), but the relative level of transcript was 40× higher in the former than in the latter suggesting a significant positive effect of *clpP* 5′ regulatory sequences on translatability, particularly in non-green plastids. These results support those obtained in expression profile analyses with total and polysomal plastid RNAs, which indicated *clpP* as one of the less down-regulated genes in tubers compared to leaves (Valkov

et al. 2009). *clpP* has multiple PEP/NEP promoters (Hajdukiewicz et al. 1997), but, in tubers, all native transcripts initiated from the strong -53 NEP promoter (Valkov et al. 2009). However, based on GFP protein accumulation in leaves, tubers and petals, it is clear that the relative performance of *rrn* and *clpP* regulatory sequences, and their potential applicability in plastid transformation approaches, vary with plastid types. In recent experiments in transplastomic *N. benthamiana* plants expressing the *gfp* gene under the control of the *rrn* promoter, decreasing transcript and protein expression levels were observed in leaves, petals and roots (Davarpanah et al. 2009). The *clpP* 5′-UTR was previously used in combination with the *rrn* promoter to express the NPTII protein in tobacco leaves, but a mutant (chlorotic) phenotype was observed, even at low accumulation levels (Kuroda and Maliga 2002).

Since protein accumulation in tubers of plants containing constructs with the *rrn* promoter is generally accompanied by high expression in leaves, a potential use of the *clpP* 5′ regulatory sequences can be envisaged in cases where recombinant protein accumulation is required in amyloplasts, but not in chloroplasts, nor in bacteria. The expression levels achieved in tubers may be sufficient to manipulate the expression of enzymatic proteins for metabolic engineering purposes, but are still too low to exploit tubers of transplastomic plants as a production platform for proteins with pharmaceutical or industrial interest. Other plastid and nuclear regulatory gene sequences could be exploited to increase (trans)gene expression in amyloplasts (Valkov et al. 2009). Anyway, the low expression in such organelles is not a limiting factor, and can be considered beneficial, if the target of the expression of recombinant proteins is the upper part of the plant, as, for instance, in the case of peptides directed against leaf pests. Hence, we think that the results reported in this manuscript showing a significant improvement of plastid transformation efficiency in potato are likely to be of considerable value for future implementation of this technology in potato breeding and biotechnology.

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