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Comparison of transplastomic *Chlamydomonas reinhardtii* and *Nicotiana tabacum* expression system for the production of a bacterial endoglucanase

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Abstract The bulk production of recombinant enzymes by either prokaryotic or eukaryotic organisms might contribute to replace environmentally non-friendly chemistry-based industrial processes with enzyme-based biocatalysis, provided the cost of enzyme production is low. In this context, it is worth noting that the production of recombinant proteins by photosynthetic organisms offer both eukaryotic (nuclear) and prokaryotic (chloroplast) alternatives, along with the advantage of an autotrophic nutrition. Compared to nuclear transformation, chloroplast transformation generally allows a higher level of accumulation of the recombinant protein of interest. Furthermore, among the photosynthetic organisms, there is a choice of using either multicellular or unicellular ones. Tobacco, being a non-food and non-feed plant, has been considered as a good choice for producing enzymes with applications in technical industry, using a transplastomic approach. Also, unicellular green algae, in particular *Chlamydomonas reinhardtii*, have been proposed as candidate organisms for the production of recombinant proteins. In the light of the different features of these two transplastomic systems, we decided to make a direct comparison of the efficiency of production of a bacterial endoglucanase. With respect to the amount obtained, 14 mg g^{-1} of biomass fresh weight equivalent to 8–10% of the total protein content and estimated production cost, 1.5–2€ kg^{-1}, tobacco proved to be far more favorable for bulk enzyme production when compared to *C. reinhardtii* which accumulated this endoglucanase at 0.003% of the total protein.

Keywords *Chlamydomonas reinhardtii* · Tobacco · Chloroplast · Cellulase · Molecular farming

Introduction

Recombinant DNA technology offers a unique opportunity to produce proteins of commercial interest that can meet the needs of the industrial, medical, and pharmaceutical sectors. For many years, the production of recombinant proteins by either prokaryotic or eukaryotic organisms has been a reality, and advantages and disadvantages of both systems has been evaluated in depth (Houdebine 2009; Demain and Vaishnav 2009; Surzycki et al. 2009; Hacker et al. 2009; Fernández-Robledo and Vasta 2010; Potvin and Zhang 2010). Remarkably, plant organisms offer both eukaryotic (nuclear) and prokaryotic (chloroplast) expression systems and have been proven to be suitable for the production of antibodies, enzymes, and vaccines (Leelavathi et al. 2003; Daniell et al. 2009; Verma et al. 2010; Scotti et al. 2012; Pantaleoni et al. 2014; Longoni et al. 2015; Jin and Daniell 2015). Compared to nuclear transformation, chloroplast transformation typically allows a higher accumulation of the protein of interest, which, for certain

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heterologous genes, may be favored by the higher plastome copy number, independent plastidial gene expression, and protein-folding machineries, along with other features linked to its prokaryotic origin (Reddy et al. 2002).

In *Nicotiana tabacum*, as in many higher plant species, plastome inheritance is maternal thus avoiding transgene dispersion by pollen. A major issue for regulatory clearance for commercial activities is potential interference with the food chain (Watson et al. 2004). Thus tobacco, being a non-food and non-feed plant, is ideal as a recombinant protein expression system.

The production of recombinant proteins using plant organisms is popularly known as molecular farming, or molecular pharming, if the recombinant product has pharmaceutical relevance. Easily scalable massive cultivation of transplastomic tobacco plants in greenhouses or fields opens up the possibility of producing recombinant enzymes on an industrial scale and at low costs (Verma et al. 2010; Verma et al. 2013). There is an urgent need to replace high energy-consuming and/or polluting chemical processes with enzyme-based ones that are environmentally friendly. Microalgae might be an interesting alternative to higher plants for the production of certain recombinant proteins, as large biomass can be obtained easily either by growing microalgae in open ponds or in basic fermenters. For instance, it is possible to envisage the growth of microalgae on cheap media such as wastewater or agro-industrial waste (Doria et al. 2011; Giovanardi et al. 2014) with the production of recombinant proteins. On the other hand, whenever required, as in the case of therapeutic applications, microalgae can be grown axenically in a photobioreactor.

C. reinhardtii was proposed as a candidate for the expression of recombinant proteins due to its ability to grow both autotrophically or heterotrophically, the presence of single chloroplast and the availability of well-established nuclear and chloroplast transformation procedures that allow to obtain and characterize transformed lines in a short time, sometimes as low as a few months (Mayfield and Franklin 2005). Instead, the time needed to obtain tobacco transplastomic plants amounts to about 6 months since several dedifferentiation-regeneration steps are required to obtain a satisfactory level of homoplasticity. Conversely, the low cultivation cost of tobacco allows scaling up the production of recombinant enzymes by simply increasing the cultivated area.

In the light of the different features of the two transplastomic systems, we decided to assess the efficiency of expression of a recombinant gene encoding a bacterial endoglucanase (CelK1, Glyco-hydrolase, family 5) in chloroplasts of *C. reinhardtii* and tobacco, in which the gene was successfully expressed previously (Longoni et al. 2015). In view of its potential applications in agro-industrial processes such as bioethanol and biogas production, the level of accumulation and the production cost of this important enzyme were evaluated in both transplastomic expression systems.

Materials and methods

DNA sequences, vectors, and bacterial strains

The DNA sequence of bacterial origin used in this study for the expression in chloroplasts encodes an endoglucanase (Glyco-Hydrolase family 5, *celK1*, endoglucanase) (GenBank acc. no. AAL83749) from *Paenibacillus* sp. KCTC8848P (CelK1). The sequence was codon-optimized for the chloroplast of *Chlamydomonas* and synthetized (GeneArt) (GenBank acc. no. KY307865).

The sequence was cloned via the *Nco*I and *Sac*I restriction sites into the atpB-int vector, and subsequently, the whole cassette containing the *psaA* promoter/5′UTR and the *rbcL* 3′ end was cloned into the IR-int vector via the *Cla*I and *Sma*I restriction sites (Michelet et al. 2011). The same sequence was cloned via the *Nco*I and *Hin*dIII sites into the pET28a vector (AddGene) for the expression of the recombinant protein in *Escherichia coli* strain BL21 (DE3). The protein expression was obtained after overnight incubation at 28 °C in auto-inductive media (Studier 2005).

C. reinhardtii chloroplast transformation

The *C. reinhardtii* strains used for chloroplast transformation were wild type (derived from strain 137c CC-125) and FUD50 (deletion of *atpB* CC-1185) (Michelet et al. 2011).

Before transformation, cells were grown to a density of 3–5 × 10^6 cells mL^{-1} in TAP medium at 25 °C under constant fluorescent light (60 μE m^{-2} s^{-1}) on a rotary shaker, harvested by centrifugation and resuspended in TAP medium.

Following helium gun bombardment, the cells were plated on the appropriate selection media.

The FUD50 transformants, complemented by the atpB-Int vector, were selected on high salt medium (HSM**)** under fluorescent light (80–100 μmol m^{-2} s^{-1}) at 25 °C. IR-int transformants were selected on Tris acetate phosphate medium (TAP) (Michelet et al. 2011) supplemented with 100 μg mL^{-1} spectinomycin under fluorescent light (80–100 μmol m^{-2} s^{-1}) at 25 °C.

In order to obtain homoplasmic lines, selected colonies were subcultured several times (at least four for *atpB* complementation and up to six for Spectinomycin selection increasing its concentration up to 1000 μg mL^{-1}). The transformation status of the colonies was checked by PCR as previously described (Michelet et al. 2011). The homoplasticity of atpB-Int-transformed lines was verified by the absence of the amplification with the primer pair Fud50_Screen_Rev and EcoRI_AtpB_For; the homoplasticity of IR-int transformants was confirmed by the absence of the specific amplification product with the primers IR For1 and IR_psaB_Luc Rev3. Both the primer pairs span across the insertion site for the transgene. Finally, the presence of the

insert was confirmed using the primer pair SR181 and CCs for aligning to the *RbcL* terminator and the CDS of the cellulase respectively. The sequences of the oligonucleotides and the PCR conditions are reported in the supplemental table S1.

Preparation of *C. reinhardtii* protein extract

Three different protein extraction protocols were tested for obtaining a CelK1 preparation from a fresh culture of transplastomic *C. reinhardtii*. Cells were resuspended in 200 μL of lysis buffer solution (containing 4% SDS, 20 mM EDTA and 100 mM Tris–HCl pH 6.8) vortexed at 4 °C for 20 min and incubated 30 min at 37 °C; for the second, cells were resuspended in 200 μL of 50 mM citrate buffer pH 6 plus Tween 0.5%, then vortexed with glass beads (Retsch FR0069) at 4 °C for 20 min followed by incubation at 37 °C for 30 min. For the last protocol, cells were resuspended in 200 μL of 50 mM citrate buffer pH 6 plus Tween 0.5% and sonicated for three intervals of 10 s using a microtip at 30% amplitude/power and stored in ice (Sonifer 450 Branson) (Specht and Mayfield 2013). After that, cells were incubated at 37 °C for 30 min. After each extraction protocol, the insoluble part was removed by centrifugation (14,000×*g* for 15 min) and the supernatant was recovered and stored in ice.

In order to verify the presence of the recombinant protein, aliquots of 100 μL of total protein extract obtained from the atpB-int C1 *C. reinhardtii* strain were analyzed by western blot after separation on 12% acrylamide gel by SDS-PAGE (Laemmli 1970). Briefly, proteins were blotted on a nitrocellulose membrane (Nitrobind GVS North America) and incubated with monoclonal anti-HA (Covance, MMS-101R) as primary antibody. The antibodies were diluted 1:10,000 in 1% BSA (Applichem) in TBS-T buffer. Quantification of the band intensity was performed with ImageJ (Schindelin et al. 2012). For the protein degradation assay, the cells were treated with 200 μg mL^{-1} of chloramphenicol as previously reported (Michelet et al. 2011).

Tobacco transplastomic lines

The *N. tabacum* transplastomic line of the Petit-Havana cv. used in this study was previously obtained (Longoni et al. 2015). Transplastomic and wild-type seeds (T3) were sterilized and geminated on semi-solid half-strength MS medium (Murashige and Skoog 1962) with or without 500 μg mL^{-1} spectinomycin and then grown at 23 °C with 80 μmol photons m^{-2} s^{-1}, 16 h per day. The percentage of germination under selection varied between 64 and 82. After 10 days, spectinomycin-resistant seedlings were transferred to soil and grown in a growth chamber at 25 °C with 80 μmol photons m^{-2} s^{-1}, 16 h per day. Alternatively, seedlings were transferred to peat pots (Jiffy) and 50 days later transferred to a containment greenhouse (200–1400 μmol photons m^{-2} s^{-1}) (45° 11′ 7″ 44 N latitude) with an average day-time length depending on the season (average of 13 h of light day^{-1}).

Preparation of tobacco crude leaf extract

Different homogenization procedures were tested with respect to tissue disruption mode and extraction buffer. Initially, leaf samples were ground in a mortar with liquid nitrogen and the resulting powder suspended in 4 volumes of buffer A (50 mM Tris–HCl pH 7.0, 5 mM DTT, 1 mM EDTA, 0.1% SDS, 1% Triton-X100) (Leelavathi et al. 2003) or in 4 volumes of buffer FL (50 mM Tris-HCl pH 7.0, 5 mM DTT, 1 mM EDTA). Alternatively, collected leaves were homogenized for 1 min in an equal volume (*v*/*w*) of 100 mM phosphate buffer pH 7.0 using a domestic blender (Braun, Germany). Total soluble protein (TSP) determination was performed according to Bradford (1976). Quantification of the amount of recombinant Celk1 was performed by Coomassie staining comparing the Celk1 band intensity with a standard curve of BSA (Applichem) using ImageJ software (Schindelin et al. 2012).

Enzymatic activity assays

Cellulase activity was assayed incubating for 60 min at 60 °C 40-μl samples of the plant extracts with 40 μL of 1% carboxy-methyl cellulose (CMC) as substrate. The amount of released reducing sugars was determined by 3,5-dinitrosalicylic acid (DNS) method (Ghose 1987). A glucose calibration curve (0.62–5 mg mL^{-1}) was used to determine the amount of released reducing sugars (mg mL^{-1} of glucose-equivalent). A cellulase unit was defined as the number of micromoles of reducing sugar-equivalents released per minute at 60 °C.

SDS-PAGE and zymogram

Proteins were separated by SDS-PAGE according to Laemmli (1970), with minor modifications. The enzyme substrate was copolymerized in the gel (0.1% CMC, 10% acrylamide). Enzyme extracts for the zymogram assay were obtained grinding, in a mortar in the presence of liquid nitrogen, each one of the first eight expanded leaves of a transplastomic plant. The frozen tissue powders (150 mg) were then resuspended in 600 μL of buffer FL (50 mM Tris–HCl pH 7.0, 5 mM DTT, 1 mM EDTA, without the presence of surfactants). For each sample, an amount of 3.5 μg total soluble proteins (TSP) was mixed with a 1/4 volume of a 4× protein loading buffer (250 mM Tris–HCl, pH 6.8, 8% SDS, 30% glycerol, 5% β-mercapto-ethanol and 0.04% bromophenol blue) and heated at 70 °C for 5′ before being loaded on the gel. After electrophoresis, the gel was washed extensively with 1% Triton X-100 in 0.05 M Tris–HCl buffer pH 8.4 for 30 min and then incubated at 60 °C for 1 h in the presence of phosphate buffer

(pH 6.0). In order to detect enzyme activity, the gel was stained with 0.1% Congo red for 30′ and destained with several washes in 1 M NaCl. The positions of enzyme bands correspond to the unstained area against the red background. The protein size was determined using Gel Analyzer 1.6 (http://www.gelanalyzer.com/), with reference to prestained protein markers (Fermentas).

Results

Production and analysis of transplastomic *C. reinhardtii* lines

For expression of CelK1 in the *C. reinhardtii* chloroplast under the control of the *psaA* promoter and 5′UTR, two vectors were used with selection based either on *atpB* mutant complementation (atpB-int) or on spectinomycin resistance (IR-int) (Michelet et al., 2011). Genotyping of homoplasmic transformants obtained with both constructs (IR-int CelK1, AtpB-int CelK1) was performed by PCR (Supplemental Fig. S1). The presence of the recombinant protein was determined by western blot using anti-HA antibodies (Fig. 1). However, the enzymatic activity was undetectable in cell extracts using the dinitrosalicylic acid (DNS) assay. Therefore, other extraction methods were tested in an attempt to reduce or change the detergent input that may possibly inhibit enzyme activity (Supplemental Fig. S2). Although the protein was detected by immunoblotting in similar amount with the different extraction protocols, the enzymatic activity was always below the level of detection by the DNS method. In order to reveal the presence of the active enzyme, we employed the zymogram method, which is expected to have a higher sensitivity since after separation by SDS-PAGE, the protein is concentrated in a single band and any possible effect due to soluble inhibitory components is avoided as well. Indeed, this experiment revealed the presence of an active recombinant enzyme with carboxy methyl cellulase (CMCase) activity (Fig. 1). A possible cause of the low accumulation level of the protein observed could be the instability of CelK1. In order to evaluate this aspect, proteins were extracted in different growth phases of the culture and the amount of the recombinant protein assessed by immunoblotting. As shown in Fig. 2, CelK1 proved to accumulate to similar levels. The stability of the recombinant enzyme was also investigated by treating the cells with an inhibitor of chloroplast protein synthesis. The results of this experiment show that the recombinant protein level is stable, suggesting that it has a long half-life and that transcription or translation may be the limiting steps (Supplemental Fig. S3). Indeed, the level of expression is low, since less than 1 ng of recombinant protein was detected in 30 μg of total soluble protein (TSP). Altogether, these data confirm that it is possible to produce an active recombinant endoglucanase within the *C. reinhardtii* chloroplast, albeit in low amounts.

Analysis of recombinant CelK1 accumulation level in tobacco leaves

Several months are needed to obtain tobacco transplastomic plants. Apart from the time required for engineering the chloroplast transformation vector, which is essentially similar for both tobacco and *C. reinhardtii*, the selection of resistant calli and the regeneration of heterotransplastomic plantlets require 2–3 months. The attainment of homoplasticity requires 2–3 additional dedifferentiation-regeneration cycles and, preferably, a seed-setting passage for a total of 5–7 months. Thus, 7–10 months are required to obtain a tobacco homotransplastomic line vs. less than a couple of months to reach the same goal with *C. reinhardtii* (Mayfield and Franklin 2005). Once homoplastic seeds are available, 3–4 additional months are required to obtain tobacco plants with fully expanded leaves.

Preliminary experiments showed that addition of detergents to the extraction buffer, aimed at improving the solubilization

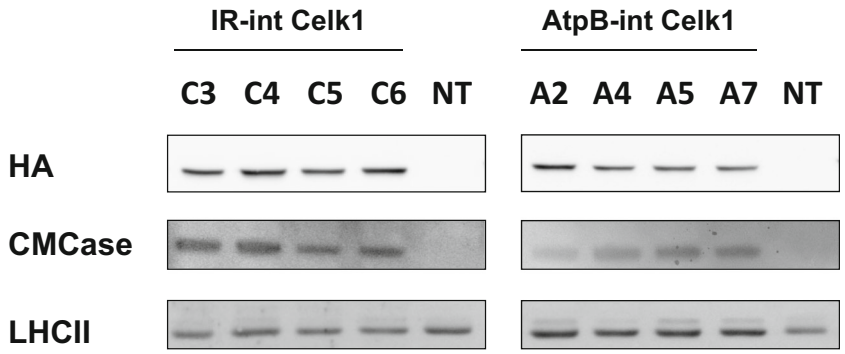

Fig. 1 Recombinant protein detection and in-gel activity assay (zymogram). Total protein extract of transformed *C. reinhardtii* strains with IR-int CelK1 (C3-C6) and with AtpB-int CelK1 (A2-A7) were analyzed by SDS-PAGE and the endoglucanase band detected by immunoblotting with anti-HA antibodies. The activity of the enzyme was confirmed by in-gel digestion of carboxy-methyl cellulose (CMC). The image is a negative of the polysaccharide staining. An immunoblot against the major LHCII (using anti-Lhcb2 antibody) was performed as loading control. *NT lane* corresponds to the untransformed parental strain

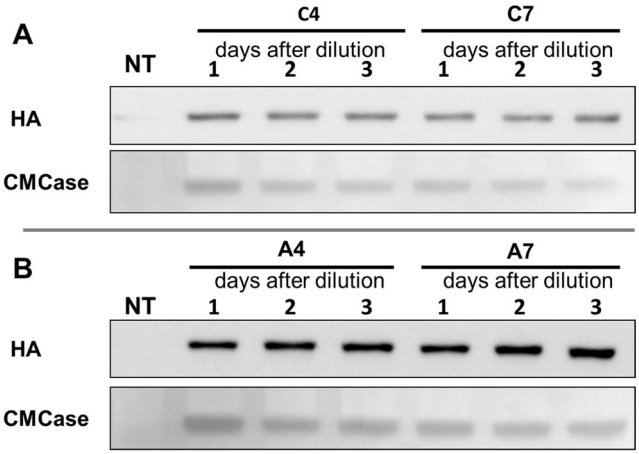

Fig. 2 Recombinant protein stability. Cultures of transformed *C. reinhardtii* strains with IR-int CelK1 (C4-C7) (**a**) and with AtpB-int CelK1 (A4-A7) (**b**) were diluted to 0.5 × 10^6 cells mL^{-1} and protein samples collected at different days of growth under constant light (60 μmol s^{-1} m^{-2}). The accumulation of CelK1 cellulase was evaluated by immunoblotting with anti-HA antibody. The activity of the enzyme was confirmed by in-gel digestion of CMC. The image is a negative of the polysaccharide staining. The *NT lane* corresponds to the untransformed parental strain

of the enzyme, had a detrimental effect on the activity (Fig. 3) and therefore a detergent-free FL buffer was used. The level of accumulation of the endoglucanase CelK1 in expanded leaves (from the 6th to 13th, Fig. 4a) collected from tobacco plants grown in a growth chamber was determined. Single leaves were homogenized in a mortar in the presence of liquid nitrogen and the homogenate analyzed by SDS-PAGE. The result of this analysis showed the accumulation of a band of the expected size (42 kDa) in all tested transplastomic leaves but not in the wild-type line, used as control (Fig. 4b). The band was identified as CelK1 by mass spectrometry (Faè et al. manuscript in preparation). In order to quantify the endoglucanase-specific activity in each single leaf, the amount of reducing sugars released was evaluated using the DNS method (Ghose 1987), incubating the crude homogenate with 1% CMC for 1 h at 60 °C, which is the optimal temperature for CelK1 activity (Longoni et al. 2015). The analyzed leaves (Fig. 4a) had an average weight of 2.67 g (6th–8th) and 4.91 g (9th–13th). As shown in Fig. 5a, CelK1 specific activity was similar in expanded leaves (7th–13th), thus indicating that a high level of active endoglucanase was retained also in mature leaves. Instead, the specific activity is lower in the sixth leaf, which is still expanding (Figs. 4a and 5a). Accordingly, as shown in Fig. 4b, L6, a less intense protein band corresponding to Celk1 is observed. This was also confirmed by zymogram analysis, as revealed by the large unstained area in each lane, using samples of equal volume (Fig. 5b). Although the latter assay associates physically the cellulase activity to a specific gel band, it is sensitive to the proteins surrounding the enzyme in the gel. Therefore, the intensity of the band is not directly comparable with the amount of the endoglucanase in samples having a different protein background.

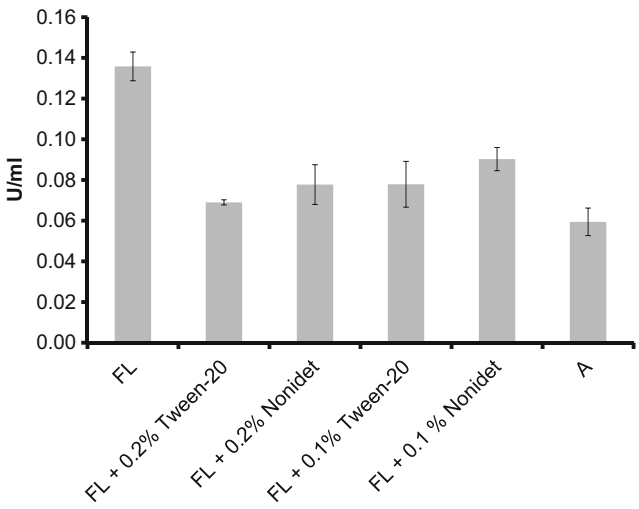

Fig. 3 CMCase activity (expressed as U mL^{-1}) of CelK1 leaf extracts obtained with FL buffer containing different detergents: +0.1 or 0.2% Tween-20, FL + 0.1% or 0.2% Nonidet, A (50 mM Tris–HCl pH 7.0, 5 mM DTT, 1 mM EDTA, 0.1% SDS, 1% Triton X-100)

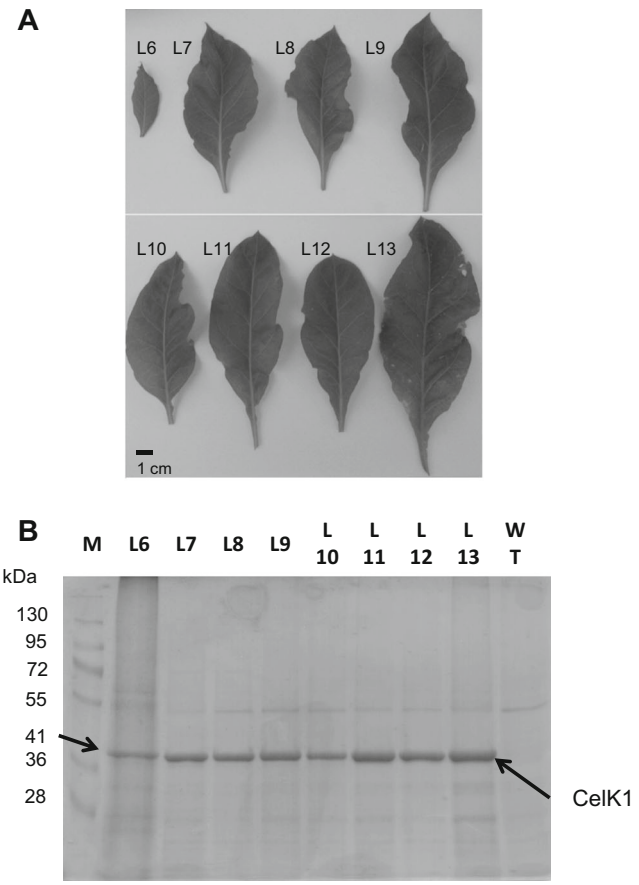

Fig. 4 **a** Size of the first eight expanded leaves (L6-L13) of a CelK1 plant. **b** SDS-PAGE analysis of leaf extracts (homogenized with buffer FL) obtained from the first eight expanded leaves of a CelK1 transplastomic *N. tabacum* plant. An extract obtained from a wild-type tobacco plant was used as control

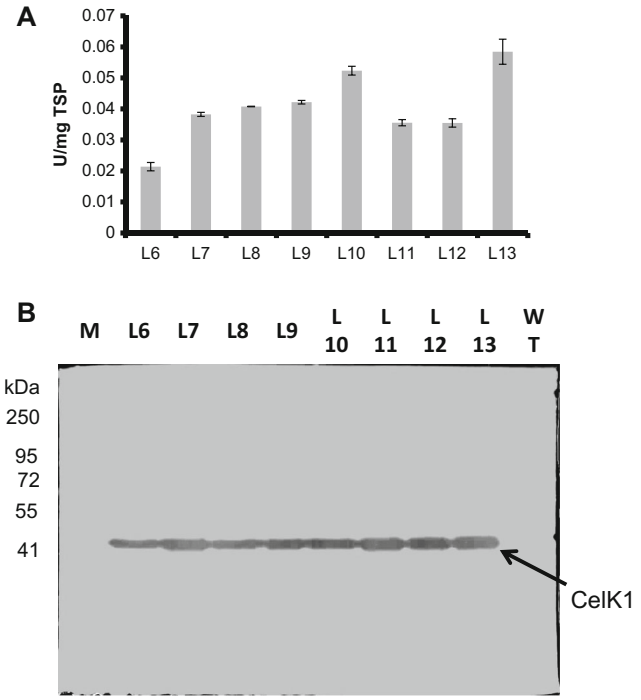

Fig. 5 **a** Zymogram analysis of CelK1 leaf extracts (homogenized with buffer FL) obtained from the first eight expanded leaves of a transplastomic *N. tabacum* plant (L6–L13). The activity of the enzyme was confirmed by in-gel digestion of CMC. Extract obtained from a wild-type tobacco plant was used as control (WT). Destained areas indicate cellulase (CMCase) activity against a stained background. **b** Cellulase (CMCase)-specific activity per leaf is expressed as U mg^{-1} of TSP. Extracts obtained with buffer A were assayed. Values are the average of three measurements

Cost of production of CelK1 by molecular farming with tobacco transplastomic plants

In order to evaluate whether the use of recombinant enzymes produced by transplastomic tobacco plants might be economically advantageous, we made an estimate of the production cost of CelK1 by plant molecular farming. The yield per harvest of tobacco (cv. Petit-Havana) grown in a containment greenhouse was found to be about 34 t per hectare, 18 of which were leaves. In view of an agro-industrial scale-up, tobacco leaves were homogenized with an equal volume (*w*:*v*) of 100 mM phosphate buffer pH 7.0, an economically sustainable option as compared to FL buffer. The concentration of CelK1 in the extract was determined by SDS-PAGE using bovine serum albumin (BSA) as standard (Supplemental Fig. S4) and found to be 14 mg g^{-1} of biomass FW. The cost for the production and processing of 1 t of tobacco biomass was calculated to be between 20 and 32€ in open field (Tusé et al. 2014). In this scenario, the cost of the CelK1 production would be between 1.5 and 2€ kg^{-1}. However, the growth of tobacco plants in a greenhouse has a high impact on the cost with respect to the field. Considering an investment of 110 € m^{-2} for the greenhouse (Battistel 2014), the impact on the production would be around 20,000€ per hectare per harvest considering a 20 years of useful life and three harvests per year. Thus, the CelK1 production price would be 41–43 € kg^{-1}. CMCase-specific activity of crude leaf extract was 1200 U L^{-1}. In the proposed extraction system, 2 L of crude extract is obtained per kg of biomass, and the cost of 1 L of CelK1 tobacco crude extract is 0.6€. Dividing this value by the enzyme concentration (U L^{-1}), we deduce that the cost of a unit of CelK1 amounts to 0.0004€.

Discussion

The production of recombinant proteins and enzymes by prokaryotic organisms is a milestone that has revolutionized both health (insulin produced by *E. coli*) and industrial (subtilisin produced by *B. subtilis*) sectors. Also, eukaryotic organisms, among which heterotrophic fungi, microalgae, and plants have been engineered to produce recombinant proteins and enzymes. Particularly, photosynthetic eukaryotes appear to offer an additional advantage over microorganisms, as they have both eukaryotic (nuclear) and prokaryotic (chloroplast) expression systems (Leelavathi et al. 2003; Mayfield and Franklin 2005; Harun et al. 2010; Verma et al. 2010; Scotti et al. 2012; Harun et al. 2014; Pantaleoni et al. 2014).

In the last decade, *C. reinhardtii*, as well as other microalgae, have attracted attention as recombinant protein expression systems and as platforms for producing compounds of commercial interest (Harun et al. 2010). Particularly, the production of heterologous proteins by *C. reinhardtii* offers the advantage of a short time for obtaining transplastomic lines (weeks) and a short generation time. Apart from other considerations, the rapidity in producing a recombinant protein might be a remarkable advantage to satisfy market needs. However, according to our study related to the production of CelK1, its percentage of accumulation was around 0.003% TSP. This low production level needs to be investigated with respect to the mechanisms involved in transcription and translation of this endoglucanase-encoding recombinant gene. It is worth noting that other recombinant enzymes accumulate at much higher level up to 3% of the TSP (Zedler et al. 2016). This suggests that in *C. reinhardtii*, the accumulation of CelK1 is especially low. Moreover, we also had difficulties with the extraction of the active enzyme. Taken together, these data underline some negative aspects of *C. reinhardtii* molecular farming. On the other hand, the higher accumulation capacity of recombinant functional proteins by transplastomic tobacco chloroplasts, which has been reported in the literature to be in the 6–16% range without any phenotypical consequence, appears a promising option. In fact, the leaf biomass produced by CelK1 transplastomic lines during a greenhouse growth cycle of 50 days amounts to 18 t of leaves ha^{-1}, namely, a total of 4×10^7 endoglucanase units.

In our homogenization conditions, we obtained 14 mg of CelK1 g^{-1} FW, namely, 240 kg ha^{-1} growth $cycle^{-1}$. Taking into account that at 45°N latitude it is possible to have three growth cycles, the total amount of CelK1 produced in a year is 720 kg ha^{-1}. This enzyme amount would suffice for the production of 72.000 L of biofuel considering the estimated input of 10 g L^{-1} (Tschofen et al. 2016).

As the accumulation of the enzyme in *Chlamydomonas* was below the level of detection using the DNS assay, the specific activity of the enzyme could not be established. However, we assume that the specific activity of the enzyme produced by *Chlamydomonas* and tobacco will be similar, as protein synthesis machinery in the chloroplast is highly conserved across the organisms. Considering that the amount of *Chlamydomonas* proteins produced is around 0.04 g day^{-1} L^{-1}, the enzyme production, at this accumulation level, would be around 1.3 μg day^{-1} L^{-1}. Therefore, to reach a production comparable to that achieved with tobacco, 10^{12} L ha^{-1} of culture should be grown, which is a value 6 times higher than the volume contained in commercial photobioreactor systems (Zittelli et al. 2013). It was estimated that the industrial cost for the production of algal biomass is between 2.5 and 10€ per kilogram in a photobioreactor, and that the total soluble proteins constitutes the 25% of the biomass (Rasala et al. 2010; Slade and Bauen 2013). Thus, the cost of this recombinant enzyme would be 100–400€ g^{-1}.

On the other hand, even taking into account the accumulation level obtained for other recombinant proteins in *C. reinhardtii* chloroplast, which is three to four orders of magnitude higher depending on the promoter and gene pair employed (Zedler et al. 2016), the estimated cost of producing CelK1 in *C. reinhardtii* (0.2–0.8€ g^{-1}) is higher than that of tobacco molecular farming (0.01–0.04€ g^{-1}). Therefore, for an industrial enzyme such as CelK1, the estimated cost of production by *C. reinhardtii* prior to purification appears economically incompatible with the downstream processes where this endoglucanase might be employed. In fact, for an enzyme to be cost-effective for industrial-scale cellulosic ethanol production, its cost was recently estimated between 0.01 and 0.06 € g^{-1}, the upper value also being the calculated market price for the commercial cellulase (Liu et al. 2016). Nevertheless, an algal molecular farming approach might be desirable for the production of high value proteins for medical purposes that might require completely sterile production procedures.

The low cost of enzymes produced by tobacco transplastomic plants might contribute to increase the economic sustainability of some agro-industrial processes. For instance, the high cost of commercial enzymes hampers their use in biodigestors since the increase in biogas production poorly compensate the cost of enzymes. Also, in the case of bioethanol production, where the cost of enzymes used for saccharification is one of the three crucial parameters for the economical sustainability of biofuel production (Wooley et al. 1999; Lunin et al. 2012), the use of recombinant enzymes produced by transplastomic tobacco lines might contribute to reduce the overall cost of the process. In addition, large-scale production at low cost of recombinant enzymes might contribute to transform chemically based industrial processes into new ones based on biocatalysis, which are generally recognized as less polluting.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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