# Stable expression and characterization of a fungal pectinase and bacterial peroxidase genes in tobacco chloroplast

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Edward Alexander Espinoza-Sánchez [a], Marianela Hazel Álvarez-Hernández [a], Jorge Ariel Torres-Castillo [b], Quintín Rascón-Cruz [c], Adriana Gutiérrez-Díez [a], Francisco Zavala-García [a], Sugey Ramona Sinagawa-García [a,*]

[a] Laboratorio de Biotecnología, Campus de Ciencias Agropecuarias, Universidad Autónoma de Nuevo León, Francisco Villa S/N Col. Ex hacienda El Canadá, General Escobedo, Nuevo León, México
[b] Instituto de Ecología Aplicada, Universidad Autónoma de Tamaulipas, División del Golfo No. 356. Colonia Libertad, Cd. Victoria, Tamaulipas, México
[c] Laboratorio de Biotecnología I, Facultad de Ciencias Químicas, Universidad Autónoma de Chihuahua, Circuito 1, Nuevo Campus Universitario, Chihuahua, México

### A R T I C L E   I N F O

### A B S T R A C T

Background: The high capacity of chloroplast genome response to integrate and express transgenes at high levels makes this technology a good option to produce proteins of interest. This report presents the stable expression of Pectin lyase (PelA gene) and the first stable expression of manganese peroxidase (MnP-2 gene) from the chloroplast genome.
Results: pES4 and pES5 vectors were derived from pPV111A plasmid and contain the PelA and MnP-2 synthetic genes, respectively. Both genes are flanked by a synthetic rrn16S promoter and the 3′UTR from rbcL gene. Efficient gene integration into both inverted repeats of the intergenic region between rrn16S and 3′rps′12 was confirmed by Southern blot. Stable processing and expression of the RNA were confirmed by Northern blot analysis. Enzymatic activity was evaluated to detect expression and functionality of both enzymes. In general, mature plants showed more activity than young transplastomic plants. Compared to wild type plants, transplastomic events expressing pectin lyase exhibited enzymatic activity above 58.5% of total soluble protein at neutral pH and 60°C. In contrast, MnP-2 showed high activity at pH 6 with optimum temperature at 65°C. Neither transplastomic plant exhibited an abnormal phenotype.
Conclusion: This study demonstrated that hydrolytic genes PelA and MnP-2 could be integrated and expressed correctly from the chloroplast genome of tobacco plants. A whole plant, having ~470 g of biomass could feasibly yield 66,676.25 units of pectin or 21,715.46 units of manganese peroxidase. Also, this study provides new information about methods and strategies for the expression of enzymes with industrial value.

## 1. Introduction

The plant cell wall is an important structure consisting of residues of cellulose, lignin, pectin and other polymers that support and confer impermeability to the cells; it also functions to prevent microbial attack [1,2,3]. From these polymers, the pectate and lignocellulosic residues figure among the main wastes produced by the human activity in the form of wood, urban solid wastes, agricultural, grass and forestry wastes, although they are not toxic. Considering that the plant cell wall comprises more than 90% of dry weight, the accumulation of these products represents a focus of environmental deterioration and a loss of material potentially useful material [4,5,6].

The cell wall components could be used if there were efficient extraction methods [5]. However, the heterogeneous nature of such

wastes is the main obstacle for their use so new technological approaches are necessary to exploit these resources [7,8,9]. In this sense, processes based on microorganisms are an alternative because their high efficiency in the processing of these materials, microorganisms have become an important source of enzymes used in genetic engineering [10].

Nevertheless, the chloroplast genetic engineering has been used to express proteins and has led to significant advances in plant biotechnology with a crucial role in plant genetic improvement in areas such as agriculture, food, medicine and environment [3,11]. This technology involves the transgene insertion into the chloroplast genome, which has several advantages as non-positional effects, absence of epigenetic effects and uniparental transgene inheritance [12,13,14].

Transplastomic plants like tobacco 'Nicotiana tabacum' [15], potato 'Solanum tuberosum' [16], tomato 'Solanum lycopersicum' [17], soybean 'Glycine max' [18], cauliflower 'Brassica oleracea' [19] and lettuce 'Lactuca sativa' [20,21] have been successfully tested for expression of multiple proteins. Due to the high capacity of chloroplast to

provide overexpression of up to 70% of total soluble protein (TSP), the possibility to express multiple transgene in operons as well as their efficiency in transgene contention, chloroplast transformation is a promising technology to obtain high level of enzymes with environmental and biotechnological impact − including lignocellulolytic enzymes such as Pectin lyase (EC 4.2.2.2) [3,22,23].

Pectin lyase is a $Ca^{2+}$dependent endoglucanase with multiples subfamilies (PL1, PL2, PL3, and PL9) [23,24,25] which act as the virulence factors of microbial plant pathogens and are responsible for the $\alpha(1 \rightarrow 4)$-bond degradation of polygalacturonic acid, a principal component of pectin. This reaction releases 4,5-unsaturated di and trigalacturonate from various pectates [26,27].

In the case of lignin, the principal component of secondary cell wall, some fungi have been shown to produce an enzyme cocktail (laccase 'Lac', lignin peroxidase 'LiP' and manganese peroxidase 'MnP') capable of degrading lignin efficiently. The primary sequence of MnP exhibits high homology with other ligninases [28,29] removing an electron of phenol moiety of lignin to generate a phenoxy radical and oxidize $Mn^{2+}$ to $Mn^{3+}$ which is stabilized by organic acid chelators, facilitating the degradation of phenolic compounds in the presence of $H_2O_2$ [30]. In this sense, lignin is the most important source of aromatic polymers in nature and its decomposition is necessary for carbon recycling, and therefore MnP activity is important for environmental conservation [31]. Although the expression of these enzymes has been reported in bacterial systems, there are few studies that focus on the production of hydrolytic enzymes in chloroplast compartments [3,5,32,33,34].

In the present report, plastid biotechnology was used to express cell wall hydrolytic enzymes to study their expression, stability and biochemical activity in chloroplast compartments and the effect that would have on the metabolism and growth of plants as well as the segregation of the stable transgene.

## 2. Materials and methods

### 2.1. Cloning genes of interest into chloroplast expression vector

Two genes of cellulolytic enzymes were used: the pectin lyase gene (*PelA*) family 3 from *Streptomyces thermocarboxydus* [34] and the manganese peroxidase isozyme H3 gene (*MnP-2*) from *Phanerochaete chrysosporium* [35]. The sequences of each gene were designed *in silico* using Serial Cloner 2.6.1 software with undesirable restriction sites eliminated by silent mutation. Sequences were designed to reflect native codon usage of the chloroplast genome [36]. The sequences of *PelA* (GenBank: AB513441) and *MnP-2* (GenBank: U10306) were synthesized by GeneScript (New Jersey, USA). The *PelA* cassette synthesized included the coding sequence (804 bp) flanked by the *rrn16S* promoter (P) and *rbcL* terminator (T), including a Shine-Dalgarno (SD) and a leader sequence (LS) of *rbcL* gene in the 5′ UTR of the coding region of the gene (P*rrn16S*:SD: LS *rbcl*: *PelA*: T*rbcL*) [37]. The complete sequence was flanked with *EcoRI*/*HindIII* sites for cloning into the same sites in the pPRV111A vector [38]; additionally, the coding region of the *PelA* gene was flanked with recognition sites to enzymes 5′*NheI*/3′*XbaI* restriction sites to allow replacement in the vector by *MnP-2* gene.

### 2.2. Plastid transformation

Tobacco plants (*N. tabacum* var. Petite Havana) were obtained from seeds germinated under sterile conditions on RM culture medium. For maintenance and propagation of plasmids *Escherichia coli* strain DH5α (Invitrogen®, Carlsbad CA, USA) was used. Plasmid DNA was obtained using QIAGEN Plasmid Maxi Kit columns (QIAGEN Inc., Valencia, CA). Gold particles of 0.6 μm (Bio-Rad®) coated with DNA were used for transformation and tobacco leaf bombardment according to Svab and Maliga [39] and Lutz et al. [40]. High-pressure gun S1000He

Bio-Rad with a Hepta adapter (Bio-RAD, Germany, Munich) was used at 1100 psi and 11 cm shooting distance. Bombarded leaves were incubated for 24 h in dark, and after were cut in sections of 3 mm × 3 mm and placed on RMOP medium supplemented with 500 mg $L^{-1}$ of spectinomycin for a first selection round [39]; spontaneous mutants were eliminated with a second selection round on RMOP supplemented with 500 mg $L^{-1}$ of spectinomycin/ streptomycin (Sigma-Aldrich, Japan) [39,41]. A third selection round was performed in RMOP medium to achieve a homoplasmy with spectinomycin. Finally, shoots from regenerated plants were placed on RM medium to promote rooting of plants. Plants of two months old were placed in pots with sphagnum moss until seed production.

### 2.3. Molecular analysis

Total DNA was extracted from leaves of tobacco plants according to Doyle [42]. PCR was performed using specific primers: *PelA* gene Fw-5′ ATGACATCCGCGACACGA3′ Rv-5′TGATGTCGGACGAGCTGTACT3′ and *MnP-2* gene Fw-5′ATGGCCTTTGCATCCTCA3′ Rv-5′TTATGCAGGGCCGT TGAAC3′, under the following conditions: 2 min of denaturing at 94°C, followed by 25 cycles of amplification (45 s at 94°C, 45 s at 60°C, 1 min at 72°C).

For Southern blot analysis 4 μg of DNA samples was digested with BamHI and then resolved in a 1% (w/v) of agarose gel at 65 V for 4 h. The agarose gels were denatured in 120 mM HCl for 30 min, followed for 30 min in 0.4 N NaOH and 0.6 M NaCl. Neutralization was performed with 0.5 M Tris pH 7.5 and 1.5 M NaCl. Transfer to nylon membranes (Blotting nylon type B positive, Fluka® Chemie GmbH, Steinheim, Germany) was performed with transference buffer (25 mM $NaPO_4$, pH 6.5) after that membranes were fixed in a CL1000 Ultraviolet crosslinker at 120,000 μJ/cm$^2$ for 12 s. Membranes were pre-hybridized at 55°C for 4 h and hybridized at 65°C for 12 h with 10 μL of a previously labeled rRNA16S probe by PCR digoxigenin-11- dUTP using Fw-5′TGAGAATGGATAAGAGGCTC3′ Rv-5′TGTTGTTCCCCT CCCAAGGG3′ primers.

To RNA analysis by Northern blot, total RNA from leaves of transplastomic and non-transplastomic plants was carried out using the LiCl protocol [43]. The Northern blots were performed as follows: 10 μg were resolved in a 0.8% (w/v) agarose/formaldehyde gel at 65 V for 2 h. The RNA was transferred overnight into nylon membranes by capillarity with 10× SSC solution. The membranes were fixed in a CL1000 Ultraviolet crosslinker at 120,000 μJ/cm$^2$ for 12 s. Pre-hybridization was performed at 50°C for 4 h followed by hybridization at 55°C for 12 h with 10 μL of labeled probe with PCR digoxigenin-11-dUTP using specific primers to each gene.

Both Southern and Northern blot membranes were washed twice during 15 min with 1× of SSC, 0.1% SDS at RT, followed by three washes of 15 min with 0.2× SSC, 0.1% SDS at 55°C. Anti-Digoxiginin-AP (Fab Fragments 'Roche, Mannheim, Germany') 1:15,000 was used. Membranes were placed in the solution for 30 min and then washed with a washing buffer (100 mM Tris–HCl, pH 9.5, 100 M NaCl). For probe detection, 500 μL of DIG High Prime DNA Labeling and Detection Starter Kit II solution (Roche, Mannheim, Germany) were placed on membranes and revealed with Lumi-Film Chemiluminescent Detection Film (Roche, Mannheim, Germany).

### 2.4. Protein extraction

Leaves from young (three weeks old) and mature (seven weeks old) transplastomic and non-transplastomic plants were collected. Total soluble protein was extracted by homogenizing leaf samples in a buffer containing 50 mM 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES)-KOH (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM potassium acetate, 5 mM magnesium acetate, 10 mM dithiothreitol (DTT) and 2 mM phenylmethylsulfonyl fluoride (PMSF) [5]. After centrifugation at

4500 rpm for 10 min and recovery of the upper phase twice, the protein concentration was determined by Bio-Rad Protein Assay Reagent Kit (Bradford's method) (Roth, Karlsruhe, Germany) using a BSA (Bovine Serum Albumin) calibration curve.

## 2.5. Enzymatic analysis

Pectin lyase activity was measured by the DNS method [44], quantifying the increase of sugar reducing groups liberated after incubation with 0.1 mL 1% Pectin from Citrus Peel (Sigma, Aldrich) as substrate dissolved in 100 mM Citrate buffer and 0.1 mL of protein extract for 1 h. The assay was carried out at pH 5, pH 7 (citrate: phosphate buffer) and pH 10 (Buffer citrate: phosphate adjust with NaOH) at 27, 45 and 60°C [45,46]. Additionally, the assay was carried out comparatively from young and mature plants. The concentration of reducing sugar present in the reaction was compared with a concentration curve by measuring the absorbance at 540 nm. A positive control of Pectinase from *Aspergillus niger* (Sigma, Aldrich) was used at the same assay condition. The enzyme activity was expressed as units per mg of TSP (U/mg), where one unit (U) of pectin lyase activity was defined as the amount of enzyme that liberates 1 μmoL of galacturonic acid per minute under the assay condition [47].

To MnP activity quantification the assay was realized by monitoring phenol red degradation [48,49]. Briefly, a final volume reaction mixture of 1 mL containing 20 mM sodium acetate-acetic acid buffer at pH 4.5, 6 and 8 was adjusted with NaOH, 25 mM lactate, 0.1 mM $MnSO_4$, 0.5 mg BSA, 0.5 mg phenol red, 0.1 mM $H_2O_2$ and 0.1 mL of enzyme solution was used incubated at 30, 50 and 65°C. Assays were carried out to young and mature plants. The reaction was stopped after 10 min incubation with 100 μL of 10% NaOH. The absorbance was recorded at 610 nm and the activity expressed in U/mg with a molar extinction coefficient of the oxidized phenol red of 22 $mM^{-1}$ $cm^{-1}$. One activity unit is defined as the amount of enzyme necessary to oxidize 1 μmoL of substrate per minute under the assay conditions.

## 3. Results

### 3.1. Vector construction with PelA and MnP-2 genes

Two vectors (pES4 and pES5) were constructed in the pPRV111A plasmid backbone, containing *PelA* and *MnP*-2 genes, respectively. These vectors have *rrn16S/3′rps12* sequences for integration via homologous recombination in the inverted repeat (IRA/IRB) of the chloroplast genome. The *aadA* gene (*aminoglycoside 3′-adenylyltransferase*) was also included as a selectable marker. The genes were cloned downstream of the *aadA* gene and flanked by 5′UTR from *rrn16S* and a 3′UTR from *rbcl*, for mRNA stabilization and to enhance gene translation [50]. Both promoter and terminator were synthetic and obtained from Kuroda and Maliga [37] performing point mutations in the 1216 bp corresponding at 3′UTR to eliminate the *XbaI* site (Fig. 1a and Fig. 1b).

### 3.2. Chloroplast transformation

pES4 and pES5 vectors containing the *PelA* and *MnP-2* genes respectively were used to transform young tobacco leaves. The bombarded leaves were placed on RMOP regeneration medium to a first selection round. Spectinomycin-resistant regenerating explant appeared at five weeks (Fig. 2a). Leaves of these regenerated clones were cut in two-pieces and submitted to a second selection round on RMOP supplemented with spectinomycin/streptomycin to identify and discard non-transformed clones arising by mutation. Regeneration on both media occurred at two weeks confirming the transformation of these explants with a transformation efficiency of 3.14% for both vector. To achieve homoplasmy in plants, leaves from explants of the second selection round were placed in a third selection round and the resultant shoots were placed on RM medium non-supplemented to rooting of plants (Fig. 2b). Putative pES4 transformed plants were less developed in vitro compared with the wild type but the pES5 plants containing the *MnP-2* gene showed larger internodes but less accumulated biomass.
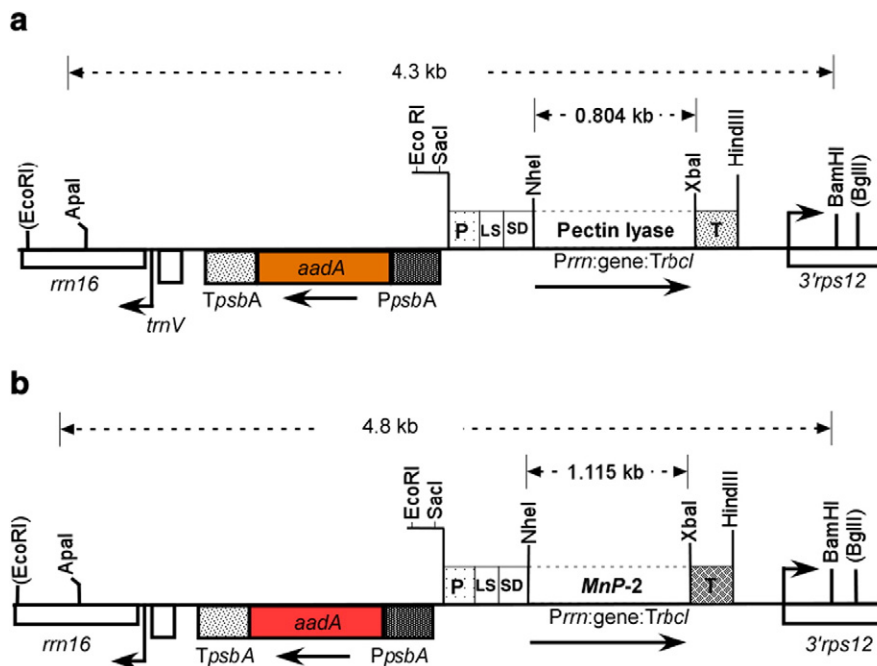


**Fig. 1.** Restriction maps of the plastid transformation vectors. (a) pES4 vector containing *PelA* gene, (b) pES5 with *MnP*-2 gene for cloning in *rrn16* and *3′rps12* homologous recombination site into chloroplast genome, these vectors contain *aadA* as selection marker gene. The genes are under control of an *rrn16S* promoter and *rbcl* terminator sequence.
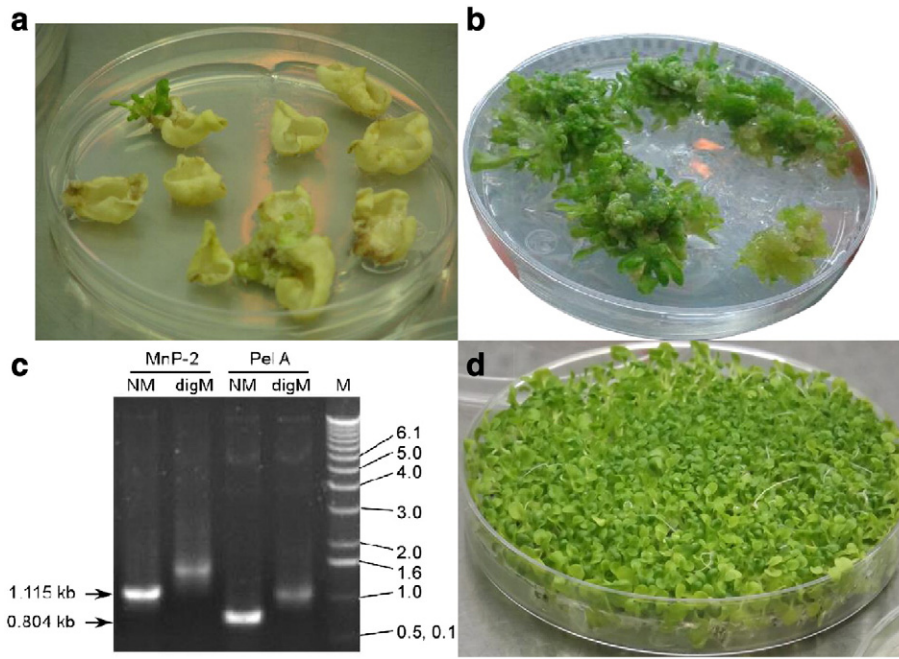
**Fig. 2.** (a) Regenerating explant spectinomycin-resistant appeared at five weeks, (b) explants from third selection placed in RMOP medium supplemented with 500 mg L$^{-1}$ of spectinomycin, (c) PCR of both transformants lines showing amplification of specific genes, also show digoxigenin integration observed by molecular weight difference (NM: non-marked, digM: digoxigenin marked), (d) seedling spectinomycin-resistant from seed in RMOP medium with spectinomycin.

### 3.3. PCR analysis and plant acclimation in the greenhouse

The putative transformants of both lines were initially tested by PCR screening to detect the presence of our genes of interest (*PelA* and *MnP-2*). Four transplastomic plants were obtained from pES4 (NtpES4A, NtpES4B, NtpES4C and NtpES4D) transformation event whereas from pES5 transformation event seven transplastomic plants were obtained (NtpES5A, NtpES5B, NtpES5C, NtpES5D, NtpES5E, NtpES5F and NtpES5G). All plants were confirmed by PCR analysis showing the 0.804 kb and 1.115 kb fragments to the *PelA* and *MnP-2* genes respectively (Fig. 2c). Transformants and non-transformed plants were transferred to pots and grown to maturity and flowering in greenhouse. The mature plants with *PelA* gene showed reduced growth compared with the wild type. However, the phenotype was similar to non-transformed plants, with respect to pigmentation, flowering time and floral phenotype (Fig. 3a and Fig. 3b). The
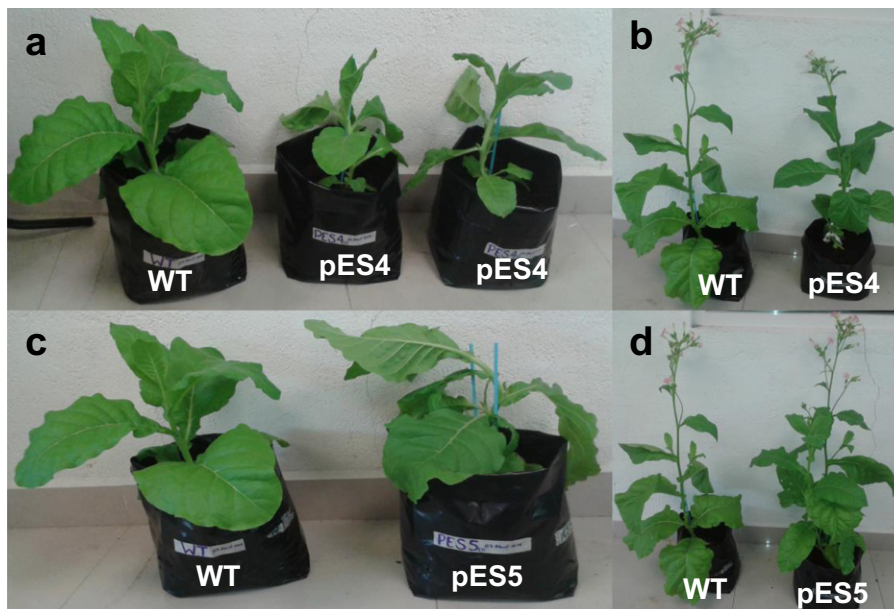


**Fig. 3.** Transformants and untransformed plants from greenhouse compared in grown and flowering. (a) pES4 young plants transformed with *PelA* gene at three weeks old showed lower growth compared with the wild type but no affected significantly, (b) pES4 mature plants at seven weeks old showed normal phenotype, non-alteration on pigmentation and non-problems in the time flowering, the phenotype in flowers was normal, (c) pES5 young plants transformed with *MnP-2* gene at three weeks old showed more growth that the wild type, with internodes more larges and higher growth rate, (d) pES5 mature plants at seven weeks old showed lower biomass production that wild type; no pigmentation alterations was showed in leaves.

transformed plants with *Mnp-2* gene interestingly showed more growth than wild type plants in young and mature stage, with larger internodes and higher growth rate but biomass production in mature plants was lower than wild type plants such as was showed in vitro conditions. In spite of this, no pigmentation alterations were showed in leaves (Fig. 3c and Fig. 3d). Both lines (*PelA* and *MnP-2* transformed plants) produced viable seed with germination at six days showing spectinomycin resistance (Fig. 2d). Seedlings of lines from both transformant lines showed fragments of similar size confirming gene segregation; this also indicates that spectinomycin-resistant phenotype is a reliable sign for the determination of transformants.

### 3.4. Confirmation of integration, homoplasmy and transcription stability

To investigate if transplastomic lines achieve a state of homoplasmy, a Southern blot analysis was performed. The *rRNA16S* probe showed a 3 kb fragment in non-transformed line. In transplastomic lines, the probe showed a single fragment of 4.3 and 4.8 kb corresponding to *PelA* and *MnP-2* lines confirming the efficient gene integration in the chloroplast genome. The absence of 3 kb fragments in both transformed lines suggests that all chloroplast genomes were transformed achieving homoplasmy (Fig. 4a). Also, to confirm the mRNA processing, Northern blot analysis was performed with total RNA from leaves of all transformed and non-transformed plants which confirmed stable monocistronic mRNA processing with transcripts of 0.804 kb and 1.115 kb corresponding to *PelA* and *MnP-2* genes respectively (Fig. 4b and Fig. 4c).

### 3.5. Effect of pH, temperature on PelA and MnP-2 enzyme activity

For enzymatic activity assays, three plants from pES4 line (NtpES4A, NtpES4B, NtpES4C) and three plants of pES5 line (NtpES5A, NtpES5B, NtpES5C) were used and the results are shown as the average of each transplastomic line. Protein extracts were obtained from young and mature *PelA* and *MnP-2* transformed plants; *PelA* activity was measured using Pectin from Citrus Peel as substrate; likewise, MnP activity was measured by monitoring phenol red degradation. In both transplastomic lines the enzymatic activity was upper in mature plants than young plants (Fig. 5a and Fig. 5d); in this respect, the leaves of mature plants were used to enzymatic assays. The enzymatic activity of *PelA* from transplastomic plants was higher than 19.72 ± 2.3 U/mg with respect to commercial pectinase from *A. niger*.

Enzymatic activity from *PelA* showed that the activity of recombinant enzyme is high at neutral pH (60.6 ± 3.6 U/mg) decreasing at alkaline pH (pH 10) to 30.29 ± 1.85 U/mg. However, slightly acid pH is unfavorable with a high decreased activity to 12.99 ± 1.0 U/mg (Fig. 5b). On another hand, the temperature also plays a role in the activity performance with more activity at 60°C with 66.011 ± 4.9 U/mg decreasing to 53.65 ± 4.1 U/mg at 27°C but at 45°C had less activity decreasing 19.14 ± 2.1 U/mg, a difference of 46.86 U/mg with respect to optimum temperature (Fig. 5c). Enzymatic activity in MnP increased activity from acid pH (pH 4.5) (91.65 ± 5.6 U/mg) to pH 6 (103.47 ± 7.0 U/mg), but showed unfavorable activity in alkaline pH (pH 8) decreasing to 39.51 ± 7.3 U/mg (Fig. 5e). The temperature also had an influence on the activity performance showing increment activity according to the increase of temperature, of 42.40 ± 5.4 U/mg at 30°C until 113.0 ± 6.1 U/mg at 65°C (Fig. 5f). Both transplastomic lines showed higher levels of enzymatic activity with regard of non-transplastomic plants.

### 3.6. Levels of PelA and MnP-2 activity in leaves

Pectin lyase protein activity (304 U $g^{-1}$ mature fresh leaves) was 55-fold more in transplastomic NtpES4 line than non-transformed plants (5.5 U $g^{-1}$ mature fresh leaves) with a yield of 66,676.25 units per plant. MnP protein activity (84.0 U $g^{-1}$ mature fresh leaves) was 22-fold more in transplastomic NtpES5 line than UT plants (3.7 U $g^{-1}$ mature fresh leaves) with a yield of 21,715.46 units per plant (Table 1).

## 4. Discussion

The production of multiple enzymes has been realized by different methods mainly chemical or through bacterial heterologous expression. In this sense, genetic industry can play an important role as a supplier of large amounts of proteins at low cost. Due to the high capacity of chloroplast genome response to direct the efficient expression of transgenes, multiple proteins of interest in agricultural sector have been expressed at high levels. In this study we report the stable expression of two hydrolytic genes (*PelA* and *MnP-2*) from the chloroplast genome. The *rrn16S* and *3'rps12* regions used as homologous recombination sites in the pPRV111A-derived expression vectors (pES4 and pES5) [38], efficiently promoted integration of the transgenes, as has been previously reported [3]. Moreover, the 5', 3' UTR and SD sequence were sufficient to express and stabilize of the
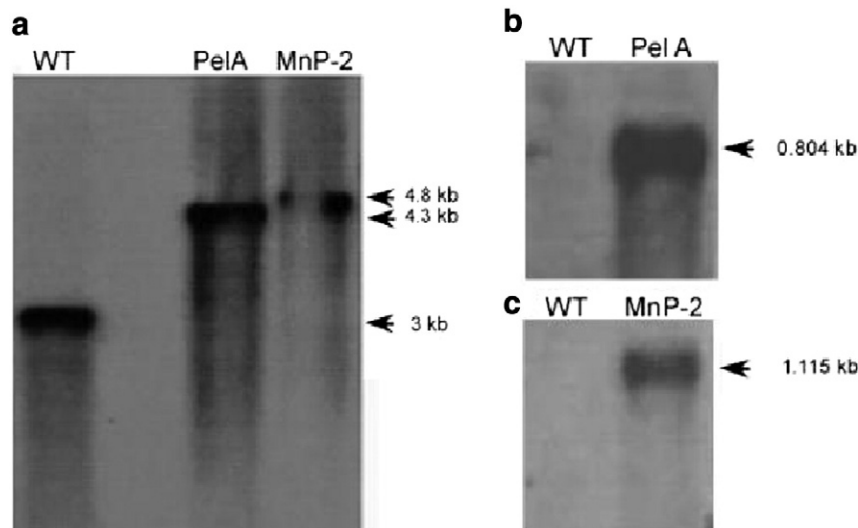


**Fig. 4.** (a) Southern blot analysis from transplastomic plants (NtpES4A and NtpES5A) shown an efficient genes integration and homoplasmy into chloroplast genome, a 4.3 kb fragment of *PelA* gene and 4.8 kb fragment of *MnP-2* gene were detected, untransformed plants a single 3 kb fragment was detected. (b, c) Northern blot analysis from transplastomic plants (NtpES4A and NtpES5A) shown transcription of mRNA in both genes '*PelA*: 0.804 kb and *MnP-2* 1.115 kb'.
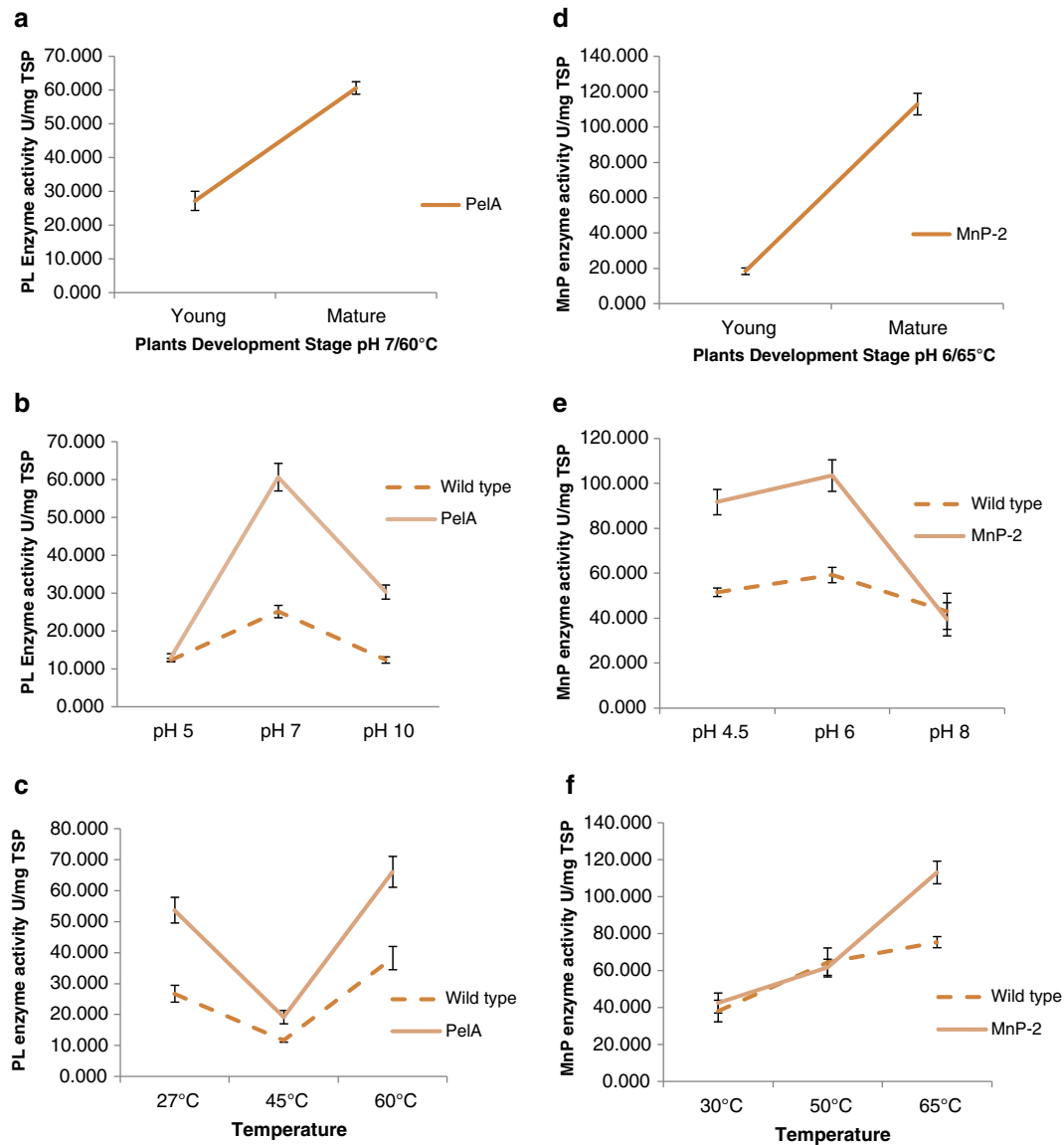
**Fig. 5.** Enzymatic activity of tobacco transplastomic plants. (a, d) Enzymatic activity of *PelA* and *MnP-2* in young (three weeks old) and mature (seven weeks old) plants, (b, c) Enzymatic activity of *PelA* protein from the leaves of mature plants subjected at different pH and temperature conditions. (d, e) Enzymatic activity of *MnP-2* protein from leaves of mature plants subjected at different pH and temperature conditions. The analysis showed more activity in transplastomic plants with respect at wild type plants in all conditions. The results are shown as average of three transplastomic plants (NtpES4A, B, C) and (NtpES5A, B, C).

mRNA. Therefore, these synthetic regulator elements were useful in directing the stable gene expression as reported by Kuroda and Maliga [37].

It has been reported that pectin lyases are involved in different roles within plants as cleavage pectin or defense against plant pathogens [3,26,51]. Although there are reports of pectin lyase expression in different organisms [52,53,54] there are few studies related to its expression in tobacco chloroplast; in this sense, we evaluated the enzymatic activity of a pectin lyase from *S. thermocarboxydus* strain B1 using commercial substrates finding high activity. The enzymatic activity was higher in transplastomic plants with respect to wild type, but the activity in different stages of transplastomic plants was also

**Table 1**
*PelA* and *MnP-2* enzyme yield in transplastomic tobacco plants according to Jin et al. [22]. One unit of pectin lyase activity was defined as the amount of enzyme that liberates 1 μmoL of galacturonic acid per minute under the assay condition. One activity unit to MnP is defined as amount of enzyme necessary to oxidize 1 μmoL of substrate per minute under the assay conditions. The results are shown as average of three transplastomic plants (NtpES4A, B, C) and (NtpES5A, B, C).

| Enzyme | Leaf Age | mg/mL | mg/g | U/mg | Average of leaves per plant | Average weight per leaf (*g*) | Unit g$^{-1}$ in leaf | Units | | Whole plant yield |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Per leaf | Per age group | |
| PelA | Young | 1.31 ± 0.07 | 5.2 ± 0.3 | 27.1 ± 2.8 | 8.3 ± 1.15 | 1.37 ± 0.28 | 142.7 ± 13.9 | 195.98 | 1632.53 | *Units* |
| | Mature | 2.0 ± 0.17 | 8.3 ± 0.69 | 36.5 ± 2.2 | 12.3 ± 1.52 | 17.31 ± 4.43 | 304.6 ± 20 | 5275.24 | 65,043.72 | 66,676.25 |
| MnP | Young | 0.29 ± 0.01 | 1.1 ± 0.07 | 18.3 ± 1.8 | 6.3 ± 2.5 | 1.27 ± 0.16 | 21.5 ± 3.4 | 27.44 | 173.70 | 21,715.46 |
| | Mature | 0.6 ± 0.06 | 2.6 ± 0.2 | 31.9 ± 2.4 | 15.3 ± 1.52 | 16.75 ± 1.25 | 83.8 ± 8.0 | 1405.20 | 21,541.75 | |

different with more activity in mature transplastomic when compared with transformed young plants as reported by Verma et al. [3]. Pectin lyase encoded by *PelA* gene showed more activity at neutral pH whereas at acid pH (pH 5) the enzyme activity was decreased until 78.5% contrary to the reported by Nakano [55]. Although it has been reported that alkaline pH is optimal for bacterial pectin lyases [45,56] our findings showed a decrease around 50% of activity at pH 10. Our results were similar to those reported by Verma et al. [3] who expressed the *PelA*, *PelB* and *PelD* genes from *Fusarium solani* in tobacco chloroplast and *E. coli*, and showed that optimal pH in *E. coli* was alkaline, whereas in plants the optimal pH was mildly acidic to neutral (6 to 7) with a 38% decline in activity at pH 10. Previously, Tonouchi et al. [34] expressed this *PelA* gene in *E. coli* with lower activity than the native enzymes; also, it is reported that heterologous protein was inactivated at 30°C. However, in this study using synthetic sequences 5′ and 3′UTR (P*rrn16S*: SD: LS *rbcl*: GOI: T*rbcL*), we obtained more protein expression such as proposed by Kuroda and Maliga [37]. Furthermore, not only the expression was improved, the enzymatic activity was more efficient with stable activity at 60°C same as was showed to be the optimum temperature, these results adds at the reports of different optimal temperatures to pectin lyases [3,45,58]. Nevertheless our results are higher than that reported by Tonouchi et al. [34] in *E. coli*, and also are less than native PL I (177 U/mg) [33].

Moreover, manganese peroxidase has been reported to intervene in the degradation of lignin facilitating the rupture of phenolic compound in $H_2O_2$ presence [30,57,58]. Lignin-degrading enzymes from different organisms have been reported [58,59] and to our knowledge this is the first report about the expression of a Manganese Peroxidase isozyme H3 encoded by *MnP-2* gene from *P. chrysosporium* in tobacco chloroplast. *MnP-2* gene was inserted in the same region than *PelA* gene with stable expression. Plants of these lines showed higher activity in mature stage than in young stage, in a similar manner as we reported for *PelA* expression. The activity of this enzyme was evaluated at three different pH levels. We observed high activity at pH 6, as was reported by Jiang et al. [58] but in contrast with different reports showing high activity at pH 5.5 and pH 4.5 [60,61]. Alkaline pH was unfavorable with a decrease of 61.8% at pH 8, whereas a decrease of 11.4% was observed at acid pH. The enzymatic activity exhibited an optimum temperature at 65°C which decreased to 62.4% at 30°C. This differs from the previously reported temperatures of 37°C and 30°C in bacterial systems [58,60,61].

Previously, the MnP gene from *Coriolus versicolor* controlled by double CaMV 35S-Promoter was transformed into tobacco using *Agrobacterium*, and resulted in 54-fold more activity than non-transformed plants [59], whereas in our study the lignin-degrading plant was 22-fold higher in transplastomic plants than wild type without differences in growth, flowering and seed production, unlike what has been previously seen in alfalfa [62] and tobacco [3] when this type of gene in expressed at this level.

The expression of different hydrolytic cell-wall genes in tobacco chloroplast has been achieved with different results. In this sense, some reports showed low effect in pigmentation with expression of a laccase gene [63] or severe retardation in plant growing when xylanase was expressed in the chloroplast genome [64]. In contrast, Petersen and Bock [5] found that expression of celluloses showed phenotypes with severe pigmentation loss followed by plant death. Beneficial results has been obtained with the hydrolytic gene expression such as the reported by Jin et al. [22] with expression of $\beta$-glucosidase, resulting in elevated phytohormone levels, increase of biomass, increase trichomes and protection against aphids or whiteflies. Because of this, the absence of negative effects in growth and reproduction in transformed plants with both expressed genes in this study might be an advantage to express these genes in industrial and agricultural systems.

In this study, we demonstrated that hydrolytic genes such as *PelA* and *MnP-2* could be integrated and expressed correctly in chloroplast genome tobacco plants. The resulting yield of hydrolytic enzymes demonstrated

the viability harvesting protein from chloroplast-expressed genes: because a one plant has ~470 g of biomass, it is feasible to expect a yield of 66,676.25 units of pectin lyase and 21,715.46 units MnP per plant. These observations are in agreement with preliminary reports by Verma et al. [3] and Jin et al. [22] on hydrolytic enzymes. Likewise, genes with low expression in bacterial systems can be efficiently expressed with new concomitant sequences in plants and these systems might be used to produce hydrolytic enzyme involved in cell-wall degradation to treatment of residues as municipal wastes, agricultural or paper production. This study establishes the basis to develop novel methods and strategies to over-express enzymes implicated in pectin and lignin degradation, a potentially useful tool for biotechnological applications using transplastomic plants.

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## Conflict of interests

The authors declare that there are no conflict of interest with Consejo Nacional de Cinecia y Tecnologia.

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