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

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ABSTRACT

*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

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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