Farran, I., Fernandez-San Millan, A., Ancin, M. et al. Mol Breeding (2014) 34: 457.
https://doi.org/10.1007/s11032-014-0047-x

Increased bioethanol production from commercial tobacco cultivars overexpressing thioredoxin f grown under field conditions

Inmaculada Farran*, Alicia Fernandez-San Millan, Maria Ancin, Luis Larraya and Jon Veramendi

Instituto de Agrobiotecnología, Dpto. Producción Agraria, Universidad Pública de Navarra-CSIC-Gobierno de Navarra, Campus Arrosadia, 31006-Pamplona (Spain)

***Corresponding author**: Inmaculada Farran. E-mail: farran@unavarra.es; Phone: (+34) 948 168 034; Fax: (+34) 948 232 191.

Abstract

Bioethanol is mainly produced from food crops such as sugar cane and maize while it has been held partly responsible for the rise of food commodity prices. Tobacco, integrated in biorefinery facilities for the extraction of different compounds, could turn into an alternative feedstock for biofuel production. When grown for energy production, using high plant densities and several mowings during the growing season, tobacco can produce large amounts of inexpensive green biomass. We have bred two commercial tobacco cultivars (Virginia Gold and Havana 503B) to increment the carbohydrate content by the overexpression of thioredoxin f in the chloroplast. Marker-free transplastomic plants were rescued and their agronomic performance under field conditions was evaluated. These plants were phenotypically equivalent to their wild types yet showed increased starch (up to 280%) and soluble sugar (up to 74%) contents in leaves relative to their control plants. Fermentable sugars released from the stalk were also higher (up to 24%) for transplastomic plants. After a heat pretreatment, enzymatic hydrolysis and yeast fermentation of leaf and stalk hydrolysates, an average of 20-40% more ethanol was obtained from transplastomic plants in relation to their control wild types. We propose an integral exploitation of the entire tobacco plant managed as a forage crop (harvesting sugar and starch-rich leaves and lignocellulosic stalks) that could considerably cheapen the entire production process.

Keywords: tobacco; chloroplast transformation; thioredoxin; bioethanol; field trial; carbohydrate

Introduction

Transportation fuels depend basically on petroleum, a non-renewable fossil hydrocarbon. Oil consumption has grown continuously, being the USA the foremost end user followed by China with a rising economic growth rate. It has been predicted that petroleum resources will be drastically constrained in the mid-term, with a consequent steady price increase. In addition to a high petroleum cost, the release of polluting gases and their implication in the global climate change have fostered the use of renewable resources for the production of transportation fuels, such as for instance ethanol. These alternative fuels could substantially reduce the worldwide dependence on petroleum.

Ethanol can be blended with gasoline in any ratio, thus obtaining fuels with different properties. Extensive international experience has already demonstrated that blends containing up to 10% ethanol do not require further engine modifications (Rutz and Janssen 2007). In Brazil, all brands of gasoline engines may run on 20-25% ethanol. Currently, flexible fuelled vehicles are manufactured with engines which can run on any type of blend ranging up to 85% of ethanol, while even completely dedicated ethanol vehicles are commercialised with an efficient use of absolute ethanol (Rutz and Janssen 2007). Consequently, all in all, it is not surprising that the fuel ethanol industry has been growing extensively worldwide, with a global production of 82,600 million litres recorded in 2012, being the USA and Brazil the main producers, representing more than 85% of the world production (http://ethanolrfa.org/pages/World-Fuel-Ethanol-Production).

First-generation bioethanol production is mainly based on food crops. Sugar (sugar cane and sugar beet) and starch (maize and wheat) crops are the most widely used for ethanol production. To state the point, grain ethanol refineries consumed almost up to 40% of the total USA maize production pertaining to the 2010/11 crop season (Du and McPhail 2012). Due to competition with the food supply, the biofuel boom has been held partly responsible for food commodity price increases (OECD 2008; Food and Agricultural Organization 2011; Kretschmer et al. 2012). Hence, a future increase of ethanol production will need to rely on exploiting other non-food/feed associated biomass products such as lignocellulosic feedstock (Hahn-Hägerdal et al. 2006; Sarkar et al. 2012), food wastes (Uncu and Cekmecelioglu 2011) and alternative plant species which are well adapted to particular agroecosystems and comprise a developed

infrastructure for crop management and harvest processing. Tobacco (*Nicotiana tabacum* L.) could become one of these dedicated energy crops given the highly plastic nature of the species, grown in over 125 countries and across 4 million hectares of land, a third of which are located in China alone. The tobacco plant is a high biomass producer, yielding up to 160 tonnes per hectare through multiple harvests (Wildman 1979). Production of tobacco in developed countries has lately declined due to the weak demand of both the internal and the export markets. The cultivated area in the last ten years has been reduced by 31% in the USA and 45% in Europe (http://faostat3.fao.org/home/index.html). This tendency has sharply increased in the European Union due to the reduced incentives derived from the Common Agricultural Policy (Food and Agricultural Organization 2003). Part of this arable land area could be dedicated to tobacco biomass production and ethanol conversion. In fact, tobacco was commercially used as a feedstock for ethanol production in the 1980s in the USA (Floyd Agricultural Energy Corporation, Virginia) and has currently been proposed as the ideal crop for biobased products including ethanol (NewAgriculture Inc., http://www.newagriculture.com/home.html). For a cost-effective ethanol production, tobacco could be integrated into a biorefinary facility for the extraction of other compounds (e.g. proteins and xhantophylls).

Genetic engineering provides a tool to improve plant characteristics in order to derive raw biomass materials appropriate for use as biofuel feedstocks. Improvement includes increasing the overall biomass, rising polysaccharide content and inducing cell wall modifications so as to diminish the need for pretreatments (expressing deconstructing enzymes in plants or down-regulating lignin biosynthesis) (Vanholme et al. 2008; Verma et al. 2010). Recently, it has been shown that the overexpression of plastidial thioredoxin f (Trx f) from the chloroplast genome leads to an enhanced starch accumulation in leaves of the cultivar Petite Havana, a rather low biomass producer (Sanz-Barrio et al. 2013). Thioredoxins are oxidoreductases that mediate the thiol-disulfide exchange of Cys residues. Many starch metabolizing enzymes have been reported to be redox regulated (Kötting et al. 2010; Glaring et al. 2012). Therefore, Trx f could act as a reductant of the redox-regulated enzymes involved in the carbohydrate metabolism and, as a consequence, allow to increase the rate of carbohydrate biosynthesis (Sanz-Barrio et al. 2013). In the present work, we have overexpressed Trx f in the plastids of two commercial tobacco cultivars of high biomass production. Transplastomic plants

grown under field conditions showed in leaves increased starch and soluble sugar contents. The integral exploitation of tobacco biomass (leaves and stalks) was studied. Pretreatment experiments, enzymatic hydrolysis and yeast fermentation evidenced a better performance in these transplastomic plants as feedstock for ethanol production compared to their wild type relatives.

Materials and methods

Development of chloroplast transgenic plants overexpressing the *trx f* gene

Two commercial cultivars of *Nicotiana tabacum* (Virginia Gold and Havana 503B) were used for the plastid transformations. *In vitro* grown tobacco leaves were transformed with the pL3-PrrnG10LTrxf chloroplast transformation vector (Sanz-Barrio et al. 2013), where the tobacco *Trx f* gene (Sanz-Barrio et al. 2012) was expressed under the control of the *rrn* promoter fused to the leader sequence of the bacteriophage T7 gene *10*. The *aadA* gene, conferring resistance to spectinomycin and streptomycin, was used as a selectable marker gene. The Bio-Rad PDS-1000/He biolistic device was utilised for the integration of the transgenes into the plastid genome as previously described (Daniell 1997). Two rounds of selection and shoot development on RMOP medium containing 500 mg/L of spectinomycin were performed. Regenerated transformants were transferred to soil.

Southern and western blot analysis

Total plant DNA (10 µg) was digested with *Bgl*II, separated in a 0.8% (w/v) agarose gel and transferred to a nylon membrane. A 0.8-kb probe (P1), homologous to the flanking sequences, was used for hybridization (Farran et al. 2008). Probe labelling and hybridization were performed using the chemiluminescent Dig High Prime DNA labelling and detection kit (Roche, Indianapolis, USA). After Southern blot analysis confirmation of the T_0 generation, selected plants were grown in the greenhouse for seed production.

For western blot analysis, proteins from transformed and untransformed leaf samples were extracted and blotted using a specific Trx f antibody as previously described (Sanz-Barrio et al. 2012).

Removal of the antibiotic resistance gene by the Cre/*lox* site-specific recombination system

The transit peptide of the tobacco Rubisco small subunit was translationally fused to the Cre recombinase from the P1 bacteriophage. The chimeric gene was introduced into a pBin20 binary vector under the control of the CaMV 35S promoter and the nopaline synthase terminator. The *Agrobacterium tumefaciens* GV3101 strain was used for nuclear transformation of the transplastomic plants. Briefly, nuclear gene transformants were selected by kanamicin resistance on regeneration medium containing 50 mg/L kanamycin and 250 mg/L cefotaxime. Kanamycin-resistant shoots were subcultured to a rooting medium containing 50 mg/L kanamycin. Removal of the *aadA* gene in the regenerated plants was confirmed by Southern blot analysis (using P1 and aadA probes) and by the *in vitro* seed germination of the T_2 generation in a medium containing 500 mg/L spectinomycin. Additionally, loss of the nuclear *nptII* gene in seed progeny (T_3 generation) was confirmed by kanamycin sensitivity.

Field trial

Transplastomic and wild-type Virginia Gold and Havana 503B tobacco plants were grown under standard field conditions in the Experimental Station of the INTIA (Sartaguda, Spain). The notification for the release of genetically modified higher plants (B/ES/12/16) was authorised by the Ministerio de Agricultura, Alimentación y Medio Ambiente. A randomized complete block design with three replications was used. For each transplastomic and wild-type lines, 15 plants were cultured per block (plantation density of 18,500 plants/ha; 90 cm between rows and 60 cm between plants). Field transplantation was undertaken on the 23rd of May of 2012. Topping (removal of the flower head and the first few top leaves to prevent the production of seeds) was performed 55 days after transplanting. Plant height and relative leaf chlorophyll content (measured with SPAD 502 chlorophyll meter, Minolta Optics Inc, Tokyo, Japan) were registered 60 days after transplanting. Total weight per plant and specific leaf weight (SLW) were measured at the date of harvest (71 days after transplanting). For the calculation of the SLW five disks per leaf were cut with a cork borer. Disks from all plant leaves were collected and dried at 80 ºC for two days. Total dry weight refers to the total disk area and is expressed as mg/cm^2.

Total leaves and stalks derived from two plants per block (yielding a total of six individual wild-type or transplastomic plants per cultivar) were harvested, pooled, and stored at 4 ºC until sample conditioning (less than a week).

Conditioning of leaf and stalk samples

The pooled leaf and stalk samples were dried separately in an air-dry oven at 45 ºC to further stop any biological reactions and possible contaminations. The dried plant material was milled to a size of 1 mm particles using an ultra-centrifugal mill (Retsch ZM200, Haan, Germany) and stored in hermetic plastic bags at 45 ºC until further experimental usage. Representative samples obtained from the pools were processed and analysed.

Compositional characterization

Leaf and stalk samples were dried at 105±2 ºC up to a constant weight and compositional values refer to dry weight. Ash content was determined after air combustion during 1 h at 550±10 ºC in a muffle. Starch content was quantified by an enzymatic method including α-amylase, protease and amyloglucosidase (Total Dietary Fibre AOAC 985.29, Megazyme, Bray, Ireland) and subsequently sugars were quantified by HPLC as described below.

For the quantification of soluble sugars, 0.5 g of dried samples were mixed with 100 mL water and incubated during 1 h at 70 ºC. Samples were centrifuged at 6000 g during 10 min and the supernatant stored at -20 ºC until analysis. Monomeric sugars (soluble monosaccharides), cellobiose and total sugars (monosaccharides and oligosaccharides) were determined according to the procedure published by the National Renewable Energy Laboratory (NREL, Golden, Colorado, USA) (www.nrel.gov/biomass/pdfs/42632.pdf). Oligomeric sugars were subjected to acid hydrolysis by adding 72% H_2SO_4 (w/w), adding enough to bring the acid concentration of an aliquot of 10 mL up to 4%. Samples were stored at -20 ºC until analysis.

Sugar analysis was carried out by HPLC. The content of monosaccharides (d-glucose, d-xylose-fructose-galactose, l-arabinose), disaccharides (cellobiose, maltose) and trisaccharides (maltotriose) were quantified by an HPLC system (Agilent Technologies, model 1200) equipped with a refractive index detector. The separation was performed with an ICSep

ION 300 column (7.8 x 300 mm, Transgenomics, Glasgow, United Kingdom) at 72 ºC, with the mobile phase consisting of 8.5 mM H_2SO_4 diluted in water.

The contents of cellulose, hemicellulose (xylans and arabinans) and lignin were analysed using a two-step acid hydrolysis method according to the procedure published by the NREL (www.nrel.gov/biomass/pdfs/42618.pdf). Dried samples (0.3 g) were treated with 3 mL of 72% H_2SO_4 (w/w) and placed in a water bath at 30 ºC for 1 h. Samples were diluted with 84 mL of water to give a H_2SO_4 concentration of 4%. Samples were autoclaved at 121 ºC during 1 h. After cooling, the samples were vacuum filtered through a 0.22 µm mesh and an aliquot was collected for the analysis of sugars and the acid soluble lignin (ASL) was measured at 320 nm in a spectrophotometer. Insoluble acid solids were washed with water, dried at 105 ºC, weighted and finally muffled at 575 ºC ± 25 ºC for acid insoluble lignin content determination.

Protein content was determined by the Dumas method, which consists of sample high temperature combustion (about 900 °C) in the presence of oxygen, leading to the release of carbon dioxide, water and nitrogen. Nitrogen is separated from the rest of the components using a conductivity column. A conversion factor of 6.25 was used for protein content determination.

Pretreatment and enzymatic hydrolysis of leaf samples

Dried leaf samples were mixed with 0.1 M citrate buffer, pH 4.8, in a 10% (w/w) proportion of solid:liquid, and pretreated at 110 ºC for 1 h. The final weight of the sample was approximately 28 g.

Four different enzyme cocktails (Novozymes A/S, Bagsvaerd, Denmark) were used for the enzymatic hydrolysis. Concentration of enzymes is expressed as % (w/w dry sample): mixture 1 (α-amylase, 0.001; β-glucoamylase, 0.002; cellulases Cellic® Ctec 3, 10.0), mixture 2 (α-amylase, 0.001; β-glucoamylase, 0.002; cellulases Cellic® Ctec 3, 10.0; carbohydrolase + protease, 1), mixture 3 (α-amylase, 0.001; β-glucoamylase, 0.002; cellulases Cellic® Ctec 2, 10.0), and mixture 4 (cellulases Cellic® Ctec 3, 10.0). Samples were incubated in an orbital shaker (180 rpm) during 72 h at 50 ºC. With regard to mixtures 1, 2 and 3, samples were kept at 80 ºC during the first hour. Cellulases were always added after the incubation at 80 ºC. Soluble sugars were quantified by HPLC as described above.

The efficiency of the enzymatic hydrolysis was calculated as follows: (glucose released after hydrolysis * hydrolysis volume / sample initial dry weight) * 100 / [% celullose * 1.11 (conversion factor of cellulose to glucose) + % starch * 1.11 (conversion factor of starch to glucose) + % fermentable soluble sugars] contained in the initial biomass.

Pretreatment and enzymatic hydrolysis of stalk samples

Stalk samples were mixed with water in pressurized stirred-tank reactors (Autoclave Engineers, model M010SS) with a solid:liquid proportion of 10% (w/w dry sample) reaching a final weight of 50 g, and subjected to three different temperature regimes (170, 190, 210 ºC) during 30 min. In addition to temperature, acid (1% w/w of H_2SO_4 with respect to dry sample) and pressure (20 bars, supplied from 200 bars pressurized pure nitrogen bottle) treatments were also applied. Nine different combinations were assayed: temperature alone, temperature plus acid and temperature along with acid plus pressure, for each of the 3 temperature regimes.

After pretreatment, samples were weighted to calculate the recoveries of the solid (insoluble) and liquid fractions. Previous to enzymatic hydrolysis, solid fractions had been dried at 45 ºC up to a constant weight. Soluble sugars and sugar degradation products (acetic acid and furfurals) of the liquid fractions were also determined. Acetic acid was quantified by HPLC as described above for the soluble sugars. Furfural and 5-hydroxymethylfurfural (HMF) were quantified by HPLC by way of a DAS index detection using a Zorbax column (250 x 4.6 mm, Agilent Technologies) at 50 ºC, and a mobile phase consisting of water:acetonitrile in a proportion of 80:20.

Enzymatic hydrolysis was performed as described above for leaf samples however only using enzyme mixture 4 (cellulases Cellic® Ctec 3, 10% (w/w dry sample)). Soluble sugars were quantified by HPLC as has been described above. The efficiency of the enzymatic hydrolysis was calculated as follows: (glucose released after hydrolysis * hydrolysis volume / sample initial dry weight) * 100 * pretreatment recovery factor / [% celullose * 1.11 (conversion factor of cellulose to glucose) of the initial biomass].

Fermentation

After enzymatic hydrolysis, plant samples were subjected to fermentation with *Saccharomyces cerevisiae* (strain Ethanol Red, Fermentis, Marcq-en-Baroeul, France) with an inoculum concentration of 1 x 10^7 cfu/mL of hydrolysed material, in a final volume of approximately 28 mL and 4 mL, for leaves and stalks, respectively. The process was conducted at 37 ºC with a constant orbital agitation (180 rpm) during 24 h. Ethanol production was determined by a Gas Chromatography-Flame Ionization Detector using a DB Wax 15m column (Agilent Technologies, model 7890A) and 1-propanol as the internal standard.

Results

Generation and characterization of the transplastomic tobacco plants overexpressing Trx f

The pL3-PrrnG10LTrxf vector (Fig.1a) was used for the plastid transformation (Sanz-Barrio et al. 2013). This vector includes the *Trx f* coding sequence under the control of the *rrn* promoter fused to the leader sequence of the phage T7 gene *10* (*G10L*), containing also the selectable spectinomycin resistance gene (*aadA*) flanked by two *lox* sequences suitable for Cre-mediated gene marker excision (Corneille et al. 2001). The plastid transformation vector targets the insertion of transgenes in the duplicated inverted repeat region, between the *16S3´/trnV* and *3´rps12* genes.

Plastid transformation efficiency was assessed in six high-biomass tobacco cultivars with *gfp* as the reporter gene. Taken into account the number of transformed buds per bombarded leaf, shoot development, root formation and leaf fluorescence (data not shown), Virginia Gold and Havana 503B cultivars were selected (thereafter Virginia and Havana, respectively). Leaves from *in vitro*-grown plantlets of these two cultivars were transformed by biolistics. Single regenerated shoots selected after two rounds of spectinomycin application were rooted and transplanted. Transformation efficiency was similar to that of the well-established Petite Havana cultivar: 5.2 and 3.9 transformed shoots were obtained per bombarded leaf in Havana and Virginia cultivars, respectively. In the case of Petite Havana, usually 4-5 shoots are observed per bombarded leaf (Molina et al. 2004).

Southern blot analysis was performed to verify the site-specific integration and to confirm homoplasmy. Total plant DNA was digested with *Bgl*II. The flanking region probe (P1,

Fig.1a) identified a 4.5-kb fragment in wild-type plants whereas a 6.7-kb fragment in the plastid transgenic plants (Fig.1b). The absence of 4.5-kb bands in the transformed lines indicated homoplasmy.

Overexpression of Trx f was confirmed by western blot with a specific Trx f antibody. Trx f was detected in both transplastomic cultivars (Virginia and Havana) (Fig.1c). No signal was detected in the wild-type plants. A lack of signal was also observed in wild-type Petite Havana plants when 10 µg of total leaf protein were loaded per lane (Sanz-Barrio et al. 2013). The spot intensity of Virginia and Havana transplastomic plants was equivalent to that of the transplastomic Petite Havana cultivar (Fig.1c), indicating a high Trx f expression level.

Development of marker-free transplastomic plants

Since the Cre-lox site-specific recombination system has already been efficiently used to obtain marker-free transplastomic plants (Corneille et al. 2001), transplastomic T_1 plants were nuclearly transformed with the *cre* gene from the P1 phage via *Agrobacterium tumefaciens*. The transit peptide of the tobacco Rubisco small subunit (SSU) fused to the N-terminus of Cre recombinase (SSU-Cre) targeted Cre to the plastid (Fig.2a), allowing the excision of the *lox*-flanked *aadA* gene from the plastid genome. Specific excision of the *aadA* gene was confirmed by Southern blot. The flanking sequence P1 probe identified the expected 5.4-kb fragment in the nuclear *cre* transformed transplastomic plants (VIR-TCre and HAV-TCre) (Fig.2b), which was lower than the 6.7-kb fragment identified in their related transplastomic plants (VIR-T and HAV-T). Confirmation of the *aadA* gene removal was obtained via the *aadA* probe. VIR-T and HAV-T showed the specific band, whereas VIR-TCre and HAV-TCre lacked the signal (Fig.2b). The nuclear *nptII* gene was subsequently removed by segregation in the T_2 seed progeny of the VIR-TCre and HAV-TCre plants. *In vitro* germination in the presence of kanamycin allowed for the identification of the sensitive seedlings. These seedlings were rescued, cultured in kanamycin-free medium and transplanted to obtain seeds. T_3 generation plants were again analysed to confirm the antibiotic sensitivity by *in vitro* germination assays (Fig.2c, d). As expected, VIR-TCre and HAV-TCre were sensitive to both spectinomycin and kanamycin, equivalent to the wild-type plants. An unrelated nuclear transgenic plant (NUC) was used as a control for kanamycin resistance.

Phenotypic and compositional characterization of Trx f-overexpressing plants grown under field conditions: increased leaf starch and soluble sugar levels

The field trial was performed in Sartaguda (Navarre, Spain). Transplastomic plants did not differ from their respective wild types with respect to plant size, total weight and relative chlorophyll content (Table 1). HAV-Trxf plants showed an increase in specific leaf weight (SLW) of more than 20% compared to the wild-type plants. A slight non-statistically significant increase in SLW was also observed in VIR-Trxf (*P*=0.093; Table 1). Increased levels of SLW had been previously observed in the Petite Havana cultivar overexpressing Trx f (Sanz-Barrio et al. 2013).

The leaf starch content of field grown transplastomic plants was monitored throughout the cultivation period with samplings at 55, 62, 71 and 93 days post-transplantation. The highest starch content corresponded to day 71 (data not shown). At that moment, harvested plants accumulated large quantities of starch in the leaves of both transplastomic cultivars, showing a 130% and 280% increase (Virginia and Havana, respectively) relative to their wild types (Table 2). This could likely explain the higher SLW values observed for these plants (Table 1). Plant material was harvested in the morning since levels of starch would be expected to run high during the whole light period, as had previously been demonstrated for the Petite Havana cultivar (Sanz-Barrio et al. 2013), making irrelevant the time of harvest. Additionally, high levels of soluble sugars were measured in the transplastomic plants of both cultivars (13% and 74% in Virginia and Havana, respectively; Table 2). No changes were observed in the hemicellulose content (measured as xylans and arabinans) between transplastomic and wild-type plants. However, a slight decrease of cellulose, lignin, proteins and ash were noted in the transplastomic plants compared to their wild types.

Generally, Trx f overexpression in tobacco plastids did not seem to affect the stalk composition (Table 2). Cellulose was the most abundant fraction (~30% weight/dry weight (w/dw)), followed by proteins (~18%), hemicellulose (~15%, mainly xylans plus a small proportion of arabinans) and lignin (~11%). Starch was not detected and the content of soluble sugars was very low, approximately 3% (w/dw). These values were similar to those that have been previously reported for tobacco stalks (Martin et al. 2008; Akpinar et al. 2010).

Increased ethanol production from leaves of Trx f-overexpressing plants

In previous experiments, we had observed that a relatively mild pretreatment (110-130 ºC), followed by an enzymatic hydrolysis, were sufficient for the total hydrolysis of starch and cellulose of tobacco leaves. Therefore, leaf samples were subjected to 110 ºC during 1 h. To assess the efficiency of different hydrolytic enzyme combinations, four mixtures were assayed. Irrespective of the mixture used, similar levels of total and fermentable sugars were obtained (Supplementary Table S1). In addition, fermentation of the hydrolysed sugars yielded equivalent levels of ethanol. Hence, the simplest mixture 4 (including just cellulases Cellic® Ctec 3) was used for further experiments. The high hydrolytic efficiency of this mixture can be explained by the presence of enhanced cellulase, hemicellulase and β-glucosidase activities (http://www.bioenergy.novozymes.com/en/cellulosic-ethanol/CellicCTec3/product-description/Pages/default.aspx). Leaf hydrolysates of transplastomic plants of both cultivars showed higher levels of total and fermentable sugars with regard to their controls (Table 3). These results agree with those obtained by the initial compositional characterization (Table 2). Enzymatic hydrolysis was complete, with efficiencies higher than 100%. The hydrolysis yield overestimation could be explained by an underestimation of fructose, xylose and galactose (they showed the same retention time in HPLC) and an interference with the malic acid peak. More than 95% of the total sugars were fermentable in all of the lines analysed, implying an optimal composition of tobacco leaves for yeast fermentation and ethanol production. The fermentation efficiency was high, with observed conversion ratios of fermentable sugars to ethanol ranging 82-89% relative to the theoretical maximum production. The highest ethanol production was obtained after 16-20 h of fermentation (data not shown). Ethanol yields were 24% and 44% higher (Virginia and Havana, respectively) in transplastomic plants in comparison to their relative wild types (Table 3).

Preparation of hydrolysates from tobacco stalks for ethanolic fermentation

In preliminary experiments, we had observed that relatively mild hydrothermal pretreatments (up to 130 ºC) were not sufficient for an efficient degradation of stalk cellulose. Therefore, we analysed the effect of harsher pretreatments based on higher temperatures (170-210 ºC) combined with diluted acid or diluted acid plus pressure in relation to the production of sugar-

enriched hydrolysates. To monitor the sternness of the pretreatments, some degradation products were quantified (Supplementary Table S2). The maximum levels recorded were 5.19, 0.96 and 0.24 g/L of acetic acid, furfural and HMF, respectively. It has been demonstrated, working with different feedstocks, that these concentrations do not decrease ethanol yields (Larsson et al. 1999; Palmqvist et al. 1999; Pienkos and Zhang 2009; Erdei et al. 2010).

Comparing the different pretreatments, no clear effects were detected with regard to total sugars (Supplementary Fig.S3a). In contrast, a positive effect of temperature on the production of fermentable sugars after enzymatic hydrolysis of the solid fraction was observed (Supplementary Fig.S3b), which was not enhanced by the combination of temperature with diluted acid or pressure. Increased heat treatments of 170 to 190 ºC showed an increment of ethanol production after fermentation, which did not further increase at 210 ºC (Supplementary Fig.S3c). However, when data referred to the liquid and solid fractions (considering ratios of recovery), no remarkable differences between the pretreatments were observed, especially as to final ethanol production (Table 4), thus showing that the addition of acid and the use of a higher pressure were unnecessary. The lack of differences with regard to ethanol production between the pretreatments could be explained by a reduction of the solid fraction's recovery with increasing temperatures. Differences between the total and fermentable sugar fractions within each sample were mainly due to hemicellulose hydrolysis, rendering xylans and arabinans that cannot be fermented by *Saccharomyces cerevisiae*. The enzymatic hydrolysis efficiency of the cellulase mixture (cellulases Cellic® Ctec 3) was far from complete (55-63%, Table 4). Hence, this is an important aspect for further improvement. Taking these results altogether, the pretreatment at 190 ºC was selected for further experiments as the enzymatic hydrolysis ratio was one of the highest. Besides, taking into consideration a scaled-up industrial process, in which there is no precise way to control temperature, 190 ºC would seem to guarantee ethanol yields similar to those obtained in the present experiment.

Tobacco stalks are a relevant biomass source for ethanol production

Stalk samples from transplastomic and wild-type plants of both cultivars were subjected to a 190 ºC pretreatment. Soluble sugars in the liquid fraction obtained after the pretreatment were determined (Table 5). The solid fraction was subjected to enzymatic hydrolysis with cellulases

Cellic® Ctec 3 and a further fermentation of sugar hydrolysates with *Saccharomyces cerevisiae*. The content of total and fermentable sugars in both, liquid and solid fractions, together with ethanol production were higher in transplastomic than in wild-type plants (Table 5). The ethanol production increase of cultivar Virginia (23%) was statistically significant. Rates of enzymatic hydrolysis were in the same order as those previously obtained for the pretreatment assay (Table 4). Fermentation efficiency was high (91-100% relative to the theoretical maximum production), while the highest ethanol production was obtained after a 24 h period of fermentation (data not shown).

The relative leaf and stalk proportions of the transplastomic plants were calculated. The leaf biomass (based on fresh weight) was 60% and 68% in the Virginia and Havana cultivars, respectively. These values are in accordance with a previous field experiment carried out in Cadreita (Navarre, Spain) in which 17 commercial cultivars were grown and evaluated under standard conditions, with a mean leaf biomass corresponding to 62%. Considering the leaf and stalk biomass, the proportion of the final ethanol yield derived from each plant organ was calculated. For the Virginia cultivar, 47% of ethanol was derived from stalks and 53% from leaves. The figures in the Havana cultivar corresponded to 36% (stalks) and 64% (leaves). These results reflect the importance of stalks for biomass processing when tobacco is cultivated for ethanol production: around 35-50% of the final ethanol yield was derived from stalks of plants grown under standard conditions.

Discussion

Although bioethanol is mainly produced from sugar cane, maize or other grain cereals, alternative starchy and lignocellulosic species are under investigation. Cassava, alfalfa, poplar and miscanthus are just few examples of biomass crops that have been proposed for ethanol production. Crop yields strongly depend on local agro-ecological conditions which hence influence the choice of the best feedstock for ethanol production. For instance, the performance of cassava in some tropical countries out yields that of maize, favouring cassava cultivation in such environments (Sánchez and Cardona 2008). In addition, the genetic modification of herbaceous plants to increase carbohydrate contents could facilitate their use as feedstocks for

biofuel production. Hence, well-adapted plants to local conditions and with an improved carbohydrate content could foster the geographical expansion of bioethanol production.

Here, we propose that tobacco, a high biomass crop, with enriched carbohydrate content could develop into an alternative energy crop, particularly in some developed countries with dedicated infrastructure yet with a continuous reduction of cultivated area. Tobacco has been previously proposed as a production platform for biofuel by increasing the amount of lipids in leaves which would favour use for biodiesel production (Andrianov et al. 2010). The integration of tobacco biomass in biorefinery facilities for the extraction and purification of additional compounds could facilitate a more cost-effective ethanol production. Tobacco produces large protein amounts (18-30%, Table 2). These proteins are nutritionally complete with all of the human essential amino acids and could be used as a supply source for human diets or animal feed, inclusive with a higher protein efficiency rating than that of either milk or soy (Wildman 1979). A protein yield of up to 3 tonnes/ha has been estimated of which approximately half is extractable and consists of food grade protein (Long 1984). Nicotine content should not be a problem since tobacco biomass would be harvested very young, well before significant amounts of nicotine had been formed. Other valuable coproducts could consist of solanesol (used in the synthesis of vitamins E and K) and xanthophylls (additive in poultry food).

We have obtained marker-free transplastomic plants of two commercial cultivars overexpressing Trx f. These plants were phenotypically indistinguishable from their wild type relatives although showed an increased content of leaf starch (up to 280%) and soluble sugars (up to 74%) relative to the control plants (Table 2). These results are in accordance with those previously obtained for cultivar Petite Havana, a low-biomass producer (Sanz-Barrio et al. 2013). Moreover, fermentable sugar levels released from the stalk after enzymatic hydrolysis were higher in the transplastomic compared to the control plants (Table 5). However, soluble sugar differences were not observed for the initial stalk compositional characterization (Table 2), probably due to the mild pretreatment applied which impeded the solubilisation of stalk components. The observed differences after a harsher pretreatment at 190 ºC could be explained by a less organised cell wall matrix structure of the transplastomic plants that would favour the enzymatic hydrolysis and release of sugars. In previous studies, a reduction of the

cell wall recalcitrance was achieved by modifying the cellulose structure (Harris and DeBolt 2010), for instance by expression of a foreign gene such as a bacterial endoglucanase (Brunecky et al. 2011) or by using synthetic biology to decrease lignin content (Yang et al. 2013). Thioredoxins catalyse redox reactions and control a wide range of biological pathways. It has so far been unknown whether Trx f affects the cell wall structure yet there are some evidences connecting the redox status with cellulose synthesis. DTT treatment stimulated carbon flux to cell wall components, especially cellulose (Kolbe et al. 2006), and cellulose synthase has been reported to be redox regulated (Kurek et al. 2002). Therefore, we hypothesise that Trx f overexpression might mediate in the modification of the cell wall structure, with an ease of hydrolytic enzyme access. Finally, after fermentation of the leaf and stalk hydrolysates, an average of 20-40% more ethanol was obtained from the transplastomic plants compared to the control wild types (Tables 3 and 5).

The efficiency of enzymatic hydrolysis of the pretreated leaves was absolute. However, the efficiency of the pretreated stalks with the cellulase mixture used (including cellulase, hemicellulase and β-glucosidase activities) was about 60%. Due to the fact that the carbohydrate composition of tobacco stalks (including mannans, galactans and arabinans) is similar to that of hardwoods, an increase in pretreatment sternness has been proposed to improve the enzymatic hydrolysis (Martin et al. 2002). In our experiments, temperatures of up to 210 ºC combined with 1% H_2SO_4 or 20 bars of pressure were applied. Acid or pressure actions did not improve the pretreatment efficiency (Table 4). Steam pretreatment at 205 ºC (Martin et al. 2002) and wet oxidation at 195 ºC (Martin et al. 2008) have been previously used for tobacco stalks with similar results to those obtained in our experiments. A broad range of other chemical pretreatments such as alkali, organosolv and ionic liquids have been used with different lignocellulosic materials to increase cellulose digestibility and an efficient lignin solubilization (Alvira et al. 2010; Zhu and Pan 2010), which could also be appropriate for tobacco biomass. Additional physicochemical pretreatments include liquid hot water (up to 240 ºC) and ammonia fiber explosion (AFEX) (Alvira et al. 2010). Particularly, AFEX could be well suited for agricultural feedstocks as it reduces lignin content, removes some hemicellulose components and decrystallizes cellulose. It consists also of a very promising method with regard to operating costs (Hu et al. 2008).

Enzymatic saccharification improvement could also be achieved by increasing the enzyme loading (Sánchez and Cardona 2008) or by the addition of specific xilanases and β-glucosidases to the enzyme cocktail. For example, a cellulose conversion of over 90% was achieved in wheat (Saha and Cotta 2006) or barley (Saha and Cotta 2010) straw using preparations which included the following three enzymes: cellulase, xilanase and β-glucosidase. A cost-effective way to perform the enzymatic hydrolysis would be the expression of cell-wall degrading enzymes in the same plant species which is used for biomass production. The chloroplast is an ideal subcellular compartment for this purpose with very high levels of foreign protein accumulation. Exo- and endoglucanases, β-glucosidases (Gray et al. 2009; Ziegelhoffer et al. 2009; Verma et al. 2010; Gray et al. 2011; Petersen and Bock 2011), endo-β-mannanase (Agrawal et al. 2011), hemicellulases (Kolotilin et al. 2013) and additional enzymes (pectate lyase, cutinase, swollenin, xylanase, acetyl xylan esterase, and lipase) (Verma et al. 2010) have been successfully expressed in transplastomic tobacco plants in their active forms. In some cases, plant crude-extract enzyme cocktails showed a higher activity level and yielded more glucose in contrast to commercial cocktails (Verma et al. 2010), demonstrating the great potential associated to plant-derived enzyme production.

In order to efficiently use tobacco as a feedstock for biofuel production, maximum biomass yields of green tissue should be generated. Growing tobacco for biomass or either cigarette production comprises very different crop management practices. In the function of a biomass crop, tobacco should be managed more as a forage crop with a very high plant density, developing a kind of grass lawn. Plants would be harvested by mowing when they reached a height of 50 cm, leaving back the stumps to resprout thus forming a dense tangle of new shoots and leaves within days (van Beilen et al. 2007). Multiple harvests could be possible under optimal environmental conditions. These young shoots comprise the sugar-richest and highest protein stage of the plant's growth cycle (van Beilen et al. 2007) which would make the process of ethanol production easier. It has been estimated that a cropping season of 6-8 months could generate up to 160 tonnes/ha of green tissue based on wet weight, which is equivalent to 16-32 tonnes of dry biomass (Wildman 1979). Tobacco farming as a biomass crop is expected to be cheap (sowing instead of planting, reduced weed management and mechanised harvesting by mowing) (van Beilen et al. 2007). Integral exploitation of the whole

plant (sugar and starch-rich leaves and lignocellulosic stalks) would be performed in a single production process. Analogous to the tobacco leaf and stalk scenario, this idea of mixed feedstocks has already been investigated to facilitate the introduction of second generation technology for bioethanol production by mixing wheat straw and meal (Erdei et al. 2010).

In conclusion, the overexpression of Trx f in tobacco chloroplasts led to high-starch and sugar phenotypes in field trials. These improved tobacco commercial cultivars, grown in very high plant densities for a maximum biomass yield, could be an alternative production platform for biofuel, with up to a 40% increase of ethanol production in relation to control plants. To our knowledge, this is the first field trial of genetically modified plants via chloroplast transformation for biofuel production. Furthermore, this technology also brings about the possibility to develop alternative crops potentially useful for improved biofuel generation.

Acknowledgements

The authors are grateful to Irantzu Alegría and co-workers of the Biomass Energy Department (Renewable Energy National Centre, Spain) for technical assistance with regard to biomass processing and for the critical reading of the manuscript; Lorea Villanueva for the collaboration undertaken with plant transformations; Jaime Zabaleta (INTIA, Spain), Esther Molinero and Amaia Altuna for field experiment collaborations. This work was supported by Grant AGL2010-15107 from the Ministerio de Ciencia e Innovación (Spain).

References

Agrawal P, Verma D, Daniell H (2011) Expression of Trichoderma reesei β-Mannanase in Tobacco Chloroplasts and Its Utilization in Lignocellulosic Woody Biomass Hydrolysis. PLoS ONE 6:e29302.

Akpinar O, Erdogan K, Bakir U, Yilmaz L (2010) Comparison of acid and enzymatic hydrolysis of tobacco stalk xylan for preparation of xylooligosaccharides. LWT - Food Science and Technology 43:119–125.

Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresour Technol 101:4851–4861.

Andrianov V, Borisjuk N, Pogrebnyak N, et al. (2010) Tobacco as a production platform for biofuel: overexpression of Arabidopsis DGAT and LEC2 genes increases accumulation and shifts the composition of lipids in green biomass. Plant Biotechnol Journal 8:277–287.

Brunecky R, Selig MJ, Vinzant TB, et al. (2011) In planta expression of A. cellulolyticus Cel5A endocellulase reduces cell wall recalcitrance in tobacco and maize. Biotechnol Biofuels 4:1.

Corneille S, Lutz K, Svab Z, Maliga P (2001) Efficient elimination of selectable marker genes from the plastid genome by the CRE-lox site-specific recombination system. Plant Journal 27:171–178.

Daniell H (1997) Transformation and foreign gene expression in plants by microprojectile bombardment. Methods Mol Biol 62:463–489.

Du X, McPhail LL (2012) Inside the black box: The price linkage and transmission between energy and agricultural markets. Energy Journal 33:171–194.

Erdei B, Barta Z, Sipos B, et al. (2010) Ethanol production from mixtures of wheat straw and wheat meal. Biotechnol Biofuels 3:16.

Farran I, Río-Manterola F, Iñiguez M, et al. (2008) High-density seedling expression system for the production of bioactive human cardiotrophin-1, a potential therapeutic cytokine, in transgenic tobacco chloroplasts. Plant Biotechnol Journal 6:516–527.

Food and Agricultural Organization (2011) Price Volatility in Food and Agricultural Markets: Policy Responses.

Food and Agricultural Organization (2003) Projections of tobacco production, consumption and trade to the year 2010. In: ftp://ftp.fao.org/docrep/fao/006/y4956e/y4956e00.pdf. Accessed 14 Mar 2013

Glaring MA, Skryhan K, Kötting O, et al. (2012) Comprehensive survey of redox sensitive starch metabolising enzymes in Arabidopsis thaliana. Plant Physiol Biochem 58:89–97.

Gray BN, Ahner BA, Hanson MR (2009) High-level bacterial cellulase accumulation in chloroplast-transformed tobacco mediated by downstream box fusions. Biotechnol Bioeng 102:1045–1054.

Gray BN, Yang H, Ahner BA, Hanson MR (2011) An efficient downstream box fusion allows high-level accumulation of active bacterial beta-glucosidase in tobacco chloroplasts. Plant Mol Biol 76:345–355.

Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, et al. (2006) Bio-ethanol--the fuel of tomorrow from the residues of today. Trends Biotechnol 24:549–556.

Harris D, DeBolt S (2010) Synthesis, regulation and utilization of lignocellulosic biomass. Plant Biotechnology Journal 8:244–262.

Hu G, Heitmann JA, Rojas OJ (2008) Feedstock pretreatment strategies for producing ethanol from wood, bark, and forest residues. BioResources 3:270–294.

Kolbe A, Oliver SN, Fernie AR, et al. (2006) Combined transcript and metabolite profiling of Arabidopsis leaves reveals fundamental effects of the thiol-disulfide status on plant metabolism. Plant Physiology 141:412–422.

Kolotilin I, Kaldis A, Pereira EO, et al. (2013) Optimization of transplastomic production of hemicellulases in tobacco: effects of expression cassette configuration and tobacco cultivar used as production platform on recombinant protein yields. Biotechnol Biofuels 6:65.

Kötting O, Kossmann J, Zeeman SC, Lloyd JR (2010) Regulation of starch metabolism: The age of enlightenment? Curr Opin Plant Biol 13:321–329.

Kretschmer B, Bowyer C, Buckwell A (2012) EU Biofuel Use and Agricultural Commodity Prices: A Review of the Evidence Base. Institute for European Environmental Policy (IEEP), London

Kurek I, Kawagoe Y, Jacob-Wilk D, et al. (2002) Dimerization of cotton fiber cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains. Proc Natl Acad Sci USA 99:11109–11114.

Larsson S, Palmqvist E, Hahn-Hägerdal B, et al. (1999) The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. Enzyme and Microbial Technology 24:151–159.

Long RC (1984) Edible tobacco protein. Crops and Soil Magazine 36:13–15.

Martin C, Fernandez T, Garcia A, et al. (2008) Wet oxidation pretreatment of tobacco stalks and orange waste for bioethanol production. preliminary results. Cellulose Chemistry and Technology 42:429–434.

Martin C, Fernandez T, Garcia R, et al. (2002) Preparation of hydrolysates from tobacco stalks and ethanolic fermentation by Saccharomyces cerevisiae. World J Microb Biot 18:857–862.

Molina A, Hervás-Stubbs S, Daniell H, et al. (2004) High-yield expression of a viral peptide animal vaccine in transgenic tobacco chloroplasts. Plant Biotechnology Journal 2:141–153.

OECD (2008) Biofuel Support Policies: An Economic Assessment. 14:409–412.

Palmqvist E, Grage H, Meinander NQ, Hahn-Hägerdal B (1999) Main and interaction effects of acetic acid, furfural, and p-hydroxybenzoic acid on growth and ethanol productivity of yeasts. Biotechnol Bioeng 63:46–55.

Petersen K, Bock R (2011) High-level expression of a suite of thermostable cell wall-degrading enzymes from the chloroplast genome. Plant Mol Biol 76:311–321.

Pienkos PT, Zhang M (2009) Role of pretreatment and conditioning processes on toxicity of lignocellulosic biomass hydrolysates. Cellulose 16:743–762.

Rutz D, Janssen R (2007) Biofuel technolgy handbook. WIP Renewable Energies

Saha BC, Cotta MA (2006) Ethanol production from alkaline peroxide pretreated enzymatically saccharified wheat straw. Biotechnol Prog 22:449–453.

Saha BC, Cotta MA (2010) Comparison of pretreatment strategies for enzymatic saccharification and fermentation of barley straw to ethanol. N Biotechnol 27:10–16.

Sanz-Barrio R, Corral-Martínez P, Ancin M, et al. (2013) Overexpression of plastidial thioredoxin f leads to enhanced starch accumulation in tobacco leaves. Plant Biotechnol Journal 11:618–627.

Sanz-Barrio R, Fernández-San Millán A, Carballeda J, et al. (2012) Chaperone-like properties of tobacco plastid thioredoxins f and m. Journal of Experimental Botany 63:365–379.

Sarkar N, Ghosh SK, Bannerjee S, Aikat K (2012) Bioethanol production from agricultural wastes: An overview. Renewable Energy 37:19–27.

Sánchez OJ, Cardona CA (2008) Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol 99:5270–5295.

Uncu ON, Cekmecelioglu D (2011) Cost-effective approach to ethanol production and optimization by response surface methodology. Waste Manag 31:636–643.

van Beilen J, Möller R, Toonen M, et al. (2007) Industrial crop platforms for the production of chemicals and biopolymers. CPL Press, Berks (United Kingdom)

Vanholme R, Morreel K, Ralph J, Boerjan W (2008) Lignin engineering. Curr Opin Plant Biol 11:278–285.

Verma D, Kanagaraj A, Jin S, et al. (2010) Chloroplast-derived enzyme cocktails hydrolyse lignocellulosic biomass and release fermentable sugars. Plant Biotechnol Journal 8:332–350.

Wildman SG (1979) Tobacco, a potential food crop. Crops and Soil Magazine 31: 7-9.

Yang F, Mitra P, Zhang L, et al. (2013) Engineering secondary cell wall deposition in plants. Plant Biotechnology Journal 11:325–335.

Zhu JY, Pan XJ (2010) Woody biomass pretreatment for cellulosic ethanol production: Technology and energy consumption evaluation. Bioresour Technol 101:4992–5002.

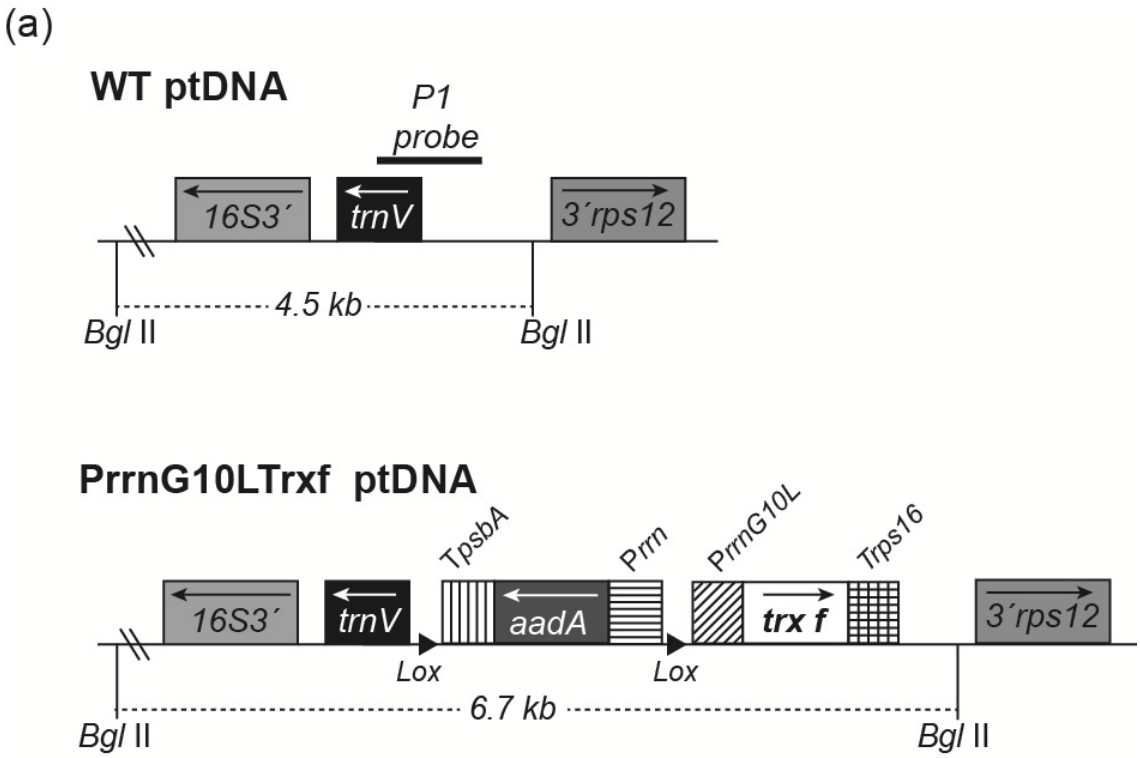
Ziegelhoffer T, Raasch JA, Austin-Phillips S (2009) Expression of Acidothermus cellulolyticus E1 endo-beta-1,4-glucanase catalytic domain in transplastomic tobacco. Plant Biotechnolo Journal 7:527–536.

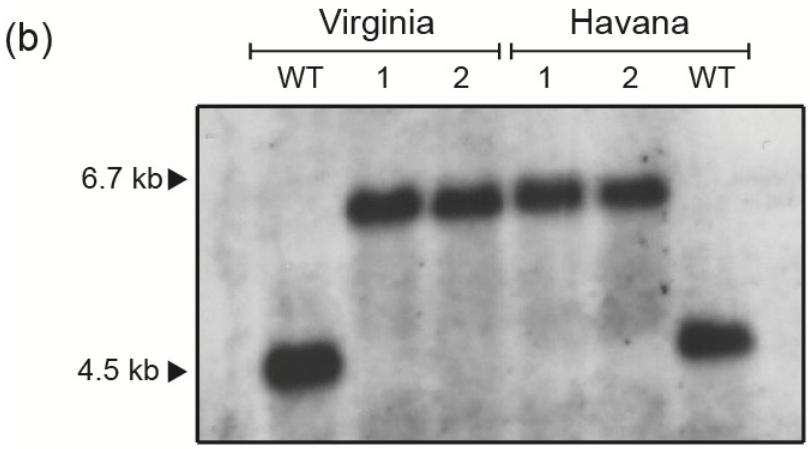
Figure captions

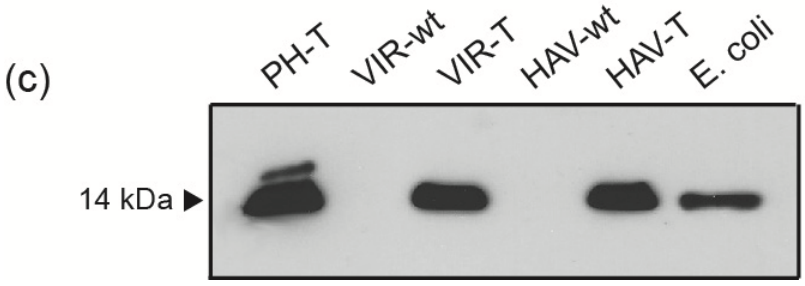
Fig.1 Integration of the *Trx f* gene into the plastid genome. (a) Wild-type and transformed plastid genomes. The P1 probe of the targeting region for homologous recombination is shown over the wild-type plastid genome. *16S3´, trnV, 3´rps12*: original sequences of the plastid genome; *aadA*: aminoglycoside 3-adenylyltransferase gene; P*rrn*: *16S* rRNA promoter and 5´-untraslated region; P*rrnG10L*: *16S* rRNA promoter fused to the leader region of the bacteriophage T7 gene *10*; T*rps16*: 3´-untranslated region of the plastid *rps16* gene; T*psbA*: 3´-untranslated region of the plastid *psbA* gene; *lox*, recognition sites for Cre recombinase. Dotted lines under the maps indicate the expected size of the fragments after restriction enzyme digestion. (b) Confirmation of homoplasmy by Southern blot analysis. Total DNA (10 µg) was digested with *Bgl*II and probed with P1. Two lines per tobacco cultivar (Virginia and Havana) and their corresponding wild-type controls are shown. (c) Immunoblot analysis of Trx f expression by transplastomic plants with a specific Trx f antibody. Ten µg of total protein were loaded per lane. VIR-wt and HAV-wt, wild-type Virginia and Havana plants; VIR-T and HAV-T, transplastomic plants from both cultivars. As controls, a Trx f overexpressing sample (10 µg of total protein) of the tobacco Petite Havana cultivar (PH-T) and 10 ng of Trx f expressed in *Escherichia coli* (*E. coli*) were used

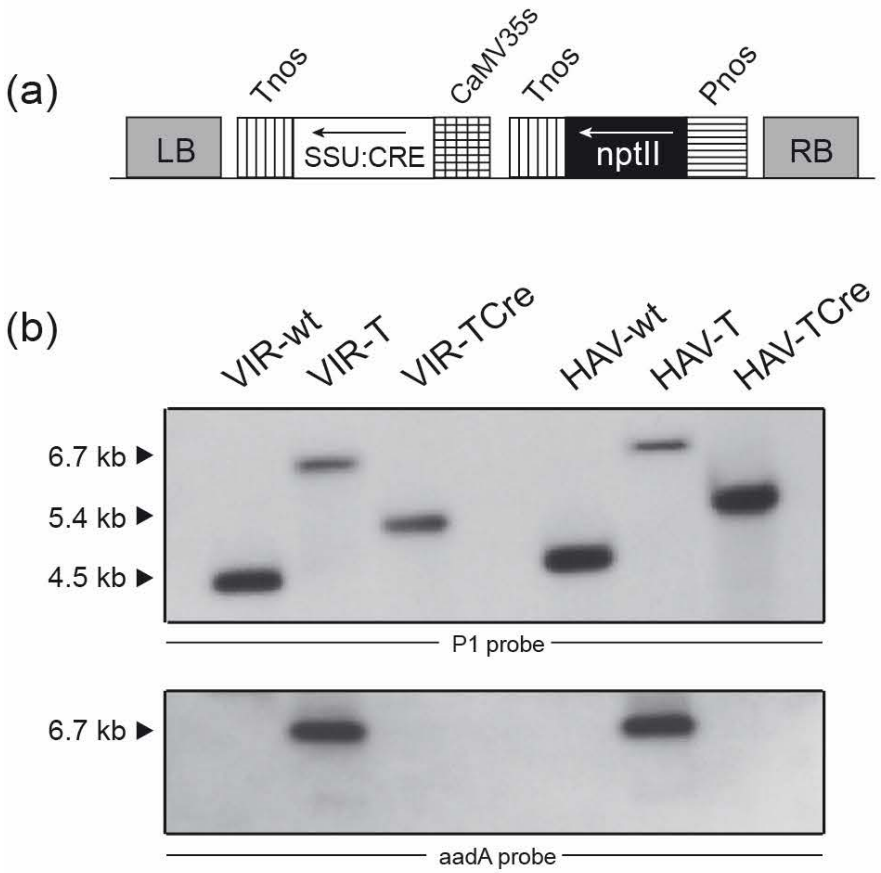
Fig.2 Removal of the antibiotic marker gene from transplastomic plants. (a) Map of the vector for nuclear transformation via *Agrobacterium tumefaciens*. LB, RB: left and right borders; *nptII*, coding sequence of the neomycin phosphotransferase type II gene; P*nos*, nopaline synthase gene promoter; T*nos*, nopaline synthase gene terminator; P*35S*, promoter of the *35S* gene from the cauliflower mosaic virus; SSU: transit peptide of the Rubisco small subunit from tobacco; *cre*, coding region of the Cre recombinase from the P1 bacteriophage. (b) Southern blot analysis to confirm loss of the *aadA* gene in transplastomic plants. VIR-wt and HAV-wt, wild-type samples from Virginia and Havana cultivars; VIR-T and HAV-T, initial transplastomic plants from both cultivars; VIR-TCre and HAV-TCre, regenerated plants after nuclear transformation with the Cre recombinase. Samples were probed with the P1 targeting region (Fig.1) and *aadA* probes. (c, d) *In vitro* germination assay of sensitivity to (c) spectinomycin (500 mg/L) and (d)

kanamycin (100 mg/L) by T_3 generation seeds. NUC, unrelated nuclear transgenic plant used as a control for kanamycin resistance

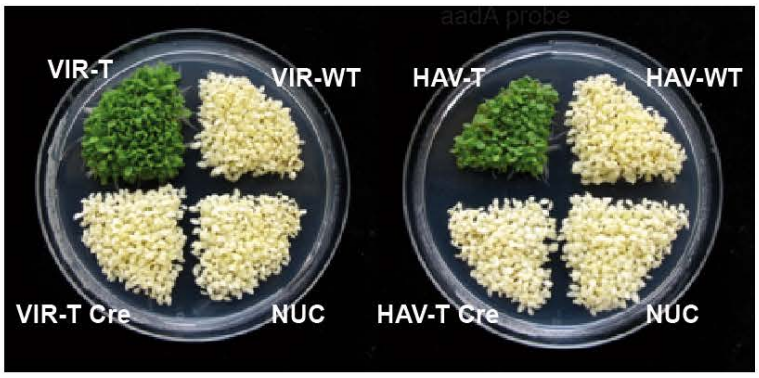








(c) Spectinomycin



(d) Kanamycin

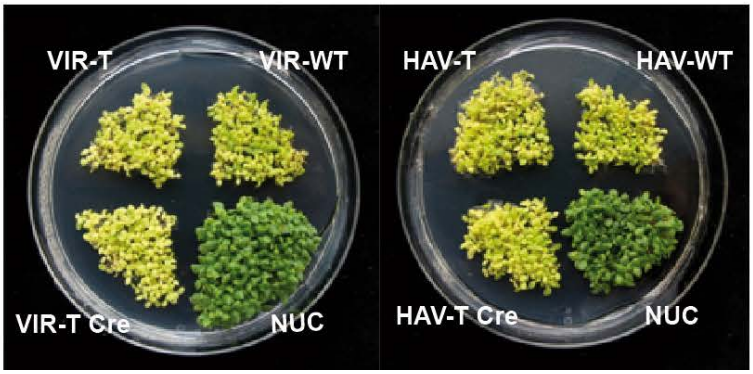


Table 1 Phenotypic analysis of Trx f-overexpressing field grown tobacco plants (cultivars Virginia and Havana) relative to their wild types

Phenotypic character	VIR	VIR-Trxf	HAV	HAV-Trxf
Total height (cm)	140±1.4	144±1.2	143±2.3	137±3.2
Total weight/plant (g)	2940±380	2750±410	1980±350	1880±250
Specific Leaf Weight (SLW, mg/cm^2)	4.24±0.2	4.66±0.2	4.85±0.3**a**	5.99±0.2**b**
Chlorophyll (SPAD)	36.3±0.8	35.3±0.7	39.1±0.5	41.3±0.7

VIR: Virginia wild type. VIR-Trxf: Trx f-overexpressing Virginia plants. HAV: Havana wild type. HAV-Trxf: Trx f-overexpressing Havana plants. Values are the mean ± SE of 15 individual plants. Different letters, within each cultivar, indicate statistically different values at a $P \leq 0.05$ (t-te

Table 2 Compositional characterization of leaf and stalk samples

	Leaf				Stalk			
Parameter (% w/dw)	VIR	VIR-Trxf	HAV	HAV-Trxf	VIR	VIR-Trxf	HAV	HAV-Trxf
Residual humidity	5.01±0.04	4.87±0.11	4.10±0.02	5.00±0.16	6.37	5.57	5.14	4.84
Soluble sugars	6.66±0.01**a**	7.60±0.13**b**	7.20±0.04**a**	12.50±0.03 **b**	3.2	3.4	3.5	3.4
Starch	1.88±0.01**a**	4.32±0.05**b**	0.94±0.00**a**	3.41±0.06**b**	nd	nd	nd	nd
Cellulose	5.27±0.18**b**	4.30±0.02**a**	4.83±0.09**a**	4.86±0.22**b**	28.7	29.1	27	27.4
Xylan	2.29±0.04	2.13±0.00	2.57±0.05	2.15±0.11	13.3	13.8	14.1	13.9
Arabinan	0.49±0.02	0.43±0.03	0.49±0.00	0.47±0.02	0.82	0.87	1.11	0.8
Lignin	3.37±0.02**b**	2.85±0.02**a**	3.26±0.07	2.80±0.03	10.9	11.4	12.7	12.4
Protein	30.20±0.44	28.60±0.19	26.90±0.04 **b**	24.12±0.25 **a**	17.8	17.3	17.1	18.2
Ash	19.03±0.04 **b**	17.98±0.07 **a**	20.13±0.01 **b**	18.69±0.04 **a**	13.6	13.5	12.5	12.7

Values are the mean ± SE (n = 4, leaf; n = 2, stalk). Different letters, within each cultivar, indicate statistically different values at a $P \leq 0.05$ (ANOVA). nd, not detected. w/dw: weight/dry weight

Table 3 Leaf sample quantification of total and fermentable sugars released after pretreatment and enzymatic hydrolysis followed by ethanol production after fermentation

Parameter	VIR	VIR-Trxf	HAV	HAV-Trxf
Total sugars (% w/dw)	16.6±0.1**a**	20.3±0.0**b**	17.9±0.7**a**	23.9±0.0**b**
Fermentable sugars (% w/dw)	15.9±0.1**a**	19.8±0.0**b**	17.1±0.6**a**	23.2±0.0**b**
Efficiency of enzymatic hydrolysis (%)	109.9	115.0	126.6	106.8
Fermentation efficiency (%)	88.7	85.1	81.9	86.2
Ethanol (% w/dw)	6.9±0.2**a**	8.6±0.1**b**	7.1±0.2**a**	10.2±0.2**b**

Values are the mean ± SE (n = 2-4). Different letters, within each cultivar, indicate statistically different values at $P \leq 0.05$ (ANOVA). w/dw: weight/dry weight

Table 4 Effect of nine different pretreatments on stalk samples of VIR-Trxf plants with regard to the concentration of total and fermentable sugars along with ethanol production based on the liquid and the recovered solid fractions

	Pretreatment								
Parameter	170 ºC	190 ºC	210 ºC	170 ºC + acid	190 ºC + acid	210 ºC + acid	170 ºC + acid + pressure	190 ºC + acid + pressure	210 ºC + acid + pressure
Recovery of solid fraction (%)	61.2	52.4	48.9	57.5	51.0	47.7	57.2	50.3	47.9
Total sugars (% w/dw)	35.1±0.9**a**	29.7±0.4**bc**	22.6±0.2**e**	31.6±0.1**b**	26.9±0.1**cd**	22.0±0.0**e**	31.9±0.2**b**	26.2±0.5**d**	20.6±0.2**e**
Fermentable sugars (% w/dw)	21.5±0.6**ab**	22.2±0.3**a**	21.6±0.2**ab**	19.4±0.1**b**	20.4±0.1**ab**	21.1±0.1**ab**	19.6±0.2**b**	20.6±0.5**ab**	19.8±0.2**b**
Efficiency of enzymatic hydrolysis (%)	60.7±1.8**ab**	62.8±0.9**a**	61.0±0.6**ab**	54.8±0.3**b**	57.6±0.3**ab**	59.6±0.1**ab**	55.2±0.5**b**	58.3±1.3**ab**	55.8±0.5**b**
Ethanol (% w/dw)	10.1±0.3	10.0±0.0	10.2±0.3	9.4±0.1	9.9±0.1	9.5±0.6	9.5±0.2	10.3±0.2	9.2±0.1

Values are the mean ± SE (n = 2-4). Different letters, within each line, indicate statistically different values at a $P \leq 0.05$ (Tukey test). w/dw: weight/dry weight

Table 5 Stalk sample quantification of total and fermentable sugars released after pretreatment at 190 ºC and enzymatic hydrolysis together with ethanol production after fermentation

Parameter	VIR	VIR-Trxf	HAV	HAV-Trxf
Recovery of solid fraction (%)	51.8	52.4	53.5	52.0
Total sugars (% w/dw)	20.9±0.7**a**	29.7±0.4**b**	23.4±0.3	24.1±0.7
Fermentable sugars (% w/dw)	17.9±0.1**a**	22.2±0.3**b**	18.3±0.2	20.1±0.6
Efficiency of enzymatic hydrolysis (%)	51.5±0.3**a**	62.8±0.9**b**	55.5±0.7	60.1±1.9
Fermentation efficiency (%)	90.9	99.8	93.2	91.7
Ethanol (% w/dw)	8.1±0.0**a**	10.0±0.0**b**	8.5±0.2	9.2±0.1

Values are the mean ± SE (n = 2-4). Different letters, within each cultivar, indicate statistically different values at a $P \leq 0.05$ (ANOVA). w/dw: weight/dry weight