High-level expression of a suite of thermostable cell wall-degrading enzymes from the chloroplast genome

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Abstract The biological conversion of plant biomass into fermentable sugars is key to the efficient production of biofuels and other renewable chemicals from plants. As up to more than 90% of the dry weight of higher plants is fixed in the cell wall, this will require the low-cost production of large amounts of cell wall-degrading enzymes. Transgenic plants can potentially provide an unbeatably cheap production platform for industrial enzymes. Transgene expression from the plastid genome is particularly attractive, due to high-level foreign protein accumulation in chloroplasts, absence of epigenetic gene silencing and improved transgene containment. Here, we have explored the potential of transplastomic plants to produce large amounts of thermostable cell wall-degrading enzymes from the bacterium Thermobifida fusca. We show that a set of four enzymes that are required for efficient degradation of cellulose (and the hemicellulose xyloglucan) could be expressed successfully in transplastomic tobacco plants. However, overexpression of the enzymes (to between approximately 5 and 40% of the plant’s total soluble protein) resulted in pigment-deficient mutant phenotypes. We demonstrate that the chloroplast-produced cellulolytic enzymes are highly active. Although further optimization is needed, our data indicate that transgenic plastids offer great potential for the production of enzyme cocktails for the bioconversion of cellulosic biomass.

Keywords Chloroplast · Plastid transformation · Nicotiana tabacum · Cellulose · Cell wall degradation · Cellulose degradation · Biofuel · Cellulosic ethanol · Thermostable enzyme

Introduction

Stimulated by the steady increase in the price of petroleum and growing concerns about global climate change, there is considerable interest in the use of renewable (plant-derived) liquid energy carriers as transportation fuels, such as ethanol (Bayer et al. 2007; Gomez et al. 2008). As the cell wall comprises up to more than 90% of the dry weight of higher plants, any economically viable technology for conversion of plant biomass into fuels faces the challenge of efficiently hydrolyzing the cell wall polymers to release soluble compounds (sugars) that are fermentable by industrial microbes. Currently, thermal and/or chemical pretreatments are required to make cellulosic biomass accessible to subsequent biological conversion. These pretreatments have a significant impact on costs, involve chemicals that are harmful to the environment (e.g., sulfuric acid, sulfur dioxide) and are not universally applicable to all sources of plant biomass. Therefore, to achieve sustainable and cost-competitive biofuel production, it will be necessary to develop technologies that facilitate the dismantling of plant cell walls without thermochemical pretreatments.

Although plants have evolved a strong natural resistance to microbial breakdown of their life-saving cell walls, a number of fungi and bacteria are known to be capable of overcoming this resistance and initiating the degradation of even tough (e.g., highly lignified) cellulosic substrates. These (parasitic or saprophytic) microbes provide a rich source of cellulolytic enzymes that potentially could be used...
for enzymatic hydrolysis of plant biomass into simple sugars for fermentation into biofuels, such as cellulosic ethanol. To largely inhibit competing microbial activities, it is highly desirable to conduct the biological conversion of cellulosic biomass into fermentable sugars at high temperatures. Therefore, thermostable cell wall-degrading enzymes have attracted particular attention as potential tools for efficient bioconversion of cellulosic biomass (Wei et al. 2009). Their industrial production by microbial fermentation is relatively expensive, because it requires synthetic or semisynthetic media and sterile growth conditions. Recent work has shown that the expression of microbial cellulolytic enzymes in plants is feasible and could provide an inexpensive production platform for suites of enzymes converting plant biomass into simple sugars (Taylor II et al. 2008). Given the enormous capacity of chloroplasts to accumulate foreign proteins (Oey et al. 2009a), plastid transformation seems to be a particularly promising technology to engineer plants that produce high levels of cell wall-degrading enzymes (Yu et al. 2007; Ziegelhoffer et al. 2009; Verma et al. 2010).

_Thermobifida fusca_, formerly known as _Thermomonospora fusca_, is a rod-shaped, thermophilic bacterium belonging to the Actinobacteria group. It is a major microbial degrader of plant cell walls and is frequently found in decaying organic matter. The preferred habitat of _Thermobifida fusca_ are self-heated organic materials (e.g., compost heaps, dunghills, rotting haystacks or bales of straw) that can reach the bacterium’s optimal growth temperature of 55°C. Here we report the use of transgenic chloroplasts for the high-yield production of a set of thermostable cellulolytic enzymes from _Thermobifida fusca_ (Irwin et al. 2003; Chen et al. 2003), which is suitable to break down the cellulose and the hemicellulose xyloglucan of plant cell walls into simple sugars for fermentation.

Materials and methods

Plant material and growth conditions

Sterile tobacco (_Nicotiana tabacum_ cv. Petit Havana) plants were grown on agar-solidified Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 30 g/l of sucrose. Regenerated shoots from homoplasmic transplastomic lines were rooted and propagated on the same medium under low-light conditions (5 μE m⁻² s⁻¹). Rooted homoplasmic plants were transferred to soil and grown to maturity under glasshouse conditions.

Vector construction

The plastid transformation vectors constructed in this study are based on the previously described transformation vector pKP9 (Zhou et al. 2008). Transgene sequences (bgI1, c6, c6B, c6A and xeg74 genes from _Thermobifida fusca_) were taken from the respective GenBank entries (Bgi1C: AAZ54975; C66B: AAA62211; C6A: AAB42 155; Xeg74: AAZ55647). The putative signal peptides for protein secretion from the bacterial cell were identified using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and the corresponding DNA sequences were removed. The coding regions of all genes were codon optimized by adjusting the codon usage to the preferred triplets in the tobacco plastid genome. Subsequently, selected restriction sites needed in later cloning steps were eliminated by silent mutations, NdeI and NheI restriction sites were added to the 5' end and an XbaI restriction site (harbouring the stop codon) was introduced at the 3' end. The genes were chemically synthesized (Genscript Corporation, NJ, USA) and cloned as NdeI/XbaI fragments into vector pHK20 (Kuroda and Maliga 2001). The expression cassettes were then excised as SacI/HindIII fragments and cloned into the similarly digested vector pKP9 (Zhou et al. 2008), generating the pKMP plastid transformation constructs illustrated in Fig. 1a.

Plastid transformation and selection of homoplasmic transplastomic tobacco lines

For biolistic chloroplast transformation, young tobacco leaves were harvested from aseptically grown plants and bombarded with plasmid DNA-coated 0.6 μm gold particles (BioRad, Salt Lake City, UT) using the DuPont PDS1000He biolistic gun (BioRad). Primary spectinomycin-resistant lines were selected on plant regeneration medium containing 500 μg/ml of spectinomycin (Svab and Maliga 1993). Spontaneous spectinomycin-resistant plants were eliminated by double selection on medium containing spectinomycin and streptomycin (500 μg/ml each; Svab and Maliga 1993; Bock 2001). For each transformation construct, several independently generated transplastomic lines were subjected to two to three additional rounds of regeneration on spectinomycin-containing medium to enrich the transplastome and select for homoplasmic tissue.

Isolation of nucleic acids and hybridization procedures

Total plant DNA was isolated from fresh leaf tissue by a rapid cetyltrimethylammoniumbromide-based mini-prep procedure (Doyle and Doyle 1990). For Southern blot analysis, DNA samples (5 μg total cellular DNA) were digested with the restriction enzyme BamHI, separated by gel electrophoresis in 1% agarose gels and transferred onto Hybond XL membranes (GE Healthcare, Buckinghamshire, UK) by capillary blotting using standard protocols. A 550 bp PCR product generated by amplification of the
The psaB coding region (Wurbs et al. 2007) was used as RFLP probe to verify chloroplast transformation.

Total cellular RNA was extracted using the peqGOLD TriFast™ reagent (Peqlab GmbH, Erlangen, Germany) according to the manufacturer’s protocol. RNA samples (3.5 μg total RNA) were electrophoresed in formaldehyde-containing 1% agarose gels and blotted onto Hybond XL membranes. Gene-specific probes for northern blot analyses were produced by PCR amplification of the corresponding DNA sequences with the following specific primers: bgl1C (Pbg1Cfor 5'-CTTGTCCAAAAATTCCTCCATT-3'; Pbg1Crev 5'-GGGCTATTGATCTACTGGATTATA-3'); cel6B (Pcel6Bfor 5'-TGAGCCAGAAACCATTGTC-3'; Pcel6Brev 5'-TGATGGGAGGACACTA-3'); and xeg74 (Pxeg74for 5'-GGGATGACGAAAACACCATTGTC-3'; Pxeg74rev 5'-TGATGGGAGGACACTA-3').
ATAG-3′), cel9A (Pcel9A for 5′-CTAAAGCACAAGGTT CTCCATTA-3′; Pcel9Arev 5′-GCTGGAATGTATCT CAAAGATCTA-3′) and xeg74 (Pxeg74for 5′-CTAGCCA GCAGCAGAACAAAG-3′; Pxeg74rev 5′-TGGACCTCTTC CTAACCTA-3′). PCR products were purified by agarose gel electrophoresis following extraction of the DNA fragments of interest from excised gel slices using the Nucleospin Extract II kit (Macherey–Nagel) and radiolabeled by random priming with 32P-dCTP using the MegaPrime DNA labeling system (GE Healthcare). Hybridizations were performed for 4–12 h at 65°C according to standard protocols.

Protein extraction and immunoblot analyses

Total soluble protein was extracted from leaf samples homogenized in a buffer containing 50 mm N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES)-KOH (pH 7.5), 10 mM potassium acetate, 5 mM magnesium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1× Complete proteinase inhibitor (Roche, Darmstadt, Germany) and 1% β-mercaptoethanol. The protein concentration of the extracts was determined using the Bradford assay (Roth, Karlsruhe, Germany) using known concentrations of bovine serum albumin (BSA) as standard. Alternatively, a phenolic extraction method was used (Cahoon et al. 1992). Briefly, protein extracts of transplastomic tobacco leaves were obtained by homogenization of tissue in a buffer containing 0.7 M sucrose, 0.5 M Tris/HCl, 50 mM EDTA and 0.1 M KCl, pH 9.4, with the addition of 2% β-mercaptoethanol and 1× Complete proteinase inhibitor (Roche, Darmstadt, Germany) prior to use. After addition of 1 vol. phenol and centrifugation at 3000×g for 10 min, the upper phase was recovered and the proteins were precipitated by the addition of 0.1 M ammonium acetate in methanol and incubation at −20°C overnight. The protein pellet obtained after centrifugation was washed, air-dried and redissolved by the addition of sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. The protein concentration of the extracts was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Protein samples were separated by electrophoresis in 12% SDS-containing polyacrylamide gels and the proteins in the gel were either directly visualized by Coomassie blue staining or transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare) using the Trans-Blot Cell (BioRad) and a standard transfer buffer (192 mM glycine, 25 mM Tris pH 8.3). Immunoblot detection was performed with anti-Bgl1C, anti-Cel6B, anti-Cel9A, and anti-Xeg74 antibodies (kindly provided by Dr. D. B. Wilson, Cornell University, Ithaca, NY, USA) using the enhanced chemiluminescence system (ECL® PLUS system; GE Healthcare).

Enzyme activity assays

β-Glucosidase activity was measured with the colorimetric p-nitrophenyl-β-D-glucopyranosid (pNPG) test. Defined amounts of total soluble protein (TSP) extracted in HEPES extraction buffer were adjusted to a total volume of 740 μl with 50 mM HEPES–NaOH (pH 7.0) and allowed to equilibrate to the assay temperature of 50°C for 5 min. The reaction was started by addition of 20 μl of 100 mM pNPG (Sigma) and terminated by the addition of 250 μl of 2 M Na2CO3 after 10 min. The absorbance at 400 nm was measured spectrophotometrically and enzyme activities were calculated by using the molar extinction co-efficient for p-nitrophenol (18.6 mM⁻¹ cm⁻¹).

Exoglucanase and endoglucanase activities were determined using carboxymethylcellulose (CMC, Sigma) as substrate. Samples of HEPES buffer-extracted TSP were diluted in 50 μl HEPES extraction buffer and added to 300 μl CMC solution (2 mg/ml CMC in 50 mM sodium acetate, 15 mM CaCl2, pH 6.0) and incubated for 1 h at 60°C. Reducing sugars were measured using the p-hydroxybenzoic acid hydrazide (PAHBAH) reagent (Lever 1972). To this end, 50 μl of the assay mix were added to 1.5 ml PAHBAH reagent and incubated at 100°C for 6 min. The absorbance at 410 nm was measured spectrophotometrically and a reference curve was prepared with glucose as standard (0–25 μg).

Xyloglucanase activity was measured using xyloglucan (Megazyme) as substrate. Samples of HEPES buffer-extracted TSP were diluted in 50 μl HEPES extraction buffer and added to 1 ml xyloglucan solution (2 mg/ml in 50 mM NaH2PO4/K2HPO4, pH 7.0) and incubated for 2 h at 60°C. Reducing sugars were quantified using the PAHBAH reagent (Lever 1972).

To determine enzyme activity on a natural substrate, particles of dried wheat straw (average size: 1 mm²) were incubated in 2% H2SO4 or in H2O at 121°C for 45 min, subsequently washed and diluted to give a 5% water-straw mixture. The pH value was adjusted to 6.0 with NaOH (or HCl for the untreated water-straw mixture). To this end, 50 μl of the assay mix were added to 1.5 ml PAHBAH reagent and incubated at 100°C for 6 min. The absorbance at 410 nm was measured spectrophotometrically and a reference curve was prepared with glucose as standard (0–25 μg).
Results

Integration of genes for thermostable cellulolytic enzymes into the tobacco plastid genome

Cellulolytic enzymes (cellulases) catalyze the hydrolysis of cellulose. Different subclasses of cellulases are known that differ in their substrate specificities. Endocellulases cleave internal bonds in cellulose fibers, thereby disrupting the crystalline structure of cellulose and exposing individual polysaccharide chains. Exocellulases cleave off disaccharide or tetrasaccharide units from the ends of polysaccharide chains. These products are further hydrolyzed by β-glucosidases into monosaccharides (glucose). In addition to these three enzyme activities, the efficient degradation of plant cell walls requires enzymes that hydrolyze hemicellulose. Xyloglucan is the most abundant hemicellulose in the primary cell wall of vascular plants and can be degraded by xylol glucanases. However, for degradation of the most abundant components of hemicellulosic biomass in nature, acetylgalacturonoxylans in wood and arabinoxylans in the cell wall of grasses, endoxylanase enzymes from other glycohydrolase families are required.

To produce an effective cocktail of thermostable cellulolytic enzymes suitable to degrade plant cell walls, we selected genes encoding the four key enzymatic activities from the thermophilic biomass-decomposing bacterium Thermobifida fusca: cel9A encoding an endocellulase (endoglucanase), cel6B encoding an exocellulase (exoglucanase), bg11C encoding a β-glucosidase and xeg74 encoding a xyloglucanase (Fig. 1a). The gene sequences were codon-optimized in silico by choosing the most preferred triplets according to the codon usage table for the tobacco plastid genome (http://www.kazusa.or.jp/codon/) and then chemically synthesized (GenBank accession numbers bg11C: JF290397; cel6B: JF290398; cel9A: JF290399; xeg74: JF290400). To maximize expression of the transgenes, their coding regions were fused to the strong ribosomal RNA operon-derived Pror promoter in combination with the 5′ untranslated region (5′ UTR) from the gene 10 leader of bacteriophage T7 (T7g10). Previous work had established that this promoter-UTR combination confers high-level foreign protein accumulation in transgenic plastids (Ye et al. 2001; Kuroda and Maliga 2001; Oey et al. 2009a, b).

The four transgene cassettes were integrated into the previously constructed plastid targeting vector pKp9 (Zhou et al. 2008) generating chloroplast transformation vectors pKMP16 (harboring bg11C), pKMP17 (harboring cel6B), pKMP18 (harboring cel9A) and pKMP19 (harboring xeg74; Fig. 1a). All vectors contain a chimeric aadA gene as a selectable marker gene for chloroplast transformation (Svab and Maliga 1993), which confers resistance to the aminoglycoside antibiotic spectinomycin.

Plastid transformation experiments in tobacco using particle gun-mediated (biolistic) transformation (Svab and Maliga 1993) and selection of spectinomycin-resistant cell lines produced several candidate transplastomic lines for all four constructs. To eliminate spontaneous antibiotic-resistant mutants, all primary spectinomycin-resistant lines were tested for double resistance to spectinomycin and streptomycin (Svab and Maliga 1993; Bock 2001). Doubly resistant lines were subjected to two to three additional regeneration rounds on spectinomycin-containing medium to isolate homoplasmic tissue and two to three independently generated lines per construct were subsequently analyzed in detail. First, successful transformation of the chloroplast genome and correct integration of the transgenes into the plastid genome by homologous recombination was confirmed by Southern blot analyses. The results clearly demonstrated that homoplasmic transplastomic lines had been obtained for all four constructs (Fig. 1b).

Phenotype of transplasmatic tobacco plants expressing cell wall-degrading enzymes

Phenotypic comparison of homoplasmic pKMP plants with wild-type plants revealed that all transplastomic lines displayed strong mutant phenotypes, the most obvious aspect of which was severe pigment deficiency (Fig. 2). When grown in soil, transplastomic plants either grew very slowly or did not survive, apparently due to their insufficient photosynthetic performance (Fig. 2a). Heteroplasmic plants that had a sufficient proportion of green tissue (containing wild-type plastids) could be grown to maturity and produced seeds (Fig. 2a). Depending on the phenotype of the flower-producing tissue, the seeds gave rise to either homoplasmic or heteroplasmic progeny.

When kept under heterotrophic conditions on sucrose-containing medium, all transplastomic lines grew well, but retained their pale phenotypes even under low-light conditions (Fig. 2b). Comparison to previously generated photosynthesis mutants, which are green under low-light conditions (Hager et al. 1999, 2002), suggests that the pigment deficiency is not only the result of photooxidative damage. To minimize interference from starvation effects, wild-type and transplastomic lines raised on sucrose-containing medium were used in all subsequent analyses.

Analysis of transgene expression in transplastomic lines expressing cellulolytic enzymes

We next wanted to analyze the expression of the four transgenes at the RNA and protein levels. To this end, we first conducted northern blot experiments using transgene-specific probes. Transcripts for all four transgenes could be readily detected and accumulated stably in transplastomic
plants (Fig. 3a). This is in line with a large body of literature reporting high-level mRNA accumulation from plastid transgenes driven by the rRNA operon promoter. Western blot analyses using specific antibodies against the Thermobifida fusca enzymes confirmed expression of all transgenes at the protein level (Fig. 3b). For all four enzymes, the antibodies recognized a specific protein of the expected molecular mass that was absent from wild-type plants. All enzymes were detectable at high sensitivity, with 75–500 ng of total soluble protein (TSP) being sufficient for specific detection in Western blot experiments (Fig. 3b).

To determine whether this degradation occurs in vivo or during protein isolation, we tested alternative protein extraction protocols. When a phenol-based protein isolation method was used, almost no protein degradation products were present suggesting that their appearance in the HEPES buffer was the result of proteolytic degradation during protein extraction.

In the absence of the pure overexpressed proteins, it was not possible to quantitate the expression levels from Western blots. However, the high sensitivity, at which all four enzymes were detectable (Fig. 3b), suggested to us that the expression levels were very high. We, therefore, attempted to directly detect the foreign proteins by Coomassie staining of electrophoretically separated total soluble plant protein extracts. Interestingly, all transplastomic lines showed an additional strongly stained protein band that corresponded in size to the expressed foreign protein (Fig. 4). Considering that, in wild-type plants, Rubisco, the most abundant protein on Earth, accumulates to approximately 50–60% of TSP, we roughly estimated the foreign protein accumulation levels in our transplastomic plants to lie between approximately 5% (exoglucanase) and 40% (endoglucanase) of TSP.

Enzymatic activity of chloroplast-produced cell wall-degrading enzymes

Having obtained extraordinarily high expression levels for all four cell wall-degrading enzymes from Thermobifida fusca, we next wanted to determine, if the chloroplast-produced proteins are enzymatically active. To this end, we performed enzyme activity assays with total protein extracts from wild-type and transplastomic plants (Fig. 5). The results demonstrated that all four enzymes were highly active and hydrolyzed their synthetic test substrates in a dose-dependent manner (Fig. 5a–d).

As, in chloroplasts, the protein biosynthesis capacity declines with leaf age, the age-dependent decrease in foreign protein accumulation provides a suitable proxy for protein stability (Birch-Machin et al. 2004; Zhou et al. 2008). To determine the stability of the Thermobifida enzymes in planta, we therefore analyzed a developmental series of 14 leaves from a pKMP16 transplastomic plant. pKMP16 was chosen, because, compared to the other transplastomic plants, homoplasmic pKMP16 plants had the least severe phenotype and grew autotrophically in soil reasonably well (Fig. 2). Interestingly, only a very subtle age-dependent decline in β-glucosidase activity was detectable and, even in old leaves, enzyme activities were nearly as high as in young and mature leaves (Fig. 5e). This suggests that the high accumulation levels of the cell wall-degrading enzymes are, at least in part, due to their high stability in chloroplasts.
We also tested our chloroplast-produced cellulolytic enzymes on a natural substrate that could be used for cellulosic ethanol production. As a model substrate for agricultural wastes, we used wheat straw and prepared an enzyme cocktail from the four *Thermobifida* enzymes expressed in our transplastomic plants. As expected, plain straw could not be hydrolyzed by the enzymes, due to its extreme hydrophobicity and recalcitrance to enzymatic deconstruction (Fig. 5f). However, following a standard thermochemical pretreatment (with 2% H$_2$SO$_4$ at 121°C for 45 min), the enzyme cocktail triggered efficient sugar release from straw (Fig. 5f), indicating that the chloroplast-produced enzymes are also active on natural sources of cellulosic biomass.

Finally, we wanted to confirm that the enzymatic properties of the chloroplast-produced cell wall-degrading enzymes are similar to those of the bacterial enzymes. We, therefore, exemplarily investigated the temperature dependence and pH dependence of the enzymatic activity of the β-glucosidase expressed in the pKMP16 transplastomic lines. The enzymatic properties of the β-glucosidase from *Thermobifida fusca* have been extensively studied (Spiridonov and Wilson 2001). The pH optimum was found to be 7.0 and the temperature optimum 50°C. Very similar values were determined for the plastid-produced enzymes (Fig. 5g, h). This suggests that expression in the chloroplast does not result in significantly different enzymatic properties, consistent with the prokaryotic nature of the plastid genetic system.

**Fig. 3** Analysis of transgene expression in transplastomic pKMP plants. a Analysis of *bgllC*, *cel6B*, *cel9A* and *xeg74* mRNA accumulation in homoplastic transplastomic tobacco plants. Specific hybridization probes detect major transcripts of the expected sizes in all pKMP lines. Two to three independently generated transplastomic lines per construct are shown along with a wild-type control. RNA species that are 0.5 or 1.0 kb larger than the expected size are the result of read-through transcription terminating either at the 5’ or 3’ loxP site flanking the *aadA* marker gene (Zhou et al. 2008). As expected, no hybridization occurs with RNA from the wild-type control. To control for equal RNA loading, the ethidium bromide-stained gels prior to blotting are shown below each blot. b Western blot analyses to detect the cell wall-degrading enzymes expressed in transplastomic pKMP plants. The enzymes are detected with specific antibodies. The loaded amounts of total soluble protein (TSP) or total cellular protein (TP) are indicated. Note that the xyloglucanase expressed in pKMP19 lines is not stable upon protein extraction with HEPES-based extraction buffer (accumulation of putative degradation products of 60 kDa and smaller), but remains stable upon protein extraction with phenol. The upper hybridizing band in the β-glucosidase blot (pKMP16) results from cross-reaction with a protein present also in the wild type. Molecular masses of marker bands are indicated in kDa.
The possibility to engineer the plastid genome of higher plants has created new opportunities for plant biotechnologists (Bock 2007; Daniell et al. 2009). Transgene expression from the plastid genome offers unique attractions, including high-level foreign protein expression (Oey et al. 2009a), absence of epigenetic effects and greatly increased transgene containment due to the maternal inheritance of plastids in most crop species (Ruf et al. 2007; Svab and Maliga 2007). In the course of this work, we have examined expression of various thermostable cellulolytic enzymes from plastid transgenes. The choice of enzymes from the thermophilic cell wall-degrading bacterium Thermobifida fusca was based on the consideration that their combined action in an enzyme cocktail will degrade cellulosic materials at high efficiency. All enzymes could be successfully produced in transplastomic tobacco plants, accumulated to very high levels and were enzymatically active. We estimated foreign protein accumulation levels to reach a minimum of 5% of the total soluble protein (exoglucanase) and a maximum of approximately 40% TSP (endoglucanase). The extreme expression levels obtainable with the endoglucanase (Fig. 4) are particularly encouraging, because this enzyme initiates cellulose degradation by internal cleavage of the polysaccharide chains and, therefore, will represent a key component of any enzyme cocktail that is to be utilized for efficient hydrolysis of lignocellulosic biomass.

The high expression levels of all enzymes caused severe mutant phenotypes of the transplastomic plants (Fig. 2). In previous experiments, plastid transgenes were expressed to similarly high or even higher levels than the cellulolytic enzymes in this study. Although protein accumulation levels of up to more than 70% TSP were obtained and the transgenes were targeted to the identical location in the tobacco plastid genome (Zhou et al. 2008; McCabe et al. 2008; Oey et al. 2009a, b), the transplastomic plants did not display similarly severe mutant phenotypes as the pKMP transplastomic lines described here. This makes it clear that the high protein accumulation levels alone cannot explain the mutant phenotypes (Fig. 2). We tentatively attribute the severity of the phenotypes of the pKMP plants to the carbohydrate-binding activity of the enzymes produced in their chloroplasts. As the chloroplast represents the center of carbohydrate metabolism in the cell, it harbors numerous enzymatic reactions involving sugars, oligosaccharides and polysaccharides. It, therefore, seems conceivable that the extraordinarily high levels of cellulolytic enzymes accumulating in the chloroplasts of our transplastomic plants exert their deleterious effects through sequestration or degradation of intermediates in plant carbohydrate metabolism.

It appears likely that lower enzyme accumulation levels would result in plants with less severe phenotypes, which can grow autotrophically at reasonable rates, as has been demonstrated for other transgenes with deleterious effects on chloroplast function (Tregoning et al. 2003; Glenz et al. 2006). Also, in recent studies reporting expression of thermostable cell wall-degrading enzymes by chloroplast transformation (Gray et al. 2009) or nuclear transformation (and targeting of the protein to the chloroplast; Jung et al. 2010), no similarly severe mutant phenotypes of the transgenic plants have been observed. Therefore, considering the extremely high accumulation levels achieved in this work (Fig. 4), a simple solution would be to tether the transgenes to expression signals that are less active. In this study, we have used the strongest known promoter and translation initiation signal (Shine-Dalgarno sequence), because we wanted to explore the maximum potential of chloroplasts to accumulate the thermostable cell...
wall-degrading enzymes. As a wide spectrum of plastid promoters and Shine-Dalgarno sequences is available that trigger lower transgene expression levels than the combination of the ribosomal RNA operon promoter with the phage T7 gene 10 leader used here (Staub and Maliga 1994; Herz et al. 2005; Bohne et al. 2007), it would be a relatively simple exercise to test expression signals of different strengths and, in this way, identify the optimum compromise between high transgene expression levels and normal plant growth. Alternatively, inducible expression systems could be used, which have recently become available for plastids (Mühlbauer and Koop 2005; Verhounig et al. 2010).

Together with previous studies (Yu et al. 2007; Ziegelhoffer et al. 2009; Verma et al. 2010), the high-level expression of a set of thermostable cellulolytic enzymes reported here provides a basis for the inexpensive production of cellulolytic enzyme cocktails in plants. However, the efficient degradation of cellulose, as shown for the set of enzymes from Thermobifida fusca expressed in this study, is alone insufficient to achieve cost-effective production of cellulosic biofuels. Current biomass conversion technologies use thermal and/or chemical pretreatment to make the cell walls more amenable to subsequent enzymatic degradation (Fig. 5f; da Costa Sousa et al. 2009). As these pretreatments have a significant impact on costs, solutions are needed that solely involve biological treatments. Also, most of the biomass currently available for biofuel production is highly lignified and the degradation of lignified cell walls remains a major challenge. However, with the current acceleration of world-wide research on genetic engineering of cell wall composition (Li et al. 2008; Weng et al. 2008) and enzymatic delignification of plant cell walls (Martínez et al. 2009), new strides forward are expected in the near future.


References


