Inducible Trans-activation of Plastid Transgenes: Expression of the *R. eutropha phb* Operon in Transplastomic Tobacco

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Deleterious effects of constitutive transgene expression can occur if gene products are harmful to the transformed plant. Constraints such as growth inhibition and male sterility have been observed in plastid transformants containing the *phb* operon encoding the genes required for the production of the polyester polyhydroxybutyric acid (PHB). In order to induce PHB synthesis in tobacco in a well-timed manner, we have constructed a trans-activation system to regulate transcription of the *phb* operon in plastids. This system consists of a nuclear-located, ethanol-inducible T7RNA polymerase (T7RNAP) which is targeted to plastids harboring the *phb* operon under control of T7 regulatory elements. Following treatment with 5% ethanol, moderate induction of PHB synthesis was found. PHB amounts reached 1,383 ppm in dry weight, and an overall background activity of 171 ppm was measured in uninduced tissues. On the transcriptional level, T7RNAP induction was proven and we found that the *phb* operon is transcribed into at least two mRNAs. Without ethanol induction, development of flowers and fertile seeds was possible. Thus, the main problem of inhibitory transgene expression was solved. Our results show that this inducible trans-activation system could serve as an alternative to constitutive expression of transgenes in the plastome.

Keywords: Inducible transcription — Plastid Transformation — Polyhydroxybutyrate — Regulation — Renewable resources — Trans-activation system.

Abbreviations: PHB, polyhydroxybutyric acid; T7RNAP, RNA polymerase of bacteriophage T7.

Introduction

Plastome transformation bears many advantages mainly with regards to the precision of transgene insertion and gene containment. The potential to produce high levels of protein by plastid expression has recently increased interest in using plastids as biological factories (Daniell et al. 2002, Maliga 2004). However, constitutive expression of transgene products can be deleterious to the plants' health because of toxicity or interference with metabolism (Daniell et al. 2001, Tregoning et al. 2003). Interactions between heterologous products and metabolism in different growth stages reduce potential plant productivity or even inhibit selection of primary transformants. In addition, constitutive expression may cause different problems of even greater relevance if the products are harmful, for example by unintended human consumption of pharmaceutical transgene products (Staub et al. 2000).

Missing functional regulation of plastid gene transcription poses the problem that transplastome technology alone does not allow any tuning of transgenes inserted into the plastome. Thus, it is required to express a transgene at a particular developmental stage, or to harmonize the timing of expression with ecological concerns.

Several regulatory systems using exogenously applied activators have been explored for use in plants. Inducibility, the main requirement for the promoter, can be achieved by various chemicals such as alcohols, steroids, antibiotics, pesticides, phytohormones or metallic ions (Gatz 1997, Caddick et al. 1998, Padidam 2003). Also light conditions (Boetti et al. 1999), pathogen infection (Lebel et al. 1998, Johnson et al. 2003) or growth stage dependent-conditions such as senescence or stress (Hennig et al. 1993, Hoff et al. 2001) can be used for induction. However, most of these systems only work in the nuclear compartment. An alternative approach is the induction of chloroplast transgene expression through a trans-activating factor, which is induced in the nucleus by a chemically controlled promoter and targeted to the plastids.

In our plastome transformation experiments, the need for such a regulating system became obvious when we produced the polyhydroxyalkanoate polyhydroxybutyric acid (PHB) in tobacco chloroplasts (Lössl et al. 2000, Lössl et al. 2003). Experiments with the *phb* operon under the control of constitu-
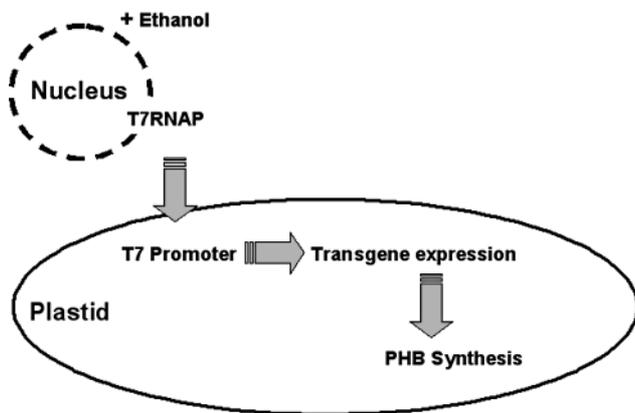
Fig. 1 Inducible trans-activation in plastids. A nuclear-located T7 RNAP gene was fused to a plastid targeting sequence. The T7RNAP was expressed under control of an inducible promoter. Induction by ethanol application leads to import of the T7RNAP into the plastids. Transcription of plastid transgenes under control of polymerase-specific regulatory elements occurs and the PHB pathway becomes established in the chloroplasts.

Fig. 2 Vector for nuclear transformation of tobacco. Transformation via *Agrobacterium tumefaciens* was carried out with the Ti plasmid pSRNTPSST7 as detailed in the text. LB, left border; P*alc*A, 5′ promoter of the *alc*A gene from *Aspergillus nidulans* for ethanol induction; TPSS, DNA sequence for the transit peptide of the small subunit of RuBisCo from *Pisum sativum* for plastid targeting (Nawrath et al. 1994, Dasgupta et al. 1998); KB1 and KB2, primers for amplification of the T7RNAP; T7RNAP, RNA polymerase gene from λ bacteriophage T7; T*ocs*, T*nos*, downstream untranslated region of the octopin and nopalin synthase genes from *A. tumefaciens*; ALCR, transcription factor necessary for induction at the *alc*A promoter; P35S, promoter of cauliflower mosaic virus for constitutive expression; NPTII, kanamycin resistance T*nptII* terminator; P*nos*, promoter of the nopalin synthase gene; RB, right border.

tive promoters revealed that PHB synthesis in tobacco chloroplasts is in principle possible. This shows that chloroplast transformation is a valid alternative to nuclear transformation (Poirier et al. 1992, Nawrath et al. 1994, Bohmert et al. 2000, Bohmert et al. 2002, Menzel et al. 2003). However, expression of the *phb* pathway was coupled with serious physiological constraints and the transformants suffered from early growth reduction and pollen sterility (Lössl et al. 2003).

To circumvent these growth-inhibiting effects, induction of PHB expression was performed late during plant development when the plant was already regenerated and when it had reached maturity. Due to late induction, growth retardation and sterility could be avoided and seed production was enabled.

Regulation of transplastomic gene expression was accomplished by a combination of two components, as shown in Fig. 1. The first component was a polymerase controlled by an inducible promoter and targeted to the plastids. This first component was introduced into the nuclear genome. The second component was the transgene inserted into the plastome and expressed under control of polymerase-specific regulatory elements.

For this purpose, we chose the T7 phage RNA polymerase (T7RNAP) which had already been successfully used for constitutive transgene expression (McBride et al. 1994). This group demonstrated that the T7RNAP could be targeted into tobacco plastids, and that, following plastid import, the polymerase directed the expression of a *uid*A reporter gene cloned downstream of the T7 gene 10 promoter and 5′-untranslated region (UTR) (McBride et al. 1994). However, the missing link for inducibility is a promoter, which allows activation through chemical regulators. An adjustable trans-activation expression using the inducible PR-1a promoter was proposed in a review by Heifetz (2000) who referred to work described
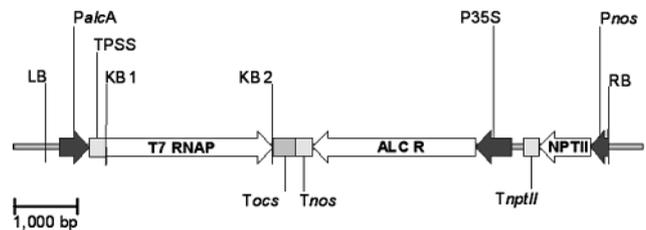
in a patent application. Magee et al. (2004) used this salicylic acid-inducible promoter; however, transcription was unspecific and the transgene product was not expressed at detectable levels in plants.

In our approach, we use the ethanol-inducible gene control mechanism based on the *alc*A promoter and transcription factor ALCR for the alcohol dehydrogenase regulon of *Aspergillus nidulans* as described by Caddick et al. (1998), Salter et al. (1998) and Roslan et al. (2001).

Using this regulon, we constructed a trans-activation system to regulate *phb* gene transcription in plastids. Ethanol application triggered the expression of the *phb* operon. Northern analysis showed that the *phb* operon is transcribed into two mRNAs. Induction of the PHB pathway after ethanol induction was demonstrated, yet a certain background activity was detectable before induction. However, the problem of flower sterility, described earlier for tobacco harboring a constitutively expressed *phb* operon in the plastome, was solved. The propagation of seeds from PHB-synthesizing plants is now feasible. To our knowledge, this is the first report on inducible trans-activation of transgenes introduced into the plastome.

Results

For regulation of plastid-located transgenes, it was necessary to transform two genetic compartments: on the one hand, the nucleus with the gene for an ethanol-inducible T7RNAP targeted to the plastids (Fig. 2); and, on the other hand, the plastid genome, which needed to be supplied with a transgene under control of T7RNAP corresponding motifs (Fig. 3).
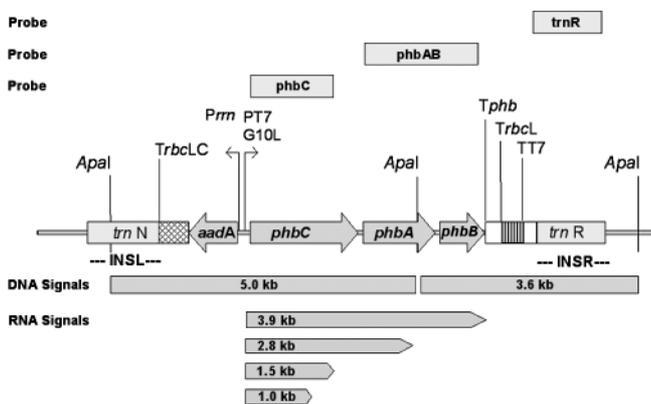
Fig. 3 Map of the *phb* operon under T7 regulatory elements integrated into the plastome. The map shows the insertion cassette, targeted to the plastome by homologous flanks INSL and INSR containing *trn*N and *trn*R. For the test of the homoplastomy of the transformants, we used as probe 'trnR', a plastome-specific fragment containing *trn*R positioned at nucleotides 109,230–110,348 within the direct repeat of the tobacco plastome. The restriction sites for *Apa*I are indicated. Probe 'phbAB' used for Southern analysis consisted of a 2.3 kb *Pst*I-cut fragment (nucleotides 1,968–4,290 from the start codon). Expected hybridization fragments for transformant lines are given at the bottom of the map. Intact transformant lines contained a 5,032 and a 3,550 bp fragment, when hybridized with the *Apa*I overlapping probe 'phbAB'. The inserted *phb* operon was under control of the T7 leader and promoter sequence (G10L, PT7 promoter) with a termination sequence, containing a terminator from *Ralstonia eutropha* (T*phb*), a *rbc*L 3′ region from tobacco (T*rbc*L) and a T7 terminator (TT7). The *aad*A cassette used for spectinomycin selection was expressed constitutively by the 16S rRNA promoter with the termination region from a *Chlamydomonas rbc*L gene (T*rbc*LC).

Insertion of the phb operon under T7 regulatory elements into the plastome

To generate transgenic plastids for PHB synthesis, we transferred the complete *phb* operon, consisting of the *phb*C, *phb*A and *phb*B genes, from *Ralstonia eutropha* to the plastid genome of tobacco. The polyester PHB is derived from acetyl-CoA by a sequence of three enzymatic reactions (Schubert et al. 1988): at first, condensation of two molecules of acetyl-CoA is catalyzed by β-ketothiolase (EC 2.3.1.9) to form acetoacetyl-CoA. Acetyl-CoA reductase (EC 1.1.1.36) then reduces acetoacetyl-CoA to β-hydroxybutyryl-CoA, which is then polymerized by PHB synthase (EC 2.3.1.-) to PHB. In *Ralstonia eutropha*, the genes for these enzymes are organized in a single operon.

To achieve inducibility, we constructed a chimera with the *phb* operon containing the promoter, 5′ leader and termination sequences of gene 10 from bacteriophage T7, as shown in Fig. 3.

Integration of genes into the chloroplast genome occurs by homologous recombination and requires sequence homology between the transformation vector and the chloroplast genome. We used the tobacco transformation vector pKCZ (Zou et al. 2003), which targeted the *phb* operon to a plastome locus within the plastid inverted repeat. The map of the plastome insertion is diagrammed in Fig. 3. In the present experiments, the construct was inserted between the plastid genes *trn*N and *trn*R. Transformations were carried out by particle gun-mediated gene delivery.

DNA analysis of transformants

After transformation, correct insertion and the total length of the operon in the plastome of transformants were confirmed by Southern hybridizations. The locations of the probes and
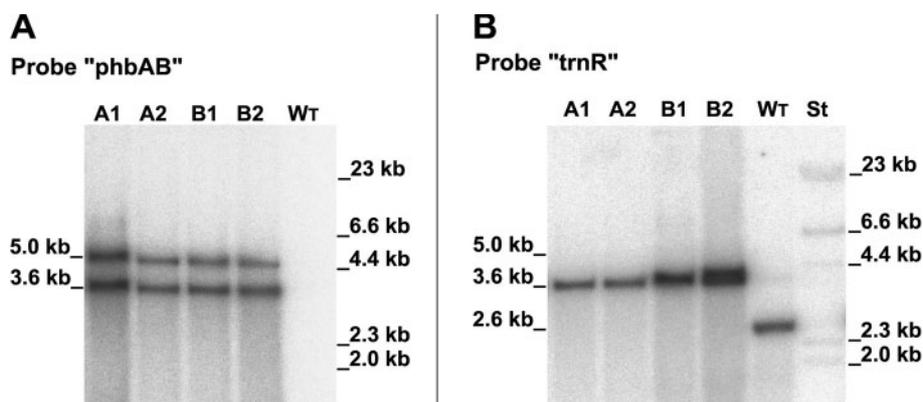

Fig. 4 Proof of correct insertion and homoplasmy by Southern hybridization. (A) In order to verify correct insertion of the *phb* operon, the transformant's DNA was cut by *Apa*I. As indicated in Fig. 3, a specific overlapping fragment containing the *phb*A and *phb*B gene was used as probe, 'phbAB' (nucleotides 1,968–4,290 from the start codon). Lanes 1–4, DNAs of transformant A1, A2 and B1, B2. Lane 5 contains wild-type DNA. (B) For proof of homoplastomy, a second hybridization was performed. In order to test for residual wild-type plastome copies in lower expressing plants, we used as probe a plastome-specific fragment containing *trn*R positioned at nucleotides 109,230–110,348 within the direct repeat, as indicated in Fig. 3. Plant DNA of A1, A2 and B1, B2 was cut by *Apa*I. For intact homoplastomic transformant lines, a single signal of 3.6 kb was detected. If the transformants were heteroplastomic, they would exhibit an additional 2.6 kb fragment specific for a non-disrupted wild-type plastome insertion site. Lanes 1–4, DNAs of transformant A1, A2 and B1, B2; lane 5, wild-type DNA; lane 6, standard DNA.
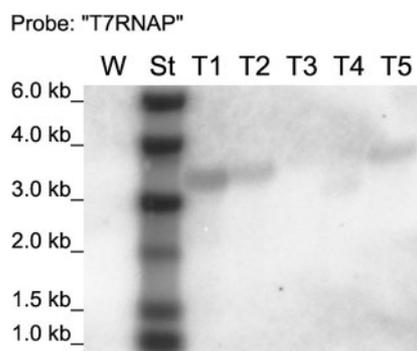
Fig. 5 Northern hybridization for T7RNAP mRNA. Following nuclear transformation by *Agrobacterium tumefaciens*, the transcription of T7RNAP was confirmed by probing with the 2.7 kb probe containing the T7RNAP gene. The analysis detected products of 3–4 kb length. After wild-type and RNA standard, lanes 1–5 were loaded with RNA of the nuclear transformants T1–T5. T3 and T4 did not show the expected signal, but were resistant to kanamycin.

Fig. 6 Serial order of successive double transformation steps, yielding transformants A1, A2, B1 and B2.

expected hybridization signals of *phb* cassettes are given in Fig. 3. Results of the Southern analysis are shown in Fig. 4A. For correct insertions, we expected two signals of 5.0 and 3.6 kb, when hybridized with the 'phbAB' probe. Plastome transformants A1, A2 and B1, B2 showed the expected fragment pattern.

As the degree of heteroplastomy could affect PHB synthesis, it was necessary to analyze for residual wild-type plastome. For selection of a homoplastomic stage, the transformants were regenerated on spectinomycin-containing medium in four cycles of shoot formation. In order to confirm maintenance of the *phb* operon in the plastome once spectinomycin selection pressure is removed, we transferred the transformants to the greenhouse. Following a 3-month greenhouse period, a further Southern analysis with the plastomic probe '*trn*N' revealed that plants were homoplastomic with respect to the operon insertion. Wild-type plastome was not detectable when hybridized with plastid DNA flanking the operon insertion (Fig. 4B).

Nuclear transformation with an ethanol-inducible T7RNA polymerase gene

For construction of the corresponding nuclear component, we inserted a T7RNAP gene into Ti plasmid pSRN (Zeneca®) containing an ethanol-inducible promoter (Caddick et al. 1998, Salter et al. 1998), which was fused in-frame with a plastid-targeting transit peptide sequence (Nawrath et al. 1994). This construct conferring kanamycin resistance was introduced into the tobacco plants by *Agrobacterium tumefaciens*-mediated transformation. Nuclear transformation of the construct into the tobacco plants was confirmed by PCR with primers specific for the T7RNAP gene.

For further confirmation, the primary transformants were tested for segregation of antibiotic resistance in their progeny. Kanamycin germination assays were conducted on the seeds
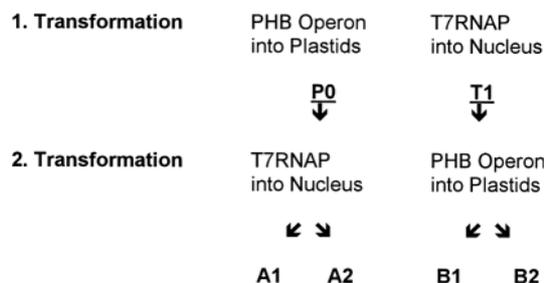
from self-pollinated transformant plants to determine those segregating 3 : 1 for the linked resistance gene. The plants, which met this criterion and thus contained a single T DNA integration, were used for Northern analysis as shown in Fig. 5. Transcript sizes of T7RNAP in the transformants T1–T5 were variable. They showed a length between approximately 3.5 and 4 kb. Agrobacterial transformation sites usually are not identical and different position effects give rise to variable susceptibility to RNA degradation. The highest expressing line 'T1' was used for further plastid transformations.

Sequential order of doubletransformations

In order to trace back possible effects of the two transformation steps individually, we carried out the *phb* transformations in two successive steps, as given in Fig. 6.

In the first approach, a plastome transformant ('P0') was generated prior to the nuclear transformation, which resulted in lines A1 and A2. Secondly, a selected nuclear transformant ('T1') was double transformed by plastid transformation yielding lines B1 and B2.

Thus we obtained two types of transformants: two lines with identical plastome transformation but different location of the T7RNAP gene in the nucleus (A1 and A2) and two lines with identical nuclear component but derived from different plastome insertion events (B1 and B2).

For subsequent double transformation of A1 and A2 with the corresponding T7RNAP construct, the most vigorous transformant (P0) was selected.

Growth and fertility

After regeneration, transformants were tested for ethanol-inducible PHB synthesis. In the absence of ethanol application, no phenotypic alterations have been detected in respect of leaf color. However, transformants were growing more slowly than wild-type tobacco plants. Spraying of a 5% ethanol solution onto wild-type tobacco did not cause any visible changes. However, ethanol application to transformants for induction of PHB synthesis caused a bleaching effect in newly developing leaf tissues (Fig. 7). In contrast to the pollen-sterile plastome transformants which expressed the *phb* operon constitutively (Lössl et al. 2003), the ethanol-inducible transformants exhib-
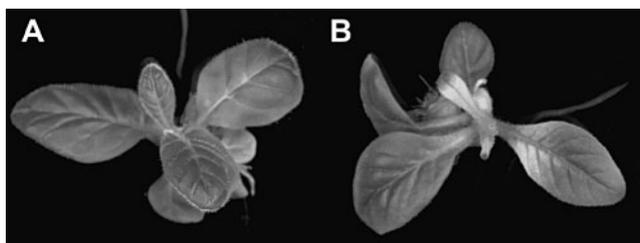
Fig. 7 Bleaching. Following spraying with a 5% ethanol solution, the wild type (A) did not exhibit symptoms. Only transformant lines showed severe bleaching on ethanol induction; here genotype B2 is depicted (B).

ited fertile flowers in the T_0 generation and yielded seeds following self-pollination. For further propagation, seeds of the T_1 generation were selected on B5 medium containing both kanamycin and spectinomycin.

Following transformation, the lines A1 and A2 as well as the tobacco ('P0') which they originated from, and the transformants B1 and B2 were regenerated and analyzed for their mRNA levels and subsequently for their PHB contents.

Northern analysis of PHB expression following ethanol induction

In order to test ethanol-mediated induction of PHB synthesis, we carried out a transcription analysis of the T7RNAP gene and the *phb* operon in the different transformants.

For this purpose, a Northern hybridization was performed with total RNA of 4-week-old in vitro-grown transformants with the T7RNAP gene, using the 'phbC' and the downstream located 'phbAB' probe. We found that spraying 5% ethanol triggered transcription of the T7RNAP and subsequently the *phb* operon in transformants A1, A2, B1 and B2 (Fig. 8). The probes detected the mRNA of the T7RNAP including the fused transit peptide with a length of about 3.5 kb. Wild-type ('W') and the transplastomic tobacco ('P0') without T7RNA polymerase did not show transcription of the *phb* operon. Transcription of the T7RNAP gene followed ethanol induction within 24 h and resulted in *phb* operon transcription and accumulation of PHB.

Hybridization with probe 'phbC' revealed that there were several transcripts within the 5′ region of the *phb* operon. They covered a range of approximately 3.9, 2.8, 1.5 and 1 kb. The longer transcripts of approximately 3.9 and 2.8 kb have also been detected with probe *phb*AB. The large 3.9 kb transcript of the *phb* operon is already terminated at the bacterial termination motif of *Ralstonia eutropha* T*phb*. Transcripts shorter than 3.9 kb and background signals were mapped as primary transcripts which were either degraded or terminated at different sites within the *phb* operon (Fig. 3).

PHB formation following induction

Four weeks after ethanol induction, genotypes A1, A2, B1, B2 and their parental transformants were analyzed by gas
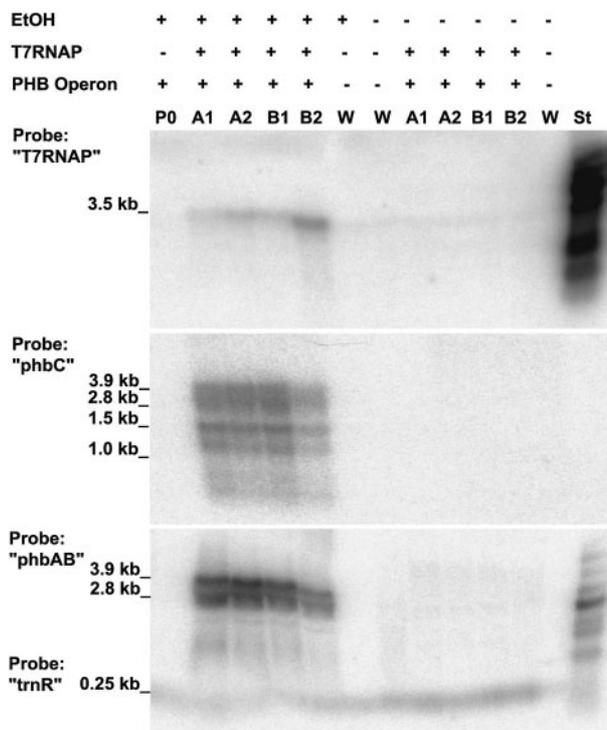

Fig. 8 Inducibility of operon transcription in the plastids by ethanol shown by Northern analysis. Following a 3 d application of 5% ethanol, mRNA signals were detected in tobacco lines with the plastome-located *phb* operon under control of the T7RNAP. The Northern blots contained RNA from the following plants: lane 1, primary plastome transformant 'P0′ with the *phb* operon, without T7RNAP; lanes 2 and 3, double transformants A1 and A2, both derived from 'P0′ with T7RNAP and the *phb* operon with ethanol application; lanes 4 and 5, double transformants B1 and B2, containing both T7RNAP and the *phb* operon, with ethanol application; lane 6, wild type (W) with ethanol application; lane 7, wild type (W) without ethanol application; lanes 8–11, double transformants A1, A2, B1 and B2, with T7RNAP and the *phb* operon without ethanol application; lane 12, wild type (W) without ethanol application. The Northern gel contained 10 μg of total plant RNA each. The sizes of RNA standard (St) are as follows: 6, 4, 3, 2, 1.5, 1, 0.5 and 0.2 kb. Signals of T7RNAP transcripts were identified in transformants with high *PHB* expression upon ethanol induction. Probes 'phbC' and 'phbAB' consisted of the respective DNA regions of the *phb* operon. RNA hybridization with 'phbC' detected transcripts in the range from 3.9 to 1 kb in the transformants A1, A2 and B1, B2. Probe 'phbAB' showed only a 3.9 and a 2.8 kb transcript. Hybridization of 'phbAB' was carried out together with the RNA probe *trn*R as an internal standard. The *trn*R signal of about 0.25 kb shows that the gel loading was balanced. The lanes of the Northern gel contained 5 μg of total plant RNA each.

chromatography for their PHB contents. The measurements were repeated eight times per genotype. The average PHB content of transformants after induction was 509 ppm DW. The highest level was detected in leaf tissue of line B2. PHB accumulation in uninduced mature plants carrying both the nuclear and plastid components of the trans-activation system revealed an overall background level of about 171 ppm Genotype B1 had the highest background PHB content of 320 ppm DW. In
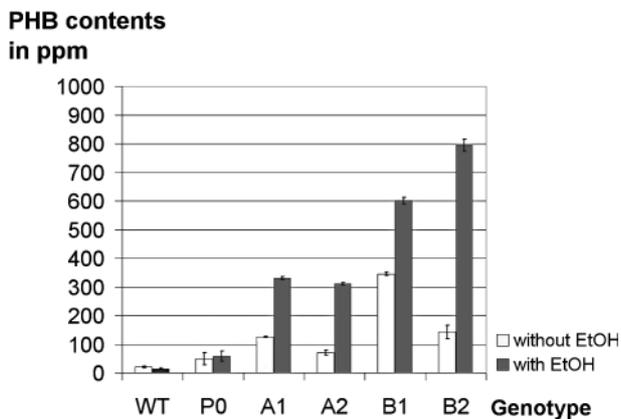
PHB contents in ppm

Fig. 9 Average PHB contents of transformants. Leaf material of each transformant line was analyzed by gas chromatography 4 weeks following 5% ethanol induction. PHB values were determined in dry matter of the transgenic tobacco lines. Standard deviations are indicated within the bars. Measurements were repeated eight times for each genotype.

PHB contents in ppm

Fig. 10 PHB contents of F_1 plants in a time course. Following a 5% ethanol induction, tobacco leaf material of F_1 progeny was collected for gas chromatographic analysis after 7, 14, 21 and 28 d. PHB values were determined in dry matter of the plants. Standard deviations are indicated within the bars. Measurements were repeated six times for each state of genotype F1C, and three times for F1-A2 and F1-B2.

wild-type tobacco without induction, we measured an average background level of 15 ppm DW with a standard error of 4 ppm Fig. 9 shows the PHB contents in ppm DW for wild-type tobacco and plastome transformants with and without ethanol application.

Comparison of the two pairs A1, A2 and B1, B2, which were derived from different primary transformants, revealed differences in PHB synthesis on a highly significant level ($P <$ 0.01).

Double transformants B1 and B2 which were derived from the nuclear transformant 'T1' yielded significantly higher PHB contents than the other genotypes derived from the plastid transformant 'P0'.

In order to test the stability of the inducible system, progeny of the second generation of transformants were used for further tests on their PHB contents. To find out the optimal induction time for PHB synthesis, contents were measured after four different points in time following ethanol spraying. The tested genotype F1-C has shown the most representative response (Fig. 10). Bleaching occurred about 10 d after ethanol application. The affected leaves were supposed to contain more PHB than phenotypically normal material. However, after 21 and 28 d, clones with extremely bleached leaves did not yield significantly higher PHB contents than tissues harvested 14 d after induction. In a selected progeny of the F1-generation of A2 and B2, we found the highest PHB amounts with 937 and 1,383 ppm when measured after 21 d (Fig. 10).

Discussion

In recent experiments, it has been shown that PHB synthesis in transplastomic chloroplasts in principle is feasible; however, constitutive expression was coupled with serious
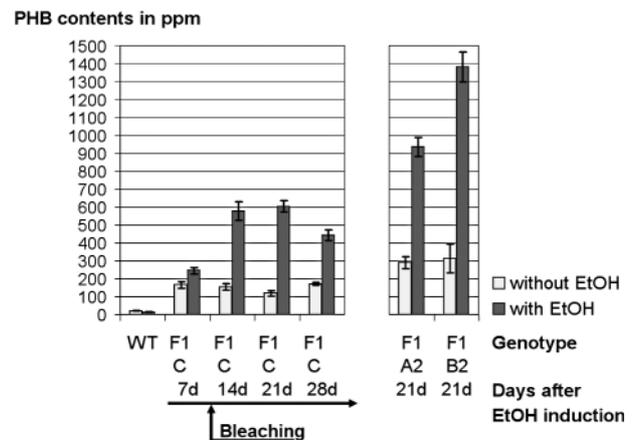
deleterious effects in different growth stages (Lössl et al. 2003). Transplastomic plants suffered from early growth retardation and male sterility, and thus production of seeds became nearly impossible. Such problems are not limited to the synthesis of PHB, but also arose when different pharmaceuticals were synthesized in transgenic plants (Daniell et al. 2001, Tregoning et al. 2003). An additional constraint occurs if the transgenic product interferes with the plant metabolism or is even toxic, because expression during the in vitro regeneration stage hinders complete segregation to homoplastomy or exerts harmful selection pressure on primary transformants.

Therefore, it became necessary to develop a trans-activation system for the functional regulation of plastid gene expression. We demonstrated regulation of PHB synthesis mediated by an alcohol-inducible promoter (Caddick et al. 1998, Salter et al. 1998) with a nuclear located T7RNAP gene (McBride et al. 1994). This T7RNAP was targeted to a plastome-localized *phb* operon under control of T7 regulatory elements. The double transformants which carried these expression constructs showed T7RNAP-mediated transgene transcription in the plastids. The transformants produced hydroxybutyric acid following induction with a 5% ethanol solution. Gas chromatographic determination of the leaf material showed a PHB content of up to 1,383 ppm DW. Although we observed leakiness of the system, the basic problems of constitutive transgene expression, which can lead to growth retardation and male sterility, were surmounted by this trans-activation system, and seeds were generated for further propagation.

The PHB contents exceeded the values achieved with constitutively expressing bacterial promoters (Lössl et al. 2000, Nakashita et al. 2001) by more than two orders of magnitude, and the system competes with the efficiency of constitutively expressing plastid promoters (Lössl et al. 2003, Arai et al.

2004). However, we realized that inducible expression stagnated on a lower level than expected.

The yield of the desired PHB product depends on the availability of metabolic precursor molecules as well as on the amounts and activities of the introduced enzymes.

It has been claimed that recombinant proteins can accumulate to >40% total soluble protein in transformed chloroplasts (De Cosa et al. 2001), if the gene or reaction products have no toxic effects on the cells.

To exploit fully the synthetic capacity of the plastid gene expression machinery, it is necessary to consider transgene integration, transcription activity as well as post-transcriptional events such as RNA turnover, translation, proper folding of the amino acid chain or protein turnover.

Expression may be influenced negatively if the transformation construct was not integrated correctly or if the material remains heteroplastomic, as segregation events may result in a reduction of the copy number of the recombinant plastome in the absence of selective pressure. However, in our experiments, Southern analysis of tissue culture material grown under spectinomycin selection showed that the PHB operon was inserted correctly. An additional hybridization experiment following a 3 month growth period in the greenhouse revealed that the plants were homoplastomic. Hence, heteroplastomy cannot be the reason for low PHB contents.

Even though transcriptional activity is usually only a minor determinant of plastid gene expression (Eberhard et al. 2002), it becomes critical if used as a control point for externally applied gene regulation. In our transgenic plants, PHB transcript levels are a function of T7 promotor activity and T7 polymerase accumulation in the plastids after induction and stability of the PHB transcripts. It is known that the T7 promoter mediates a strong transcriptional activity in the presence of sufficient amounts of T7RNAP. However, Northern analysis revealed very low transcript abundance for T7RNAP after ethanol induction, indicating a potential weak point for T7RNAP expression. Improving nuclear T7RNAP transcription together with optimizing the T7RNAP import into the plastids could contribute to a higher transcription of the *phb* operon. According to the nuclear segregation ratio of 3 : 1 determined by the kanamycin-resistant phenotype, the T7RNAP gene in the primary transformants existed as a singular insertion with heterozygous inheritance. Hence, further crossing steps might allow generation of multiallelic homozygous genotypes, which express the T7RNAP in higher concentrations, although silencing might occur in some lines. It may also be possible to select nuclear transformants with improved T7RNAP expression.

RNA hybridization experiments probing the plastid-located *phb* operon showed that the *phb* operon was transcribed following ethanol induction. Interestingly, we found that the variability in PHB synthesis did not correlate with transcript abundance. Transcription of the *phb* operon through the T7RNAP yields a variety of transcripts (Fig. 8). For transcription of the complete *phb* operon, the primary transcripts should

cover a length of 3877 bp. RNA hybridization also detected a series of smaller transcripts. Plastid RNA is influenced by a considerable extent of instability, and transcripts are subject to various degradation events (Sugita and Sugiura 1996). Also the *phb* transcripts are affected by these processes. However, eventually PHB was formed in these plants, and therefore we suppose that there were sufficient intact primary transcripts containing the complete set of *phb* genes.

The regulatory elements derived from the phage T7 gene 10 (Lehmeier and Amann 1992) not only provide a very strong promoter, but are also capable of mediating strong translation activity, which together with protein stability has been shown to be the most important determinant for plastid expression levels (Eibl et al. 1999, Staub et al. 2000, Zou et al. 2003). However, only the first cistron in the *phb* operon encoding the PHB synthase was under control of the T7 leader sequences, whereas the other two cistrons, *phb*A and *phb*B, were under control of the unmodified bacterial spacer elements, which may result in an insufficient translation of the β-ketothiolase (*phb*A) and acetoacetyl-CoA reductase (*phb*B). The supply of the 3-butyric acid substrate for the PHB synthase may therefore be suboptimal. One means of further improving PHB synthesis could be to determine and optimize the accumulation of the β-ketothiolase and acetoacetyl-CoA reductase enzymes, e.g. by using spacer elements from strongly expressed plastid operons.

Finally, plastid proteases can exert a high impact on every step of plant development (Huffaker 1990, Estelle 2001, Kuroda and Maliga 2003). Such plastid proteases possibly could recognize the operon-encoded enzymes, β-ketothiolase, acetoacetyl-CoA reductase and PHB synthase, or also the T7RNAP and degrade them as foreign proteins (Adam and Clarke 2002). Low production could then also be due to degradation of one or all of the PHB-forming enzymes directly after translation. The problem could be circumvented by using suitable protein fusion tags such as, for example, ubiquitin, which have been shown to exert a protein-stabilizsing effect, thus increasing recombinant protein concentrations (Staub et al. 2000). It is to be expected that the recombinant enzymes accumulate in different amounts, and each of them could be rate limiting, if they were present in insufficient amounts. Any further approach for optimizing enzyme levels should therefore be preceded by a determination of recombinant protein accumulation and pool sizes of the metabolic precursors (acetyl-CoA) and intermediates (acetoacetyl-CoA and hydroxybutyric acid).

We observed an induction of PHB steady-state levels of up to 6-fold; however, the trans-activation system was affected by a certain degree of leakiness. This was not expected, since, according to Kapoor and Sugiura (1999), promoters from T7 bacteriophage are not recognized by the tobacco plastid transcriptional apparatus. Also McBride et al. (1994) did not report any expression of T7-regulated genes in the tobacco plastid genome until the heterologous T7RNAP was introduced.

A background expression of the three enzymes introduced into the plastids could be the result of three independent

undesired activities: (i) leaky expression of the nuclear-encoded T7RNAP even in the absence of ethanol; (ii) leaky plastid transcription from the T7 promoter by any other RNA polymerase present in the plastids; and (iii) read-through transcription from any upstream plastid promotor. In order to identify these activities, we independently analyzed PHB levels of both mere plastid transformants as well as of nuclear and plastid double transformants.

The double transformant line B1 showed a high background level of PHB synthesis independent of ethanol application. The leakiness could be traced back to an T7RNAP background activity or to an unspecific activity of plastid RNA polymerases. Analysis of the mere plastome transformant 'P0′' (without a nuclear component) revealed a PHB background synthesis of about 50 ppm, which nearly reached the level of the non-induced double transformants. Therefore, we conclude that there is a non-specific plastid background transcription activity, which is consequently also present in all double transformants, A1, A2, B1 and B2. Another reason for leakiness is very often read-through transcription due to the generally weak termination of plastid transcription (Stern and Gruissem 1987, Hayes et al. 1996). Although the PHB operon is inserted in the opposite direction compared with the *aad*A selection marker, it cannot be excluded that the operon is to a certain degree co-transcribed from an endogenous upstream promoter. In this case, the degree of leakiness could be influenced by the insertion site.

However, additionally, in the non-induced double transformants, we found that the PHB levels exceeded that of the non-inducible 'P0′'. Therefore, it is obvious that this surplus PHB synthesis is due to the single additional factor—the nuclear-located T7RNAP, despite T7RNAP transcripts being only detectable in ethanol-treated transformants. Explanations for this leakiness could be position effects in the nuclear genome or traces of acetaldehyde. Acetaldehyde can arise from plant metabolism (Kimmerer and MacDonald 1987), which could accidentally lead to an induction of the *alc*A regulon (Junker et al. 2003).

Therefore, we suppose two sources for leakiness of this trans-activation system acting simultaneously: on the one hand, promiscuous recognition by the nuclear encoded RNA polymerase (NEP), and, on the other hand, background activity of T7RNAP due to unspecific transcription at the *Alc*A promoter.

Differences in PHB levels as a consequence of different nuclear insertion events should also be considered. We have carried out transformations in two different sequences and observed higher PHB amounts in the two B clones. Tissue culture defects as a source for lower synthesis rate of A1 and A2 are unlikely, as we have selected the most vigorous transformants for super-transformation. Since the plastome insertion site is identical for all four genotypes, the reason for these differences most probably consists of a more advantageous localization of the nuclear component in the two B genotype transformants. The variability traces back to the agrobacterial

transformation, which can lead to a spectrum of different expression levels due to position effects resulting in silencing or even enhancement.

In conclusion, we found that amounts of PHB produced via T7RNAP induction were higher than when bacterial promoters and 5′-UTRs were used, but did not exceed PHB amounts achieved with the plastid *psb*A 5′ regulatory elements, which were used for constitutive expression (Lössl et al. 2003). Essential difficulties arising from constitutive expression were successfully resolved. Therefore, it seems feasible to deploy the chloroplast gene expression machinery for PHB production in plants and make use of its superior advantages, especially the precision of site-specific transgene integration and the decrease of unwanted gene escape to the environment. Through this ethanol-inducible trans-activation system, we obtained fertile flowers from the transformants. The problem of male sterility, early growth reduction and seed propagation was solved. Thus it was shown that the ethanol-induced PHB synthesis is functional; however, the system needs further development for economical use. By our estimation, the system can also be used for the expression of other growth-inhibiting enzymes.

Materials and Methods

Plastid transformation vector

To generate the plastid transformation vector pT7PHB-N containing the *phb* operon under the control of T7RNAP regulatory elements (Fig. 3), a 252 bp fragment was synthesized by PCR. The sequence generated contained the T7RNAP promoter, the T7RNAP 5′-UTR and the T7RNAP termination region and all the restriction sites necessary for further vector construction. These sites were, in order: *Apa*I, *Nhe*I, T7RNAP promoter and T7RNAP 5′-UTR, *Nco*I, *Eco*RV, *Eco*RI, *Nde*I, *Sac*I, *Kpn*I, T7RNAP terminator, *Bam*HI, *Sgf*I, *I-Sce*I and *Nsi*I. The restriction sites *I-Ppo*I, *Pac*I, *Nde*I, *Sac*I, *Kpn*I and *Sgf*I were included for further experiments. The 252 bp product was generated using three pairs of overlapping primers: the primer F1, a 75mer, was used in combination with F_con, a 34mer. The second pair of primers included F2, a 69mer, and R2, a 73mer. The third PCR amplification was performed using R_con, a 37mer, and R1, a 66mer. The PCRs were performed with *Pfu* polymerase. They led to three products which were combined for a final PCR amplification using the terminal primers F1 and R1. The 252 bp product generated was inserted into a pGEM vector digested with *Apa*I and *Nsi*I. The resulting construct was called pT7.

The *phb* operon was inserted in two steps. The 5′ region from *phb*C was integrated as an *Eco*RI–*Nco*I fragment from pUC_psbA_phbC3100, an intermediate vector for constitutive PHB expression (Lössl et al. 2003), to yield pT7C. The *phb*C 3′ region, *phb*A and *phb*B and the bacterial termination region were ligated into pT7C as an *Not*I–*Eco*RI fragment. The resulting plasmid pT7CAB contained a cassette bearing the *phb* operon under control of the T7RNAP promoter, the T7RNAP 5′-UTR of T7RNAP and the bacterial terminator.

In a final step, this cassette was integrated into the plastid transformation vector pKCZ (Zou et al. 2003). This vector bore the *aad*A gene leading to spectinomycin and streptomycin resistance. The resistance marker was expressed under control of the 16S rRNA promoter. The 3′ end (450 bp) of the *Chlamydomonas reinhardtii rbc*L gene

(Koop et al. 1996) was used as terminator. Vector pKCZ contained the flanking regions INSL and INSR, homologous to the respective loci *trn*N and *trn*R in the inverted repeats of the tobacco plastome (Fig. 3). The flanking sequences included nucleotides 109,230–110,348 and 110,349–111,520 of IR-A, as well as nucleotides 131,106–132,277 and 132,278–133,396 of IR-B. The *phb* operon was transferred as an *Nhe*I–*Bam*HI fragment. This fragment was introduced into pKCZ, and cut with *Xba*I–*Bgl*II to yield the final transformation vector pT7PHB-N that was 11,131 bp long. The vector backbone was pBluescript-IISK including ampicillin resistance and the origin of replication. The integrity of the reading frames of the operon created was verified by sequencing. All cloning procedures were carried out using standard methods described in Sambrook et al. (1989). Nucleotide positions for transgene insertions are given according to the plastome sequence data in EMBL GenBank accession no. Z00044 (Shinozaki et al. 1986).

Ti plasmid for nuclear transformation

As depicted in Fig. 2, the Ti plasmid contained a backbone with the *npt*II gene for kanamycin selection in plants and an ethanol-inducible regulon (Caddick et al. 1998, Salter et al. 1998, Roslan et al. 2001) in combination with the nucleotide sequence for the transit peptide of the RuBisCo small subunit as developed by Zeneca Seeds. By a further vector cloning step, we fused the RNA polymerase-coding region of bacteriophage T7 N-terminally to the nucleotide sequence of the transit peptide, for the import of the polymerase into the plastids (Coruzzi et al. 1984, Dasgupta et al. 1998). This was done with plasmid DNA clone HC-PTO-5T2 containing the T7RNAP-coding sequence, which was kindly provided by Professor Y. Gleba (Icon Genetics AG, Germany). The T7RNAP sequence was amplified by *Taq* and *Pfu* polymerases in a mixture ratio of 1 : 2. The forward primer KB1 (CATCCCGGGTGAACACGATTAACATCG) contained a terminal *Sma*I restriction site and the reverse primer KB2 with the sequence

ATTGAGCTCTTACGCGAAGTC contained a terminal *Sac*I site used for ligation into the Ti plasmid.

Plastid transformation

Nicotiana tabacum L. cv. Petit Havanna plantlets (Surrow Seeds, 4990 Sakskøbing, Denmark) were grown from seeds in vitro at 25°C (0.5–1 W m^{-2} Osram L85 W/25 Universal-White fluorescent lamps).

For particle gun-mediated transformation (Svab et al. 1990), DNA on the surface of gold particles (0.6 mm) was introduced into leaf chloroplasts using the biolistic gun DuPont PDS1000He. Selection and regeneration of transgenic calli were carried out on RMOP medium (Svab et al. 1990). After 4–6 cycles of repetitive shoot regeneration on spectinomycin and streptomycin (500 mg l^{-1} each), shoots were transferred to B5 medium (Gamborg et al. 1968) with spectinomycin for root formation.

Ethanol treatment

Transcription of T7RNAP in tobacco was induced in 3- to 4-week-old in vitro-grown plants on B5 medium containing 1.5% ethanol or by spraying with a 5% ethanol solution only once.

Analysis of transgenic lines by PCR and Southern hybridization

DNA was extracted from 100–150 mg of in vitro- or greenhouse-grown plant material using the Qiagen DNeasy plant DNA isolation kit (Hilden, Germany). PCR was carried out with the sense primer located in the transgene and the antisense primer positioned in the plastome outside the vector flanks. For Southern analysis, 3 μg of digested total plant DNA was separated on 0.8% agarose gels. Blots were prepared by transfer to nylon membranes (Hybond-N, Amersham). Specific probes were random prime labeled with α-^{32}P using Klenow fragment and hybridized to the membranes. Hybridization was carried out over-night at 65°C in hybridization buffer (0.5 M sodium phosphate, pH 7.5 and 7% SDS). Filters were also hybridized simultaneously with a probe derived from λ DNA in order to detect size marker bands. Blots were washed twice at 50°C in 0.1% SDS and 2× SSC, pH 7 for 30 min and once at 65°C in 0.1% SDS and 2× SSC for 30 min. Filters were exposed on imaging plates for 8 h and signals were detected using a phosphoimager (BAS-1500, Fuji, Tokyo).

Analysis of transgenic lines by Northern analysis

Total RNA was isolated from 30–100 mg of plant material (leaves) using the Qiagen RNeasy plant mini kit (Hilden, Germany). About 5 μg of RNA were separated on 1.2% formaldehyde–agarose gels and transferred to nylon membranes. Blotting was carried out as described by Sambrook et al. (1989). Random primed α-^{32}P-labeled DNA probes were hybridized to the membranes as described for Southern blotting. Washing of membranes was done with 0.1× SSC, 0.1% SDS at 65°C. Signal strength was determined using the phosphoimager.

Gas chromatography

PHB contents were measured in tobacco leaf material as recently described (Lössl et al. 2003, Menzel et al. 2003). PHB contents were measured by gas chromatography using a 10m-CP-WAX-52CB column with a diameter of 100 μm and 0.2 μm liquid phase. The procedure was adapted to small volumes and a short gas chromatography column according to Brandl et al. (1988). If not indicated otherwise, contents are given as proportions of DW.

Acknowledgments

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