Published online 30 March 2015

Nucleic Acids Research, 2015, Vol. 43, No. 10 ***e66***
doi: 10.1093/nar/gkv165

Boosting riboswitch efficiency by RNA amplification

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Received October 14, 2014; Revised February 17, 2015; Accepted February 19, 2015

ABSTRACT

Riboswitches are RNA sensors that regulate gene expression in response to binding of small molecules. Although they conceptually represent simple on/off switches and, therefore, hold great promise for biotechnology and future synthetic biology applications, the induction of gene expression by natural riboswitches after ligand addition or removal is often only moderate and, consequently, the achievable expression levels are not very high. Here, we have designed an RNA amplification-based system that strongly improves the efficiency of riboswitches. We have successfully implemented the method in a biological system for which currently no efficient endogenous tools for inducible (trans)gene expression are available: the chloroplasts of higher plants. We further show that an HIV antigen whose constitutive expression from the chloroplast genome is deleterious to the plant can be inducibly expressed under the control of the RNA amplification-enhanced riboswitch (RAmpER) without causing a mutant phenotype, demonstrating the potential of the method for the production of proteins and metabolites that are toxic to the host cell.

INTRODUCTION

Since most natural riboswitches control the expression of metabolic enzymes (1–6), their efficiency is limited in that they do not confer high expression levels in the on-state. It is mainly for this reason that the enormous theoretical potential of riboswitches as universal tools in biotechnology and synthetic biology has not been fully deployed. The plastid (chloroplast) exemplifies a system that would greatly benefit from the development of an efficient riboswitch-based inducible (and repressible) gene expression system. Chloroplasts represent a highly promising production platform for the inexpensive high-level synthesis of biopharmaceuticals, industrial enzymes and metabolites, in which transgene expression levels of up to 70% of the total soluble protein can be reached (7–12). Unfortunately, chloroplasts lack endogenous systems for inducible gene expression (that could be engineered to regulate transgenes) and their prokaryote-derived gene expression machinery prohibits the implementation of eukaryotic systems (that are based on transcription factors that bind small molecules). Chloroplast gene expression can be controlled via nuclear transgenes (13–15), for example, via a nucleus-encoded, ethanol-inducible and plastid-targeted T7 RNA polymerase (16). However, any technology involving nuclear genetic engineering would abrogate one of the most attractive features of plastid genome engineering: transgene containment conferred by maternal inheritance of plastids (8,12). Although pollen transmission of a transgene for an inducer protein (such as T7 RNA polymerase) does not conceivably pose an environmental risk, its introduction into the nuclear genome usually requires a selectable marker (typically an antibiotic resistance gene). Also, for a number of (largely non-scientific) reasons, not only antibiotic resistance genes but also transgenic sequences that are assumed to be neutral (in that they do not confer a selective advantage to any potential recipient organism) are undesired. Therefore, a chloroplast-only inducible expression system that does not depend on nuclear transgenes is highly desirable. Previous attempts to develop such a system have met with limited success in that some inducibility could be obtained, but the achievable expression levels were rather low (17–19).

The inducible expression of plastid (trans)genes requires switches that act at the level of translational control (20). A synthetically designed theophylline-responsive riboswitch was capable of conferring theophylline-induced translation in plastids, but its efficiency and, hence, the transgene expression level that could be reached, was very low (19). Here, we describe an RNA amplification-based system that strongly enhances riboswitch-regulated expression and its implementation as a tool for high-level inducible (trans)gene expression in the chloroplasts of higher plants

MATERIALS AND METHODS

Plant material and growth conditions

Sterile tobacco plants (*Nicotiana tabacum* cv. Petit Havana) were grown on Murashige & Skoog (MS) medium (21) containing 3% sucrose. Regenerated shoots from homoplasmic transplastomic lines were rooted and propagated on MS medium supplemented with 500 mg/l spectinomycin, then transferred to soil and grown to maturity under standard greenhouse conditions in a diurnal cycle of 16 h light at

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25°C and 8 h darkness at 20°C. Inheritance assays were performed by sowing surface-sterilized seeds on MS medium containing 500 mg/l spectinomycin.

For induction of gene expression by theophylline treatment under aseptic conditions, the MS medium was supplemented with 2.5 mM theophylline. For induction of gene expression in greenhouse-grown plants, 6-week-old transplastomic plants were watered with 200 ml of 15 mM theophylline solution for 4 consecutive days.

Construction of transformation vectors

The plastid transformation vectors constructed in this study are based on the previously described plasmids pAV6 (19), pBSU0 (22), pHK20 (23), pZF6 (24) and pRB96 (25). The nucleotide sequence of the theophylline riboswitch used for vector construction was published previously (19).

To construct a *gfp* cassette driven by T7 expression elements, the sequence of the T7 RNA polymerase terminator was added to the 3′ UTR of the plastid *atpA* gene from *Chlamydomonas reinhardtii* by polymerase chain reaction (PCR) with primer pairs CrAtpAT7Tfor and CrAtpAT7Trev (Supplementary Table S1). The PCR product was inserted into the cloning vector pUC18 as KpnI/XbaI restriction fragment, resulting in plasmid pDK190. The coding region of *gfp* was excised from vector pBSU0 (22) by digestion with the enzymes PstI and XbaI and cloned into pDK190, generating plasmid pDK191. The sequence of the T7 RNA polymerase promoter was added to the *T7g10* 5′ UTR (23) by PCR with primers T7Prog10for and T7Prog10rev (Supplementary Table S1) using pHK20 as DNA template. The resulting PCR product was cloned into pDK191 as PstI/NcoI fragment, generating plasmid pDK192.

To produce a riboswitch-controlled T7 RNA polymerase gene, vector pAV6 (19) was digested with the restriction enzymes SacI and HindIII, and the resulting P_{rrn}-*riboswitch*-*gfp*-T_{rps16} fragment was ligated into cloning vector pUC18, generating plasmid pUC18-AV6. The rRNA operon promoter (P_{rrn}) was then exchanged by the *psbA* promoter from *C. reinhardtii*. The promoter was amplified by PCR with primers CrpsbAfor and CrpsbArev (Supplementary Table S1) using *Chlamydomonas* DNA as template. The PCR product was cloned into pUC18-AV6 as SacI/BamHI fragment, producing plasmid pDK193. The T7 RNA polymerase gene was amplified by PCR with primers T7Polfor and T7Polrev using total DNA from *Escherichia coli* BL21 (DE3) as template. The PCR product was digested with NsiI and AvrII and ligated into pDK193 cut with NsiI and XbaI, generating plasmid pDK197.

To assemble the final transformation vector pME16, the T7 RNA polymerase expression cassette obtained by digestion of pDK197 with HindIII and PstI was cloned into the similarly cut vector pDK192, resulting in pME1. Subsequently, pME1 was digested with HindIII and SacI, and the released insert transferred into plastid transformation vector pRB97 cut with the same enzymes, resulting in pME16 (Figure 1). pRB97 is similar to pRB96 (25), with the only difference being the orientation of the *aadA* cassette.

For construction of a plastid transformation vector for RAmpER-inducible expression of the HIV antigen Nef, the *nef* coding region was amplified by PCR with primers neffor (replacing the start codon-containing NdeI site by NcoI) and nefrev (Supplementary Table S1) using pZF6 as DNA template (24). The resulting PCR product was digested with XbaI and NcoI, and ligated into the similarly cut vector pME16, producing plastid transformation vector pME17 (Figure 3).

Chloroplast transformation and selection of transplastomic lines

Plastid transformation was carried out using the biolistic protocol (26). Young leaves from aseptically grown tobacco plants were bombarded with vector DNA-coated 0.6 μm gold particles with a helium-driven particle gun (PDS-1000/He equipped with the Hepta adaptor; Bio-Rad). Primary spectinomycin-resistant lines were selected on an MS-based regeneration medium (26) containing 500 mg/l spectinomycin. For each construct, several independent transplastomic lines were selected and subjected to three additional rounds of regeneration on spectinomycin-containing medium to obtain homoplasmic plantlets.

Isolation of nucleic acids and gel blot analyses

Leaf tissue frozen in liquid nitrogen was used to isolate total plant DNA by a cetyltrimethylammoniumbromide (CTAB)-based method (27). Total cellular RNA was extracted using the peqGOLD TriFast reagent (Peqlab GmbH). For Southern blot analysis, samples of 5 μg total DNA were digested with the restriction enzyme BglII, separated by gel electrophoresis in 0.8% agarose gels, and transferred onto Hybond nylon membranes (GE Healthcare) by capillary blotting (28). For northern blot analysis, samples of 3.5 μg total cellular RNA were electrophoresed in formaldehyde-containing 1.5% agarose gels and blotted onto Hybond nylon membranes (29). Hybridizations were performed at 65°C in Church buffer (30). Hybridization probes 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, Düren, Germany). A 550-bp PCR product generated by amplification of a portion of the *psaB* coding region (25) was used as an RFLP probe to verify plastid transformation and assess homoplasmy. Transgene-specific probes for northern blot analyses were generated by PCR using the primers listed in Supplementary Table S2.

Protein isolation and immunoblot analyses

Total cellular protein extracts from tobacco leaves were prepared by a phenol-based method (31). The protein pellets were dissolved in 1% sodium dodecyl sulphate (SDS) and the protein concentration was determined with the BCA Protein Assay kit (Pierce, Rockford, IL, USA).

For western blot analysis, protein samples were separated by electrophoresis in 15% SDS-containing polyacrylamide gels. Afterward, proteins were visualized by Coomassie blue staining or blotted onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare). Membranes were treated with blocking buffer (20 mM Tris–HCl, pH 7.6; 137 mM

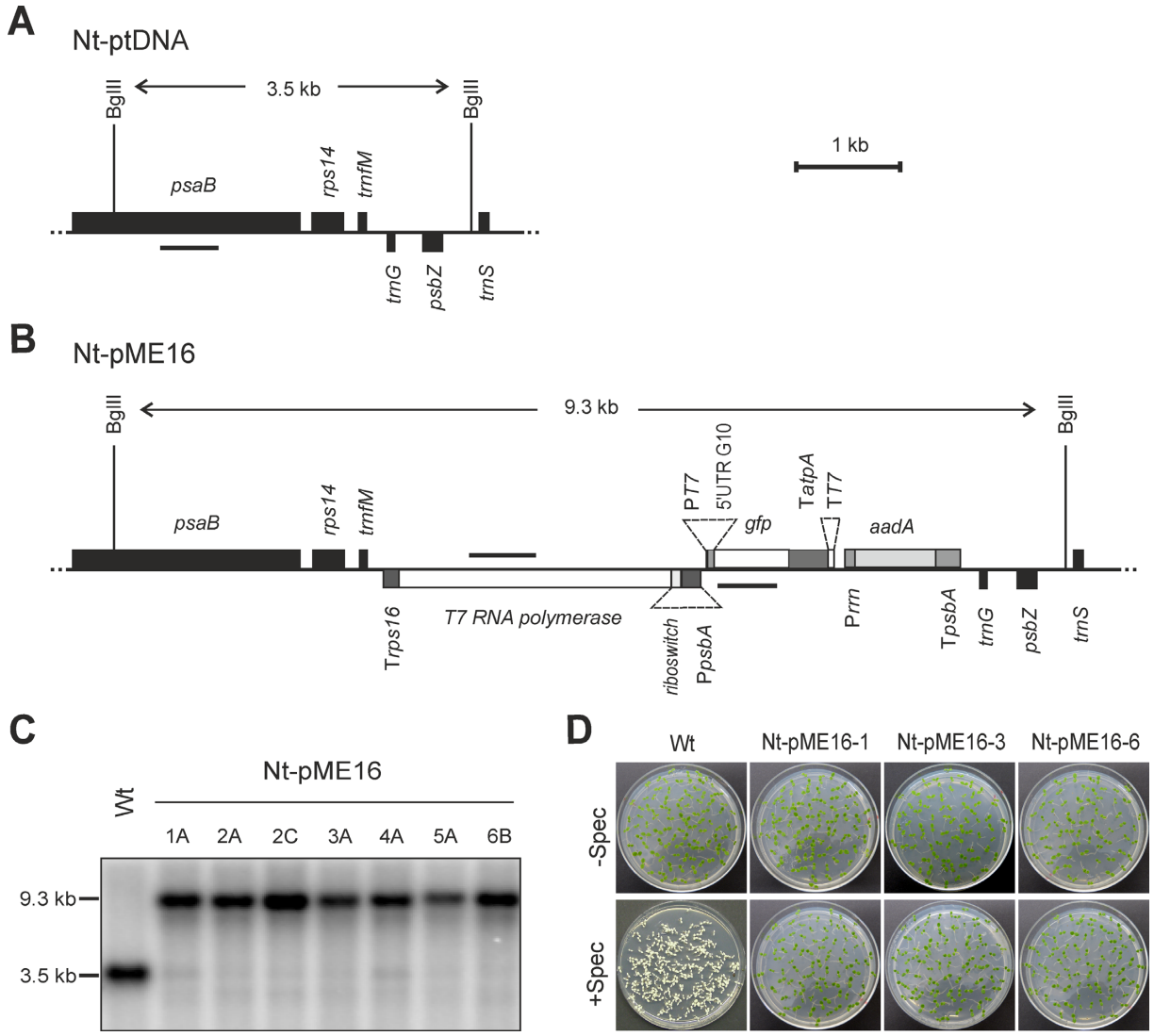

Figure 1. Generation of transplastomic tobacco (*Nicotiana tabacum*) plants that express GFP under the control of the RNA amplification-enhanced riboswitch (RAmpER). (**A**) Physical map of the region in the plastid genome to which the RAmpER-driven *gfp* construct is targeted. The transgenes are inserted into the intergenic region between the *trnfM* and *trnG* genes. (**B**) Map of the transgene-containing region in Nt-pME16 transplastomic plants. The plastid transformation vector designed for RAmpER regulation of *gfp* expression carries the selectable marker gene *aadA,* the *gfp* reporter gene and the gene for T7 RNA polymerase under the control of the theophylline-responsive synthetic riboswitch (19,32). The expected sizes of DNA fragments in RFLP analyses with the restriction enzyme BglII are indicated in panels (A) and (B). Probes used for RFLP and RNA gel blot analyses are indicated as black bars. P_{psbA}: *psbA* gene promoter; T_{rps16}: 3′ UTR from the *rps16* gene; P_{T7}: T7 RNA polymerase promoter; 5′ UTR G10: leader sequence from *gene10* of bacteriophage T7; T_{atpA}: 3′ UTR from the *atpA* gene; P*rrn*: plastid rRNA operon promoter; T_{psbA}: 3′ UTR from the *psbA* gene. (**C**) RFLP analysis of transplastomic tobacco lines generated with vector pME16. DNA samples were digested with BglII and hybridized to a probe detecting the *psaB* gene which flanks the transgene insertion site (cf. panel A). Numbers (1–6) indicated independently generated transplastomic lines, letters (A–C) designate individual regenerated plants. The faint band of wild-type size observed in the mutants most likely represents promiscuous plastid DNA in the nuclear genome (28). (**D**) Inheritance assays confirming homoplasmy of Nt-pME16 lines. Wild-type (Wt) seeds and T1 seeds from three independent transplastomic lines were germinated on synthetic medium in the presence of spectinomycin. Absence of antibiotic-sensitive progeny indicates the homoplasmic state of all three transplastomic lines. The assay was performed in five repeats for each homoplasmic line isolated.

NaCl; 0.5% bovine serum albumin (BSA)) for 1 h and then incubated with a mouse polyclonal anti-GFP antibody (JL-8; Clontech, Mountain View, CA, USA). The Nef protein was detected with a mouse anti-Nef antibody (NIBSC, Herts, UK). Detection was performed with the ECL Prime system (GE Healthcare) and an anti-mouse secondary antibody (Sigma). Purified recombinant GFP (Roche Applied Science) and Nef protein (NIBSC; Herts, UK) were used as standards for protein quantification. All western blots for protein quantification were performed in duplicate or triplicate.

Transformation of *E. coli*, induction of GFP expression with theophylline, and GFP detection by western blotting were performed as described previously (19).

Fluorescence microscopy

GFP fluorescence in seedlings was detected with a Leica stereo-fluorescence motorized microscope (MZ16 FA with

DFC 300 FX, Germany). Fluorescence was excited with light of 450–490 nm. Emitted fluorescence was monitored using a filter permeable for wavelengths >510 nm. Image analysis was performed using the LAS software version 4.2 (Leica).

RESULTS

Design of the RAmpER system

To boost riboswitch functionality in plastids and to develop a generally applicable tool for high-level transgene expression under riboswitch control, we tested the idea of introducing an RNA amplification step into riboswitch-regulated transgene expression. RNA amplification is mediated by the RNA polymerase from bacteriophage T7 which is placed under the control of the synthetic theophylline-responsive riboswitch, a translational on-switch (19,32). The transgene of interest (which, for the purpose of establishing the system, was *gfp* encoding the green fluorescent protein) is driven by the T7 promoter (Figure 1). Thus, treatment of plants with theophylline should result in induction of T7 RNA polymerase expression at relatively low levels (19), which however would be sufficient to trigger strong transcriptional activation of the *gfp* transgene. Low-level synthesis of T7 RNA polymerase is desirable, because the presence of high levels of T7 RNA polymerase in chloroplasts has been shown to cause mutant phenotypes (33). We will subsequently refer to this system as RAmpER (for RNA amplification-enhanced riboswitch).

It is important to note that the designed RAmpER switch still represents a translational riboswitch. However, the amplification of the induction signal occurs at the transcriptional level, by triggering strong RNA synthesis from the transgene of interest.

RAmpER-driven reporter transgene expression from the plastid genome

The RAmpER construct was introduced into tobacco chloroplasts by stable transformation of the plastid genome using the biolistic protocol (34). Transplastomic lines were selected on spectinomycin-containing regeneration medium and purified to homoplasmy (see 'Material and Methods' section). Plastid genome transformation, transgene integration by homologous recombination and homoplasmy of the transplastomic lines were confirmed for more than 10 independently generated lines by RFLP analysis (Figure 1C) and inheritance assays (Figure 1D). To assess RAmpER-induced transgene expression, the transplastomic plants (Nt-pME16 lines) were compared with previously generated transplastomic lines that harbor a *gfp* transgene under direct control of the theophylline riboswitch (Nt-pAV6; Figure 2). While GFP accumulation under direct riboswitch control was insufficient to be detectable by UV microscopy, theophylline induction of GFP expression by RAmpER led to strongly fluorescing plants (Figure 2A). Western blot analysis of plants shifted from theophylline-free to theophylline-containing synthetic medium confirmed high-level GFP accumulation to 1.75% of the total protein (TP) of the plant, almost 100-fold higher than upon direct riboswitch control of GFP expression (Figure 2B). The GFP expression level comes reasonably close to what has been obtained previously with constitutive expression of GFP from the strongest known chloroplast promoter (35). Northern blot analyses revealed strong transcriptional induction of *gfp* expression by theophylline, while accumulation of the T7 RNA polymerase mRNA was nearly unchanged. The latter was expected, because the theophylline-responsive riboswitch controlling T7 RNA polymerase expression acts as a translational switch (Figure 2C). However, there was a small increase in the abundance of the 3 kb full-length T7 RNA polymerase transcript after theophylline induction (Figure 2C). We tentatively attribute this to an increased RNA stability due to active translation. It is known that translating ribosomes can protect mRNAs from endoribonucleolytic attack and, in this way, enhance their stability (36).

In previous work, the theophylline riboswitch was only tested under *in vitro* culture conditions (19). To test the RAmpER system upon plant growth in soil, we analyzed the dependence of the induction of GFP accumulation on the theophylline concentration by watering greenhouse-grown plants with different theophylline concentrations for three days (Figure 2D). Both before and after watering with theophylline, the transplastomic plants were indistinguishable from wild-type plants, indicating that expression of the RAmpER construct and its induction by theophylline are phenotypically neutral (Supplementary Figure S1). Watering with 20 mM theophylline was sufficient to give full induction (Figure 2D), and inducibility by watering of large plants in the greenhouse was similarly effective as transfer of seedlings to theophylline-containing synthetic medium (~7-fold as judged by comparison with a dilution series of recombinant protein in two independent western blots; Figure 2B and E). Induction to maximum GFP accumulation levels was virtually complete 4 days after watering with theophylline solution and even the youngest leaves (that were most distant from the watered soil) were efficiently induced (Supplementary Figure S2), indicating that uptake and distribution of theophylline occur rapidly.

Low-level GFP accumulation is detectable in the non-induced state (Figure 2). This leakiness of the system could be due to one of the two plastid T7-like RNA polymerases (37) transcribing the transgene promoter or, alternatively, to some leakiness of the riboswitch (in that some translation occurs in the absence of theophylline).

RAmpER facilitates expression of a cytotoxic vaccine protein

A significant number of recombinant proteins cannot be expressed to high levels in chloroplasts because they cause severe mutant phenotypes or even lethality (e.g. 38–40). Having established that the RAmpER system strongly improves inducible transgene expression from the plastid genome, we next wanted to apply the system to facilitate the expression of a pharmaceutical protein that cannot be expressed constitutively. For this purpose, we chose the Nef protein of HIV-1 (24; Figures 3 and 4A). Nef is a non-structural HIV gene product regarded as a promising target for the development of a multi-component AIDS vaccine able to induce durable antiviral immunity (41,42). Previous attempts to express it from the chloroplast genome led to mutant

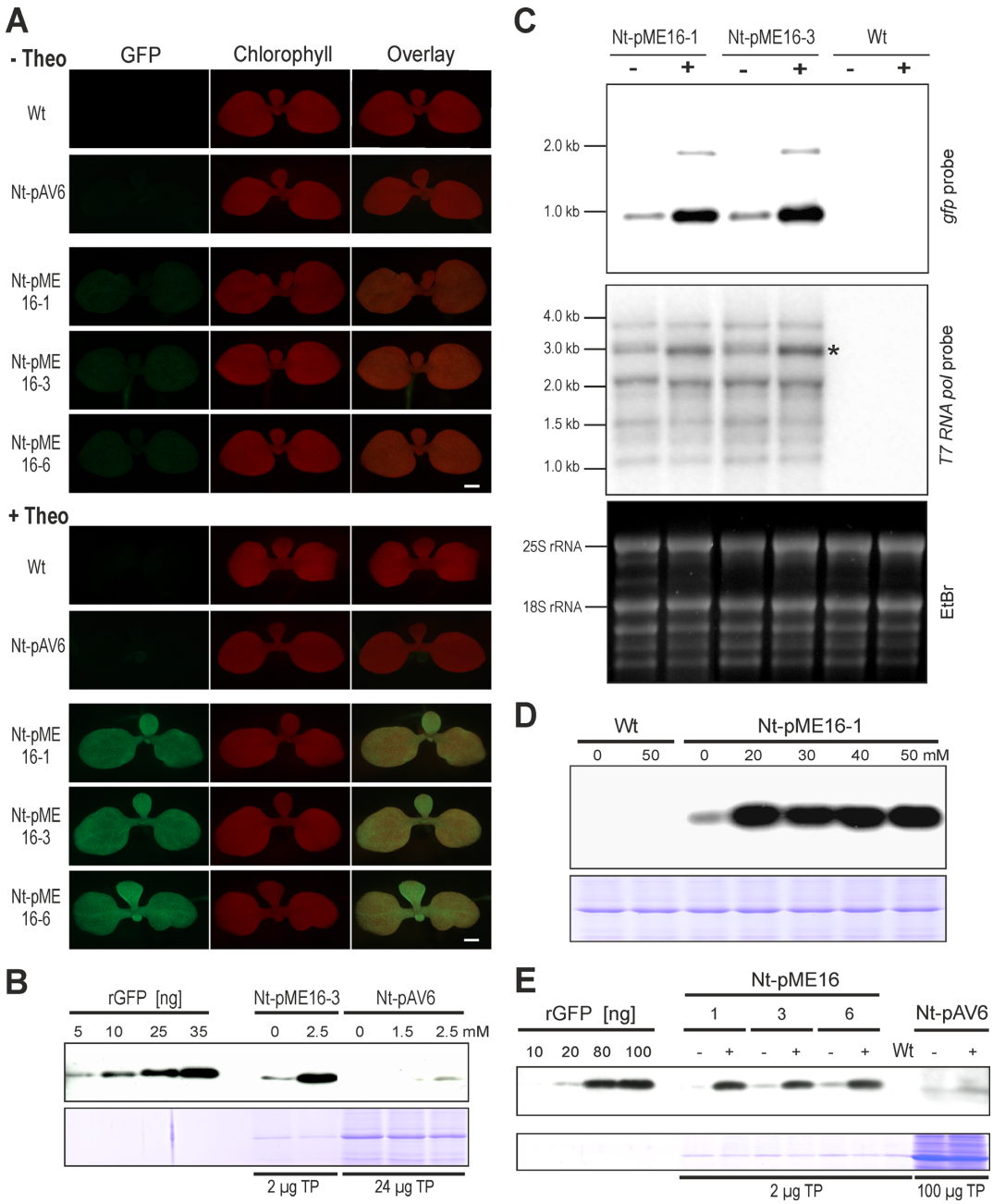

Figure 2. Theophylline-dependent GFP expression in transplastomic tobacco plants. (**A**) Induction of GFP expression in seedlings grown under aseptic conditions. Seeds from transplastomic plants expressing GFP under direct riboswitch control (Nt-pAV6; 19) or under RAmpER control (Nt-pME16; Figure 1) were germinated on synthetic medium in the absence of theophylline (− Theo), then transferred to medium supplemented with 2.5 mM theophylline (+ Theo) and grown for an additional week. GFP expression was monitored by fluorescence microscopy (see 'Materials and Methods' section; $n > 20$). Scale bars: 1 mm. (**B**) Comparison of GFP accumulation in transplastomic Nt-pME16 and Nt-pAV6 seedlings by western blotting. Protein accumulation is strongly induced (∼7-fold) after transfer to medium containing 2.5 mM theophylline in Nt-pME16 seedlings and reaches ∼1.75% of the total protein (TP) of the plant. By contrast, only a weak induction of GFP expression (of approximately 0.02% of TP) is visible in Nt-pAV6 seedlings. (Note that, from Nt-pAV6 plants, 12 times more protein was loaded.) (**C**) Induction of *gfp* expression at the mRNA level in leaves of Nt-pME16 transplastomic plants by watering with 15 mM theophylline in the greenhouse. Northern blots were hybridized to radiolabeled probes specific to *gfp* (upper panel) and the T7 RNA polymerase gene (middle panel). 3.5 µg of total cellular RNA was electrophoretically separated under denaturing conditions. The ethidium bromide-stained gel prior to blotting is shown as a control for equal loading (bottom panel). The major (full-length) mRNA of the T7 RNA polymerase gene is indicated by an asterisk. The additional 2 kb band recognized by the *gfp* probe in the induced transplastomic lines is likely due to read-through transcription, which is common in plastids (9,29). The smaller-than-expected T7 RNA polymerase transcripts are likely degradation products, the larger transcript of ∼3.7 kb likely comes from read-through transcription that terminates upstream of *trnfM* (the antisense transcript of which also folds into a stable cloverleaf-like secondary structure and therefore can act as RNA processing signal; 29). (**D**) Concentration dependence of the induction of GFP accumulation in transplastomic Nt-pME16 plants. Six-week old greenhouse-grown plants were watered with different theophylline concentrations for three days. Samples of 10 µg TP were analyzed by western blotting. (**E**) Comparison of theophylline-inducible GFP accumulation in greenhouse-grown Nt-pME16 and Nt-pAV6 plants. Leaf number 11 of 6-week old plants was harvested after 4 days of watering with 15 mM theophylline. (Note that, from Nt-pAV6 plants, 50 times more protein needed to be loaded to facilitate GFP detection.)

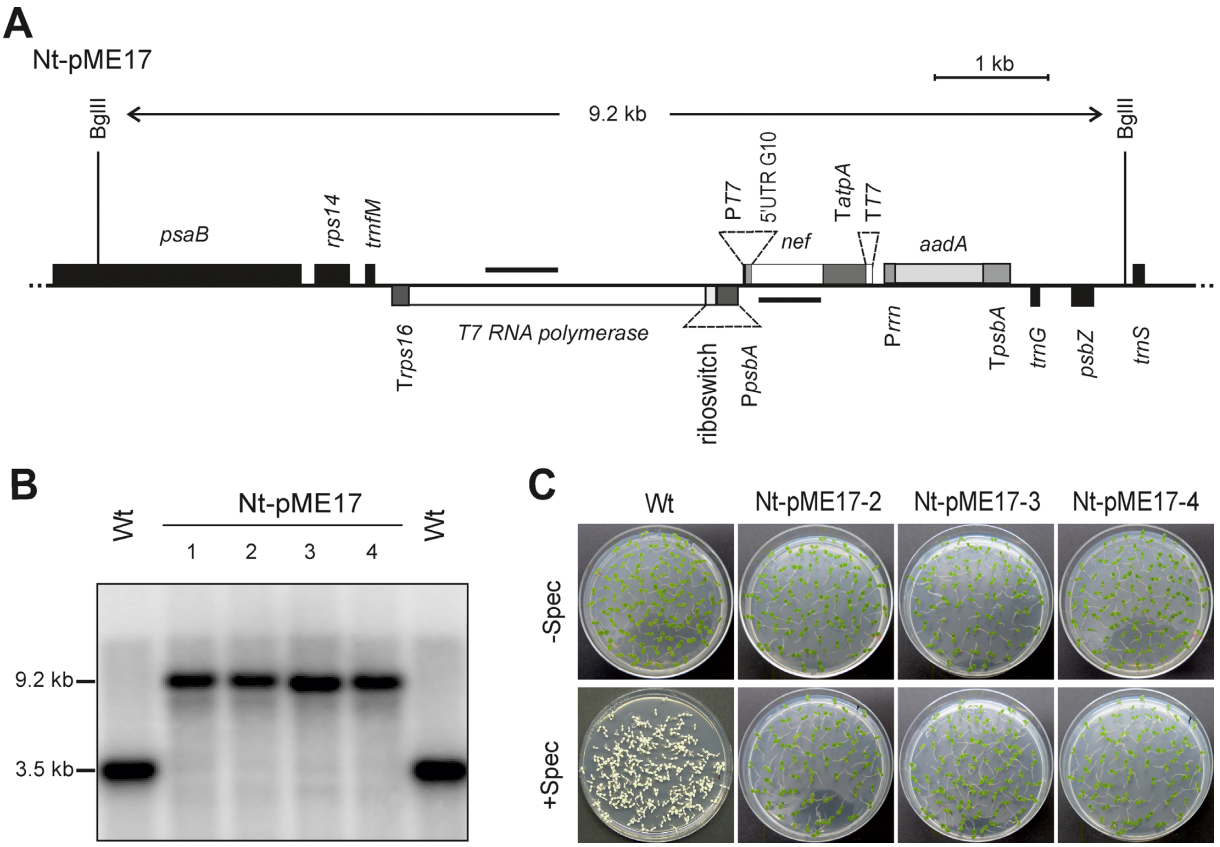

Figure 3. Generation of transplastomic plants expressing the HIV-1 antigen Nef under the control of the RNA amplification-enhanced riboswitch. (**A**) Map of the transgene-containing region in Nt-pME17 transplastomic plants. The plastid transformation vector designed for RAmpER regulation of *nef* expression (pME17) carries the selectable marker gene *aadA,* the *nef* transgene and the gene for T7 RNA polymerase under the control of the theophylline-responsive synthetic riboswitch. For a physical map of the region in the plastid genome to which the RAmpER-driven *nef* construct was targeted, see Figure 1A. The expected sizes of DNA fragments in RFLP analyses with the restriction enzyme BglII are indicated (cf. also Figure 1A). Probes used for RNA gel blot analyses are indicated as black bars, the RFLP probe is shown in Figure 1A. P_{psbA}: *psbA* gene promoter; T_{rps16}: 3′ UTR from the *rps16* gene; P_{T7}: T7 RNA polymerase promoter; 5′ UTR G10: leader sequence from *gene10* of bacteriophage T7; T_{atpA}: 3′ UTR from the *atpA* gene; P*rrn*: plastid rRNA operon promoter; T_{psbA}: 3′ UTR from the *psbA* gene. (**B**) RFLP analysis of transplastomic tobacco lines generated with vector pME17. DNA samples were digested with BglII and hybridized to a probe detecting the *psaB* gene which flanks the transgene insertion site (cf. panel A and Figure 1A). Numbers (1–4) indicate independently generated transplastomic lines. (**C**) Inheritance assays confirming homoplasmy of Nt-pME17 lines. Wild-type (Wt) seeds and T1 seeds from three independent transplastomic lines were germinated on synthetic medium in the presence of spectinomycin. Absence of antibiotic-sensitive progeny demonstrates homoplasmy of all three transplastomic lines. The assay was performed in four repeats for each homoplasmic line isolated.

plants that displayed a pigment-deficient phenotype and hardly grew photoautotrophically (24; Figure 4A), presumably due to the propensity of the protein to associate with membranes and the resulting interference with thylakoid biogenesis. A RAmpER construct for inducible Nef expression was assembled by replacing the *gfp* coding region with a codon-optimized *nef* gene (Figure 3A). Biolistic transformation of the tobacco plastid genome produced more than 10 independent transplastomic lines (Nt-pME17 lines) that were purified to homoplasmy, as confirmed by Southern blotting and seed assays (Figure 3B and C).

When grown under greenhouse conditions, Nt-pME17 plants were indistinguishable from the wild type, both in the non-induced state and after theophylline induction of transgene expression (Figure 4B and C), indicating that the mutant phenotype is fully suppressed by placing *nef* under RAmpER control. Northern blot analyses confirmed transcriptional induction of *nef* expression by theophylline (Supplementary Figure S3), as had been seen before with *gfp* (Figure 2C). Immunoblot analysis with Nef-specific antibodies revealed strong induction of Nef protein accumulation after watering with theophylline solution for four days. Interestingly, the transplastomic lines producing Nef under RAmpER control accumulate approximately three times more Nef protein in the induced state than the plants expressing Nef constitutively under the strongest known chloroplast promoter and 5′ untranslated region (5′ UTR; 24). This suggests that the RNA amplification-enhanced riboswitch does not only prevent the development of the mutant phenotype caused by Nef production *in planta*, but also significantly increases the attainable expression level. The accumulation level of Nef was lower than that of GFP (∼0.2% of the total protein), presumably because the protein is less stable than GFP. The stability of Nef can be easily improved by protecting its N-terminus. Previous work has shown that two orders of magnitude higher accumulation levels of Nef are achieved when the N-terminus of the protein is fused to a stable polypeptide (24).

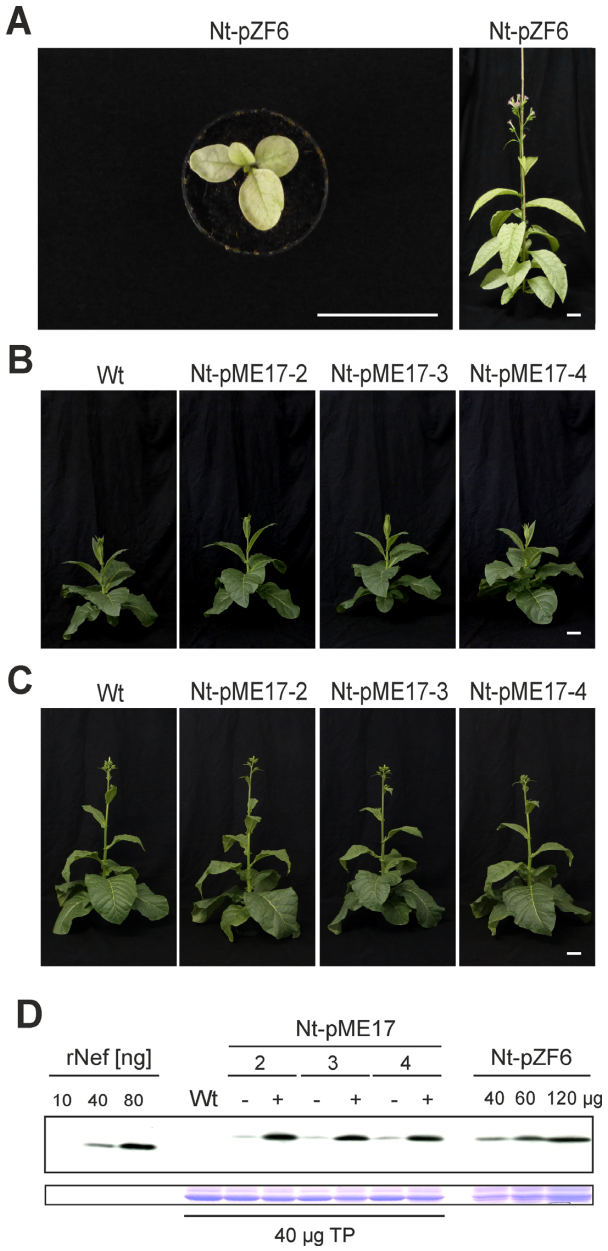

Figure 4. Phenotype of Nef-expressing transplastomic plants and inducible expression of Nef under RAmpER control. (**A**) Severe mutant phenotype of 6- and 15 week-old transplastomic tobacco plants expressing Nef constitutively (24). The plants grow extremely slowly and display a pale, strongly pigment-deficient phenotype. (**B**) Phenotype of 6-week-old transplastomic Nt-pME17 plants harboring the RAmpER-inducible *nef* expression construct. The transplastomic lines (irrigated with water) are phenotypically indistinguishable from wild-type (Wt) controls. (**C**) Phenotype of the same plants after 4 days of watering with 15 mM theophylline solution. The phenotype of the transplastomic lines remains indistinguishable from that of the wild-type control plants. Scale bars: 5 cm. (**D**) Induction of Nef accumulation in transplastomic mutants by watering with theophylline. Leaf number 11 of 6-week-old tobacco mutants was harvested after 4 days of watering with 15 mM theophylline solution and used for protein extraction and western blot analysis. For comparison, different amounts of total protein samples from a transplastomic line producing Nef constitutively (Nt-pZF6) and a dilution series of purified Nef protein (recombinantly produced in bacteria) were also loaded. Note that the inducible transplastomic lines (Nt-pME17) produce approximately three times more Nef protein than the plants expressing Nef constitutively under the strongest known plastid promoter and 5′ UTR (24).

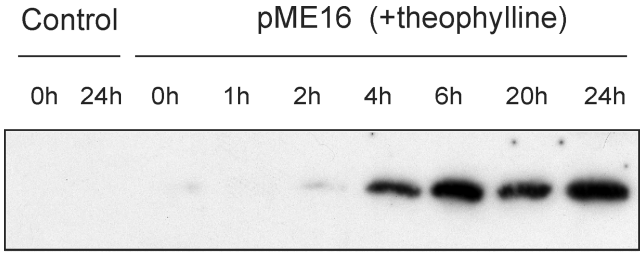

Figure 5. Test for RAmpER functionality in the bacterium *Escherichia coli*. Bacteria were transformed with plasmid pME16 and induced by addition of theophylline as described previously (19). GFP accumulation was detected by western blotting. Control: bacteria transformed with a vector harboring an unrelated insert.

RAmpER also functions in bacteria

Due to the prokaryotic origin of plastids, chloroplast promoters, translation signals in the 5′ UTR and transcript-stabilizing elements in the 3′ UTR often function to at least some extent also in bacteria (e.g. 43,44). This enabled us to test our RAmpER construct (pME16; Figure 1) directly in bacteria by transforming it into *E. coli*. Time-resolved induction experiments with theophylline (19) followed by detection of GFP accumulation by western blotting confirmed RAmpER functionality in bacteria and revealed that full induction of GFP expression is reached after ∼6 h (Figure 5).

DISCUSSION

In this work, we have designed a new molecular tool that strongly enhances riboswitch function. We show that the level of riboswitch-induced transgene expression can be enhanced by almost two orders of magnitude through introducing a simple amplification step. We have developed the technology for chloroplasts, because (i) chloroplasts exemplify a system that still requires the development of efficient tools for inducible (trans)gene expression, and (ii) chloroplasts provide a superb production platform in biotechnology (8,10,45) and also represent the most attractive target for future synthetic biology applications in plants (46). This is because their genome is small and easy to engineer by homologous recombination. Moreover, transgene expression from the chloroplast genome can produce enormous amounts of foreign protein, is not hampered by epigenetic transgene silencing and provides greatly increase gene containment due to maternal inheritance of plastids in most crops. However, in a number of cases, plastid transgene expression has caused severe mutant phenotypes, either because of extremely high overexpression levels of recombinant proteins or because of the recombinant proteins interfering with chloroplast biogenesis (9,38–40,47). The latter can be due to association of the synthesized foreign protein with thylakoid membranes which disturbs photosystem biogenesis and results in pigment-deficient plants that grow very slowly or not at all (24,39). Interestingly, although RAmpER-driven expression of the HIV-1 Nef antigen produced three times more Nef protein in the induced state than the plants expressing Nef constitutively (and suffering from a severe mutant phenotype), induction of expression by watering with theophylline did not cause a mutant phe-

notype (Figure 4). This is most likely due to Nef accumulation occurring after completion of thylakoid biogenesis, when the photosystems are fully assembled and, therefore, Nef cannot do any harm to membrane protein insertion and protein complex assembly.

A previously reported inducible expression system for plastids used the Lac repressor from *E. coli* and the chemical inducer IPTG (17,18). Since IPTG is toxic and prohibitively expensive, this system is unlikely to be useable at the commercial scale. By contrast, theophylline is an inexpensive, non-toxic compound that is naturally produced by plants (e.g. cocoa beans) and, therefore, can be used as an inducer even in large-scale greenhouse culture or in the field.

In summary, the RNA amplification-enhanced riboswitch developed in the course of this work provides a novel tool for high-level inducible transgene expression. It also provides a valuable tool for reverse genetics in that it will facilitate the functional analysis of essential genes (by withdrawal of the inducer). Thus, RAmpER will significantly expand the range of applications of the transplastomic technology. Moreover, since the system only requires a riboswitch and an RNA polymerase gene, it should be generic and easily adaptable to both other organisms (Figure 5) and other small molecules as switching agents.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Claudia Hasse and Dr Stephanie Ruf (MPI-MP) for help with plastid transformation. We are grateful to the EU Programme EVA Centralised Facility for AIDS Reagents, NIBSC, UK (AVIP Contract Number LSHP-CT-2004–503487) for kindly providing an anti-Nef antibody.

FUNDING

Max Planck Society; European Union (EU-FP7 DISCO 613513 to R.B.). Funding for open access charge: Max Planck Society.
Conflict of interest statement. None declared.

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