

Inducible gene expression from the plastid genome by a synthetic riboswitch

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Riboswitches are natural RNA sensors that regulate gene expression in response to ligand binding. Riboswitches have been identified in prokaryotes and eukaryotes but are unknown in organelles (mitochondria and plastids). Here we have tested the possibility to engineer riboswitches for plastids (chloroplasts), a genetic system that largely relies on translational control of gene expression. To this end, we have used bacterial riboswitches and modified them *in silico* to meet the requirements of translational regulation in plastids. These engineered switches were then tested for functionality *in vivo* by stable transformation of the tobacco chloroplast genome. We report the identification of a synthetic riboswitch that functions as an efficient translational regulator of gene expression in plastids in response to its exogenously applied ligand theophylline. This riboswitch provides a novel tool for plastid genome engineering that facilitates the tightly regulated inducible expression of chloroplast genes and transgenes and thus has wide applications in functional genomics and biotechnology.

chloroplast | plastid transformation | translational regulation

Riboswitches are natural RNA sensors that mediate control of gene expression via their capacity to bind small molecules (metabolites). They fold into RNA secondary structures whose conformation switches between an “on” state and an “off” state in response to ligand binding (reviewed, e.g., in refs. 1–4). Riboswitches can be divided into two distinct structural domains: an aptamer and an expression platform. The aptamer domain binds the metabolite and this triggers conformational changes in the expression platform, which either permit or prevent gene expression. Depending on the nature of the response to metabolite binding, “on” switches and “off” switches are distinguished. In bacteria, riboswitches reside mainly in the 5′ untranslated regions (UTRs) of mRNAs and an emerging theme is that anabolic genes are mainly controlled by “off” switches (5–7), whereas catabolic genes are controlled by “on” switches (8), thus providing an efficient mechanism of feedback control of metabolic pathways. In bacteria, most riboswitches act at the transcriptional level [e.g., by resolution of rho-independent transcription termination hairpins (8, 9)], but a few translational switches have also been identified (5, 7). Translational riboswitches usually function via sequestration of the ribosome-binding site (Shine-Dalgarno (SD) sequence), thereby blocking translation initiation in a metabolite-dependent manner. The increasing understanding of the functional principles of bacterial riboswitches and the possibility to produce novel aptamers by *in vitro* evolution of RNA molecules has facilitated attempts to design synthetic switches (10–12), which potentially can provide versatile tools for genetic engineering and synthetic biology applications.

While, over the past years, numerous riboswitches have been discovered in prokaryotes (13) and also a few in eukaryotes including plants (14, 15), no riboswitches are known in organellar (plastid and mitochondrial) genomes. Plastids (chloroplasts) and mitochondria are derived from formerly free-living bacteria and have retained a largely prokaryotic gene expression machinery. However, in contrast to prokaryotes and eukaryotes, chloroplasts have largely abandoned transcriptional control and switched to predominantly translational control of their gene expression

(16–18). Although some transcriptional regulation is known to occur (19, 20), its importance for determining plastid protein levels appears to be very much limited (16, 17). Translational control is much more prevalent (21, 22) and, in many cases, appears to largely override any changes that are seen in transcript abundance (17, 20). The posttranscriptional mechanisms of gene regulation in plastids are mediated by intricate plastid-nuclear interactions (18) and are still far from being fully understood. Their complexity has also hampered the development of tightly controlled inducible gene expression systems for plastids. Although recent progress has made it possible to genetically engineer the chloroplast genomes of an increasing number of higher plants (23), transgene expression from the plastid genome is usually constitutive and attempts to construct inducible gene expression systems have been afflicted with the requirement for additional nuclear transgenes and/or a substantial leakiness of gene expression in the uninduced state (24–26). This is highly unfortunate, because tightly controllable expression systems represent versatile tools in both functional genomics and biotechnology. The possibility to express transgenes from the plastid genome has stirred considerable excitement among plant biotechnologists (23, 27, 28), mainly due to the attainable enormous expression levels (29) and the increased transgene containment provided by maternal inheritance of plastids in most crops (30, 31). However, constitutive plastid transgene expression can cause severe mutant phenotypes (e.g., 32, 33) and, also for this reason, the development of tightly controllable systems for inducible gene expression is of utmost importance. Here we have explored the possibility of engineering riboswitches to function as translational regulators of gene and transgene expression in plastids.

Results

Design of Synthetic Riboswitches for Plastids. To engineer translational switches for inducible gene expression from the plastid genome, several riboswitches known to function in bacteria were selected and modified to meet the requirements of plastid translational regulation. From the relatively few translational riboswitches identified so far, we chose the *Escherichia coli* thiamine pyrophosphate (TPP) riboswitch [translational “off” switch (5, 34)] and the synthetic theophylline (theo) riboswitch [translational “on” switch (12)]. We also selected two transcriptional “on” switches and attempted to convert them into translational switches: the glycine (gly) switch (8) and the adenine (ade) switch (9) (Fig. 1 and Fig. S1). Both are from *Bacillus subtilis* and operate by a transcriptional antitermination mechanism (1, 13). The riboswitches were first engineered *in silico* to improve the translation initiation signals for expression in plastids (Fig. 1

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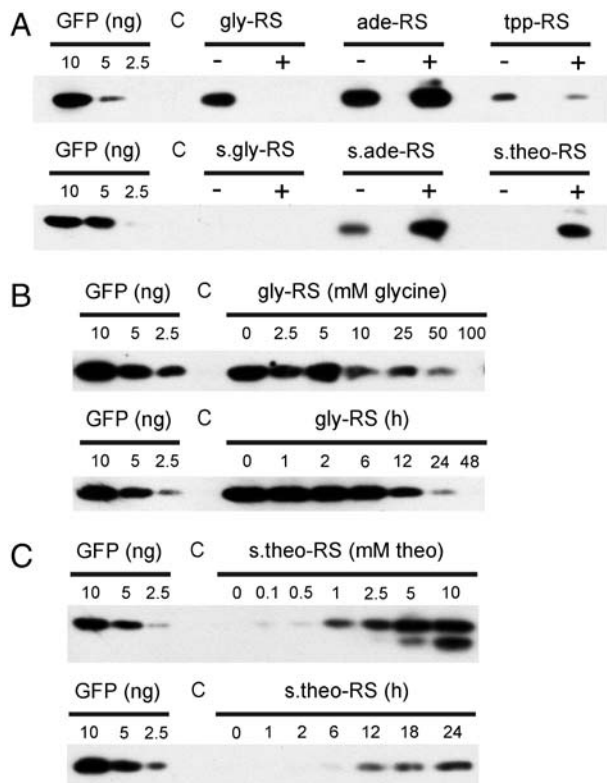


Fig. 2. Test of metabolite dependence of GFP expression from riboswitch constructs in *E. coli* by western blotting. Cells were grown in minimal medium. An *E. coli* strain harboring a luciferase gene (*51*) instead of *gfp* was used as negative control. To allow for quantitative comparisons, a dilution series of purified GFP (10 ng, 5 ng, 2.5 ng) was included in all blots. (A) GFP accumulation in the absence (–) versus the presence (+) of the regulatory metabolite. In the upper panel, 3 μ g of total soluble protein (TSP) were loaded for the gly-RS, ade-RS, tpp-RS, and the control strain (C). In the lower panel, 9 μ g TSP were loaded for the s.gly-RS, s.ade-RS, s.theo-RS, and the C strain. Cells were grown to midexponential phase. For metabolite-induced switching, the medium was supplemented with 100 mM glycine, 5 mM adenine, 1 mM thiamine pyrophosphate, or 10 mM theophylline. (B) GFP expression controlled by the glycine riboswitch. In the upper panel, the GFP accumulation in dependence on the glycine concentration is shown. The lower panel shows the time course of GFP accumulation following addition of 100 mM glycine. 3 μ g TSP were loaded in all lanes. (C) GFP expression controlled by the theophylline riboswitch. The upper panel shows the GFP accumulation in dependence on the theophylline concentration in the medium. The lower panel shows the time course of GFP accumulation following addition of 10 mM theophylline. 9 μ g TSP were loaded in all lanes.

Analysis of Riboswitch Function in *E. coli*. To analyze the functionality of the riboswitches in bacteria, we introduced the six constructs into *E. coli* cells. We first tested the switch properties on minimal medium with or without the regulatory metabolite (glycine, adenine, TPP, or theophylline). No GFP expression was detectable with the synthetic glycine switch, possibly indicating that the designed secondary structure masking the SD is too stable to be melted by metabolite binding (Fig. 2A). The natural adenine switch showed relatively poor inducibility by adenine, whereas the synthetic adenine switch responded much stronger, possibly suggesting that we have successfully converted the ade switch from a transcriptional into a translational riboswitch (Fig. 2A). To confirm the translational nature of the induction of GFP accumulation by adenine in the s.ade-RS construct, we compared RNA accumulation in the presence vs. the absence of adenine (Fig. S3A). No adenine dependence of *gfp* mRNA accumulation was detectable, confirming that the designed s.ade-RS functions at the level of translation initiation. As expected, the TPP riboswitch showed a repression of GFP accumulation in the presence

of TPP, and the s.theo-RS showed efficient and fast induction of GFP expression by theophylline (Fig. 2A and C). Unexpectedly, the natural gly riboswitch, acting as a transcriptional “on” switch in *Bacillus subtilis*, behaved like an “off” switch when expressed from our construct in *E. coli* (Fig. 2A). gly-RS switching required relatively high concentrations of glycine (10–100 mM) and became evident ≥ 12 h after metabolite addition (Fig. 2B). Analysis of a control construct lacking a riboswitch sequence confirmed that addition of none of the four metabolites appreciably affected GFP expression (Fig. S3B).

As a control, we also analyzed all riboswitch constructs in bacterial cultures grown in Luria-Bertani (LB) medium. LB is a rich medium, which contains most bacterial metabolites in significant concentrations. As expected, this resulted in loss of switchability of all riboswitches, with the notable exception of the s.theo-RS (Fig. S3C). This is because theophylline is a product of plant metabolism and, therefore, is not present in LB medium (which is produced from bacterial and yeast cell extracts).

Integration of Riboswitches into the Plastid Genome. To analyze riboswitch function in plastids, we introduced all six constructs into tobacco plants by stable transformation of the chloroplast genome (38, 39). As the functioning of riboswitches depends on a multitude of factors, including endogenous metabolite concentrations, proper RNA folding, RNA stability, and ribosome-binding to the mRNA, we decided to test also those riboswitches in plastids that were not properly regulated by metabolite addition in *E. coli*.

Plastid transformation was carried out by particle bombardment followed by selection of spectinomycin-resistant cell lines. Spectinomycin resistance is conferred by the chimeric selectable marker gene *aadA* (38), which is driven by plastid expression signals (Fig. 3A and B). For each construct, several putative transplastomic lines were obtained and subjected to additional rounds of regeneration under antibiotic selection to eliminate residual wild-type copies of the plastid genome and isolate homoplasmic lines (39). Successful plastid transformation and integration of the transgenes into the chloroplast genome by homologous recombination was verified by restriction fragment length polymorphism (RFLP) analyses (Fig. 3C). Inheritance assays revealed homogeneous populations of spectinomycin-resistant seedlings in all crosses using transplastomic lines as a maternal crossing partner, thus ultimately demonstrating that homoplasmic lines had been obtained and all wild-type copies of the plastid genome had been successfully eliminated.

Analysis of Riboswitch Function in Plastids. Having obtained stable homoplasmic transplastomic plants, we next wanted to test metabolite dependence of GFP expression from the chloroplast genome. To this end, we germinated seeds on medium with or without the regulatory metabolite and comparatively analyzed GFP accumulation by western blotting using a GFP-specific antibody. GFP was undetectable in the tpp-RS and s.gly-RS lines (Fig. 4A), although *gfp* transcripts accumulated to normal levels (Fig. 4D). This may suggest that, neither in the presence nor in the absence of the metabolite, the tpp-RS and s.gly-RS structures permit access of the plastid ribosome to the SD sequence in the 5'UTR. In contrast, GFP accumulated in a metabolite-independent manner in the gly-RS, ade-RS, and s.ade-RS transplastomic lines, suggesting that these riboswitches either do not function in plastids or, alternatively, the endogenous metabolite levels of glycine and adenine are too high to allow suppression of gene expression in the absence of exogenously supplied metabolite. Testing of a wide range of metabolite concentrations confirmed the insensitivity of GFP expression to the regulatory metabolite in these transplastomic lines (Fig. 4B). Interestingly, the s.theo-RS lines showed strong induction of GFP expression by theophylline and no background expression in the absence of the metabolite

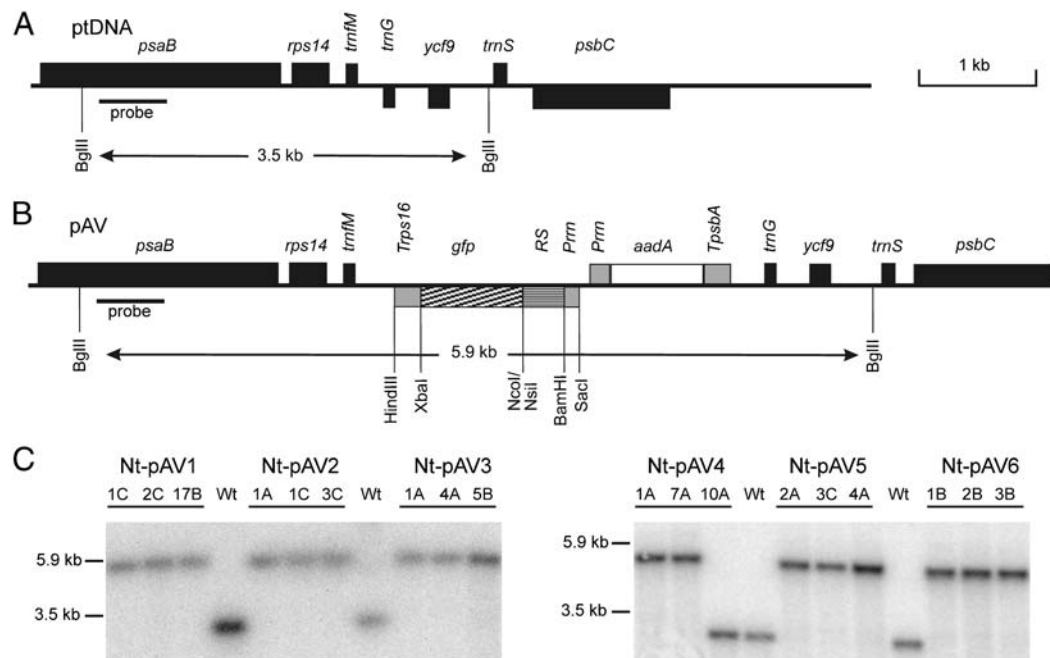


Fig. 3. Generation of plastid transformants with vectors carrying *gfp* gene constructs under the control of riboswitch elements. (A) Physical map of the targeting region in the plastid genome. (B) Structure of plastid transformation vectors of the pAV series harboring riboswitch-containing GFP expression cassettes. Relevant restriction sites are marked. The transgenes are targeted to the intergenic region between the *trnFM* and *trnG* genes (37). The GFP expression cassette consists of the ribosomal RNA operon promoter (*Prrn*) fused to the riboswitch (*RS*) element (see Fig. 1 and Fig. S1) and the 3'UTR from the plastid *rps16* gene (*Trps16*). The expected sizes of DNA fragments in restriction fragment length polymorphism analyses with the enzyme *BglIII* are indicated. The location of the RFLP probe is shown as a black bar. The selectable marker gene *aadA* is driven by a chimeric ribosomal RNA operon promoter (*Prrn*) and fused to the 3'UTR from the plastid *psbA* gene [(*TrpsbA*) (38)]. (C) RFLP analysis of transplastomic tobacco lines. Total cellular DNA was digested with *BglIII* and hybridized to a radiolabeled probe detecting the region of the plastid genome that flanks the transgene insertion site. Fragment sizes for the wild-type and the transplastomic lines are indicated. Absence of a hybridization signal for the wild-type genome indicates homoplasmy of the transplastomic lines. Line Nt-pAV4-10A shows the hybridization signal for the wild-type genome suggesting that this line represents a spontaneous antibiotic-resistant mutant. The transplastomic lines harbor the following riboswitches: Nt-pAV1: gly-RS; Nt-pAV2: ade-RS; Nt-pAV3: tpp-RS; Nt-pAV4: s.gly-RS; Nt-pAV5: s.ade-RS; Nt-pAV6: s.theo-RS.

(Fig. 4A). Induction was strongest with a theophylline concentration of 2.5 mM (Fig. 4C). Theophylline concentrations of 5 mM and higher were toxic to tobacco plants (as evidenced by aberrant growth and a pale-green phenotypic) and resulted in a concomitant decline in GFP expression. When theophylline was sprayed onto plants raised in the absence of the metabolite, nearly full induction was seen after 24 h (Fig. 4C). RNA gel blot analyses revealed theophylline-independent accumulation of *gfp* transcripts (Fig. 4D) confirming that the s.theo-RS acts as a translational switch also in plastids.

Discussion

In the course of this work, we have tested the possibility to adapt bacterial riboswitches to the gene expression system in plastids. Although plastids have a prokaryotic past, the regulatory mechanisms of their gene expression are very different from those operating in bacteria (18). Plastid gene expression is extensively regulated at the posttranscriptional level, and translational regulation can override even big changes in transcription and/or RNA stability (17). Because of the small number of natural riboswitches acting at the translational level, we attempted to convert transcriptional riboswitches into translational riboswitches. Although the s.ade-RS appears to function as a translational switch in *E. coli* at least to some extent (Fig. 2A), the limited success of this approach indicates that conversion of transcriptional into translational riboswitches is far from straightforward and much is yet to be learned about the structural and sequence requirements determining riboswitch efficiency. However, as transcription initiation from the plastid *Prrn* promoter has not been characterized in *E. coli*, and we also cannot exclude the possibility that aberrant long-range secondary structural in-

teractions prevented proper RNA folding in *E. coli* and/or in plastids; the exact reasons for the lack of metabolite responsiveness currently remain unknown.

When tested in chloroplasts of tobacco plants, the synthetic theophylline riboswitch displayed excellent switching properties. No basal expression was detectable in the absence of the metabolite and expression was rapidly inducible by application of theophylline. The tightness of the switch in both bacteria and chloroplasts may be attributable to the fact that, unlike the other regulatory metabolites tested here, theophylline is not endogenously produced by either *E. coli* or tobacco plants. The GFP accumulation level reached by induction with theophylline in tobacco chloroplasts was in the range of 0.01–0.02% of the total soluble protein (Fig. 4C). This is significantly lower than upon constitutive expression of GFP from the same promoter [which is at least 10-fold higher (40)] and may be due to the Shine-Dalgarno sequence within the theophylline switch being less efficient and/or less accessible.

The identification of a riboswitch that functions in plastids provides opportunities for both functional genomics and plastid biotechnology. The highly efficient homologous recombination system in plastids has been successfully employed to study the functions of plastid genome-encoded genes and open reading frames by reverse genetics (e.g., refs. 41–43). However, the plastid genome of higher plants contains many essential genes that are not amenable to functional analysis by targeted inactivation (i.e., knockout by insertional or deletional mutagenesis). These essential plastid genes include components of the gene expression machinery (44–46), essential metabolic enzymes (47), as well as several open reading frames of unknown function (48). The synthetic theophylline riboswitch can now be used to construct

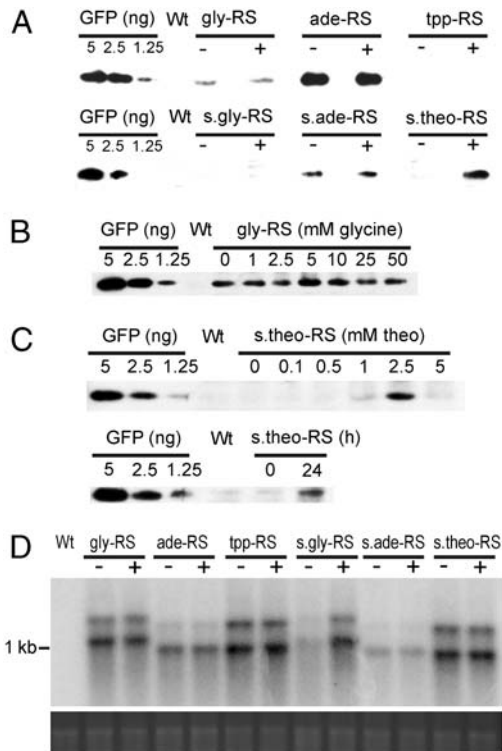


Fig. 4. Metabolite-dependent mRNA and foreign protein accumulation in transplastomic plants. (A) GFP accumulation in transplastomic tobacco plants. Total soluble protein was extracted from pools of 10–20 seedlings (28 d old) grown on medium without inducers (–) or medium supplemented with 10 mM glycine, 5 mM adenine, 0.1 mM thiamine pyrophosphate, or 2.5 mM theophylline (+). 10 μ g TSP were loaded for the gly-RS, tpp-RS, and s.ade-RS lines; 1 μ g TSP for the ade-RS; and 20 μ g TSP for the s.gly-RS and s.theo-RS lines. For comparison, a dilution series of purified GFP was included. Wt: wild-type. (B) GFP expression in transplastomic lines harboring the glycine riboswitch (Nt-pAV1) in dependence on the glycine concentration in the medium. 10 μ g TSP were loaded in all lanes. As glycine in high concentrations is toxic to plants (and results in pale phenotypes), concentrations higher than 50 mM were not tested. (C) GFP expression in transplastomic lines harboring the synthetic theophylline riboswitch (Nt-pAV6). The upper panel shows the dependence of GFP accumulation on the theophylline concentration in the medium. Induction is detectable at 1 mM theophylline and peaks at 2.5 mM. Application of 5 mM theophylline is toxic to plants and results in aberrant growth and reduced induction of GFP expression. 20 μ g TSP were loaded in all lanes. The lower panel shows the induction of GFP expression 24 h after spraying with a solution of 50 mM theophylline. (D) Analysis of metabolite dependence of mRNA accumulation in transplastomic lines harboring riboswitch constructs. Plants were grown on medium without inducer (–) medium or supplemented with 10 mM glycine, 5 mM adenine, 0.1 mM thiamine pyrophosphate, or 2.5 mM theophylline (+). Samples of 2 μ g total RNA were blotted and hybridized to a *gfp*-specific probe. The size of the *gfp* transcripts varies slightly due to length differences of the riboswitches (Fig. S2). The major band represents monocistronic *gfp* mRNA and the upper band originates from read-through transcription, as observed before with transgene inserted into the same genomic location (52).

inducible knockout lines for essential plastid genes by simply placing the target gene under the control of the s.theo-RS and transforming this construct into the plastid genome to replace the resident gene copy. Selection of transplastomic lines in the presence of theophylline will keep the essential gene active and allow isolation of homoplasmic transplastomic lines. Subsequent removal of theophylline will then allow to gradually repress the gene activity and analyze the phenotype of the plant in the absence of the essential gene product.

Transgene expression from the plant's plastid genome has unique attractions to biotechnologists (28), including the plastids' potential to accumulate foreign proteins to extraordinarily high

levels [to up to more than 70% of the plant's total soluble protein (29)] and the increased biosafety provided by the maternal mode of plastid inheritance, which greatly reduces unwanted transgene transmission via pollen (30, 31). However, constitutive expression of pharmaceutical proteins or unique metabolic pathways from the plastid genome can result in mutant phenotypes and/or severe growth retardation of transplastomic plants due to metabolite toxicities, interference with photosynthesis or disturbance of the plastid endomembrane system (e.g., refs. 32, 33). Solutions to these problems have long been sought (24–26), but progress has been hampered so far by the lack of tightly regulated inducible gene expression systems for plastids that are independent of additional nuclear transgenes. The theophylline riboswitch offers a “plastid-only” solution to inducible gene expression from the chloroplast genome that does not require additional (nuclear or plastid) transgenes and thus should be widely applicable.

Materials and Methods

Bacterial Strains and Growth Conditions. *E. coli* strains Top10F' (Invitrogen) or BL21(DE3) (Stratagene) were used for transformation with plasmid constructs. Transgenic strains were grown aerobically to midexponential phase in either LB medium or M9 minimal medium with ampicillin (100 μ g/ml) at 37 °C under continuous shaking (180 rpm). For metabolite induction of riboswitches, the medium was supplemented with 100 mM glycine, 5 mM adenine, 1 mM thiamine pyrophosphate, or 10 mM theophylline, as indicated. To this end, cultures were diluted 1:30 in 3 ml of medium and grown to midexponential phase in the presence or absence of the regulatory metabolite. To determine the time course of metabolite-dependent repression or induction of gene expression, bacteria were precultured in M9 minimal medium in the absence of the metabolite and then diluted in fresh medium. The starting cell density was set to $OD_{600} = 1$. Glycine or theophylline was added to a final concentration of 100 mM or 10 mM, respectively, and samples were taken for protein isolation at the indicated time points. Cultures were continuously diluted in fresh medium supplemented with the regulatory metabolite to keep them in the midexponential phase.

Plant Material. Sterile tobacco (*Nicotiana tabacum* cv. Petit Havana) plants were grown on agar-solidified MS medium (49) supplemented with sucrose (20 g/l). Regenerated shoots from homoplasmic transplastomic lines were rooted and propagated on the same medium, then transferred to soil and grown to maturity under standard greenhouse conditions. For inheritance assays, seeds were surface sterilized and germinated on MS medium with or without spectinomycin (500 mg/l). For analysis of riboswitch function, the medium was supplemented with 10 mM glycine, 5 mM adenine, 0.1 mM thiamine pyrophosphate, or 2.5 mM theophylline.

Cloning Procedures. All vectors containing 5'UTR riboswitches from *Bacillus subtilis* or *E. coli* are based on a pBS SK(+) vector harboring a *Prrn-5'UTR-gfp-Trps16* expression cassette (50). Details are provided in *SI Materials and Methods*. The riboswitch-containing *Prrn-5'UTR-gfp-Trps16* expression cassettes were subsequently excised with *SacI* and *HindIII* and inserted into the similarly cut plastid transformation vector pRB95, resulting in plasmids pAV4 (s.gly-RS), pAV5 (s.ade-RS), and pAV6 (s.theo-RS).

Plastid Transformation. Plastid transformation was carried out by the biolistic protocol (38) using 0.6 μ m gold particles and a helium-driven biolistic gun (PDS1000He; BioRad). Transplastomic lines were selected on regeneration medium containing 500 mg/l spectinomycin (38). Primary transplastomic lines were subjected to additional regeneration rounds on spectinomycin-containing medium to obtain homoplasmic tissue.

Metabolite Induction Experiments, Protein Extraction, and Immunoblotting. Transformed *E. coli* cells of strains Top10F' or BL21(DE3) were grown to midexponential phase in either LB or M9 minimal medium supplemented with the regulatory metabolite as indicated. Cells were diluted 1:30 into 3 ml of the appropriate medium in the presence or absence of the metabolite, further grown to midexponential phase and finally brought to an OD_{600} of 1.0 (in LB medium). Cells were sedimented by centrifugation, the pellets were frozen in liquid nitrogen, resuspended in 1,000 μ l lysis buffer (50 mM HEPES, 300 mM NaCl, 0.5% SDS, pH 8.0) and incubated for 30 min on ice. Subsequently, the cells were disrupted by sonication (amplitude 10%, 15 s; Sonifier®, W-250 D, Heinemann Ultraschall & Labortechnik) and centrifuged for 12 min at 12,000 g. The protein concentration was measured by the

bicinchoninic acid protein assay (Pierce). Plant total soluble protein was extracted from 10 to 20 pooled seedlings (28 d old) grown on agar-solidified MS medium containing 2% sucrose. To test metabolite dependence of GFP expression, plants were grown on MS medium containing 2% sucrose with or without the regulatory metabolite. Total soluble protein was extracted from samples homogenized in a buffer containing 50 mM HEPES (pH 7.5), 10 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM Pefabloc. After centrifugation for 10 min at 12,000 g at 4 °C, the protein concentration in the supernatant was determined using the Bradford assay (Roth) and known concentrations of bovine serum albumin as standard. To determine the time course of theophylline-dependent induction of gene expression, transplastomic seedlings were grown on agar-solidified MS medium containing 2% sucrose for 28 d. Theophylline was sprayed (5 ml of 50 mM theophylline dissolved in water) on the plate to a final concentration of 2.5 mM and samples were taken for protein iso-

lation after 24 h. For western blotting, protein samples were separated by electrophoresis in denaturing SDS-polyacrylamide gels and transferred to Hybond P PVDF membranes (GE Healthcare). Transfer was carried out using a semidry blotting apparatus (SEDEC-M, PeqLab) and a standard transfer buffer (25 mM Tris-HCl, 192 mM glycine, pH 8.3). Protein detection was performed with a monoclonal mouse antibody raised against GFP (JL-8; Clontech) using the ECL Plus detection system (GE Healthcare). To exclude biological or technical variation, all results were confirmed by three biological replicates.

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