



An efficient dsRNA constitutive expression system in *Escherichia coli*

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Abstract

Synthetic dsRNA are valuable tools for reverse genetics research and virus silencing applications. Its synthesis can be performed both in vivo or in vitro. Whilst the latter presents the drawback of high production cost, the former has the advantage of being less expensive and suitable for scalable production. In general, dsRNAs are obtained in vivo from *Escherichia coli* heterologous systems that require the gene for the T7 RNA polymerase inducible by IPTG. The (ds)RNAs for gene of interest are then synthesized under the T7 promoter. In this work, we present a reliable vector system that includes the insulated promoter proD for the constitutive expression of dsRNA in *E. coli* that does not require any inducer and that renders elevated dsRNA yield. In tandem, the T7 and proD promoters render the highest dsRNA yield. The accumulation of dsRNA in this system entails a high metabolic cost for the cell. Bacterial RNA extractions that included dsRNAs homologous to the *m5GFP_{er}* gene and derived from both the synthetic and constitutive promoters induce silencing of GFP expression in *Nicotiana benthamiana* 16c.

Key points

- A vector system that includes a constitutive promoter and a T7 promoter in tandem for maximizing dsRNA synthesis.
- The metabolic cost for bacteria is maximum when the two promoters are operating simultaneously and results from the accumulation of dsRNA.
- Bacterial RNA extractions from both the induced and constitutive systems that include a mGFP_{5er}-derived dsRNA are capable of silencing the GFP expression in *Nicotiana benthamiana* 16c plants

Keywords dsRNA synthesis · In vivo synthesis · Constitutive promoter · *Escherichia coli* · IPTG · D-lactose · GFP silencing

Introduction

RNA interference (RNAi) refers to the natural process of exogenous double-stranded RNA (dsRNA) silencing complementary RNAs. Since being discovered in the nematode *Caenorhabditis elegans* and then through all the eukaryotic kingdom (Fritz et al., 2006), RNAi has rapidly established as a widely used tool for reverse genetics in a variety of biological systems, including plants, humans, insects, fungi, and nematodes (Bargmann, 2001; Silva et al. 2004; Zotti et al. 2018, Dubrovina and Kiselev, 2019). Silencing dsRNAs can

be produced either internally, i.e., by transgenic expression in a given organisms, or it can be externally supplied by topical adsorption as is the case of plants, infiltration, spreading, feeding in nematodes or protozoa or microinjected and ingested as is the case of insects in the form of naked dsRNA or coated with a variety of nanoparticles (Solis et al. 2009, Simón-Mateo and García, 2011; Niehl et al. 2018). In this case, the dsRNAs have to be produced in vitro or in vivo in heterologous expression systems.

One of the most widely expressed system is the production of dsRNAs in *E. coli* HT115(DE3) (Timons et al. 2001; Tenllado et al. 2003). As the dsRNAs produced in vivo should not be biologically degraded in the cell after its synthesis, it is required a bacteria defective in the RNase III (ribonuclease III) gene, for example, the Tn5 insertion that mutated the *rnc* gene present in *E. coli* HT115 (*rnc14::Tn5*) (Takiff et al. 1989). RNase III is an endonuclease that cleaves dsRNA to yield 5' phosphates and 3' hydroxyls. It is

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required for processing of ribosomal RNA, for the regulation of a number of genes, motility, and for proper function of regulatory antisense RNAs (see details in: Ecocyc database online, Keseler et al. 2017). The DE3 designation means that the respective strains contain the λ (DE3) bacteriophage that carries the gene for the T7 RNA polymerase under control of the inducible *lacUV5* promoter. Isopropyl β -D-thiogalactoside (IPTG) is required to maximally induce expression of the T7 RNA polymerase in order to express recombinant genes cloned downstream of a T7 promoter and has the advantage that it is not consumed by the cells. However, IPTG as inducer presents some disadvantages because it is prone to metabolic stress on the bacteria in addition to the production of inclusion bodies in the cells (Dvorak et al. 2015; Slouka et al. 2019). Besides, it may be also toxic to humans (Donovan et al. 1996). Consequently, some reports, including the present work, has proposed the comparably inexpensive disaccharide lactose as alternative inducer to IPTG (Viitanen et al. 2003; Papić et al. 2018). Nevertheless, other common inconveniences of inducers appear when scaling to industrial production, in addition to the abovementioned ones, such as hypersensitivity to the inductor, expenses, or variable levels of expression (Gilman and Love, 2016). Furthermore, the addition of any additional inducer to the bacterial culture, regardless of cost or availability, introduces sources of contamination into the culture and requires additional manipulations.

Inducible promoters present some advantages, in particular in genetic research (Siegele and Hu, 1997). The constitutive promoters in *E. coli* are not as strong as the T7 promoter present in many plasmids. To overcome this inconvenience, synthetic and specifically designed promoters have been proposed by the workers (Gilman and Love, 2016; Zucca et al. 2012; Song et al. 2016; Davis et al. 2011). An insulated promoter, proD, has been reported as capable of driving a high RNA synthesis yield in downstream open reading frames (Davis et al. 2011). Constitutive bacterial RNA polymerase (RNAP) binds specifically to proD, not requiring any other external factor or inducer such as IPTG or lactose. This promoter has been included in different plasmids in order to conduct the synthesis of RNA for protein production (Alkim et al, 2016). We thought it feasible that this promoter could lead to the production of dsRNA if it were included in the direct and reverse sides of a given gene or gene segment of interest.

Novel and emergent viral diseases in crops require alternative control strategies (Velasco et al. 2020). Naked or nanoparticle-protected release of virus-derived dsRNAs have been proposed as valuable tools for plant virus control inducing the RNAi mechanisms (Mitter et al. 2017; Das and Sherif, 2020). Cucumber green mild mottle virus (CGMMV) is a threat to cucurbit cultivation worldwide (Dombrovsky et al. 2017). It was detected in Spain in the early 1980s, and

more recently, another strain, more efficient in its mechanical transmission, compromises cucurbit crops in the country (Crespo et al. 2017). Since no genetic resistance to CGMMV is yet available in commercial cucurbits, we aim to control this virus using specific dsRNAs to be topically applied on vulnerable plants. The system we describe in this work allows the production of dsRNAs for silencing this virus species or any other application for which exogenous dsRNAs may be required. Transgenic *Nicotiana benthamiana* 16c is a widely used model to assess RNAi (Ruiz et al. 1998; Bally et al. 2018). In this work, we have evaluated the ability of the constitutive proD promoter in triggering dsRNA synthesis to silence the GFP expression in this plant.

Materials and methods

Bacterial strains, media, and obtention of the vectors

E. coli Top10 (F^- *mcrA* Δ [*mrr-hsdRMS-mcrBC*] ϕ 80 *lacZ* Δ M15 Δ *lacX74* *recA1* *ara* Δ 139 Δ [*ara-leu*]7697 *galU* *galK* *rpsL*[Str^R] *endA1* *nupG*) and *E. coli* HT115(DE3) (W3110 *rnc-14::* Δ Tn10 λ (DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*])) were used in this work. Plasmid L4440gtwy, a derivative of L4440 that carries a double T7 promoter at both sides of the Gateway attR1/attR2 cloning sites, was a gift from G. Caldwell (Addgene plasmid # 11,344; <http://n2t.net/addgene:11344>; RRID: Addgene_11344) and was kept in *E. coli* DB3.1. Next, we ordered the synthesis of the 160 bp proD promoter (Davis et al. 2011) from Biocat (Heidelberg, Germany) that was supplied cloned in pUC57 and included a *XbaI* site at the 5' end. Afterwards, we performed the Gibson assembly of the proD promoter at both sides of L4440gtwy. For that, a two-step process was followed. First, we digested L4440gtwy with *KpnI* in addition to PCR amplification using the Biotools PCR master mix (Biotools, Madrid, Spain) of the proD insert present in pUC57-proD with primers K-proD-F1 and K-proD-R1 (Table S1). Gibson assembly allowed the cloning of proD at one side of L4440gtwy, obtaining plasmid L4440-proD-gtwy that was selected in *E. coli* DB3.1 cells in LB supplemented with ampicillin. In a second step, L4440-proD-gtwy was digested with *BglII*, and the proD segment from pUC57-proD was amplified using primers B-proD-F1 and B-proD-R1. The linearized plasmid and the PCR product were Gibson assembled, and plasmid L4440-2XproD-gtwy was obtained and selected again in *E. coli* DB3.1 supplemented with ampicillin. This plasmid, in addition to the double proD promoters, has upstream of these, the T7 promoter (Fig. 1a). In order to obtain a T7 promoter-free plasmid, we released the *XbaI* fragment that contained the attR1/R2 cloning sites, the double proD promoters and the bacterial selection genes *cat* and

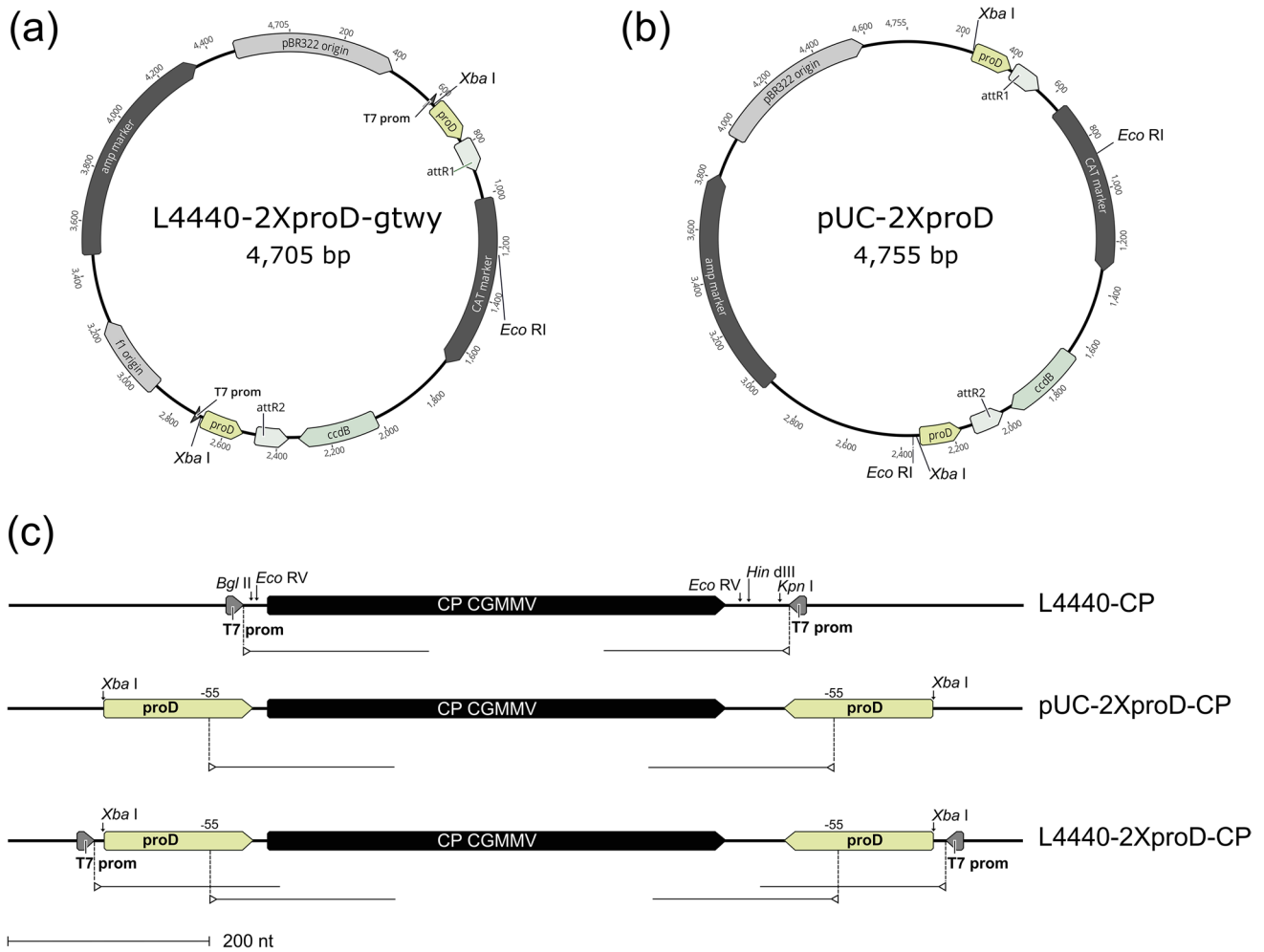


Fig. 1 Map of plasmids **a** L4440-2XproD-gtwy and **b** pUC-2XproD. **c** relative positions of the T7 and proD transcription start sites in the expression plasmids used in this work for the synthesis of the dsRNAs from the CGMMV CP gene (see text for descriptions)

ccdB and was ligated into pUC19 previously digested with *Xba*I. The resulting plasmid, pUC-2XproD, lacking the T7 promoters, was used for the transformation of *E. coli* DB3.1 and selected with ampicillin and X-gal (Fig. 1b). Correctness of these plasmids was checked by restriction analysis and Sanger sequencing. The sequences of pUC-2XproD and L4440-2X-proD-gtwy were deposited in GenBank under accession numbers MT333852 and MT333853.

Obtention of specific constructs for dsRNA production

Cucumber plants infected with CGMMV strain Cu16 (Crespo et al. 2017) were used for RNA extraction using the Sigma Plant RNA extraction kit (Sigma, St. Louis, USA). Next, the RNA was reverse transcribed using the high capacity cDNA synthesis kit using random primers according to manufacturer’s recommendations (Thermofisher, USA). A PCR fragment that included a 464 bp segment of

the coat protein (*cp*) gene of CGMMV was amplified from the cDNA using primers attB1-CGM-CP and attB2-CGM-CP (Table S1) and the Biotools PCR master mix. These primers included the attB1/attB2 flanking sequences for Gateway cloning. The resulting amplicon was then cloned in pDONR221 using the BP clonase (Invitrogen, Carlsbad, USA), thus obtaining plasmid pENT-CP that was used for the transformation of *E. coli* Top10 cells selected in LB supplemented with kanamycin. Plasmid pENT-CP that includes the attL1/L2 flanking sites was used for the LR cloning of the CP gene in plasmids L4440gtwy, L4440-2XproD-gtwy, and pUC-2XproD that allowed the obtention of plasmids L4440-CP, L4440-2XproD-CP, and pUC-2XproD-CP, respectively. These plasmids were selected in a first stage in *E. coli* Top10 in LB supplemented with ampicillin, given that HT115(DE3) are resistant to the *ccdB* toxic gene and colonies from the LR recombination cannot be directly selected in these cells. Afterwards, the plasmids obtained from Top10 cells were

Sanger sequenced for checking correctness and used to transform HT115(DE3).

In a similar way, we obtained constructions for GFP silencing. For that, we used plasmid p35S-GFP that included the *mGFP5er* gene from *Aequorea victoria* (Niedz et al. 1995). Next, we amplified a 550 bp fragment of the GFP gene included in the plasmid using primers attB1-mgfp5-ER-154F and attB2-mgfp5-ER-683R. The amplicon was cloned to pDONR221 to obtain pDNR-mgfp5-550 that was used to obtain plasmids L4440-GFP and L4440-2XproD-GFP after LR reactions with plasmids L4440gtwy and L4440-2XproD-gtwy, respectively. Plasmids L4440-GFP and L4440-2XproD-GFP were used to transform HT115(DE3) cells.

Growth of bacteria for in vivo synthesis of dsRNA

E. coli HT115(DE3) cells carrying L4440-CP, L4440-2XproD-CP, or pUC-2XproD-CP were grown overnight at 37 °C in flasks containing 50-mL LB broth (Miller) supplemented with carbenicillin (100 µg/mL). Next, the culture was diluted in LB up to OD₆₀₀ = 1.0 as determined in the GeneQuant pro spectrophotometer (Amersham, UK) and 0.5 mL of this dilution were used to inoculate flasks containing 35-mL LB supplemented with carbenicillin (100 µg/mL) and their respective inducers. Depending upon the experiment, we supplied the culture with 100-µg/mL IPTG (Sigma), 100-µg/mL D-lactose (Sigma), or none. Then, 630 min after inoculation, the culture was stopped by putting the flasks on ice followed by the harvest of the cells in falcon tubes for immediate processing or storage at –20 °C. OD₆₀₀ was the parameter used for estimating the bacterial growth with time. For that, OD₆₀₀ values were determined at regular intervals of 90 min and plotted against time. The growth curves were fitted to the four-parameter (4P) logistic curve, defined as (Zwietering et al. 1990): $y(t) = Y_{max} + \left[\frac{Y_{max} - Y_{min}}{1 + \left(\frac{t}{C}\right)^h} \right]$, where Y_{max} represents the maximum theoretical growth value, Y_{min} is the baseline, C is the inflection point (IP) and h is the Hill coefficient that represents the slope of the curve at the inflection point. The values of the four parameters were estimated using the solver add-in in Microsoft Excel. For comparison of the growth curves in the different conditions, we used the inflection points and the slopes. Alternative culture cycles were set up to solve the inconvenience of D-lactose metabolism when used as inducer. For that, culture flasks of 50 mL were inoculated with 1 mL of OD₆₀₀ = 2.0 from overnight culture and grown for 360 min. Culture media were supplemented with the antibiotics, D-lactose, or IPTG in the same conditions as the long cycle cultures. Cells

were collected by centrifugation and frozen or used immediately for RNA extractions.

Total RNA and dsRNA extractions and quantitation

For total RNA extraction, 10-mL cells were collected in falcon tubes and then centrifuged at 8000 g for 10 min at 4 °C. The pellet was resuspended and mixed in 2-mL Trisure reagent (Bioline, Memphis, USA) and allowed to stay for 5 min at room temperature. Next, 0.4 mL of chloroform was added to each sample, hand agitated, and let at room temperature for 3 min. After that, the mixture was centrifuged at 12,000 g for 10 min at 4 °C, and the supernatant was transferred to clean tubes. Total RNAs were precipitated using ethanol and resuspended in DEPC water, followed by quantitation using the Nanodrop ND-1000 (Wilmington, DE, USA). For dsRNA purification, total RNA extracts were digested with DNase I (1 U) (Sigma) and S1 nuclease (2 U) (Takara, Otsu, Japan) for 5 min at 37 °C and further recovered by precipitation with cold ethanol, NaCl 0.05 M, and glycogen (Sigma). Next, the dsRNAs were resuspended in DEPC water, quantified with the Nanodrop, and visualized in 2% agarose gels stained with RedSafe (Intron Biotechnology, South Korea). Comparison of full-length CP dsRNA yield in cultures was made after agarose gel electrophoresis and image density analysis of dsRNA bands with GelAnalyzer 19.1 (www.gelanalyzer.com, Lazar, I.). Five biological replicas were used in these experiments for each condition.

Biomass and cytosolic protein determination

Cells from 5 mL of the flask cultures were harvested for biomass measurement after centrifugation at 4 °C and dried in Eppendorf tubes in the Accublock (Labnet, Madrid, Spain) for 30 min at 80 °C. For protein determination, the pellets were resuspended in 20 µL of lysis buffer consisting of 0.1% SDS and 0.2 N NaOH and heated at 80 °C for 3 min. Debris-free supernatants were recovered by precipitation of the cell lysate at 12,000 × g for 5 min at 4 °C. Protein determination was done following the Bradford assay protocol (Sigma). For that, we diluted 5 µL of the cytosolic extracts in 495 µL of water and add 500 µL of Bradford reagent for colorimetric OD reading at 595 nm in the MultiSkan spectrophotometer (Thermo Fisher, Ratastie, Finland). Serial dilutions of 1 mg/mL of bovine serum albumin (Sigma) used as a reference were prepared for the protein standard calibration and calculations.

Northern blot of dsRNAs

DsRNAs (2 µL of total RNA extracts) treated with nuclease S1 and DNase I were denatured in MOPS 10X:formamide:formaldehyde solution (1:1.6:5) for

10 min at 65 °C followed by electrophoresis in a denaturing 6% formaldehyde and 1.5% agarose gel (MOPS 1X). Next, the denatured dsRNAs were transferred by capillarity onto a positively charged nylon membrane (Roche, Mannheim, Germany) in 10 X SSC buffer (1.5 M NaCl, 0.15 M sodium citrate) using the 785 Vacuum Blotter (Bio-Rad). Membranes were UV cross-linked and hybridized at 50 °C with a digoxigenin-dUTP (Roche)-labeled DNA probe obtained with the CGMMV specific primers CP5771F and CP6332R, using plasmid L4440-CP as template for the PCR (Table S1). To detect the hybridization bands by chemiluminescence, we followed manufacturer's recommendations (Roche).

RT-qPCR quantitation of dsRNA production in HT115(DE3) cells

We aimed to quantify the specific *cp* dsRNAs by RT-qPCR. For that, the RNA extractions (100 ng) obtained from HT115(DE3) cells harboring L4440-CP, L4440-2XproD-CP, or pUC-2XproD-CP supplemented with lactose or IPTG were reverse transcribed with the high capacity reverse transcriptase kit (Thermo Fisher) in 20 µL of final reaction volume. Prior to cDNA synthesis, the RNAs were denatured with the random primers for 5 min at 95 °C. A standard curve was prepared to estimate the number of dsRNA molecules (Figure S3). For that, we linearized the L4440-CP plasmid in independent reactions with either *Bgl*III or *Sal*I. The linearized plasmids were purified and used as templates in a single reaction for dsRNA synthesis using the HiScribe T7 high yield RNA synthesis kit (NEB, Ipswich, USA) (Figure S3a). For plasmidic DNA removal, the synthetic dsRNA was treated with DNase I for 10 min at 37 °C and purified by precipitation. Synthetic dsRNA (100 ng) was serially diluted in MilliQ and reverse transcribed, as described above, for obtaining the qPCR standard curve. qPCRs were performed in white 96-well PCR plates using the Bio-Rad iQ5 Thermal cycler. Each reaction (20-µL final volume) contained 2 µL of cDNA, 10 µL of KAPA SYBR Green qPCR mix (KAPA Biosystems, Wilmington, USA), and 500 nM of primers CP197F and CP305R (Table S1). The cycling conditions consisted of an initial denaturation of 3 min at 95 °C and 40 cycles of 30 s at 94 °C and 15 s at 60 °C. The efficiency (*E*) of the amplifications was calculated from the standard curve using the equation: $E = 10^{(-1/\text{slope})}$. In percentage, it is expressed by the formula $\% = (E - 1) \times 100$. Considering the molecular weight of the synthetic dsRNA, the number of dsRNA copies in each dilution could be calculated. For the quantitative analysis, we used six biological and three technical replicates per sample. The absolute number of CP-dsRNA molecules in each sample was thus inferred by interpolation in the standard curve (Figure S3b).

Application of GFP-dsRNA to *N. benthamiana* 16c plants

Plants of *N. benthamiana* 16c carrying a single *35S::mGFP5er::nos* transgene (Ruiz et al. 1998) were used for the subsequent experiments. Total RNA that included GFP-specific dsRNAs were obtained from crude extracts of HT115(DE3) cells harboring plasmids L4440-GFP and L4440-2XproD-GFP (Fig. S6). For that, cells carrying L4440-GFP were grown overnight and the next day, 1 mL was used to inoculate 30-mL LB supplemented with ampicillin. When cells reached 0.5 OD, IPTG was added as inducer to the culture. In addition, an overnight culture of cells harboring L4440-2XproD-GFP was used to inoculate 30 mL of LB and ampicillin and let grown without inducer. Cells were harvested 8 h after inoculation, and the total RNAs were extracted with as described above and quantified.

Total RNA extracts (30 µg/leaf) were applied to plants of *N. benthamiana* 16c using an artist airbrush (2.5 bar) covering 4 cm² in the center of the leaf. Five plants (replicas) were used for each assay that was grown in a growth room at 25 °C and 16 h/8 h light/dark cycles. GFP silencing was examined using a fluorescent black UV light lamp (40 W), and pictures were taken with a Sony Nex-5 camera using long exposure (3 s) and Ø49 mm Tiffen filters green 11 and yellow 12.

Quantitative and statistical analysis

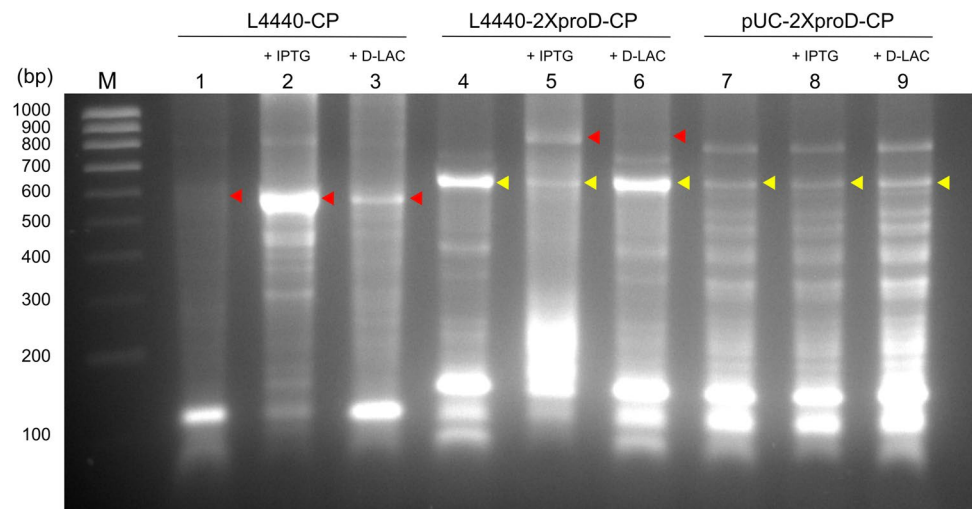
HT115(DE3) cells harboring plasmids L4440-CP, L4440-2XproD-CP, or pUC-2XproD-CP were grown in LB and induced with IPTG, lactose, or none. Bacterial dry weight, cytosolic protein, total RNA, and dsRNA, in addition to the growth parameters, were quantified, and the results were compared using ANOVA followed by mean separation using the post hoc Tukey HSD tests as available in JAMOMI v.1.2.12 statistical suite (Jamovi Project, 2020: <https://www.jamovi.org>).

Results

DsRNA is synthesized in *E. coli* HT115(DE3)

A dsRNA band of the expected size (~570 bp) was observed in RNA extracts from HT115(DE3) cells that harbor plasmid L4440-CP and were induced by IPTG or lactose, while no dsRNAs were observed in non-induced cells (Fig. 2, lanes 1, 2, and 3). In addition to the high molecular weight dsRNA, low molecular weight dsRNAs were observed, as the probable result of incomplete extension of RNA synthesis. Cells carrying plasmid L4440-2XproD-CP without inducer, showed a main dsRNA band of ~640 bp,

Fig. 2 Agarose gel (1.5%) of total RNAs treated with DNase I and S1 nuclease showing the dsRNA bands produced in HT115(DE3) cultures harboring the different plasmids and supplemented with IPTG, D-lactose, or none. M: molecular weight marker (NZYDNA Ladder V). A double dsRNA band appears in lane 5 because of the simultaneous synthesis of dsRNA driven by the T7 and proD promoters. Triangles indicate the predicted sizes of the CP dsRNAs according to the T7 promoter (red) or proD (yellow)

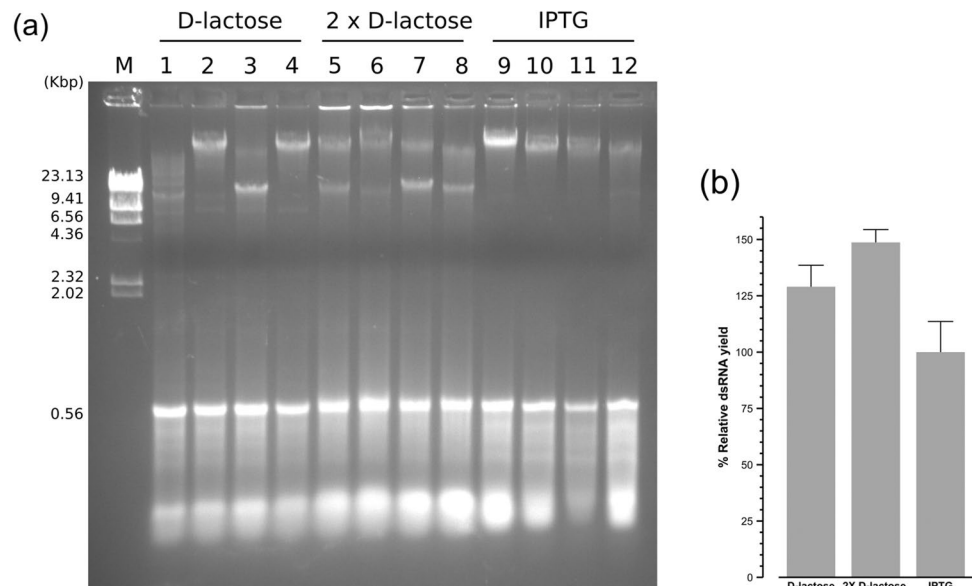


corresponding to RNA synthesis driven by the constitutive proD promoter that leads transcription start at position -55 (Fig. 1c; Fig. 2, lane 4). When IPTG was included, two high molecular bands appeared, because of the simultaneous expression by the T7 promoter (~850 bp) that is now upstream the proD promoter, and by the proD promoter (~640 bp) when induced by IPTG (Fig. 2, lane 5). In the presence of D-lactose, the dsRNA band corresponding to the proD promoter is intense, and the band corresponding to the T7 promoter results barely visible. (Fig. 2, lane 6). It can be observed in the figure (lane 5) that a significant fraction of low molecular weight dsRNA bands appeared, plausibly because of synthesis of incomplete RNA molecules when the T7 and proD promoters are in tandem. In HT115(DE3) cells harboring plasmid pUC-2XproD-CP, a dsRNA of the expected size appeared (~640 bp),

in addition to a ~800 bp dsRNA band that may be the product of extended termination of RNA synthesis as the plasmid lacks RNAP terminator sequences (Peters et al. 2011). Besides the high molecular weight dsRNAs, there is a collection of small dsRNA molecules possibly product of premature terminations of RNA synthesis (Fig. 2, lanes 7, 8, and 9). Top10 cells that have a functional RNase III that degrades dsRNA, were unable to produce dsRNA with any of the plasmid constructs regardless the use of inducers (not shown) Fig. 3.

We also compared the induction of dsRNA synthesis by IPTG and D-lactose in HT115(DE3) cells harboring L4440-CP when the culture cycles were short (360 min) and inoculated with high bacterial density (Fig. 3a). On the contrary to the extended (630 min) cultures, in short-time cultures, D-lactose showed a stronger inducer activity of the

Fig. 3 **a** 1% agarose gel electrophoresis of dsRNA produced in *E. coli* HT115(DE3) carrying L4440-CP induced by either D-lactose (1–4), D-lactose supplemented twice (5–8), and IPTG (9–12) in cultures growing for 360 min starting with high density of inoculum; M: lambda *Hind*III molecular weight marker. **b** Relative dsRNA yield from the densitometry quantitation of the 0.6 Kbp dsRNA bands with respect to IPTG as inducer (100%)



T7 RNA polymerase than IPTG. When D-lactose was supplied at the start of the culture, dsRNA amount was 29% higher than that of IPTG (Fig. 3b). Moreover, when we supplied D-lactose again at $t = 180$ min, dsRNA production was 48% higher than that of IPTG.

DsRNA yield in *E. coli* HT115(DE3)

In order to check that the dsRNA bands, including those of low molecular weight, that appear in the extractions are produced by specific synthesis and correspond to the CP gene of CGMMV, a northern blot was performed (Fig. 4). Prior to electrophoresis, samples were denatured with formaldehyde and formamide at 65 °C as described above. The electrophoresis was run in 6% formaldehyde, 1.5% agarose gel. After the hybridization with the CP probe, strong chemiluminescent signals appeared in all the samples, except for the sample corresponding to L4440-CP without inducer. There appear some bands of intense signal that correspond to the high molecular weight dsRNA bands observed in agarose gels, but the strongest hybridization signals with the specific CP probe corresponded to the low molecular weight dsRNA bands observed in the gels (~150–200 bp). A parallel northern blot run in denaturing 0.9% formaldehyde agarose but with no previous denaturalization of dsRNAs, the lane corresponding to L4440-2XproD-CP induced with IPTG, showed the strongest signal that covered a wide range of RNA/dsRNA sizes (Figure S2, lane 5).

In another approach, we compared the dsRNA yield in the different conditions by RT-qPCR that allowed us to determine the number of dsRNA molecules obtained per mL of culture (Fig. 5). As expected, dsRNAs were obtained from HT115(DE3) cells harboring L4440-CP when the inducers were included in the media, and only a scarce number of molecules were obtained in their absence. HT115(DE3)

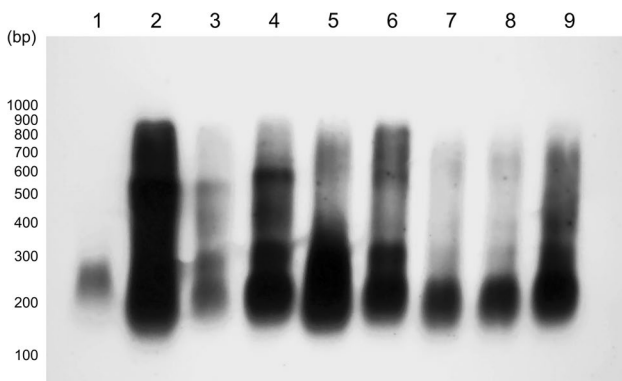


Fig. 4 Northern blot of denatured dsRNA after 6% formaldehyde gel electrophoresis. Lanes: (1) L4440-CP; (2) L4440-CP + IPTG; (3) L4440-CP + D-lactose; (4) L4440-2XproD-CP; (5) L4440-2XproD-CP + IPTG; (6) L4440-2XproD-CP + D-lactose; (7) pUC-2XproD-CP; (8) pUC-2XproD-CP + IPTG; (9) pUC-2XproD-CP + D-lactose

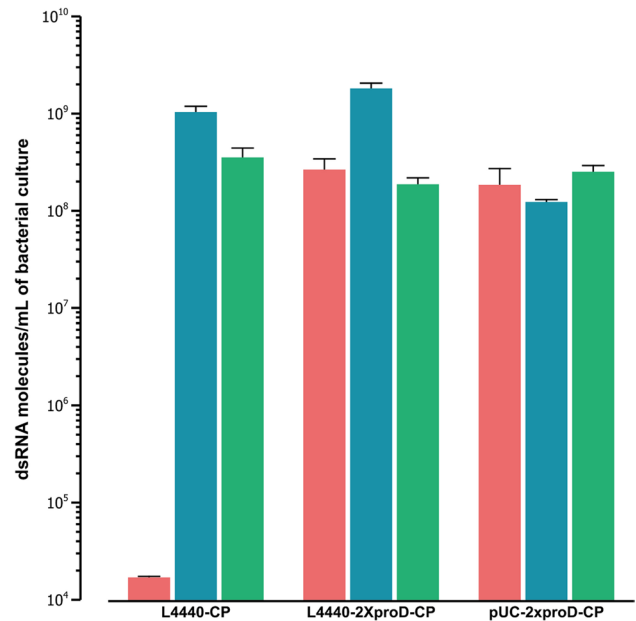


Fig. 5 RT-qPCR quantitative analysis of dsRNA molecules produced by HT115(DE3) cells per mL of culture at $t = 630$ min with different plasmids and inducers. No inducer (red), IPTG (blue) and D-lactose (green). Error bars represent the standard error of the means

cells carrying L4440-CP produced 9.36×10^9 dsRNA molecules per mL of cells when induced with IPTG, three times more than lactose-induced cells in these culture cycles. When HT115(DE3) cells carried the L4440-2XproD-CP with both types of promoters in tandem, dsRNA yield was 2.4×10^8 , 1.6×10^9 molecules/mL, and 1.7×10^8 molecules/mL for no inducer, IPTG, and lactose, respectively. Finally, when the constitutive promoter was the only available for dsRNA production in pUC-2XprpD-CP, yield was in the same magnitude order irrespectively the use of inducers (1.7×10^8 – 2.3×10^8 molecules/mL).

Given that at $t = 630$ min, IPTG-induced cells have not reached the growth of other cultures when harboring L4440-CP or L4440-2XproD-CP (Fig. 6a); we estimated dsRNA yield considering the mg of bacterial dry weight (biomass). When the production of dsRNA was referred to molecules per mg, in IPTG-induced HT115(DE3) cells yield was 3.94×10^7 , 8.86×10^7 , and 3.06×10^6 molecules/mg for L4440-CP, L4440-2XproD-CP, and pUC-2XproD-CP, respectively. In the absence of inducer, L4440-CP dsRNA production was negligible whilst L4440-2XproD-CP and pUC-2XproD-CP yielded 7.75×10^6 and 4.60×10^6 molecules/mg, respectively.

Another estimation of dsRNA production was focused on the molecules corresponding to full-length CP segment. For that, we perform the quantitation of the dsRNA bands using known amounts of a DNA molecular weight marker and a gel analysis software package (Supp. Figure 4). The

result showed that the amount of the ~ 570 bp dsRNA in cells carrying L4440-CP in IPTG-supplemented medium was 62-ng dsRNA/ μ g total RNA. Calculated in terms of the bacterial culture, we obtained 1.24- μ g dsRNA/mL of bacterial culture. When D-lactose was used, the result was 51-ng dsRNA/ μ g total RNA, equivalent to 1.02- μ g dsRNA/mL. In absence of inducers, the constitutive synthesis driven by L4440-2XproD-CP was 39-ng dsRNA/ μ g total RNA for the band of ~ 640 bp, or 0.78- μ g dsRNA/mL of culture. In the presence of IPTG, the bands corresponding to the constitutive (~ 640 bp) and inducible (~ 850 bp) promoters were 14.8-ng/ μ g total RNA and 13-ng/ μ g total RNA, respectively (0.28 μ g/mL and 0.25 μ g/mL, respectively). Finally, the expression of L4440-2XproD-CP in the presence of D-lactose was 37-ng/ μ g total RNA, although there is hardly any production from the inducible promoter (7-ng dsRNA/ μ g total RNA). To remove bacterial endogenous nucleic acids and the incomplete dsRNAs or ssRNAs product of promoter-directed synthesis, RNA extracts were treated with a high concentration of the S1 nuclease. The result showed bands corresponding to intact dsRNA products, although there were losses due to the action of the nuclease (Sup. Fig. S5).

Growth dynamics and metabolic parameters in *E. coli* HT115(DE3)

Growth curves of the bacteria showed differences depending upon the plasmid and inducer used in the media (Fig. 6a). Statistical support came from the comparison of inflection points (IP) (Fig. 6b) and slopes (Fig. 6c) of the growth curves. In cells harboring L4440-CP, non-induced cells grew at a higher rate than induced cells, and IPTG-induced cells grew at a lower rate than lactose-induced cells. Cells harboring vector L4440-2XproD-CP that includes the two types of promoters showed significant delayed growth compared with the other conditions. In particular, IPTG-induced cells delayed the growth at maximum (lowest slope and IP), plausibly indicating that the dsRNA production imposes elevated metabolic stress because of the simultaneous production of dsRNA driven by both types of promoters. In HT115(DE3) cells that produce dsRNA under the constitutive promoter present in pUC-2XproD-CP, growth parameters showed non-significant differences among the different conditions.

Two additional parameters were compared at the terminus of the cultures, cytosolic protein, and total RNA (Fig. 7). The lowest protein and total RNA in HT115(DE3) cells production corresponded to cells harboring L4440-2XproD-CP when inoculated with IPTG, in agreement with the growth curve. Strikingly, cells that carried only the proD promoter did not decrease their protein production relative to cells carrying the other plasmids; however, total RNA production

was significantly reduced (Fig. 7). Finally, Top10 cells harboring the plasmid with the T7 and the proD promoters grew in a similar manner to non-induced HT115(DE3) cells carrying L4440-CP and at a higher rate than HT115(DE3) cells carrying L4440-2XproD-CP when supplemented with lactose (Figure S1).

GFP silencing in *N. benthamiana* 16c plants with bacterial RNA extracts

RNA extractions that included dsRNAs homologous to the *mGFP5er* gene were applied onto *N. benthamiana* 16c plants. Silencing of the GFP fluorescence was observed with RNA extractions from IPTG-induced HT115 cells harboring L4440-GFP (Fig. 8B–E) and non-induced L4440-2XproD-GFP (Fig. 8F–H). Control plants supplied with RNA extracts from non-induced cells harboring L4440-GFP did not show GFP silencing (Fig. 8A). Besides, GFP silencing was not observed in plants supplied with a mock control that consisted of RNA extracts from IPTG-induced cells harboring L4440-CP (not shown). We observed that GFP silencing started in plants 3 days post application (dpa) when the L4440-GFP RNA extraction was used, reaching other parts of the plant at 4–5 dpa. Leaves, in which the RNA extract was applied, were fully silenced on average at 6 dpa. Instead, when applying RNA extractions from non-induced cells harboring L4440-2XproD-GFP, silencing started at 5 dpa, reaching other parts of the plant after 8 dpa. GFP fluorescence resulted silenced on the whole leaf used for the application of the RNA extract at 10 dpa. At 25 dpa, plants inoculated with both RNA extractions showed silencing even at the flowers. Thus, a delay of 3–4 days was observed when applying RNA extracts derived from cells harboring L4440-2XproD-GFP with respect to L4440-GFP RNA extracts.

Discussion

RNAi-mediated control of pests and diseases in agriculture faces a number of challenges that include the production of dsRNA and siRNA, the application of these to the plants and the delivery using, e.g., nanoparticles and surfactants that may favor the entry of the active silencing components into the plant (Das and Sherif 2020; Christiaens et al. 2020).

Expression of dsRNA in bacteria offers several advantages, including scalability and lower cost than in vitro production. Hence, we came up with the idea of a constitutive bacterial production system of dsRNA, which does not need an inducer. Once the system was developed, we wanted to quantify dsRNA production by several methods. DNase I and S1 nuclease treatment of nucleic acids allows in principle to quantify dsRNAs by spectrophotometer by degrading all the ssRNAs and DNA, but treatment with

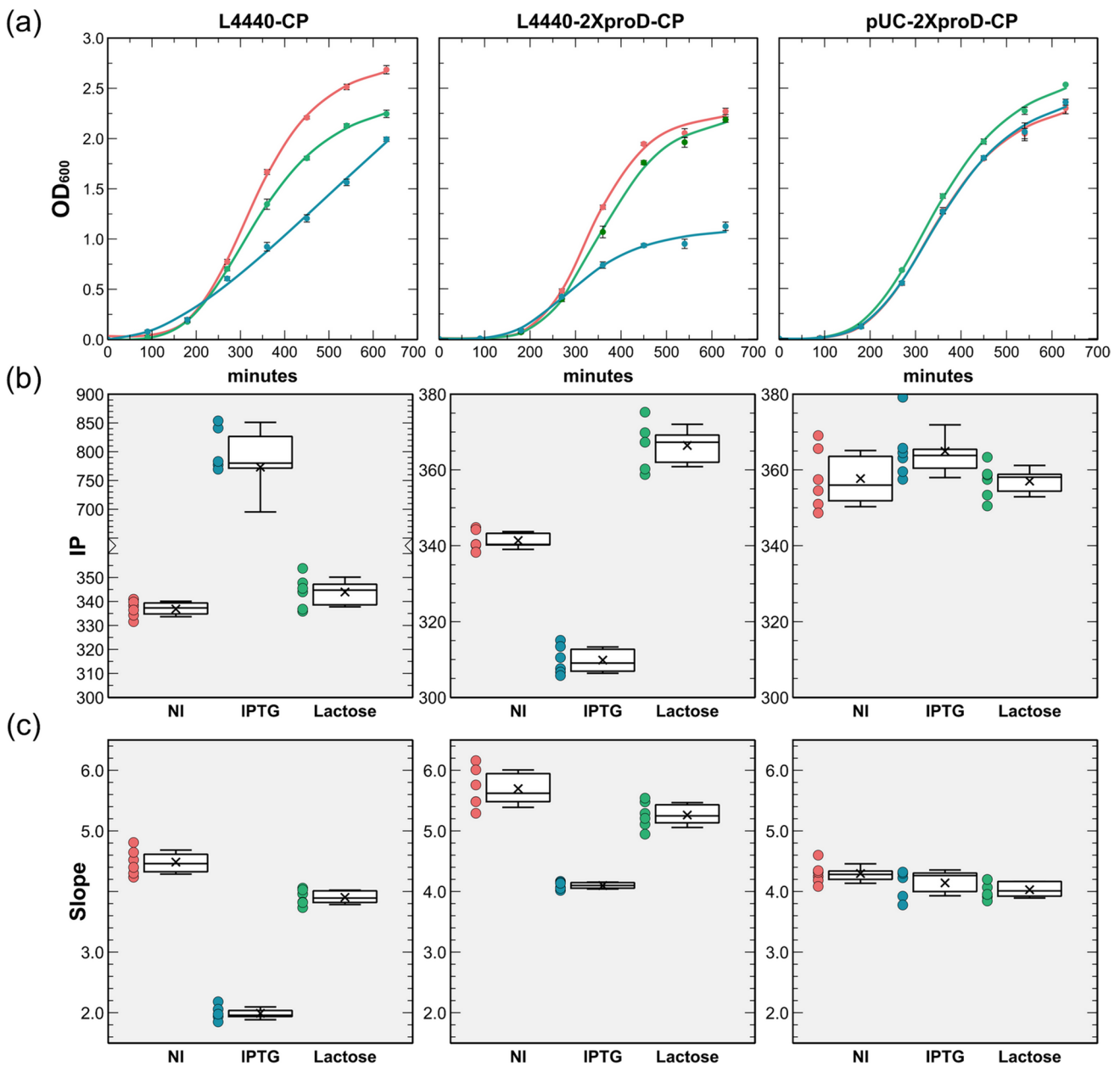


Fig. 6 **a** Growth dynamics of *E. coli* HT115(DE3) cells carrying L4440-CP, L4440-2XproD-CP, or pUC-2XproD-CP in LB supplemented with different inducers: NI, no inducer (red), IPTG (blue), and D-lactose (green). Points were adjusted to the 4P-logistic curve. Bars represent the standard deviation. **b** Box plots of points

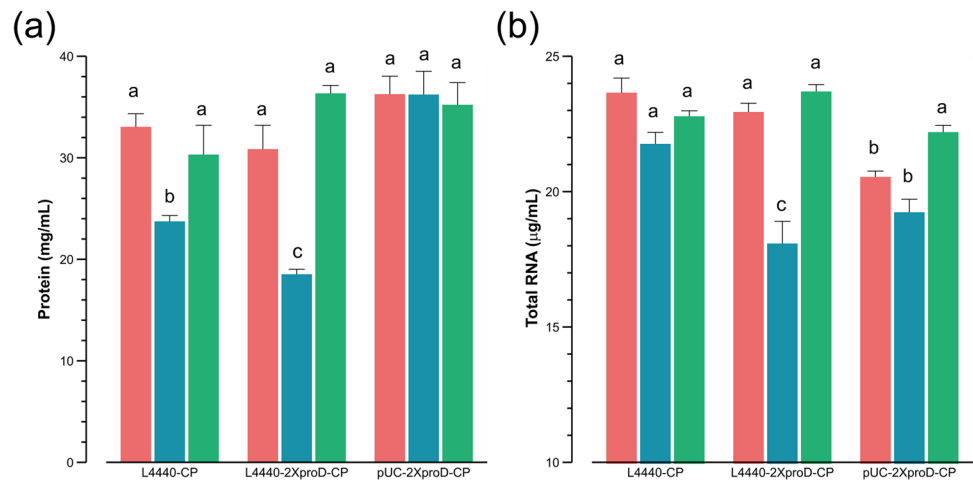
of inflection (IP) and **c** slopes at the point of inflection according to the adjusted 4P logistic growth curves for the different plasmids and inducers used in cultures of HT115(DE3) cells. Bars refer to the standard deviation of the values

high concentrations of S1 nuclease also degrades part of the dsRNA. For this reason, the option of quantifying dsRNA using gel analysis proved to be a viable alternative. On the other hand, from the agarose gels, it became evident that most of the dsRNA molecules produced were not of full size. The extension of RNA transcription from both the T7 and proD promoters resulted partially incomplete, rendering small dsRNA molecules, or hybrid molecules with partial

dsRNA and single stranded RNA (ssRNA) tails. The northern blot confirmed the abundance of synthetic low molecular weight dsRNA in all the cases.

Quantitative RT-qPCR was used as another approach to compare dsRNA production and, therefore, the relative strengths of the T7 and proD promoters. A primer set that matched an internal 108 bp sequence in the middle of the CP insert was used for the amplifications.

Fig. 7 **a** Cytosolic protein and **b** total RNA yield in HT115(DE3) cells grown for 630 min with plasmids L4440-CP, L4440-2XproD-CP, or pUC-2XproD-CP and inducers: IPTG (blue), lactose (green), or none (red). Error bars represent the standard error of the means. Bars with the same letter are not significantly different at $P < 0.05$



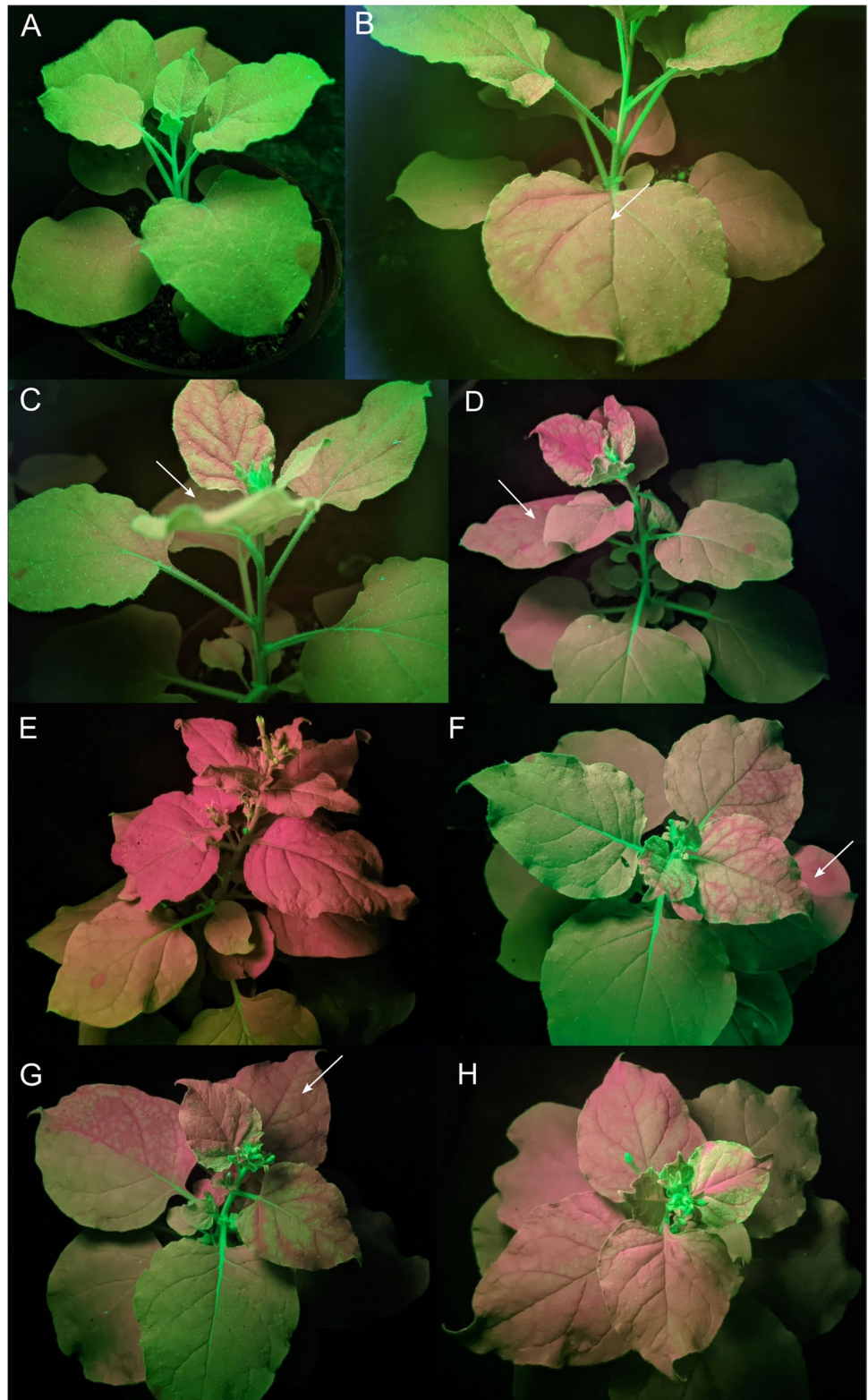
This selection restricted the quantitation of dsRNA to molecules of at least 370 bp for L4440-CP, 390/510 bp for L4440-2XproD-CP, and 390 bp in the case of pUC-2XproD-CP. All the dsRNAs of lower molecular size could not be amplified and used in the quantitation. Despite this, the RT-qPCR allowed a comparison, albeit restricted, of the dsRNA synthesis. According to RT-qPCR when supplied with IPTG, the T7 promoter present in L4440-CP resulted in a fivefold higher performance compared to the proD promoter in pUC-2XproD-CP. Interestingly, in L4440-2XproD-CP, the synthesis of dsRNA was around twofold with respect to L4440-CP when supplemented with IPTG, showing that the simultaneous presence of the T7 and proD promoters increased the yield. Quantitation of full-length CP-dsRNAs was done using known amounts of molecular weight marker and gel analysis software. Results showed that in the presence of IPTG the T7 promoter in L4440-CP doubles the dsRNA production when compared with the proD promoter in L4440-2XproD-CP in absence of inducers. DsRNA production in L4440-CP/HT115(DE3) was in the same range as those reported by Ma et al. (2020) in an equivalent system, although lower than the produced using the *rnc*⁻ BL21(DE3). Finally, in short culture cycles (360 min), we observed that D-lactose was a better inducer of dsRNA synthesis than IPTG, even though the latter is not metabolized by the cell. A similar observation was reported previously (Donovan et al. 1996). In long culture cycles, D-lactose metabolization has prevented it from being used as an effective inducer. Top10 cells carrying L4440-2XproD-CP can plausibly produce dsRNA using the constitutive promoter, but results are immediately degraded by the RNase III and do not accumulate. Although RNase III is a key enzyme in those cellular processes, its activity is not essential as other alternative enzymes and systems are present in the bacteria (Court et al. 2013). However, lack of RNAase III

in HT115(DE3) results in lower metabolic efficiency in these cells, as reported elsewhere (Takiff et al. 1992). On the other hand, accumulation of dsRNA in the cells may inhibit bacterial growth. Recently, it has been reported that the growth inhibition observed in bacteria producing recombinant proteins is mainly due to RNA transcription rather than protein translation (Li and Rinas, 2020). Thus, our results suggest that dsRNA accumulation entails a high cost to the cell.

Given that the pUC-2XproD vector system does not require the λ DE3 lysogen that contains the T7 RNA polymerase in the bacterial chromosome, it is suitable to produce dsRNA in any cells defective in the RNase III gene (*rnc*) that do not degrade dsRNA, such as the original *E. coli* HT115 (W3110 *rnc*-14:: Δ Tn10) or the modified BL21(DE3) obtained by Ma et al. (2020). Although the strength of the T7 promoter resulted higher than the proD promoter, when in tandem, the yield of dsRNA synthesis increases. Moreover, a constitutive promoter for dsRNA synthesis in microbial fermenters allows production on an industrial scale by overcoming some of the drawbacks of inducers.

In our system, the proD promoter drives the RNA synthesis from a virus gene segment, and our results have shown a significant fraction of incomplete dsRNAs using as template plasmid pUC-2XproD-CP and, to a lesser degree, L4440-2XproD-CP that seems more stable for RNA transcription. The most probable cause is constraining in RNA transcription elongation by the bacterial RNAP. RNA elongation does not occur at a uniform rate as the RNAP movement and binding to DNA can be disrupted by various constraints such as DNA sequence, topology, and secondary RNA structures (Lee and Borukhov, 2016). In our case, the differences in plasmid topology between pUC-2XproD-CP and L4440-2XproD-CP may explain the differences in RNAP-dependent dsRNA transcription. It is also possible that a different gene fragment used as template for dsRNA

Fig. 8 GFP silencing on *N. benthamiana* 16c plants supplied with different (ds)RNA extracts: **A** L4440-CP; **B–C–D–E** L4440-GFP at 3, 6, 12, and 20 days post application (dpa); **F–G–H** L4440-2XproD-GFP dsRNA extract at 10, 12, and 20 dpa. Arrows indicate the leaves on which the (ds)RNA extracts were applied with the airbrush



synthesis yields a bigger fraction of long dsRNAs under the proD promoter.

Recently, a novel IPTG-inducible plasmid system in RNase III deficient *E. coli* BL21(DE3) strain has shown

a high production of dsRNA, doubling in comparison the production with respect to HT115(DE3) (Ma et al. 2020). Given that BL21 seems to be more efficient than HT115, it is plausible that our constitutive system would be capable

of producing higher amounts of dsRNA in the BL21(DE3) *rnc⁻* strain. Obtaining high amounts of dsRNA in vivo has also been achieved in *Pseudomonas syringae* engineered with components of the bacteriophage phi6 (Niehl et al. 2018). Although the systems described here produce a significant fraction of dsRNAs of low molecular weight with respect to the gene segment targeted, they can still be useful for RNAi applications (Kakiyama et al. 2019; He et al. 2020). In ongoing experiments, we are studying the effect in CGMMV control after foliar application of different dsRNA extracts obtained by the methods described in this work. Finally, the dsRNAs produced in HT115 driven by either the induced T7 promoter in L4440-GFP or the constitutive proD promoter in L4440-2XproD-GFP are capable of silencing GFP expression in *N. benthamiana* 16c. The effectiveness of dsRNA produced by pUC-2XproD-GFP remains to be tested.

In conclusion, the synthetic promoter proD that was originally designed for constitutive RNA transcription in *E. coli*, can drive the constitutive production of specific dsRNAs in *E. coli* HT115(DE3), and plausibly in other RNase III-deficient strains, when allocated in plasmids in opposite directions and flanking a gene of interest. The systems described here allow the easy cloning by the Gateway system of any gene of interest for RNAi applications.

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Author contribution L.V. designed the research; J.D. and L.V. performed researches; J.D. and L. V. analyzed the data and results; L.V. wrote the original draft; J.D. and L.V. wrote and edited the final manuscript; L.V. provided funds for the project.

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Data availability Data available on request from the authors.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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