Central to the study and engineering of plants is their transformation, the genes required for the transfer of T-DNA have been stably integrated into the genome and expressed (Chilton 1989). The induction of crown galls is induced by the transfer of T-DNA (Thomashow et al. 1980), a segment of a tumor-inducing plasmid (Van Larebeke et al. 1974) that is resident in A. tumefaciens, into the nucleus of infected plant cells, wherein DNA can be readily cloned and manipulated between the borders of T-DNA, prior to transfer into E. coli. The mechanism by which pGreenII perturbs E. coli growth appears to be dysregulation within the ColE1 origin of replication.

An Improved Binary Vector and Escherichia coli Strain for Agrobacterium tumefaciens-Mediated Plant Transformation

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ABSTRACT The plasmid vector pGreenII is widely used to produce plant transformants via a process that involves propagation in Escherichia coli. However, we show here that pGreenII-based constructs can be unstable in E. coli as a consequence of them hampering cell division and promoting cell death. In addition, we describe a new version of pGreenII that does not cause these effects, thereby removing the selective pressure for mutation, and a new strain of E. coli that better tolerates existing pGreenII-based constructs without reducing plasmid yield. The adoption of the new derivative of pGreenII and the E. coli strain, which we have named pViridis and MW906, respectively, should help to ensure the integrity of genes destined for study in plants while they are propagated and manipulated in E. coli. The mechanism by which pGreenII perturbs E. coli growth appears to be dysregulation within the ColE1 origin of replication.

KEYWORDS Agrobacterium plant transformation pGreen E. coli growth defects
Plasmids: sources, propagation, and analysis
The plasmid pGreenII (version 0179; http://www.pgreen.ac.uk/) was obtained from the John Innes Centre (Norwich Research Park, UK). The pMET1-03 plasmid contains the cDNA of MET1 under the control of the 35S promoter. The MET1 cDNA sequence was obtained as a GACGGGTTCG-3 fragment from pGreenII (ver. 0000; http://www.pgreen.ac.uk/). The introduction of plasmid DNA via the process of transformation into cells made competent by treatment with calcium chloride, the isolation of plasmid via alkaline lysis, and the analysis of plasmid using restriction enzyme in combination with agarose gel electrophoresis were done using widely used protocols (Sambrook and Russell 2001). To estimate the yield, plasmid was isolated from 2 OD₆₀₀ units of culture, resuspended in 40 µl of sterile deionized water, and a 2 µl aliquot was analyzed by agarose gel electrophoresis.

E. coli: growth and measurement of colony forming units (cfu)
E. coli DH5α cells containing derivatives of pGreenII (ver. 0179) as described were grown in Luria Bertani broth (Sigma) with kanamycin selection (50 µg/ml) and shaking (200 rpm) at 37°C. Cultures of 50 ml were incubated in 250 ml Erlenmeyer flasks, while cultures of 5 ml were grown in 50 ml Falcon conical centrifuge tubes held vertically. Growth was monitored by measuring the optical density of the culture at 600 nm (OD₆₀₀ reading). When the OD₆₀₀ of cultures exceeded 1.0, samples were diluted to ensure that readings were well within the linear range of the spectrophotometer. To determine the number of cfu values, samples of E. coli cultures were collected, diluted serially by 10-fold, aliquots spread on the surface of LB agar plates containing kanamycin (50 µg/ml), and incubated overnight. After confirming that the number of colonies on the plates corresponded to the expected 10-fold dilution, the precise number of colonies on a plate with 10–100 colonies was counted and used to determine the number of cfu/ml after correcting for dilution and sample volume spread on plates. To correct for growth, this value was divided by the corresponding OD₆₀₀ value of the culture to give the value of cfu/OD₆₀₀ unit. An OD₆₀₀ unit of 1.0 is the biomass in a 1.0 ml sample with an OD₆₀₀ reading of 1.0. Values of cfu/OD₆₀₀ unit were determined during exponential growth and following overnight culture. Independent measurements were made at least thrice to allow values of average and standard deviation to be calculated. Inoculums to initiate culturing in liquid broth were either cells scraped from individual or multiple colonies derived by the process of transformation, which was started the previous day.

DNA sequencing: plasmid and chromosome
Plasmids were isolated as described above and sequenced as part of a service provided by Beckman Coulter Genomics (Essex, UK). Chromosomal DNA was isolated as part of a protocol usually used by us to isolate total RNA from E. coli (Kime et al. 2008). Upon the addition of ethanol to precipitate nucleic acids, chromosomal DNA in the form of a stringy aggregate was removed using a pipette tip, pelleted by brief centrifugation (2 min) in a microfuge, washed with 70% [v/v] ethanol, and dried. It was sequenced to ~35× coverage using an Illumina MiSeq as part of a service provided by the Next Generation Sequencing Facility (St James’s University Hospital, Leeds). E. coli strain DH10B was used as the reference genome (GenBank: X0000024) and DH5α was sequenced to establish its allelic differences prior to scanning for mutations in the genomes of spontaneous mutants of DH5α using NextGENe software.

Arabidopsis transformation: confirmation of plasmid transfer
Arabidopsis (Col-0) was transformed by floral dip (Clough and Bent 1998). 0.6 g of seeds was spread on the surface of MS plates (4.4 g/l Murashige and Skoog plus vitamins; 10 g/l sucrose; 5.5 g/l agar; pH 5.8) containing hygromycin (15 µg/ml) to select transformants (i.e., resistant seedlings), from which DNA was isolated and analyzed by PCR. The sequences of the primer pairs were 5′-GGTTGTAGTTGA GAGGTTG-3′ plus 5′-GTCAGAGGCTCAAGGAGAG-3′, and 5′-TGCCATGCCCAGAAGTTATG-3′ plus 5′-TGTGTAATCCCCAG CAGCAGT-3′. These produced amplicons of 649 bp and 435 bp in the presence of the genes encoding elongation factor 1a and green fluorescent protein, respectively. The Agrobacterium culture used for the floral dip (Clough and Bent 1998) was grown at 28°C in Luria Bertani (LB) broth (Sigma) containing kanamycin (50 µg/ml), tetracycline (12.5 µg/ml), and gentamycin (40 µg/ml), until an OD₆₀₀ of 1.0 was reached. Cells were pelleted and resuspended in 5% sucrose; 0.05% Silwet-L77 to an OD₆₀₀ of 0.8. Arabidopsis plants were grown at 25°C under long day conditions for 4 wk and then inverted into the resuspended culture for 1 min. Seeds were harvested and dried.

Data availability
The vector pVirids and strain MW906 are available upon request from BCCM (http://bccm.belspo.be/). Accession numbers are held by laboratory of P. M. (p.meyer@leeds.ac.uk).

RESULTS
Adverse effects of pGreenII on E. coli growth
The vector pGreenII (version 0179) has an adverse effect on the growth of E. coli as evidenced, for example, by extensive filamentation (i.e., incomplete septation) of cells during exponential growth (Figure 1,
was also able to force the selection of mutants (see below). For this small indels or nucleotide substitutions. pGreenII without any insert some mutations, which we exploited (see below), or plasmids with obvious changes in restriction fragment length either have chromo-plants (Figure 1, panel D). Mutants that contain plasmids without MET1-03, the pGreenII-derived construct that carries involving the these mutants revealed that many (4 of 16) had obvious rearrangements with improved growth. The analysis of the plasmids from a selection of extended to 3 d, but when aliquots were spread on agar plates the cubation. Turbid cultures could be produced when the incubation was noticeably smaller than those of cells containing pET28a during exponential growth (Table 1). The selection of spontaneous mutations indicated that the combined effects of pGreenII and the majority of the resulting colonies were larger (data not shown). This edge of DH5α (pGreenII) colonies also appeared to undulate and be less regular, respectively. The above experiments were conducted using DH5α, a derivative of E. coli K-12 used widely for the purpose of recombinating DNA (BRL 1986). However, the effects of pGreenII on growth do not appear to be strain specific. For example, a sharp drop in viability following overnight incubation was observed using BL21 (DE3) (Table 1), a derivate of E. coli B used extensively for protein production (Studier et al. 1990). The cfu values obtained for DH5α (pET28a) cells during growth were in agreement with those typically reported in the literature (Ausubel 1995). DH5α cells containing pGreenII, in comparison to those containing pET28a, produced slightly smaller colonies on agar plates (Figure 1, panel C), consistent with their longer doubling time and reduced viability after exit from exponential growth (Table 1). The surface and edge of DH5α (pGreenII) colonies also appeared to undulate and be less regular, respectively. The selection of spontaneous mutations The growth defects caused by pGreenII can be compounded when this vector carries an insert. In comparison to DH5α (pGreenII), cells containing pMET1-03, the pGreenII-derived construct that carries MET1 (for details, see Materials and Methods), produced colonies that were noticeably smaller than those of cells containing pGreenII (Figure 1, panel C). More remarkably, it proved impossible to produce reproducibly turbid cultures of cells containing pMET1-03 with overnight incubation. Turbid cultures could be produced when the incubation was extended to 3 d, but when aliquots were spread on agar plates the majority of the resulting colonies were larger (data not shown). This indicated that the combined effects of pGreenII and the MET1 insert were sufficiently severe to force the selection of spontaneous mutants with improved growth. The analysis of the plasmids from a selection of these mutants revealed that many (4 of 16) had obvious rearrangements involving the MET1 cassette (4865 bp EcoRI fragment) destined for plants (Figure 1, panel D). Mutants that contain plasmids without obvious changes in restriction fragment length either have chromosome mutations, which we exploited (see below), or plasmids with small indels or nucleotide substitutions. pGreenII without any insert was also able to force the selection of mutants (see below). For this reason, all of the colonies we have shown (Figure 1, panel A) correspond to primary transformants. Moreover, the cultures used for the measurement of the doubling times, and cfu values during exponential growth and following overnight culture, (Table 1) were inoculated using cells obtained from multiple colonies of primary transformants and not overnight cultures.
As a step toward negating the deleterious effects of pGreenII on *E. coli* growth, we selected spontaneous mutants by independently passaging multiple transformants through three cycles of culture using cells from an overnight incubation as the inoculum for the next (for details, see *Materials and Methods*). This led to the isolation of a plasmid that no longer causes such a dramatic drop in cfu values following overnight incubation, even when retransformed into a fresh batch of cells (Table 1). This plasmid was named pGreenII-SS, as sequencing revealed that it had acquired IS5, a 1.2 kbp transposable element (Szybalski 1977; Engler and van Bree 1981), in the region between the ColE1 origin of replication and the stop codon of npt1 (Figure 2, panel A). This is the location implicated previously in the instability of the original pGreen plasmid (see *Introduction*).

### Complete negation of the growth defects caused by the original pGreenII

Next, to explore the capacity of further changes in the region upstream of the ColE1 origin to reverse the growth defects caused by pGreenII, we introduced deletions using Bal31 into pGreenII-SS at the NcoI and MscI sites within IS5 and at the two flanking DnaI sites (Figure 2, panel A) and then screened > 40 mutants. The cfu values following overnight incubation, as well as the time it took cultures to reach early-exponential growth (using an overnight culture as the inoculum), were determined. In addition to identifying deletions that abolished or left unaffected the beneficial effects of IS5, we identified one that increased the viability of overnight cultures above that provided by the IS5 insertion. Sequencing revealed that the deletion was 1.3 kbp, removed much of the IS5 element, and extended into the ColE1 origin of replication (Figure 2, panel A). The corresponding plasmid was named pViridis.

In comparison to DH5α (pViridis) cells (Figure 1, panel A), DH5α (pViridis) cells did not produce obvious filaments (Figure 2, panel B) and were straightforward to passage using an overnight culture as the inoculum (Figure 2, panel C). Moreover, their doubling time during exponential growth was 1.50-fold shorter, and the cfu values during exponential growth and following overnight incubation were 14 and 15,600-fold higher, respectively (Table 1). Indeed, the actual values for DH5α (pViridis) cells were very similar to those of DH5α (pET28a) cells (Table 1). Cells containing pGreenII-SS, the intermediate in the construction of pViridis, produced filaments (Figure 2, panel B) and were still significantly delayed in reaching the exponential phase of growth, although not to the same extent as cells containing pGreenII (Figure 2, panel C and Table 1). The colony morphology of cells containing pViridis was indistinguishable from those of pET28a (data not shown). The pViridis vector has been successfully used by us to clone a number of fragments (M. R. Watson and P. Meyer, unpublished results). Moreover, pViridis constructs have been introduced successfully into Arabidopsis (*Col-0*) (Figure 3).

### A strain that better tolerates pGreenII

As outlined above, we were able to select spontaneous mutations in pGreenII that reduced the deleterious effects of this plasmid on cell growth. During the screening, we also detected chromosomal mutations, *i.e.*, the improved growth characteristics were not linked with the resident plasmid when introduced by transformation into fresh DH5α cells. A strain that better tolerates pGreenII would be beneficial in the propagation and manipulation of the large number of existing constructs based on this vector. Therefore, we repeated the screen (for further details, see *Materials and Methods*), but increased the selection pressure by using cells that carried pMET1-03. The MET1 insert compounds the effects of pGreenII (Figure 1) by a mechanism that is not dependent on the production of a functional cytosine-DNA-methyltransferase (data not shown). Mutations located in chromosome were identified by showing that improved growth was not transferred with the resident plasmid, and persisted when the strain was cured of the resident plasmid (via culture in the absence of antibiotic) and retransformed with a fresh batch of pGreenII.

Next, we assayed chromosomal mutants for plasmid yield to avoid mutations that alleviated the deleterious effects of pGreenII by reducing its copy number. This revealed a mutant strain, now designated MW906, which yielded an amount of pGreenII at least comparable to that obtained from DH5α (Figure 4, panel A). Included in this analysis were DH5α cells containing pET28a and pViridis, and MW906 cells containing pGreenII and pMET1-03. Sequencing of MW906 located the spontaneous mutation to the *penB* gene (Liu and Parkinson 1989), which encodes an RNA poly(A) polymerase (Cao and Sarkar 1992). The mutation caused a glycerine to serine substitution at position 67 (*i.e.*, a G67S mutation). In comparison to the equivalent data for DH5α (pMET1-03) cells (Figure 1), MW906 (pMET1-03) cells took considerably less time to reach early-exponential growth using an overnight culture as the inoculum (Figure 4, panel B) and did not appear to produce filaments, at least to the same extent (see inset, Figure 4, panel B). The latter interpretation is consistent with the finding that the cfu values during exponential growth were higher (Table 1). The doubling time during exponential growth and viability following overnight incubation were also improved (Table 1). More importantly, with regard to maintaining the integrity of DNA intended for plants, turbid cultures of MW906 (pMET1-03) cells could be produced readily by overnight incubation, and so far the analysis of plasmids from clones isolated at the end of overnight incubation has failed to identify any rearrangement of the plasmid (for examples, see Figure 4, panel C).

### Discussion

The plasmid vector pGreenII, which is widely used in the production of stable plant transformants, is shown herein to predispose constructs to the acquisition of mutations (Figure 1) despite its earlier revision (Hellens and Mullineaux 2000). This predisposition arises from pGreenII...
having an adverse effect on the growth of *E. coli*. It perturbs normal cell division resulting in the production of long filaments (Figure 1), a phenomenon associated with stressed cells (Justice et al. 2008), and causes a dramatic reduction in cell viability following overnight incubation (Table 1). This is far from ideal as the insertion of DNA into plasmids can itself affect the growth of *E. coli* (cf. DH5α) cells containing pGreenII based constructs without causing a decrease in plasmid yield. DH5α was chosen as the background strain for the selection as it is commonly used for DNA cloning in *E. coli* (BRL 1986). The viability of MW906 (cf. DH5α) cells containing pGreenII increased by two orders of magnitude, while the filamentous phenotype was reduced, although not completely eliminated. The superiority of strain MW906 was also demonstrated by the relative ease with which it can be propagated when carrying pMET1-03 and the greater stability of pMET1-03. With regard to the latter, we failed to identify any isolates containing plasmid with obvious rearrangements at the end of batch culture (Figure 4). However, given the choice of using the combination of MW906 and pGreenII or DH5α and pViridis for the construction of new cassettes for delivery into plants, we would suggest the latter combination. This is because MW906 (pGreenII) does not grow as well as DH5α (pViridis); consequently, the selective pressure for mutation has not been completely removed using MW906 (pGreenII). However, it should be noted that while the G67S mutation does not affect the yield of constructs based on pGreenII, it does reduce the yield of constructs based on pViridis (data not shown). Thus, we recommend always using DH5α for the propagation of constructs based on pViridis. Other strains that are wild-type with regard to the *pcnB* gene might also be suitable.

At the present time, we can only speculate on the cause of the growth defects conferred by pGreenII. However, as part of our investigation, we...
found that the spontaneous insertion of IS5, a 1.2 kbp transposable element, in the region upstream of the ColE1 origin of replication (and downstream of the npt1 gene) which confers kanamycin resistance increased the viability of cells following overnight culture by around three orders of magnitude. The cfu values for DH5α (pGreenII-IS5) were lower than those for PET28a, but no more than could be explained by filamentation, which was not suppressed. The location of IS5 suggests that some aspect of the replication of pGreenII is abnormal. This is supported by the fact that the deletion which produced pViridis extended 103 bp into the ColE1 origin of replication, removing the endogenous promoter and 5’ end of the RNAII, which is the primer of plasmid replication (Polisky 1988). In addition, the chromosomal mutation that permits better tolerance of pGreenII-based constructs causes an amino acid substitution within pcnB (Lopilato et al. 1986), which controls plasmid copy number by promoting the degradation of RNAI (Xu et al. 1993; He et al. 1993), the antisense RNA regulator of RNAII activity (Cesareni et al. 1991). The genes encoding RNAI and RNAII overlap; thus, the deletion in pViridis causes truncation of 45 nucleotides from the 3’ end of RNAI, as well as the 5’ end of RNAII. The deletion also illustrates the functional plasticity of the RNA components of the ColE1 replication machinery. We have found that other mutations in pcnB can alleviate the growth defects conferred by pGreenII, but not without reducing plasmid yield (data not shown). Thus, the G67S mutation in MW1053 appears to be unique in correcting a defect in the replication of pGreenII, while managing to provide a normal balance between the activities of RNAI and RNAII. Consistent with this notion, when the proposed imbalance is corrected in the case of pViridis, the effect of the pcnB mutation in MW906, instead of establishing balanced regulation, causes a reduction in plasmid copy number (data not shown).

The source of the original instability reported for pGreen might also have been related to plasmid replication (Hellens and Mullineaux 2000). It was reported that several ‘enlarged’ plasmids had *E. coli* chromosomal DNA inserted in the ColE1 ori, and reasoned that the selective pressure may have been the inefficient termination of transcription from the convergent nptI gene (Figure 2). As indicated previously, the region between the ColE1 origin and the stop codon of the nptI gene in pGreenI was replaced with the corresponding sequence from pBlueScript to produce pGreenII. However, we can report that the insertion of a strong transcriptional terminator (RBa_B0010, iGEM Registry of Standard Biological Parts; Campos 2012) between the ColE1 ori and the 3’ end of the nptI gene in pGreenII had no discernible effect on the ability of pGreenII to cause growth defects (data not shown). Indeed, in pViridis, transcription from nptI may contribute to the transcription of RNAII in the absence of the promoter that normally produces the primer of replication.

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Author contributions: K.J.M. and P.M. supervised the overall study, which was conducted largely by Y.-F.L. and M.R.W. with input from R.E.D. E.H. performed the *Arabidopsis* transformation and analysis. K.J.M. wrote the article with input from P.M. and M.R.W. All authors read and approved the final manuscript. The authors declare that they have no competing interests of a financial or nonfinancial nature.

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**Figure 4** Characterization of strain MW906. (A) Plasmid yield. Labeling at the top of the panel indicates the combinations of strains and plasmids. For each combination, plasmid DNA was isolated from 2 OD<sub>600</sub> units of an overnight culture using a standard protocol (for details, see Material and Methods). The marker was the 1 kb Plus DNA Ladder (Life Technologies). The gel used for electrophoresis was composed of 0.8% [w/v] agarose and stained with ethidium bromide. (B) Growth in liquid culture using overnight cultures as an inoculum. The crosses correspond to data-points for MW906 cells containing pGreenII. Compare with the diamonds and squares corresponding to data-points for DH5α cells containing pET28a (Figure 1) and pGreenII (Figure 3), respectively. The inset shows the morphology of MW906 (pGreenII) cells, as imaged in Figure 1, panel A. (C) Restriction enzyme analysis of plasmid from a selection of clones isolated from a culture of MW906 (pMET1-03) following overnight incubation (labeled 1–16). The lane labeled M contains the 1 kb Plus DNA Ladder (Life Technologies). Numbering on the panel indicates the expected sizes of the two fragments produced by EcoRI digestion of pMET1-03. The smaller of the two fragments corresponds to the MET1 cassette destined for plants. The gel used for electrophoresis was composed of 0.8% [w/v] agarose and stained with ethidium bromide.
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