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A Comparison of Two Selectable Marker Gene Systems Used in the Transformation of Chickpea (*Cicer arietinum* L.)

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ABSTRACT

Different selectable marker genes are used in the genetic transformation of plants, but the efficiency of these genes in the selection of transgenic plants is different. In this study, we have used two different selectable marker gene systems in an established chickpea transformation protocol, and have compared the effectiveness of each selection system based on transformation efficiency. Using the herbicide resistance *bar* gene, together with a low concentration of the selective agent phosphinothricin, results in greater transformation efficiency than using the antibiotic resistance *nptII* gene with a high concentration of the selective agent kanamycin. In addition, modification of the rooting media and the use of a grafting method further improved the transformation efficiency of both selection systems. The transformation efficiency using the *nptII* and *bar* genes as selectable markers was 0.37% and 4.3%, respectively.

Keywords: *bar*, marker gene, *nptII*, transformation, transgenic chickpea

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most widely grown grain legume in worldwide, but yields have generally remained low because of its susceptibility to several biotic and abiotic stresses (Ignacimuthu and Prakash 2006). Improvement via genetic modification is an attractive option, because of the lack of desirable genes in current chickpea germplasm, and sexual incompatibility with wild native species. However, chickpea transformation efficiency is generally low due to the recalcitrant nature of chickpea, genotype, *Agrobacterium* strain and selectable marker (Popelka and Higgins 2007).

Most researchers have used the neomycin phosphotransferase II (*nptII*) gene as the selectable marker for chickpea transformation experiments, using constant or increasing Kan (Kanamycin, Sigma Co.) concentrations in the media for the selection of putative transgenic plants: 50 mg/l (Fontana *et al.* 1993), 200 mg/l (Sarmah *et al.* 2004), 50 to 150 mg/l (Polowick *et al.* 2004), 100 to 200 mg/l (Sanyal *et al.* 2005). However, the survival of non-transgenic plants or "escapes" has been observed, and sometimes many selection cycles are needed for effective selection.

The transformation percentage of chickpea with the *nptII* gene as selectable marker has been low in most reports. Transformation percentage was usually between 0.4 to 3% (Polisetty *et al.* 1997; Krishnamurthy *et al*. 2004; Sarmah *et al*. 2004; Sanyal *et al*. 2005). Further, Kan was not recommended as selective agent for pea transformation due to the high level of tolerance to this antibiotic (Puonti-Kaerlas *et al*. 1990; Schroeder *et al*. 1993), and as high Kan concentrations are known to impair shoot vigor and rooting capability.

There are a few reports about using herbicide resistance genes, particularly the *bar* gene (Senthil *et al.* 2004), which confers resistance to the active compound phosphinotricin, used in the commercial herbicide "Basta". A high transformation efficiency of 5.1% was obtained in this study. Singh *et al.* (2009) were used *bar* gene as a selectable marker gene for transformation of chickpea using shoot meristem and the average transformation frequency was between 1.29-3.33% with different *Agrobacterium* strains. It was interesting that the high frequency of transformation was obtained with *Agrobacterium* strain AGL0 and the frequency was lower with other strains such as LBA4404 and EHA105. Ease of herbicide application and low cost of phosphinotricin makes using *bar* gene for transformation of plants is a good alternative for antibiotic selection.

In this study, the transformation efficiencies of two selectable marker gene systems, *bar* and *nptII,* were compared, with the purpose of improving an existing transformation protocol for chickpea. This work was carried out at the CSIRO Division of Plant Industry laboratories, Canberra, Australia.

MATERIALS AND METHODS

Plant materials

The commercially available chickpea cultivar 'Jimbour' was used in this study. Seeds of the chickpea cv 'Jimbour' were obtained from Mr Ted Knights, NSW Agriculture, Tamworth, NSW, Australia.

Bacteria and plasmids

Two constructs were available at the CSIRO laboratories at the time of this study, and were selected on the basis of their selectable marker genes. The construct *pBSF16* contains the *Bar* selectable marker gene (**Fig. 1A**), and its design and construction are detailed in Molvig *et al.* (1997). The *nptII* selectable marker gene was available in the construct *pCry1Ac-nptII* (**Fig. 1B**). The design and construction of this plasmid are as yet unpublished. However, a sister plasmid, identical except for the *Cry1Ac* coding region being replaced by the *Cry2Aa* coding region, is described in Acharjee *et al.* (2010).

The *Agrobacterium tumefaciens* strain AGL1 was used as a vehicle to transfer the transgenes into chickpea genomic DNA.

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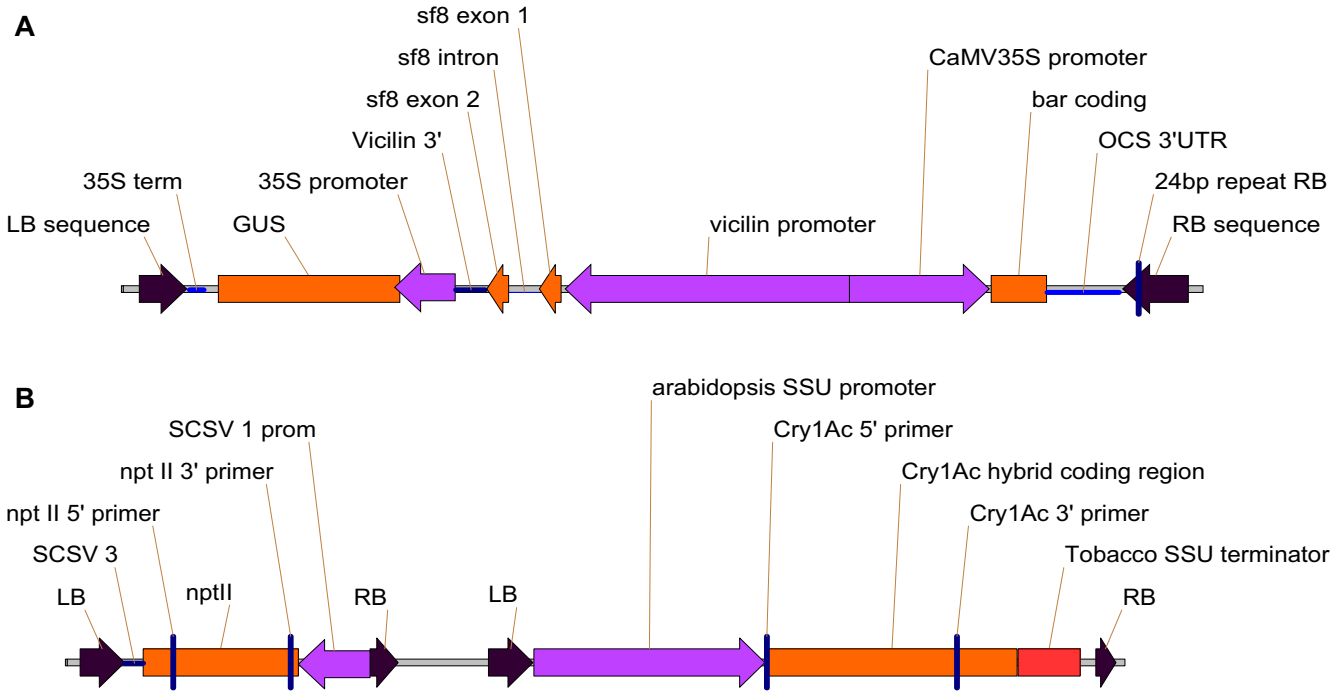

Fig. 1 Constructs of T-DNAs. (**A**) Fragment of pBSF16, 10793bp length. (**B**) Fragment of pCry1Ac-nptII, 7864 bp length.

Plant transformation

Plant transformation was done according to Sarmah *et al*. (2004) protocol with minor modifications. Explants were prepared by removing the seed coat and bisecting the embryonic axis. Co-cultivation of explants with *Agrobacterium* solution was done for 45 minutes. After that, the explants transferred directly to B5 co-cultivation medium (Gamborg *et al*. 1968) containing 10 mM MES (2-*N*-morpholino-ethanesulfonic acid); 1 mg/l NAA (1-naphthalene acetic acid); 1 mg/l BA (6-benzyl adenine); 100 µM coniferyl alcohol; 0.8% (w/v) agar. All *in vitro* plant culture was performed at 24°C with a photoperiod of 16 hrs day length. After 72 hrs of co-cultivation, explants were plated with the cut surface down on RS medium containing basal MS medium (Murashige and Skoog 1962); 0.5 mg/l BA; 0.5 mg/l kinetin; 0.05 mg/l NAA; 10 mM MES; 5 mg/l phosphinotricine in first experiment or 200 mg/l Kan in second experiment; 150 mg/l timentin (Beecham Research Laboratories, Dandenong, Victoria, Australia); 0.8% agar; pH 5.8. After two weeks, explants producing green shoots were sub-cultured (removing the cotyledon and any roots) onto SS medium (containing basal MS medium; 0.5 mg/l BA; 0.5 mg/l kinetin; 10 mM MES; 5 mg/l phosphinotricin or 200 mg/l Kan; 150 mg/l timentin; 0.8% agar; pH 5.8. After 14 days, explants surviving on Kan or phosphinotricin selection were subcultured onto TS medium (containing basal MS medium; 0.1 mg/l BA; 0.1 mg/l kinetin; 10 mM MES; 5 mg/l phosphinotricin or 200 mg/l Kan; 150 mg/l timentin; 0.8% (w/v) agar; pH 5.8. Shoots surviving selection were sub-cultured every 10 to 14 days for a minimum of 3 cycles onto fresh TS medium. From the forth cycle, the shoots from each explant were separated and cultured individually. Any bleached (in Kan) or brown (in phosphinotricin) portion of a shoot was cut off and only the green portion was subcultured.

Rooting and establishment

To establish root growth on of surviving shoots, three media were compared: MR medium (B5 media + 1 mg/l IBA [indole-3-butyric acid, Sigma Co.] with or without Kan), MY medium (half strength of MS salts and B5 vitamins + 2 mg/l IBA), and IAA medium (MS salts + B5 vitamins + 0.1 mg/l IAA [indole-3-acetic acid, Sigma]). Once roots were established, the plants were transferred to light sandy soil for acclimatization. Also, 10 shoots were grafted on 6 days old seedlings from the parental line, following the protocol from Sarmah *et al*. (2004).

GUS assay

To identify the presence of transformed plant cells, a *gus* assay on plant tissue was carried out using the Jefferson *et al*. (1987) protocol, at five different stages during the transformation protocol: stage 1, after two days of cocultivation; stage 2, after two weeks on SS medium; stage 3, after two weeks on RS medium; stage 4, after two weeks on TS medium and finally stage 5, after the establishment of regenerated plants in the glasshouse. 12 explants were randomly selected from each stage for the assay.

PCR analysis

The presence or absence of the *nptII*, *bar*, *Cry1Ac*, and *gus* genes in putative transgenic plants was determined by PCR analysis. Genomic DNA was extracted from young folded leaf tissue (Doyle and Doyle 1987; Cullings 1992). The sequence of the primer sets is shown in **Table 1**.

Table 1 primer sequences in different PCR reactions.

gene	Primer sequence
bar	5′ATTACCATGAGCCCAGAACG3′
	5′TCAGCAGGTGGGTGTAGAGC 3′
gus	5′AATAACGGTTCAGGCACAGC3′
	5′CCCTTACGCTGAAGAGATGC 3′
cry1Ac	5′-GACACAATGGACAACAACCCAAA-3′
	5′-TCACTGCAGGGATTTGAGTAATA-3′
nptII	5′-ATCGGGAGCGGCGATACCGTA-3′
	5′-GGCTATTCGGCTATGACTG-3′

NPTII assay

An NPTII assay identifying the expression of neomycin phosphotransferase in green leaves of plants transformed with the *nptII* gene was used. Protein extraction from young leaves and NPTII assay were done according to the McDonnell (1987) protocol. Membranes were exposed for 12 hrs with radioactive film to detect positive samples.

Western blotting

To determine the expression of *cry1Ac* protein in putative transgenic plants, total soluble protein was extracted from young leaves in PEB buffer (0.2 M NaCl, 0.1 M TES, 10 mM EDTA, 1 mM

Table 2 Comparisons of relative recovery of transgenic plants in two separate experiments.

No* of Experiment	No of explants	No of established plants in glasshouse	Positive plants in PCR	Positive plants in protein assay			Recovery percentage of transgenic plants	Percentage of escapes
				GUS assay	Western blotting	NPTII assay		
1	300	14	13	9	--	--	4.3	7
2	11000	76	38	--	30	35	0.37	50

PMSF). Protein concentration was determined by the Bradford (1976) procedure. 50 µg of protein was loaded on SDS PAGE gel (NuPAGE® 10% Bis-Tris precast gel, Invitrogen) and blotted onto a nitrocellulose membrane by wet transfer (Burnette 1981). Primary antibody rose in rabbits against purified Cry1Ac bacterial protein, (kindly provided by Dr William Moar, Auburn University, Auburn, AL, USA) and AP-conjugated secondary antibody raised in goats against rabbit IgG (Promega) was used for detection. The blot was developed in BCIP/NBT (Sigma) solution for 15-20 min and the reaction was stopped by washing the membrane with distilled water.

RESULTS

Shoot regeneration and rooting

58% of explants produced shoots within 2 weeks of cocultivation. Each explant produced 5-10 shoots, but most of them were bleached on Kan, or brown on phosphinotricin selection medium, in these early stages. In the *nptII* gene group, 624 shoots (5.7%) from a total of 11,000 explants survived after 5 selection cycles and all of them transferred to different rooting media but only 76 of them (0.69%) were established in glasshouse . In the *bar* gene group, 14 shoots (4.7%) survived from 300 explants after 3 cycles of selection.

Rooting percentage was different in the three media groups. For the MR media containing Kan, only 3% of shoots established roots, and those roots turned brown after 2 weeks and then died. For the MR medium without Kan, only 5% of shoots developed roots. These shoots were survived but they did not acclimate in glasshouse. In contrast, the rooting percentage on MY and IAA media was equally 37%. However, the success in establishing these rooted shoots in the glasshouse was different. 20% of rooted shoots from MY medium were successfully established in the glasshouse, whereas rooted shoots from IAA medium had 62% establishment and survival. This difference may be due to the different morphology of roots in the two media. Roots in MY media were condensed and thick, but roots in IAA media were long and narrow, apparently giving them more success in establishing in soil (**Fig. 2A**). Grafting was also successful for establishing shoots in glasshouse (**Fig. 2B**). From 10 shoots grafted, 8 of them survived in glasshouse, and appeared to have more vigorous growth than those rooted in media.

Gus assay

Gus assay was done on explants in five stages: 100% of explants tested in stage 1 had blue spots but no blue spots (0%) was observed on shoots in second stage. 17 and 28% of explants detected blue spots respectively in stage 3 and stage 4. At last 64% of established plants in glasshouse had blue spots in their leaves.

Although, results showed that blue spots were observed after cocultivation on all of explants (**Fig. 3A**) but it is not clear that these regions can regenerate shoots or not. At the end, of 300 explants were used for transformation with p*BSF16*, approximately 14 plants were regenerated and transferred to glasshouse but 9 of them showed different expression of β-glucoronidase in Gus assay. Approximately most of the shoots appeared in first weeks (first and second cycles) were not blue but most of the shoots survived after 3 selection cycles in phosphinotricin were blue in Gus assay, so putative transgenic shoots usually appear after 3 selection cycles in selection medium (**Fig. 3B**).

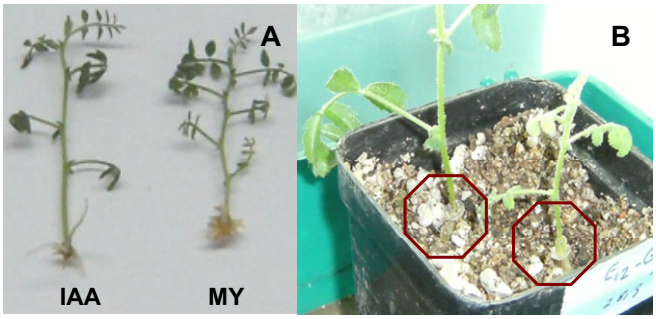

Fig. 2 (**A**) root morphology difference after 2 weeks in MY and IAA media, (**B**) grafting.

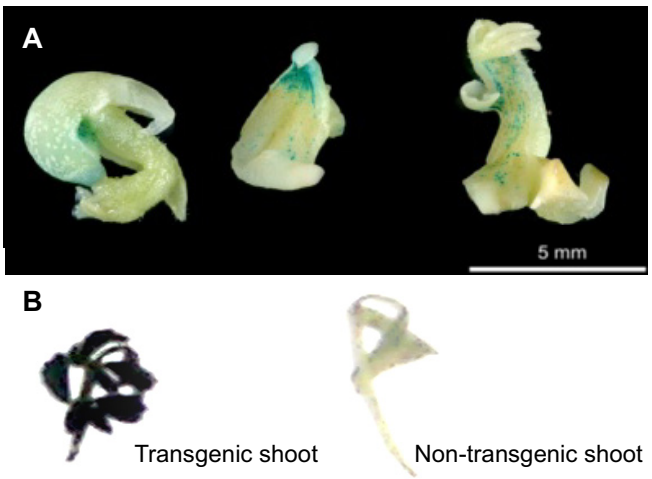

Fig. 3 Gus assay (**A**) after cocultivation. (**B**) Gus assay in the the leaves of transgenic and non-transgenic shoots.

PCR analysis

14 plants from the *bar* selection group were regenerated in glasshouse, and of these, 13 plants were found to contain both the *bar* and *gus* coding regions, by PCR analysis. 36 plants from the *nptII* selection group, from a total of 76, were found to contain the coding regions of both the *nptII* and *cry1Ac* genes. In addition, two plants were found to contain only the *nptII* coding region, indicating that only the *nptII* gene was successfully transferred into the plant genome of these particular plants. Transformation percentage using the *bar* gene (first experiment) and the *nptII* gene (second experiment) was 4.3 and 0.37%, respectively (**Table 2**).

NPTII assay and western blotting

76 plants from the *nptII* selection experiment were established in the glasshouse, and of these, 35 plants, showed positive results on the nptII assay, indicating both presence and expression of the *nptII* gene (**Fig. 4**).Western blotting was carried out using leaf protein from established plants from the *nptII* selection group. Of the 36 plants identified by PCR as containing the *Cry1Ac* coding region, 30 plants were identified as having detectable levels of Cry1Ac protein by western blot (**Fig. 5**).

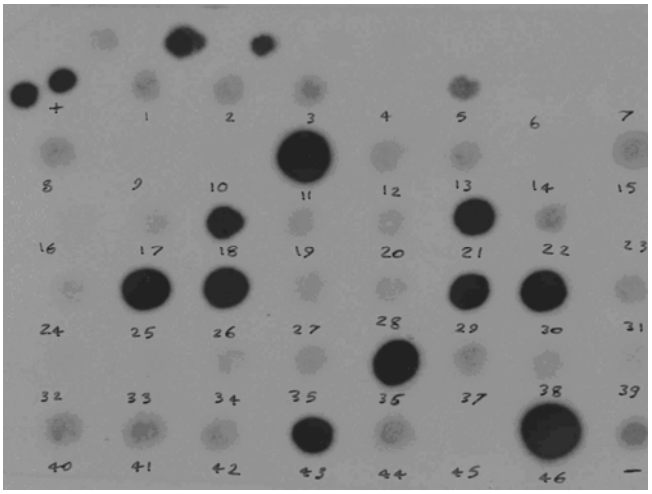

Fig. 4 nptII assay, showing dark spots indicating nptII expression on radioactive film.

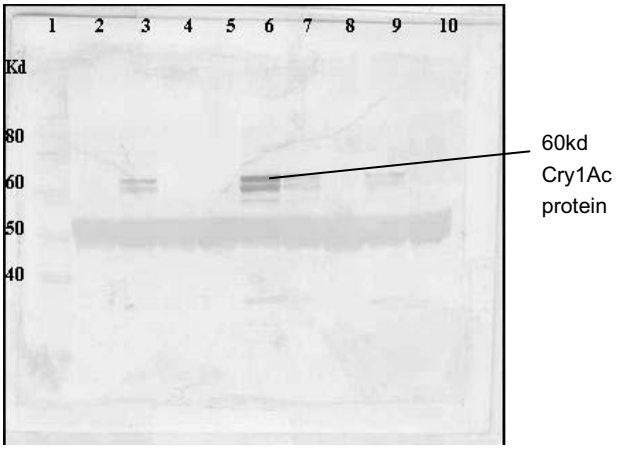

Fig. 5 Western blotting. Cry1Ac protein bands on nitrocellulose membrane after interaction with antibodies.

All of the comparisons between these two selection groups are brought in **Table 2**.

DISCUSSION

In this study, two selectable marker genes, *nptII* and *bar*, were compared as to their effectiveness in the recovery of transgenic plants from a chickpea transformation system. Number of shoots surviving before rooting was 14 (4.7%) and 624 (5.7%) shoots respectively for *bar* and *nptII* genes. Thus selection with phosphinotricin gives a 1% higher of shoots before the rooting stage, so maybe it is a better selection system, but the problem is in recovering rooted plants and also half of these shoots were escapes.

Totally, the results showed that transformation percentage was relatively low in chickpea, 4.3 and 0.37%, respectively in *bar* and *nptII* selection systems, indicating recalcitrant nature of legumes especially in chickpea. Transformation percentage of chickpea was reported between 1 to 1.5% (Kar *et al*. 1996), 1.5% (Sanyal *et al*. 2005), 3.1% (Polisetty *et al*. 2004) and 5% (Senthil *et al*. 2004). Although different attempts have been done for optimization of regeneration and transformation in chickpea but regeneration and transformation are depended on genotypes (Yousefiara *et al*. 2008), preparing explants (Sanyal *et al*. 2005) and culture condition (Kar *et al*. 1996; Krishnamurthy *et al*. 2000; Polowick *et al*. 2004). Maximum transformation percentage (5%) was observed in one report by Senthil *et al*. (2004) with *bar* gene. In other hand, existence protocols don't have reproducibility in different experiments.

Application of 200 mg/l Kan cannot remove non-transgenic shoots rapidly and shoots were green in top and a little yellow in bottom. Production of chimeric shoots is reported by Kar *et al*. (1996). *Bar* selection may give established transgenic plants quicker than *nptII* selection. As in *bar* experiment shoots are ready for rooting after 3 cycles of selection and with *nptII*, the shoots are ready for rooting after 5 cycles of selection, but 50% are escapes. As a result *bar* selection produces transgenic plants faster than *nptII* selection, and with fewer escapes. For pea transformation, Kan was not recommended as selective agent due to the high level of tolerance to this antibiotic (Puonti-Kaerlas *et al*. 1990; Schroeder *et al*. 1993) as high Kan concentrations are known to impair shoot vigor and rooting capability. So using of another agent selection as alternative of Kan is recommended for increasing of selection efficiency in short time.

In addition, observations showed that application of Kan in rooting media has negative effect on rooting. In this study, Kan was used in rooting media at first but none of shoots produced roots or roots were died at first stages, so Kan was removed from rooting media. Fontana *et al*. (1993) used MS medium with low concentration of IAA and Kin for rooting and have 50% rooting in shoots. They have observed that Kan severely inhibited rooting, so Kan was removed from rooting media. Polowick *et al*. (2004) transferred shoots to medium containing Kan at first but removed it immediately after appearance of root tip because Kan inhibit the root growth.

One of the main problems in chickpea regeneration is rooting and undesirable acclimatization of plantlets in glasshouse. Considering low rooting percentage in some studies (Kar *et al*. 1997), so optimization of *in vitro* culture for rooting and acclimatization in glasshouse is recommended. In this experiment using IAA medium for rooting gave 37% rooting and 62% acclimatization in glasshouse so it can be a good improvement in chickpea regeneration. Different rooting media has an effect on the number of shoots that develop roots and go on to be established plants. IAA medium gives 37% shoots produced roots, and of these, 62% were established. Thus with or without Kan, MR media gives poor results. The best result is from the IAA media. Shoot rooting therefore has less to do with the effect of Kan in the earlier selection media, and more to do with the type of rooting media used. Grafting has been done on a few shoots but 80% of them were survived so it can be a good alternative for rooting as in media.

The results showed that the *bar* gene can be a suitable selectable marker for getting transgenic plants with high transformation percentage and is more quickly than the *nptII* selection system. But the *bar* gene is owned by the multinational company Bayer, who will only approve its use in research facilities, and will not approve its use in commercial transgenic crops. However, if the rooting issue in *nptII* selection system could be resolved, then transformation efficiency using *nptII* could be a lot closer to that of the *bar* group, but would still take longer.

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