Mungbean yellow mosaic virus-Vi Agroinfection by Codelivery of DNA A and DNA B From One *Agrobacterium* Strain

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ABSTRACT

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Agroinfection of bipartite geminiviruses is routinely done by mixing two *Agrobacterium* strains that independently harbor partial tandem repeats of DNA A and DNA B. We report here an improved agroinfection method for bipartite geminiviruses that utilizes one strain of *Agrobacterium* that harbors DNA A and DNA B partial tandem repeats on two compatible replicons. A cointegrate vector, pGV2260::pGV1.3A, with the partial tandem repeat of *Mungbean yellow mosaic virus*-Vi (MYMV-Vi) DNA A and a binary vector, pGA1.9B, with the partial tandem repeat of MYMV-Vi DNA B gave an agroinfection efficiency of 24% when harbored in two *Agrobacterium* strains and an efficiency of 61% when harbored in one *Agrobacterium* strain. A combination of binary vectors, pGA1.9A with MYMV-Vi DNA A partial tandem repeat and pGA1.9B with DNA B partial tandem repeat, gave an agroinfection efficiency of 74% when harbored in two strains. But pGA1.9A and pPZP1.9B (a partial tandem repeat of DNA B), when present in the same *Agrobacterium* strain, gave 100% agroinfection. Accumulation of viral DNA was shown by Southern blotting. The single-strain method using two compatible replicons consistently gave 100% agroinfection efficiency.

Additional keywords: blackgram, cotransformation, *Vigna mungo*

A geminivirus isolated and cloned from infected blackgram (*Vigna mungo* (L.) Hepper) plants (34) (EMBL Accession Numbers: VMYMV DNA A-AJ132575 and DNA B-AJ132574) has been named *Mungbean yellow mosaic virus*-Vi (MYMV-Vi; 12). The family *Geminiviridae* comprises viruses with circular, single-stranded DNA genomes. MYMV-Vi is classified under the genus *Begomovirus* and has a bipartite genome with DNA A and DNA B. Agroinfection is an effective method by which infectious viral clones are introduced into plants using *Agrobacterium tumefaciens* (14). A partial tandem repeat of a virus is constructed and placed in the T-DNA of a binary vector and introduced into *A. tumefaciens*. Upon inoculation of plants with the *Agrobacterium* strain and transfer of the T-DNA, the viral genome is released in the plant cells from the partial tandem repeat of the virus. Agroinfection with a geminivirus was first reported for *Tomato golden mosaic virus* (TGMV; 29).

Two mechanisms have been proposed to explain the release of unit-length circular replicative forms in plants from the partial tandem repeats of geminiviruses delivered by agroinfection. In the absence of a duplication of viral origin of replication, homologous recombination between the tandem repeats leads to the release of unit-length double-stranded circles of DNA (27). Replicational release, a process in which the viral replication-associated protein replicates the full-length viral genome from the partial tandem repeat portion of the T-DNA, is favored when the viral replication origin is duplicated (27,32). For agroinfection of bipartite geminiviruses, two strains of *Agrobacterium* are mixed and used for inoculation (coagroinoculation). The DNA A and DNA B partial tandem repeats are independently cloned in Ti plasmid-based vectors and mobilized into separate *Agrobacterium* strains (3,10,11,13, 22,23,28,35). The strains are grown individually, mixed, and used for agroinfection.

The feasibility of simultaneous delivery of DNA A and DNA B of a bipartite geminivirus (TGMV) from a single T-DNA in one *Agrobacterium* strain has been demonstrated (18). A similar approach was deployed recently for agroinfection of *Ageratum yellow vein virus* (AYVV; 30), where partial tandem repeats of DNA A and recDNA-Aβ17 (a recombinant of DNA A and DNA β) and a tandem dimer of DNA β were placed in one T-DNA of a binary vector. This approach increased the probability of delivering all three components to the same plant cell. Cloning of partial tandem repeats of both DNA A and DNA B in one T-DNA is a complex procedure. In addition, DNA A and DNA B need not be linked on the same T-DNA for the release of circular unit-length replicative forms inside the plant cell. Therefore, we tested a simple alternate approach of placing DNA A and DNA B partial tandem repeats in the T-DNAs of two separate but compatible vectors that coexist in *A. tumefaciens.*

The efficiency of agroinfection is dependent on the *Agrobacterium* strain employed (2), the *vir* functions on the Ti plasmid (15), and the strength of promoters upstream of the viral replication-associated protein (24). We report that the efficiency of agroinfection with a bipartite geminivirus can be increased by using one *Agrobacterium* strain that harbors both DNA A and DNA B partial tandem repeats in compatible replicons. The single-strain method is simple to perform and more efficient than the two-strain method routinely used for agroinfection of bipartite geminiviruses.

MATERIALS AND METHODS

Construction of partial tandem repeat clones of MYMV-Vi. The plasmid pGA1.3A (19), harboring the partial tandem repeat of DNA A (2.7-kb *Pst*I fragment representing the full-length 1-mer + a 0.74-kb *Pst*I/*Bam*HI fragment representing the 0.3-mer) of MYMV-Vi in the plasmid pGA472 (1), has a single viral origin of replication (Fig. 1a). It was digested with *Sac*II and the partial tandem repeat was subcloned into the *Sac*II site of pGV1500 (an intermediate vector that cointegrates into the Ti plasmid derivative pGV2260 by recombination in the pBR322 sequence present in both; 7) to produce pGV1.3A (Fig. 1b). A second partial tandem repeat clone of DNA A, pGA1.9A, harbors a 5.1-kb *Sac*I/*Xho*I viral insert comprising a 2.7-kb *Pst*I fragment representing the full-length 1-mer and a 2.4-kb *Pst*I/*Hin*dIII fragment representing the 0.9-mer in pGA472 with RK2 replicon (Fig. 1c). The viral replication origin is duplicated in pGA1.9A.

The DNA B partial tandem repeat, a 5.1-kb *Sac*I/*Xho*I fragment, harbors duplicated viral replication origin in pGA1.9B (33). It

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includes a full-length (2.65-kb) DNA B with a 2.5-kb 0.9-mer (Fig. 1d). The plasmid pPZP1.9B (Fig. 1e) with pVS1 replicon was constructed as follows: the 5.1-kb DNA B partial tandem repeat was excised as a *Sac*I/*Xho*I fragment from pBS1.9B (33) and placed between the *Sac*I/*Sal*I sites of the binary vector, pPZP201 (16).

Mobilizations of partial tandem repeat clones of MYMV-Vi into *A. tumefaciens*. The partial tandem repeats in binary or cointegrate vectors were introduced into the respective *Agrobacterium* strains (Table 1) by triparental mating (9) or by electroporation. *A. tumefaciens* into which binary vectors were introduced by electroporation (transformants) or by triparental mating involving conjugation (transconjugants) were selected on AB minimal medium (pH 7.0) (4) supplemented with the appropriate antibiotics (Table 1). All *Agrobacterium* transformants and transconjugants were confirmed by Southern blot analysis for the presence of the partial tandem repeat clones (*data not provided*). For the construction of the cointegrate vector pGV2260::pGV1.3A, the intermediate plasmid pGV1.3A was introduced into C58C1 (pGV2260) by triparental mating. The recipient strain harbors pGV2260 (6), a derivative of an octopine type Ti plasmid, pTiB6S3, from which the T-DNA region has been deleted. The integration of pGV1.3A into pGV2260 will lead to the growth of *Agrobacterium* strains on a medium supplemented with carbenicillin, streptomycin, spectinomycin, and rifampicin (Table 1). The confirmation of cointegration, a process involving a recombination of pBR322 sequences of pGV2260 (6) and pGV1.3A, was performed by detailed restriction analysis with *Hin*dIII, *Kpn*I, *Sal*I, and *Eco*RI followed by Southern blot analysis (*data not shown*).

Agroinfection. The agroinfection procedure of Mandal et al. (23) was followed with a few modifications. Briefly, *A. tumefaciens* cultures were grown in AB minimal medium (pH 7.0) at 28°C to an optical density of 1 at 600 nm. The cultures were centrifuged at 1,100 × *g* for 10 min at 25°C. The pellet was resuspended in AB minimal medium (pH 5.6) supplemented with 100 µM acetosyringone. In the case of the strains harboring only DNA A or DNA B, equal volumes of the two corresponding cultures were mixed and used (coagroinoculation). *V. mungo* seeds germinated for 12 h were immersed in the respective cultures after the hypocotyl was punctured four times with a 30-G needle. The infection was carried out at 25°C for 12 h in the dark. Subsequently, the seedlings were washed with sterile single distilled water and grown in sterile vermiculite wetted with half-strength Murashige-Skoog me-

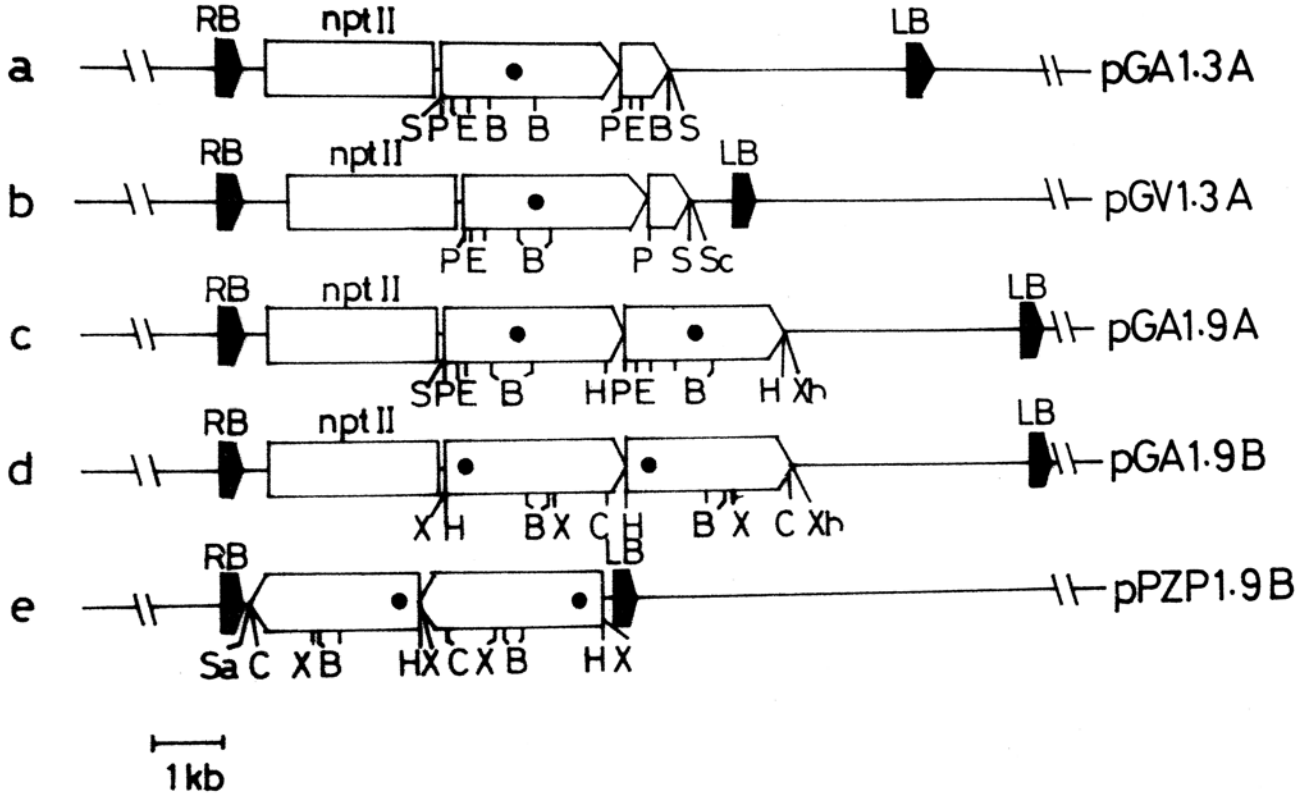

Fig. 1. Linear maps of *Mungbean yellow mosaic* virus-Vi (MYMV-Vi) partial tandem repeat regions of DNA A and DNA B. The full-length 1-mer portion and the 0.3-mer or 0.9-mer repeat portions of the virus are shown as boxed arrows; ● = common region, RB = right T-DNA border, LB = left T-DNA border, *npt*II = neomycin phospho-transferase II, B = *Bam*HI, C = *Cla*I, E = *Eco*RI, H = *Hin*dIII, P = *Pst*I, S = *Sac*I, Sa = *Sal*I, Sc = *Sac*II, X = *Xba*I, and Xh = *Xho*I.

Table 1. List of *Agrobacterium* transconjugants and plasmids with partial tandem repeats harbored

A. tumefaciens recipient strains	Reference or source	Mobilized plasmids	Drugs used for transconjugant selection (µg/ml)
C58	31	pGA1.9B	Rifampicin (10), Tetracycline (5).
C58	31	pGA1.9A	Rifampicin (10), Tetracycline (5).
C58	31	pGA1.3A	Rifampicin (10), Tetracycline (5).
Ach5	17	pGA1.9A	Tetracycline (5).
Ach5 (pGA1.9A)	This study	pPZP1.9B	Tetracycline (5), Spectinomycin (100).
C58C1 (pGV2260)	6	pGV1.3A	Rifampicin (10), Carbenicillin (100), Streptomycin (300), Spectinomycin (100).
C58C1 (pGV2260::pGV1.3A)	This study	pGA1.9B	Rifampicin (10), Carbenicillin (100), Streptomycin (300), Spectinomycin (100), Tetracycline (5).

dium (25). The plants were placed in a growth room with a light and dark cycle of 16 and 8 h, respectively, at 25°C. After a period of 8 days, the plants were potted in a 1:1 mixture of sand and vermiculite and transferred to a greenhouse. Symptoms were scored based on the appearance of yellow mosaic pattern on the trifoliate leaves. The leaves with yellow mosaic symptoms were harvested 24 days after inoculation for DNA analysis.

DNA extraction and Southern analysis of agroinfected *V. mungo* plants. DNA was extracted from trifoliate leaves of control plants and symptomatic plants as previously described (26). DNA was estimated using Hoechst dye No. 33258 in a fluorometer. Total DNA (3 µg) was fractionated in 1% agarose gels containing 1× TNE buffer (40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA, pH adjusted to 7.5 with acetic acid), blotted onto Zeta-Probe membrane (Bio-Rad Laboratories, Hercules, CA), and used for Southern analysis. For S1 nuclease treatment, 3 µg of DNA was digested for 30 min at 37°C with 4.5 units of S1 nuclease. Probes were prepared with a random primer labeling kit (Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, UK) using (α-^{32}P)dCTP (BRIT, Mumbai, India).

RESULTS

Single-strain method using a cointegrate vector. The cointegrate vector system offers a simple system to accommodate a compatible binary vector so that both DNA A and DNA B partial tandem repeat plasmids can be placed in the same *Agrobacterium* cell. A comparative analysis of agroinfection frequencies between the two-strain method and the single-strain method was first made using a cointegrate plasmid and a binary plasmid (Fig. 1). A mixture of *A. tumefaciens* strains C58 (pGV2260::pGV1.3A), harboring a cointegrate plasmid with DNA A, and C58 (pGA1.9B), harboring a binary plasmid with DNA B, caused 33 and 24% agroinfection in two independent experiments (Table 2). However, the use of a single-strain method using C58C1 (pGV2260::pGV1.3A, pGA1.9B), harboring both DNA A and DNA B, resulted in the elevation of agroinfection frequency from 33 to 78% and from 24 to 61% in two independent experiments. Thus, deployment of a single *Agrobacterium* strain harboring a cointegrate plasmid with DNA A partial tandem repeat and a binary plasmid with DNA B partial tandem repeat markedly increased the agroinfection frequency. Symptoms did not appear on control plants and on the plants infected with C58C1 (pGV2260::pGV1.3A) that carried DNA A alone. Symptoms appeared only when both DNA A and DNA B were used, confirming the bipartite nature of MYMV-Vi.

Single-strain method using two binary vectors. A comparison of agroinfection frequency of the single-strain and two-strain methods was made using binary vectors harboring both DNA A and DNA B partial tandem repeats. When the cultures of Ach5 (pGA1.9A) with DNA A and C58 (pGA1.9B) with DNA B were mixed and used in the two-strain method, agroinfection frequencies of 63 and 74% were obtained in two independent experiments (Table 2). However, in the single-strain method using Ach5 (pGA1.9A, pPZP1.9B), agroinfection frequency increased from 63 to 100% and from 74 to 100% in two independent experiments. The single-strain method using Ach5 (pGA1.9A, pPZP1.9B) consistently gave 100% agroinfection. These results indicate that partial tandem repeats of DNA A and DNA B harbored on two binary vectors in a single *Agrobacterium* strain gave higher frequencies of agroinfection than when two strains are mixed and used in the conventional way.

Analysis of viral DNA in agroinfected *V. mungo* plants. DNA was extracted from representative agroinfected plants and subjected to Southern blot analysis to detect single-stranded and double-stranded MYMV-Vi DNA. Total DNA samples extracted from the trifoliate leaves of uninfected controls, plants infected with DNA A alone, and symptomatic trifoliate leaves infected with DNA A and DNA B were analyzed by Southern blotting. DNA A- and DNA B-specific probes (without the common region) were used to confirm the accumulation of the respective viral DNA in plants exhibiting yellow mosaic virus symptoms. S1 nuclease sensitivity of single-stranded DNA was used to distinguish between single-stranded virion DNA and double-stranded replicative forms.

Control plants (Figs. 2A and B and 3A and B, lane C) and plants infected with C58C1 (pGV2260::pGV1.3A) with DNA

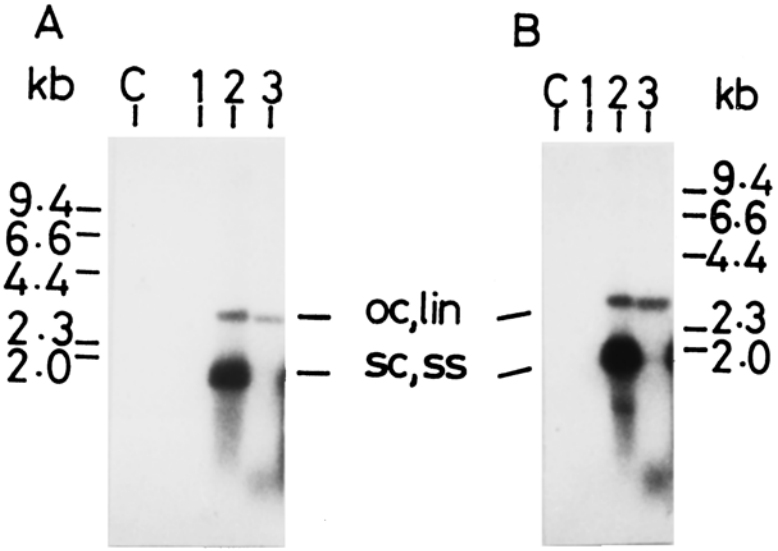

Fig. 2. Southern blot analysis of *Vigna mungo* agroinfected with cointegrate and binary vectors in the same *Agrobacterium* strain. **A,** DNA A-specific probe and **B,** DNA B-specific probe used were without common regions. Undigested total DNA (3 µg/lane) was fractionated in 1% agarose gels with 1× TNE buffer (40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA, pH adjusted to 7.5 with acetic acid). DNA from control plants (lane C), plants inoculated with C58C1 (pGV2260::pGV1.3A) (lane 1), and plants inoculated with C58C1 (pGV2260::pGV1.3A, pGA1.9B) (lanes 2 and 3) were analyzed. DNA was subjected to S1 nuclease treatment in lane 3; oc = open circular, lin = linear, ss = single-stranded, sc = supercoiled (20). Positions of λ*Hin*dIII fragments are marked.

Table 2. Comparison of *Vigna mungo* agroinfection efficiencies between single-strain and two-strain methods

Serial no.	*Agrobacterium tumefaciens* strains with partial tandem repeat clones	Plants (%) with yellow mosaic symptoms (symptomatic plants/total plants)	
		Experiment I	Experiment II
Two-strain method			
1	C58 (pGV2260::pGV1.3A) + C58 (pGA1.9B)	33 (10/33)	24 (9/37)
2	Ach5 (pGA1.9A) + C58 (pGA1.9B)	63 (17/27)	74 (14/19)
Single-strain method			
3	C58C1 (pGV2260::pGV1.3A, pGA1.9B)	78 (35/45)	61 (26/43)
4	Ach5 (pGA1.9A, pPZP1.9B)	100 (22/22)	100 (15/15)

A alone (Fig. 2A and B, lane 1) did not show any signal corresponding to the viral DNA using both the probes. Southern blot analysis of agroinfection experiments involving a combination of cointegrate vector with DNA A and a binary vector with DNA B is presented in Figure 2. Plants infected by the single-strain method (C58C1 [pGV2260::pGV1.3A, pGA1.9B]), which exhibited yellow mosaic symptoms, showed the presence of viral DNA (lanes 2 and 3). Signals for both DNA A (Fig. 2A) and DNA B (Fig. 2B) were detected. An intense band at the 1.8-kb position corresponds to the virion single-stranded DNA because it is not seen following S1 digestion. A weak 1.8-kb signal left after S1 nuclease digestion may represent the supercoiled replicative form (20). A band at the 2.7-kb position, remaining after S1 nuclease digestion, represents double-stranded replicative form in either open circular or linear form (20). *V. mungo* plants agroinfected by single-strain and two-strain methods involving two binary vectors with DNA A and DNA B also were subjected to Southern blot analysis (Fig. 3). Viral DNA corresponding to DNA A (Fig. 3A) and DNA B (Fig. 3B) was detected in all plants exhibiting yellow mosaic symptoms. An intense signal was seen at 1.8 kb for single-stranded virion DNA (plus supercoiled double-stranded replicative form). A signal at 2.7 kb corresponded to the linear and open circular forms of double-stranded replicative forms.

DISCUSSION

A simple strategy involving a single *Agrobacterium* strain harboring DNA A and DNA B partial tandem repeats in T-DNAs of two compatible binary vectors yielded a high frequency (100%) of agroinfection of the bipartite *Begomovirus* MYMV-Vi. Codelivery of DNA A from pGV2260::pGV1.3A (a cointegrate vector) and DNA B from pGA1.9B (a binary vector) in one *Agrobacterium* strain increased agroinfection from 33 to 78% and from 24 to 61% in two independent experiments (Table 2). Similarly, codelivery of DNA A from pGA1.9A (a binary vector) and DNA B from pPZP1.9B (a binary vector) increased agroinfection from 63 to 100% and from 74 to 100% in two independent experiments. In both cases, the single-strain strategy yielded higher agroinfection frequencies. The strategy of using compatible binary vectors to carry both DNA A and DNA B is advantageous over the strategy involving a cointegrate vector for DNA A, because the former yielded 100% agroinfection frequency consistently.

The relatively lower agroinfection efficiency of the cointegrate vector pGV2260::pGV1.3A could be due to two reasons: (i) the copy number of cointegrate vector in an *Agrobacterium* cell is one, whereas the binary vectors exist in multiple copies (5) and (ii) The 0.3-mer of the partial tandem repeat is relatively short in pGV1.3A and lacks the replication origin. We could not succeed in constructing cointegrate vectors harboring longer partial tandem repeats with the replication origin, because such plasmids exhibited extensive deletions of viral sequences when the intermediate plasmids were mobilized into *Agrobacterium* by triparental mating (*data not shown*). Agroinfection, involving replicational release of viral DNA from partial tandem repeats, requires the duplication of replication origin (32).

The single-strain method provided a higher efficiency of agroinfection with cointegrate vectors and with binary vectors. The use of a single *Agrobacterium* strain for codelivery of two T-DNAs has been advocated for cotransformation by Depicker et al. (8). They observed a higher cotransformation efficiency in tobacco when two T-DNAs present on the same nopaline Ti plasmid (one wild type and the other with *nptII*) were cotransferred from a single strain of *A. tumefaciens*. This was reaffirmed by Komari et al. (21), who showed that cotransformation efficiency increased from 35 to 52% in tobacco and from 14 to 47% in rice after adopting a single-strain strategy, with two T-DNAs on a single binary vector.

Many efforts have been made to improve agroinfection efficiencies of geminiviruses. In the case of a bipartitie geminivirus, TGMV, mixing of two *Agrobacterium* strains, one with DNA A dimer, and the second with DNA B dimer, yielded 70 and 80% agroinfection frequencies in *Nicotiana benthamiana* and *N. tabacum,* respectively (18). However, upon placing both DNA A and DNA B dimers of TGMV in the same T-DNA, agroinfection frequencies increased from 70 to 95% in *N. benthamiana* and from 80 to 92% in *N. tabacum*.

Saunders et al. (30) attempted to coagroinfect *Ageratum conyzoides* using *Ageratum yellow vein virus* (AYVV) DNA A and DNA β cloned in separate binary vectors and harbored in two strains of *Agrobacterium tumefaciens*. Utilizing the same strategy, they also used a combination of AYVV DNA A and recDNA-Aβ17 for agroinfection studies. This yielded agroinfection frequencies of 13 and 6%, respectively. However, when a single strain of *A. tumefaciens* harboring AYVV DNA A and DNA β in one T-DNA was used, agroinfection increased from 13 to 100%. Similarly, the use of a single *Agrobacterium* strain harboring AYVV DNA A and recDNA-Aβ17 in one T-DNA increased agroinfection from 6 to 86%. The single T-DNA-based cotransfer of DNA A and DNA B components (18) of TGMV and DNA A, DNA β and recDNA-Aβ17 of AYVV (30), were very effective and yielded almost 100% agroinfection. Though very effective, the above method involves insertion of multiple DNA components, each with partial tandem repeats or dimers, into a single vector. This is a tedious and difficult exercise. The single-strain method reported here requires a relatively simple cloning strategy, which utilizes two binary vectors with compatible replicons. Cloning of partial tandem repeats of DNA A and DNA B of a bipartite geminivirus is independently performed in separate binary vectors. The mobilization of both plasmids

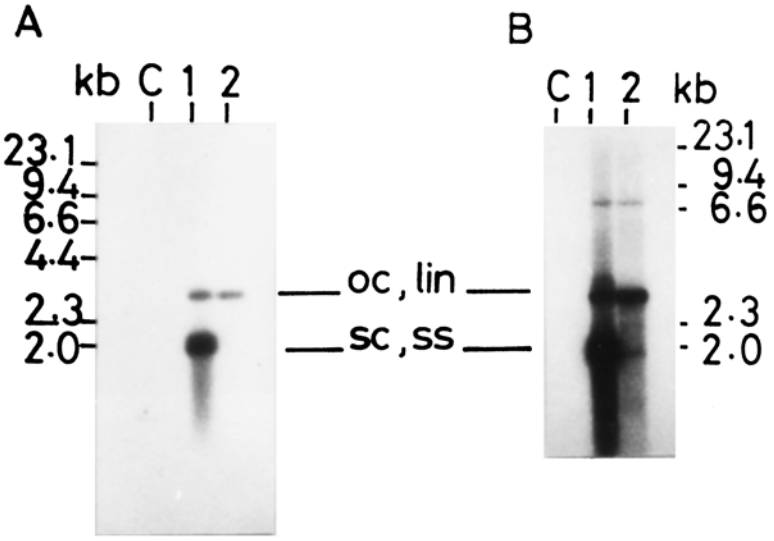

Fig. 3. Southern blot analysis of *Vigna mungo* agroinfected with two binary vectors in the same *Agrobacterium* strain. **A,** DNA A-specific probe and **B,** DNA B-specific probe without common regions were used. Undigested total DNA (3 µg/lane) was fractionated in 1% agarose gels with 1× TNE buffer (40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA, pH adjusted to 7.5 with acetic acid). DNA from control plants (lane C), and plants inoculated with Ach5 (pGA1.9A, pPZP1.9B) (lanes 1 and 2) was analyzed. DNA was subjected to S1 nuclease treatment in lane 2; oc = open circular, lin = linear, ss = single-stranded, sc = supercoiled. Positions of λ*Hin*dIII fragments are marked.

into one *Agrobacterium* strain facilitates cotransfer of DNA A and DNA B from a single *Agrobacterium* strain. This modified approach resulted in 100% agroinfection. Besides the convenience of cloning and achieving a high frequency agroinfection, the single-strain strategy described here helps in performing studies with pseudorecombinants with a high efficiency of agroinfection.

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