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ARTIFICIAL GENE-CLUSTERS ENGINEERED INTO PLANTS USING A VECTOR SYSTEM BASED ON INTRON- AND INTEIN-ENCODED ENDONUCLEASES

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Summary

The ability to create artificial gene-clusters for genetic transformation could facilitate the development of crops with multiple engineered traits, or with traits which result from the expression of multiple genes. A simple method to assemble artificial gene-clusters was developed by designing a multiple cloning site consisting of an array of homing endonuclease cleavage sites into a single vector. These enzymes are also known as intron- or intein-encoded endonucleases, and have very long recognition sequences, which makes them very rare cutters. The resulting vectors are pUGA for microprojectile-mediated transformation, and pUGA2 for *Agrobacterium*-mediated transformation. In addition, a series of unidirectional shuttle vectors containing various combinations of homing endonuclease restriction sites was constructed. Gene cassettes can be cloned into individual shuttles, and then transferred to either pUGA or pUGA2 to construct artificial gene-clusters. To test the feasibility of this approach, a six-gene cluster was constructed and transformed into soybean via microprojectile bombardment and into tobacco via *Agrobacterium*. The genes were assayed for expression in both the T_0 and T_1 generations for three independent transgenics. Up to five of the six genes were expressed. Additional changes to the construction of individual gene cassettes may improve the frequency with which all genes in the cluster are expressed.

Key words: Agrobacterium; gene stacking; metabolic engineering; microprojectile bombardment; soybean; tobacco; transformation.

Introduction

The multiple cloning sites (MCSs) of vectors commonly used for plant transformation employ the standard bacterial type II restriction endonucleases and predominantly contain hexameric rather than the rarer octameric homing sites. Due to random chance alone, the same restriction sites in the MCSs of vectors also occur often in the coding or control sequences of genes inserted into the cloning site. Thus, additional genes cannot be inserted without also digesting previously inserted genes (Überlacker and Werr, 1996). This technical difficulty has been a limiting factor to the placement of multiple genes in one vector.

Nevertheless, transformation of crops with multiple genes is necessary to produce crops with multiple traits, or when multiple genes are necessary to obtain the trait of interest (Halpin et al., 2001). Alternatives exist to placing various genes in one vector. For example, co-transformation can be used (Bower et al., 1996; Komari et al., 1996), but this makes it impossible to synchronize the number of integration events attributable to the individual plasmids. Co-transformation also does not permit control of the orientation of integration of the various different genes. Finally, the ability to obtain all the necessary plasmids in one plant is not efficient; for example, when three plasmids were co-bombarded into wheat, only 35% of the transgenic plants had all three plasmids (Campbell et al., 2000).

Polycistronic messages can also be used (Hunt and Maiti, 2001), but unless a suitable pre-assembled set of cistrons can be found (Nakashita et al., 2001), the necessary set of coding sequences still has to be assembled together. Alternatively, plants can be individually transformed and crossed together. However, having various independently segregating transgenes complicates the downstream breeding of transgenic cultivars, as each segregating transgene increases the necessary segregating population size exponentially. Technology that permits removal of the genetic marker, and thus permits successive transformation cycles is also available (Sugita et al., 2000), but such technology would require extended periods to engineer several genes.

The need for a convenient method to ligate multiple pieces of DNA together has been noted (Shibata and Liu, 2000). Such a method can be obtained with the use of a growing subset of restriction endonucleases known as intron- or intein-encoded endonucleases, or homing endonucleases. These catalyze the sequence-specific cleavage of double-stranded DNA, resulting in four-nucleotide, 3′-OH extensions (Lambowitz and Belfort, 1993), and contain highly specific large recognition sequences of 10–19 nucleotides in length, which are often asymmetrical. They have been found in group I introns, and inteins of lower eukaryotes, archaea, and proteobacterial phages (Mueller et al., 1993; Colston and Davis, 1994; Perler et al., 1994; Belfort et al., 1995). Currently

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TABLE 1

LINKERS USED TO INTRODUCE HOMING ENDONUCLEASE SITES INTO pMECA

Enzyme	Linker
I-*Ceu*I	5′-pTAACTATAACGGTCCTAAGGTAGCGA-3′
I-*Ppo*I	5′-pATGACTCTCTTAAGGTAGCCAAA-3′
PI-*Psp*I	5′-pTGGCAAACAGCTATTATGGGTATTATGGGT-3′
PI-*Sce*I	5′-pATCTATGTCGGGTGCGGAGAAAGAGGTAATGAAATGGCA-3′
I-*Tli*I	5′-pGGTTCTTTATGCGGACACTGACGGCTTTATG-3′

five homing endonucleases are commercially available. These are I-*Ppo*I from *Physarum polycephalum* (Muscarella and Vogt, 1989); I-*Ceu*I from *Chlamydomonas eugametos* (Lemieux and Turmel, 1991); I-*Tli*I from *Thermococcus litoralis* (Perler et al., 1992); PI-*Sce*I from *Saccharomyces cerevisae* (Colleaux et al., 1986); and PI-*Psp*I from *Pyrococcus* spp. (Xu et al., 1993).

Here we present a new vector system that allows for the simple creation of Artificial Gene-Clusters (AGCs), that can be at least nine genes long, through the use of homing endonucleases and an associated set of shuttle vectors, and show its use for both *Agrobacterium*- and microprojectile-mediated transformation. This work was conducted to determine whether a vector system of this nature was sufficiently functional to merit further studies.

Materials and Methods

To create a MCS consisting of homing endonuclease restriction sites, linkers (Table 1) were synthesized and PAGE- and reverse HPLC-purified by New England Biolabs (Beverly, MA). Reactions to add non-templated dATP to the 3′-OH ends of the linkers used 1 p*M* of each linker incubated with 200 μ*M* dATP and 2.5 U *Taq* DNA polymerase at 72°C for 3 h. Linkers were ligated into either *Hpa*I, *Eco*RV, *Msc*I, *Sma*I, or *Stu*I-digested pMECA (Thomson and Parrott, 1998) which had the non-templated addition of dTTP 3′-OH ends as described above, to generate pUGA (3011 bp). Ligated plasmids were transformed into DH5α and plated on Luria-Bertani agar plates containing 100 mg l^{-1} ampicillin. Initial recombinants were determined by large-colony screening (Thomson and Parrott, 1998) and subsequently confirmed by PCR. Initially, PCR was performed using the T3 and T7 promoter primers, followed by combinations of T3 or T7 with linker-specific primers. The *Agrobacterium*-based vector, pUGA2 (7096 bp), was generated by digesting pUGA with *Eco*RI and *Hin*dIII to liberate the multiple cloning site, which was subsequently cloned into the corresponding sites of pPZP201BK, which is a version of pPZP201 (Hajdukiewicz et al., 1994) wherein the spectinomycin resistance gene was replaced with *aph* for kanamycin resistance (GenBank Y00452). Finally, a series of shuttle vectors was created by adding homing endonuclease sites in the *Eco*RV and *Hpa*I sites of pMECA. Each shuttle incorporates either two identical homing endonuclease sites flanking the pMECA MCS, or two different homing endonuclease sites flanking the MCS (heteroshuttles). Positioning the intron-encoded endonucleases at the ends of the pMECA MCS preserves the availability of 40 bacterial type II restriction sites, nine of which are octamers.

An AGC containing six genes was built to test the vector system (Fig. 1). For assembly, pGPTV-bar (Becker et al., 1992) was cut with *Xba*I and *Bam*HI to release a fragment containing *bar* driven by the *nos* promoter (*nos*P-*bar*). To add the *Arabidopsis actin 2* terminator (An et al., 1996) to *nos*P-*bar*, and to replace the *nos* promoter with the potato *ubiquitin 3* (Garbarino and Belknap, 1994) promoter, as well as to obtain a cassette for hygromycin resistance, the fragment was ligated into pAPCH6 cut with *Spe*I and *Bam*HI to give pBarHyg. pBarHyg was cut in turn with *Asc*I/*Pac*I to release a fragment containing both *ubi*3P-*hph*-*nos*T and *nos*P-*bar*-*act*2T. This fragment was then ligated to the *Sce*/*Psp* forward heteroshuttle cut with *Asc*I/*Pac*I to create pMHBar. This plasmid was cut next with PI-*Sce*I/PI-*Psp*I to shuttle the BarHyg construct to pUGA, to create pU2. pGPTV-ble (Becker et al., 1992) was cut with *Xba*I and *Bam*HI to release a CaMV35S-*Ble* fragment, which was ligated into pAPC6 cut with *Spe*I and *Bam*HI, yielding pBle. pBle was then cut with *Sna*BI/*Asc*I to release a CaMV35S-*ble*-*act*2T fragment which was ligated into the *Msc*I/*Asc*I sites of the Ceu/Ppo forward shuttle to create pMBle. Digestion of pMBle with I-*Ppo*I and I-*Ceu*I allowed the fragment to be shuttled into the corresponding sites of pU2, to create pU3. To obtain the green fluorescent protein (GFP) construct, plasmid smRS-GFP (Davis and Vierstra, 1998) was cut with *Hin*dIII and *Eco*RI, the ends blunted with Klenow and dNTPs, and the CaMV35S-RS*GFP*-*nos*T fragment ligated into the *Stu*I site of the Tli/Ceu forward shuttle. Following digestion with PI-*Tli*I and I-*Ceu*I, the GFP construct was shuttled into pU3 to create pU4. Next, pGPTV-kan (Becker et al., 1992) was cut with *Xba*I and *Bam*HI to release a *nos*P-*nptII* fragment, which was subsequently ligated into pAPC6 cut with *Spe*I/*Bam*HI to yield pKan. pKan was then cut with *Sna*BI/*Asc*I to release a *nos*P-*nptII*-*act*2T fragment, which was ligated into the Ceu forward homoshuttle cut with *Msc*I and *Asc*I, thus permitting transfer of the *nptII* cassette into pU4, to create pU5. Finally, pCAMBIA 1301 was cut (intron-containing β-glucuronidase gene) with *Bst*EII and *Nco*I, and the ends blunted with Klenow and dNTPs. The resulting intron-*gus*A fragment was ligated into pAPC6 cut with *Eco*RV, yielding pActGUS. This last plasmid was then cut with *Spe*I and *Asc*I, to release an *act*2P-i*Gus*A-*act*2T cassette, which was ligated into the *Spe*I/*Asc*I sites of the Ppo/Sce forward shuttle. Digesting with I-*Ppo*I and PI-*Sce*I then permitted the cassette to be shuttled into pU5 to create pU6. Cutting pU6 with PI-*Tli*I and PI-*Psp*I made it possible to transfer the AGC into the corresponding sites of pUGA2, thus creating pU62 (20.9 kb).

Agrobacterium-mediated transformation of tobacco with pU62 and microprojectile-mediated transformation of soybean somatic embryos with pU6 were as described by Schardl et al. (1987) and Trick et al. (1997), respectively. Southern analysis verified integration of the AGC into both tobacco and soybean cell lines (Fig. 2; for the sake of brevity, only the tobacco blot is shown), while antibiotic resistance and histochemical assays (Jefferson, 1989; Kramer et al., 2002) were used to determine the expression of the remaining genes.

Gene expression was also tested in soybean. NPTII activity was measured using the Agdia kit (Elkhart, Indiana) as per the manufacturer's instructions. GUS was tested as described previously, while resistance to hygromycin, glufosinate, and bleomycin was tested by growing tissues on 20, 8, or 100 mg l^{-1}, respectively, for 2 mo. GFP was detected using a Nikon TE inverted microscope equipped with the appropriate filter set and a mounted CCD camera, in conjunction with IP Lab Spectrum imaging software.

Tobacco genomic DNA was isolated with a DNeasy Plant kit (Qiagen, Valencia, CA) and quantified. *Hin*dIII was used to cut DNA for the *hph*, *bar*, and *gusA* blots, and *Eco*RI was used for the *gfp*, *ble*, and *nptII* blots. Ten μg were run on a 0.8% agarose gel and transferred to a Hybond-N + membrane (Amersham-Pharmacia Biotech, Arlington Heights, IL) using a downward capillary blotting technique (Chomczynski, 1992). PCR, with primers specific for coding regions of each gene, was employed to obtain DNA fragments to be used as hybridization probes. The PCR products were purified on low-melting agarose gels. The ^{32}P-labeled probes were prepared using a Prime-a-Gene labeling system (Promega, Madison, WI). Hybridization and signal detection were performed according to published protocols (Sambrook et al., 1989). A 1-kb DNA ladder (Invitrogen, Carlsbad, CA) was used as a molecular weight marker.

All restriction enzymes and linkers were from New England Biolabs, *Taq*DNA polymerase was from Promega or Applied Biosystems (Foster City, CA), dNTPs were from Amresco (Solon OH), and primers were from Quiagen-Operon (Alameda, CA) or Integrated DNA Technologies (Coralville,

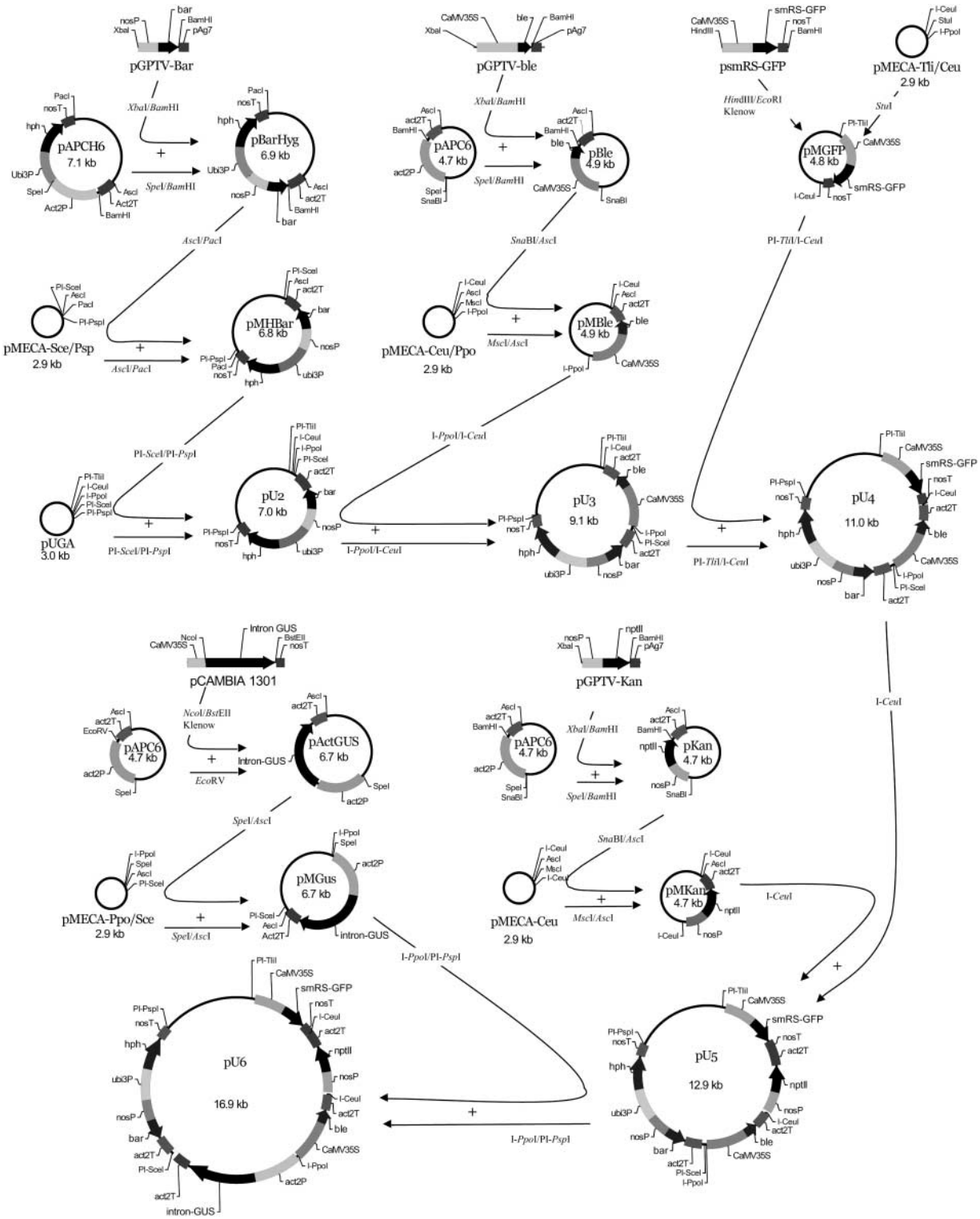

FIG. 1. Construction of the six-gene AGC. A description of the process is in the text.

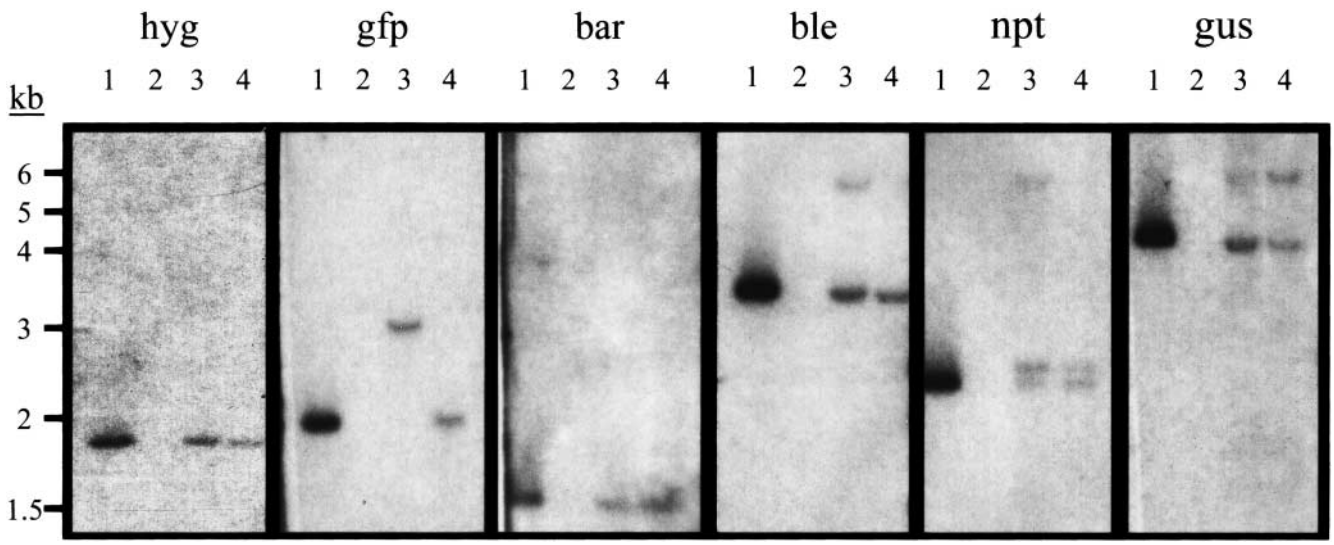

FIG. 2. Southern blot of tobacco plants transformed with pU62 containing the six-gene AGC. *Lane 1*, plasmid control; *lane 2*, untransformed tobacco; *lanes 3 and 4* are two independent transformants. The *labels* at the *top* of each *panel* indicate the probe used for that blot. *Hind*III was used to cut DNA for the *hph*, *bar*, and *gusA* blots, and *Eco*RI was used for the *gfp*, *ble* and *nptII* blots. The two transformants shown were from independent transformation attempts performed 2 mo. apart.

IA). DNA fragments were purified from gels as described by Thomson and Compton (1998). Conditions for the enzymatic manipulation of the homing endonucleases are similar to those for standard bacterial type II enzymes. The exception was that PI-*Psp*I required an overnight digestion to give complete cutting.

Results and Discussion

Asselbergs and Rival (1996) previously demonstrated the utility of homing endonucleases for vectors, while Lebel et al. (1995) demonstrated the use of shuttle vectors to link independent genes together. pUGA and its associated shuttle vectors incorporate these two principles. We currently designed nine different shuttles. At least one gene cassette can be placed in each shuttle, thus adding homing endonuclease sites to the cassette. These homing endonuclease sites are then used to clone the cassette into pUGA. The process is repeated with the other shuttles, thus assembling a gene cluster. As the recognition sites for these endonucleases can be expected to occur at random anywhere from 4^{10} bp for I-*Ppo*I, PI-*Psp*I, and I-*Tli*I to 4^{19} for I-*Ceu*I, there is little risk that the same sites will occur in the genes being added to the vector. The sequence of assembly is important. If a homing endonuclease site will be used more than once to assemble an AGC, the heteroshuttles must be utilized first. Otherwise, cloning into a single site eliminates the uniqueness of that site.

A consequence of assembling AGCs via multiple shuttles, each containing up to 40 unique sites, is that there is not a suitable enzyme that will cut only once in the introduced DNA region. The only enzymes that would cut once are the homing endonucleases, but the very large genomic fragments produced precludes distinguishing different sized fragments from independent transformants. Verification of transgenic status therefore requires other techniques, such as segregation in the progeny, while the number of gene insertion sites can be calculated with real-time PCR (Schmidt and Parrott, 2001).

The six-gene AGC was successfully transformed into plants using both microprojectile and *Agrobacterium*-mediated transformation. In the case of tobacco, two independent transgenic lines were tested, and in each case, five genes of the six genes were expressed, with GUS being the gene not expressing (data not shown). Segregation in the progeny, as determined by resistance to both kanamycin ($300\,mg\,l^{-1}$) and hygromycin ($25\,mg\,l^{-1}$), fitted the expected 3:1 ratio (χ^2 ranged from 0.06 to 0.42 for the various tests). One transgenic soybean line was tested using remnant embryogenic tissue in culture, and all genes but *ble* were expressed (Fig. 3).

This is the first study that has tested the expression of all genes used in a transformation attempt. The previous record was the study by Chen et al. (1998), who monitored expression of four genes in rice plants co-transformed with 9–11 genes. In the case of the six-gene AGC used in this study, the use of the same promoter multiple times in the same construct could have contributed to lack of expression of one gene. Besides avoiding the repeated use of the same promoter, it is possible that additional design features, such as the use of additional polyadenylation sites (Ingelbrecht et al., 1991; Breyne et al., 1992) or other transcription blockers (Padidam and Cao, 2001), can stabilize expression of all genes within a cluster. Likewise, the orientation of genes relative to each other within the transformation cassette has been shown to affect transient expression (Padidam and Cao, 2001). Because four of the five homing endonuclease recognition sites are not symmetrical, the shuttles come in two versions, with the homing endonuclease sites added in either the forward or the reverse orientation, thus making it easier to control the orientation of each gene within the cluster. The use of matrix attachment regions may also help stabilize gene expression (Allen et al., 2000).

The results show that the use of AGCs can facilitate the introduction and expression of multiple genes into plants, though questions still remain as to the optimal arrangement of individual genes within a cluster. As more homing endonucleases become commercialized, it will be possible to build ever larger AGCs. The upper size limit for these clusters remains unknown. Thus far,

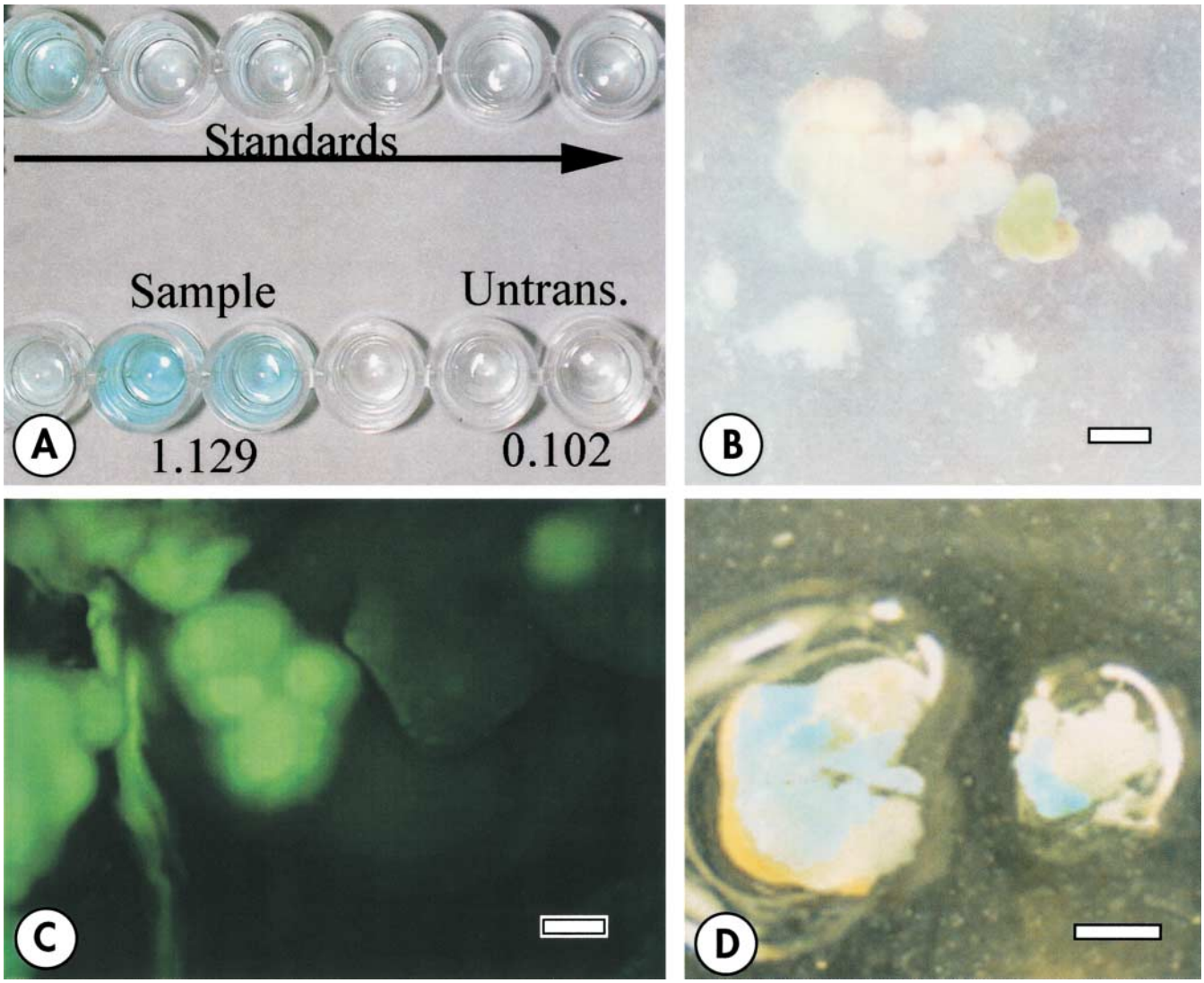

FIG. 3. Assays used to evaluate transgene expression in soybean. *A*, ELISA measurements of NPTII activity in transgenic soybean. Numbers under the wells indicate the absorbance readings. *B*, Survival of transgenic tissue after 2 mo. on 20 mg l^{-1} hygromycin. *C*, Embryogenic clusters of soybean expressing GFP. *D*, GUS-staining in soybean tissue. *Bar* = 1 mm.

Agrobacterium-mediated transformation has been used to transfer 150-kb-long YAC DNA into plant cells (Hamilton et al., 1996), while microprojectile bombardment has been likewise successful with YAC DNA of the same size (Mullen et al., 1998). Finally, whereas these vectors were designed for plant transformation, there is no reason why the vectors cannot be adapted for transformation of any type of organism.

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