Plant Cell Reports (1993) 12:165-169

Plant Cell Reports

© Springer-Verlag 1993

Stable transformation of *Phaseolus vulgaris* via electric-discharge mediated particle acceleration

D. R. Russell, K. M. Wallace, J. H. Bathe, B. J. Martinell, and D. E. McCabe

Agracetus, Inc., 8520 University Green, Middleton, Wisconsin 53562, USA

Received May 27, 1992/Revised version received August 31, 1992 – Communicated by R. Gilbertson

Summary. Transgenic *Phaseolus vulgaris* or common bean has been produced using electric-discharge particle acceleration. The method uses particle acceleration to introduce DNA into bean seed meristems. Multiple shoots are then generated and screened to recover transgenic plants at a rate of 0.03% germline transformed plants/shoot. We have been able to recover transgenic plants using both GUS and herbicide screening to introduce the *gus*, *bar*, and bean golden mosaic virus coat protein genes into the navy bean cultivar, Seafarer. The transgenic plants have been characterized over 5 generations of self-fertilization with no loss of introduced genes or expression. In addition, several families have been crossed with non-transgenic parents and these plants also show expected inheritance patterns. The introduced *bar* gene has been shown to confer strong resistance in transgenic beans to basta herbicide application in the greenhouse.

Key words: *Phaseolus vulgaris* - Bean - Transformation -Regeneration - Particle acceleration

Abbreviations: BGMV, bean golden mosaic virus; PAT, phosphinothricin acetyltransferase.

Introduction

Phaseolus vulgaris or common bean is represented by a wide variety of seed types, and is consumed both as an edible pod and as a dry seed. Bean cultivation and use is currently constrained by a number of agronomic problems such as viral, bacterial, and fungal diseases and pests, and nutritional limitations such as protein quality and digestibility. To provide a tool to address those constraints, we have developed a system to produce transgenic beans.

Efforts to produce transgenic beans have suffered from a lack of efficient DNA delivery and regeneration systems. Several systems have been described for the micropropagation of beans from apical or axillary meristems (Rubluo and Kartha, 1985; Saam et al., 1987), from cotyledonary nodes via organogenesis (McClean and Grafton, 1989), and from leaf (Malik and Saxena, 1991). Susceptibility of various bean cultivars to strains of *Agrobacterium* has been demonstrated, but the infected tissues did not produce transgenic plants (McClean et al., 1991). Mariotti et al., (1989) reported using *Agrobacterium* to infect beans, and observed GUS expression and kanamycin resistance in *Agrobacterium*-infected plants. However, there was no DNA or progeny analysis to confirm transformation or transmission of introduced traits.

A critical step in the development of a plant transformation procedure is to deliver DNA to tissues that can regenerate into intact, fertile plants. This step was overcome in soybean by developing an electric-discharge particle acceleration method to produce transgenic soybeans (McCabe et al, 1988). This protocol uses ACCELL™ technology (electric-discharge particle acceleration) to deliver the DNA directly into the mature seed apical meristem. Transgenic plants are then recovered through de novo shoot formation. Since the DNA is delivered to organized tissue many of the problems associated with recovering plants from protoplasts or callus are avoided, and the procedure is rapid and cultivar independent. This method has been used to produce many transgenic soybeans from a variety of elite cultivars and those soybeans are currently undergoing field evaluations. We have adapted the procedure used for soybean to produce transgenic beans and have been able to produce fertile, transgenic *Phaseolus vulgaris*.

Correspondence to: D. R. Russell

Material and methods

DNA. We have introduced pWRG2204 into beans in the transformation experiments described here (see Fig 1). pWRG2204 contains a pUC19 backbone with the *gus* (Jefferson et al., 1986), *bar* (White et al., 1990), and BGMV coat protein genes. The *gus* and *bar* genes are both expressed using the CaMV 35S promoter and a 5' untranslated leader from alfalfa mosaic virus (Barton et al., 1987). The *gus* gene has a 3' poly A addition site from the *Agrobacterium nos* gene while the *bar* gene has a 3' poly-A addition site from a soybean *ssu* gene (Berry-Lowe et al., 1982). The coat protein gene has been modified to contain the CaMV 35S promoter, the coat protein leader sequence, the coat protein coding sequence, and a 3' *nos* poly A site (D. Maxwell et al., in preparation).

Bead Prep. DNA-coated gold particles were prepared by resuspending gold particles (Alfa Chem. Co.) in TE (10 mM Tris-HCl, 0.1 mM EDTA, pH8) and adding DNA to a 0.5 mg DNA/mg Au concentration. Spermidine and $CaCl_2$ were added to a final concentration of 33 mM spermidine and 0.83 M $CaCl_2$. The DNA-coated Au is precipitated and resuspended in ethanol to a final concentration of 1 mg Au/ml ethanol. 163 ul of the DNA-coated gold slurry is layered on a 18 mm X 18 mm mylar sheet, allowed to dry and then used in particle acceleration as described previously (McCabe et al.,1988).

Transformation. Navy bean seeds (Seafarer, Michigan Crop Improvement Association) were surface sterilized by soaking in 2% hypochlorite, and rinsing in sterile water. The seeds were placed on MS basal media and incubated at 28° overnight. The meristems were exposed by removing the seed coat, cotyledons, and primary leaves. They were then plated on OR [MS basal media with 13 uM benzylaminopurine (BAP), (Barwhale et al., 1986)] and incubated at 15° overnight. The following day the meristems were arranged 16 explants/5 cm plate and subjected to 2 particle accelerations, using a discharge of 19 Kv under partial vacuum, about 4 hours apart. After particle acceleration, the meristems were incubated on fresh OR for an additional 2 days at 15°. The meristems were then transferred to MSR (similar to OR above except for a lower cytokinin concentration, 1.7 uM BAP) and incubated at 28° for 7 days. The meristems were then transferred to woody plant media (WPM, McCown and Lloyd (1981) and incubated at 28°C and 16 hour light to allow for shoot growth. As soon as shoots began to form on the explant and became 2-10 cm in length they were removed and a small portion of each shoot was assayed to assess transformation by the GUS histochemical stain (Christou and McCabe, 1992). Shoots that contained substantial regions of GUS activity were transferred to bean rooting media (MS basal media with vitamins, 3% sucrose and 0.1 mg/l IAA, Saam et al., 1987) and allowed to form roots. Once the plantlets formed roots, the R_0 plants were transferred to soil and grown in the greenhouse where they were allowed to flower, set seeds, and mature.

Herbicide screening and application. At the stage when shoots became 2-10 cm long, the GUS-negative shoots were removed from the explant, and placed in WPM + 5 mg/l Bialaphos. The shoots were incubated at 28 ^{o}C, 16h light until the majority of the shoots died (within 2-3 weeks). The surviving shoots were transferred from the selective media onto bean rooting media. The plants were allowed to root and grow to maturity. Plants were sprayed in the greenhouse using an aerosol applicator using water control or Basta at a rate equivalent to 1000 g/ha.

Enzyme analysis. GUS histochemical analysis was used to screen for transformants and to confirm enzyme activity in the progeny (Christou and McCabe, 1992). GUS enzyme activity was measured using a fluorometric MUG assay (Jefferson, 1987). *bar* gene activity was detected by a Bialaphos acetylation assay (De Block et al., 1987).

Nucleic acids analysis. Southern, northern (Christou et al., 1989), and PCR analysis (Gilbertson et al., 1991) were performed as previously described .

Results and discussion

Production of transgenic plants

Electric-discharge particle acceleration was used to introduce pWRG2204 into the navy bean cultivar Seafarer, and many independent transgenic families were produced. Two methods were used to screen the shoots for transformation events. In all the experiments described here the shoot segments were first screened for GUS enzyme activity (Christou and McCabe, 1992). Shoots showing significant GUS activity were transferred to rooting medium. The rooted R_0 plants were transferred to soil, and grown in the greenhouse. R_1 seed was collected from the transformed R_0 plants and planted. The R_1 plants were assayed to confirm that the introduced genes were passed on to the progeny. The presence of the DNA was confirmed by both PCR and Southern analysis.

About 75% of the germline transformed R_0 bean plants were chimeric. For example, KW46 showed only a portion of the R_0 KW46 plant was expressing GUS; of the 39 R_1 seed produced, 8 were found to be transgenic for the introduced genes (*gus*, *bar*, and BGMV coat protein). For the R_0 plant KW43, 13 of 17 progeny were transgenic and contained all of the introduced genes., suggesting KW43 was fully transformed. In any method to produce transgenic seed plants, the critical aspect is whether or not the plants can pass the introduced traits to the progeny. In all of the chimeric R_0 plants described here, the plants were fertile and passed the traits to their progeny. The progeny were fully transformed, clonal plants. In these experiments, an average of 2 shoots/explant were harvested. Approximately 0.5% of those shoots showed significant GUS-expressing transformed sectors and about 0.03% of the shoots harvested lead to germline transformed plants. We have used this procedure on a limited number of additional *Phaseolus vulgaris* cultivars. During the early stages of protocol development we produced several fully transformed shoots from the snap bean cultivar, Top crop. These shoots were destructively analyzed for GUS expression, and the expression patterns clearly predicted the transgenic shoots would have produced transgenic progeny (data not shown). We have also tried several cultivars that did not yield transgenic progeny. These included a snap bean cultivar, Bountiful (Willhite Seed), and two Latin American cultivars, Carioca and A295 (provided by Centro International de Agricultura Tropical, Cali, Colombia). We have not yet determined why certain cultivars are recalcitrant.

A second transformation screening system exploited

the ability of the enzyme phosphinothricin acetyltransferase (PAT, encoded by the *bar* gene) to detoxify the herbicides Basta or Bialaphos. About 10,000 non-GUS expressing shoots were screened for *bar* gene expression by selecting for growth on 5 mg/l Bialaphos in WPM media. Most of the non-GUS expressing shoots died within several weeks. However several shoots remained green and healthy and were removed from selection and allowed to flower and set seed. Two of these plants gave rise to transgenic progeny containing the *bar* gene. Expression of the PAT enzyme was confirmed by herbicide spraying. The remaining Bialaphos-selected shoots did not have any detectable PAT activity or *bar* DNA in the leaves of the R_0 plants and were presumably escapes from the selective regime. DNA analysis has showed that an intact copy of the *gus* gene was not present in either of these herbicide-selected plants.

Analysis of transgenic plants

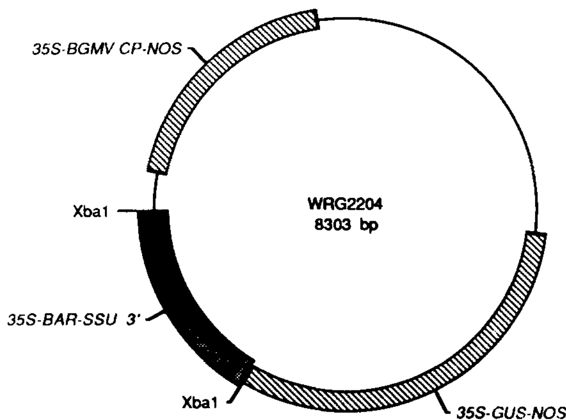


Fig. 1. Restriction map of pWRG2204. pWRG2204 contains CaMV 35S P-AMV 5' leader-*gus*-nos terminator construct transcribing clockwise, CaMV 35S P-AMV 5' leader-*bar*-soybean *ssu* terminator construct transcribing counter clockwise, and CaMV 35S P-BGMV coat protein gene-*nos* terminator construct transcribing counter clockwise.

Figure 2A shows the results of a Southern hybridization of a *gus* gene fragment to DNA from several R_1 progeny of two independent, transgenic parents. The progeny from parent KW46 had about 3 to 4 copies of the inserted DNA with only one copy at the position for full-length fragment (7 kb) inserted, while the other fragments were slightly larger than the expected size. The progeny from KW43 also showed multiple inserts with one band smaller than full-length, and many copies at the full-length size. Southern analysis of the independent transgenic families showed that plants derived from the same parent transgenic plant generally have the same pattern of integration, suggesting that even though the parent plants may have been chimeric for transgenic tissue, each set of progeny must have been

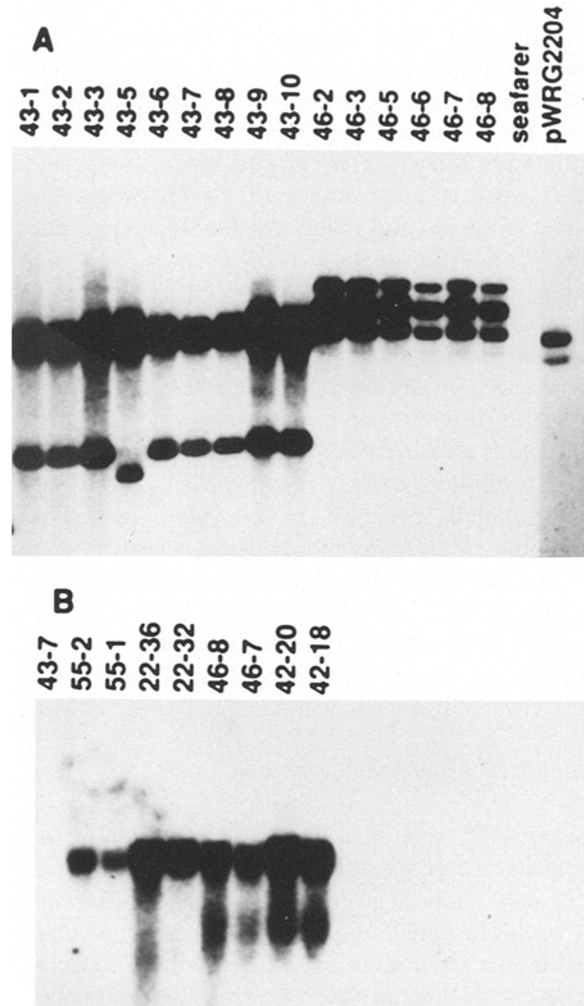


Fig. 2. (A) Southern blot bean genomic DNA. DNA from R_1 progeny plants was digested with XbaI, transferred to nylon membrane, and probed with radioactively labeled *gus* gene DNA. Lanes 1-9 are DNAs from individual progeny from the R_0 transgenic parent plant 43. Lanes 10-15 are DNAs from individual progeny from the transgenic parent plant 46. Lane 16 is DNA from wildtype navy bean (Seafarer). Lane 17 is pWRG2204 plasmid DNA at approximately 1 copy/genome digested with XbaI (the upper band migrates with the expected 7 KB XbaI fragment, the lower band migrates with undigested, supercoiled pWRG2204).

(B) Northern blot. RNA from R_1 progeny was isolated from leaves of transgenic R_1 progeny, transferred to nylon membrane, and probed with radioactively labeled *bar* gene DNA.

derived from the same transformation event. One of the

progeny, KW43-6 has a slightly altered pattern that may represent additional rearrangement of the introduced DNA. During segregation the genes appeared to co-segregate together through several generations suggesting multiple or duplicated copies may have been inserted in the same locus (data not shown). Further analysis of the results of the crosses of the transgenic beans with non-transgenic plants will be needed to confirm this point.

After an initial screening for GUS or PAT activity, transformed plants were analyzed to determine if the other genes were present. About 75% of the transgenic bean families we have examined so far contain at least one copy of each gene found on the transformation vector (data not shown). This co-integration frequency of linked genes is very similar to the co-integration frequencies observed for soybean callus (Christou and Swain, 1990) and for hundreds of independent soybean transformations (data not shown).

Northern analysis of the R_1 progeny was also performed to assess the level of RNA expression of genes inserted. A northern blot of total RNA from members of 5 different transgenic families is shown in Fig 2B. The level of *bar* RNA expression was similar for each pair of plants derived from the same parent, but the level varied between independent transgenic families. Four of these families had abundant RNA by northern and showed strong tolerance to Basta herbicide application (KW22, KW42, KW46, and KW55). KW43 *bar* RNA could be detected only by an overexposure of the blot, and the plant showed very weak resistance to Basta application.

Stable inheritance and gene expression

We examined the patterns of gene inheritance and gene expression in five of the transgenic bean families, and observed stable inheritance and segregation patterns through multiple generations. We advanced several families for five generations of self-fertilization with no loss of genes. We have also crossed the transgenic navy beans to the Brazilian dry bean variety, Carioca. In 7 different crosses using various transgenic navy beans as either male or female parent we have observed the transgenic traits are transmitted through both parents.

We have also examined the GUS expression. While all of the transgenic families that contained the *gus* gene expressed detectable GUS protein, the level and tissue-specificity varied widely between transgenic families. For example, Table 1 (A) shows GUS assay data for 5 different transgenic families. Families with the highest expression in the seed had rather low leaf expression while the high leaf expressing families had lower seed expression. These differences have also been observed using the GUS histochemical assay (data not shown). While there is wide variability between transgenic families, the level and specificity of expression is relatively

Table 1 GUS assays of transgenic beans.

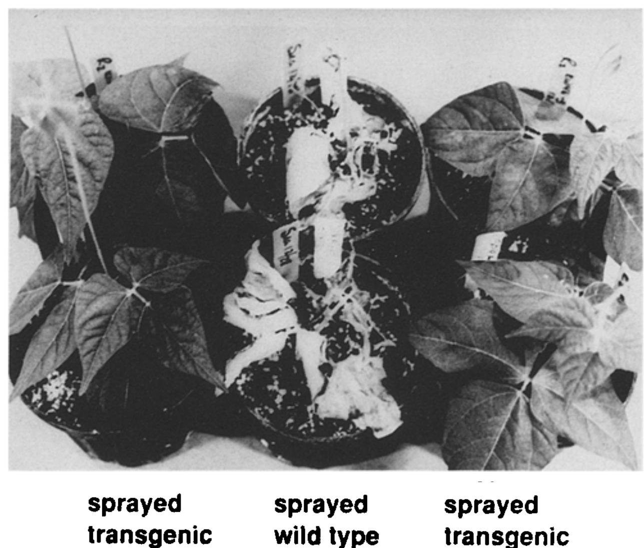
(A) MUG assay comparison of transgenic bean families.

	seed nmol/min/mg	leaf nmol/min/mg
KW41 R_2	22 (+/- 16)	171 (+/- 98)
KW42 R_2	214 (+/- 77)	3 (+/- 4)
KW43 R_2	3 (+/- 1)	1 (+/- 1)
KW46 R_2	248 (+/- 37)	3 (+/- 2)
KW47 R_2	66 (+/- 36)	286 (+/- 133)
Seafarer	< 0.2 (+/- 0.2)	< 0.2 (+/- 0.2)

(B) MUG assay comparison through several generations.

	seed nmol/min/mg
KW42 R_1	187 (+/- 82)
KW42 R_2	214 (+/- 77)
KW42 R_3	203 (+/- 61)
KW42 R_2xCarioca R_2	108 (+/- 8)
KW46 R_2	248 (+/-37)
KW46 R_3	191 (+/- 50)
KW46 R_4	131 (+/- 20)
KW46 R_5	256 (+/- 88)
KW46 R_2xCarioca R_2	101 (+/- 9)

Mug assay values are the average of assays of 3 to 5 individual seeds or plants from each group. Standard deviation is given in parenthesis. All plants are from self-fertilization except the Carioca crosses. The crosses were seed produced by selfing the F1 plant that resulted from the cross of transgenic navy bean with Carioca.



sprayed transgenic | sprayed wild type | sprayed transgenic

Fig. 3. Herbicide resistance in transgenic beans. 17 day old transgenic KW46 R_2 and wildtype Seafarer bean seedlings were sprayed at a rate of 1000 g/ha Basta or with water. The photograph was taken 6 days after treatment.

stable within a family (data not shown). Table 1 (B) shows GUS assays of seeds from several subsequent generations of 2 different transgenic families. GUS expression appears to be stable for multiple generations of self-fertilization. For both families the GUS expression level decreased in the selfed progeny of the cross with Carioca. The lower GUS expression may be due to a gene dosage effect but analysis of additional generations will be required to address this observation.

Herbicide resistant beans

We used the *bar* gene to screen for transgenic shoots and to confer strong resistance to Basta spray applications. Transgenic navy bean R_2 seed (KW46) and wildtype Seafarer seeds were planted and grown in the greenhouse for 17 days, at which stage they had produced several sets of trifoliolate leaves. The plants were sprayed at a rate of 1000 g/ha Basta or with a water control. Within 2 days necrotic spots were evident on the control leaves, and within 6 days the control plants were dead while the transgenic plants were healthy and indistinguishable from the water sprayed transgenic plants (see Fig 3.). Similar resistance to Basta in the greenhouse has also been observed using the same *bar* gene in transgenic soybeans and those transgenic soybeans have been shown to confer Basta resistance under field conditions (D. Russell, et al., in preparation). It is likely that the level of resistance observed in the transgenic navy beans described here would also be sufficient to confer resistance under field conditions.

In summary, we have developed an electric-discharge particle acceleration procedure to produce transgenic navy beans. The transgenic bean plants were healthy and fertile and were shown to transmit the transgenic traits through both pollen and ovum. The method has proven to be applicable to a very diverse group of plants including common bean, soybean (McCabe et al. 1988), poplar (McCown et al., 1991), cranberry (Serres et al., 1992), rice (Christou et al., 1991), and cotton (McCabe et al., in preparation).

Acknowledgments. We thank Douglas Maxwell for helpful discussions, Robert Rand for assistance in crossing, and James Fuller and Jon Cooley for excellent technical assistance.

References

Barton K, Whiteley H, Yang N-S. (1987) Plant Physiol 85: 1103-1109.

Barwhale U, Kerns H, Widholm J (1986) Planta 167: 473-481.

Berry-Lowe SL, McKnight T, Shah D, Meagher R (1982) J Mol Appl Genet 1:483-498

Birkett CR, Foster K, Johnson L, and Gull K (1985) FEBS Letters 187:211-218.

Christou P, McCabe D (1992) Plant Journal 2:283-290.

Christou P, Ford T, Kofron, M. (1991) Bio/Technology 9:957-962.

Christou P, Swain W (1990) Theor Appl Genet 79:337-341.

Christou P Swain WF, Yang N-S, McCabe DE (1989) Proc Natl Acad Sci USA 86:7500-7504.

De Block M, Botterman J, Vandewiele M, Dockx J, Thoen C, Gosselé V, Rao Movva N, Thompson C, Van Montagu M, Leemans J (1987) EMBO J 9:2513-2518.

Gilbertson R, Rojas M, Russell D, Maxwell D (1991) J Gen Virol 72:2843-2848.

Jefferson RA, Burgess SM, Hirsh D (1986) Proc Natl Acad Sci USA 83:8447-8451.

Jefferson RA (1987) Plant Mol Biol Rep 5:387-405.

McCabe DE, Swain WF, Martinell BJ, Christou P (1988) Bio/Technol 6:923-926.

Malik KA, Saxena DK (1991) Planta 184:148-150.

Mariotti D, Fontana G, Santini L (1989) J Genet Breed 43: 77-81.

McClean P, Chee P, Held B, Simental J, Drong RF, Slightom J (1991) Plant Cell, Tissue and Organ Culture 24:131-138.

McClean P, Grafton KF (1989) Plant Sci 60:117-122.

McCown B, McCabe D, Russell D, Robison D, Barton K, and Raffa K (1991) Plant Cell Rep 9:590-594.

McCown and Lloyd (1981). Proc. International Plant Propagation Soc. 30:421.

Rubluo A, Kartha KK (1985) J Plant Physiol 119:425-433.

Saam MM, Hosfield GL, Saunders JW (1987) J Amer Soc Hort Sci 112(5):852-855.

Serres R, Stand E, McCabe D, Russell D, Mahr D, McCown B (1992) J. Hort. Sci. 17:174-180.

White J, Chang SYP, Bibb MJ, Bibb MJ (1990) Nucl Acid Res 18(4):1062