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Recovery of primary transformants of soybean *

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ABSTRACT

Three transformants of soybean, Glycine max (L.) Merr., have been recovered among a total of 18 plants regenerated by somatic embryogenesis from immature cotyledon tissues after cocultivation with Agrobacterium strains carrying a 15 kD zein gene (pH5PZ3D). DNA from upper leaves hybridized to a synthetic RNA probe specific for the zein sequence at a level equivalent to at least one copy per haploid genome. Hybridization to a vir G/C probe, however, was negligible, indicating that sequestration of whole bacteria or even persistence of plasmids within the tissues could not account for the zein hybridization signals. Progeny of all plants were uniformly untransformed. Since most somatic embryos have a multicellular origin in the regeneration system used, it is believed that the primary transformants were chimeric. The results indicate that somatic embryogenesis may be adaptable to Agrobacterium-mediated transformation in soybean, but that greater numbers of mitotic cycles under selection before embryo initiation will be required if somatic embryogenesis is to be used efficiently for production of plants with transformed germ-line cells.

ABBREVIATIONS

NAA, 1-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

INTRODUCTION

The leaf disc transformation system developed by Horsch et al (1985) has facilitated the Agrobacterium-mediated genetic transformation of those dicotyledonous species able to regenerate from leaf tissue, most notably certain members of the Solanaceae. This development has led to the transformation of various crop species, with soybean (Glycine max (L.) Merr.) being a major exception among dicotyledonous crop plants.

Soybeans will regenerate from leaf tissue only if axillary meristem tissue is present (Wright et al., 1987), and this tissue has not so far been susceptible to transformation by Agrobacterium tumefaciens. In addition, soybeans have not always been considered a host for Agrobacterium tumefaciens (DeCleene and DeLey, 1976; Matthysse and Gurlitz, 1982). It is possible, however, to obtain galls from seedlings (e.g. Kudirka et al. 1986, Byrne et al., 1987), indicating that soybean should be amenable to Agrobacterium transformation. This has been confirmed recently by Hinchee et al. (1988) who achieved Agrobacterium-mediated gene transfer and plant regeneration from mature cotyledon tissues. Soybean protoplasts have also been transformed by cocultivation with Agrobacterium (Baldes et al., 1987), or by electroporation (Christou et al., 1987; Lin et al., 1987). A recently reported protocol for regeneration from protoplasts (Wei and Xu, 1988) may therefore be adaptable as an alternative technique for gene transfer.

De novo regeneration of soybean has been shown to occur from immature cotyledons, which undergo somatic embryogenesis when exposed to moderately high auxin levels. Lazzeri et al. (1985) and Barwale et al. (1986) developed a regeneration system using NAA as the inducing auxin, while Ranch et al. (1985, 1986) developed a system based on 2,4-D. Subsequent studies have revealed that NAA-induced embryos more closely resemble zygotic embryos in morphology (Hartweck et al., 1988) and in the profile of lipids and storage proteins (Dahmer, 1988). These characteristics are associated with germination efficiency (Lazzeri, et al., 1987a; Parrott et al., 1988). In this work we demonstrate that regeneration from immature embryos by somatic embryogenesis also shows potential for development as a gene transfer system for soybean.

MATERIALS AND METHODS

Plants of soybean genotypes Century, Cobb, Douglas, Harosoy, Heilongjiang 10, Heilongjiang 26, J103, Lee, Manchu, Manitoba Brown, McCall, Peking, PI 283332, and Williams 82 were grown in the greenhouse. Plants were grown, 5 to a pot, using a 2:2:1 mix of soil, sand, and Promix, in ten-inch pots. The photoperiod was supplemented, when necessary, to 14 hours using high pressure sodium lighting. Pods were surface sterilized by immersion in 70% 2-propanol for

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30 seconds, then in 25% commercial chlorine bleach solution for 12 minutes. Cotyledons were removed from immature seeds 3 to 5 mm in length, by removing the end containing the embryonic axis and pushing the cotyledons out of the seed coat (Lazzeri et al., 1985).

Not all cells of the immature cotyledons are capable of forming somatic embryos. Cells with embryogenic potential following exposure to NAA are limited to a narrow crescent near the distal perimeter of the immature cotyledons when these are placed abaxial side down on the medium (Hartweck et al., 1988). To wound this region and permit direct contact between Agrobacterium and the cells with embryogenic potential, twenty immature cotyledons were placed on a 2 x 2 cm square of 500 μm stainless steel or nylon mesh and pressed into the mesh with a spatula. The mesh squares, containing the macerated cotyledons, were placed on N10 medium (MS salts, B5 vitamins, 1.5% sucrose, 10 mg/l NAA, adjusted to a pH of 5.8, with 0.2% Gelrite as a solidifying agent; Lazzeri et al., 1985). The medium was dispensed into plastic disposable 100 x 20 mm petri dishes in 35 ml aliquots. The macerated cotyledons were inoculated with an overnight suspension of LBA4404 (pH5PZ3D) or EHA101 (pH5PZ3D). pH5PZ3D is a binary Ti plasmid that contains the 15 kD zein coding region behind the β-phaseolin promoter (Hoffman et al., 1987). Neomycin phosphotransferase was used as the selectable marker (Fraley et al., 1983). Following overnight incubation at 28^{o}C the mesh squares with the cotyledon tissue were transferred to N10 medium supplemented with 500 mg/l of Mefoxin (cefoxitin) to remove the Agrobacterium. Some mesh squares with cotyledon tissue were transferred to medium additionally supplemented with G418 at 10 mg/l as a selection agent. Somatic embryos were removed at 30 days and placed on BKZN medium, which contains MS salts, B5 vitamins, 3% sucrose, 0.017 mg/l each of benzyladenine, kinetin, and zeatin, 0.05 mg/l of NAA, adjusted to pH 5.8, and solidified with 0.2% Gelrite (Lazzeri et al., 1985). The BKZN medium was supplemented with Mefoxin at 500 mg/l. Embryos were transferred on this medium at monthly intervals until germination occurred. Germinating embryos were transferred to medium with HPN salts (Lazzeri et al., 1987b) solidified with 0.2% Gelrite. All cultures were maintained at 25^{o}C with a 23 hour photoperiod provided by cool white fluorescent tubes supplemented with 50W incandescent bulbs (c. 15 $\mu Em^{-2}S^{-1}$). Rooted plants were transferred to soil and allowed to set seed in the greenhouse. DNA was extracted from leaf tissue of the primary regenerants and their progeny, as described by Hoffman et al. (1987) and digested with restriction endonuclease Eco R1. The DNA fragments were separated by electrophoresis in a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled synthetic RNA probe specific for the 15 kD zein sequence (Hoffman et al., 1987). DNA from tobacco plants transformed with the zein gene was used as a positive control.

To check the possibility that apparent transformation might result from the presence of persistent whole bacteria or free plasmids sequestered in the plant tissue, Southern hybridizations were also performed using a 6.3kb BAM H1 probe covering part of the Vir G and most of the Vir C region of pTi 15955.

RESULTS AND DISCUSSION

All the transformation attempts are summarized in Table 1. Both embryo recovery and conversion of somatic embryos to plants were very low following cocultivation with Agrobacterium. A total of 9800 cotyledons from 14 different soybean genotypes were cocultivated with Agrobacterium following maceration on either nylon or stainless steel mesh. Early attempts at using Agrobacterium strain LBA4404 were discontinued because recovery of somatic embryos following cocultivation with LBA4404 was consistently lower than when strain EHA101 was used. Somatic embryos were not recovered from all soybean genotypes. PI 283332 is especially amenable to this protocol, as evidenced by the high number of somatic embryos recovered. It was possible to regenerate a total of 18 plants using this protocol. Of these, three plants contained T-DNA, as determined by Southern hybridization. One plant was obtained from the cultivar Peking after cocultivation with LBA4404 (pH5PZ3D), and the other two were obtained from PI 283332 using EHA101 (pH5PZ3D). Tissue samples collected from these plants were surface-sterilized and placed on LB agar to test if Agrobacterium might be present in the tissues. Agrobacterium was never recovered.

Table 1. Summary of soybean genotypes and number of cotyledons cocultivated with Agrobacterium strains carrying plasmid pH5PZ3D, the number of somatic embryos recovered in the presence (+) or absence (-) of G418, the number of plants regenerated, and the number of transformed plants obtained.

Genotype	Strain	G418	# Cot	# Embryos	# Plants	# Trans.
Century	EHA101	+	680	0	--	--
Cobb	EHA101	+	300	0	--	--
Douglas	EHA101	+	580	5	0	--
Douglas	EHA101	-	100	0	--	--
Harosoy	EHA101	+	300	0	--	--
Heil. 10	EHA101	+	460	0	--	--
Heil. 26	EHA101	+	100	1	0	--
J103	EHA101	+	2160	83	0	--
J103	EHA101	-	340	31	0	--
J103	LBA4404	+	200	0	--	--
J103	LBA4404	-	200	1	0	--
Lee	EHA101	+	30	0	--	--
Manchu	EHA101	+	380	0	--	--
Manchu	EHA101	-	180	18	1	0
Man. Br.	EHA101	+	20	0	--	--
Man. Br.	EHA101	-	20	30	1	0
McCall	EHA101	+	680	13	0	--
Peking	EHA101	+	960	1	0	--
Peking	EHA101	-	100	0	--	--
Peking	LBA4404	+	200	0	--	--
Peking	LBA4404	-	200	10	1	1
PI 283332	EHA101	+	820	237	14	1
PI 283332	EHA101	-	10	1	1	1
Williams	EHA101	+	480	7	0	--
Williams	EHA101	-	300	0	--	--

Heil. 10 = Heilongjiang 10; Heil. 26 = Heilongjiang 26; Man. Br. = Manitoba Brown; Williams = Williams 82

A Southern hybridization analysis was performed using DNA extracted from upper leaves (stage R3-R4) of the first putatively transformed regenerant (cv. 'Peking') treated with LBA4404 (pH5PZ3D). The pH5PZ3D probe hybridized with a 2.7 kb Eco R1 band from the transformed plant (Fig. 1, lane 4), rather than the expected 4.7 kb band. Hybridization to a typical 4.7 kb Eco R1 zein band was seen in the positive-control tobacco plants transformed with the same construct via Agrobacterium at an earlier time (Fig. 1, lanes 1 & 2). This indicated that a deletion or other rearrangement had occurred in the T-DNA. The alteration of the zein sequence was later found to have occurred in pH5PZ3D in the Agrobacterium strain LBA4404, between the time at which this strain was used to produce transgenic tobacco plants expressing a functional 4.7kb zein gene (Hoffman et al., 1987), and the time of coculture with soybean cotyledons in the present work. The occurrence of this mutation emphasizes the need for constant monitoring of T-DNA sequences with each plant transformation experiment.

The zein probe hybridized to the equivalent of at least one copy per haploid genome equivalent (Fig 1), as did probes for NPT II and octopine synthase (data not shown). Hybridization to the Vir G/C probe, however, as assessed by laser densitometry, represented only 0.03 of a genome equivalent (data not shown). Sequestration of whole Agrobacterium cells or free

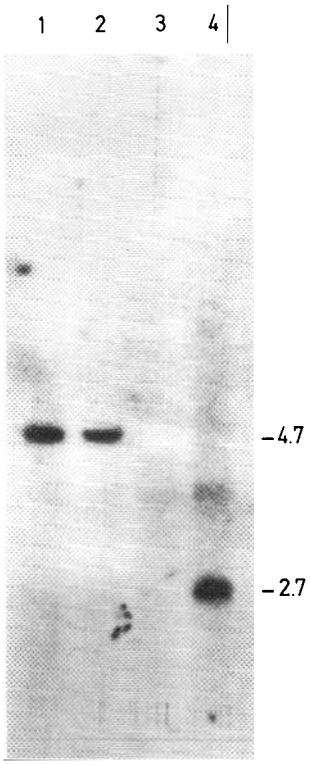

Fig. 1. Southern hybridization analysis of the first transformant plant [(cv. Peking treated with LBA4404 (pH5PZ3D))]. Lanes 1 and 2 contain tobacco DNA with three copies and one copy respectively of pH5PZ3D T-DNA; lane 3 contains DNA from non-transformed soybean; lane 4 contains DNA from the transformed soybean regenerant. The probe is a ^{32}P-labelled synthetic RNA specific for the 15 kD zein sequence (5μg DNA loaded per lane.)

plasmids within the soybean tissues therefore could not account for the zein hybridization signals. Two additional putatively transformed regenerants [P.I. 283332 treated with EHA101(pH5PZ3D)] contained NPT II-hybridizing sequences, but again not on a restriction fragment of the expected size.

No progeny of the three plants were transformed. Taken together with the strong evidence for transformation, this suggests that the regenerated plants were chimeric. Chimeric plants are consistent with the histological observation of a predominantly multicellular origin of somatic embryos in soybean (Hartweck et al. 1988), and with preliminary observations (unpublished) of differentiated β-glucuronidase-positive and -negative layers in embryonic structures regenerating from soybean cotyledons cocultivated with Agrobacterium carrying the β-glucuronidase (GUS) reporter gene (Jefferson 1987) within the T-DNA. Hartweck et al. (1988) found that, when NAA is used as the inducing auxin, most early formed somatic embryos originate from small groups of cells isolated from the rest of the cotyledon by necrotic tissue. These groups of cells appear to act coordinately as a group to form a globular stage somatic embryo, with little or no intervening cell divisions before they become embryogenic. It is this phenomenon that most distinguishes soybean regeneration from that of other species whose transformation has been reported. Agrobacterium probably transforms only one or a few of the cells that will act coordinately to form a single embryo. The lack of intervening cell division cycles before the cells become embryogenic has presumably prevented the selection of uniformly transformed embryos and shoots.

These results indicate that immature embryonic tissues of soybean are amenable to Agrobacterium-mediated transformation. Both soybean genotype and Agrobacterium strain are expected to influence the efficiency of any soybean transformation protocol. Modifications of the regeneration protocol to promote cell division cycles before the initiation of embryos, or to stimulate secondary embryogenesis from transformed embryo sectors (McGranahan et al., 1988), should permit the recovery of plants that are not chimeric, or at least have larger transformed sectors. This will increase the likelihood that in primary transformants the L2 layer of the apical meristem (which ultimately gives rise to the gametes) will be transformed.

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