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Genotype screening for proliferative embryogenesis and biolistic transformation of short-season soybean genotypes

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Abstract Eighteen of 20 short-season soybean (Glycine max (L.) Merrill) genotypes (maturity group 0 and 00) screened for proliferative embryogenic capacity formed secondary globular embryos, at rates of 1–70% of cultured immature cotyledons. Five genotypes produced embryogenic cultures which were proliferative for at least 6 months. Proliferative embryogenic cultures of AC Colibri and X2650–7–2–3 were bombarded using a Bio-Rad PDS-1000/He particle gun. Co-bombardments with plasmid pairs pHyr (encoding a type IV aminoglycoside phosphotransferase; aphIV) and pRD300pat (encoding a phosphinothricin N-acetyltransferase; pat) or pRD300pat and pFF19G (β-glucuronidase; uidA or gus) resulted, respectively, in 12 hygromycin-selected lines with multiple insertions of aphIV and pat, and two l-phosphinothricin-selected lines plus three β-glucuronidase-positive lines recovered without selection. Although fertile plants were recovered from young proliferative cultures, transgenic plants, which were derived from cultures 12–14 months of age, were sterile.

Key words Soybean · Proliferative · Embryogenic · Bombardment · Transformation

Abbreviations AMV: alfalfa mosaic virus · aphIV: Type IV aminoglycoside phosphotransferase · CaMV: cauliflower mosaic virus · 2,4-D: 2,4-Dichlorophenoxyacetic acid · gus: uidA or β-glucuronidase · L-ppt: L-Phosphinothrinic · MS: Murashige and Skoog · pat: Phosphinothrinic N-acetyltransferase · PEG: Polyethylene glycol · X-gluc: 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Introduction

Soybean acreage in short-season areas of Canada has risen due to breeding for long-day insensitive genotypes that mature under short days (Beversdorf et al. 1995). In addition to introduction of new traits by breeding, novel traits for further cold and short-season adaptation may now also be introduced by transformation. In other soybean genotypes, transformation has resulted in herbicide-resistant soybeans (Padgette et al. 1995), and tolerance to lepidopteran insects (Stewart et al. 1996). However, existing transformation methods are inefficient and genotype-dependent and their applicability to short-season adapted genotypes is unknown. Three approaches resulting in fertile transgenic soybean include electric discharge particle gun transformation of immature embryonic axes (McCabe et al. 1988), biolistic particle gun transformation of proliferative embryogenic cultures (Finer and McMullen 1991), and Agrobacterium-mediated transformation of cotyledonary explants (Hinchee et al. 1988). In the first method, use of the proprietary electric discharge gun device was shown to be essential for delivery of particles to the appropriate cell layer of the target tissue (Sato et al. 1993). In this paper we evaluate the short-season soybean genotypes using the second, or biolistic, approach. Our results for Agrobacterium-mediated transformation of these genotypes are reported in Donaldson and Simmonds 1999.

Production of transgenic soybean via biolistic transformation uses proliferative embryogenic cultures as targets (Finer and McMullen 1991; Sato et al. 1993; Parrott et al. 1994). These cultures undergo continuous embryogenesis to the globular stage from secondary globular embryos initially formed on immature cotyledons (Finer and Nagasawa 1988) and thus differ from primary somatic embryos of soybean which have been studied more extensively (Komatsuda and Ohyama 1988; Parrott et al. 1989). Unfortunately, formation of the proliferative embryogenic cultures is genotype-dependent (Bailey et al. 1993a, 1993b) and transgenic

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plants obtained following bombardment of the cultures are often sterile (Kostow et al. 1995; Parrott et al. 1995). Factors influencing genotype-dependency for formation of proliferative cultures are not understood, as only three of eight maturity group III-VI soybean genotypes formed proliferative embryogenic cultures (Bailey et al. 1993a). High rates of primary somatic embryogenesis are reported to possibly correlate with the presence of certain genotypes, including the Swedish genotype 840–7–3 (PI487477) and AK (Harrow) (Parrott et al. 1989; Tian et al. 1994). It is not known, however, if these genotypes, which are predominant in the pedigree of many of the short-season genotypes, are also correlated with high rates of proliferative embryogenesis. Factors affecting the efficiency of transformation are also not understood since transgenic lines cannot always be recovered (Parrott et al. 1994). In this study, we wished to examine the capacity for proliferative embryogenesis and transformation efficiency amongst short-season soybean genotypes. Twenty maturity group 0 and 00 genotypes were screened for their ability to form proliferative embryogenic cultures using established methods. Several resulting cultures were co-bombarded with plasmids carrying the transgenes aphIV and pat or pat and uidA and either selected on hygromycin or phosphinothricin, or screened for β-glucuronidase (gus) activity. Transgenic lines were analysed for their patterns of integration of selected and unselected genes and for the fertility of the regenerated plants.

**Materials and methods**

**Plant material**

*Glycine max* (L.) Merrill cultivars AC Colibri, Maple Arrow, Maple Amber, Maple Donovan, Maple Glen, AC Brant, AC Alpha, and natto-type cultivars Canatto, Commander, Nattawa and Nattosan were developed at the Eastern Cereal and Oilseed Research Centre (ECORC). Nine other ECORC breeding lines and *G. soja* PI342–619B and PI342–621A were also used. Donor plants for immature cotyledons were grown in the greenhouse using a 16-h photoperiod, with lighting supplemented by high pressure sodium lamps when required.

Induction of proliferative embryogenic cultures and regeneration of plants

Culture initiation, proliferation, and plant recovery essentially followed the methods of Finer (1988) and Finer and Nagasawa (1988). Immature cotyledons between 4 and 5.9 mm in size were placed axially side-down on MSD40 medium (Finer and Nagasawa 1988) containing MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 6% (w:v) sucrose, 10 mM asparagine, pH 5.8 and cultured at 27°C under a 16-h photoperiod with a light intensity of 70 µmol m–2 s–1. Embryos were desiccated in 85% relative humidity as described by Bailey et al. (1993b) and germinated on B5 medium with 3% sucrose and 0.6% Phytagar or 0.2% Gelrite, pH 5.8. Plants with two trifoliate leaves were transplanted to soil and grown under a 16-h photoperiod prior to transfer to a 12-h photoperiod for seed formation.

Plasmids, co-bombardments and selection and/or screening for transformed cultures

Plasmid pRD300pat encoding a sequence-modified *phosphinothricin N-acetyl transferase gene* (pat) from *Streptomyces viridochromogenes* (Wohleben et al. 1988) linked to the AMV enhancer of transcription, the cauliflower mosaic virus (CaMV) 35S enhanced promoter and the 3′ polyadenylation sequence from the *nos* gene (3′-nos-ter) in pUC9, was co-bombarded with either plasmid pHygR (kindly provided by J. Finer, Ohio State University) encoding *aphIV* linked to the CaMV 35S promoter and 3′-nos-ter in pUC119, or plasmid pFF19G (Timmermans et al. 1990) encoding the *uidA* or β-glucuronidase (gus) gene linked to the CaMV 35S enhanced promoter and 3′ polyadenylation regions in pUC120. Plasmids were isolated from *Escherichia coli* using an alkaline lysis midi-prep/PEG purification procedure according to Sambrook et al. (1989). Gold particles (3 mg, 1.6 µm diameter) were mixed with 2.5 µg of each plasmid, 50 µl of 2.5 µm CaCl2 and 1 ml spermidine and precipitated using Klett (1991). Embryogenic culture tissue (100–300 mg) was blotted dry on sterile filter paper prior to bombardment with the biolistic particle delivery system (Bio-Rad) and cultured in 10A40N medium as above. After 2 weeks, cultures were divided into two and 30 mg/l hygromycin or 80 mg/l L-ppt was added to the liquid medium for selection. Green colonies forming in the presence of hygromycin or L-ppt selection were cultured as separate transgenic lines. Alternatively, after an additional 2 weeks, unselected tissue was screened for gus activity in a ‘semi-non-destructive’ manner by incubation in fresh medium (at pH 7.0) containing 1 mg/l X-gluc (Diagnostic Chemicals, P.E.I., Canada). Incubation varied between 4 h to overnight and dark blue sectors, and adjacent tissue was ‘rescued’ and grown separately in fresh medium (pH 5.8) without X-gluc in multi-well dishes until they were approximately 2 mm in diameter. Cultures were then transferred to flasks and subsequently these cultures were sampled using a destructive gus assay and any chimeric cultures were re-screened using the ‘semi-non-destructive’ method until homogeneous gus-positive cultures were obtained. Destructive histochemical gus assays of fresh tissue followed the procedure of Jefferson et al. (1987) using 1 mg/ml X-gluc.

Southern analysis of selected transgenic embryogenic suspension cultures

Genomic DNA was isolated from transformed embryogenic suspension cultures using a modified SDS-protease K method (Draper and Scott 1988) in which fresh tissue was crushed in pre-warmed extraction buffer. Approximately 5 µg of DNA was cleaved with restriction endonucleases HindIII, or *SstI*, and after gel electrophoresis, restriction fragments were transferred to nylon membranes using standard solutions and protocols (Sambrook et al. 1989). Southern blots were probed with a 1.0 kb *BamH1* fragment within the coding region of the *aphIV* gene or a 0.6-kb *SalI* fragment from pRD300pat encoding the *pat* gene. Probes were random-primer labelled with 32P-dCTP according to