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ORIGINAL PAPER

Plant development inhibitory genes in binary vector backbone improve quality event efficiency in soybean transformation

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Abstract Conventional *Agrobacterium*-mediated plant transformation often produces a significant frequency of transgenic events containing vector backbone sequence, which is generally undesirable for biotechnology applications. We tested methods to reduce the frequency of transgenic plants containing vector backbone by incorporating genes into the backbone that inhibit the development of transgenic plants. Four backbone frequency reduction genes, bacterial levansucrase (*sacB*), maize cytokinin oxidase (*CKX*), *Phaseolus* GA 2-oxidase (*GA 2-ox*), and bacterial phytoene synthase (*crtB*), each expressed by the enhanced CaMV 35S promoter, were placed individually in a binary vector backbone near the left border (LB) of binary vectors. In transformed soybean plants, the lowest frequency of backbone presence was observed when the constitutively expressed *CKX* gene was used, followed by *crtB*. Higher backbone frequencies were found among the plants transformed with the GA 2-oxidase and *sacB* vectors. In some events, transfer of short backbone fragments appeared to be caused by LB readthrough and termination within the backbone reduction gene. To determine the effect of the backbone genes on transformation frequency, the *crtB* and *CKX* vectors were then compared to a control vector in soybean transformation experiments. The results revealed that there was no significant transformation frequency difference between the *crtB* and control vectors, but the *CKX* vector showed a significant transformation frequency decrease. Molecular analysis revealed that the frequency of transgenic plants containing one or two copies of the transgene and free of backbone was significantly increased by both the *CKX* and *crtB* backbone reduction vectors, indicating that there may be a correlation between transgene copy number and backbone frequency.

Keywords Soybean transformation · T-DNA, Vector backbone · Cytokinin oxidase · *crtB* · GA 2-oxidase · *sacB*

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Introduction

Binary vectors used for *Agrobacterium*-mediated plant transformation usually consist of a transfer DNA (T-DNA) flanked by right border (RB) and left border (LB) 25 bp imperfect repeats. A plant selectable marker gene and at least one gene of interest are usually contained within the T-DNA. Vector sequences outside the T-DNA are referred to as the vector "backbone", which contains genetic elements

such as bacterial replication origins and antibiotic resistance genes for maintenance in both *E. coli* and *Agrobacterium.* In the process of *Agrobacterium*-mediated transformation, the *Agrobacterium* virulence protein virD2 cleaves one strand of the border sequences to initiate the production of a single stranded T-DNA (T-strand) with virD2 covalently attached to its 5′ end (Zupan et al. 2000). Both the RB and the LB can initiate T-strand formation, but the RB is more efficient at initiation, possibly due to the presence of one or more cis-active T-DNA transfer enhancer sequences adjacent to the RB (van Haaren et al. 1987). The LB is usually the site of T-strand termination (van Hareen et al. 1987, 1988). The backbone sequence can be introduced into cells when the T-strand is not terminated at the LB, or when T-strand initiates from the LB. (Kononov et al. 1997; Ramanathan and Veluthambi 1995; Wenck et al. 1997; van der Graaff et al. 1996, Huang et al. 2004). Depending on the plant species, *Agrobacterium* strains, and vectors used, 20–80% of the transgenic plants may contain the backbone sequence. (De Buck et al. 2000; Hanson et al. 1999; Olhoft et al. 2004; Rommens et al. 2004). Although backbone transfer can actually be exploited to generate useful transgenic plants (Huang et al. 2004), in most cases vector backbone sequences are not necessary for the utility of the transgenic plants, and may not be desirable for some commercial applications.

Methods have been tested to reduce the frequency of transfer of backbone sequences during plant transformation. In one approach, multiple tandem LB repeats were used to suppress the transfer of vector backbone in rice transformation (Kuraya et al. 2004). In another approach, a plant expression cassette is placed in the vector backbone, where the expression of the gene provides a phenotype if the gene, i.e. the vector backbone, is transferred into the plant cell. In one example, the phytotoxic gene barnase, which encodes a general RNase activity, was inserted into the binary vector backbone and used to produce backbone-free transgenic plants (Hanson et al. 1999). In this case, primary transgenic cells containing backbone sequences could not develop due to the lethal effect of barnase expression, while transgenic plants without the backbone sequence containing the barnase gene developed normally. However, transformation frequency (TF) decrease was observed in tobacco and tomato (Hanson et al. 1999). In the same way, the cytokinin synthesis gene *ipt* from *Agrobacterium tumefaciens* was inserted into a binary vector backbone for potato transformation, which produced an abnormal stunted phenotype in backbone containing events with less efficient for backbone event elimination (Rommens et al. 2004).

We tested the impact of placing non-lethal genes that interfere with plant development in a binary vector backbone in soybean transformation experiments to reduce the frequency of transgenic plants containing backbone sequences. We selected genes whose expression would be expected to disrupt normal plant development and/or cause severe aberrant phenotypes such as dwarfism, less elongated shoots and unexpanded leaves. Thus, only the primary transformed cells without such genes in the vector backbone can develop into normal shoots.

We tested four genes: levensucrase (*sacB*) from *Bacillus subtilis* (Steinmetz et al. 1985), maize cytokinin oxidase (*CKX*, Huang et al. 2003), *Phaseolus* GA 2-oxidase (*2-ox*, Thomas et al. 1999) and *Erwinia* phytoene synthase (*crtB*, Shewmaker et al. 1999). All four of these genes have been reported to produce pleiotropic phenotypes in various plant species when they were constitutively expressed (Fray et al. 1995; Huang et al. 2003; Ye et al. 2001; Werner et al. 2003), suggesting they may be useful for backbone frequency reduction.

The *sacB* gene in many bacteria such as *Bacillus* spp., *Erwinia* spp. etc, encodes levansucrase, which is responsible for levan (neutral polyfructan) synthesis using sucrose as a substrate (See review by Cairns 2003). Tobacco or ryegrass plants expressing the *sacB* gene with a vacuole targeting sequence driven by CaMV 35S promoter showed stunted phenotype (Ye et al. 2001). The native *sacB* gene without the vacuole targeting sequence could only be expressed in a tissue-specific manner. In corn, the *sacB* expressing kernels disturbed grain filling in later stage and resulted in shrunken seeds with very low germination frequency (Caimi et al. 1996). In potato, the expression of the native *sacB* in tubers leads to smaller tubers (Röber et al. 1996). These results suggested that expression of the native *sacB* gene inhibits plant cell development. Overexpression of the *CKX* in plants results in cytokinin deficiency which led to stunted shoots with smaller apical

meristems in tobacco (Werner et al. 2001), and diminished activity of the vegetative and floral shoot apical meristems and leaf primordial in *Arabidopsis* (Yang et al. 2003; Werner et al. 2003). Constitutive expression of a phytoene synthase causes dwarfism in tomato (Fray et al. 1995) and both dwarfism and infertility in rice (Burkhardt et al. 1997). Overexpression of GA oxidases created severe dwarfism in many plant species due to significant drop of GA level (Huang et al. 1998; Sakamoto et al. 2003).

We present results here using these four genes in a binary vector backbone to reduce the frequency of transgenic soybean plants that contain vector backbone sequences, and thus increase the frequency of quality plants in transgenic soybean populations.

Materials and methods

Binary vector construction

The four pleiotropic phenotype producing, or backbone reduction genes (BRGs), were cloned individually into an expression cassette with an enhanced CaMV 35S promoter (Kay et al. 1987) and a transcriptional terminator sequence from the *nos* gene (Depicker et al. 1982) according to standard methods (Sambrook et al. 1989). The resulting expression cassettes were further subcloned into a unique *Xho*I site adjacent to the LB region in the backbone of the control vector pMON83898 by blunt fragment ligation (Fig. 1). The T-DNA border regions were previously described by Horsch and Klee (1986). The RB region is a 357 bp DNA sequence that was originally isolated from *A. tumefaciens* plasmid pTiT37 (Depicker et al. 1982). The LB region is a 456 bp DNA sequence that was originally isolated from *A. tumefaciens* plasmid pTi15955 (Barker et al. 1983). The RK2 replicon oriV (Cross et al. 1986) is used for *Agrobacterium* maintenance and ori-322 for *E. coli* (Huang et al. 2004).

The control vector pMON83898 contains the *EPSPS-CP4* (Barry et al. 1992) gene conferring resistance to glyphosate driven by an FMV 35S promoter (Sanger et al. 1990) and terminated with a pea *rbcS E9* polyadenylation signal (Schardl et al. 1987), as well as an intron-containing gus gene (Vancanneyt et al. 1990) driven by the enhanced

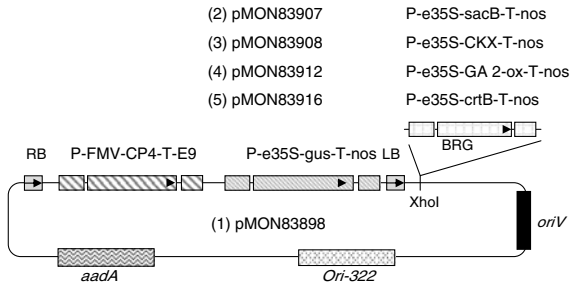

Fig. 1 Five binary vectors used for soybean transformation. (1) pMON83898: the control vector with the selectable marker *EPSPS-CP4* and *gus* genes. The *Xho*I site after the LB was used to insert the backbone reduction genes (BRGs). (2) pMON83907: the levensucrase (*sacB*) expression cassette was inserted into *XhoI* site of pMON83898. (3) pMON83908: the maize cytokinin oxidase (*CKX*) expression cassette was inserted into *Xho*I site of pMON83898. (4) pMON83912: the GA 2-oxidase (*2-ox*) expression cassette was inserted into *Xho*I site of pMON83898. (5) pMON83916: the *Erwinia* phytoene synthase (*crtB*) cassette was inserted into *Xho*I site of pMON83898. *oriV*: RK2 vegetative replication origin for vector maintenance in *Agrobacterium*; Ori-322: *E. coli* plasmid replication origin; *aadA*: spectinomycin resistance gene

CaMV 35S promoter (Kay et al. 1987) and terminated with the *nos* transcription terminator (Depicker et al. 1982).

Agrobacterium preparation and plant transformation

Agrobacterium tumefaciens strain ABI derived from the C58 strain (Koncz and Schell 1986) was used for soybean transformation. The binary vectors were transformed into the ABI strain by electroporation with the Bio-Rad Gene Pulser II according to the manufacturer's instruction (Bio-Rad Laboratories, Hercules, CA). Soybean cultivar A3525 was used for *Agrobacterium*-mediated transformation as described (Martinell et al. 2002). Briefly, soybean seeds were imbibed for less than 14 hours at room temperature in Bean Germination Medium (BGM) (Martinell et al. 2002) and meristem explants were excised from imbibed seeds. The *Agrobacterium* strain containing each binary vector was grown in LB medium (Samsbrook et al. 1989) with 75 mg/l of spectinomycin and 50 mg/l of kanamycin in a gyratory shaker at 200 rpm for about 20–24 h at 28°C. The culture was spun down and directly re-suspended in inoculation medium (INO), which

contains 2/5× of B5 macro elements and 1/10× of B5 micro elements (Gamborg et al. 1968), supplemented with 30 g/l glucose, 3.9 g/l MES, pH 5.4. The final optical density of *Agrobacterium* inoculum was adjusted to approximately 0.3 at OD660.

For each transformation experiment approximately 500 explants were placed in a Plantcon® (MP Biomedicals, Inc. Cat. No. 26-720-02) lid and mixed with 100 ml of *Agrobacterium* suspension and sonicated in a W-113 Sonicator for 20 s (Martinell et al. 2002). After sonication, the explants were co-cultured with *Agrobacteria* on a sterile filter paper within a Plantcon® for 2–4 days at 23°C. Explants were then transferred onto the surface of solid WPM medium (Lloyd and McCown 1980) purchased from Phytotech Lab (Shawnee Mission, KS, Cat. No. L449) with supplement of 20 g/l sucrose, 1.29 g/l calcium gluconate, 200 mg/l cefotaxime, 100 mg/l Ticarcillin, 200 mg/l carbenicillin, 12.68 mg/l glyphosate (Sigma, St. Louis, Missouri, Cat. No. PS 1051), and 4 g/l Agargel, pH 5.6. After 2 weeks, explants were transferred to the fresh WPM medium with the primary radicle inserted in the medium, and each Plantcon® contained about 25 explants.

Glyphosate resistant shoots, which were elongated with fully expanded trifolia, were identified 6–10 weeks post-inoculation. They were detached from original explants and transferred to Bean Rooting Medium (BRM) for rooting. The BRM contains 1/2× MS salts and 1× MS vitamins (Murashige and Skoog 1962) supplemented with 30 g/l sucrose, 100 mg/l cysteine, 0.1 mg/l IAA, 100 mg/l Ticarcillin and 4.2 mg/l glyphosate, pH 5.6 and solidified with 8 g/l washed agar. After 2–3 weeks on BRM, the rooted plantlets were transplanted to soil and grown in greenhouse for maturation and further analysis. The TF was defined as the number of *EPSPS-CP4* positive plants/the number of initial explants.

Molecular analyses

Leaf samples of approximately 200 mg were collected from each plant. Total DNA was extracted as described by Dellaporta et al. (1983). The transgene copy numbers were estimated by Invader quantitative analysis using a probe specific to the *EPSPS-CP4* gene according to the manufacturer's instruction (http://www.twt.com/, Third Wave Technologies Inc. Madison, WI). The Invader DNA copy number assay was extensively validated previously by comparison with known copy samples and its accuracy is comparable to real time PCR (Taqman) or southern blot (Pielberg et al. 2003).

The presence of backbone sequence in transgenic plants was determined by Taqman assay using probes specifically for the oriV sequence or for LB sequence that is 3′ to the LB nicking site. In other experiments (data not shown) we tested the accuracy of the oriV Taqman assay results by the use of multiple probes across vector entire backbone in Southern blots. The Taqman assay result corresponded with presence of the entire backbone at least 95% of the time. Approximately 10 ng of genomic DNA was used for a qualitative Taqman reaction according to the manufacturer's instructions (Applied Biosystems, Forster City, CA). The primers 5′ AACGCCT GATTTTACGCGAG 3′ (forward) and 5′ CAATAC CGCAGGGCACTTATC 3′ (reverse), which amplify a 70 bp fragment and further detected with MGB (minor grove binding) Taqman probe 6-FAM-CCCACAGATGATGTGGAC, were used to analyze the *oriV*-containing backbone sequence. The LB primers 5′ GCACCCGGTGGAGCTT 3′ (forward) and 5′ TCTGCCTAACCGGCTCAGT 3′ (reverse), which amplify a 55 bp fragment, with the probe 6FAM-CATGTTGGTTTCTACGCAG were used to detect sequence 35 bp downstream from the LB nicking site.

The BRG presence was also analyzed by PCR. The following primer pairs were used to detect the BRG respectively: (1) 5′ GACTTCGGCAACATCACGTC 3′ (*CKX* forward) and 5′ AGGCTCTGGTTCACG AACAC 3′ (*CKX* reverse) to amplify a 712 bp *CKX* fragment in pMON83908; (2) 5′ GTCTCAGCCAG-CATTGAACC 3′ (*GA 2-ox* forward) and 5′ ACCTG TAGAGCGTCACCAAC 3′ (*GA 2-ox* reverse) to amplify a 737 bp *GA 2-ox* fragment in pMON83912; (3) 5′ TAGCGTGCTGATGCTCTACAC 3′ (*crtB* forward) and 5′ AGGAGATGTAGTACGGCTCTG 3′ (*crtB* reverse) to amplify a 600 bp *crtB* fragment in pMON83916; (4) 5′ ACGGCACTGTCGCAAAC-TATC 3′ (*sacB* forward) and 5′ AGCTCAATCATAC CGAGAGCG 3′ (*sacB* reverse) to amplify a 673 bp *sacB* fragment in pMON83907.

Experiment design and statistical analysis

The soybean transformation experiments were carried out in two sets: the first set including all four BRG constructs and the control vector was transformed individually to screen the most effective BRG genes and was designated as preliminary experiment in context, while the second set with two promising BRG constructs and the control vector was transformed in parallel with identical conditions and was designated as parallel experiment. Statistical analysis was performed with generalized linear model which appears to allow more flexibility in terms of analysis (http://www.ats.ucla.edu/STAT/sas/glimmix.pdf).

Results

Determination of BRG efficacy in preliminary experiments

The four BRG vectors and the control vector were used to transform soybean in multiple transformation experiments to determine the impact of the BRG on the frequency of plants containing vector backbone, as well as transgene copy number. In all BRG transformation experiments, no obviously aberrant shoots were observed. Normal shoots from the BRG containing vectors were harvested and rooted in the rooting medium in the same time frame as the control, indicating there is no delay with the BRG vector. Transgenic plants were screened for the presence or absence of the backbone sequence (Fig. 2). In preliminary experiments, the different construct transformations were conducted at different times (nonparallel experiments, Fig. 2). In the second set of experiments, all constructs were transformed at the same time under identical conditions (Fig. 3). The oriV sequence-specific probe (probe c, Fig. 2A) was used in a Taqman assay to detect the presence of backbone sequences that extend beyond the BRG gene. The BRG-specific fragment (probe b, Fig. 2A) was used in a PCR assay to detect the presence of backbone fragments that may not have extended beyond the BRG.

As shown in Fig. 2B, the *CKX*, *GA 2-ox* or *crtB* constructs significantly reduced the frequency of transgenic plants with vector backbone when the oriV backbone fragment was probed. The *CKX* vector had the lowest frequency, with no oriV backbone-containing plants, followed by *GA 2-ox* (8.8%) and *crtB* vector (9.7%). The vector with *sacB* in backbone had the same backbone frequency as the control vector.

To determine if the shorter backbone fragments terminated before oriV were present, a second assay using PCR to test for the presence of the BRG (probe b, Fig. 2A) was conducted in the transgenic plants from the preliminary experiments. Transgenic plants containing BRG sequences but no oriV sequence were detected in transformants from three vectors (*sacB*, *GA 2-ox* and *CKX*). The frequencies observed for such plants were 13% (9 events out of 70) for the *sacB* vector, 15.6% (7 out of 45) for the *GA 2-ox* vector, 2.1% (2 events out of 92) for the *CKX* vector, and no such plants for the *crtB* vector after analyzing 31 plants (Fig. 2B). In the *GA 2-ox* and the *sacB* vector transformations, these additional plants with short vector backbone sequence significantly contributed to the total number of plants with backbone. The overall frequencies of the plants containing backbone with the oriV and/or BRG region were 24.4 and 33%, respectively, for *GA 2-ox* and *sacB* transformants. In addition, a few events that were BRG negative but oriV positive were also observed, albeit at lower frequencies: 3.4% (2/70) for the *sacB*, 4.4% (2/45) for the *GA 2-ox* and 3.2% (1/31) for the *crtB* construct.

Transgenic soybean plants from the preliminary experiments were subjected to the Invader assay to estimate the *EPSPS-CP4* transgene copy number. As shown in Table 1, the frequencies of plants with low copy (1–2 copies) numbers for the *EPSPS-CP4* transgene were 68.6% for *sacB* vector, 73.5% for the control, 75.6% for *GA 2-ox*, 74.7% for *CKX* and 83.9% for *crtB*.

The production of transgenic plants with low transgene copy number and absence of vector backbone sequences is important for basic research as well as many biotechnology applications. The most useful plants have 1–2 copies of the transgene, and are backbone-free. In the preliminary experiments, two BRG vectors, pMON83908 (*CKX*) and pMON83916 (*crtB*) showed frequencies of 74.7 and 83.9% respectively, of plants that meet that definition of useful plants, which was significantly higher (Fig. 2C) than the control vector with (63.3%). The other two BRG vectors, pMON83907 (*sacB*) and pMON83912 (*GA 2-ox*), had lower frequencies of

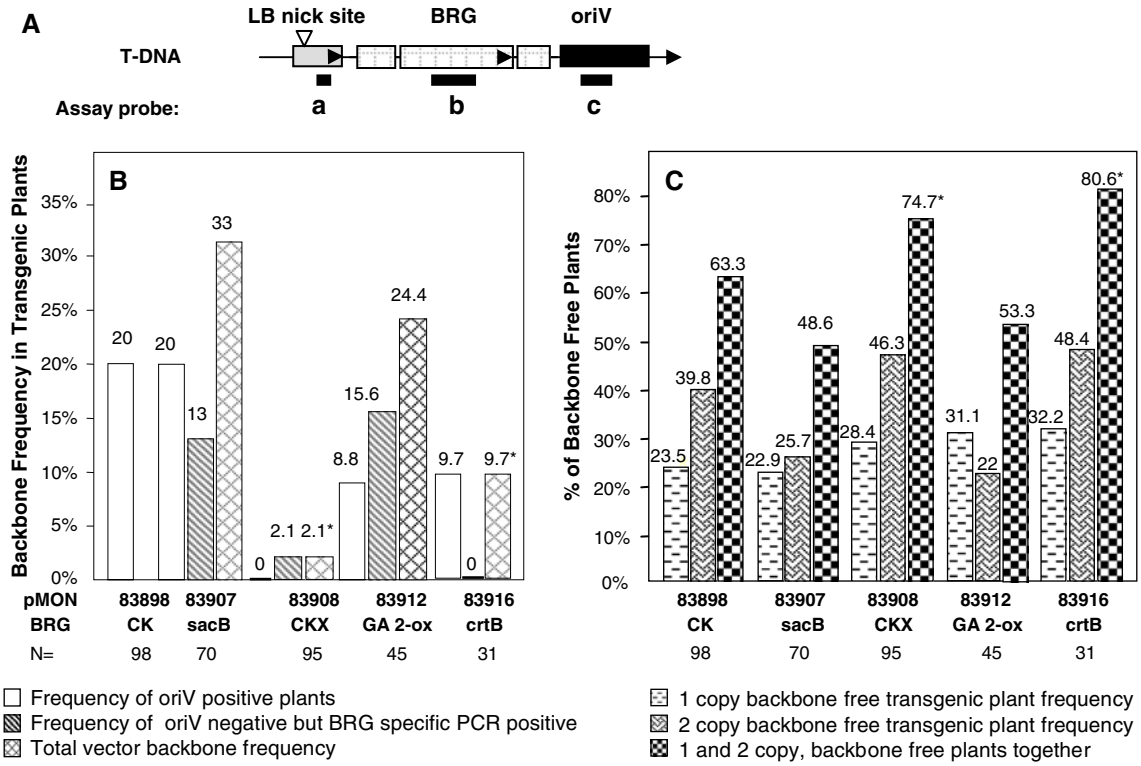

Fig. 2 Molecular analysis of vector backbone frequency and copy number in transgenic soybean plants from preliminary experiments. (**A**) Three backbone assay probes in the vector backbone after the LB nicking site are indicated. a: LB 3′ Taqman probe detecting backbone sequence directly downstream of the LB nic site; b: BRG specific PCR primers detecting BRG presence; c: oriV Taqman probe detecting longer backbone sequence. (**B**) backbone frequency analysis in transgenic soybean plants transformed by the four BRG and the control vectors from the preliminary experiments. The vector backbone presence was analyzed by Taqman assay of oriV (probe c in **A**) and BRG specific PCR (probe b in **A**). (**C**) effect of BRGs in binary vector backbone on usable (1 or 2 copies, backbone-free) soybean transgenic plant frequencies. CK: control vector pMON83898; sacB: pMON83907; CKX: pMON83908; GA 2-ox: pMON83912; crtB: pMON83916. The percentage of backbone frequency in **B** or backbone-free frequency in **C** of transgenic soybean plants was indicated on the top of bars. *Statistically significant ($P = 0.05$)

Table 1 *EPSPS-CP4* copy number of the R_0 transgenic soybean plants with different BRG in binary vector backbones from the preliminary non-parallel transformation experiments

Invader *EPSPS-CP4* copy #	Number of plants (%)				
	pMON83907 (*sac*B)	pMON83908 (*CKX*)	pMON83912 (*2-ox*)	pMON83916 (*crt*B)	pMON83898 (Control)
1	22 (31.4%)	27 (28.4%)	17 (37.8%)	8 (25.8%)	24 (24.5%)
2	26 (37.1%)	44 (46.3%)	17 (37.8%)	18 (58.1%)	48 (49.0%)
>2	22 (31.4%)	24 (25.3%)	11 (24.4%)	5 (16.1%)	26 (26.5%)
Total plants	70	95	45	31	98

useful plants due to significantly higher frequency of plants with backbone sequences, including both longer backbone fragments (oriV positive) and short backbone fragments (BRG positive but oriV negative) (Fig. 2C).

Determination of impact and BRG efficacy in parallel experiments

To further investigate the impact of the *crtB* and *CKX* BRG constructs on TF, frequency of plants with

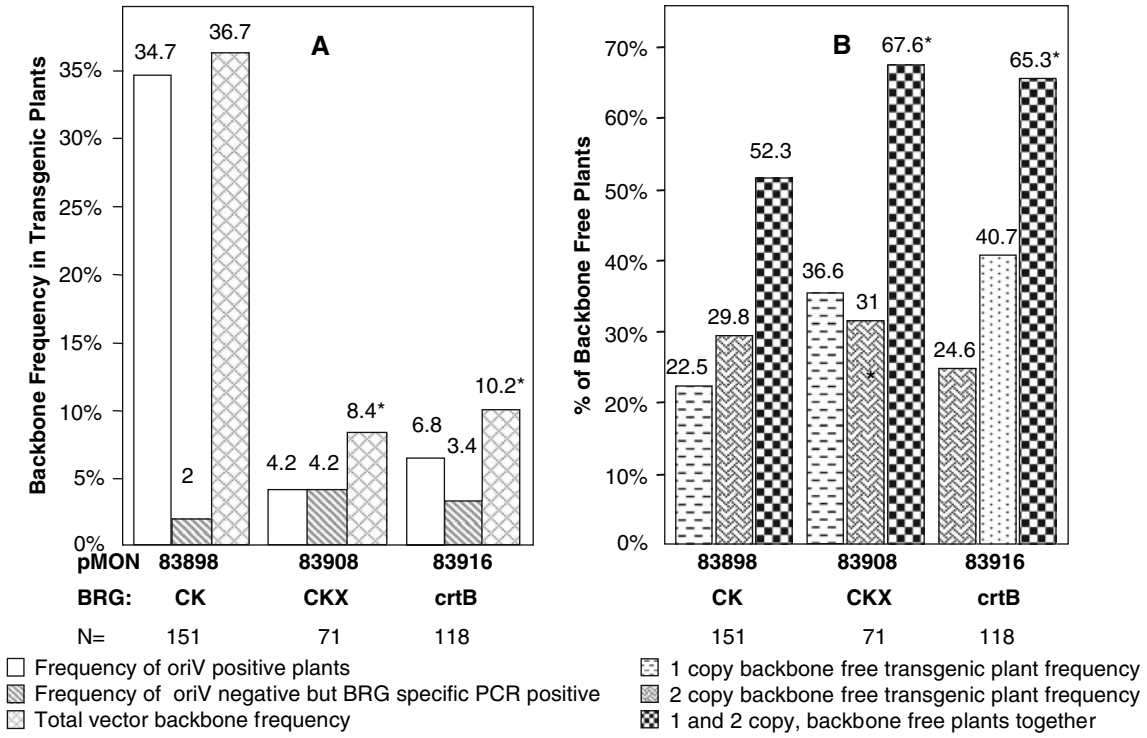

Fig. 3 Molecular analysis of vector backbone frequency and copy number in transgenic soybean plants from the in parallel transformation experiments. (**A**) Backbone frequency analysis in transgenic soybean plants transformed by the *crtB* or *CKX* BRG and the control vectors. The vector backbone presence was analyzed by Taqman assay of oriV (Fig. 2A probe c), BRG specific PCR (Fig. 2A probe b) or LB-specific Taqman probe (Fig. 2A probe a). (**B**) Effect of *crtB* or *CKX* BRGs in binary vector backbone on usable (1 or 2 copies, backbone-free) soybean transgenic plant frequencies. CK: control vector pMON83898; CKX: pMON83908; crtB: pMON83916. The percentage of backbone frequency in **A** or backbone-free frequency in **B** of transgenic soybean plants was indicated on the top of bars. * Indicating statistically significant ($P = 0.05$) from the control plasmid

backbone sequences, and transgene copy number, we conducted additional transformation experiments, carrying out all steps for each construct in parallel, under identical conditions. Ten experiments were conducted with a total of approximately 5,000 explants for each vector. The average TF for *crtB* construct was 2.33 ± 0.76%, which was not significantly different from the TF of the control vector (2.80 ± 0.70%, $P = 0.05$). In contrast, the *CKX* vector resulted in a significant drop in TF (1.47 ± 0.31%, $P = 0.05$).

All plants generated from these experiments were assayed to determine the frequency of plants that contain vector backbone sequences. The oriV-specific probe (probe c, Fig. 2A) was used in the Taqman assay to detect the presence of long backbone sequences that extend into the oriV region. The probe specific for the BRGs (probe b, Fig. 2A) was used in PCR assays for detecting shorter backbone fragments that extended into the BRG, but terminated before oriV region. In these experiments, an additional probe, designed to detect backbone sequences adjacent to the LB nic site (probe a, Fig. 2A) was used in Taqman assay for detecting the presence of very short backbone fragments, beginning 18 bp from the nic site that may not have included the BRGs or the oriV region.

The results obtained from these experiments were consistent with the results from the preliminary transformation experiments. The frequency of plants with backbone sequences, determined with the oriV Taqman assay, decreased from 34.7% with the control vector to 4.2 *and* 6.8% with the *CKX* and the *crtB* BRG vectors, respectively (Fig. 3A). Only a few additional short LB 3′ events were observed in both *crtB* and *CKX* BRG constructs and the control vector (Fig. 3A).

Transgenic soybean plants from the parallel experiments were subjected to the Invader assay for estimates of the *EPSPS-CP4* transgene copy number. Consistent results were observed with the *CKX* and *crtB* BRG constructs (Table 2) compared to the preliminary transformation experiments (Table 1), where the frequency of plants with 1 or 2 copies of the transgene for the *crtB* construct was very close to that in the control vector. However, the frequency of plants with a single copy of the transgenes (43.7%) was significantly higher for the *CKX* construct than the control and *crtB* constructs ($P = 0.05$).

In addition, in the latter comparison experiments, 1–2 copy, backbone-free plant frequencies for the *CKX* construct (67.6%) and the *crtB* construct (65.3%) were significantly higher than the control vector (52.3%) (Fig. 3B), which was consistent with previous non-parallel experiments.

Discussion

We tested the utility of inserting four pleiotropic phenotype-producing genes in the binary vector backbone to reduce the frequency of transgenic soybean plants that contain vector backbone sequences. All four BRG vectors produce transgenic soybean plants within the same timeframe. A binary vector containing the *CKX* gene was the most effective in reducing vector backbone frequency in soybean transformation. Cytokinin is an essential phytohormone for plant cell differentiation and plays a major role in plant morphogenesis. The *CKX* gene plays an opposite role in plant morphogenesis by catabolizing cytokine to fine tune the cytokine level for proper plant development (Armstrong 1994). Severe aberrant phenotypes were observed in *CKX* overexpressing plants (Werner et al. 2001, 2003; Yang et al. 2003). When the *CKX* gene is transferred into plant cells as part of the backbone sequences, it likely results in cytokinin deficiency in the transformed cells. Since the soybean transformation system used in our experiments is a shoot apical meristem-based organogenic system, which is highly dependent on endogenous cytokinin levels for shoot formation (Martinell et al. 2002), plants containing backbone sequence with the *CKX* expression cassette were presumably impaired in normal cell development by cytokinin depletion, and thus deficient in normal shoot formation.

The *CKX* binary vector resulted in unexpected TF decrease compared to the control vector. It seems unlikely that the TF drop with the *CKX* vector is due to the elimination of the events with the *CKX* backbone since the *crtB* BRG vector showed no significant TF drop even though the frequency of backbone-containing plants is the same as *CKX* vector. Frequently, many T-DNAs are transferred into multiple cells in the same meristem as we observed in *gus* transient expression assays (data not shown). It appeared that the *CKX* backbone T-DNAs not only degraded cytokinin in the transformed cells but also depleted cytokinin in the adjacent cells of the same meristem, which further inhibited shoot regeneration from the adjacent cells transformed with GOI alone. Evidence was shown that the *CKX* encodes a potentially secreted protein containing a putative transit peptide and glycosylation signal (Morris et al. 1999; Huang et al. 2003), which might indicate the *CKX* proteins were translocated into some adjacent cells.

The binary vector with the *crtB* gene in the backbone resulted in a TF equivalent to the control vector in soybean. It also resulted in a significant reduction in vector backbone frequency in transgenic soybean plants and thus an increase in the overall frequency of usable transgenic plants. Other studies also showed that constitutive expression of a

Table 2 *EPSPS-CP4* copy number of the R_0 transgenic soybean events from in parallel comparison experiments

Invader *CP4* copy #	Number of plants (%)		
	pMON83908 (CKX)	pMON83916 (crtB)	pMON83898 (Control)
1	31 (43.7%)*	30 (25.4%)	37 (24.5%)
2	28 (39.4%)	51 (43.2%)	60 (39.7%)
>2	12 (16.9%)	37 (31.4%)	54 (35.8%)
Total plants	71	118	151

* Statistically significant ($P = 0.05$)

phytoene synthase causes dwarfism in tomato (Fray et al. 1995) and both dwarfism and infertility in rice (Burkhardt et al. 1997). Phytoene synthase converts geranyl-geranyl diphosphate (GGDP) to phytoene and is a key enzyme for carotenoid biosynthesis. The precursor GGDP is also a key product for biosynthesis of gibberellins (GA), tocopherols, phytol, quinnones etc. GA is an important phytohormone for cell expansion and shoot elongation. Indeed, overexpression of a phytoene synthase in tomato resulted in 30-fold reduction in GA content in dwarf plants (Fray et al. 1995). It was proposed that the overexpression of the phytoene synthase depleted the GGDP precursor and thus down-regulated GA and phytol biosynthesis (Fray et al. 1995).

The importance of GA in shoot formation was further demonstrated with the GA 2-oxidase BRG construct. We observed a similar reduction in backbone frequency with this construct to the *crtB* construct if the plants with short backbone sequence (15.6% *GA 2-ox* PCR positive but oriV negative), i.e. those that probably did not contain an intact *GA 2-ox* gene, were excluded. The GA 2-oxidase oxidizes GA and thus regulates GA level to control plant cell development.

Unexpectedly, the use of the *sacB* gene in the binary vector backbone did not reduce the frequency of plants containing backbone sequences in this study. The high backbone frequency with the *sacB* BRG construct consisted of combinations of entire backbone (19%) and short backbone (12.7% *sacB* PCR positive but *oriV* negative). The *sacB* backbone-containing soybean transgenic plants could have resulted from low expression of the *sacB* gene. The localized regions of AT richness resembling plant introns, the presence of mRNA destabilizing ATTTA sequences and rare codon usage found in the *sacB* from *Bacillus* sp. were proposed to be associated with the low expression of the *sacB* gene in plants (Ye et al. 2001). It was suggested that codon optimization or another source of the *sacB* gene may improve the *sacB* gene expression in plants (Ye et al. 2001) and may thus enhance its utility for backbone reduction.

A significant number of transgenic plants with the short backbone containing the BRG but not oriV were recovered with the GA 2-oxidase and *sacB* constructs (12.7 and 15.6% for *sacB* and *GA 2-ox* construct, respectively), indicating that the T-DNA truncation occurred during T-DNA process or integration. The oriV sequences tested in the above experiments are positioned at 455 bp relative to the LB nic site in the control vector pMON83898 and at 3075 bp in the largest *CKX* BRG vector pMON83908. Since high frequency of transfer of short backbone sequences occurred in the same region of the two BRG plasmids, the results may suggest that T-DNA border-like sequences might be present in these regions. It was recently observed that plant genome derived border-like sequences could replace the RB and LB for *Agrobacterium*-mediated transformation (Rommens et al. 2004) and the *Agrobacterium* protein virD2 can recognize an oriT sequence from pTF-FC2 in an in vitro assay (Dube et al. 2004). Aligning the oriT and pTiC58 border sequences revealed a consensus TCCTG sequence adjacent to the nick site (Dube et al. 2004). Several TCCTG motifs are present between the BRG reverse primer and the oriV sequence, with one in *nos* transcriptional terminator that is part of all BRG cassettes. In addition, border nicking efficiency may be affected by flanking sequence AT content, and AT-rich upstream flanking is required for LB termination function (Rommens et al. 2004). Both *sacB* and *GA 2-ox* genes are AT-rich sequences containing 58.86 and 52.95% AT content, respectively, while *CKX* and *crtB* are GC-rich sequences with 30 and 34% AT content correspondingly. The upstream AT-rich sequences combined with the border-like consensus sequence may facilitate the termination of backbone transfer that occurs before oriV, especially for the *sacB* and *GA 2-ox* vectors. Whether there is indeed a cryptic border-like sequence present in these BRG cassettes remains to be determined. Removal of the potential border-like sequences in these BRG cassettes may eliminate events with short sequence transfer and increase the efficiency of overall backbone reduction.

Due to the significant short backbone presence in *sacB* and *GA 2-ox* BRG constructs, the rest backbone presence after the oriV backbone detection point may be questioned and more probes may be required for determining an ideal BRG for backbone frequency reduction. The short T-DNA presence is often due to T-DNA truncations occuring close to RB or LB during T-DNA process or integration. Middle T-DNA deletion is rare, which is very often associated with repeats by homologous recombination (Ye et al. unpublished observation) or potential cryptic border

sequence, as indicated by plant derived T-DNA (Rommens et al. 2004). Intact over 20 kb backbone T-DNAs after LB are frequently integrated in the rice transformation with the single LB control vector (Kuraya et al. 2004). Our oriV detection represents at least 95% accuracy for entire backbone since multiple backbone probes including a probe before RB nicking site were extensively used to validate the detection in other earlier experiments (data not published).

The reliability of BRG strategy is not only dependent on the BRG expression, but it may also depend on the gene sequence context, which was not mentioned in previous studies (Hanson et al. 1999; Kuraya et al. 2004; Rommens et al. 2004). Furthermore, it is also dependent on the impact on TF. Potential negative effect on TF was observed in *CKX* gene and also in the lethal gene (Hanson et al. 1999).

Though there is minor difference of 1–2 copy frequency between the preliminary and the parallel experiments for *CKX* and *crtB* BRG constructs, the relative tendency of the 1–2 copy backbone-free frequency (Figs. 1 and 2) are consistently higher than the control, indicating that the backbone frequency reduction may help to increase the low copy usable plant frequency. The Invader copy number assay used in this research provides comparable accuracy to the real time Taqman or Southern blot within a 70–90% range for single copy transformants (Pielberg et al. 2003). The minor difference of single copy frequency between the preliminary and the parallel transformation experiments for *CKX* BRG construct (Tables 1 and 2) may be due to the assay discrepancy or transformation condition variation in the preliminary screening experiment, which was not performed in parallel.

In experiments with the control vector we observed a correlation between the presence of vector backbone and the presence of multiple copies of the transgene (data not shown). Transgenic plants with one or two copies of the transgene were approximately 90 and 80% backbone-free, respectively. The majority of backbone-containing plants contained multiple copies of the transgene. Thus, a reduction of the frequency of plants with vector backbone would be expected to increase the frequency of plants with low copy numbers of the transgene. This is indeed what we observed. The higher frequency of backbone-free plants with one or two copies in experiments using the *CKX* and *crtB* constructs is consistent with the fact that the multiple copy, backbone-containing events were efficiently eliminated in the two BRG constructs.

Previously the lethal gene, barnase, containing an intron was placed in the vector backbone for reducing backbone frequency in transgenic plants (Hanson et al. 1999). The *Agrobacterium* isopentenyl transferase (*ipt*) was also used in the vector backbone for backbone frequency reduction, which is dependent on the phenotype production with much less efficiency (Rommens et al. 2004). The *CKX* and *crtB* as BRGs as shown in this study provided an alternative method for vector backbone reduction in soybean and other crop transformation. Although the genes we used had the potential to form transgenic shoots with abnormal phenotypes, we did not use phenotypes as a screen for transgenic plants that did or did not contain vector backbone. The same criteria for advancement of putative transgenic shoots were used regardless of the construct. The *CKX* and *crtB* genes appear to block the development of shoots, so there was no requirement for additional time or resources to screen for backbone-free shoots based on phenotypes.

The use of non-lethal genes, such as *CKX* and *crtB*, to eliminate transgenic plants vector backbone sequences offers a potential advantage over the use of lethal genes. Depending on the gene used, a lethal gene may express in cells into which the vector backbone has been transformed, perhaps as part of an additional T-strand transferred to the plant cell, but has not and will not integrate. Such a potentially useful transformed cell would be eliminated by the use of a lethal gene, but may proliferate when a non-lethal gene is used. Indeed, transformation frequencies decreased in experiments that used the barnase gene (Table 1 in Hanson et al. 1999) for backbone reduction.

The use of multiple LB border elements has also been applied to reduce the frequency of vector backbone transfer in rice (Kuraya et al. 2004) to levels that are as low as the levels we have observed using a non-lethal gene in the backbone. We wanted to avoid possible complications arising from initiation of T-strands at the LB (Podevin et al. 2006) which could lead to transfer of short segments that initiate at the first LB and terminate at the second LB, which would be very difficult to detect.

Our strategies were designed to reduce the transfer of binary vector backbone in *Agrobacterium*-mediated transformation methods. Several other methods for delivery of DNA to plant cells exist, including direct DNA transfer methods using particle guns. Under optimal conditions these methods can efficiently produce transgenic plants with low copy number (Agrawal et al. 2005, also see Alterpeter et al. 2005 for recent review). Moreover, in the case of direct DNA transfer, it is relatively straightforward to purify transgene cassettes from vector backbone using, for example, gel purification prior to DNA delivery (Fu et al. 2000). Thus, the BRG strategy is not necessary for most direct DNA transfer methods.

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