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Development of quantitative competitive PCR for determination of copy number and expression level of the synthetic glyphosate oxidoreductase gene in transgenic canola plants

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Abstract

Background: For successful *in vitro* plant regeneration, plant cell lines with multiple transgene integration and low transgene expression levels need to be ruled out. Although real-time polymerase chain reaction (real-time PCR) is a rapid way to accomplish this, it is also expensive and typically limits the size of the target sequence. Quantitative competitive PCR (QC-PCR) is proven to be a safe and accurate method for determination of both copy number and quantification of transcript levels of synthetic transgenes in transformed plants. **Results:** The glyphosate oxidoreductase gene was chemically synthesized and used to transform *Brassica napus* L. via *Agrobactrium*-mediated transformation. A construct containing the mutated form of a synthetic glyphosate oxidoreductase (*gox*) gene (internal standard) was prepared. Gene copy number was estimated in nine independent transgenic lines using QC-PCR as well as the standard method of Southern blot analysis. By quantitative competitive reverse transcriptase PCR (QC-RT-PCR), transcript levels were also determined in these lines. High (> 3), medium to high (2.2-3), medium to low (1-2.2), and low (< 1) levels of transcript were detected**. Conclusions:** No direct relationship was found between copy number and transgene expression levels. QC-PCR method could be implemented to screen putative transgenic plants and quickly select single T-DNA inserts. QC-PCR methods and the prepared competitor construct may be useful for future quantification of commercial transgenic food and feed.

Keywords: *Brassica napus* L., competitive quantitative PCR, transcript level, transgene copy number

INTRODUCTION

Genetic transformation is widely used for plant improvement and basic research (Huang et al. 2003). In this method, foreign DNA is randomly inserted into the plant genome, and multiple transgenic events may occur at one or several chromosomal locations. Transgenic plant lines with multiple gene integration events show lower or unstable transgenic expression, which usually leads to gene silencing (Kooter et al. 1999; Iyer et al. 2000). Current transformation methods do not allow for the control of the number of transgene integration into the plant genome, but this should be estimated as early as possible after transformation. Southern blot analysis is the traditional method generally used for determining the copy number of a transgene. However, this method is laborious and time-consuming, particularly when a large number of samples need to be determined, and each assay requires relatively

large amounts of DNA from fresh or frozen samples (Ingham et al. 2001; Mason et al. 2002). In addition, the accurate quantification of Southern blot images is difficult, especially in rearranged transgene copies lacking the relevant restriction sites, and in cases of concatemers (Mason et al. 2002). For determination of transgene messenger RNA (mRNA), Northern blot analysis and reverse transcription followed by the polymerase chain reaction (PCR) are often used, but the results obtained with these methods are usually only qualitative or semi-quantitative (Beltrán et al. 2009).

To overcome these limitations, several sensitive and effective quantitative PCR–based methods (Piatak et al. 1993; Al-Robaiy et al. 2001; Livak and Schmittgen, 2001), including quantitative competitive PCR (QC-PCR) (Callaway et al. 2002) and real-time PCR (qPCR) (Ingham et al. 2001;Mason et al. 2002; Song et al. 2002) have been developed. In real-time PCR, quantification takes place within the exponential phase of the amplification curve; therefore the sensitivity of this method is high (Raeymaekers 2000; Ingham et al. 2001). However, in typical real-time PCR-based techniques, only small amplicons of about 100 base pairs (bp) in size are used (Ludwig and Schleifer, 2000). In addition, these methods are rather expensive and personal expertise is required.

In QC-PCR, the expression level and copy number of target genes are determined in comparison to a known amount of a mutant form of the same gene (the competitor). The competitor fragment is derived from the same region of DNA, but differs slightly in size so it can be distinguished from the target DNA by agarose gel electrophoresis. For each PCR reaction in QC-PCR, a constant amount of target DNA and a known dilution series of competitor DNA (internal standard) are used. The mutant gene (either cDNA or DNA) can compete with the native gene for the same primers. At the point where the concentration of both sequences are the same, the band intensities will be equal. Visual assessment of band intensities or digital analysis of gel images and generation of a regression line can be used to determine this point (Raeymaekers, 2000).

In this study, a synthetic glyphosate oxidoreductase (*gox*) gene was designed and optimized, based on canola (*Brassica napus* L.) plant codon preferences, to optimize translation in the plant host. This synthetic gene was transferred into the canola genome using*Agrobacterium*-mediated transformation, and putative transgenic lines were analyzed with molecular methods. Here, we describe our attempts to identify an efficient alternative approach to quantify copy number and estimate expression levels of the synthetic *gox* (*synth-gox*) gene in transgenic canola plants using a QC-PCR based protocol.

MATERIALS AND METHODS

The synthetic *gox* gene

An open reading frame (ORF; EMBL Bank: GU214711.1) of 1296 bp was previously reported as a *gox* gene sequence. The sequence was optimized based on the canola plant codon preference, and the regulatory Kozak sequence was added before the start codon to improve the efficiency of transcription and translation (Kozak, 1989; Gustafsson et al. 2004). The *Bam*HI and *Sac*I restriction sites were introduced at the 5’ and 3’ ends of the synthetic gene, respectively. The *synth-gox* gene was synthesized by Shine Gene Molecular Biotech, Incorporated (Shanghai, China), and the sequence was submitted to GenBank (Accession Number, HQ110097).

Construction of plant expression vector

The *synth-gox* gene was used to replace the *gus* fragment in the binary vector pBI121 (Clontech) using *Bam*HI and *Sac*I restriction sites. By this strategy, the *synth-gox* gene came under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (nos) terminator (pBI-*synth-gox*) (Figure 1). Authentic clones harboring recombinant plasmids were selected by PCR and restriction enzyme analysis. The recombinant plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 cells by the freeze-thaw transformation procedure (Sambrook et al. 2001). Recombinant cells were confirmed by PCR analysis and used for plant transformation.

Canola transformation and regeneration

Canola transformation was carried out based on the procedure described by Kahrizi et al. (2007) with some modifications. In brief, the seeds of *B. napus* cultivar, Hyola 308, were surface-sterilized in 10% chlorine solution and germinated in MS media (Murashige and Skoog, 1962). Canola cotyledonary explants were excised from 4-day old seedlings, and recombinant*A. tumefaciens* harboring the pBI-*synth-gox* was grown overnight at 28ºC in liquid LB medium containing 100 mg/l rifampicin and 50 mg/l kanamycin. The bacterial culture was centrifuged (3000 rpm/ 4ºC/5 min) and the pellet was suspended in MS medium (pH 5.2). Two days after infection of cotyledons with recombinant *Agrobactrium* (co-cultivation), the cotyledons were sub-cultured in selective medium supplemented with 200 mg/l cefotaxime and 15 mg/l kanamycin. The culture was incubated for 14 days at 22ºC under a 16 hrs light/8 hrs dark photoperiod. After primary selection, the explants were transferred to soil and grown under greenhouse conditions.

DNA and RNA extraction

Genomic DNA was extracted according to the protocol described by Dellaporta et al. (1983), using 300 mg of leaf tissue pulverized with liquid nitrogen. The quantity and quality of extracted DNA were determined by spectrophotometer and agarose gel electrophoresis, respectively. RNA was extracted using the Plant Total RNA Kit (Roche), analyzed on a 0.8% agarose gel to confirm its quality, and quantified using a spectrophotometer. The extracted RNA was treated with DNase I (Invitrogen) to eliminate DNA residues.

Southern blot analysis

The *Bam*HI enzyme was used to digest 40 µg of genomic DNA from wild-type and transgenic lines. The digested DNA was electrophoresed on 1% agarose gels and blotted on Hybond N membranes (Roche) following standard procedures (Sambrook et al. 2001). A DIG-labeled probe (1314 bp) was prepared with the PCR DIG Labeling Mix using the *synth-gox* gene as a template with specific primers (Table 1). Hybridization was performed using the PCR DIG detection kit following the supplier's instructions (Roche).

Primer design for QC-PCR

Two sets of primers (*sg* F and *sg* R) were designed for amplification of a 210 bp fragment of the*synth-gox* gene as a target sequence. The mutated form of the *synth-gox* gene, which was 370 bp in length, was made by adding 160 bp to the target sequence using *msg* F and *msg* R primers. These primers contained an extra fragment of about 80 bp at their 5' ends, and were used for production of two intermediate fragments (179 bp and 201 bp) with a 10-nucleotide overlap. These two large fragments were fused together by splicing by overlapping extension PCR (SOE-PCR (Table 1)).

Amplification and cloning of mutant *synth-gox* gene

PCR was carried out in a 25 µl volume containing 1X PCR buffer, 1.5 mM $MgCl_2$, 2 mM dNTP mixture, 0.5 unit *Taq* DNA polymerase, 20 ng template DNA, and 1 pmol of each primer (*sg*F/*msg* R and *msg* F/*sg* R were used individually in PCR I and II). Thermocycling conditions started with an initial denaturation step at 95ºC for 5 min, followed by 35 additional cycles at 95ºC for 1 min, annealing temperature of 70ºC for 1 min, and extension at 72ºC for 1 min. The PCR products were electrophoresed on a 1.5% agarose gel, and the DNA was purified using the DNA Extraction Kit (Bioneer). Two intermediate fragments (179 bp and 201 bp) were fused together by two-step PCR; in step 1 of the SOE-PCR cycle, 56ºC was established as the annealing temperature for 15 cycles; in step 2, SOE-PCR was continued by adding 1.5 pmol of*sg* FandR primers, which annealed at 63.4ºC for another 30 cycles. The reaction was set up with 0.2 mM dNTP, 1 mM $MgCl_2$, 1X PCR buffer, and 1 unit *Taq* DNA polymerase. The PCR product was purified and inserted into the pGEM-T easy vector (Promega). The resulting plasmid was named pGEM-*msg* (mutant *synth-gox*) and after sequencing was used as an internal standard in QC-PCR. The copy number of the mutant plasmid was calculated based on the concentration of plasmid using *ds copy number analyzer* online software (www.uri.edu/research/gsc/resources/cndna.html). The estimated copy number was 9×10^6 copies/µl.

Quantifying expression levels of the *synth-gox* gene using QC-RT-PCR

Two micrograms of total RNA for each sample was used to synthesize the first-strand (cDNA) using MMLV reverse transcriptase (Promega) with oligo-dT primer. Equal volumes (4 µl) of target cDNA and serially dilutions of the internal standard plasmid (9×10^6, 8.0×10^5, 7.97×10^4, 6.86×10^3, 5.0×10^3, 4.56×10^2, 3.11×10^1 copies/µl) were used in each QC-RT-PCR reaction in order to quantify the unknown mRNA expression level. PCR reactions were conducted in each QC-RT-PCR series. Each QC-RT-PCR reaction mixture contained 1 unit of *Taq*DNA polymerase, 1 mM $MgCl_2$, and 1 pmol of each primer (*sg* F andR). Nuclease-free water (Sigma) was added to bring the final volume to 50 µl. PCR cycling conditions were one cycle of 1 min at 94ºC, followed by 30 cycles of 30 sec at 94ºC, 1 min at 63.4ºC, 1 min at 72ºC, and finally, a 5 min extension at 72ºC. The amplified products were separated by gel electrophoresis, and the images were digitally recorded.

Estimation of *synth-gox* copy number by QC-PCR

Extracted genomic DNA from transgenic canola lines was diluted to 10 ng/µL (approximately 7700 haploid genomes/µL) (Arumuganathan and Earle, 1991). One microliter of DNA and 5 µl of serially diluted standard plasmid in each QC-PCR reaction were used. PCR procedures were carried out as aforementioned.

Validation of experiments based on QC-PCR

Duplex RT-PCR by co-amplification of *synth-gox* transgene and endogenous tubulin as a housekeeping gene was performed for each transgenic line (1-9). The cDNA used in QC-RT-PCR was diluted (1:10 ratio), and 2 µl was used for conventional RT-PCR for *synth-gox* and tubulin gene amplification. The same composition (2 mM Mg^{+2}, 0.5 pmol of each primer) was used in a final reaction volume of 25 µl. The PCR program for both transgenes consisted of a denaturation cycle for 5 min at 94ºC, followed by 35 cycles of amplification of 1 min at 94ºC, 1 min at 60ºC, 1.5 min at 72ºC, and an extension of 5 min at 72ºC. The amplification products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining.

Statistical analysis

The intensity of native and mutated amplification products was directly measured using an image program (http://rsb.info.nih.gov/ij), total lab and SAS (Statistical Analysis System, version 7.12; SAS, Cary, N.C.) software. To correct differences in the fluorescence of ethidium bromide-stained PCR fragments, the intensity of the competitor was multiplied by the ratio of target sequence size (210 bp) to competitor sequence size (370 bp). For determination of target copy numbers and transcription levels, the $\log_{10}$ of the ratio of fluorescence intensities of the competitor and target bands was plotted as a function of $\log_{10}$ of the concentration of competitor molecules added. Interpolation of the regression equation for a y value of 0 ($\log_{10}^1 = 0$) gives the concentration of the target template in the sample (Gilliland et al. 1990; Connolly et al. 1995).

RESULTS

Molecular analysis of transgenic canola plants

The nine transformed canola lines, named 1-9, were subjected to molecular analysis using the QC-PCR method to determine the copy number and transgene expression level. Furthermore, duplex conventional RT-PCR was used to evaluate the relative amounts of target *synth-gox*and tubulin transcript in these transgenic lines (Figure 2).

Estimation of the copy number of the *synth-gox* transgene using QC-PCR

To estimate the copy number of the transgene, genomic DNA from each transgenic line (1-9) was co-amplified with the mutated *synth-gox* plasmid (competitor) in a single tube (Figure 3a). The regression equation and R^2 were determined for the nine transgenic lines (Table 2). The copy number of *synth-*

gox in each transgenic line was calculated based on the final intensity of the *synth-gox* gene relative to the initial intensity ofthe *synth-gox* gene in QC-PCR (Table 2 andTable 3). Estimation with QC-PCR showed that five of the nine lines (64%) had one copy, and two lines (23%) represented two copies of the *synth-gox* gene. For line 1 (6.6%), one or two copy numbers of the transgene are shown, and the calculation for line 8 (6.6%) showed three copies of the *synth-gox* gene (Table 4).

Quantifying *synth-gox* transgene expression by QC-RT-PCR

QC-RT-PCR was used to examine *synth-gox* mRNA levels in the nine transgenic lines, of which*synth-gox* expression was driven by the strong constitutive CaMV35S promoter (Figure 4). According to QC-PCR regression equations and R^2, the final intensity of the *synth-gox* band in QC-RT-PCR was shown as the mRNA level of the *synth-gox* gene (Table 3). For *synth-gox* mRNA expression levels in the transgenic lines, we classified these data into four groups; namely, high (> 3), medium to high (2.2-3), medium to low (2.2-1) and low (< 1). In QC-RT-PCR analysis, four transgenic lines (9, 4, 2 and 6) showed high expression (> 3), two lines (3 and 1) showed medium to high expression (2.2-3), two lines (5 and 7) showed medium to low expression (1-2.2), and only one line (8) showed low expression levels (< 1).

Comparison between copy number and expression level by QC-PCR

Here, we showed that four transgenic lines (6, 9, 4, and 2) with the highest levels of *synth-gox*gene expression with a score of > 3 had only one copy of the transgene. Two other lines with one to two copies (lines 3 and 1) showed medium to high levels of transgene expression. The expression level in line 5 with two copies and line 7 with one copy of transgene were low to medium (1-2.2). Line 8, containing three copies of the *synth-gox* transgene, yielded the lowest level of the mRNA transcript (Table 4).

Comparison of QC-PCR and Southern blotting in gene copy number determination

To confirm the copy numbers of transgenic canola lines illustrated in QC-PCR, Southern blot analysis was performed (Figure 5). The results from this experiment and QC-PCR analysis are compared in Table 4. When we assessed QC-PCR reliability by comparing the results of this method with those from Southern blot analysis, the results of both methods were the same in eight of the nine samples.

DISCUSSION

Isolation of the *gox* gene from the *Ochrobactrum antrophi* strain LBAA was previously reported (Barry and Kishore, 1995). This gene encodes the glyphosate oxidoreductase (GOX) enzyme, which can degrade glyphosate and convert it to glyoxlate and aminomethylphosphonic acid (AMPA). The *gox* gene was used for transforming canola to generate the glyphosate tolerance trait. The GOX enzyme can reduce glyphosate injury to the crop plant by decreasing the amount of glyphosate. The herbicide inhibits EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) in the shikimate pathway, which has a key role in the biosynthesis of aromatic amino acids and is required for survival of the plant (Padgette et al. 1996; Dill, 2005).

This study reports the application of a QC-PCR technique to estimate copy number and determine expression levels of the *synth-gox* transgene in transformed canola lines. Southern blot analysis was also used in parallel with QC-PCR to estimate the copy number in nine transgenic lines. Comparison between the results of these two methods showed about 89% similarity, with only one line (line 9) showing a difference in estimated transgene copy numbers. For this line, Southern blotting revealed two copies of the transgene, whereas QC-PCR only showed one copy. This difference might be attributed to distortion of the sequence of primer alignment, rearrangements, or partial digestion of DNA, all of which could lead to wrong copy number estimation (Mason et al. 2002). Although recent real-time PCR methods are rapid and can precisely determinecopy number, they typically require suitable internal standards, limit the size of target sequences, require expensive equipment, and are not fail-safe (Callaway et al. 2002; Bubner and Baldwin, 2004).

Most studies assume that copy number determination in real-time PCR experiments is confirmed by Southern blot analysis (Ingham et al. 2001; Mason et al. 2002; Bubner et al. 2004; Shou et al. 2004). The mismatch increase in studies reporting higher copy numbers-comment (Bubner and Baldwin, 2004). The results obtained from QC-PCR were in accordance with data from Southern blot analysis; thus, QC-PCR could be used to determine transgene copy numbers in tested plants more rapidly and effectively.

Although QC-PCR can only determine transgene amount, additional analyses would be necessary to confirm gene copy number and segregation, specifically in a breeding lines. Furthermore, the independence of multiple transgene events should still be estimated by efficient methodology such as Southern blot analysis with QC-PCR serving as a complementary approach (Bubner and Baldwin, 2004).

Previous studies show an inverse relationship between copy number and transgene expression level in transformed plants (Dai et al. 2001; Vaucheret and Fagard, 2001). However here, we show that such a relationship was not the case in all of our transgenic lines; one line (7) carried only one copy number but its expression level was as low as 0.16×10^{1} (for details see Table 4). Actually, transgenic events with only one copy of a transgene may show low expression levels, which could be explained by gene silencing effects (Elmayan and Vaucheret, 1996). Some phenomena, such as methylation and chromatin structure at the insertion site known as a “positional effect”, could also explain this observation (Kumpatla et al. 1998). Our results for the QC-PCR approach suggest that an inverse relationship between copy number and expression level may be tendency rather than a rule, and this is also supported by previously published reports (Beltrán et al. 2009). According to this hypothesis, the copy number in line 9 is more reliable using QC-PCR than Southern blotting. Therefore, based on differences between the copy number and expression level of the transgene, we could develop this system as an efficient tool to quickly select transgenic plants with low copy numbers and high expression levels. Although there are some reports on the usefulness of qPCR for correlating expression levels with the numbers of insertions in transgenic plants (Shou et al. 2004; Beltrán et al. 2009), such studies are very limited.

Our data are also in agreement with the results of other studies on *Agrobacterium*-mediated transformation of canola which showed that transgenic plants with low copy numbers occur more frequently (Shou et al. 2004; Travella et al. 2005) than those with multiple copies. QC-PCR was previously recommended as a high-throughput tool for estimating copy numbers in transgenic plants (Callaway et al. 2002), but our study is the first in which QC-RT-PCR is used to estimate mRNA levels in a transgenic plant. Because individual mRNA species are expressed in extremely small quantities, PCR-based approaches are becoming more widely used for quantification of transcript levels (Callaway et al. 2002).

CONCLUDING REMARKS

We successfully used the QC-PCR method to estimate the copy number of the synthetic *gox*gene as a transgene, and measured its transcript levels in transformed canola lines. Since there is no direct relationship between copy number and gene expression level, this method could be implemented as a tool in the primary screening of putative transgenic plants. To the best of our knowledge, there are no reports in the literature on the use of QC-PCR for analyzing transgene copy number and expression in transgenic canola plants. Although finding appropriate internal standards usually complicates QC-PCR methodology (Bubner and Baldwin, 2004), in our study only one serial dilution for both determination of copy number and transcript level of a synthetic gene (without intron) was required.

As the use of genetically modified (GM) plants for food and feed has been increased rapidly, labeling systems for GM foods that guarantee consumers a choice between GM and non-GM products are required. Hence, QC-PCR methods and the prepared competitor construct developed in this study could be used more efficiently for future evaluations of commercial GM products (Hübner et al. 2001).

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FIGURES

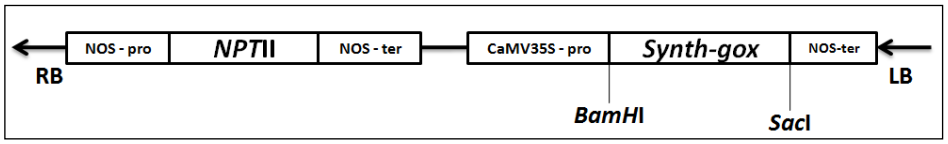

Fig 1. Schematic representation of the pBI-*synth-gox* construct. The construct contains the synthetic *gox* gene under the control of the CaMV 35S promoter and NOS terminator. The Kozak sequence is located at the 5' end. LB and RB are left and right border sequences, respectively.

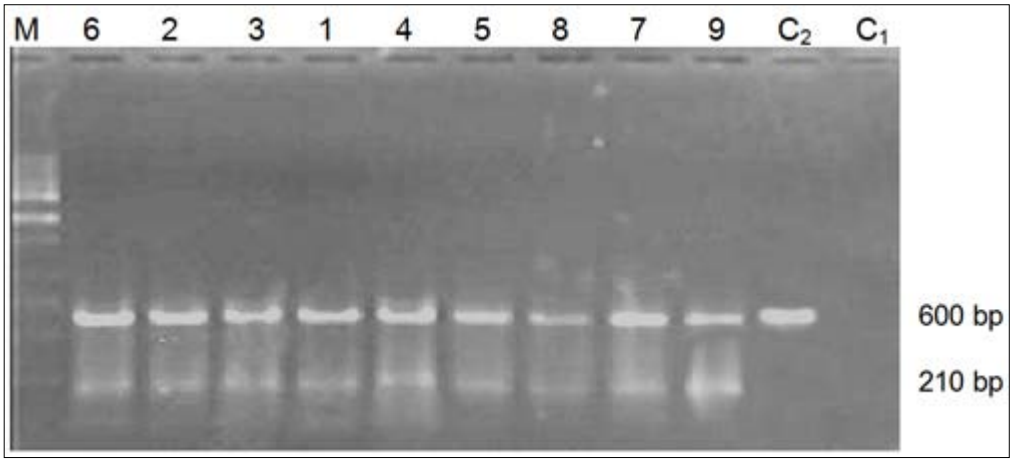

Fig 2. Conventional RT-PCR analysis for *synth-gox* and tubulin genes in transgenic canola lines. Tubulin (600 bp) was amplified as a quality and quantity control of the RNA analysis in transgenic canola lines. Individual intensities of amplification corresponded to the quantitative data obtained with QC-RT-PCR. C_1: H_2O as control; C_2: Non transgenic control; M: 1kb ladder (Fermentas). Top row of numbers refer to the transgenic canola lines.

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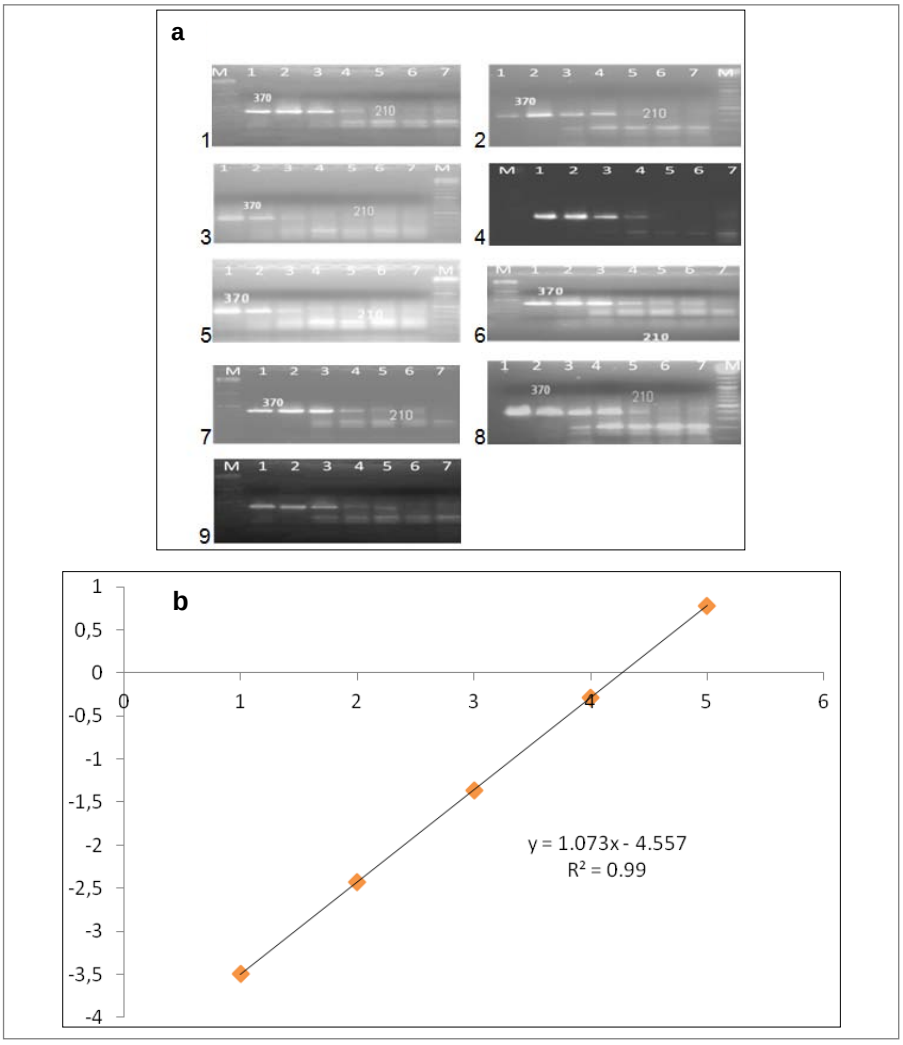

Fig 3. Quantitative analysis of genomic DNA using QC-PCR. (a) Lanes 1-7 in each figure respectively represent the QC-PCR products of the co-amplified serially diluted competitor (9 x 10^6, 8.0 x 10^5, 7.97 x 10^4, 6.86 x 10^3, 5.0 x 10^3, 4.56 x 10^2, 3.11 x 10^1copies/µl) (370 bp) and the fixed amount of target DNA (210 bp) for determination of*synth-gox* gene copy number in nine canola lines (1-9) after electrophoresis in a 2% agarose gel). (b) Example of a linear regression for calculation of the amount of *synth-gox* gene copy number in line 9. The ratio of intensities of target to competitor was plotted against the concentration of the competitor, which was serially diluted on a log scale. Each point on the plot is the mean (± standard deviation) of two replicate samples.

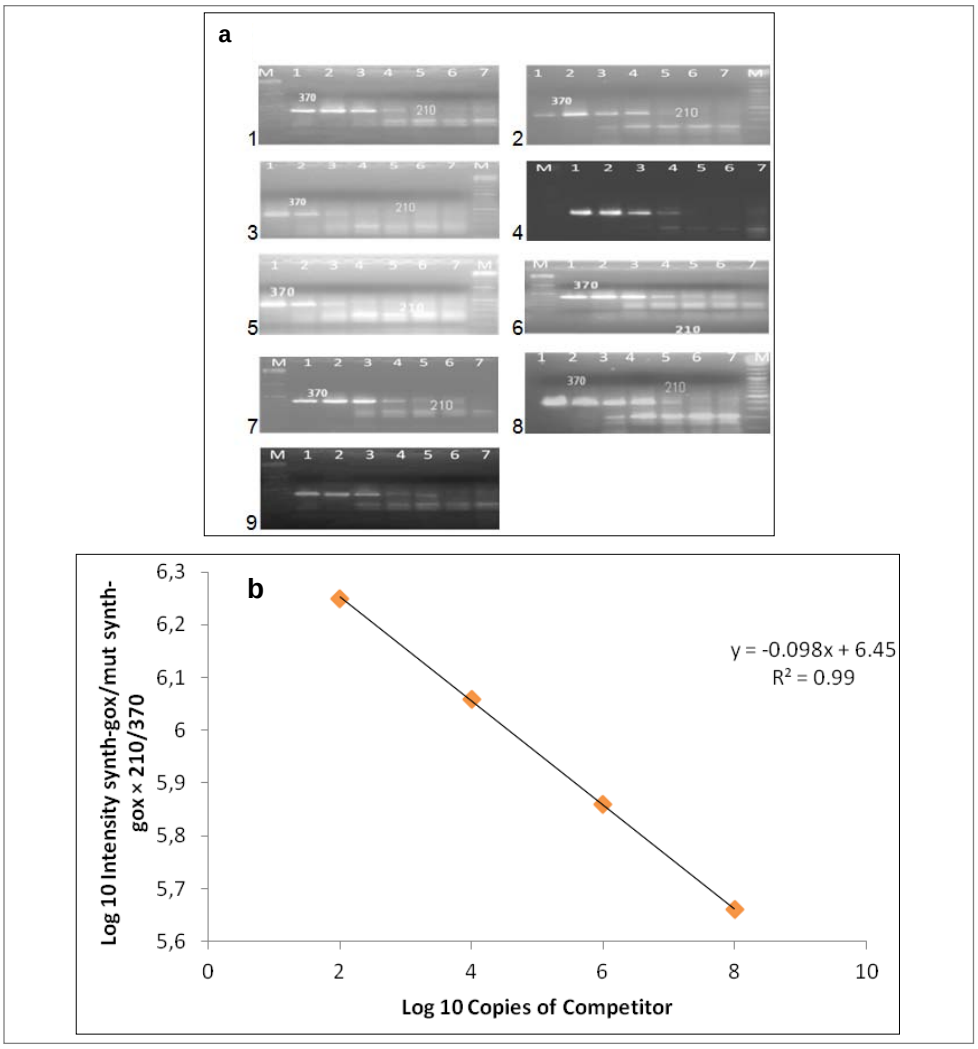

Fig 4. Quantitative analysis of mRNA expression using the QC-RT-PCR system. (a) Lanes 1-7. QC-RT-PCR products of the co-amplified serially diluted competitor (9 x 10^6, 8.0 x 10^5, 7.97 x 10^4, 6.86 x 10^3, 5.0 x 10^3, 4.56 x 10^2, 3.11 x 10^1 copies/µl) (370 bp) and a fixed amount of target cDNA (210 bp) for examination of *synth-gox* mRNA levels in nine independent transgenic canola lines (1-9). (b) Example of linear regression to calculate the amount of *synth-gox* gene transcripts in line 4. The ratio of intensities of target gene to competitor was plotted against the concentration of the competitor, which was serially diluted on a log scale.

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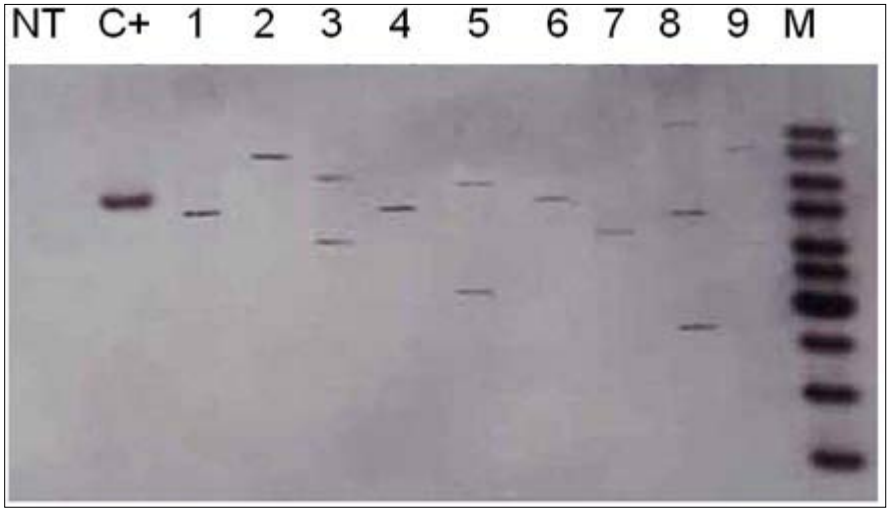

Fig 5. **Southern blots of genomic DNA from nine transgenic canola plants.** DNA from wild-type and transgenic plants were digested with *Bam*HI and hybridized with a PCR Dig-labeled *synth-gox* probe. NT: nontransgenic control; C+: positive control, (pBI-*synth-gox*); M: 100 bp Mix ladder; Top row of numbers refer to the transgenic canola.

TABLES

Table 1. Primer information used in this study.

	Gene	Sequence (5' → 3')	Amplicon (bp)
QC-PCR	*synth-gox*	***sg* F**:TTGATTGAAGAGAACGGCCACAC ***sg* R**: GAAACTCTTTGAATGCGCACCT	210
	Fragment 1 for SOEing	***msg* F**: GCTAGGCTATGGCTATCGAATCGCAATCAGGCT ACGCTACGGATAGCAGCTAGTGCATGAATCCGTACGTAACCGTCCATCCGAGGGAAGAGCCCTG ***sg* R**: GAAACTCTTTGAATGCGCACCT	179
	Fragment 2 for SOEing	***sg* F**: TTGATTGAAGAGAACGGCCACAC ***msg* R**: GCCAATGGTCGGTTACGTACGGATTCATGCAC TAGCTGCTATCCGTAGCGTAGCCTGAGCCTGATTGCGATTCGATAGCCCGGTTTCAAAACCAATAAC	201
Southern blotting	*synth-gox* probe	***synth-gox* F**: GGATCCACCACCATGTCG ***synth-gox* R**: AGCTCTCAGGAGGCAGGAC	1314
Duplex RT-PCR	Housekeeping gene (tubolin)	**F**: GCTTTCAACACCTTCCTTCAG **R**: GGGGCGTAGGAGGAAAGC	600

Table 2. Calculation of copy number of *synth-gox* gene for each transgenic canola line by QC-PCR.

Transgenic canola lines	[a]Initial intensity of *synth- gox* gene	[a]Finalintensity of *synth- gox* gene	Regression equations from QC-PCR and R^2		Copy number
			Equation	R^2	
1	*0.89 x 10^2 0.01 x 10^1	1.89 x 10^2	y = 0.91x-2.24	0.91	1-2
2	1.07(± 0.68) x10^4	1.05 x 10^4	y = 0.97x-2.94	0.97	1
3	3.81(± 0.56) x 10^8	6.13 x 10^8	y = 0.97x-6.57	0.96	2
4	2.24(± 0.68) x 10^3	2.89 x10^3	y = 0.91x-2.24	0.98	1
5	6.88(± 0.38) x 10^8	8.60 x 10^8	y = 1.03x-8.18	0.95	2
6	8.19(± 0.28) x 10^4	9.03 x 10^4	y = 0.98x-3.31	0.97	1
7	7.99(± 0.11) x 10^4	8.33 x 10^4	y = 2.25x-2.459	0.95	1
8	0.81(± 0.12) x 10^{10}	2.3 x 10^{10}	y = 1.08x-7.97	0.96	3
9	3.33(± 0.38) x 10^5	4.26 x 10^5	y = 1.07x-4.57	0.99	1

[a]Mean intensity of initial *synth-gox* gene ± standard deviation of two replicate samples.
*The data for this line in two replicate samples is not in the range of the standard deviation.

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Table 3. Quantification of *synth-gox* transcript levels for each canola transgenic line by QC-RT-PCR.

Transgenic canola lines	Transgene transcript level (Quantified by QC-RT-PCR)	Regression equations from QC-RT-PCR and R^2	
		Equation	R^2
1	0.28×10^1	y = -1.10x + 5.41	-0.94
2	0.34×10^1	y = -0.98x + 3.47	-0.95
3	2.4	y = -0.96x + 6.68	-0.94
4	0.037×10^2	y = -0.098x + 6.45	-0.99
5	1.14	y = -0.96x + 6.66	-0.96
6	0.041×10^2	y = -0.098x + 3.47	-0.94
7	0.16×10^1	y = -0.095x + 6.28	-0.92
8	0.005	y = -0.094x + 5.87	-0.94
9	0.38×10^1	y = -1.10x + 5.41	-0.94

Table 4. Comparison of copy number values estimated by QC-PCR and Southern analysis, and mRNA expression level determination by QC-RT-CR and reverse transcriptase analysis (nine lines).

Transgenic canola lines	Copy number		mRNA expression analysis	
	QC-PCR	Southern blot	QC-RT–PCR	RT-PCR
1	1-2	1	$0.2\ 8 \times 10^1$	Positive
2	1	1	0.34×10^1	Positive
3	2	2	2.4	Positive
4	1	1	0.037×10^2	Positive
5	2	2	1.14	Positive
6	1	1	0.041×10^2	Positive
7	1	1	0.16×10^1	Positive
8	3	3	0.005	Positive
9	1	2	0.38×10^1	Positive

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