BMC Biotechnology

BioMed Central

Methodology article

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Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR

Ben Bubner, Klaus Gase and Ian T Baldwin*

Address: Department of Molecular Ecology, Max-Planck-Institute for Chemical Ecology, Hans-Knöll-Str. 8, 07745 Jena, Germany

Email: Ben Bubner - bubner@ice.mpg.de; Klaus Gase - gase@ice.mpg.de; Ian T Baldwin* - baldwin@ice.mpg.de

* Corresponding author

Published: 13 July 2004

BMC Biotechnology 2004, **4**:14 doi:10.1186/1472-6750-4-14

Received: 21 February 2004
Accepted: 13 July 2004

This article is available from: http://www.biomedcentral.com/1472-6750/4/14

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Abstract

Background: After transformation, plants that are homozygous and contain one copy of the transgene are typically selected for further study. If real-time PCR is to be used to determine copy number and zygosity, it must be able to distinguish hemizygous from homozygous and one-copy from two-copy plants. That is, it must be able to detect two-fold differences.

Results: When transgenic *Nicotiana attenuata* plants which had been previously determined by Southern analysis to contain one or two copies of the transgene, were analyzed by real-time PCR ($2^{-\Delta\Delta Ct}$ method), the method failed to confirm the results from the Southern analysis. In a second data set we analyzed offspring of a hemizygous one-copy plant, which were expected to segregate into three groups of offspring in a 1:2:1 ratio: no transgene, hemizygous, homozygous. Because it was not possible to distinguish homozygous from hemizygous plants with real-time PCR, we could not verify this segregation ratio.

Conclusions: Detection of two-fold differences by real-time PCR is essential if this procedure is to be used for the characterization of transgenic plants. However, given the high variability between replicates, a detection of two-fold differences is in many cases not possible; in such cases Southern analysis is the more reliable procedure.

Background

Real-time PCR is widely used to detect and quantify DNA and cDNA in such diverse applications as the detection of transgenic contamination in food or expression studies [1,2]. Real-time PCR has been proposed for determining transgene copy number in transformed plants [3-6]. For many applications transformants are required to harbor only one copy (per haploid genome) of the transgene to increase the stability of the construct over many generations of subsequent breeding. Real-time PCR is faster and less expensive than the traditional method of determining copy number, Southern analysis, and requires less plant material.

Despite these advantages many authors speak of "estimating" copy number by real-time PCR [5,6]. How well real-time PCR performs depends on the objectives of the study. Many transformation methods, such as ballistic transformation [7] or whiskers transformation [8] yield a high proportion of transformants with highly differing numbers of transgenes and only a small proportion of primary transformants with low copy numbers. In these cases it is important to identify promising candidates for further breeding at an early stage, and hence being able to distinguish transformants with low copy numbers (one or two) from those with high copy numbers is sufficient. However, for actual experiments or commercial release,

copy number has to be established without error, and for such applications real-time PCR must be able to precisely distinguish plants with one, two, or three transgenes. In protocols using *Agrobacterium*-mediated transformation which produce predominantly only one or two-copy transformants [9,10] real-time PCR will only be useful for copy-number determination if it can distinguish one from two copies with a high degree of certainty.

To test the ability of real-time PCR to detect two-fold differences, we analyzed *Nicotiana attenuata* Torrey ex. Watson plants that were transformed with constructs containing fragments of the endogenous oxylipin genes, hydroperoxide lyase (*hpl*), allene oxide synthase (*aos*) and lipoxygenase (*lox*) in an antisense orientation. The objective of these transformations was to manipulate the oxylipin cascade of plants to facilitate an understanding of defense signaling when plants are attacked by herbivores [11]. *N. attenuata* is a diploid tobacco that propagates largely by selfing, which facilitates large-scale breeding and is rapidly becoming a model organism for the analysis of plant-herbivore interactions [12]. We compared transgene copy number in homozygous T_2 plants as determined by Southern and real-time PCR analyses. A further test of the ability of real time PCR to distinguish two-fold differences was conducted by analyzing T_2 offspring of a segregating hemizygous T_1 line consisting of plants that were null, hemizygous and homozygous for the transgene. Homozygous plants should contain twice as many transgenes as hemizygous plants.

Results and Discussion

Determining copy numbers

For determining copy numbers, we chose the $2^{-\Delta\Delta Ct}$ method with a calibrator as described by [3]. The $2^{-\Delta\Delta Ct}$ method assumes that the efficiencies for the endogenous control amplicon (ampGSP1) and the transgene amplicon (ampNAT1) are the same. Efficiencies were determined for the amplicons ampGSP1 and ampNAT1 on a 1:5 dilution series in a multiplex PCR with DNA from plant as-*hpl* A422-4-1 as template (Figure 1A,1B). The slopes of Ct/log dilution plots for the reactions were -3.41 and -3.51 respectively; thus, according to equation 5 (see Methods), both amplicons amplify with very similar efficiencies (0.96 and 0.93, Figure 1B). If ΔCt is plotted against log dilution, the slope of the graph is 0.11 (Figure 1A), which is still in the range of a maximal slope of 0.1 as recommended by ABI [13].

Two calibrator plants A434-4-2 and A300-1-2 were chosen (Table 1) because they contain only one transgene copy according to Southern analysis (Figure 2C). Thus both calibrators should yield the same copy number. As seen in the different ΔCt values (Table 1), this is clearly not the case. Theoretically, all plants with the same copy number (that is 10 of the 13 lines shown in Table 1) should have the same ΔCt values because the difference between the Ct values for the transgene and the endogenous control is independent of the amount of template as long as the copy number is invariant.

The Ct values of biological replicates were also highly variable. The analysis of plants A422-4-1 and A422-4-2 produced ΔCts of 1.76 and 3.17, respectively, which corresponds (using equation 4 in Methods) to the first plant having three times as many transgenes as the second. The consistency of the PCR results in comparison to the Southern data is summarized in Table 2. A copy number is viewed to be unambiguous if for a line all four copy numbers are the same. Thus, transgenic line A160-5 contains unambiguously one copy of the transgene, because plant 1 and 2 each have one copy by real-time PCR (0.9 and 1.2) and by Southern analysis. Using A434-4-2 as a calibrator, unambiguous copy numbers were determined for only two lines; with A300-1-2 as a calibrator, the correct values for six lines could be estimated.

We propose that the observed variation in calculated copy numbers results from variation of the PCR and that an indicator of a variable PCR is the standard deviation of triplicate measurements. Individual measurements of Ct values are given for two plants of as-*hpl* line A340 in Table 3 to demonstrate the calculation of the standard deviation of ΔCt: First the ΔCt for the two amplicons in one well is calculated and then the average and SD for all three wells. This means that even with moderate SD for the single amplicons (0.47 and 0.19 for A340-1-1) the standard deviations of ΔCt can be above 0.5 (0.64 for A340-1-1) representing already a two-fold difference ($2^{0.64 \times 2} = 2.4$). All Ct values, their averages and standard deviations used for calculation of the ΔCt standard deviations in Table 1 are derived from one PCR experiment with a 96 well plate and are listed in the Additional File 1. Because samples belonging to the same line are placed adjacent to each other on the plate (plate layout in Additional File 3) we can exclude a position effect (variation due to nonuniform heating).

Another possible reason for high standard deviation is high Ct values, because they indicate low numbers of starting molecules. However, in Figure 1B (e.g. 1:125 dilution for ampNAT1) and Additional File 1, samples with high average Ct have still low standard deviation. Thus, we do not observe a correlation between standard deviation and Ct value or standard deviation and plate position and conclude that the observed variation is a random PCR artifact.

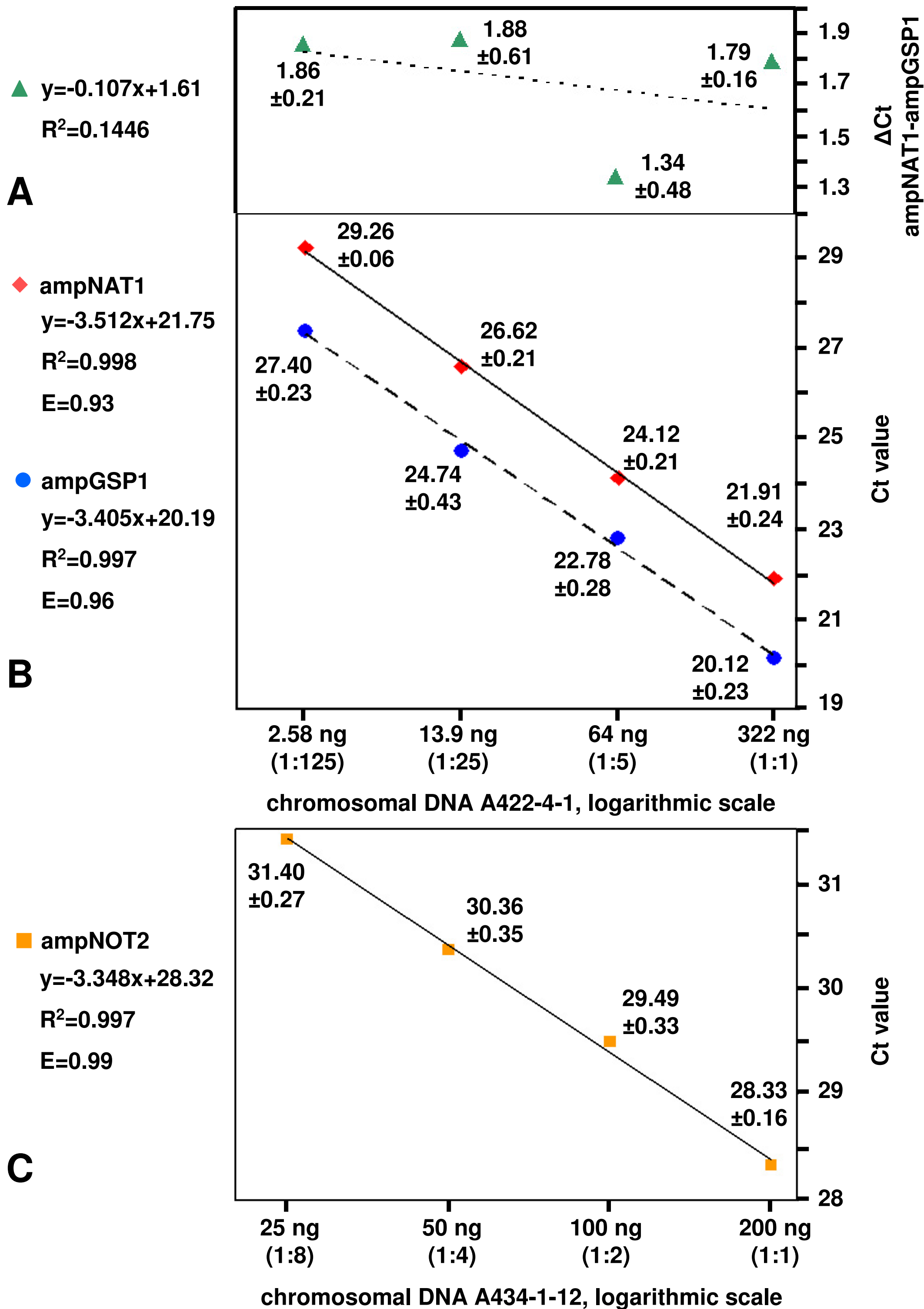

Figure 1

Calculation of efficiency A) ΔCt (ampNAT1-ampGSP1) for as-*hpl* plant A422-4-1 over a 1:5 dilution series, ampNAT1 and ampGSP1 are amplified in the same well (multiplex) with conventional TaqMan® probes, DNA extracted with a miniprep (Ariel) method. B) Ct of ampNAT1 and ampGSP1 (same PCR as A) for calculation of efficiency. C) Ct of ampNOT1 for calculation of efficiency for as-*hpl* plant A434-1-12 over a 1:2 dilution series, amplification as singleplex reaction with Minor-Groove-Binder probe.

Table 1: Copy number analysis for progeny of 13 homozygous lines (two plants for each line) with two different calibrators.

Construct	Plant	**ΔCt** (Ct FAM - Ct TET) ± SD[a]	Copy number with A434-4-2 as calibrator	Copy number with A300-1-2 as calibrator	Copy number from Southern analysis
as-*hpl*	A188-4-1	0.42 ± 0.11	4.8	1.4	1
	A188-4-2	2.26 ± 1.77	1.4	0.4	1
	A247-3-1	0.28 ± 0.07	5.3	1.6	2
	A247-3-2	0.72 ± 0.10	3.9	1.2	2
	A337-2-1	0.89 ± 0.24	3.5	1.0	1
	A337-2-2	1.80 ± 0.44	1.9	0.5	1
	A422-4-1	1.76 ± 0.63	1.9	0.6	1
	A422-4-2	3.17 ± 0.34	0.7	0.2	1
	A434-4-1	2.69 ± 1.36	1.0	0.3	1
	A434-4-2	2.69 ± 0.95	1.0	0.3	1
as-*aos*	A160-5-1	0.69 ± 0.06	4.0	1.2	1
	A160-5-2	1.13 ± 0.32	3.0	0.9	1
	A160-7-1	1.10 ± 0.07	3.0	0.9	1
	A160-7-2	1.88 ± 0.14	1.8	0.5	1
	A266-2-1	2.36 ± 0.35	1.3	0.4	1
	A266-2-2	1.91 ± 0.25	1.7	0.5	1
	A340-1-1	1.32 ± 0.64	2.6	0.8	1
	A340-1-2	1.02 ± 0.25	3.2	0.9	1
as-*lox*	A211-1-2-1	2.62 ± 0.27	1.2	0.3	1
	A211-1-2-2	2.46 ± 0.36	1.1	0.3	1
	A248-2-1	-0.17 ± 0.22	7.2	2.1	2
	A248-2-2	0.74 ± 0.73	3.9	1.1	2
	A300-1-1	1.42 ± 0.69	2.4	0.7	1
	A300-1-2	0.93 ± 0.75	3.4	1.0	1
	A363-1-1	-0.06 ± 0.39	6.7	2.0	2
	A363-1-2	-0.32 ± 0.16	8.1	2.4	2
controls	Wildtype 12	12.04 ± 0.81	0	0	-
	A340-5-1[b]	8.51 ± 0.10	0	0	-
	A340-5-2[b]	9.02 ± 0.26	0	0	-

[a] average of three measurements [b] transformed line without T-DNA (T-DNA segregated out)

Determining zygosity

To determine if the variability in measuring two-fold differences is independent of the problems associated with the choice of calibrators, we examined ΔCt values of offspring of a heterozygous line. As-*hpl* line A 443-1 (T_1) had been previously shown by classical segregation analysis to be hemizygous for the transgene [11]. By Southern analysis we confirmed that T_2 offspring segregate in the ratio 3:1 for the transgene (Figure 2B). Because of the inaccuracies of determining DNA-concentration by OD_{260} measurements we refrained from using signal intensity of the Southern blot for determining zygosity. Indeed, the strength of real-time PCR with endogenous controls is its independence from the need for exact DNA measurements.

According to Mendel's laws, the 16 positive samples should fall into two groups: one third homozygous and two-thirds hemizygous and both should be distinguished by a Ct of one, because homozygous plants contain twice as many transgenes as the hemizygous. While the plants indeed had ΔCt values around one and two, the values were continuously distributed and could not be categorized into two distinct groups (Table 4, Additional Files 2 and 4). Only the first three and the last seven plants (in ascending order: Table 4) could be designated as homozygous or hemizygous. Of the 16 positive plants, six plants could not be assigned a zygosity based on ΔCt values alone and homo- and hemizygous plants could not be distinguished.

Despite the inaccuracies in assigning homo- and hemizygous plants by real-time PCR, transgenic plants could be separated from nontransgenic plants at the ratio of 3.2:1 (16 plants with transgene, five without). The five

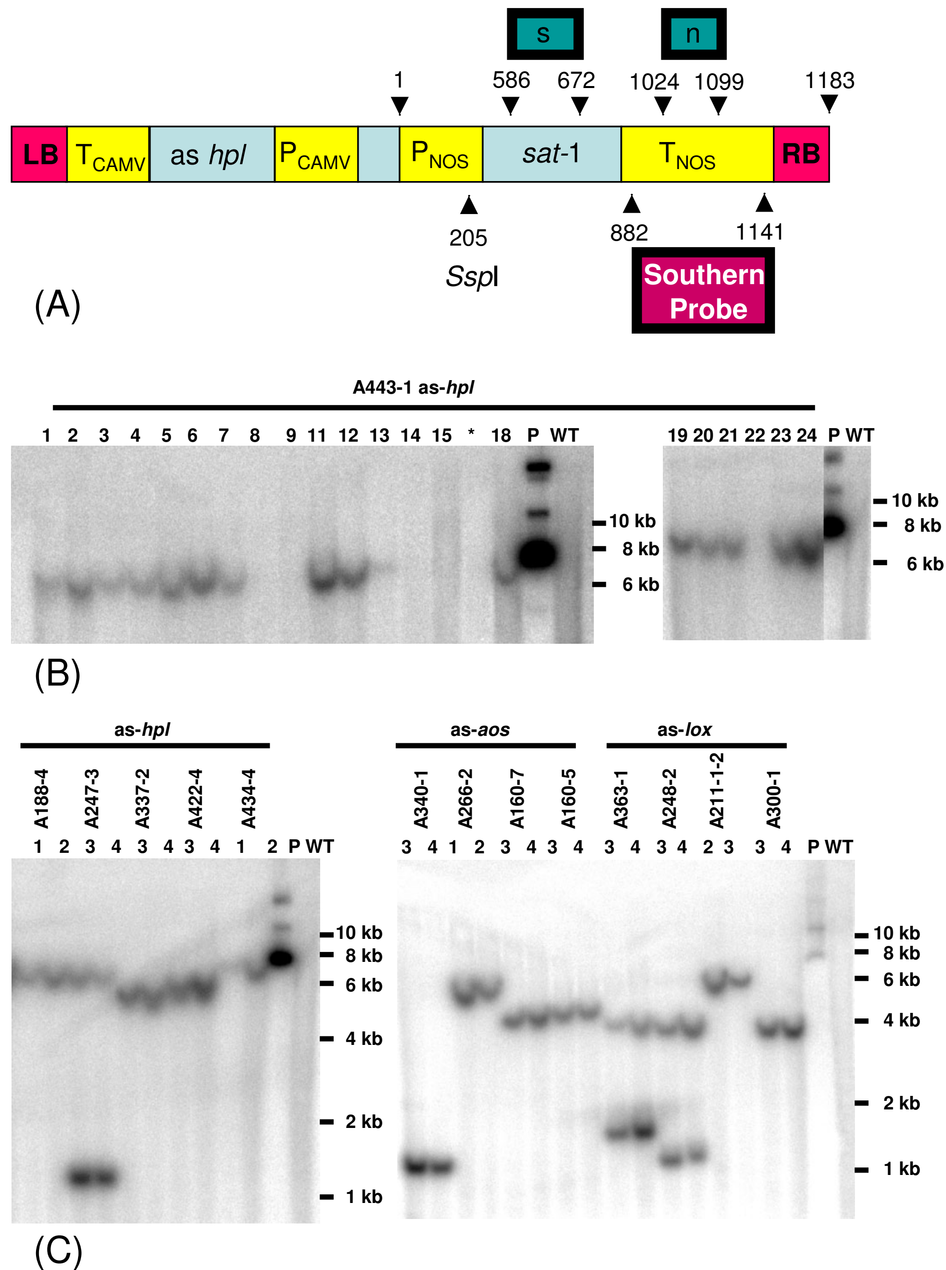

Figure 2

Southern analysis of chromosomal DNA of *N. attenuata* transformed with binary vectors containing antisense-cDNA for *N. attenuata* hydroperoxide lyase (*hpl*), allene oxide synthase (*aos*), lipoxygenase (*lox*) and digested with *Ssp*I. A) T-DNA of transformation vector pNATHPL: P_{CaMV}/T_{CaMV}, CaMV 35S promoter/terminator: P_{NOS}/T_{NOS} NOS promoter/terminator, as-*hpl*, antisense *hpl*; *sat*-1, nourseothricin resistance gene; LB/RB, left/right border of T-DNA; *SspI*, recognition site for restriction enzyme; s, amplicon ampNAT1 (conventional TaqMan® probe); n, amplicon ampNOT2 (Minor-Groove-Binder probe); Southern probe, 260 bp radioactive PCR-labeled probe for Southern blot; vectors pNATAOS, pNATLOX have the same organisation as pNATHPL except that that as-*hpl* is replaced by as-*aos* or as-*lox, respectively*. B) Southern blots of progeny of the hemizygous line A443-1 transformed with pNATHPL, 21 plants were examined (plants 1–24, except 10 and 17; *, DNA of plant Nr. 16 was blotted, but not included into the segregation ratio, because of low concentration); 5 plants without T-DNA, 16 plants with T-DNA: result corresponds to the sensitive/resistant ratio of 1:3 in segregration analysis of a hemizygous line; P (plasmid), 2 ng pNATHPL; WT, wild-type DNA. C) Southern blots for progeny of homozygous lines (2 from each line) transformed with pNATHPL, pNATAOS or pNATLOX; 1 as-*hpl* line and 2 as-*lox* lines show two bands on the blot, indicating two insertion sites for the T-DNA; P (plasmid), 2 ng pNATHPL; WT, wild-type DNA.

Table 2: Summary of Table 1: Unambiguous copy numbers, for a line all four copy numbers must be the same: real-time PCR copy numbers[a] for plant 1 and 2, Southern copy numbers for plant 1 and 2.

Calibrator A434-4-2	Calibrator A300-1-2
15% (2 o13 lines)	46% (6 of 13 lines)

[a] a calculated copy number of less than 0.5 is considered to represent no transgene

Table 3: Variation in triplicate measurements for each amplicon in two plants of as-*hpl* line A340-1: standard deviation of endogenous control adds to the standard deviation of the transgene, resulting in a ΔCt standard deviation as high as the difference (of one) to measure

Plant	Amplification of ampNAT1 (transgene)		Amplification of ampGSP1 (endogenous control)		ΔCt	
	Ct FAM	Average ± SD	Ct TET	Average ± SD	Ct FAM-Ct TET	Average ± SD
A340-1-1	21.17	21.72 ± 0.47	20.57	20.39 ± 0.19	0.60	1.32 ± 0.64
	21.99		20.42		1.57	
	21.99		20.19		1.80	
A340-1-2	21.13	21.26 ± 0.16	20.19	20.23 ± 0.12	0.94	1.02 ± 0.25
	21.20		20.37		0.83	
	21.44		20.14		1.30	

nontransgenic plants by real-time PCR (Table 4) are the same as designated by Southern analysis (Figure 2B).

Contamination with transgene results in ΔCt values between five and seven for untransformed plants with Ct values for the transgene between 24.9 and 26.8 (Additional File 2). This is high contamination. However with an *Agrobacterium*-mediated transformation method, contamination is not completely avoidable because *Agrobacteria* carrying the transgene could be growing on the plant's surfaces. This is especially true for outcrossed offspring of a transgenic line that grow together with transformed plants. For controls and wildtypes grown separately from the transgenic plants the Cts for ampNAT1 and the resulting ΔCts are much higher (Additional File 2, bottom lines Table 4). Despite this relatively high contamination, the contribution to the standard deviation is minimal. The minimum ΔCt difference between transgenic plants and nontransgenic plants in Table 4 is 2.93 and the maximum difference is 6.05. According to the $2^{-\Delta\Delta Ct}$ relationship, the nontransgenic plants contain 1/8 to 1/66 of the DNA of the transgenic plant and will contribute this amount to the Ct values of the transgenics. The contamination decreases the Ct value of the transgenic plants between 0.18 and 0.02 which corresponds to a standard deviation of approximately 0.08.

Minor groove binder amplicon

In order to test whether the standard deviation of Ct values can be reduced by using a different target sequence, amplicon ampNOT2 was designed to target the NOS-terminator. AmpNAT1 was targeted to the coding sequence of the resistance gene, because the Primer Express software version 1.0 could not find a TaqMan® amplicon in the AT rich sequence of the NOS-terminator. By using a Minor Groove Binder (MGB) probe and Primer Express 1.5 (supporting this new probe chemistry) ampNOT2 could be targeted to the NOS-terminator. MGB probes are double dye oligonucleotides featuring additional minor groove binding ligands on the 3' prime end which hyper-stabilize the duplex with the complementary DNA template [14]. This allows the probe to stay annealed to AT rich sequences at 60°C during the extension phase. Two further improvements for better signal generation is the use of VIC (a proprietary dye by ABI) as reporter dye yielding higher fluorescence than TET and the use of a dark quencher emitting transferred energy as heat (instead as fluorescence like TAMRA) and thereby reducing cross fluorescence with reporter dyes.

In addition to representing a technical improvement, ampNOT2 would have allowed direct comparison with Southern data, because the radioactive probe was also targeted to the NOS-terminator. However, although high quality DNA (originally extracted with CTAB for Southern

Table 4: ΔCt values for progeny of heterozygous T1 line A443-1 (21 plants) in ascending order

plant	**ΔCt** (Ct FAM-Ct TET) ± SD[a]
Containing the transgene according to Southern analysis (homo- and hemizygous)	
A443-1-20	1.12 ± 0.35
A443-1-12	1.25 ± 0.20
A443-1-6	1.36 ± 0.33
A443-1-11	1.50 ± 0.08
A443-1-2	1.54 ± 0.17
A443-1-23	1.55 ± 0.06
A443-1-3	1.56 ± 0.23
A443-1-13	1.60 ± 0.20
A443-1-5	1.73 ± 0.06
A443-1-24	1.82 ± 0.61
A443-1-1	1.96 ± 0.25
A443-1-21	2.03 ± 0.08
A443-1-18	2.04 ± 0.22
A443-1-4	2.06 ± 0.22
A443-1-19	2.13 ± 0.10
A443-1-7	2.15 ± 0.23
no transgene according to Southern analysis	
A443-1-9	5.08 ± 0.22
A443-1-8	5.25 ± 0.32
A443-1-14	5.98 ± 0.39
A443-1-15	6.36 ± 0.27
A443-1-22	7.25 ± 0.02
Controls	
A340-5-1[b]	8.18 ± 0.04
wildtype 12	11.18 ± 2.09

[a] average of three measurements [b] transformed line without T-DNA (T-DNA segregated out)

analysis) of as-*hpl* sample A443-1-12 was used, the Ct value standard deviation on 1:2 dilution series (Figure 1C) was not lower as compared with the conventional TaqMan® amplicon ampNAT1 (Figure 1B). The Ct values for ampNOT2 have been much higher than for ampNAT1 (Figure 1B and 1C). Because SD and Ct values did not improve, ampNOT2 is not a suitable replacement of ampNAT1. With a theoretically better probe chemistry and a purer template, SD and Ct values did not improve. In conclusion, even with the additional effort, we could not optimize real-time PCR to detect small differences.

Conclusions

Quantification by real-time PCR is an amplification-based procedure. Thus, small fluctuations in the starting conditions of a PCR assay will lead to large fluctuation of the product amount which is expressed in Ct values (or equivalents). Thus seemingly small standard deviations of Ct values (between 0.3 and 1) are amplified in the analysis because a Ct difference of one represents a two-fold difference in starting amount. In addition to the variability of the transgene measurements, the variability of the endogenous control measurement must also be considered.

We do not challenge the claim that real-time PCR is a useful quantification procedure, particularly when distinguishing plants with vastly different copy numbers. In our experiments, the plants with highest copy numbers by real-time PCR were also the plants that contained 2 copies of the transgene according to Southern analysis. Other researchers using real time PCR for copy number determination do not claim that exact determinations are possible and report considerable mismatches between determinations by real-time PCR and Southern analysis [3,6].

Our experiments demonstrate that the required two-fold difference is at the detection limit of real-time PCR due to random PCR variation. Although in our experiments contamination contributes to the observed standard deviation, its contribution is so low that copy number determination would have been possible if the random PCR effects were lower. Contamination is difficult to avoid in *Agrobacterium*-based transformation procedures and a high throughput copy number determination method must be sufficiently robust to deliver correct copy numbers with contaminated plants. In laboratories that use *Agrobacterium*-based procedures to produce many one and two-copy transformants at a relatively low output of primary transformants, Southern analysis is the preferred technique for copy number determinations.

Methods

Plant material

Nicotiana attenuata Torrey ex Watson plants were transformed with cassettes expressing the genes for hydroperoxide lyase (*hpl*), allene oxide synthase (*aos*) and lipoxygenase (*lox*) in antisense (as) orientation by an *Agrobacterium*-mediated method, and were tested for expression of the transgenes by segregation analysis of the selectable marker and oxylipin phenotypic screens [11]. All experiments were performed with T_2 plants that were progeny of homozygous T_1 lines (five as-*hpl*, four as-*aos*, four as-*lox*), plus progeny of one T_1 line (A443-1), which was hemizygous for as-*hpl*.

DNA extraction

Leaves from plants in the late rosette stage were extracted with the Ariel method for DNA extraction for quantitative PCR and cloning of chromosomal sequences. This method relies on lysis with the detergent Ariel (Procter and Gamble, Schwalbach, Germany) and subsequent phenol/chloroform extraction as described in [11]. The CTAB-method [15] was modified to remove RNA for DNA

extraction for Southern analysis. Two g of leaf material (flowering plants) were ground with mortar and pestle in liquid nitrogen and extracted in 4 ml hot (65°C) buffer (2% CTAB w/v, 100 mM Tris pH 8.0, 20 mM EDTA pH 8.0, 1.4 mM NaCl, 1% PVP M_r 40000, 1% β-mercaptoethanol) and 5 ml of chloroform/isoamyl alcohol 24:1. After being mixed and centrifuged, the supernatant was again phase-separated with 1 ml 5% CTAB (w/v in 0.35 M NaCl) solution and 5 ml chloroform/isoamyl alcohol 24:1. The nucleic acids in the resulting supernatant were precipitated with 2/3 volume ice cold isopropanol. After rehydrating the pellet in 500 μl high salt TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 1 M NaCl, 100 ng/μl RNase A; Roth, Karlsruhe, Germany), the solution was incubated at 37°C for 15 min. Following a phase separation with 500 μl chloroform/isoamyl alcohol 24:1, the supernatant was taken and the DNA was precipitated by adding 1 ml ice cold 100% ethanol. The pellet resulting from centrifugation was washed with 80% ethanol and rehydrated in 50 μl 10 mM Tris pH 8.0. OD_{260} measurements on a Biophotometer (Eppendorf, Hamburg, Germany) indicated concentrations between 0.5–2.0 μg/μl. For each homozygous line, DNA from 2 plants was extracted.

Cloning of chromosomal sequences of plastidic glutamine synthetase (endogenous control)

The cDNA sequence of *N. attenuata* plastidic glutamine synthetase (GSP), which is encoded in the nucleus, was cloned previously in our lab (accession number AY426758). Because GSP was to be used as an endogenous control, we determined its chromosomal sequence to confirm that the target sequence was not interrupted by an intron.

PCR on chromosomal DNA

PCR was performed with a Master Cycler Gradient (Eppendorf, Hamburg, Germany) in 10 μl PCR Mix containing 1 × buffer + 1 U *Taq* polymerase (Eppendorf, Germany, Hamburg), 400 μM dNTPs, 1 μM for each primer and wildtype DNA (inbred line Utah DI92) as template according to the PCR program: 2 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 64°C, 90 s at 72°C. Amplification with the primer pairs GSP1-19 (5'-GGCCGAGATATATCAGATG-3'), GSP2-18 (5'-GCGAAGGGAGAGATTCAG-3') and GSP9-19 (5'-GAGTACACCTTACTCCAAC-3'), GSP10-18 (5'-CTCAAGGATGTATCTAGC-3') yielded the fragments GS58-0 (≈1300 bp) and GS58-4s (≈550 bp), respectively. The sequences of the corresponding fragments on the cDNA overlapped.

Cloning

Fragment GS58-0 was cloned into the vector pCR2.1 using TOPO TA Cloning® kit (Invitrogen, Groningen, Netherlands) and electro-transformed into *E. coli* TOP10F' according to the manufacturer's instructions. Fragment GS-58-4s was treated with T4-DNA-Polymerase to produce blunt ends, ligated to a *Sma*I digest of pUC19 and electro-transformed into *E. coli* TOP10F'.

Sequencing

For GS58-0, two clones were sequenced on a Genetic Analyzer 3100 (Applied Biosystems, Darmstadt, Germany), each with the primers UNIFOR (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and UNIREV (5'-AGCGGATAACAATTTCACACAGGA-3'). Because the sequences of the forward and reverse sequencing reactions did not overlap, a third reaction with primer GSP19-20 (5'-GGTGACTGGAATGGTGCCGG-3') was necessary. With this information plus the sequences of forward and reverse sequencing reactions of one clone of GS58-4s, a 1700 bp sequence of chromosomal DNA coding for plastidic glutamine synthetase (accession number AY183657, bp 717-2416) was assembled.

Southern analysis

Approximately 10 μg of DNA were digested with 62.5 U *Ssp*I (NEB, Frankfurt/Main, Germany) at 37°C for 4 or 16 hrs respectively. After separation on a 0.8% agarose gel, the DNA was blotted on Gene Screen Plus® Nylon membrane (NEN, Boston, MA) by capillary transfer according to the manufacturer's instructions. A radiolabeled probe of 260 bp corresponding to the NOS-terminator was prepared by PCR using [α-^{32}P dCTP] (Perkin Elmers, Mechelen, Netherlands) the primers NOT1-29 (5'-CCCGATCTAGTAACATAGATGACACCGCG-3'), NOT2-23 (5'-CCCCGATCGTTCAAACATTTGGC-3') and the plasmid pNATLOX (Fig 1A) as template. After probe hybridization overnight at 65°C, the membranes were washed twice at 65°C in washing buffer (2 × SSC, 0.1% SDS) and analyzed on a Phosphoimager FLA 3000 (Fujifilm Europe, Düsseldorf, Germany).

TaqMan® PCR

The principles of TaqMan® chemistry for real time detection of PCR products are explained in [16] and in the handbooks for real-time PCR machines that can use the TaqMan® chemistry.

Probes and primers

Probes and primers were designed with the program Primer Express™ versions 1.0 and 1.5 (ABI, Weiterstadt, Germany) and synthesized by Eurogentec (Seraing, Belgium; TaqMan® probes), ABI (Weiterstadt, Germany; MGB probe) and Sigma-ARK (Darmstadt, Germany; primers). The sequences and the location of the fluorescent dyes on the probe are given in Table 5. Because the amplicons for the T-DNA were targeted to sequences that are common for all three transformation plasmids (Fig 1A), one PCR setup could be used to examine the three constructs as-*hpl*, as-*aos*, as-*lox*.

Table 5: Primers and probes used for the real-time PCR

Target	Amplicon: length, T_m	Oligonucleotide: Length, T_m	Sequence with dyes (for probes)
Endogenous control: Plastidic glutamine-synthetase	ampGSP1 86 bp, 79°C	Forward primer GSP20-24 24 bp, 54°C	5'TGGAAACTTTAGGGTCCTTACTAC3'
		Reverse primer GSP21-22 22 bp, 56°C	5'CAAGCCTTGTAGTGAGCATCTG3'
		Probe (binds to same strand as GSP21-22) 24 bp, 64°C	TET-5'ATGACTTATCCGCTCCAGCACCAC3'-TAMRA
Transgene: Streptothricin-acetyl transferase	ampNAT1 86 bp, 81°C	Forward primer NAT8-18 18 bp, 57°C	5'TTGTTGTGTCGCACACGC3'
		Reverse primer NAT9-20 20 bp, 56°C	5'GAGCTGTCTGCTTAGTGCCC3'
		Probe (binds to same strand as NAT8-18) 23 bp, 64°C	FAM-5'AAGGAGTCGCGCACAGTCTCATC3'-TAMRA
Transgene: NOS-terminator	AmpNOT2 76 bp, 74°C	Forward primer NOT5-24 24 bp, 59°C	5'-TGGGTTTTTATGAT TAGAGTCCCG-3'
		Reverse primer NOT6-23 23 bp, 59°C	5'-CCTAGTTTGCG CGCTATATTTTG-3'
		Probe (binds to same strand as NOT5-24) 20 bp, 69°C	FAM-5'-CATTTAATACGC GATAGAAA-3'-Q-MGB Q = dark quencher

Real-time PCR machine and chemicals

Real-time PCR was performed using the qPCR Mastermix (containing HotGoldStar *Taq* polymerase, dNTPs with dUTP replacing 1/3 of dTTP, uracil-N-glycosylase, reference dye ROX) and 96-well plates with optical caps from Eurogentec (Cologne, Germany) on a SDS 7700 Sequence detector from ABI (Weiterstadt, Germany). Some individual components of the qPCR core kit from Eurogentec were used for testing.

Reaction conditions

Concentrations and PCR program for 25 µl assays were as follows: Mastermix 1×, primers 500 nM, probe 100 nM, $MgCl_2$ 5 mM (supplied in the Mastermix), 2 min at 50°C (uracil-N-glycosylase digest), 10 min at 95°C (polymerase activation) and 45 cycles with 15 s at 95°C and 1 min at 62°C. The amount of DNA varied between 10 and 100 ng for each sample. Amplicons ampNAT1 and ampGSP1 were amplified in the same well (multiplex assay), ampNOT2 as singleplex assay. Each sample was assayed in triplicate, and analyzed with the SDS software 1.6.3 (ABI, Weiterstadt, Germany) and Microsoft Excel.

Quantification by the $2^{-\Delta\Delta Ct}$ method

Basics

The derivation of quantification by real-time PCR and the $2^{-\Delta\Delta Ct}$ method have been described elsewhere [16,17] and only the results are shown here. Real-time PCR quantification is based on the description of exponential growth:

$$X_n = X_0 \times (1+E)^n \quad (1).$$

where X_n is the number of target molecules after n cycles, X_0 the initial number of target molecules, n the number of cycles and E the efficiency of the reaction. An E of one means that the number of molecules doubles during each cycle.

Ct value

Although X_n is physically measured as fluorescence, a real-time PCR machine provides the Ct value, the cycle number C at which the PCR product triggers a certain amount of fluorescence (threshold t).

Initial target amounts and Ct value

Low Ct values indicate a high initial target amount, high Ct values the opposite. For reactions with E = 1, the amount of product doubles after each cycle and following relationship applies:

$$2 \times X_0 \Rightarrow Ct\text{-}1 \quad (2).$$

Consequently, if the initial number of target molecules X_0 doubles, the fluorescence will cross the threshold one PCR cycle earlier.

Endogenous reference

To normalize the input amount of chromosomal DNA, both the transgene sequence (ampNAT1) and a plant gene, in this case plastidic glutamine synthetase (ampGSP1), are amplified (Table 5). This plant gene is located on the chromosomal DNA and serves as an endogenous control against which the signal for the trans-

gene can be normalized. If both amplicons propagate with the same efficiency, the difference between the Ct for the transgene (Ct_t) and the Ct for the endogenous control (Ct_e) will be constant, independent of the amount of chromosomal input DNA:

$$\Delta Ct = Ct_t - Ct_e \quad (3).$$

Calibrator

The ΔCt value of a sample with known zygosity but unknown copy number can be related to the ΔCt value of a calibrator for which copy number and zygosity are known. Thus, if a one-copy homozygous calibrator and a homozygous sample have the same ΔCt, the sample contains one copy of the transgene. More generally, the ratio of the initial number of transgenes in the sample (X_s) against the initial number of transgenes in the calibrator (X_{cal}) can be calculated as follows:

$$X_s/X_{cal} = (1+E)^{-\Delta\Delta Ct} \quad (4)$$

where $\Delta\Delta Ct = \Delta Ct_s - \Delta Ct_{cal}$.

Note that E of the PCR is a simplified term and that this equation can only be used when the efficiencies of all PCRs are the same. However this equation is also valid for efficiencies lower than 1, so that the $2^{-\Delta\Delta Ct}$ method, as coined by Livak et al. [17], is actually a $(1+E)^{-\Delta\Delta Ct}$ method.

Efficiency

Ct values of dilution series are plotted against the common logarithm of the dilution. If the amount of PCR product doubles every cycle, the slope for this graph will be -3.32. With the slope and after converting equation (1), the efficiency of the reaction can be calculated as:

$$E = 10^{-(1/slope)}-1 \quad (5).$$

Another test for the validity of $2^{-\Delta\Delta Ct}$ method plots ΔCt against log dilution. Here the slope should be close to zero, because if both reactions have the same efficiency ΔCt does not depend on the dilution series.

Authors' contributions

BB carried out the Southern blotting, real-time PCR analysis and cloning. KG devised cloning procedure for getting chromosomal sequences. ITB was responsible for coordination of the study. All authors participated in the design of the experiments, and writing of the manuscript.

Additional material

Additional File 1

Original Ct values and SD of triplicate measurements. The average ΔCt values and their SD in the last two columns (both bold) appear in Table 1 and are used for copy number calculation.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1472-6750-4-14-S1.xls]

Additional File 3

Screenshot of sample designation on the SDS7700 for the copy number calculation experiment (Table 1).
Click here for file
[http://www.biomedcentral.com/content/supplementary/1472-6750-4-14-S3.ppt]

Additional File 2

Original Ct values and SD of triplicate measurements. The average ΔCt values and their SD in the last two columns (both bold) appear in Table 4 and are used for zygosity calculation.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1472-6750-4-14-S2.xls]

Additional File 4

Screenshot of sample designation on the SDS7700 for the zygosity calculation experiment (Table 4).
Click here for file
[http://www.biomedcentral.com/content/supplementary/1472-6750-4-14-S4.ppt]

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