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Research article

REAL-TIME PCR FOR THE DETECTION OF PRECISE TRANSGENE COPY NUMBER IN DURUM WHEAT

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Abstract: Recent results obtained in various crops indicate that real-time PCR could be a powerful tool for the detection and characterization of transgene locus structures. The determination of transgenic locus number through real-time PCR overcomes the problems linked to phenotypic segregation analysis (i.e. lack of detectable expression even when the transgenes are present) and can analyse hundreds of samples in a day, making it an efficient method for estimating gene copy number. Despite these advantages, many authors speak of "estimating" copy number by real-time PCR, and this is because the detection of a precise number of transgene depends on how well real-time PCR performs.
This study was conducted to determine transgene copy number in transgenic wheat lines and to investigate potential variability in sensitivity and resolution of real-time chemistry by TaqMan probes. We have applied real-time PCR to a set of four transgenic durum wheat lines previously obtained. A total of 24 experiments (three experiments for two genes in each transgenic line) were conducted and standard curves were obtained from serial dilutions of the plasmids containing the genes of interest. The correlation coefficients ranged from 0.95 to 0.97. By using TaqMan quantitative real-time PCR we were able to detect 1 to 41 copies of transgenes per haploid genome in the DNA of homozygous T_4 transformants. Although a slight variability was observed among PCR experiments, in our study we found real-time PCR to be a fast, sensitive and reliable method for the

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Abbreviations used: FISH – fluorescence *in situ* hybridization; GM – genetically modified; HMW-GS – high molecular weight glutenin subunits; MGB – minor groove binder; PCR – polymerase chain reaction; SDS – Sequence Detection System

detection of transgene copy number in durum wheat, and a useful adjunct to Southern blot and FISH analyses to detect the presence of transgenic DNA in plant material.

Key words: Durum wheat, Transgenic plants, Biolistic method, Glutenin subunits, Real-time PCR, Southern analysis, Transgene copy number, Quantitative PCR assay, Transgene stable expression, HMW-GS genes

INTRODUCTION

The estimation of gene copy number has become an important area of genetically modified (GM) crop research. This is principally for two reasons: first because transgene copy number may influence the expression level and the genetic stability of both exogenous and endogenous genes, and secondly because the sensitivity of the detection methods is important for GM crop traceability. In research labs, the method traditionally employed to determine transgene copy number was Southern blotting. While reliable, this classic molecular biology technique is laborious, time consuming, requires large amounts of starting plant material and may involve the use of hazardous radioisotopes [1]. Moreover, Southern analysis may fail to detect the exact number of transgene copies when these have been rearranged during integration into the host genome with resultant changes and/or losses of relevant restriction sites [2].

A new detection method has recently been developed to estimate transgene copy number in transformed plants based on quantitative real-time PCR [3]. Up to now this new approach has successfully been applied to several species such as bread wheat [4], maize [5], rice [6], soybean and peanut [7], *Nicotiana attenuata* [8] and tomato [2].

Real-time PCR can be employed for quantifying both DNA and RNA abundance. It is used in a huge number of research applications such as monitoring of viral load in infected plants [9, 10] or detection of genes involved in toxinogenesis [11, 12]. Today, in response to compulsory requirements for food quality and safety, real-time PCR is also being employed in testing food adulteration or contamination with transgenic DNA [13]. Interestingly, this quantitative PCR assay is recently becoming a choice for a growing number of molecular analyses including gene expression quantification, expression profiling, SNP analysis, validation of microarray data and detection of transgene copy number.

Real-time PCR allows two methods for the quantification of a target nucleic acid sequence: absolute and relative quantification. In absolute quantification (AQ), the exact level of a sequence in a sample is directly inferred from a standard curve prepared from a dilution series of control template of known concentrations. On the other hand, relative quantification is mainly used in gene expression studies to determine the up- or down-regulation of a gene of interest

relative to a calibrator. Ideally, for actual experiments or GM crop commercial release, copy number should be established without any error [8].

Because of their major role in determining the viscoelastic properties that underlie bread dough formation [14], transformation experiments with high molecular weight glutenin subunits (HMW-GS) genes in wheat have been published by several research groups in the last decade [15-17]. In order to study their effects on dough properties, HMW-GS genes have also been used to transform *Tritordeum* [18], maize [19], and rye [20]. The HMW-GS genes used were *1Dx5*, *1Ax1* or a hybrid between the *1Dy10* and *1Dx5* genes [21]. Stable integration expression of the *1Dy10* gene has been reported by Altpeter *et al.* [20] in rye and by Blechl *et al*. [16] and León *et al*. [22] in bread wheat. In one previous study, transformation of four commercial durum wheat cultivars with HMW-GS genes *1Dx5* and *1Dy10* was carried out by Gadaleta *et al*. [23] in order to study the expression of the two genes in different genotypes. Four transgenic lines were obtained: two transgenic events expressed the 1Dx5 and 1Dy10 subunits; one event expressed the 1Dx5 subunit, and one event showed co-suppression of both transgenes *1Dx5* and *1Dy10* and the native genes encoding the endogenous HMW glutenin subunits.

Reliable and stable expression of transgenes as well as the characterization and field adaptation of transgenic lines are prerequisites for the successful application of gene technology. Many papers have reported significant variability in the behaviour of the same transgene in different lines [24]. Loci that appear to be stably expressed initially can become progressively silenced over several generations [25]. The stability and the behaviour of transgenes are influenced by several factors such as chromosomal location, transgene copy number and arrangement, and interaction with the host genotype. Thus, the establishment of an efficient and fast protocol for the detection of the inserted transgene copy number would be useful. In the present work, the four durum wheat transgenic lines obtained by Gadaleta *et al*. [23] were characterized for the integrated copy number of the transgenes *Glu-D1-1b* and *Glu-D1-2b* by TaqMan real-time PCR.

MATERIALS AND METHODS

Transgenic plants and DNA preparation

Four independent transgenic lines of durum wheat (*Triticum turgidum L.* var. *durum*), previously obtained in direct transformation experiments by the biolistic method [23], were characterized by absolute real-time PCR. Lines DV1-4c, DC2-10-4, DC2-65s and DS2-127 were derived from cultivars Varano (DV1-4c), Creso (DC2-10-4, DC2-65s) and Svevo (DS2-127) co-transformed with the two bread wheat (*Triticum aestivum L.*) genes *Glu-D1-1d* and *Glu-D1-2b* encoding the HMW glutenin subunits 1Dx5 and 1Dy10, respectively [26]. As negative controls for real-time experiments, non-transformed lines of the same cultivars were used. Genomic DNA from control and transformed wheat lines was

extracted starting from 5 grams of frozen leaves using the phenol-chloroform protocol [27]. DNA concentration was determined using a UV-visible spectrophotometer detecting absorbance at a wavelength of 260 nm. Purity of extracted DNA was assessed reading the 260 nm/280 nm ratio, with a value of approximately 1.8 indicating a good quality. DNA was also checked on 0.8% agarose gel stained with Gel-Red® (Biotium, Inc., Hayward, CA) dying solution.

In order to have standard templates to build the calibration curves for the real-time experiments, DNA from plasmids pKS+Dx5 and pKS-Dy10 containing the target transgenes *1Dx5* and *1Dy10* [26] were purified from *E. coli* by using the *QIAGEN Plasmid MAXI Kit* following the manufacturer's instructions. The size and integrity of the extracted DNAs were checked by PCR amplification with primers specific for the inserted genes, and by endonuclease digestion followed by electrophoretic separation on 1.8% agarose gels.

In this study we determined the transgene copy number relative to a haploid wheat genome. With this aim we first calculated the exact DNA concentration of both transgenic and non-transformed (control) lines using a fluorimetric measurement, then we converted this value into the number of copies of haploid genome/μl as follows:

$$\text{No. copies genome}/\mu\text{l} = \frac{\text{DNA concentration[g/l]}}{\text{Molecular weight[g/mol]}} \text{ x Avogadro n. x } 10^{-6}$$

The same quantification was carried on plasmids pKs+Dx5 and pKs-Dy10 in order to prepare the dilution series of known concentrations and to obtain the standard curves for the absolute quantification.

Design and optimisation of the real-time PCR method

As *1Dx5* and *1Dy10* bread wheat genes show sequence similarity to other endogenous glutenin subunits, primers for the detection of each transgene were designed as one complementary to the vector sequence and the other one annealing to the gene of interest. The use of primer pairs encompassing the vector sequence ensured the amplification only of the transgenic insert and not of the endogenous genes. It also has to be considered that transgenesis in this case is based on the random (non-homologous) integration of the plasmid into the genome. This could lead to a DNA break between the transgene and the primer annealing site on the vector sequence. If this rare event occurs, the applied real-time system is not able to detect the integrated transgene. Such rare events could produce false negative values, so to avoid this problem we decided to design two PCR systems for each gene, one for each side of the transgenic construct. As shorter amplicons work more efficiently, primers were designed that amplified short DNA segments in a 50 to 150 bp range. As a starting point, a standard PCR reaction and a PCR with a gradient of different annealing temperatures (ranging between 56°C and 64°C) were performed on genomic DNA extracted from different transgenic lines, on plasmids as positive control and on non-transformed lines as a negative control for each designed primer

pair, in order to check for their specificity and determine the best temperature for their annealing. Different primers pairs have been tested for each gene and those giving a specific amplification product were chosen to perform real time assays. Equal volumes of amplification products were checked for the expected molecular size on 1.5% agarose gel stained with Gel-Red® dying solution (Fig. 1). Gradient PCR was carried out in the Mastercycler Gradient cycler (Eppendorf, Germany). The primers used for the real-time PCR had an optimal annealing temperature of 60°C.

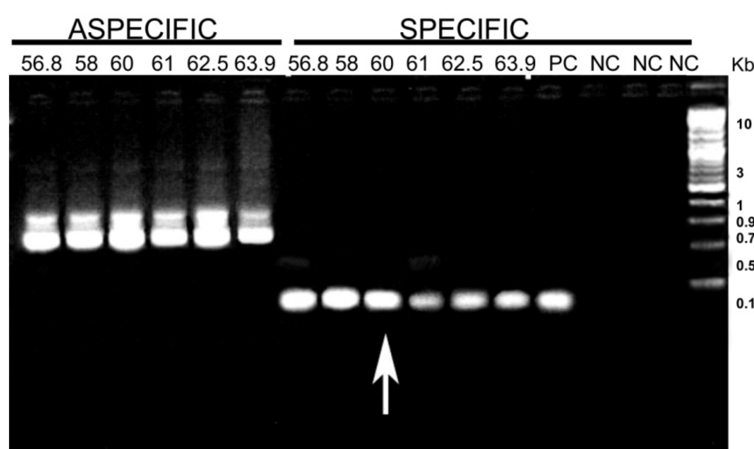

Fig. 1. Electrophoretic pattern deriving from PCR amplification of *1Dy10* gene with a gradient of different annealing temperatures (ranging between 56°C and 64°C). On the left are shown the fragments derived from the amplification with a non-specific primer pair (for details see the text). On the right is reported the amplification of the same gene with specific primers giving the expected product of 65 bp. The white arrow indicates the best annealing temperature of 60°C chosen for the real-time PCR reactions. PC= Positive control, represented by the plasmid and NC = negative control, represented by non-transformed lines.

TaqMan assays were performed using specific primer pairs and specific probes annealing to the target gene sequence slightly downstream of one of the primers. Each primer pair was used to amplify both plasmid standards and the transgenic plant sample. Primers and probes were designed by using Primer Express software (Applied Biosystems Inc., Foster City, CA, USA) and those eventually chosen are listed in Tab. 1 along with their expected amplicon sizes. For both *1Dx5* and *1Dy10*, the forward primer and probe were complementary to the gene sequence while the reverse primer was complementary to the vector sequence. For TaqMan chemistry, Minor Groove Binder (MGB) DNA probes were used (Applied Biosystems). An MGB probe consists of a 14 or 15 bp long oligonucleotide carrying FAM fluorescent dye (6-carboxyfluorescein, excitation wavelength = 494 nm, emission wavelength = 521 nm) at its 5' end, and the TAMRA quencher and MGB at its 3' end. The MGB part of the probe binds to the minor

groove of the double stranded DNA helix (consisting of the oligo part of the MGB probe and the complementary target sequence to which it is hybridized) irrespective of the nucleotide sequence, thus enhancing the stability of the probe amplicon binding. Traditionally, FAM-TAMRA is one of the most frequently used pairs for TaqMan probes, where FAM acts as the fluorophore and TAMRA as the quencher. Other quenchers can also be used, especially dark quenchers or black hole quenchers, which capture energy from an excited reporter molecule without subsequent emission of light, i.e. they do not fluoresce. TAMRA used to be the most commonly used quencher at the 3' end of a TaqMan® probe before the advent of dark quenchers. The latter are nowadays preferred because of the reduced background noise.

Tab. 1. Primers and probes employed for PCR amplifications in TaqMan assays. *F*: Forward primer; *R*: Reverse primer.

Target gene	Primer sequence (5' to 3')	MGB probe sequence (5' to 3')	Amplicon size (bp)
1Dy10	*F*: TCATTTGGAGTTGTGCAGAATAGC *R*: GCTGCAGGAATTCTGGACTGA	ATCTCTGTTGTAGCCTTT	65
1Dx5	*F*: AAATTATGATCGCACATTTGTAACG *R*: CCCGGGCTGCAGGAAT	CGCTGATTTGGTACTTG	60

TaqMan reactions were carried out in the 7300 Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA) using 96-well reaction plates. Reaction mixtures consisted of 1X Platinum Quantitative PCR SuperMix UDG (Life Technologies-Invitrogen, containing AmpliTaq Gold DNA Polymerase, dNTPs, dUTP and the enzyme uracil-N-glycosylase [UNG]), 500 nM of ROX passive reference, 250 nM of specific probe, specific primers at various concentrations, 5 ng of sample DNA and water to a 15 µl final volume. The cycling parameters used were the following: one cycle at 50°C for 2 min for activation of UNG, one cycle at 95°C for 10 min for DNA polymerase activation, followed by 40 cycles of 95°C for 15 sec (denaturation) and 60°C for 1 min (combined annealing and extension). The use of 60°C (7-10°C below the probe Tm) prevents the displacement of the probe from the template, which can occur if the temperature in the reaction is too high during the Taq polymerase extension (such as at the standard extension temperature of 72°C). Primer and probe quantities were optimised in preliminary experiments by testing nine different combinations of primer concentration (50, 300 and 900 nM for each primer). Concentrations of 300 nM for both forward and reverse primers were found to be optimal in giving a high endpoint fluorescence and a low Ct value. For the MGB probe, an optimal concentration of 250 nM was determined.

Calibration curves

As real-time absolute quantification of DNA is based on the linear correlation between starting amount of the template and fluorescence intensity at the Ct, preliminary SYBR Green Real-Time experiments were carried out to test the

linearity between Ct and log $_{\text{copy number}}$ in *1Dx5* and *1Dy10 left* and *right* systems. This allowed us to evaluate whether the amount of the template and its Ct varied linearly during real-time PCR, and build calibration curves to use for transgene quantification. Starting with known concentrations of the standard templates (plasmid pKs+Dx5 for *1Dx5* and pKs-Dy10 for *1Dy10*), accurate serial dilutions were prepared and run in triplicate. In order to accurately quantify all the samples, the least and most diluted points of the curves were appropriately adjusted so that samples' Ct values fell within the linear section of the curve. For each gene the standard curve was run on the same plate as the samples in order to ensure good reproducibility of Ct values, as these may vary if run at different times and plates.

The calibration curves were obtained for each primer-probe system by plotting the log of the starting standard DNA amounts versus the corresponding Ct. Calibration curves were optimized by adjusting the threshold and the baseline, in order to obtain an R square value (R^2 or Pearson correlation coefficient) very close to 1 and a good slope (around -3.3). The threshold is the fluorescence value slightly above the background fluorescence measured before exponential growth starts. Thresholds were either calculated according to an algorithm of the real-time PCR software or set manually.

We used a six-point calibration curve with five-fold dilution starting with 72 000 plasmid copies/ul (calculated by the formula previously reported) for the two systems of *1Dx5*. Similarly for *1Dy10* six-point standard curves we used a three-fold serial dilution starting with 56 300 plasmid copies/ul.

Real-time PCR AQ determination of transgene copy number

A total of 24 independent experiments were conducted, three experiments for each gene in each transgenic line using Absolute Quantification (AQ). For each transgenic line, after a first set of tests on different DNA extract from the same line, we chose to carry out all the subsequent experiments using the same DNA extraction for each transgenic line in order to ensure good reproducibility and to allow correct validation of the real-time PCR method. In addition to the transgenic lines, both positive and negative controls were amplified in each experiment. The positive controls were the same transgenic plasmids used to obtain the standard curves. As the negative controls, we used DNA from non-transformed cultivars Svevo, Varano and Creso and the "No-Template Control", a reaction mix including all the reaction components except for the DNA.

The exact amount of each target gene was calculated by comparison of Ct values to those of a calibration curve obtained for each experiment from a dilution series of a standard template represented by the plasmid used for the transformation experiments. Vector pKS+Dx5 was used for the quantification of *1Dx5*, while pKS-Dy10 was employed for the detection of *1Dy10* (Figs. 2, 3). The results of AQ experiments are reported in the same units as the standard curve.

The software used in real-time experiments was the *Applied Biosystems 7300/7500 Sequence Detection System* (*SDS Software*, Applied Biosystems Inc.,

Foster City, CA, USA), which determines the internal fluorescence emission of the passive reference (ROX) and that of the report dye attached to the 5' end of the TaqMan probe in order to calculate the normalized reporter (R_n) as follows:

$$R_n = \text{Report dye fluorescence emission} / \text{ROX fluorescence emission}$$

The magnitude of the signal generated by the specified set of PCR conditions is called $\Delta R_n = R_n$ - baseline (this is the line that fit the fluorescence emission during the initial cycle, usually 3-15, in which there is little change in fluorescence signal). For each sample the amplification results are reported in a ΔR_n versus Cycle view, that displays the log of dye fluorescence (ΔR_n) as a function of cycle number.
The precise number of integrated transgenic sequences was finally obtained from the ratio between the absolute quantity of each gene and the genome copy number of each wheat line in each experiment.

RESULTS

The aim of the present work was to test whether it was possible to assess the precise number of transgenes by real-time PCR in a species such as durum wheat, a tetraploid crop plant containing a complex and large genome. After preliminary experiments to determine optimum primer and probe concentrations and annealing temperatures, a total of 24 experiments (three experiments for each HMW glutenin transgene type in each transgenic line) were conducted. The absolute quantity of *1Dx5* and *1Dy10* transgenes in each wheat line fell within the range covered by the calibration curves. Correlation coefficients (R^2) varied between 0.95 and 0.97, indicating a good linearity between fluorescence output and DNA concentration over a broad range of values (Fig. 2, 3). The precise number of transgenic sequences integrated in each wheat line was obtained from the ratio between the absolute quantity of each gene and the number of haploid genome copies in each reaction. For each transgene, the averaged results of the six experiments are shown in Tab. 2 together with their standard deviation.
As the obtained data fell within the linear correlation between starting DNA amount and Ct reported in the calibration curves, they can be considered acceptable. In order to check the specificity of PCR amplifications, DNA from the non-transformed cultivars (Svevo, Varano, Creso) and the "No-Template Control" were used as negative controls, and no transgenes were detected in these samples. The lack of amplification of negative controls was one of the most important features of the quantitative PCR assay as it allows checking for external contamination or other factors that could result in non-specific increases in the fluorescence signal.
The estimated number of transgenes detected in our experiments ranged from 1 of *1Dy10* in line DV1-4c to 41 of *1Dx5* in line DC2-65s. The latter showed the highest number of inserted sequences for both the *1Dy10* and *1Dx5* genes, while the line harbouring the lowest overall transgene copy numbers was DV1-4c.

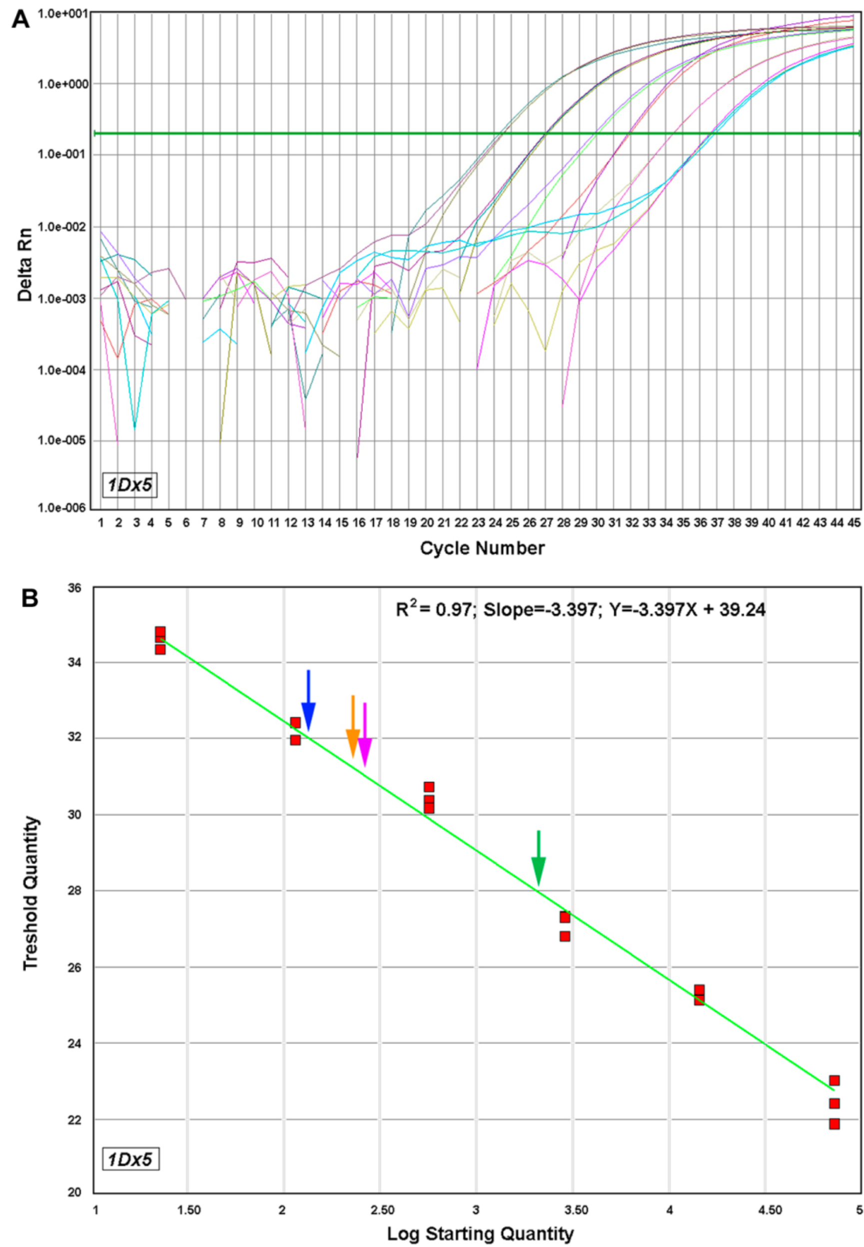

Fig. 2. Real-time PCR amplification and standard curve of *Glu-D1-1b* (*1Dx5*) transgene. A – Logarithmic plot resulting from the amplification of six five-fold dilutions of standard DNA (plasmid pKS+Dx5). Each sample was run in triplicate. Green line represents the threshold level. B – Standard curve reporting the calculated Ct values plotted against the log of each starting quantity. Correlation coefficient and slope values are indicated. Coloured arrows indicate the Ct value calculated for each of the four transgenic lines (DS-127 orange; DC2-65s green; DV1-4c blue; DC2-10-4 violet).

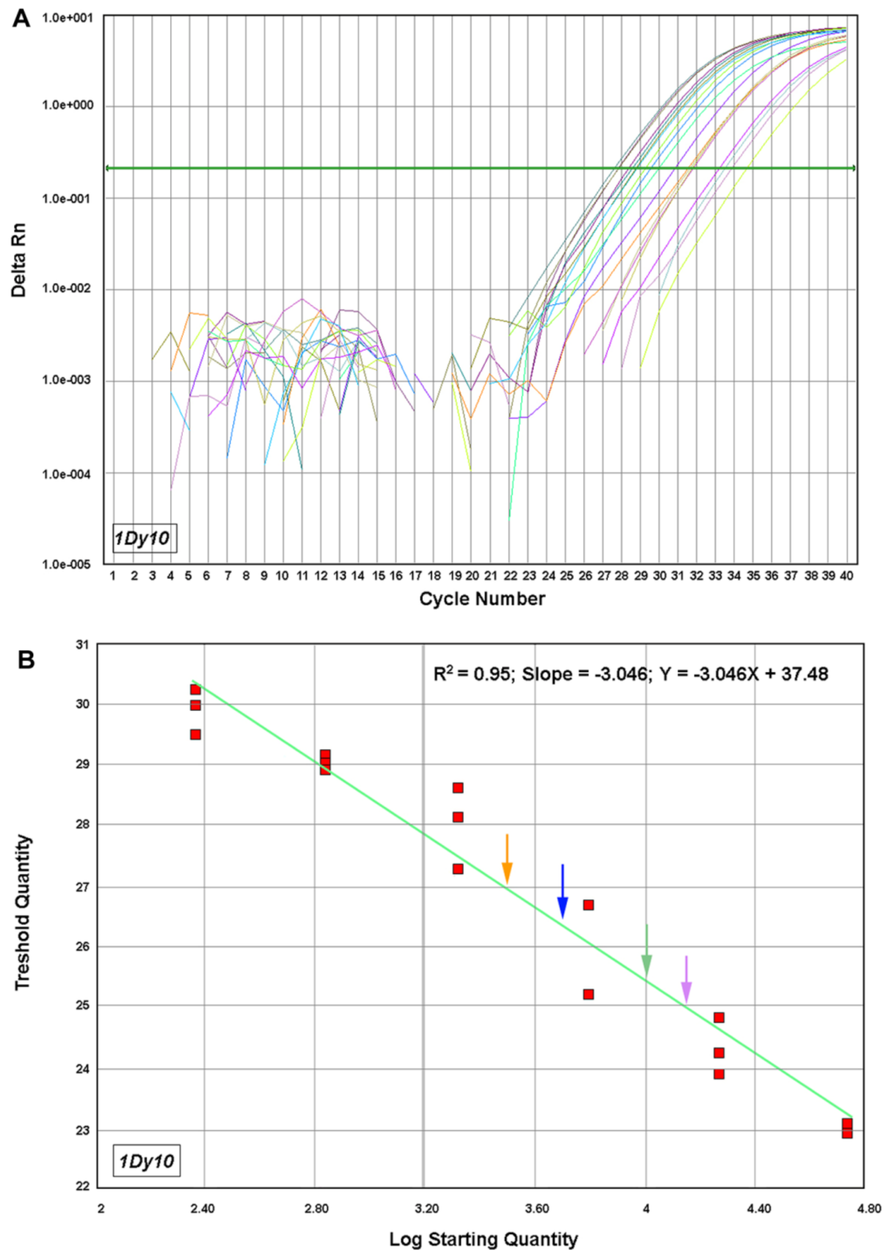

Fig. 3. Real-time PCR amplification and standard curve of *Glu-D1-2b* (*1Dy10*) transgene. A – Logarithmic plot resulting from the amplification of six three-fold dilutions of standard DNA (plasmid pKS-Dy10). Each sample was run in triplicate. B – Standard curve reporting the calculated Ct values plotted against the log of each starting quantity. Correlation coefficient and slope values are indicated. Coloured arrows indicate the Ct value calculated for each of the four transgenic lines (DS-127 orange; DC2-65s green; DV1-4c blue; DC2-10-4 violet).

Tab. 2. Copy number of *Glu-D1-1b* (*1Dx5*) and *Glu-D1-2b* (*1Dy10*) genes in wheat transgenic lines estimated from real-time TaqMan assays. Starting quantities, as calculated by the software, are expressed in arbitrary units (a.u.). For each gene the calculated number was obtained from the ratio between gene absolute quantity and genome copy number.

Line	Transgene	Initial number of genomes/ul	Starting absolute quantity (a.u.)	Standard deviation	Estimated copy number per number of genomes	Estimated copy number per haploid genome
DV1-4c	*1Dy10*	2730	3791	372	1.4	1
DC2-10-4	*1Dy10*	329	8422	375	26	13
DC2-65s	*1Dy10*	101	7206	843	71	35
DS-127	*1Dy10*	62	2470	212	40	20
DV1-4c	*1Dx5*	56	613	55	11	6
DC2-10-4	*1Dx5*	49	1359	85	28	14
DC2-65s	*1Dx5*	102	8525	723	83	41
DS-127	*1Dx5*	62	1073	144	17	8

In order to evaluate the accuracy and reproducibility of our data we calculated the efficiency of the reactions as: E = 10(-1/slope) –1). It ranged between 97% (corresponding to a slope of -3.397) and 112% (corresponding to a slope of -3.046). This value should range between 90% and 110%. A number of variables can affect the efficiency of the PCR such as length of the amplicon, presence of inhibitors, secondary structure and primer design. Although for *Dy10* we obtained an efficiency value higher than 110%, we repeated each experiment three times and we used in each experiment 6 replicates for each line with 6-point standard curves. Thus we are quite confident about the reproducibility of our data.

DISCUSSION

To test the ability of real-time PCR to detect transgene copy number in a species with a large and complex genome, we applied quantitative real-time PCR in replicate experiments on durum wheat (*Triticum turgidum* L. var. *durum*) lines transformed with *Glu-D1-1d* (*1Dx5*) and *Glu-D1-2b* (*1Dy10*) [23] genes of bread wheat encoding the HMW glutenin subunits 1Dx5 and 1Dy10. Using an optimized assay, we were able to detect even a single copy of the *1Dy10* gene. Among our four lines the highest number of transgenes detected was 41, but the high correlation coefficients for the standard curves predict that it will be possible to use this assay to reliably measure even higher copy numbers. These four transgenic lines were characterized in our previous work [23] for HMW-glutenin protein expression and by FISH for the detection of the chromosomal locations of integrated plasmids pKS+Dx5 and pKs-Dy10.

DS2-127 derived from cv. Svevo exhibited expression of both HMW-1Dx5 and HMW-1Dy10 glutenin subunits in endosperm extracts and had two transgenic

loci detected by FISH on different chromosomes. The first locus carried a co-insertion of the two plasmids, while the second locus contained only the plasmid encoding the 1Dy10 subunit. In this line, the total number of transgenes detected by real-time PCR was 8 for *1Dx5* and 20 for *1Dy10*. By comparing the results from FISH and real-time PCR, we hypothesize that the 8 copies of *1Dx*5 gene were inserted at a single locus, while the 20 copies of *1Dy10* integrated in two loci with a mean value of 10 transgenes for each locus.

Line DC2-10-4 derived from cv. Creso also accumulates both 1Dx5 and 1Dy10 in its endosperm and FISH analyses show that it carries the plasmids pKS+Dx5 and pKS-Dy10 inserted at a single locus, which as we show here by real-time PCR contains 14 copies of the *1Dx5* gene and 13 of *1Dy10*. Line DV1-4c of cv. Varano showed expression of the *1Dx5* transgene, but no detectable levels of the HMW-GS 1Dy10 in endosperm extracts. Six copies of the *1Dx5* gene were detected by real-time PCR. FISH had previously revealed that all the transgenes in this line, including *1Dy10*, were integrated at a single site. Real-time PCR confirms the presence of *1Dy10* in this line and suggests that this single-copy transgene was either silenced, altered in some way that prevented translation, or expressed at such low levels that the protein product could not be detected by SDS-PAGE conditions that readily detect the endogenous HMW glutenin subunits encoded by single copy native genes. The line DC2-65s carries the pKS+Dx5 and pKS-Dy10 plasmids in each of two insertion sites located in the telomeric and sub-telomeric region of the same chromosome, and showed co-suppression of all HMW glutenin subunits including the endogenous ones. Moreover, co-suppression was found to be genetically stable in the generations analysed from T_1 to T_4 [23]. In the present work, real-time PCR detected the highest number of transgenes among our four lines in this event's genome, i.e. 41 copies for *1Dx5* and 35 for *1Dy10*.

Genetic engineering of plants sometimes results in transgene silencing after integration into the host genome, which may be related to a defence mechanism against foreign DNA expression [28]. In fact, when analysing the effects of the surrounding genomic environment on transgene expression, one must also consider the potentially broader impact of the genome organization of the host plant with respect to the chromosomal distribution of structural genes, abundance, diversity, arrangement of repetitive DNA and the constitution of sub-genomes in polyploids [24]. Transgene silencing has been reported for diploid, autopolyploids and allopolyploids [25, 29]. These species all have distinctive genomic attributes that might influence the frequency and types of silencing effects.

The phenomenon of transgene silencing has often been reported in hexaploid wheat, and it may affect both the exogenous DNA sequences and the homologous genes, depending on a mechanism called homology-dependent gene silencing (HDGS) [30, 31]. Altpeter *et al*. [32] transformed bread wheat cultivar Bobwhite with the gene encoding HMW-GS 1Ax1 and speculated that its integration resulted in silencing of the *1Ax2* gene in one of their transformants.

Blechl and Anderson [21] transformed Bobwhite with a chimeric gene composed of *1Dy10* and *1Dx5* and observed co-suppression in one of their lines [33]. More recently, silencing of HMW-GS genes in wheat has been reported by Alvarez *et al*. [34], Uthayakumaran *et al*. [35] and He *et al*. [36]. There is surprising evidence to suggest that the expression of endogenous genes can be inhibited by the introduction of homologous constructs capable of transcribing mRNA of the same sense as the transcripts of endogenous genes [37]. In our work, line DC2-65s contained two transgene insertions on the same chromosome carrying a high total gene copy number; thus we speculate that the interaction of multiple genes with the same mRNA on the same chromosome could have made co-suppression of the *1Dy10* transgene and the endogenous homologous-more likely.

Real-time PCR has traditionally been used to quantify RNA or DNA, but very few studies have employed it to determine transgene copy number [38, 39]. Transgenic plants with a single transgene copy are desirable because multiple copies often lead to gene silencing [28]. Unfortunately, the presence of more than one transgene locus is frequently observed in plants transformed via microprojectile bombardment [21, 40, 41].

Compared with other techniques of DNA quantification, real-time PCR allows the detection of the precise amount of a given nucleic acid sequence in a rapid, specific and sensitive way. Combining amplification and detection into one homogeneous assay, it obviates the need for gel electrophoresis to determine the amount of the DNA target accumulated after a fixed number of cycles.

The estimation of precise transgene copy number by real-time PCR usually needs an endogenous control gene, but choosing an appropriate internal gene can be problematic. The *puroindoline-b* gene highly conserved in several crops such as wheat, maize and sorghum [42] has been used as an internal gene in some published reports [4], but this gene is not found in durum wheat, which lacks the D genome. In our work, we optimised the detection of each transgene copy number by using as a control the same plasmids used in the transformation. In this way, a correct concentration of plasmid copies could be made in order to obtain an accurate standard curve that is a key prerequisite for the quantitative assay. In this work, real-time PCR was shown to be a precise and sensitive method to estimate gene copy number. The data obtained confirmed and extended the results from the previous cytogenetic analyses of the same transgenic lines [23]. This research shows that in addition to other methods, real-time PCR can be considered a valid tool for characterization of transgenic lines and a sensitive method for DNA-based detection of GM crops.

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