DETECTION OF TRANSGENE COPY NUMBER BY ANALYSIS OF THE T_1 GENERATION OF TOBACCO PLANTS WITH INTRODUCED *P3* GENE OF POTATO VIRUS A

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Summary. – Real-time PCR, namely the ΔΔCt method was used to determine the relative copy number of Potato virus A (PVA) *P3* gene in the genome of the T_1 generation of 18 transgenic tobacco lines. These results were compared with segregation ratios of kanamycin (Km)-resistant phenotype in T_1 plants of each line and were found to be, in general, concordant. All the five lines with the Mendelian segregation ratio of 3:1 carried one gene copy. In 12 of 13 lines with uneven segregation more inserted gene copies were detected. Only for one line the real-time PCR and phenotype segregation differed. According to our results the real-time PCR of T_1 generation may be used as supplementary method of estimation of the number of transgene copies in the case of nonavailability of the original T_0 plants.

Key words: real-time PCR; transgenic tobacco; phenotypic segregation; Potato virus A; potyvirus

In consequence of technological progress and decrease of required charges, real-time PCR became a powerfull tool for quantitative studies of genomes and transcriptomes. Its practical applications include the estimation of the copy number (N) of transgenes in genetically modified plants. This is essential for choosing one-copy plants for preparation of homozygous progeny, because multiple gene copies may interfere with each other. Plants with several transgene copies are generally problematically characterizable and often genetically unstable (Hobbs *et al*., 1993). Until recently, Southern blot analysis together with segregation analysis were current techniques for the N estimation. Although potentially harmful radioactive probes may be replaced by non-radioactive, Southern blot analysis remains relatively costly, requires more plant material and, in the case of rearranged gene copies, it yields incorrect results. The real-time PCR is an ideal method for screening regenerated transformed plants as it is simple, fast and enables to analyze many samples at the same time. It has been used for the N estimation in different transgene/plant systems (Ingham *et al*., 2001; Mason *et al*., 2002; Li *et al*., 2004).

We applied this technique to the analysis of tobacco (*Nicotiana tabacum*) plants carrying the gene for non-structural P3 protein of PVA, an aphid-transmitted potyvirus (Nováková *et al*., 2005). These plants were prepared in 1999 to test their possible resistance against PVA. They were positively selected for the *P3* gene by PCR and the seeds were collected after self-pollination (first filial generation, T_1). However, the N was not determined and, due to technical problems with cultivation room, original (T_0) plants were eventually lost (Ľ. Mazúrová, personal communication). Here, we report the estimation of *P3* gene copy number in transgenic tobacco T_1 plants (N') by real-time PCR and discuss the possibility of calculating the N value for T_0 plants from the obtained data.

Transgenic tobacco plants cv. Petit Havana SR1 with introduced PVA *P3* gene (Nováková *et al.*, 2005) were grown

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Abbreviations: Km = kanamycin; N = transgene copy number for T_0; N' = N for T_1; PVA = Potato virus A; T_0 (generation) = transgenic plants obtained by transformation and selection; T_1 = first filial generation of transgenic plants obtained by self-pollination of T_0

Table 1. Primers used in the real-time PCR

Target gene	Orientation	Sequence (5'-3')	Location	Amplicon length (bp)
P3	Forward	TCAAGCAATAAGACATTCGCG	2951–2971[a]	516
P3	Reverse	TGAAACAGAACCACTTCTGC	3447–3466 [a]	
axi1	Forward	ATGAAGAGGAGAAAACAACGC	578–598 [b]	108
axi1	Reverse	AGGAAGGAGGTGGTGAATAC	666–685 [b]	

[a]PVA strain B11, Acc. No. NC 004039 (Puurand *et al*., 1994).
[b]*N. tabacum axi1* gene, Acc. No. X80301 (Walden *et al*., 1994).

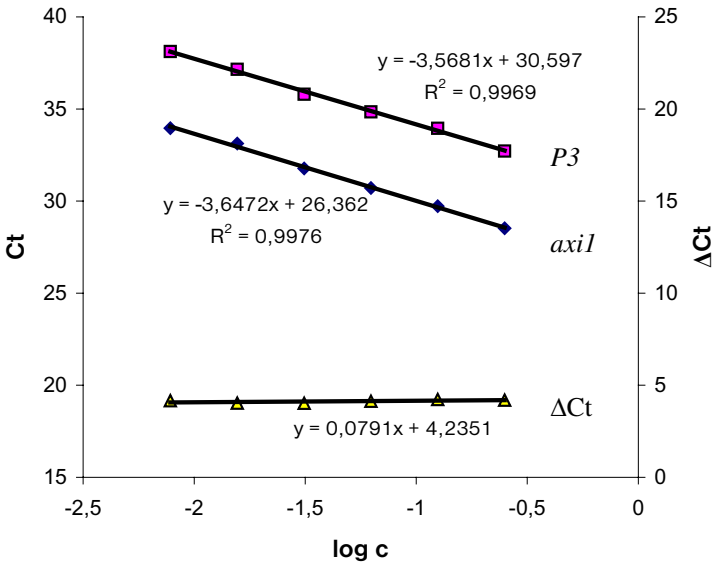

Fig. 1

Comparison of amplification efficiencies for amplicons of *P3* and *axi1* genes

Ct values are plotted in relation to log of relative template concentration (reciprocals of twofold dilutions). Slopes, intercepts and correlation coefficients for both standard curves as well as the plot of ΔCt to log of template concentration are shown.

in sterilized soil at insect-free controlled conditions (22°C, 14 hrs photoperiod). Aliquots from 10 different plants of each line were pooled (totally 300 mg) for DNA isolation by phenol-chlorophorm (Sambrook *et al*., 1989). DNA concentration was estimated spectrophotometrically by measuring A_{260}.

In the real-time PCR, the tobacco *axi1* gene (Walden *et al*., 1994; Acc. No. X80301) was used as endogenous control. Both *P3*- and *axi1*-specific primers are shown in Table 1. The output of a real-time PCR instrument is the Ct value, the cycle number when the fluorescence, directly proportional to the amplicon concentration, reaches the threshold value. For the elimination of template concentration variation an endogenous reference gene, present in all samples in constant relative amount, was used. The value of ΔCt was calculated from the equation

$$\Delta Ct = Ct(P3) - Ct(axi1)$$

As the *axi1* gene copy number per genome is constant, a change of ΔCt is linearly dependent on the change of N'. Comparison of ΔCt for particular sample with that for the control (ΔΔCt) enables to estimate N' from the equation

$$N' = 2^{-\Delta\Delta Ct}$$

(Livak and Schmittgen, 2001). The sample with the lowest obtained ΔCt value (line 200) was presumed to contain one *P3* gene copy and was used as virtual calibrator.

The reaction mixture (20 µl), pipetted automatically (Motion 5070, Eppendorf, Germany), contained 10 µl of SYBR® Green PCR Master Mix (Applied Biosystems, USA), 2 pmoles of each primer, and 100 ng or 200 ng of plant DNA (two template concentrations for each sample were applied). The reaction was performed in an ABI Prism 7900HT apparatus (Applied Biosystems) under following conditions: denaturation at 95°C for 10 mins, 40 cycles of 95°C/30 secs (denaturation), 54°C/30 secs (annealing) and 72°C/60 secs (elongation). To observe the specificity of the reaction, the melting curve of the reaction products was measured at the end of the program. Each sample was assayed in triplicate and mean values of Ct were used for copy number calculation.

The ΔΔCt method is only applicable if the target sequence of the analyzed gene and that of the endogenous control are amplified with equal or very similar efficiency (Livak and Schmittgen, 2001). Amplification efficiencies for *P3* and *axi1* genes were estimated by PCR on DNA from tobacco line 214 in the range of 4–256 ng DNA per reaction mixture. The differences among triplicates were very small (SD<0.4% of mean values). The slopes of Ct/log concentration plots for the reactions were –3.65 (*axi1*) and –3.57 (*P3*), indicating similar amplification efficiencies (Fig. 1). The slope of ΔCt/log concentration plot (0.08) was in the range of 0.1 in accord with the recommendation of the real-time PCR machine manufacturer.

Totally, 18 transgenic tobacco lines were analyzed for the *P3* gene copy number. The vector-(pBI121, Clontech, USA)-transformed line 166 (Nováková *et al*., 2005) and wild-type tobacco SR1 plants were used as negative controls. Both

of them gave no specific amplification signal for the *P3* gene. The results are summarized in Table 2. One copy of the *P3* gene was found in all five tobacco lines with a Mendelian phenotype segregation ratio 3:1 for the Km resistance linked with the transgene. Such a segregation ratio is typical for insertion of the gene in one locus in redundant DNA (Topping and Lindsey, 1997). The one copy result of the real-time PCR for the line 175 did not correlate with its T_1 phenotype segregation ratio; moreover, a low germination ability was recorded and the *nptII* gene responsible for Km resistance was not detected by conventional PCR in this line. All this indicated possible gene rearrangement events and gene damage in the course of transformation (Nováková *et al*., 2005). For the rest of the lines, N was estimated at least at 2 (including two lines with N>10), which was in agreement with their fluctuating phenotypic segregation.

Practically, N has to be determined in the regenerated T_0 plants in order to select appropriate (one-copy) lines for further work. Since, in our case, original T_0 plants were no more available, we tried to check whether plant lines with the N of 1 could be detected *ex post* by the analysis of their progeny, the T_1 plants. As these plants have been obtained by self-pollination of T_0 plants, their N' values could not be considered exact N values. N' values reflect statistical Mendelian distribution of the transferred gene in individual plants included in the experiment. Genetic heterogeneity of the T_1 generation make Southern blot analysis unsuitable for the N' estimation because of difficult evaluability of restriction profiles. Real-time PCR, however, provides quantitative numeric results. The more plants analyzed, the more precise result corresponding to the true situation is obtained. If N = 1, 50 % of T_1 plants have the same gene copy number as T_0 plants(N' = N). With increasing N value the number of T_1 plants with N' ≠ N and the probability that such plants will be sampled grow. We used bulk of the leaf pieces from 10 different plants for DNA isolation for each transgenic line. Because of limited number of analyzed plants the accuracy of the results was expected to decrease with increasing N value.

Consistency of the results with the T_1 phenotype segregation ratio of 3:1 indicated that 5 plant lines carried one *P3* gene copy in the genome. However, only slightly higher N' values were obtained for the lines 175, 167 and 212 showing different segregation ratios (Table 2). Bubner *et al*. (2004) have shown that two-fold differences constituted the detection limit for determining transgene copy numbers in plants by real-time PCR. Hence it may be difficult to distinguish plants with N = 1 and those with N = 2.

Generally, the analysis of a limited number of individual plants of T_1 generation does not allow estimation of real gene copy number. Therefore, only the bottom border of the range of potential N values may be estimated (see the last column in Table 2). Moreover, the differences among theoretical phenotype segregation ratios also rapidly decrease with rising N. The main aim of the analysis is usually selection of the lines with one transgene copy from other lines. As we showed, for this purpose, if the T_0 plants are not available, the real-time PCR of T_1 generation may be used as supplementary but not exclusive method.

Table 2. *P3* gene copy numbers and segregation ratios of Km-resistant phenotype

Plant line[a]	(Km^r/Km^s)[b]	T_1 germ.[b] (%)	*P3* gene copy number[c]	
			N' (T_1)	N (T_0)
SR1[d]	0	100	0	0
166[d]	1:5	100	0	0
200	3:1	100	1.0000±0.0369	≥ 1
181	3:1	90	1.2431±0.3151	≥ 1
214	3:1	100	1.2483±0.0345	≥ 1
188	3:1	49	1.2624±0.2381	≥ 1
171	3:1	98	1.4753±0.1820	≥ 1
175	19:1	20	2.2255±0.4712	≥ 1
167	0	1	2.4480±0.2676	≥ 2
212	24:1	100	2.5507±0.0263	≥ 2
206	100:0	100	3.1166±0.2832	≥ 2
183	100:0	100	4.5195±0.8123	≥ 2
201	100:0	100	5.3446±0.4583	≥ 2
178	1:2	100	5.6637±0.7749	≥ 3
190	0	71	9.1713±0.0652	≥ 5
208	30:1	100	9.4527±0.2707	≥ 5
169	1:2	42	10.3493±0.6346	≥ 5
207	100:0	100	13.4823±0.2895	≥ 7
150	1:2	58	23.9967±0.1237	≥ 12
186	0	74	34.6110±0.6230	≥ 17

[a]The plant lines are ordered according their ascending N' values.
[b] The ratio of Km-resistant to Km-sensitive T_1 plants and germinative capacity of T_1 seeds according to Nováková *et al*. (2005).
[c]N' values estimated by the real-time PCR and N limits approximated as N ≥ (N'/2).
[d]Negative controls.

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