QUANTITATIVE DETECTION OF CAMV- 35S PROMOTER AND T-NOS TERMINATOR IN GENETIC MODIFIED TOMATO FROM IRAQI MARKETS

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ABSTRACT

Aim of this research was quantitative detection of Increase and decrease in copy number of CaMV-35S promoter and Nos terminator in genetic modified tomato by using Real-time PCR. Twenty four samples of genetic modified tomato seeds were isolated from 84 tomato samples collected from Iraqi markets during the period from December 2016 to January 2017. The experiences were conducted in the Institute of Genetic Engineering, University of Baghdad. Go Taq®qPCR master mix kit supplied by USA Promega Company, three specific primers to CaMV-35S promoter, T-Nos terminator and β-actin housekeeping gene supplied by Canadian Alpha Company were used. To quantitative detection of increase and decrease in copy number of GM tomato samples contain CaMV-35S promoter and T-Nos terminator, comparing with the β-actin (houskeeping gene) using Multiple of Median (MoM) equation. The results showed that the lowest recorded of Ct value was (27.88) for CaMV-35S promoter gave an increase in copy number (1.1766) above the normal limit, while highest recorded of Ct value was (32.67) gave an increase in copy number (1.0350) above the normal limit. The lowest recorded of Ct value was (27.35) for T-Nos terminator gave an increase in copy number (1.1600) above the normal limit, whereas highest recorded of Ct value was (32.82) gave a decrease in copy number (0.9920) under the normal limit.

***Keywords: Tomato, quantitative detection, CaMV-35S promoter, Nos terminator and MoM.**
***Part of M.Sc. Thesis of the first researcher.**

الكشف الكمي عن المشغل CaMV-35S والفاصل T-Nos الوراثياً في الأسواق العراقية للطماطم المحورة

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المستخلص

هدف البحث الى الكشف الكمي عن الزيادة والنقصان في عدد النسخ للمشغل CaMV-35S والمنهي T-Nos في الطماطم المحورة وراثياً بأستخدام Real-time PCR. عزلت أربعٌ وعشرون عينة من بذور الطماطم المحورة وراثياً من 84 عينة طماطم التي جمعت من الأسواق العراقية في الفترة من كانون الأول لسنة 2016 ولغاية كانون الثاني لسنة 2017. نفذت التجربة في معهد الهندسة الوراثية والتقنيات الإحيائية/جامعة بغداد. أستخدم المجهز Go Taq®qPCR master mix kit من قبل شركة بروميغا الأمريكية وثلاث من البوادئ الخاصة بالمشغل CaMV-35S، الفاصل T-Nos والجين β-actin housekeeping المجهزة من قبل شركة الفا الكندية. للكشف الكمي عن الزيادة والنقصان في عدد النسخ لعينات الطماطم المحورة وراثيا التي تحتوي على المشغل CaMV- 35S و الفاصل T-Nos، مقارنتاً مع جين β-actin housekeeping باستخدام معادلة الوسيط المتعدد MoM. أظهرت النتائج أن أقل قيمة سجلت لـCt (27.88) للمشغل CaMV-35Sأعطت زيادة في عدد النسخ (1.1766) أعلى من الحد الطبيعي، في حين سجلت أعلى قيمة لـCt (32.67) أعطت زيادة في عدد النسخ (1.0350) أعلى من الحد الطبيعي. أقل قيمة سجلت لـCt (27.35) للفاصل T-Nos أعطت زيادة في عدد النسخ (1.1600) أعلى من الحد الطبيعي، في حين سجلت أعلى قيمة لـCt (32.82) أعطت تناقص في عدد النسخ (0.9920) عن الحد الطبيعي.

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INTRODUCTION

A genetically modified plant is a plant whose genetic formation has been altered by means of the recombinant DNA technology (18). The genetic modification usually involves inclusion of a piece of DNA (the insert) taken from other naturally occurring organisms, a synthetic combination of different smaller pieces of DNA into the genome of the organism to be modified through the procedure of genetic transformation (11). Area under cultivation of GM crops has already amount upto175.2 million hectares in 2013, out of which, more than 170 million hectares are of GM food or/and oil seed crops, namely, corn, soybean, cotton , canola and tomato. The first genetically modified crops approved for the commercialization was the Flavr- Savr tomato in 1994 (14). Generally, the constructs used for transformation of plants are composed of the gene(s) of interest, mostly bacterial and plant sequences, fused to promoter/enhancer sequences of bacterial, viral, or plant origin and combined with marker genes (usually consisting of antibiotic or herbicide resistance genes plus promoter sequences). Two of the most usually used regulatory elements are the constitutive 35S promoter from the *cauliflower mosaic virus* and T-Nos terminator from the *Agrobacterium tumefaciens*. These two elements are present either jointly or separately in most of the genetically modified plants (12). The most frequently used method for detecting GMO plants is screening for the CaMV-35S promoter (CaMV) and the nopaline synthase gene (T-nos) (21). The quantitative Real-time PCR a useful tool for estimation of the copy number in transgenic plants (16, 27). Aim of this study was relative quantitative detection of Increase and decrease in copy number of CaMV-35S promoter and Nos terminator in genetic modified tomato by using Real-time PCR

MATERIAL AND METHODS

DNA extraction using (CTAB) method

Tomato seed samples were planted in incubator and after two weeks plant with 3-4 young leaves stage were cut and sterilized with concentration ethanol 70%. Fifty miligrams samples of young leaf tissues were ground to a fine powder in 700 µl 2% CTAB extraction buffer [20 mM EDTA, 0.1 M Tris-HCl pH 8.0, 1.4 M NaCl, 2% CTAB, plus 0.4% b-mercaptoethanol were added just before use], they were crushed inside eppendorf 1.5 ml (2). Tubes were then incubated at 65ºC for 1 h, softly mixed every 15 min for adequate homogenization. 500 µl of chloroform-isoamylalcohol (24:1) was then added, mixed softly for 1 min, and centrifuged at 8,000 rpm for 10 min (6). 500 µl of the supernatant was then transferred to a fresh tube with 700 µl of cold isopropanol (-20ºC); samples were softly mixed by inversion and centrifuged at 12,000 rpm for 10 min, the mixture is then placed in the freezer for one hour, and so it was possible to visualize the DNA adhered to the bottom of the tube. The liquid solution was then released and the DNA spherule washed with 700 µl of 70% ethanol to remove salt residues adhered to the DNA, and set to dry for approximatel 2 h, or until the next day, with the tubes inverted over a filter paper, at room temperature (2). The spherule was then resuspended in 100 µl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) plus 5 mL ribonuclease (RNAse 10 mg mL–1) in each tube; this solution was incubated at 37ºC for 1h, and then stored at -20ºC (6).

Concentration and purity of DNA

Ultraviolet Light (UV) spectroscopy was used to esteem DNA purity by measuring a sample's absorbance spectrum between 200 and 320 nm, and calculating the A260/A280 and A260/A230 ratios (24). Proportion between 1.8 and 2.0 for A260/A280 are accepted as indicating pure DNA (22).

=DNA Purity = O.D 260 ÷ O.D 280

Concentration was measured after sample dilution 100 once with a TE buffer solution and reading the amount of absorption of the device Nano Drope

DNA Concentration (µg/ml) = (A260 reading – A320 reading) × dilution factor × 50µg/ml

For checking the DNA quality, it was migrated through 1% gel agarose electrophoresis.

Quantitative detection by Real -time PCR

Samples were tested using relative quantitative depending on the "housekeeping gene" used as a control for experimental variability (26), and quantification of copy number were accomplished used the standard curve method depending on Threshold cycle (Ct) of houskeeping gene and genetically modified

gene by calculating the Multiple of Median (MoM) for each amplicon (1). The (Ct) average of (β-actin) reference genes was calculated; the Ct value of the target gene of the average Ct value of the (β-actin) reference genes was subtracted; Multiple of Median (MoM) values were obtained by dividing the outputs to the Ct value of (β-actin) reference genes. Multiple of Median (MoM) equation was applied to 24 tomato sample containing genetic modification elements CaMV-35S promoter and T-Nos terminator.

$$MoM = \frac{\textbf{average of Ct(sc)} + [\textbf{average of Ct(sc)} - \boldsymbol{Ct\ of\ GM\ gene}]}{\textbf{average of Ct(sc)}}$$

Ct=Threshold Sc=Stander curve GM=Genetic Modification

Detection primer of GM tomato

Because most of GM products contain either the cauliflower mosaic virus (CaMV) 35S promoter or the nopaline synthase (nos) terminator, or both, most of the PCR screening methods are based on detecting these sequences in the product (8). Accordingly,the primers were relied from European Commission link. https://ec.europa.eu/commission/index_en which carries registration codes for the genebank database http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide sequences primers were illustrated in Table 1.

Stander curve houskeeping gene

Housekeeping genes are genes that required for the maintenance of basal cellular functions that are essential for the existence of a cell, regardless of its specific role in the tissue or organism. Thus, they are expected to be expressed in all cells of an organism under normal conditions, irrespective of tissue type, developmental stage, cell cycle state, or external signal. From a fundamental point of view (5), the internal control β-actin gene was used in this study. It maintains the cytoskeleton and physiology of eukaryotic plants thus stabilizing the shape of cell (19). The primer sequences of β-actin were used in Table 1.

Gradient PCR of β-actin

Gradient PCR was used in the next experiment in order to determine the optimal annealing temperature. Using the gradient function of the universal block, a gradient of 50 to 54°C (50C, 52 and 54) were set. This was achieved by setting a 3°C gradient and setting the annealing temperature at 54°C. This program was applied to two negative control samples. The gradient was also used for size primers (1µl, 0.8µl). The concentrations of PCR reaction were 11 µl and 11.4 µl d.w, 5 µl AccuPower® PCR PreMix, 1 µl and 0.8 µl (F, R β-actin Primer) and 2µl target DNA, final volume for reaction were 20 µl. And the PCR reaction conditions for β-actin were initial 5min denaturation at 95°C by 1 cycle, (1min denaturation at 95°C, 45sec annealing at 54°C and 1min elongation at 72°C by 35 cycles) and 10min final extension at 72°C by 1 cycle.

Go Taq®qPCR Master Mix protocol

The procedure of Real-time qPCR using SYBR Green was done according to Wolton, (25). The Reaction Mix volume was 25µl for CaMV-35S promoter and T-Nos terminator primers were contain 12.5µl GoTaq®qPCR Master Mix; 2X, 8.9 µl d.w, 0.8µl (F, R CaMV-35S promoter and T-Nos terminator Primers) and 2µl target DNA. The reaction conditions of Real-time PCR for CaMV-35S prometer and T-NOS terminator were 2 min hot-start activation at 95°C by 1 cycle, (15sec denaturation at 95°C and 60 sec annealing at 60°C by 40 cycles) and 7min final extension at 72°C by 1 cycle.

Table1. Sequences of primers utilized for quantitative detection and identification in Real-time PCR

NO.	Primer Name	Target sequence	Origin sequence	5'to3' Sequence	Amplicon [bp]	Primer source
1	Detection primer	CaMV - P35S Promoter	*Cauliflower Mosaic Virus*	**F**-5'-GCTCCTACAAATGCCATCA-3' **R**-5'-GATAGTGGGATTGTGCGTCA-3'	195bp	QL-ELE-00-005
2	Detection primer	NOS terminator	*Agrobacterium Tumefaciens*	**F**-5'-GAATCCTGTTGCCGGTCTTG-3' **R**-5'-TTATCCTAGTTTGCGCGCTA-3'	180bp	QL-ELE-00-007
3	Housekeeping gene	β-actin	_____	**F**-5'-TGG CAC CCG AGG AGC ACC CTG-3' **R**-5'-GCG ACG TAC ATG GCA GGA ACA-3'	118bp	(13)

RESULTS AND DISCUSSION

Quantitative detection of GM tomato by Real-time PCR: Numerous countries have implemented regulations requiring the labeling of products containing GMOs, or materials derived from GMOs, above certain thresholds, therefore emphasizing the necessity for quantification of GMO content (9). GMO content in plants and feed samples is expressed in relative terms as the ratio of the quantity of the transgene (GM target, i.e. the nucleic acid fragment introduced in the host genome) to that of the endogene (houskeeping gene in the host genome) (23). The primers of β-actin (houskeeping gene) were used for quantitative detection and determine the proportion of increase and decrease in copy number of CaMV-35S promoter and T-Nos terminator.

Determine of β-actin by conventional RCR

Temperature and size primer were gradient of two tomato samples without genetic modification. The primers specific of β-actin recorded a PCR product of 118 bp. The results show that the idealistic annealing temperature is 54ºC and the idealistic size of primers is 0.8 µl (Figure 1).

Assessment Multiple of Median (MOM) depended on (Ct) value

Quantitative Real-time polymerase chain reaction (qPCR) had been widely used for transgene of copy number studies (3). Multiple of Median (MoM) equation was used to determine the level of increase and decrease in the copy number of genetically modified genes, during the qPCR assay the target gene was amplified, simultaneously recognized and monitored by SYBR Green

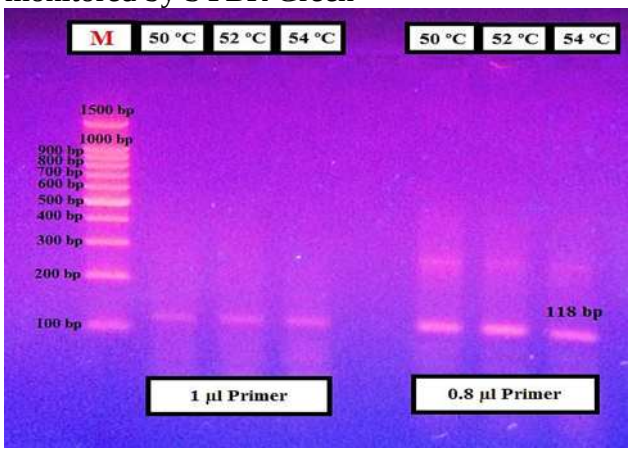

Fig 1. Gradient PCR products of β-actin of two tomato samples in three annealing temperatures and two primer size using 100 bp Lader on 2% agarose gel at 75 volt for 90 minutes

Real-time PCR was given as the threshold cycle Ct, the threshold cycle Ct is the cycle at which the fluorescence scale reaches a certain amount (the threshold) (20). The decrease in Ct value of the target gene is an indicator of increased genetic modification content.

Quantitative detection of CaMV-35S promoter

For each sample Ct values of the specific target genes CaMV-35S promoter and houskeeping gene (β-actin) were determined as mentioned by Mbongolo *et al.,* (17). To evaluate the correctness of the amplicons Real-time PCR obtained with the respective methods, the melting temperature (Tm) of each target was determined experimentally as mentioned by Kagkli *et al.,* (15). (33.86) was the average of Ct value of β-actin gene that represents standard curve, after adding the primers of β-actin gene to 15 tomato samples containing the CaMV-35S promoter. In the current study, the results showed that the lowest recorded of Ct values were 27.88, 28.99 and 29.13 for GM tomato samples (GS-12, TO saffaa and Escalibur) from Thailand, Netherland and USA low Ct value reflects a high target concentration (26). The highest recorded Ct values were 32.67, 32.50 and 32.03 of GM tomato samples (Nada, 4610 and Yamama) from Holland. Figure 2 show strong amplification curves for each of the Real-time PCR runs with specific primers, for CaMV-35S promoter.

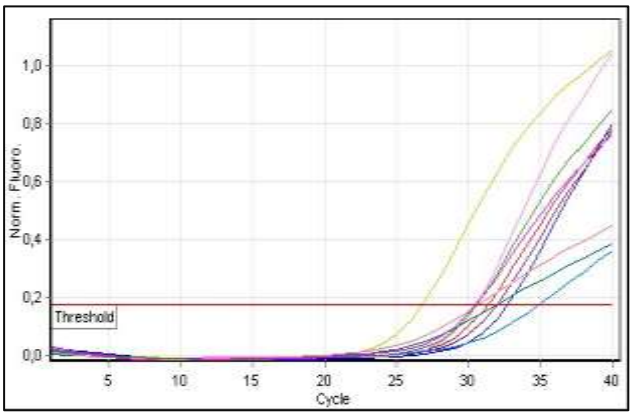

Fig 2. Amplification curves of some GM tomato samples contains CaMV-35S promoter by Real-time PCR

In an Iraqi study by Farhan (7) the Ct values were 33.73 and 38.63 of two GM rice samples contains CaMV-35S promoter. Results showed that applying the multiple of medium (MoM) equation, the lowest readings of Ct values gave the highest percentage in copy number of CaMV-35S promoter above the normal limit, the highest of MoM values are 1.1766, 1.1438 and 1.1397 respectively, as the number 1.000 represents the normal limit of copy number and any increase in the copy number of 1.000 is abnormal limit, increase the copy number of elements controlling the genetic modification of the normal limit, may lead to intended and off-target (red line with purple target section) gene silencing in the GMO (10). New dsRNA molecules are commonly created by the genetic engineering process. Indeed, most cells initially engineered used in vitro nucleic acid techniques ultimately "silence" the gene, was inserted because they cause the production of dsRNA (4). Table 2 show the increase in copy number for CaMV-35S promoter in fifteen GM tomato samples. Figure 3 show amount of increase in copy number of CaMV-35S promoter above normal limit where the horizontal axis represents the samples and the vertical axis represents of increase in Multiple of Median (MoM) value, as shows in Table 2.

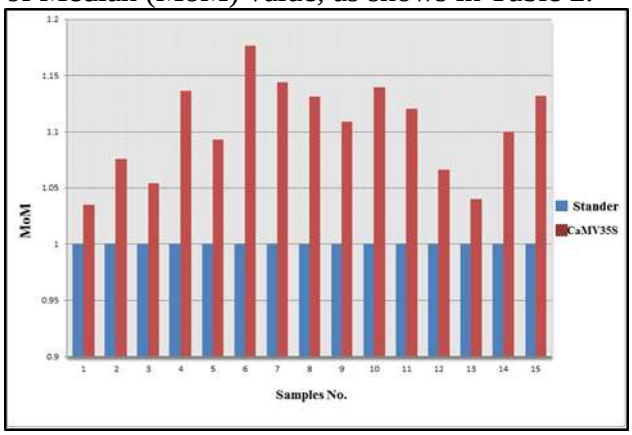

Fig 3. Increase in copy number of CaMV-35S promoter of (1.000) in GM tomato samples

Quantitative detection of T-Nos terminator

After obtaining the Ct value of specific primers for β-actin gene that were added to 13 genetically modified tomato samples which contain T-Nos terminator, Ct average was calculated for β-actin (32.56) which represents of standard curve. Ct values were calculated of specific primer of tomato samples containing genetic modification of T-Nos terminator; subtracted the Ct value of T-Nos terminator from the average Ct value of the (β-actin) reference genes; Multiple of Median (MoM) values were obtained by dividing the outputs on the Ct value of (β-actin) houskeeping

genes. The results showed that the lowest recorded of Ct values were 27.35, 28.59 and 28.83 of GM tomato samples (Anfas, Tomaland and Wogdan) from Holland and Mexico, the highest recorded of Ct values were 32.82, 32.66 and 32.21 of GM tomato samples (Omnia, Hanine and Tres Cantos) from Holland and Spain. Figure 4 show strong amplification curves for each of the Real-time PCR runs with specific primers, of T-Nos terminator.

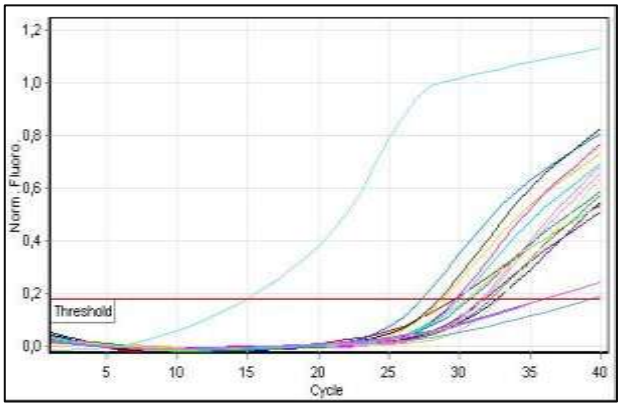

Fig 4. Amplification curves to some GM tomato samples contains T-Nos terminator by Real-time PCR

In an Iraqi study by Farhan (7) the Ct values were 30.87, 30.91, 31.00 and 33.75 of four GM rice samples contains T-Nos terminator comparison with the positive control which was 29.75 obtained with NOS terminator specific primer. The results showed that when applying the multiple of medium (MoM) equation, the lowest readings of Ct values gave the highest percentage increase in copy number of T-Nos terminator of the normal limit, the highest of MoM values are 1.1600, 1.1220 and 1.1146 respectively, and highest readings of Ct values gave the lowest percentage in copy number of T-Nos terminator of the normal limit, the lowest of MoM values are 0.9920, 0.9969 and 1.0110 respectively in GM tomato samples (Omnia, Hanine and Tres Cantos); This decrease of copy number is close of normal limit of copy number of (β-actin) houskeeping gene. Table 3 shows the increase and decrease in copy number of T-Nos terminator in thirteen GM tomato samples. Figure 5 shows amount of increase and decreases in copy number of T-Nos terminator on normal limit where the horizontal axis represents the samples and the vertical axis represents of increase and decreases in Multiple of Median (MoM) value

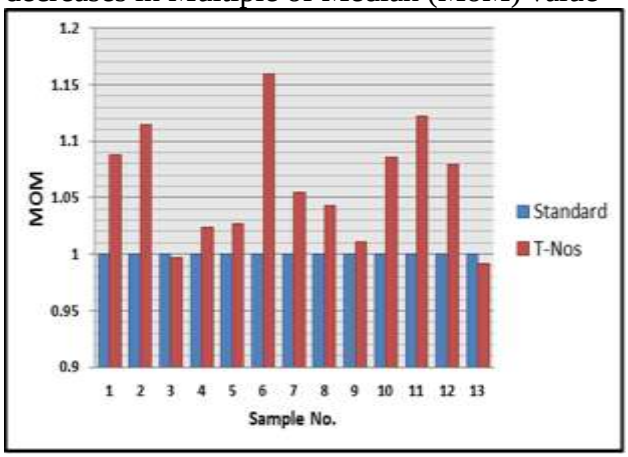

Fig 5. Increase and decrease in copy number of T-Nos terminator of (1.000) in GM tomato samples

Table 2. Multiple of Median (MoM) for CaMV-35S promoter in GM tomato samples

No.	Cultivar	Ct β-actin	Ct of CaMV-35S	Ct average-Ct(P35s)	+ Average	MoM
1	Nada	36.20	32.67	1.19	35.05	1.0350
2	Wogdan	35.72	31.30	2.56	36.42	1.0756
3	Yamama	35.45	32,.03	1.83	35.69	1.0540
4	Simona	34.90	29.23	4.63	38.49	1.1367
5	SHEFA	32.54	30.71	3.15	37.01	1.0930
6	GS-12	31.05	27.88	5.98	39.84	1.1766
7	TO saffaa	32.22	28.99	4.87	38.73	1.1438
8	Rayan	34.60	29.41	4.45	38.31	1.1314
9	MeyaMeya	31.66	30.17	3.69	37.55	1.1089
10	Escalibur	32.83	29.13	4.73	38.59	1.1397
11	Reem	32.61	29.78	4.08	37.94	1.1204
12	Flneness	34.36	31.62	2.24	36.10	1.0660
13	4610	33.09	32.50	1.36	35.22	1.0400
14	Omnia	36.88	30.56	3.30	37.16	1.1000
15	Ban	33.78	29.40	4.46	38.32	1.1317
	Average	33.86				

Table 3. Multiple of Median (MoM) of T-Nos terminator in GM tomato samples.

No.	Cultivar	Ct βactin	Ct of T-Nos	Ct average-Ct(T-Nos)	+ Average	MoM
1	Sadek	34.78	29.70	2.86	35.42	1.0878
2	Wogdan	31.19	28.83	3.73	36.29	1.1146
3	Hanine	34.17	32.66	-0.10	32.46	0.9969
4	NADA	33.48	31.80	0.76	33.32	1.0233
5	Hala21	32.49	31.70	0.86	33.42	1.0264
6	Anfas	31.05	27.35	5.21	37.77	1.1600
7	Ginan	32.11	30.77	1.79	34.35	1.0550
8	Shourouq	33.01	31.53	1.03	33.59	1.0430
9	Tres Cantos	32.65	32.21	0.35	32.91	1.0110
10	Escalibur	33.16	29.77	2.79	35.35	1.0860
11	Tomaland	30.68	28.59	3.97	36.53	1.1220
12	Ban	32.01	30.00	2.56	35.12	1.0790
13	Omnia	32.45	32.82	-0.26	32.30	0.9920
	Average	32.56				

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