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The Potential Role of Plastid DNA as a Biomarker and Biosensor Using Real-time Quantitative Polymerase Chain Reaction

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Abstract

Plastids are plant specific semiautonomous organelles, which differentiate to perform many cardinal functions including photosynthesis, energy generation, development, stress perception and response, storage, flowering, and fruit ripening. Additionally, plastids are of immense nutritional value to humans, underscoring the need to develop reliable assays to determine the plastid content of plant based products. Studies have shown that the number of plastids present in various plant organs is regulated by both internal and external factors, however determination of plastid number is time consuming and requires sophisticated instruments. Interestingly, studies have shown that the plastome number, small circular DNA molecules present in the plastids is also regulated by both internal and external stimuli and therefore can be potentially used to estimate the plastid content in a plant organ. This study hypothesizes that plastome copy number, determined by real-time quantitative polymerase chain reaction (qPCR) in different plant samples can be used as a plant biomarker and biosensor. Samples of cotton seed (CS), leaf (CL) and lint (Clt) and seeds of rice (RS), soybean (SS), maize (MS) and sesame (SeS) were obtained over a period of two years, their plastome copy number was determined by the Ct values obtained using real-time qPCR assays using chloroplast tRNA specific primers. Regression analysis was performed to ensure reliability of the real-time qPCR data. Subsequent, data analysis revealed that the mean log plastome copy number showed a statistically significant difference for the plant species including seeds of cotton, soybean, maize, and sesame suggesting that the log plastome copy number is plant species specific and therefore can be used as a biomarker. Furthermore, evidence in support of the use plastids as biosensors was obtained by calculating the range of log plastome copy number, where range represents the difference between lowest and highest log copy number values. A low range value indicated that the log copy values do not deviate from the mean copy number value, suggesting that the samples have encountered consistent and similar internal and external factors during growth and post-harvest storage and processing, Whereas, high range values indicated large deviations from mean copy value suggesting that the samples have encountered changing internal and external conditions. These conclusions are supported by observation of low range for all the seed samples including CR (0.31), CS (0.58), RP (0.05), SeS (0.31), seeds have a protective coat to withstand variations in environmental conditions. Whereas, observation of high range values for CL (4.11), Clt (3.63), and RS (3.09) could be explained by different developmental stage of CL samples, different post-harvest conditions for Clt samples and broken RS samples. In conclusion, the study present preliminary data in support of use of plastome copy number as a plant biomarker and biosensor.

Key Message

The study proposes that the range of log plastome copy number, which represents the difference between highest and lowest log copy number can be used as a biomarker for plant species determination and as a biosensor of environmental conditions encountered by plants during growth and post-harvest processing to assess quality of plant based products.

Introduction

Plastids are semi-autonomous organelles distinguishing plant cells from other eukaryotic cells, acquired by endosymbiosis of an early photosynthetic prokaryote by a primitive eukaryotic cell (Gray 1992). Although, Plastids such as chloroplast perform the critical function of carbon fixation and energy generation through photosynthesis, parts of metabolic pathways such as lipid biosynthesis and amino acid metabolism also occur in plastids (Galili 1995; Ohlrogge and Browse 1995). Other members of the plastid family play pivotal roles in starch and oil storage, fruit and flower coloration, gravitropism, stomatal functioning, and environmental perception (Rolland et al. 2018). Just like mitochondria, plastids are maternally inherited in Angiosperms and are derived initially from small, undifferentiated proplastids found in meristematic cells, which differentiate into plastid subtypes depending on the cell type. Biogenesis of plastids such as chloroplast in leaves, amyloplasts in seeds, and chromoplast in fruits and flowers have been reviewed previously (Marano et al. 1993; Thompson and Whatley 1980; Pyke and Page 1998). Developmental processes such as seed germination, flowering, and ripening involve differentiation of plastids, whereas senescence involves degradation of plastids (Waters and Pyke 2005).

Plastid biogenesis and differentiation has been shown to be associated with plant development and environmental stress (Jarvis and López-Juez 2013). Sensory plastids localized to the epidermis and vascular parenchyma have been shown to be involved in sensing of environmental stress and regulate gene expression and epigenetic circuits (Beltran et al. 2018). It has also been shown that stressed chloroplasts induce post-translational mechanisms, including autophagy that allows individual chloroplast to regulate their own degradation and turnover (Woodson 2019). Retrograde signaling coordinates the expression of nuclear genes encoding organellar proteins with the metabolic and developmental state of the organelle. The chloroplasts therefore act as sensors of environmental changes and complex networks of plastid signals coordinate cellular activities and assist the cell during plant stress responses (Fernandez and Strand 2008). Role of plastids in plant development and plant stress is supported by involvement of plastids specifically chloroplast in biosynthesis of plant hormones including abscisic acid (ABA), jasmonic acid (JA), ethylene (ET), salicylic acid (SA), which are known for their role in plant growth and development and biotic and abiotic stresses. Chloroplast is also site for reactive oxygen species (ROS) production, known for its role in pathogen killing, cell wall strengthening, activating defense gene expression, production and signaling of phytohormones (Lu and Yao 2018). Recently, it has been shown that high light intensity, *Botrytis* infection and senescence resulted in reduction of size and number of chloroplast in the leaves of Arabidopsis (Zechmann 2019).

Amyloplasts and chromoplast are the most studied non-photosynthetic plastids because of their relevance to nutritional qualities of major commodity crops. Amyloplast store starch present in roots (cassava) and tubers (potato), seed endosperm (grain of cereal crops), involved in gravitropism (Su et al. 2017) and starch biosynthesis (Dupont 2008), whereas chromoplasts, rich in carotenoids, are important component of pigments (Price et al. 1995).

The plastid genome or plastome is 120–160 kbp of about 22 to 200 circular DNA copies with 100–120 highly conserved genes (Renner et al. 1934). During the course of evolution many genes were either completely lost or transferred to the nucleus (Martin et al. 2002). Although, the plastome encodes for core proteins of photosynthetic apparatus, retrograde signaling is necessary to regulate the organelle's proteome during establishment of photoautotrophy and fluctuating environmental conditions (Fernández and Strand 2008; de Vries and Archibald 2018).

This study used real-time quantitative polymerase chain reaction (qPCR) for analyzing plastome copy number of different plant organs including seed (with and without husk), leaf, and lint. The study provides evidence that the range of log plastome copy number could be correlated to the plant species, developmental stage and environmental conditions. Observations in cotton plant organs such as seed, leaf, and lint shows that the plastome copy number is regulated developmentally and by various environmental factors, whereas observation in seed samples of different plants supports the hypothesis that plastome copy number is plant type specific. Based on our analysis we propose that the plastome can be used as a biosensor and biomarker for determining quality of plant material and plant based products.

Materials And Methods

Plant Material: *Gossypium hirsutum L.* -cotton (Event code: MON15985-7) and trade name of Bollgard II™ Cotton was acquired from The American Oil Chemists Society (AOCS) to serve as a control reference material (CRM). MON15985-7 has been genetically modified and contains cauliflower mosaic virus 35S (CaMV35S) promoter sequence. The CRM was used as positive control for GMO detection using quantitative PCR assays using CaMV35S specific primers in seed material of different genotypes including cotton, rice, soybean, maize, sesame, paddy, and cotton plant material such as lint, raw cotton, and leaf as described previously (Eede et al. 2000). Chloroplast tRNA specific primers were used as extraction control in all the GMO detection job orders received during 2015 and 2016 (Taberlet et al. 1991).

DNA Extraction: Three different protocol were used for extraction of DNA depending on the type of material used based on the IS/ISO 21569 (Reaffirmed-2012) and IS/ISO 21570:2005 (Reaffirmed-2012). CTAB Method A was used for matrices including processed food material. Cetrimonium bromide (CTAB) Method B for fiber matrices, and Qiagen kit for plant tissues such as leaves, seeds, and stem.

Reagents required for CTAB Method (A and B)

CTAB extraction buffer [CTAB (20g/l), NaCl (1.4 M/l), Tris-Cl (0.1M/l), EDTA (0.2 M/l, pH 8.0], CTAB precipitation buffer [CTAB (20g/l), NaCl (0.04 M/l)], NaCl solution (1.2 M/l), Proteinase K (20 mg/ml), RNase A (10 mg/ml), PVP (360000D), TE buffer [Tris-Cl (0.01 M/l), Na_2 EDTA (0.001 M/l), pH 8.0], Chloroform, Isopropanol, 70% Ethanol, 10% SDS, Ammonium acetate (7.5 M/l).

Extraction procedure for seeds: To 300 mg of homogenized leaves, seeds or grains added 1.5 ml of pre-warmed (65°C) CTAB extraction buffer and vortex. Added α-amylase (rice seeds), RNase A, mixed gently, and incubated at 65°C for 10 minutes. Subsequently, added Proteinase K and incubated at 65°C for 30 min., thereafter spun at 14000 rpm for 10 min. Collected the supernatant and added 1 volume of chloroform and mixed thoroughly, followed by centrifugation at 14000 rpm, and collection of aqueous phase. To this added 2 volumes of CTAB precipitation buffer and ammonium acetate solution (leaf samples) and incubated at room temperature without agitation. After spinning at 14000 rpm discarded the supernatant and dissolved the DNA pellet in NaCl and chloroform (1:1). Centrifuge at 14000 rpm for 10 min. and to the aqueous phase added 0.6 volume chilled isopropanol and incubated at room temperature for 20 min. Subsequently it was spun at 14000 rpm for 15 min. and discarded the supernatant, washed the pellet with 70% ethanol and suspended pellet in TE.

Extraction procedure for cotton lint: A 1.5 g of lint sample is homogenized in 25 ml of pre warmed (65°C) CTAB extraction buffer in a mortar and pestle, avoid frothing. To the homogenate added RNase A followed gentle mixing and incubation in a 65°C water bath for 30 minutes. Centrifuged the contents at 8000 rpm for 30 min. and transferred the supernatant to fresh tube and incubated at 65°C for 30 min. after addition of Proteinase K. Added 1 volume of chloroform and mixed thoroughly followed by centrifugation at 8000 rpm for 20 min. To the aqueous phase added 2 volumes of CTAB precipitation buffer and ammonium acetate and incubated at room temperature for 60 min. to overnight without agitation. After precipitation centrifuged the contents at 8000 rpm for 30 min. and dissolved DNA pellet in NaCl solution and chloroform (1:1). Spun the content at 14000 rpm for 15 min. followed by transferring the aqueous phase to a fresh tube. Added 0.6 volume of isopropanol and incubate at -20°C after mixing for 30 min. Centrifuged at 14000 rpm for 15 min. and washed the pellet with 70% ethanol, centrifuged at 14000 rpm for 10 min., dried the pellet, and dissolved in TE.

Extraction procedure by kit method

Procedure followed to extract DNA was essentially as mentioned in the user manual of Qiagen DNeasy Plant Mini Kit.

DNA quantitation

Shimadzu spectrophotometer with UV Probe software version 2.52 was used for assessment of DNA quantity and quality OD_{260}/OD_{280}.

Reagents required for real time-qPCR assay

Premix Ex Taq master mix, SYBR Premix Ex Taq, primers and probes for CaMV35S promoter, and primers for chloroplast gene SYBR green based (Table 1), ROX dye, sterile nuclease free water, Tris-EDTA (TE) buffer.

Real time-quantitative Polymerase Chain Reaction (*Real time-qPCR*): All the reagents are thawed, vortex briefly and centrifuged before use. A master mix contained SYBR *Premix Ex Taq* (2X), ROX dye (50X), Primers (10 µM), DNA (20 ng), Sterile water. CRM material was used as positive control and negative control had no DNA. Chloroplast tRNA primers were used as internal positive control (IPC) to demonstrate DNA extraction had occurred successfully.

The master mix for GMO detection contained *Premix Ex Taq* master mix (2X), ROX dye (50X), forward and reverse 35S primers, 35S probes (5 µM), DNA (20 ng), sterile water. CRM material was used as the positive control and for negative control no DNA was added.

The real-time qPCR was performed under the following conditions in Applied Biosystem 7300; 2 min at 50°C, 10 min at 95°C (Denaturation), 15 sec at 95°C (Denaturation), 1 min at 60°C (Annealing and Extension), 95°C /15 sec, 60°C /30 sec, 5°C / 15 min (Dissociation). Reverse and forward primers used for chloroplast and CaMV35S promoter are as described previously (Taberlet et al. 1991; Eede et al. 2000).

Statistical analysis

All analysis were performed using excel function of students TTEST (type 2, tail 2). For calculating *p* values same number of data points were used for each plant material.

Results

For plastome analysis using real-time qPCR technique, a gradient PCR was performed to determine the optimum conditions for amplification using chloroplast tRNA and CaMV35S promoter specific primers (Table 1). Amplicons of approximately 600 bp and 80 bp were obtained for cotton and rice CRMs using chloroplast tRNA and CaMV35S promoter specific primers, respectively (Fig. 1, CaMV35S data not shown). Thereafter, the 600 bp amplicon was gel extracted and sequenced to confirm the presence of chloroplast tRNA gene sequence (Data Not Shown).

Standard curves were generated for real-time qPCR assays performed in cotton CRM using chloroplast tRNA and CaMV35S promoter specific primers for determination of PCR efficiencies, slope and intercept (Fig. 2). For chloroplast tRNA specific primers an efficiency of 127% was achieved and for 35S promoter specific primers an efficiency of 83% was achieved (Data Not Shown). R^2 values of 0.99 and 0.98 were observed for chloroplast and 35S promoter specific primers, respectively (Fig. 2A and Fig. 2B). Ct value for 0.1 pg (100 femtogram) for chloroplast specific primers was 25.79, whereas 35S primers exhibited a value of 37.54 (Fig. 2A and Fig. 2B). The Ct values for PCR blank i.e. without DNA was observed as undetermined and 41.87 for chloroplast and 35S promoter specific primers, respectively (Data Not Shown). A slope of -2.87 and − 3.84 and y intercept of 28.38 and 40.58 was observed for chloroplast and 35S promoter specific primers, respectively (Fig. 2A and 2B).

Chloroplast tRNA specific primers were used for performing real-time qPCR with DNA obtained from different cotton plant organs including seed, leaves, lint and cotton raw, where raw cotton was composed

of lint and seeds, cotton lint was primarily cotton fibers, and cotton leaves were green in color and of different sizes. The Ct values for cotton seed and raw cotton were observed at the mean of 13.45 and 13.61, respectively (Fig. 3). Whereas, the Ct values for cotton leaf were in the range of 8.7 to 23.7 and cotton lint ranged from 11.25 to 25.3 (Fig. 3). However, for cotton leaf majority of samples exhibited Ct values around 13.0, similar to cotton seed and cotton raw. All samples showed statistically significant *p* values calculated by TTEST when compared to cotton seed samples (Fig. 3). The statistical significance increased in the following order; cotton raw, cotton leaf and cotton lint (Fig. 3).

For comparing the plastome copy number of various seed samples including cotton, soybean, maize, sesame and rice, real-time qPCR was performed with equivalent amount of DNA. TTEST was performed to compare Ct values of all the seed samples to cotton seed samples, excluding rice paddy, which was compared to rice seeds. An increasing statistically significant difference was observed in the following order; sesame, soybean and rice (Fig. 4). However, no statistically significant difference was observed between cotton and maize (Fig. 4). It was also observed that rice and maize seed samples exhibited Ct values ranging from 13.5 to 25.4 and 10.2 to 15.8, respectively (Fig. 4). Whereas, rice paddy showed a mean Ct value of 13.51 (Fig. 4). The mean Ct values observed for different seed samples were as follows; Cotton (13.5), Rice (17.1), Soybean (22.7), Maize (13.8), Sesame (9.7), and rice paddy (13.5) (Data Not Shown).

The log plastome copy number was determined using Ct, slope and intercept values obtained from the standard curve of real-time qPCR performed with chloroplast specific primers. The log plastome copy number values were compared to cotton seed excluding rice paddy, which was compared to rice seeds. Based on the TTEST analysis, a significant difference was observed between the log plastome copy numbers of different plant materials, excluding cotton seed and cotton raw, where cotton raw samples were observed to contain both cotton seed and cotton lint (Fig. 5A). Moreover, the range of plastome copy number, calculated as a difference between highest and lowest log plastome copy number values, was observed to be highest for cotton leaf followed by cotton lint and rice seed, whereas the observed range was narrow for cotton seed, cotton raw, sesame seed and rice paddy (Fig. 5B). Intermediate range of log plastome copy number was observed for soybean and maize seed samples (Fig. 5B). The calculated mean log copy number was different for all the plant materials studied (Fig. 5B).

Discussion

Chloroplast, a subtype of plastid, performs the crucial function of photosynthesis in green algae and majority of plants, excluding insectivorous plants. However, during the course of evolution, in higher plants, plastids have differentiated to perform diverse functions including stress perception and response, flowering, fruit ripening, endosperm development and root gravitropism (Waters and Pyke 2004; Beltran et al. 2018; Rolland et al. 2018). Besides having nutritional value, plastids have found application in the field of synthetic biology, where plastids are used for metabolic engineering, because incorporating foreign genes into the plastome results in a high-level foreign protein expression due to absence of gene silencing (Jensen and Scharff 2019). Studies have shown that the plastid number and subtype in a plant

species depends on the cell-type and environmental factors such as light, temperature, and nutrition (Solymosi et al. 2018; Sadali et al. 2019). Therefore, previously plastome has been used as a biomarker for plant species/variety identification (Zhitao et al. 2017). Interestingly, research has also shown that the environmental factors and developmental stage can affect the plastome copy number (Sakamoto and Takami 2018; Oldenburg and Bendich 2015; Greiner et al. 2020). Therefore, this study investigated existence of correlation between plastome copy number and the plant species and the environmental conditions encountered by plant material during growth or post-harvest processing by studying plant material samples acquired during a period of 2 years. Based on data analysis this study hypothesizes that the plastome copy number can be used as an indicator or biomarker of plant species and biosensor of biotic and abiotic conditions encountered by plant material.

Plastids can be studied by isolating intact plastids via centrifugation of cell homogenates and fluorescent microcopy in plant tissue, however to study plastome copy number, real-time qPCR technique has been adopted (Miflin and Beevers, 1974; Sakamoto and Takami, 2018; Greiner et al. 2020). A real-time qPCR technique is different from conventional PCR, where the later detects the amplicon in an end-point analysis and the former detects amplicon as the reaction progresses i.e. amplicon is quantified after each amplification cycle. The main advantage of real-time qPCR over conventional PCR is that former allows determination of the initial copy number of template DNA.

This study used real-time qPCR assays for determining the plastome copy number in cotton, rice, maize, soybean and sesame seeds, which are important fiber, oil and food crops. Plastome copy number for different plant materials was studied by amplifying an approximately 600 bp amplicon using chloroplast tRNA specific primers and confirmed by sequencing (Fig. 1, Sequencing Data Not Shown). An important characteristic distinguishing the plastome from the nuclear genome is its high copy number per organelle and cell. This study shows that real-time qPCR can be used for differentiating nuclear DNA from the chloroplast DNA, based on the Ct values. A higher Ct value for CaMV 35S promoter specific primers was observed, when compared to Ct values for chloroplast specific primers, at the lowest DNA concentration used (Fig. 1A and 1B). *Gossypium hirsutum L.* (MON15985-7), a tetraploid with 26 chromosomes is a genetically modified cotton with CaMV35S promoter integrated into the nuclear DNA. While a cell has a single nucleus, there are more than one plastid and each plastid contains many circular DNA molecules (Martin et al. 2012). This study provides evidence supporting the presence of many plastids in a cell, by observation of lower Ct values and higher copy number (0.9 log value) for chloroplast specific primers, compared to higher Ct values and lower copy number (0.79 log value) for CaMV35S promoter specific primers (Fig. 1A and 1B, copy number data not shown). Although, the observed plastome copy number was higher for chloroplast, the log value difference might have been greater between nuclear and chloroplast DNA if the size of the amplicon for chloroplast (600 bp) and CaMV35S promoter (80 bp) has been more comparable. The consistency of the technique and reliability of the data obtained is supported by generating standard curves, which exhibited efficiencies of 127% and 83% and R^2 values of 0.99 and 0.98 for chloroplast and CaMV35S specific primers, respectively (Fig. 1A and 1B). Efficiency of real-time qPCR reflects that with each cycle DNA is doubled and R^2 value exhibits existence of a good correlation

between the x and y axis values meaning that if either one of the value (x or y) is known the other can be correctly calculated by the linear equation (Fig. 1A and 1B). Based on these observations the data obtained over a period of two years for various plant materials was analyzed to establish possibility of existence of a correlation between plastome copy number and plant species and the environmental conditions encountered by the plant materials.

Analysis of the Ct values for cotton seed (13.45) and cotton raw (13.61) revealed little deviations from the mean values, moreover the observed log plastome copy number for cotton seed (3.85) and cotton raw (3.81) did not show a statistically significant difference indicating that the plastome copy number in seed containing samples of cotton is tightly regulated (Fig. 3), probably because seeds are vehicles for plant propagation, ensuring continuation of species and transmission of genetic information. Additionally, the plastome copy number in cotton seed (3.85) and cotton raw (3.81) exhibited a narrow range of log plastome copy number; 0.58 and 0.31, respectively and absence of statistically significant log variation (Fig. 5A and 5B). Previous studies have shown that the plastome copy number is regulated during development and DNA damage in seeds has been linked to storage under non-ambient conditions (Zoschke et al. 2007; Waterworth et al. 2019), therefore these results might suggest that cotton seeds encountered consistent and ambient environmental conditions during development and post-harvest processing and storage.

Comparison of the mean log plastome copy number values for different seed samples including cotton (3.85), rice (2.9), soybean (1.96), maize (5.05) and sesame (6.49) exhibited statistically significant differences, suggesting that the plastome copy number is plant species specific (Fig. 5B). This study shows that the seeds used for producing oil such as maize and sesame exhibited higher mean log plastome copy number values, further supporting the hypothesis that plastome copy number can be used as a biomarker for determining plant species. These results can be supported by previous studies where the plastid number and type has been shown to be regulated and is plant specific and de novo fatty acid synthesis takes place in plastids (Borisjuk et al. 2005; Solymosi et al. 2018.). The Ct values for various seed samples also exhibited statistically significant differences when compared to cotton seeds, except for maize seeds (Fig. 4). However, subsequent comparison of the mean and range of log plastome copy number values for cotton (3.85, 0.58) and maize (5.05, 1.94) seeds indicated their different origins (Fig. 5A and Fig. 5B). Observation of greater range of plastome copy number in maize can be explained by either presence of different variety of maize seeds in the samples or exposure to varying environmental conditions either during development or post-harvest processing. The former explanation is ruled out as all the maize seed samples were phenotypically similar looking.

The hypothesis that plastome copy number can be used as a biomarker and biosensor is further supported by observation of Ct values, which showed positive deviation from the mean and wide range of plastome copy number (3.09) for rice (21samples) (Fig. 4 and Fig. 5), where the rice samples contained mixture of different colored seeds and broken seed (Data Not Shown). Whereas, samples of rice paddy (rice seeds covered with husk), husk is leafy part and might contain chloroplast or gerontoplast, exhibited lower Ct values when compared to rice seeds without husk and higher log plastome copy number similar

to cotton seeds (Fig. 4 and Fig. 5). Based on these observations it can be speculated that dehusked rice is either exposed to different environmental conditions after harvest or is mixture of different varieties, which ultimately affects plastid number (Waterworth et al. 2019).

Finally, no statistically significant difference was observed between Ct values when cotton seed samples processed in the first year were compared to the cotton seed samples processed in the subsequent year (Data Not Shown), providing support for using real-time qPCR as a highly sensitive technique to differentiate various seed materials and conditions encountered by plant material, based on mean and range of log plastome copy number.

Our hypothesis that plastome copy number can be used as a biosensor of environmental conditions encountered by plant materials is supported by observation of Ct values showing deviation (both positive and negative) from mean Ct value (13.0) and greater range (4.11) observed for cotton leaf samples (Fig. 3). A greater range for cotton leaves as opposed to cotton seeds could be due to different developmental stages of leaves regulated by internal (cell type, phytohormones) and external stimuli (light, temperature) (López-Juez 2007; Grigorova et al. 2012), which supports our hypothesis that range of plastome copy number can be used as a biosensor. Similarly, observation of greater deviation from mean Ct value (16.05) and range of plastome copy number (3.63) for cotton lint (Figs. 3 and 5), which is a single-celled trichome and an extension of the epidermal cell of the ovule, indicating variation in developmental stage or environmental conditions encountered by lint post-harvest (Kawano et al. 1989). Our observations and analysis is supported by previous studies that have shown that plastome of plants is affected by various abiotic and biotic factors (Baumgartner et al. 1989; Zachman 2019; Figlioli et al. 2019).

Conclusion

Based on data analysis, it can be concluded that real-time qPCR assay can be used for determining the plastome copy number, subsequently, the mean and range of log plastome copy number can be used as biomarker for plant species determination and biosensor for various abiotic and biotic conditions that a particular plant has encountered during growth and development and post-harvest storage and processing. This method can possibly be translated into industrial application for determining the quality of commercially available oil seeds, herbs (parsley, tea, coffee), carotenoid rich eggs and cereals to name a few plant-based food product. This study further proposes use of real-time qPCR in metabolic engineering for determining the copy number of transgene and regulating conditions required for maintaining optimum plastome copy number and plastid number in a plant organs for optimum production of valuable metabolites and proteins. Additionally, with increased global usage of GMO plants and tissue culture grown plants, plastome copy number can serve as a reliable indicator of the conditions maintained for generating such plants. However, this is a preliminary study and in future experiments need to be conducted to study effect of developmental stage and external factors on the plastome copy number.

Declarations

Conflicts of interest/Competing interests: Authors have no conflict of interests.

Ethics approval: Not required

Consent to participate: All others have contributed towards the manuscript preparation.

Consent for publication: All authors have read and approved manuscript for publication.

Availability of data and material: Data is available on request.

Authors' contributions: SC conducted the experiments, AP conceived the idea, analyzed the data, and prepared the manuscript, SC, BB and AP read and approved the manuscript.

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References

1. Baumgartner BJ, Rapp JC, Mullet JE (1989) Plastid transcription activity and DNA copy number increase early in barley chloroplast development. Plant Physiol. 89(3):1011-1018. doi: 10.1104/pp.89.3.1011
2. Beltrán J, Wamboldt Y, Sanchez R, LaBrant EW, Kundariya H, Virdi KS, Elowsky C, Mackenzie SA (2018) Specialized Plastids Trigger Tissue-Specific Signaling for Systemic Stress Response in Plants. Plant Physiol. 178(2):672-683. doi: 10.1104/pp.18.00804
3. Borisjuk L, Nguyen TH, Neuberger T, Rutten T, Tschiersch H, Claus B, Feussner I, Webb AG, Jakob P, Weber H, Wobus U, Rolletschek H (2005) Gradients of lipid storage, photosynthesis and plastid differentiation in developing soybean seeds. New Phytol 167(3):761-76. doi: 10.1111/j.1469-8137.2005.01474.x.
4. de Vries J, Archibald JM (2018) Plastid genomes Current Biology. 28:R329-R341. doi: 10.1016/j.cub.2018.01.027.
5. Dupont FM (2008) Metabolic pathways of the wheat (*Triticum aestivum*) endosperm amyloplast revealed by proteomics. BMC Plant Biol. 8:39. doi: 10.1186/1471-2229-8-39. doi: 10.1186/1471-2229-8-39.
6. Eede et al., 2000. Validation of an analytical method for the detection of GMO-derived DNA in processed foodstuffs. EUR 19677 EN, Report issued by the IHCP ISPRA

7. Fernández AP, Strand A (2008) Retrograde signaling and plant stress: plastid signals initiate cellular stress responses. Curr Opin Plant Biol. 11(5):509-513. doi: 10.1016/j.pbi.2008.06.002
8. Figlioli F, Sorrentino MC, Memoli V, Arena C, Maisto G, Giordano S, Capozzi F, Spagnuolo V (2019) Overall plant responses to Cd and Pb metal stress in maize: Growth pattern, ultrastructure, and photosynthetic activity. Environ Sci Pollut Res Int. 26(2):1781-1790. doi: 10.1007/s11356-018-3743-y
9. Galili G (1995) Regulation of Lysine and Threonine Synthesis. The Plant Cell. 7:899-906. doi: 10.1105/tpc.7.7.899
10. Gray MW (1992) The endosymbiont hypothesis revisited. International Review of Cytology 141:233-357. doi: 10.1016/s0074-7696(08)62068-9
11. Grigorova B, Vassileva A, Klimchuk D, Vaseva I, Demirevska K, Feller U (2012) Drought, high temperature, and their combination affect ultrastructure of chloroplasts and mitochondria in wheat (*Triticum aestivum*) leaves. Journal of Plant Interactions. 7(3):204-213. doi:org/10.1080/17429145.2011.654134
12. Jarvis P, López-Juez E (2013) Biogenesis and homeostasis of chloroplasts and other plastids. Nat Rev Mol Cell Biol. 14(12):787-802. doi: 10.1038/nrm3702
13. Jensen PE, Scharff LB (2019) Engineering of plastids to optimize the production of high-value metabolites and proteins. Curr Opin Biotechnol. 59:8-15. doi: 10.1016/j.copbio.2019.01.009
14. Kawano SS, Tano S, Kuroiwa T (1989) Preferential digestion of chloroplast nuclei (nucleoids) during senescence of the coleoptile of *Oryza sativa*. Protoplasma. 152:65-68. doi:10.1007/BF01323063
15. López-Juez E (2007) Plastid biogenesis, between light and shadows. Journal of Experimental Botany. 58(1):11-26. doi: 10.1093/jxb/erl196
16. Lu Y, Yao J (2018) Chloroplasts at the Crossroad of Photosynthesis, Pathogen Infection and Plant Defense. Int J Mol Sci. 19(12):3900. doi: 10.3390/ijms19123900
17. Magdy M, Ou L, Yu H, Chen R, Zhou Y, Hassan H, Feng B, Taitano N, van der Knaap E, Zou X, Li F, Ouyang B (2019) Pan-plastome approach empowers the assessment of genetic variation in cultivated Capsicum species. Hortic Res. 6:108. doi: 10.1038/s41438-019-0191-x
18. Marano MR, Serra EC, Orellano EG, Carrillo N (1993) The path of chromoplast development in fruits and flowers. Plant Sci. 94:1-17. doi:10.1016/0168-9452(93)90002-H
19. Martin W, Rujan T, Richly E, Hansen A, Cornelsen S, Lins, Leister TD, Stoebe B, Hasegawa M, Penny D (2002) Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. Proc Natl Acad Sci U S A. 99(19):12246-51. doi: 10.1073/pnas.182432999
20. Mellor SB, Behrendorff JBYH, Nielsen AZ, Jensen PE, Pribil M (2018) Non-photosynthetic plastids as hosts for metabolic engineering. Essays Biochem. 62(1):41-50. doi: 10.1042/EBC20170047
21. Miflin BJ, Beevers H (1974) Isolation of intact plastids from a range of plant tissues. Plant Physiol. 53(6):870-4. doi: 10.1104/pp.53.6.870
22. J Ohlrogge 1, J Browse (1995) Lipid biosynthesis. Plant Cell 7(7):957-70. doi: 10.1105/tpc.7.7.957

23. Oldenburg DJ, Bendich AJ (2015) DNA maintenance in plastids of plants. Plant Sci. Plant Sci. 6:883. doi.org/10.3389/fpls.2015.00883
24. Price CA, Hadjeb N, Newman LA, Reardon EM (1995) Chromoplasts. Methods Cell Biol. 50:189-207.
25. Pyke KA, Page AM (1998) Plastid ontogeny during petal development in Arabidopsis. Plant Physiol. 116(2):797-803. doi: 10.1104/pp.116.2.797
26. Rolland N. et al. 2018. The main functions of plastids. Methods Mol. Biol. 1829:73-85
27. Sakamoto W, Takami T (2018) Chloroplast DNA Dynamics: Copy Number, Quality Control and Degradation. Plant Cell Physiol. 59(6):1120-1127. doi: 10.1093/pcp/pcy084
28. Sadali NM, Sowden RG, Ling Q, Jarvis RP (2019) Differentiation of chromoplasts and other plastids in plants. Plant Cell Rep. 38(7):803-818. doi: 10.1007/s00299-019-02420-2
29. Greiner S, Golczyk H, Malinova I, Pellizzer T, Bock R, Börner T, Herrmann RG (2020) Chloroplast nucleoids are highly dynamic in ploidy, number, and structure during angiosperm leaf development. Plant J. 102(4):730-746. doi: 10.1111/tpj.14658.
30. Solymosi K, Lethin J, Aronsson H (2018) Diversity and Plasticity of Plastids in Land Plants. Methods Mol Biol. 1829:55-72. doi: 10.1007/978-1-4939-8654-5_4
31. Su S-H, Gibbs NM, Jancewicz AL, Patrick H Masson PH (2017) Molecular Mechanisms of Root Gravitropism. Curr Biol 27(17):R964-R972. doi: 10.1016/j.cub.2017.07.015.
32. Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol. 17(5):1105-9. doi: 10.1007/BF00037152
33. Thomson WW, Whatley JM (1980) Development of Nongreen Plastids. Annual Review of Plant Physiology. 31:375-394. doi.: 10.1146/annurev.pp.31.060180.002111
34. Woodson JD (2019) Chloroplast stress signals: regulation of cellular degradation and chloroplast turnover. Curr Opin Plant Biol. 52:30-37. doi: 10.1016/j.pbi.2019.06.005
35. Waters M, Pyke K (2018) Plastid Development and Differentiation. Annual Plant Reviews book series, Volume 13: Plastids. doi: 10.1002/9781119312994.apr0122
36. Sakamoto W, Takami T (2018) Chloroplast DNA Dynamics: Copy Number, Quality Control and Degradation. Cell Physiol 59(6):1120-1127. doi: 10.1093/pcp/pcy084
37. Zechmann B (2019) Ultrastructure of plastids serves as reliable abiotic and biotic stress marker. PLoS One 14(4):e0214811. doi: 10.1371/journal.pone.0214811
38. Zhitao N, Shuying Z, Jiajia P, Ludan L, Jing S, Xiaoyu D (2017) Comparative analysis of Dendrobium plastomes and utility of plastomic mutational hotspots. Scientific Reports 7: 2073
39. Zoschke R, Liere K, Börner T (2007) From seedling to mature plant: arabidopsis plastidial genome copy number, RNA accumulation and transcription are differentially regulated during leaf development. Plant J 50(4):710-22. doi: 10.1111/j.1365-313X.2007.03084.x

Tables

Table 1: Primers and probes used for amplification chloroplast and CaMV 35S promoter sequences in cooton and rice CRMs and plant based samples processed over a period of two years.

Chloroplast tRNA gene	5'-GGG GAT AGA GGG ACT TGA AC-3'
	5'-CGA AAT CGG TAG ACG CTA CG-3'
CaMV 35S promoter	5'-GCC TCT GCC GAC AGT GGT-3'
	5'-AAG ACG TGG TTG GAA CGT CTT C-3'
35S probe	5'-[6FAM]CAAAGATGGACCCCCACCCACG[TAM]-3'

Figures

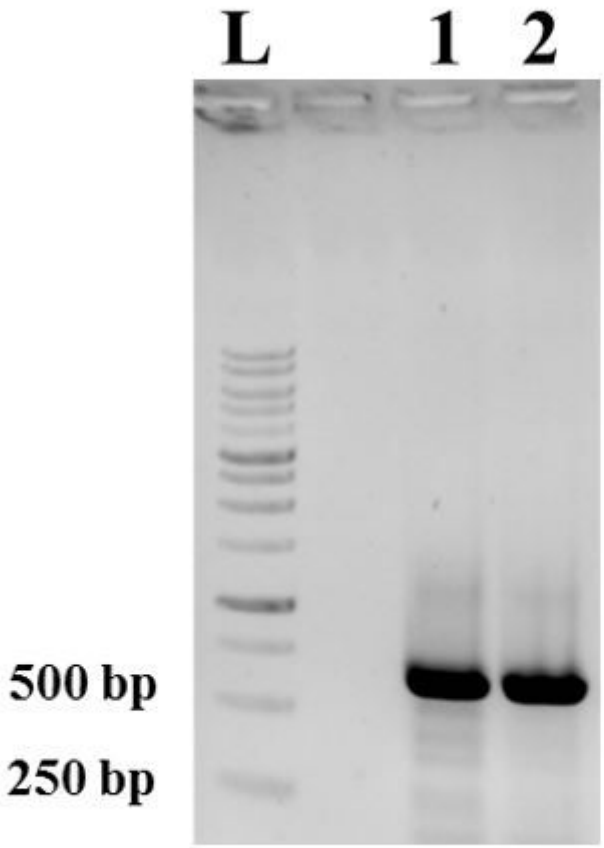


Figure 1

Agarose gel electrophoresis of chloroplast amplicon: 1ng of rice and cotton genomic DNA each was used for polymerase chain reaction (PCR). The amplified products were size separated by agarose gel

electrophoresis (0.8%). Amplicons were observed for both cotton (lane 1) and rice (lane 2), a 1 kb DNA ladder (lane L) was used for size determination.

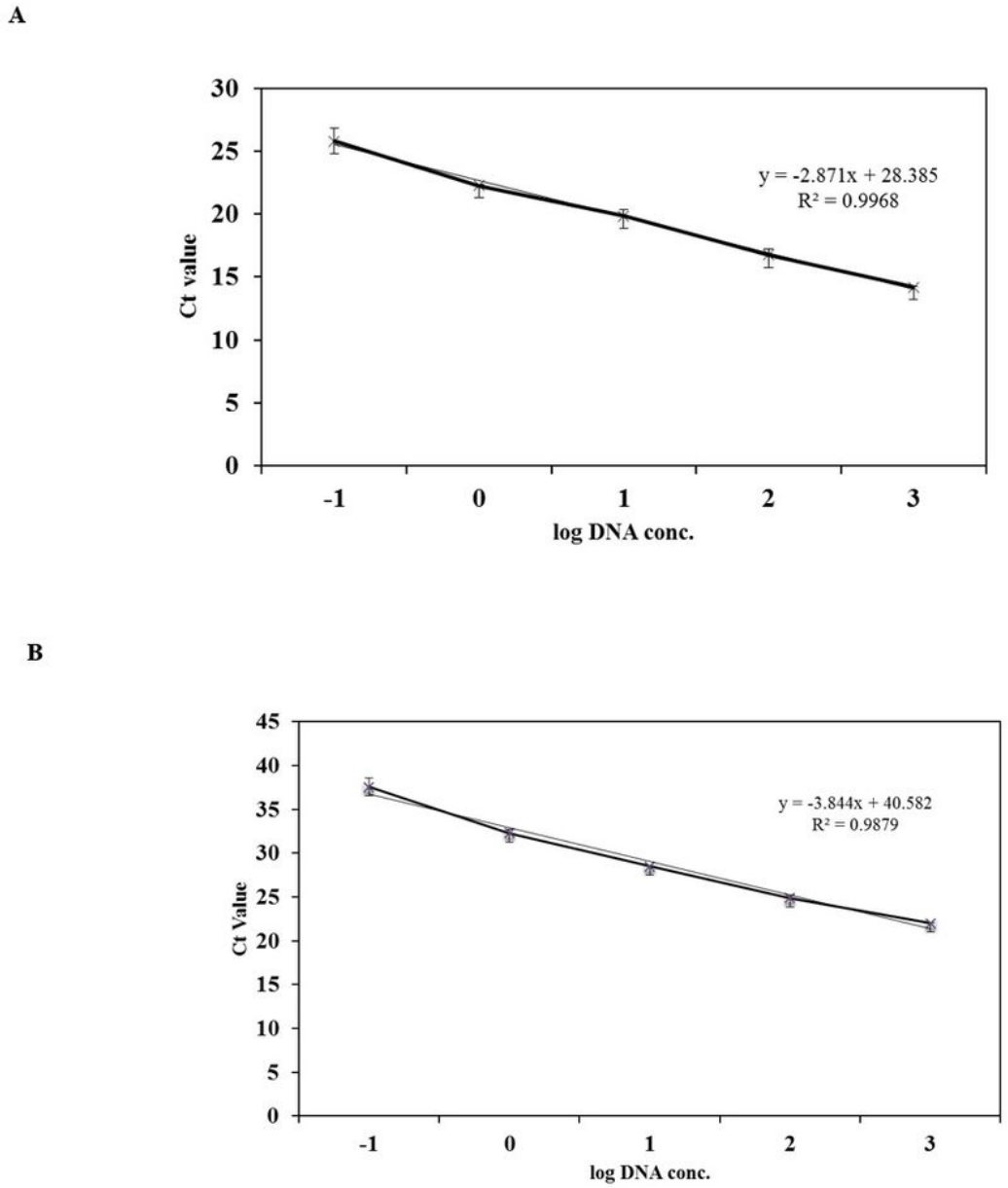


Figure 2

Standard curves for chloroplast tRNA and CaMV 35S promoter for cotton reference material: Five log DNA concemtrations corresponding to 0.1 picogram (pg) (-1 log), 1 pg (0 log), 10 pg (1 log), 100 pg (2 log), and 1000 pg (3 log) were used for generation of the standard curves. (A) Standard curve for

chloroplast tRNA showing a slope of -2.871 and a y intercept of 28.285. (B) Standard curve for CaMV 35S promoter showing a slope of -3.844 and a y intercept value of 40.582. Real-time qPCR assays were performed in Applied Biosystem 7300 under following conditions; 2 min at 50°C, 10 min at 95°C (Denaturation), 15 sec at 95°C (Denaturation), 1 min at 60°C (Annealing and Extension), for a total of 45 cycles. SYBR green was used as dye for detection of chloroplast amplicons and 35S FAM probe was used for 35S promoter (Table 1). Regression analysis was performed to determine the reliability of method and PCR efficiencies were calculated as follows: 10(-1/slope)*100.

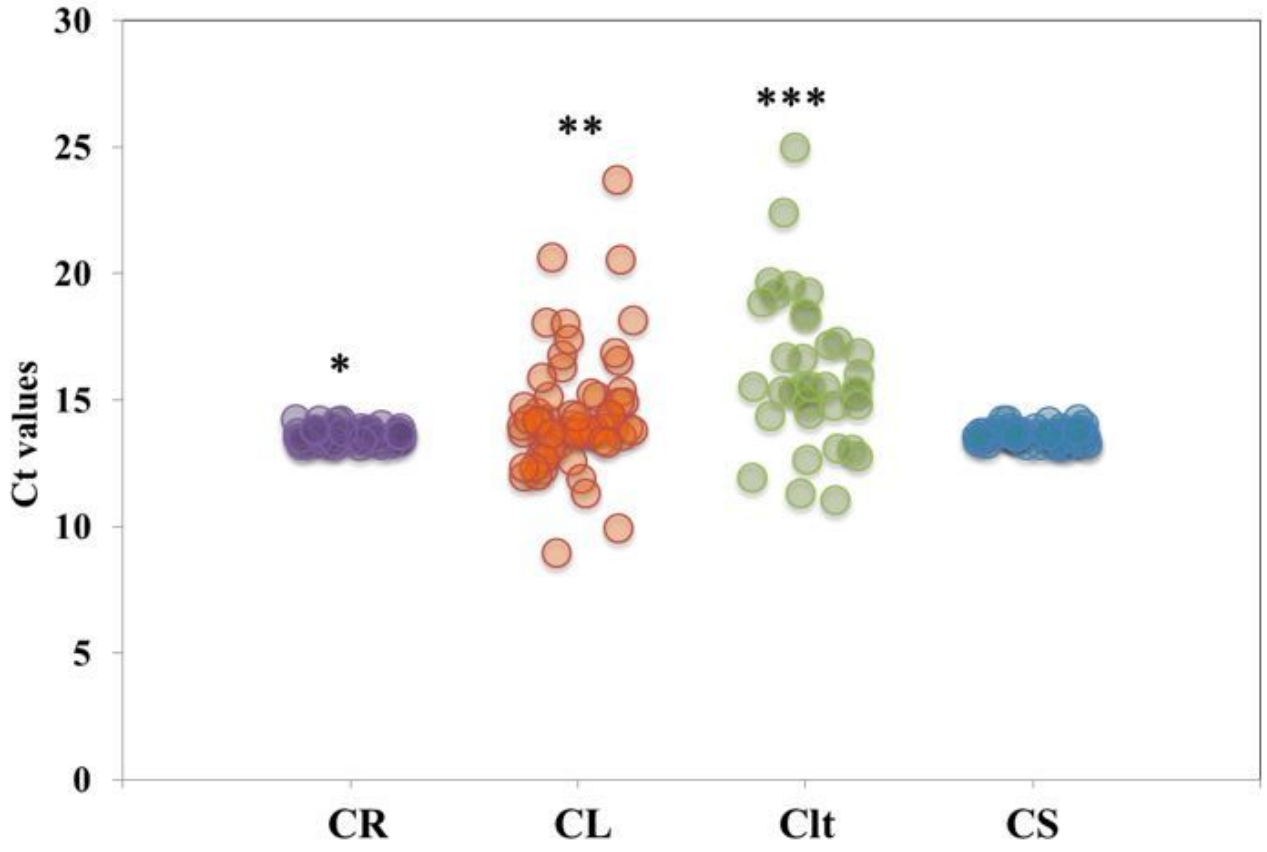


Figure 3

please see the manuscript file for the full caption

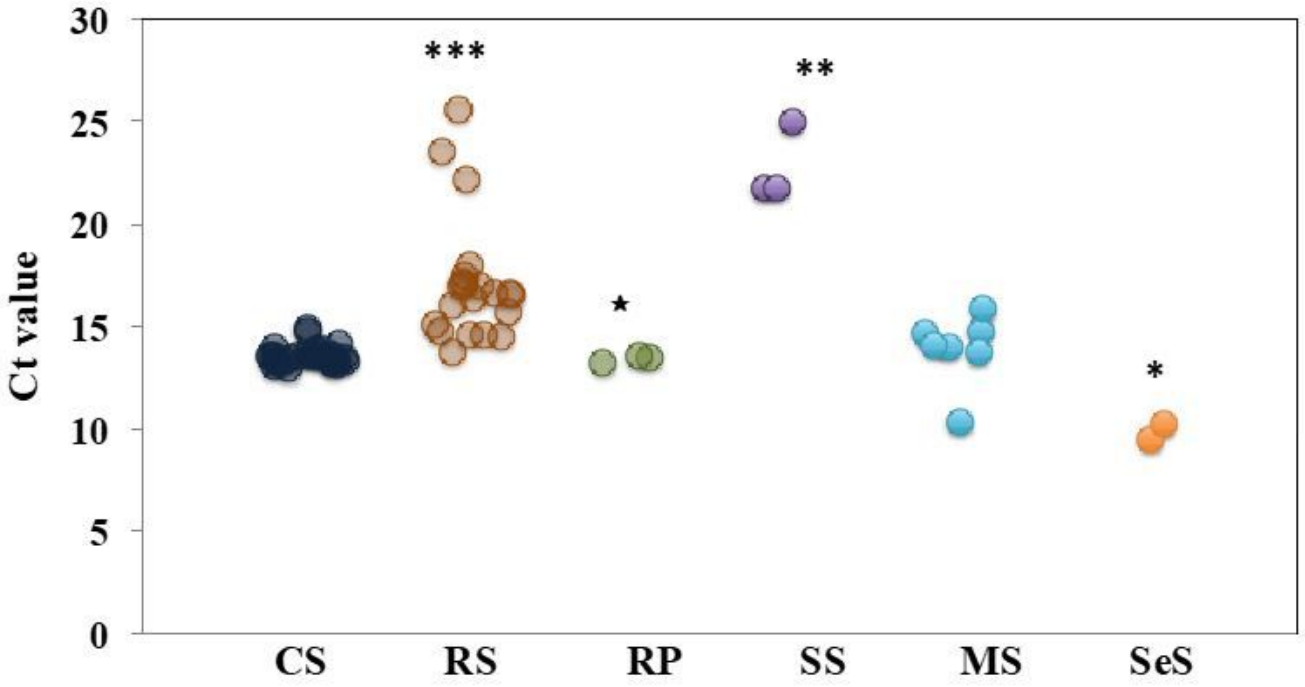
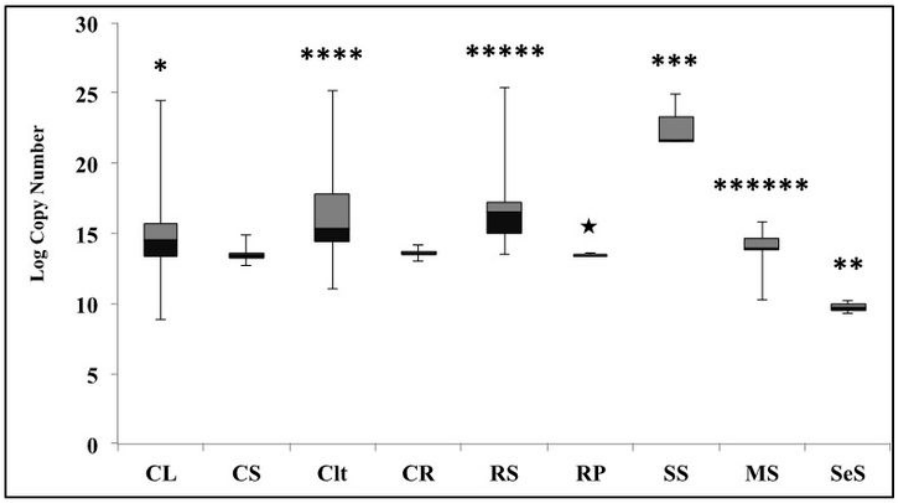


Figure 4

please see the manuscript file for the full caption

A



B

	CR	CL	Clt	CS	RS	RP	SS	MS	SeS
Mean	3.81	4.18	3.22	3.85	2.90	3.86	1.96	5.05	6.49
Range	0.31	4.11	3.63	0.58	3.09	0.05	1.17	1.94	0.31

Figure 5

please see the manuscript file for the full caption