Current Genomics, 2022, *23*, 289-298

RESEARCH ARTICLE

The Potential Role of Plastome Copy Number as a Quality Biomarker for Plant Products using Real-time Quantitative Polymerase Chain Reaction

Amita Pandey[1,*], Shifa Chaudhary[1] and Binu Bhat[1]

[1]*19 University Road, Shriram Institute for Industrial Research, Analytical Science Division - Biology, Molecular Biology Laboratory, New Delhi, India*

ARTICLE HISTORY

Received: January 21, 2022
Revised: February 28, 2022
Accepted: March 18, 2022

DOI:
10.2174/1389202923666220513111643

Abstract: ***Background*:** Plastids are plant-specific semi-autonomous self-replicating organelles, containing circular DNA molecules called plastomes. Plastids perform crucial functions, including photosynthesis, stress perception and response, synthesis of metabolites, and storage. The plastome and plastid numbers have been shown to be modulated by developmental stage and environmental stimuli and have been used as a biomarker (identification of plant species) and biosensor (an indicator of abiotic and biotic stresses). However, the determination of plastome sequence and plastid number is a laborious process requiring sophisticated equipment.

***Methods*:** This study proposes using plastome copy number (PCN), which can be determined rapidly by real-time quantitative polymerase chain reaction (RT-qPCR) as a plant product quality biomarker. This study shows that the PCN $\log_{10}$ and range PCN $\log_{10}$ values calculated from RT-qPCR data, which was obtained for two years from leaves and lint samples of cotton and seed samples of cotton, rice, soybean, maize, and sesame can be used for assessing the quality of the samples.

***Results*:** Observation of lower range PCN $\log_{10}$ values for CS (0.31) and CR (0.58) indicated that the PCN showed little variance from the mean PCN $\log_{10}$ values for CS (3.81) and CR (3.85), suggesting that these samples might have encountered ambient environmental conditions during growth and/ or post-harvest storage and processing. This conclusion was further supported by observation of higher range PCN $\log_{10}$ values for RS (3.09) *versus* RP (0.05), where rice seeds in the RP group had protective hull covering compared to broken hull-less seeds in the RS group. To further support that PCN is affected by external factors, rice seeds treated with high temperatures and pathogens exhibited lower PCN values when compared to untreated seeds. Furthermore, the range PCN $\log_{10}$ values were found to be high for cotton leaf (CL) and lint (Clt) sample groups, 4.11 and 3.63, respectively, where leaf and lint samples were of different sizes, indicating that leaf samples might be of different developmental stage and lint samples might have been processed differently, supporting that the PCN is affected by both internal and external factors, respectively. Moreover, PCN $\log_{10}$ values were found to be plant specific, with oil containing seeds such as SeS (6.49) and MS (5.05) exhibiting high PCN $\log_{10}$ values compared to non-oil seeds such as SS (1.96).

***Conclusion*:** In conclusion, it was observed that PCN $\log_{10}$ values calculated from RT-qPCR assays were specific to plant species and the range of PCN $\log_{10}$ values can be directly correlated to the internal and external factors and, therefore might be used as a potential biomarker for assessing the quality of plant products.

Keywords: Plastids, plastome, plastome copy number, real-time qPCR, biomarker, plant product quality.

1. INTRODUCTION

Plastids are semi-autonomous organelles distinguishing plant cells from other eukaryotic cells, which are acquired by endosymbiosis of a photosynthetic prokaryote by a eukaryotic cell [1]. 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 [2, 3]. Members of the plastid family play pivotal roles in storage, coloration, gravitropism, stomatal functioning, and perception [4]. Like mitochondria, plastids are maternally inherited and are derived from small, undifferentiated proplastids found in the meristematic tissue, which differentiate into subtypes depending on the cell-type and developmental processes such as seed germination, flowering, and fruit ripening [5]. Plastids contain their own genome/DNA also called plastome, which is 120-160 kbp of about 22 to 200 circular DNA copies with 100-120 highly conserved genes [6, 7].

*Address correspondence to this author at the 19 University Road, Shriram Institute for Industrial Research, Analytical Science Division - Biology, Molecular Biology Laboratory, New Delhi, India; Tel: 09811810147;
E-mail: ampandey@shriraminstitute.org

1875-5488/22 $65.00+.00

Plastid biogenesis and differentiation have been associated with environmental stress [8, 9]. They have been shown to be involved in the biosynthesis of stress-related phytohormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and the production of reactive oxygen species (ROS) [10-13]. Studies in Arabidopsis have shown that plastids undergo a process of degradation due to high light intensity, *Botrytis* infection and senescence resulting in reduction in size and number of chloroplast in the leaves of Arabidopsis [14]. Moreover, chloroplast regulates their own degradation during stress conditions by inducing autophagy [15]. Similarly, the PCN has been shown to vary during plant development when compared to the nuclear genome [16, 17].

Chloroplast, amyloplast and chromoplast are the most studied plastid subtypes because of their direct relevance to the nutritional quality of crops. While chloroplast are the main photosynthetic plastids affecting the overall quality of plant growth and ultimately plant products, amyloplast, which store starch in roots, tubers, and seed endosperm and chromoplast, rich in carotenoids, present in vegetables and fruits are equally beneficial for human health [18]. Drought and high temperature affect the seed filling process, affecting the final quality and quantity of seed yield [19, 20]. Despite the fact that dry seeds are metabolically quiescent, exposure to higher moisture and temperature can induce ROS production and lipid peroxidation, damaging cell organelles and DNA [21-23]. Seeds are not only stored for human consumption but also for the preservation of biodiversity in seed banks. Therefore, it is critical to regularly monitor seed viability and quality by seed germination activities. Nucleic acid amplification-based assays have been used for assessing seed health/ quality by detecting seed pathogens [24, 25]. However, these assays are not designed to assess the damage caused by abiotic stresses.

This study proposes the application of PCN determined by RT-qPCR in plant organs, including seed (with and without husk), leaf, and lint, in assessing the effect of biotic and abiotic stresses on seed quality/health. This technique for the assessment of plant product quality is proposed based on previous research that shows that plastid biogenesis and PCN are affected by plant stresses. This study involves RT-qPCR data mining, which was collected over a period of two years for genetically modified organism (GMO) event detection using cauliflower mosaic virus (CaMV) 35S promoter and chloroplast tRNA specific primers. The role of PCN as a quality biomarker was correlated with the observed PCN $\log_{10}$ and range PCN $\log_{10}$ values. Additional experimental support to the hypothesis was provided by treating rice seeds with biotic and abiotic stresses. A decrease in PCN was observed for rice seeds treated with high temperature and pathogen when compared to untreated seeds.

2. MATERIALS AND METHODS

2.1. Plant Material

Seed samples of cotton (78), rice (20), soybean (2), maize (7), sesame (3), and paddy (3) and cotton plant products such as lint (34), cotton raw (48), and leaf (61) were obtained for detection of GMO event over a period of two years. The visual appearance of the samples was noted and was as follows; some of the rice samples were composed of broken de-hulled seeds, cotton raw was composed of lint covered seeds, cotton leaves were green and of different sizes, and rice paddy had seeds covered with the protective hull.

2.2. Abiotic Stress Experiments

Rice seeds were exposed to high temperature (40°C - 50°C) and pathogen (at room temperature) for 7 and 11 days, respectively. For pathogen treatment, rice seeds were placed in an unlidded petri plate, inside a glass dish partially filled with water, and covered with cling wrap to maintain the humid conditions required for microbial growth. Total DNA (tDNA) was isolated from 170 mg seed tissue using DNeasy Plant Mini Kit (Qiagen). Subsequently, 20 ng of the DNA was used for real-time qPCR with CaMV 35S promoter and chloroplast tRNA-specific primers.

2.3. Extraction Procedure for Lint and Seeds

To 300 mg of homogenized fiber, leaves, seeds or grains, added 1.5 ml of pre-warmed (65°C) CTAB extraction buffer [CTAB (20g/l), NaCl (1.4 M/l), Tris-Cl (0.1M/l), EDTA (0.2 M/l, pH 8.0] and vortexed. 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 minutes. Collected the supernatant and added 1 volume of chloroform and mixed thoroughly, followed by centrifugation at 14000 rpm and collection of the aqueous phase. To this added 2 volumes of CTAB precipitation buffer [CTAB (20g/l), NaCl (0.04 M/l)] 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 (1.2 M) and chloroform (1:1). Centrifuged at 14000 rpm for 10 min and to the aqueous phase added 0.6 volume chilled isopropanol followed by incubation at room temperature for 20 min. Subsequently, centrifuged at 14000 rpm for 15 min. and discarded the supernatant, washed the pellet with 70% ethanol and suspended in TE [Tris-Cl (0.01 M), Na_2EDTA (0.001 M), pH 8.0].

2.4. Extraction Procedure by Kit Method

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

2.5. Reagents Required for the Real time-qPCR Assay

Premix Ex Taq master mix (Takara), SYBR and TB green Premix Ex Taq (Takara), primers and probes for CaMV 35S promoter, and primers for chloroplast tRNA gene (Table **1**), ROX dye, sterile nuclease free water, Tris-EDTA (TE) buffer.

2.6. Real time-qPCR (RT-qPCR)

All the reagents were thawed, vortexed briefly and centrifuged before use. A master mix contained SYBR *Premix Ex Taq* (1X), ROX dye (1X), Primers (0.3 μM), and sterile

Table 1. Sequences of primers and probes: The table shows sequences of primers and probes used for amplification of chloroplast tRNA and cauliflower mosaic virus (CaMV) 35S promoter sequences obtained from different plant materials.

Primer Name	Primer Sequence
Chloroplast tRNA	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 3'
CaMV 35S Probe	5' [6FAM] CAA AGA TGG ACC CCC ACC CAC G [TAM] 3'

water. Certified Reference Material (CRM) was obtained, including cotton (MON15985-7) and rice (LLRice62) from The American Oil Chemists Society (AOCS), which served as a positive control. CaMV 35S specific primers and chloroplast tRNA primers were used for GMO detection and internal positive control, respectively to demonstrate DNA extraction had occurred successfully [26]. Chloroplast tRNA primers were used since the tRNA gene is highly conserved in various plastid subtypes.

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, 1 cycle); 15 sec at 95°C and 1 min at 60°C (Annealing and Extension, 45 cycles); 95°C /15 sec, 60°C /30 sec, 5°C / 15 min (Dissociation, 1 cycle).

2.7. Statistical Analysis

Statistical analysis was performed using one-way ANOVA when mean values of more than two groups were compared. '*p*' values were calculated with F*statistic*, Df*b*, and Df*w* values. Students TTEST was performed for pairwise analysis. '*p*' values of < 0.05 were considered to be statistically significant.

3. RESULTS

For plastome analysis using the RT-qPCR technique, a gradient PCR was performed to determine the optimum conditions required for amplification of the desired amplicon. Subsequently, a product of approximately 600 bp was observed for both cotton and rice CRM (Supplementary Fig. **S1**), which was verified by sequencing and blast (Data not shown).

Standard curves were generated for cotton CRM with chloroplast tRNA and CaMV 35S promoter-specific primers to determine PCR efficiency, slope and intercept (Fig. **1A** and **1B**). PCR efficiencies calculated on the basis of the slope were observed as 127% and 83% for chloroplast and CaMV 35S promoter-specific primers, respectively (Data Not Shown). R^2 values of 0.99 and 0.98 and slope of -2.87 and -3.84 were observed for chloroplast and CaMV 35S promoter specific primers, respectively (Fig. **1A** and **1B**). The limit of detection (LOD) was found to be 1 picogram of DNA for chloroplast specific primers (Ct value 25.79) and CaMV 35S primers (Ct value of 37.54) (Fig. **1A** and **3B**). The Ct values for PCR blank, which did not contain DNA was observed as undetermined and 41.87 for chloroplast and CaMV 35S promoter-specific primers pairs, respectively (Data Not Shown).

Chloroplast tRNA specific primers were used for performing RT-qPCR with tDNA obtained from different cotton sample groups including cotton seeds (CS), leaves (CL), lint (Clt) and cotton raw (CR). The mean Ct values observed for CS (13.45), CR (13.61), CL (14.42), and Clt (16.05) groups exhibited low percentage of variance for CS (2.47%) and CR (1.79%) and higher variance for CL (16.7%) and Clt (18.75%) within samples of each group. Low variance indicated that the percentage of seeds with Ct values close to the mean Ct value was high (Fig. **2** and Table **S1**). A one-way ANOVA analysis was performed, which showed statistically significant difference, *i.e.* p <0.05, between all the four cotton sample groups (Data not shown). Whereas, no statistically significant difference *i.e.* p >0.05 was observed between CR and CS groups.

The observed mean Ct values of for seed sample groups were as follows; SeS (9.76) < RP (13.42) < CS (13.45) < MS (13.82) < RS (17.11) < SS (22.72) (Fig. **3**). It was also observed that RS (17.66%) and MS (12.51%) exhibited highest percentage of variation within the group, although the observed variance was within the acceptable range *i.e.* CV <30% (Table **S1**). One-way ANOVA analysis showed a statistically significant difference (p <0.05) between the mean Ct values of different seed groups.

The PCN value was determined by using a linear quadratic equation obtained from the cotton standard curve generated with chloroplast specific primers. The observed mean PCN $\log_{10}$ values for seed sample groups were as follows; SeS (6.49) > MS (5.05) > CS (3.85) > RS (2.90) > SS (1.96) (Fig. **4**). Similarly, the observed mean PCN $\log_{10}$ values for cotton plant samples were as follows; CL (4.18) > CR (3.85) > CS (3.81) > Clt (3.22) (Fig. **4**). The number of plastome copies were observed to be highest in SeS (305492.1) and lowest in SS (91.90) (Table **S2**). Moreover, comparison of mean PCN $\log_{10}$ values using one-way ANOVA analysis exhibited a statistically significant difference *i.e.* p <0.05 for the seed sample groups. The observed variance was within the acceptable limit *i.e.* CV <30% for all the groups with the exception of SS (34.55%) (Data Not Shown).

In order to determine the variation in PCN for samples in each group, the range PCN $\log_{10}$ was calculated by subtracting minimum PCN $\log_{10}$ value from maximum PCN $\log_{10}$ value (Fig. **4** and Table **S2**). The range PCN

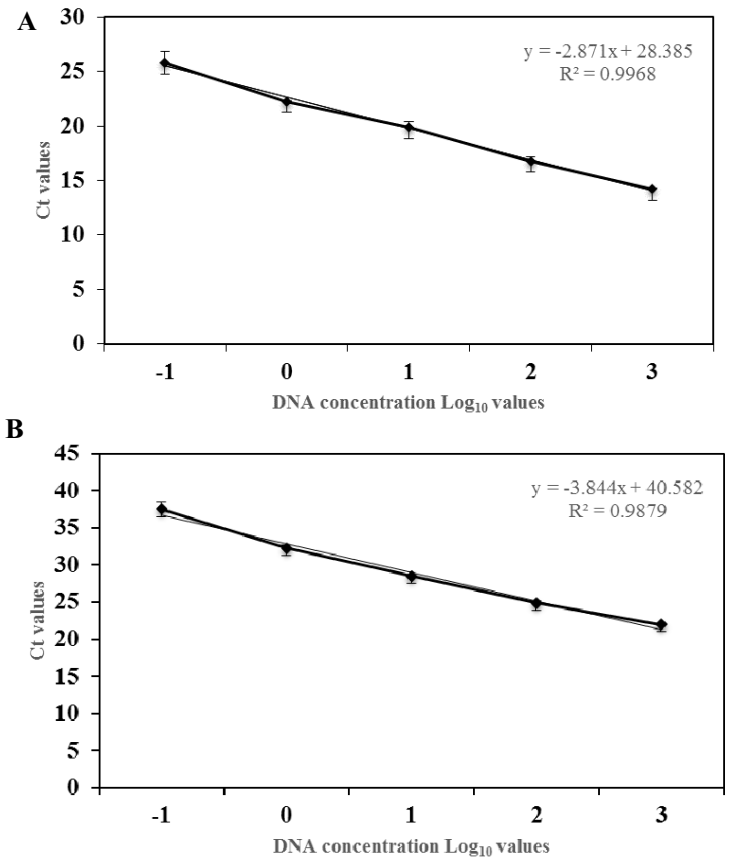

Fig. (1). Standard curves for real-time qPCR assays performed with chloroplast tRNA and CaMV 35S promoter specific primers for cotton reference material (CRM): Five log DNA values corresponding to 0.1 picogram (pg) (-1 log), 1 pg (0 log), 10 pg (1 log), 100 pg (2 log), and 1000 pg (3 log) of DNA concentration were used for generation of the standard curves. Real-time qPCR was performed in Applied Biosystem 7300. SYBR green and FAM probe were used for detection of chloroplast and CaMV 35S amplicons, respectively (Table **1**). PCR efficiencies and R^2 values were calculated from the linear equation. (**A**) The limit of detection (LOD) for chloroplast specific primers was 0.1 pg at a Ct value of 25.79 and PCN of 7.99. (**B**) The limit of detection (LOD) for CaMV 35S specific primers was 0.1 pg at a Ct value of 37.54 and PCN of 5.97. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

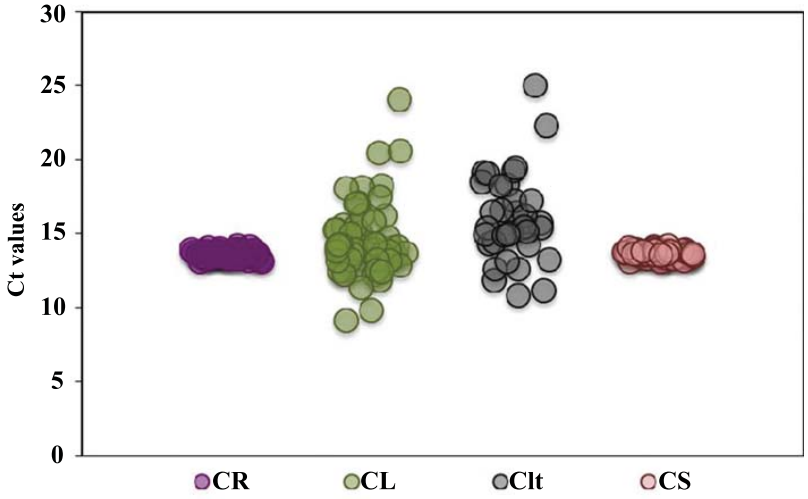

Fig. (2). Scatter plot comparing mean Ct values and variance for cotton sample groups: 20 ng total DNA was used for real-time qPCR using chloroplast tRNA specific primers from different cotton organs including 78 cotton seed (CS), 61 cotton leaf (CL), 34 cotton lint (Clt) and 48 cotton raw (CR) samples. Real-time qPCR was performed in ABI 7300 real-time machine using SYBR green as fluorescent dye. One-way ANOVA analysis was performed, where a significant difference of was observed in the mean Ct values (indicated above each group *i.e.* $p < 0.05$. CL and Clt samples exhibited high variance in Ct values when compared to CS and CR samples (Table **S1**). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

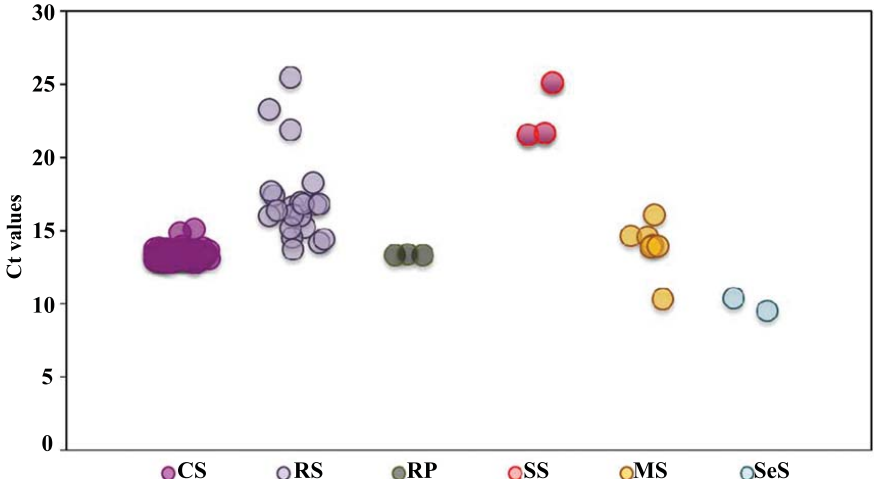

Fig. (3). Scatter plot comparing mean Ct values for seed sample groups: Equal amount (20 ng) of total DNA from 78 cotton seed (CS), 20 rice seed (RS), 3 soybean seed (SS), 7 maize seed (MS), 2 sesame seed (SeS) and 3 rice paddy (RP) samples was used for real-time qPCR using chloroplast tRNA specific primers. Plot showing a mean cycle threshold (Ct) value was generated. Real-time qPCR was performed in ABI 7300 real time machine using SYBR green as fluorescent dye to detect amplification. One-way ANOVA analysis was performed, where a significant difference of was observed in the mean Ct values (indicated above each group) *i.e.* $p < 0.05$. Highest variance in the Ct values was observed for RS samples. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

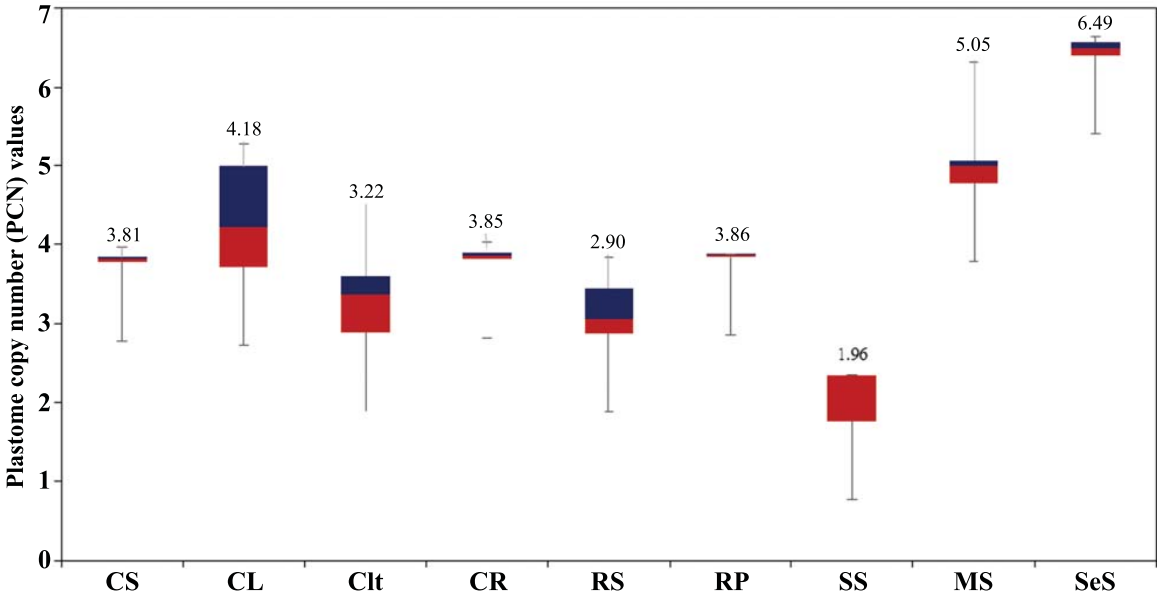

Fig. (4). Box plot exhibiting comparison of the mean log plastome copy number (PCN) for different sample groups: Cotton seed (CS), cotton leaf (CL), cotton lint (Clt), cotton raw (CR), rice seed (RS), rice paddy (RP), maize seed (MS), soybean seed (SS), sesame seed (SeS) mean log PCN values were calculated from Ct values using the formula, copy number (CN) = $10^{(\text{Ct-Intercept})/(\text{Slope})}$. Blue and red boxes represent the samples above and below the mean log PCN value. A statistically significant difference ($p < 0.05$) was observed between different sample groups calculated by performing one-way ANOVA analysis. To determine variance within a sample group, log range PCN was calculated, which revealed that maximum variance was observed for CL and Clt sample groups (Table **S2**). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

$\log_{10}$ values observed for different groups were as follows: CL (4.11) > Clt (3.63) > RS (3.09) > MS (1.94) > SS (1.17) > CR (0.58) > CS (0.31), SeS (0.31) > RP (0.05) (Table **S2**). The range PCN $\log_{10}$ was observed to be highest for CL, Clt and RS, which is equivalent to a maximum variation of up to 13000 plastome copies within a group (Table **S2**). Whereas the observed range was lowest for CS, CR, SeS and RP (Table **S2**), which is equivalent to a maximum variation of up to 4 plastome copies within a group (Table **S2**).

Rice seeds treated with the pathogen (T-1) for 11 days and high temperature (T-2) for 7 days showed an increase in mean Ct value T-1 (27.23) and T-2 (28.28) when compared to untreated seeds (24.61) (Fig. **5A**). A standard curve was generated using rice seed tDNA (Fig. **S2**) and the linear equation was used to obtain the PCN values. Treatment of rice seeds with pathogen and high temperature resulted in statistically significant *i.e.* $p < 0.05$ difference in PCN values; T-1 (1728.56) and T-2 (1339.88) when compared to untreated rice (9182.79) seeds (Fig. **5B**).

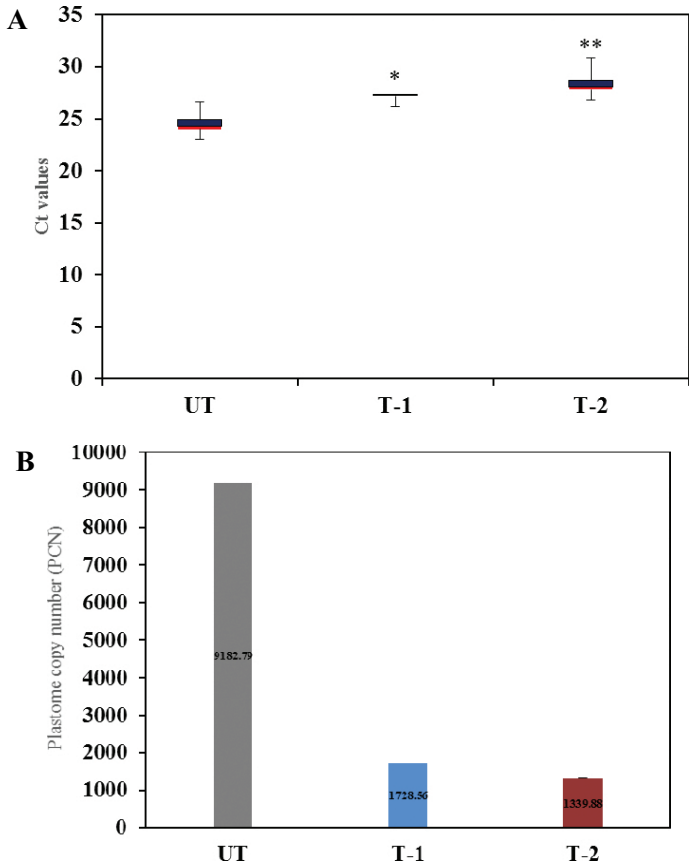

Fig. (5). Bar graph depicting the mean Ct and PCN values for rice seeds treated with pathogen and high temperature: Total DNA was isolated from rice seeds treated with pathogen (T-1) and high temperature (T-2) and untreated seeds (UT). Real-time qPCR was performed using 20 ng DNA using chloroplast specific primers in ABI 7300 machine. SYBR green was used as fluorescent dye for detecting amplification. (**A**) TTEST revealed a significant difference when T-1 (*****$p = 0.02$) and T-2 (******$p = 0.0$) was compared to UT. (**B**) TTEST revealed a significant difference when T-1 (*****$p = 0.04$) and T-2 (******$p = 0.01$) was compared to UT. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. DISCUSSION

During the course of evolution, plastids have differentiated to perform functions other than photosynthesis and storage including stress perception and response, flowering, fruit ripening, endosperm development and root gravitropism [4, 5, 27, 28]. Studies have shown that the plastid number and subtype in a plant species are modulated by both internal and environmental factors such as cell type, developmental stage, light, temperature, and nutrition [29, 30]. Moreover, development and plant stress have been shown to affect the plastome number, which are circular DNA molecules present inside plastids [16, 17, 31, 32]. In addition to being indispensable for green plants, plastids has found application in the field of synthetic biology for the expression of proteins [33, 34], as a biomarker for plant species identification [4, 35], in differentiating ploidy levels [36], studying transgene introgression [37], as a metabarcoding marker for plant dietary analysis [38], and study of phylogenetic relationships between species [39].

Plastid dynamics can be studied by isolating intact plastids *via* centrifugation of cell homogenates and fluorescent microcopy, which requires specialized equipment and is time-consuming. Recently, PCR and RT-qPCR techniques have been developed to study plastome and PCN, which have proved to be far more convenient [17, 32, 40].

Plant products such as seeds lose viability and fruits and vegetables lose nutritional value if exposed to biotic and abiotic stresses during growth and post-harvest storage, underscoring requirement for a rapid and affordable method for preventive maintenance. Therefore, this study investigated the potential role of PCN as a biomarker for assessing the effect of abiotic and biotic factors on the quality of plant material including seeds, leaves, and lint, by analyzing data obtained from RT-qPCR assays. PCN was selected for this study because PCN dynamics is affected by environmental factors compared to static nuclear DNA ploidy, the PCN per cell is higher when compared to the nuclear DNA requiring minute amount of starting material for analysis, use of universal primers eliminates the requirement for prior sequence information of the plastome, plastid DNA isolation is rapid and convenient when compared to plastid isolation and plastome sequencing, and rapid quantification of plastome is possible using RT-qPCR assay.

The PCN was determined for cotton sample groups including seed (CS), leaf (CL), raw (CR) and lint (Clt) and seed sample groups including rice (RS), rice paddy (RP), maize (MS), soybean (SS) and sesame (SeS), which are im-

portant fiber, oil and food crops by performing RT-qPCR using chloroplast tRNA specific primers. DNA isolated from GMO cotton, *Gossypium hirsutum L.* was used for standard curve generation using CaMV 35S and chloroplast specific primers. This study shows that RT-qPCR can be used for differentiating nuclear DNA from the plastome, based on the Ct values. Mean Ct values of 37.54 and 25.74 were observed for CaMV 35S promoter and chloroplast specific primers, respectively at LOD of one picogram DNA concentration (Fig. **1A** and **1B**), indicating that plastome exists at a higher copy number compared to nuclear DNA. These results were supported by studies which show that while a cell has a single nucleus, there are numerous plastids and each plastid contains many circular DNA molecules or plastomes [41]. Although, the observed PCN (7.99) for chloroplast specific primers was higher than CaMV 35S promoter (5.97) specific primers (Data Not Shown), the difference would have been more significant if the amplicon for chloroplast (600 bp) and CaMV35S promoter (82 bp) had been of comparable sizes. The consistency of the technique and reliability of the data obtained was supported by calculating PCR efficiency and performing regression analysis. PCR efficiencies of 127% for chloroplast and 83% for CaMV 35S promoter specific primers and R^2 values of 0.99 and 0.98 for chloroplast and CaMV 35S specific primers were observed, 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 axes values implicating 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**).

Analysis of the mean Ct values for CS (13.45) and CR (13.61) samples tested over a period of two years exhibited little variance CS (2.37%) and CR (1.79%), respectively (Table **S1**), implicating that the observed Ct values for 78 CS and 41 CR samples were close to the mean Ct values, suggesting that the plastome in seed containing samples of cotton analyzed over the period of two years was highly constant (Fig. **2**). Moreover, these results could also indicate that the accuracy and precision of RT-qPCR assays and that the samples had not been exposed to abiotic and biotic stresses during growth, harvesting or storage stages. These conclusions were further supported by the observation of a narrow range PCN $\log_{10}$ values for CS (0.31) and CR (0.58) sample groups indicating a variation of up to 4 plastomes between different samples processed and procured from various sources during the two year time period (Fig. **5** and Table **S2**). One-way ANOVA analysis was performed to compare the mean PCN $\log_{10}$ values, which revealed no statistically significant difference ($p = 0.391$), indicating that CS and CR samples had been exposed to similar environmental conditions during post-harvest storage and processing and probably during plant growth. All these observations support the use of RT-qPCR assay and PCN for assessing seed quality, which can be modulated by non-ambient conditions during plant growth, seed filling, and processing and storage of seeds. Our conclusions corroborate with previous findings that have shown that the PCN is regulated during development and DNA damage in seeds has been linked to storage under non-ambient conditions [22, 42].

The conclusion that PCN could be used as a biomarker to assess seed quality was supported by one-way ANOVA analysis of the mean Ct values for RS (17.11) and RP (13.42) rice seed groups, which showed a statistically significant difference ($p < 0.05$) (Fig. **3**). Moreover, RS (17.66%) group showed higher variance within the group when compared to RP (0.86%) (Table **S1**). Similarly, range PCN $\log_{10}$ was higher for RS (3.09) than RP (0.05) (Fig. **4**, Table **S2**). Although, both RS and RP groups were rice seed samples, a statistically significant difference was observed for the mean Ct, PCN $\log_{10}$ and range PCN $\log_{10}$ values, which might be due to damaged seeds in the RS sample group as they were composed of broken grains. Whereas, grains were covered with a protective hull in the RP sample group and hence were not damaged during post-harvest processing and storage. These results support the use of RT-qPCR and PCN as an indicator of seed quality, which has been shown to be affected by various biotic and abiotic stresses during growth and storage [43, 44].

To investigate specificity of RT-qPCR assay, the study conducted a comparative analysis of the mean PCN $\log_{10}$ values of different seed groups by performing a one-way ANOVA analysis. A statistically significant difference *i.e.* $p < 0.05$ was observed for the mean Ct values, CS (13.45), RS (17.11), RP (13.42), SeS (9.76), MS (13.85), SS (22.72), which indicated that the PCN might be specific to the seed type (Fig. **3**). The specificity of PCN to seed type was further supported by an observed CV of less than 30% for the mean Ct values; CS (2.47%), RS (17.66%), SeS (6.38%), MS (12.51%), SS (8.59%) (Table **S1**). However, compared to CS sample group rest of the seed sample groups exhibited higher percent variance either due to fewer samples with in a group or quality of samples like RS group containing hull-less and broken seeds. Furthermore, one-way ANOVA analysis revealed a statistically significant difference ($p < 0.05$) between the mean PCN $\log_{10}$ values for different seed samples including cotton (3.85), rice (2.9), paddy (3.86), soybean (1.96), maize (5.05) and sesame (6.49) (Fig. **4**). Our rationale for using RT-qPCR for determining PCN was further supported by the observations, where the oil-producing seeds, including SeS (6.49), MS (5.05) and CS (3.81) exhibited higher mean PCN $\log_{10}$ values when compared to RS (2.10) and SS (1.96) (Fig. **4**, Table **S2**). These observations are supported by studies which have shown that triacylglycerols (TAGs), the major storage form of seed oil, are synthesized and stored in plastids [45].

The hypothesis that PCN determined by RT-qPCR can be used as a quality biomarker for plant products was further supported by analysis of mean Ct and PCN $\log_{10}$ values for CL (14.36, 4.18) and Clt (15.68, 3.22) sample groups. The Ct values showed high percent variance from the mean Ct values for CL (18.75%) and Clt (16.7%), similarly, the two groups also exhibited the highest range PCN $\log_{10}$ values, CL (4.11) and Clt (3.63) of all the sample groups analyzed. These variations in the range PCN values could be supported by visual examination of samples, where leaf samples processed over a period of two years were of variable sizes, indicating different developmental stages and lint, which is an extension of a single epidermal cell, were of different lengths probably due to differential processing. Studies have shown that the PCN is modulated by cell type and developmental stage [5].

To provide experimental support for the use of PCN $\log_{10}$ as a quality biomarker, rice seeds were exposed to biotic and abiotic stresses, including pathogen infection and high temperature, respectively. The results obtained showed that abiotic and biotic stresses affected the PCN, as a statistically significant difference was observed for the mean Ct and PCN values for high temperature (28.28, 1339.8) and pathogen treated (27.23, 1728.56) seeds when compared to untreated seeds (24.61, 9182.79) (Fig. **5A** and **5B**). These preliminary experiments indicated that seeds, when stored under non-ambient conditions, undergo a change in PCN and support its use as a quality biomarker for plant products.

CONCLUSION

Biomarkers are molecules found in living organisms, which can be used to identify and discriminate pathological/ abnormal states from healthy/ normal states. Several computational methods have been used for screening biomarkers, including Density Functional Theory (DFT) , Random forests *etc*. [46-48]. This study based on omics data analysis and preliminary experiments, concluded that RT-qPCR can be reliably used to determine the PCN, which is plant specific and can be subsequently used to assess quality of plant products such as seeds, acting as a quality biomarker.

Quantitative DNA fluorescence and qPCR studies have shown that the number of plastids and plastome in the leaf tissue is highly dynamic during development in plants such as *Triticum aestivum* (wheat), *Beta vulgaris* (Sugar beet), *Arabidopsis thaliana*, *Zea mays* (Maize), *Nicotiana tabacum* (Tobacco), *Medicago truncatula*, *Pisum sativum* (Pea) [49-53]. Plastome number has also been shown to be modulated by various abiotic stresses because plastids are involved in providing stress tolerance [54]. However, most of these studies have been performed on tissues other than seeds. This study determined the range PCN $\log_{10}$ in seed samples using qPCR and proposes that it can be used as an indicator of conditions under which plant products have been stored after harvesting or exposed during plant growth. This method can possibly find industrial application in determining the quality of commercially important oil seeds, herbs (parsley, tea, coffee), and cereals, to name a few plant-based food products. Additionally, this method can be used to monitor the viability of seeds in the seed bank, replacing the laborious seed germination process.

KEY MESSAGE

For the first time, the study proposes the use of plastome copy number (PCN) determined by real-time quantitative PCR (RT-qPCR) using chloroplast tRNA specific primers to assess the quality of plant products.

AUTHORS' CONTRIBUTIONS

Shifa Chaudhary conducted some of the experiments, Amita Pandey conceived the idea for the manuscript, analyzed the data, did some experiments and prepared the manuscript. Binu Bhat read the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval of this study was obtained from the Directors office (SRI-MS # 20211004-01) at the Shriram Institute for Industrial Research, Delhi.

RESEARCH INVOLVING PLANTS

The plant species used are not endangered.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Real-time qPCR data is available on request.

FUNDING

The authors extend sincere thanks to the management of Shriram Institute for Industrial Research for providing financial support and infrastructure facility for bringing the manuscript (SRI-MS # 20211004-01) to its present form.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

REFERENCES

[1] Gray, M.W. The endosymbiont hypothesis revisited. *Int. Rev. Cytol.,* **1992**, *141*, 233-357. http://dx.doi.org/10.1016/S0074-7696(08)62068-9 PMID: 1452433

[2] Galili, G. Regulation of lysine and threonine synthesis. *Plant Cell,* **1995**, *7*(7), 899-906. http://dx.doi.org/10.2307/3870045 PMID: 12242392

[3] Ohlrogge, J.; Browse, J. Lipid biosynthesis. *Plant Cell,* **1995**, *7*(7), 957-970. PMID: 7640528

[4] Rolland, N.; Bouchnak, I.; Moyet, L.; Salvi, D.; Kuntz, M. The main functions of plastids. *Methods Mol. Biol.,* **2018**, *1829*, 73-85. http://dx.doi.org/10.1007/978-1-4939-8654-5_5 PMID: 29987715

[5] Waters, M.; Pyke, K. Plastid development and differentiation. *Plastids*; Møller, S.G., Ed.; Blackwell: Oxford, UK, **2004**, pp. 30-59.

[6] Renner, O. Die pflanzlichen plastiden als selbstandige elemente der genetischen konstitution. Ber. math.-phys. Klasse S€achs. *Akad. Wiss. Leipzig,* **1934**, *86*, 241-266.

[7] de Vries, J.; Archibald, J.M. Plastid genomes. *Curr. Biol.,* **2018**, *28*(8), R336-R337. http://dx.doi.org/10.1016/j.cub.2018.01.027 PMID: 29689202

[8] Fernández, A.P.; Strand, A. Retrograde signaling and plant stress: plastid signals initiate cellular stress responses. *Curr. Opin. Plant Biol.,* **2008**, *11*(5), 509-513.

http://dx.doi.org/10.1016/j.pbi.2008.06.002 PMID: 18639482

[9] Jarvis, P.; López-Juez, E. Biogenesis and homeostasis of chloroplasts and other plastids. *Nat. Rev. Mol. Cell Biol.,* **2013**, *14*(12), 787-802. http://dx.doi.org/10.1038/nrm3702 PMID: 24263360

[10] Rekhter, D.; Lüdke, D.; Ding, Y.; Feussner, K.; Zienkiewicz, K.; Lipka, V.; Wiermer, M.; Zhang, Y.; Feussner, I. Isochorismate-derived biosynthesis of the plant stress hormone salicylic acid. *Science,* **2019**, *365*(6452), 498-502. http://dx.doi.org/10.1126/science.aaw1720 PMID: 31371615

[11] León, J. Role of plant peroxisomes in the production of jasmonic acid-based signals. *Subcell. Biochem.,* **2013**, *69*, 299-313. http://dx.doi.org/10.1007/978-94-007-6889-5_16 PMID: 23821155

[12] Seo, M.; Koshiba, T. Complex regulation of ABA biosynthesis in plants. *Trends Plant Sci.,* **2002**, *7*(1), 41-48. http://dx.doi.org/10.1016/S1360-1385(01)02187-2 PMID: 11804826

[13] Lu, Y.; Yao, J. Chloroplasts at the Crossroad of Photosynthesis, Pathogen Infection and Plant Defense. *Int. J. Mol. Sci.,* **2018**, *19*(12), 3900. http://dx.doi.org/10.3390/ijms19123900 PMID: 30563149

[14] Zechmann, B. Ultrastructure of plastids serves as reliable abiotic and biotic stress marker. *PLoS One,* **2019**, *14*(4), e0214811. http://dx.doi.org/10.1371/journal.pone.0214811 PMID: 30946768

[15] Woodson, J.D. Chloroplast stress signals: Regulation of cellular degradation and chloroplast turnover. *Curr. Opin. Plant Biol.,* **2019**, *52*, 30-37. http://dx.doi.org/10.1016/j.pbi.2019.06.005 PMID: 31442733

[16] Oldenburg, D.J.; Bendich, A.J. DNA maintenance in plastids and mitochondria of plants. *Front. Plant Sci.,* **2015**, *6*, 883. http://dx.doi.org/10.3389/fpls.2015.00883 PMID: 26579143

[17] Greiner, S.; Golczyk, H.; Malinova, I.; Pellizzer, T.; Bock, R.; Börner, T.; Herrmann, R.G. Chloroplast nucleoids are highly dynamic in ploidy, number, and structure during angiosperm leaf development. *Plant J.,* **2020**, *102*(4), 730-746. http://dx.doi.org/10.1111/tpj.14658 PMID: 31856320

[18] Price, C.A.; Hadjeb, N.; Newman, L.A.; Reardon, E.M. Chromoplasts. *Methods Cell Biol.,* **1995**, *50*, 189-207. http://dx.doi.org/10.1016/S0091-679X(08)61031-6 PMID: 8531794

[19] Otegui, M.E.; Slafer, A. Increasing cereal yield potential by modifying developmental traits. *Proceedings for the 4th International Crop Science Congress,* 26 September - 1 October 2004, Brisbane, Australia.

[20] Prasad, R.; Bhattacharyya, A.; Nguyen, Q.D.; Nguyen, Q.D. Nanotechnology in sustainable agriculture: Recent developments, challenges, and perspectives. *Front. Microbiol.,* **2017**, *8*, 1014. http://dx.doi.org/10.3389/fmicb.2017.01014 PMID: 28676790

[21] McDonald, M.B. Seed deterioration: Physiology, repair and assessment. *Seed Sci. Technol.,* **1999**, *27*, 177-237.

[22] Waterworth, W.M.; Bray, C.M.; West, C.E. The importance of safeguarding genome integrity in germination and seed longevity. *J. Exp. Bot.,* **2015**, *66*(12), 3549-3558. http://dx.doi.org/10.1093/jxb/erv080 PMID: 25750428

[23] Kurek, K.; Plitta-Michalak, B.; Ratajczak, E. Reactive Oxygen species as potential drivers of the seed aging process. *Plants,* **2019**, *8*(6), 174. http://dx.doi.org/10.3390/plants8060174 PMID: 31207940

[24] Thierry, M.; Chatet, A.; Fournier, E.; Tharreau, D.; Ioos, R. A PCR, qPCR, and LAMP toolkit for the detection of the wheat blast pathogen in seeds. *Plants,* **2020**, *9*(2), 277. http://dx.doi.org/10.3390/plants9020277 PMID: 32098075

[25] Kamber, T.; Malpica-López, N.; Messmer, M.M.; Oberhänsli, T.; Arncken, C.; Alkemade, J.A.; Hohmann, P. A qPCR Assay for the Fast Detection and Quantification of *Colletotrichum lupini. Plants,* **2021**, *10*(8), 1548. http://dx.doi.org/10.3390/plants10081548 PMID: 34451593

[26] Taberlet, P.; Gielly, L.; Pautou, G.; Bouvet, J. Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.,* **1991**, *17*(5), 1105-1109. http://dx.doi.org/10.1007/BF00037152 PMID: 1932684

[27] Su, S-H.; Gibbs, N.M.; Jancewicz, A.L.; Masson, P.H. Molecular mechanisms of root gravitropism. *Curr. Biol.,* **2017**, *27*(17), R964-R972. http://dx.doi.org/10.1016/j.cub.2017.07.015 PMID: 28898669

[28] Beltrán, J.; Wamboldt, Y.; Sanchez, R.; LaBrant, E.W.; Kundariya, H.; Virdi, K.S.; Elowsky, C.; Mackenzie, S.A. Specialized plastids trigger tissue-specific signaling for systemic stress response in plants. *Plant Physiol.,* **2018**, *178*(2), 672-683. http://dx.doi.org/10.1104/pp.18.00804 PMID: 30135097

[29] Solymosi, K.; Lethin, J.; Aronsson, H. Diversity and plasticity of plastids in land plants. *Methods Mol. Biol.,* **2018**, *1829*, 55-72. http://dx.doi.org/10.1007/978-1-4939-8654-5_4 PMID: 29987714

[30] Sadali, N.M.; Sowden, R.G.; Ling, Q.; Jarvis, R.P. Differentiation of chromoplasts and other plastids in plants. *Plant Cell Rep.,* **2019**, *38*(7), 803-818. http://dx.doi.org/10.1007/s00299-019-02420-2 PMID: 31079194

[31] Baumgartner, B.J.; Rapp, J.C.; Mullet, J.E. Plastid transcription activity and DNA copy number increase early in barley chloroplast development. *Plant Physiol.,* **1989**, *89*(3), 1011-1018. http://dx.doi.org/10.1104/pp.89.3.1011 PMID: 16666609

[32] Sakamoto, W.; Takami, T. Chloroplast DNA dynamics: Copy number, quality control and degradation. *Plant Cell Physiol.,* **2018**, *59*(6), 1120-1127. http://dx.doi.org/10.1093/pcp/pcy084 PMID: 29860378

[33] Jensen, P.E.; Scharff, L.B. Engineering of plastids to optimize the production of high-value metabolites and proteins. *Curr. Opin. Biotechnol.,* **2019**, *59*, 8-15. http://dx.doi.org/10.1016/j.copbio.2019.01.009 PMID: 30798145

[34] Sameeullah, M.; Yildirim, M.; Aslam, N.; Baloğlu, M.C.; Yucesan, B.; Lössl, A.G.; Saba, K.; Waheed, M.T.; Gurel, E. Plastidial Expression of 3β-Hydroxysteroid Dehydrogenase and Progesterone 5β-Reductase genes confer enhanced salt tolerance in tobacco. *Int. J. Mol. Sci.,* **2021**, *22*(21), 11736. http://dx.doi.org/10.3390/ijms222111736 PMID: 34769166

[35] 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. Pan-plastome approach empowers the assessment of genetic variation in cultivated *Capsicum* species. *Hortic. Res.,* **2019**, *6*, 108. http://dx.doi.org/10.1038/s41438-019-0191-x PMID: 31645963

[36] Gulsen, O.; Ceylan, A. Elucidating polyploidization of bermudagrasses as assessed by organelle and nuclear DNA markers. *OMICS,* **2011**, *15*(12), 903-912. http://dx.doi.org/10.1089/omi.2011.0100 PMID: 22106951

[37] Woo, H-J.; Lim, M-H.; Shin, K-S.; Martins, B.; Lee, B-K.; Cho, H-S.; Mallory-Smith, C.A. Development of a chloroplast DNA marker for monitoring of transgene introgression in *Brassica napus* L. *Biotechnol. Lett.,* **2013**, *35*(9), 1533-1539. http://dx.doi.org/10.1007/s10529-013-1236-0 PMID: 23690044

[38] Mallott, E.K.; Garber, P.A.; Malhi, R.S. trnL outperforms rbcL as a DNA metabarcoding marker when compared with the observed plant component of the diet of wild white-faced capuchins (*Cebus capucinus*, Primates). *PLoS One,* **2018**, *13*(6), e0199556. http://dx.doi.org/10.1371/journal.pone.0199556 PMID: 29944686

[39] Zhang, Q; Zhao, L; Folk, RA; Zhao, J-L; Zamora, NA; Yang, S-X; Soltis, DE; Soltis, PS; Gao, L-M; Peng, H; Yu, X-Q Phylotranscriptomics of Theaceae: generic level relationships, reticulation and whole-genome duplication. *Ann. Bot.,* **2022**, mcac007. http://dx.doi.org/10.1093/aob/mcac007

[40] Miflin, B.J.; Beevers, H. Isolation of intact plastids from a range of plant tissues. *Plant Physiol.,* **1974**, *53*(6), 870-874. http://dx.doi.org/10.1104/pp.53.6.870 PMID: 16658807

[41] Martin, W.; Rujan, T.; Richly, E.; Hansen, A.; Cornelsen, S.; Lins, T.; Leister, D.; Stoebe, B.; Hasegawa, M.; Penny, D. Evolutionary analysis of Arabidopsis, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc. Natl. Acad. Sci. USA,* **2002**, *99*(19), 12246-12251. http://dx.doi.org/10.1073/pnas.182432999 PMID: 12218172

[42] Zoschke, R.; Liere, K.; Börner, T. From seedling to mature plant: arabidopsis plastidial genome copy number, RNA accumulation and transcription are differentially regulated during leaf development. *Plant J.,* **2007**, *50*(4), 710-722. http://dx.doi.org/10.1111/j.1365-313X.2007.03084.x PMID: 17425718

[43] Wang, W.; He, A.; Peng, S.; Huang, J.; Cui, K.; Nie, L. The effect of storage condition and duration on the deterioration of primed rice seeds. *Front. Plant Sci.,* **2018**, *9*, 172. http://dx.doi.org/10.3389/fpls.2018.00172 PMID: 29487612

[44] Wijewardana, C.; Reddy, K.R.; Krutz, L.J.; Gao, W.; Bellaloui, N. Drought stress has transgenerational effects on soybean seed germination and seedling vigor. *PLoS One,* **2019**, *14*(9), e0214977. http://dx.doi.org/10.1371/journal.pone.0214977 PMID: 31498795

[45] Li, N.; Meng, H.; Li, S.; Zhang, Z.; Zhao, X.; Wang, S.; Liu, A.; Li, Q.; Song, Q.; Li, X.; Guo, L.; Li, H.; Zuo, J.; Luo, K. Two plastid fatty acid exporters contribute to seed oil accumulation in arabidopsis. *Plant Physiol.,* **2020**, *182*(4), 1910-1919. http://dx.doi.org/10.1104/pp.19.01344 PMID: 32019874

[46] Piacenza, M.; Zambianchi, M.; Barbarella, G.; Gigli, G.; Della Sala, F. Theoretical study on oligothiophene N-succinimidyl esters: Size and push-pull effects. *Phys. Chem. Chem. Phys.,* **2008**, *10*(35), 5363-5373. http://dx.doi.org/10.1039/b803963a PMID: 18766232

[47] Wong, B.M.; Piacenza, M.; Della Sala, F. Absorption and fluorescence properties of oligothiophene biomarkers from long-range-corrected time-dependent density functional theory. *Phys. Chem. Chem. Phys.,* **2009**, *11*(22), 4498-4508. http://dx.doi.org/10.1039/b901743g PMID: 19475168

[48] Huang, X.; Lin, X.; Zeng, J.; Wang, L.; Yin, P.; Zhou, L.; Hu, C.; Yao, W. A computational method of defining potential biomarkers based on differential sub-networks. *Sci. Rep.,* **2017**, *7*(1), 14339. http://dx.doi.org/10.1038/s41598-017-14682-5 PMID: 29085035

[49] Boffey, S.A.; Leech, R.M. Chloroplast DNA levels and the control of chloroplast division in light-grown wheat leaves. *Plant Physiol.,* **1982**, *69*(6), 1387-1391. http://dx.doi.org/10.1104/pp.69.6.1387 PMID: 16662409

[50] Shaver, J.M.; Oldenburg, D.J.; Bendich, A.J. Changes in chloroplast DNA during development in tobacco, *Medicago truncatula*, pea, and maize. *Planta,* **2006**, *224*(1), 72-82. http://dx.doi.org/10.1007/s00425-005-0195-7 PMID: 16362324

[51] Oldenburg, D.J.; Rowan, B.A.; Zhao, L.; Walcher, C.L.; Schleh, M.; Bendich, A.J. Loss or retention of chloroplast DNA in maize seedlings is affected by both light and genotype. *Planta,* **2006**, *225*(1), 41-55. http://dx.doi.org/10.1007/s00425-006-0329-6 PMID: 16941116

[52] Rowan, B.A.; Oldenburg, D.J.; Bendich, A.J. A multiple-method approach reveals a declining amount of chloroplast DNA during development in Arabidopsis. *BMC Plant Biol.,* **2009**, *9*(9), 3. http://dx.doi.org/10.1186/1471-2229-9-3 PMID: 19128504

[53] Rauwolf, U.; Golczyk, H.; Greiner, S.; Herrmann, R.G. Variable amounts of DNA related to the size of chloroplasts III. Biochemical determinations of DNA amounts per organelle. *Mol. Genet. Genomics,* **2010**, *283*(1), 35-47. http://dx.doi.org/10.1007/s00438-009-0491-1 PMID: 19911199

[54] Hameed, A.; Ahmed, M.Z.; Hussain, T.; Aziz, I.; Ahmad, N.; Gul, B.; Nielsen, B.L. Effects of salinity stress on chloroplast structure and function. *Cells,* **2021**, *10*(8), 2023. http://dx.doi.org/10.3390/cells10082023 PMID: 34440792