4-24-2011

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Recommended Citation

Ruiz, O. N., Alvarez, D., Torres, C., Roman, L., & Daniell, H. (2011). Metallothionein Expression in Chloroplasts Enhances Mercury Accumulation and Phytoremediation Capability. *Plant Biotechnology Journal, 9* (5), 609-617. http://dx.doi.org/10.1111/j.1467-7652.2011.00616.x

At the time of publication, author Henry Daniell was affiliated with the University of Central Florida. Currently, he is a faculty member at the School of Dental Medicine at the University of Pennsylvania.

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Abstract

Genetic engineering to enhance mercury phytoremediation has been accomplished by expression of the merAB genes that protects the cell by converting Hg[II] into Hg[0] which volatilizes from the cell. A drawback of this approach is that toxic Hg is released back into the environment. A better phytoremediation strategy would be to accumulate mercury inside plants for subsequent retrieval. We report here the development of a transplastomic approach to express the mouse metallothionein gene (*mt1*) and accumulate mercury in high concentrations within plant cells. Real-time PCR analysis showed that up to 1284 copies of the *mt1* gene were found per cell when compared with 1326 copies of the 16S *rrn* gene, thereby attaining homoplasmy. Past studies in chloroplast transformation used qualitative Southern blots to evaluate indirectly transgene copy number, whereas we used real-time PCR for the first time to establish homoplasmy and estimate transgene copy number and transcript levels. The mt1 transcript levels were very high with 183 000 copies per ng of RNA or 41% the abundance of the 16S rrn transcripts. The transplastomic lines were resistant up to 20 µm mercury and maintained high chlorophyll content and biomass. Although the transgenic plants accumulated high concentrations of mercury in all tissues, leaves accumulated up to 106 ng, indicating active phytoremediation and translocation of mercury. Such accumulation of mercury in plant tissues facilitates proper disposal or recycling. This study reports, for the first time, the use of metallothioniens in plants for mercury phytoremediation. Chloroplast genetic engineering approach is useful to express metal-scavenging proteins for phytoremediation.

Keywords

plastid genome, real-time PCR, bioremediation, genetic engineering, chelator, environmental biotechnology

Disciplines

Dentistry

Comments

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HHS Public Access

Author manuscript

Plant Biotechnol J. Author manuscript; available in PMC 2015 August 03.

Published in final edited form as:

Plant Biotechnol J. 2011 June ; 9(5): 609–617. doi:10.1111/j.1467-7652.2011.00616.x.

Metallothionein expression in chloroplasts enhances mercury accumulation and phytoremediation capability

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Summary

Genetic engineering to enhance mercury phytoremediation has been accomplished by expression of the merAB genes that protects the cell by converting Hg[II] into Hg[0] which volatilizes from the cell. A drawback of this approach is that toxic Hg is released back into the environment. A better phytoremediation strategy would be to accumulate mercury inside plants for subsequent retrieval. We report here the development of a transplastomic approach to express the mouse metallothionein gene (*mt1*) and accumulate mercury in high concentrations within plant cells. Real-time PCR analysis showed that up to 1284 copies of the *mt1* gene were found per cell when compared with 1326 copies of the 16S *rrn* gene, thereby attaining homoplasmy. Past studies in chloroplast transformation used qualitative Southern blots to evaluate indirectly transgene copy number, whereas we used real-time PCR for the first time to establish homoplasmy and estimate transgene copy number and transcript levels. The mt1 transcript levels were very high with 183 000 copies per ng of RNA or 41% the abundance of the 16S rrn transcripts. The transplastomic lines were resistant up to 20 μ_M mercury and maintained high chlorophyll content and biomass. Although the transgenic plants accumulated high concentrations of mercury in all tissues, leaves accumulated up to 106 ng, indicating active phytoremediation and translocation of mercury. Such accumulation of mercury in plant tissues facilitates proper disposal or recycling. This study reports, for the first time, the use of metallothioniens in plants for mercury phytoremediation. Chloroplast genetic engineering approach is useful to express metal-scavenging proteins for phytoremediation.

Keywords

plastid genome; real-time PCR; bioremediation; genetic engineering; chelator; environmental biotechnology

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Introduction

Phytoremediation, the use of plants to restore contaminated environments, offers a potentially low cost and environmentally friendly approach. However, toxic heavy metal elements, such as mercury (Hg), cannot be detoxified into non-toxic metabolites by plants. Genetic engineering can be used to integrate genes from other organisms into plant genomes to enhance Hg phytoremediation capabilities. A method of mercury phytoremediation by the reduction of ionic-Hg (Hg^{2+}) and subsequent volatilization of elemental mercury (Hg^0) has been developed (Rugh et al., 1996; Ruiz *et al*., 2003; Hussein *et al*., 2007; Ruiz and Daniell, 2009). The disadvantage of this approach, which is based on the use of the mer genes, is that Hg^0 is released back into the environment where it accumulates and can be converted back into highly toxic forms.

Alternatively, molecules known as chelators have been proposed as suitable heavy metal-scavenging agents that can be expressed in organisms with the purpose of providing resistance to heavy metals, while allowing accumulation inside the cell in an inert form. Metallothioneins and polyphosphates are two examples of chelator molecules that have been expressed in bacteria. Metallothioneins are low-molecular-weight, metal-sequestering proteins rich in cysteines that can bind metal ions in a biologically inactive form (Hamer, 1986). Metallothioneins are encoded by the *mt* genes and are found in most eukaryotes. Metallothioneins are some of the most studied metal chelator agents and their effectiveness has been shown in transformed bacteria and plants for several heavy metals including Zn, Cd, Cu and Ni. Its role in Hg detoxification, though, is less clear. So far there is no report on the use of metallothioneins in Hg phytoremediation.

In regard to mercury, few attempts have been made to genetically engineer plants for the expression of metal chelators with the goal of enhancing mercury phytoremediation. Some reports have shown that tobacco plants genetically modified via the nuclear genome to express the *ppk* gene have increased polyphosphate production and enhanced tolerance and accumulation of Hg up to 10 μM (Nagata *et al*., 2006a,b). Others have shown that expression of the *merP* gene in the nuclear genome of transgenic *Arabidopsis thaliana* provided resistance up to 10 μM Hg by chelation (Hsieh *et al*., 2009; Ruiz and Daniell, 2009).

Most attempts to enhance Hg phytoremediation have been made by using nuclear genetic engineering to achieve the genetic modifications (Bizily *et al*., 1999). An alternate approach is the use of chloroplast genetic engineering to express the bacterial *mer* genes; this approach provided higher Hg resistance and phytoremediation from contaminated soil than nuclear genetic engineering (Ruiz *et al*., 2003; Hussein *et al*., 2007; Ruiz and Daniell, 2009). Chloroplast or plastid genetic engineering presents several advantages over nuclear transformation including site-specific integration of transgenes by homologous recombination (Verma and Daniell, 2007), multiple transgene integration in a single transformation event (DeCosa *et al*., 2001; Lossl *et al*., 2003; Quesada-Vargas *et al*., 2005), very high levels of transgene expression because of >10 000 copies of transgene per plant cell (up to 72% of the total leaf protein, Ruhlman *et al*., 2010) and transgene containment because of the maternal inheritance of the chloroplast genomes (Daniell, 2007) or cytoplasmic male sterility (Ruiz and Daniell, 2005). Most importantly, because the

chloroplast is the essential metabolic centre for photosynthesis, fatty acids, amino acids, pigments, vitamins and starch biosynthesis, it is important to protect it from the toxic effects of Hg. The chloroplast compartment is the primary target for Hg toxicity in plants affecting electron transport and photosynthesis (Sen and Mondal, 1987; Bernier *et al*., 1993; Kupper *et al*., 1996). Because of this, it is important to express the detoxifying enzymes in the chloroplast to efficiently protect its metabolic integrity.

The main objective of this investigation is to demonstrate that metallothionein can be expressed efficiently in plants via the chloroplast genome using optimal gene constructs to promote mercury phytoremediation. The accumulation of mercury in the roots, stalk and leaves of tissues of transgenic plant was also determined. Here, we report that the expression of mouse metallothionein in transplastomic plants increases mercury resistance, accumulation and phytoremediation by the mechanism of chelation. This is the first report where metallothionein has been efficiently expressed to provide mercury phytoremediation by chelation and the first instance where real-time PCR analysis has been used to quantify transgene copy number, homo-plasmy and transcript abundance in transplastomic plants.

Results and discussion

Chloroplast transformation vector construction

Chloroplast transformation vectors harbouring the *mt1* gene required for mercury detoxification and chelation were developed. These chloroplast transformation vectors were used to genetically modify *Nicotiana tabacum* plants via particle bombardment, which were then selected and regenerated *in vitro*. Initially, polymerase chain reaction amplification primers were developed for gene amplification and vector construction (Table 1). Amplification primers for mouse *mt1* gene and bacteriophage T7 gene 10 (g10) 5′ translational enhancer elements were developed using GenBank sequences BC036990 and 71534864, respectively. After PCR amplification, the *mt1* amplicon was subcloned and verified to be correct by restriction enzyme and DNA sequence analysis. The functional expression construct was cloned into the pLD-ctv chloroplast transformation vector to produce the final transformation vector pLD-g10-mt1-rps-5′UTR-merB-3′UTR. A schematic representation of the final chloroplast transformation vector is shown in Figure 1a. The pLD-ctv provides the 16S rRNA promoter (Prrn), which drives the transcription of the selectable marker gene *aadA*, which confers resistance to spectinomycin. The pLD-ctv vector also contains the *trnI* and *trnA* genes from the inverted repeat region of the tobacco chloroplast genome as flanking sequences for homologous recombination. Translation of the *mt1* gene is regulated by the gene10 5′ UTR from bacteriophage T7 that is known to enhance translation in chloroplasts up to 100-fold when fused to endogenous promoters (Dhingra *et al*., 2004; Kumar *et al*., 2004; Staub *et al*., 2000). In these expression constructs, *mt1* is transcribed dicistronically in the chloroplasts because the gene10 5′ UTR (g10) is promoterless; the P*rrn* promoter is the only promoter upstream of the *aad*A and *mt1* genes. The *g*10-*mt1* construct was fused to the 3′UTR from the chloroplast *rps* 16 gene. This region has been shown to increase transcript stability and abundance (Shiina *et al*., 1998).

Chloroplast transformation and selection

The final chloroplast transformation vector containing the *mt1* gene was used to transform tobacco plants by particle bombardment. To accomplish this, several tobacco plant leaves were bombarded with the chloroplast transformation vector pLD-g10-mt1-rps-5′UTR-merB-3′UTR and selected in regeneration media of plants (RMOP) with 500 μg/mL spectinomycin to select for the cells containing the transgenic chloroplasts. After 4–6 weeks in selection media, multiple putative transgenic shoots were observed growing green from a background of bleached untransformed leaf tissue. Transgenic plants from the first selection were tested for transgene integration by PCR, and positive clones were then moved through two additional rounds of selection to increase the number of transgenic chloroplasts. Several transplastomic clones were obtained after three rounds of selection for the expression construct.

Molecular characterization of chloroplast transgenic plants

Several mt1 transgenic plants growing in soil were tested by polymerase chain reaction (PCR) using PCR primer sets that confirm the correct integration of the transgenes into the spacer region between the chloroplast trnI and trnA genes (Figure 1b). The forward integration primer annealed within the native 16S *rrn* gene at position 1085–1109 of the gene. The 16S *rrn* gene is 1492 bp in length and is upstream of the *trnI* gene. The *trnI* and *trnA* genes are the chloroplast flanking sequences in the chloroplast transformation vector. The reverse primers annealed within the construct *aadA* gene and at the 3′ end of the *mt1* genes. The *aadA* reverse primer lands within the construct *aadA* gene at position 173–197 of that gene. By using this primer set, a PCR amplicon will only be obtained when site-specific integration occurs within the *trnI* and *trnA* gene region. The schematic representation in Figure 1a shows the PCR primer sets used for analysis, their respective annealing sites in the transformed chloroplast genome and the expected amplification products (amplicons). PCR products were resolved by agarose gel electrophoresis. We observed several transplastomic plants with site-specific integration of the *mt1* gene (Figure 1b). Site-specific integration takes place by the action of the chloroplast recombinase RecA acting on the homologous recombination sequence trnI/trnA. Obtaining site-specific integration eliminates detrimental position effects and transgene silencing. Site-specific integration was analysed by using PCR primer pairs where the forward primer annealed with the native plastid genome upstream of the integration site and the reverse primer annealed with the gene construct within the *aadA* gene or at the 3′ end of the *mt1* gene. These primer combinations generated 1.75- and 2.5-kb PCR products (Figure 1b). The formation of these amplicons showed that the expression cassette integrated at the specified location in the chloroplast genome. The presence of the *mt1* gene was further confirmed by using gene-specific PCR primers. The expected 221-bp *mt1* gene amplicon was observed (Figure 1b).

Real-time PCR studies were performed to determine the abundance of transgenes in the transplastomic lines and evaluate homoplasmy. Homoplasmy is achieved when every chloroplast genome contains the transgene. Homoplasmy have been related to increased expression level of transgenes. So far, many questions have not been addressed regarding the process of achieving transgene homoplasmy in the chloroplast genome through the *in vitro* selection process. Southern blot analysis has been the preferred method to test for

homoplasmy. One drawback with the use of Southern blot is the lack of quantification and reduced sensitivity when compared with PCR amplification. To address some of these concerns, we developed real-time PCR assays with synthetic oligonucleotide standards (Table 1) that allow the quantification of transgene copies, homoplasmy and transgene expression. An important characteristic of real-time PCR is its linearity because of the use of extremely short amplicons that are usually <150 bp. This ensures that the PCR exponential process is maintained for an accurate quantification.

Quantitative real-time PCR analysis performed on mt1 transgenic plant lines showed that transplastomic lines have higher gene copy number per ng of total DNA (Figure 2a). Mt1 transplastomic lines had between 50 904 and 64 842 copies of the *mt1* gene per nanogram of DNA (Figure 2a). In the literature, it has been reported that mature leaves from plants growing in soil under optimal conditions can have as many as 10 000 copies of the chloroplast genome per cell (Bendich, 1987). In order to estimate the transgene copy number per cell in the transplastomic lines, we made a mathematical calculation by converting genomic DNA concentration into copy number and subsequently to cell number (See Experimental section). The results showed that the mt1 transplastomic lines have between 1004 and 1284 copies per cell (Figure 2c). After determining the transgene abundance, we performed analyses to determine the degree of homoplasmy achieved in the transgenic plants. This was possible by comparing the transgene quantification data against the quantification of the chloroplast endogenous 16S *rrn* gene. It is expected that if homoplasmy is achieved, there would be an equal number of 16S *rrn* gene and transgene copies because both genes are located within inverted repeat region. It has been well documented that homoplasmic transplastomic lines are very similar in transgene expression and functionality. For example, several homoplasmic transplastomic lines expressing the *merA* and *merB* genes showed similar levels of resistance and mercury phytoremediation (Ruiz *et al*., 2003; Hussein *et al*., 2007). Because of this, we have chosen to study a homoplasmic and heteroplasmic lines for comparison purposes. By comparing these two transplastomic lines, we can demonstrate the potential of real-time PCR analysis. Furthermore, by comparing these two lines, we can help demonstrate the effect of different degrees of heteroplasmy on transgene expression.

Our real-time PCR assay results showed that the levels of the *mt1* gene in the transplastomic lines 1 and 3 were very high with 6.48×10^4 and 5.09×10^4 gene copies per nanogram of genomic DNA, respectively (Figure 2b). These values were equivalent to 1284 and 1004 transgene copies per cell, respectively (Figure 2c). No *mt1* gene was detected in the untransformed plants. In the same study, the levels of the16S *rrn* gene in untransformed and transplastomic lines 1 and 3 were also measured. The results showed that transgenic lines 1 and 3 and the untransformed plants had similar levels of 16S *rrn* gene, which accounted for 6.70×10^4, 7.02×10^4 and 7.42×10^4 gene copies per ng of genomic DNA, respectively. These values were equivalent to 1326, 1390 and 1486 copies of 16S *rrn* gene per cell, respectively. When the mt1 gene copy levels were compared against the16S *rrn* gene levels, we were able to determine that the transgenic line 1 achieved homoplasmy, while the transgenic line 3 was heteroplasmic with 72% of the genomes containing the transgene (Figure 2b). This indicated that 28% were untransformed genomes. Thus, real-time PCR, for

the first time, facilitated the quantitation of homoplasmy or heteroplasmy. Previous estimates of 10 000 copies of chloroplast genome per cell performed by DNA-specific 4′,6-diamidino-2-phenylindole (DAPI) fluorescence analysis (Bendich, 1987) may be an overestimation. Also, plastid genome abundance has been shown to vary greatly depending on light and developmental conditions (Sugiura, 1992; Oldenburg *et al*., 2006; Shaver *et al*., 2008).Therefore, real-time PCR may be used to estimate chloroplast genome copy numbers in crop species at different developmental stages, growth conditions or in different tissues.

The quantitative real-time PCR is a simpler method than Southern blot because it does not require radioisotopes and can be performed in just a few hours. Southern blot can be performed using non-radioactive probes, but these are not sensitive enough to demonstrate the absence of untransformed genomes in transplastomic lines. Southern blots have been used as the gold standard in the transplastomic field to establish homoplasmy, but accurate quantitation has been difficult to demonstrate with this method. Some of the factors that limit quantification by Southern blots are <100% transfer from gels to membranes, saturation of binding of the probe and denaturation of single-stranded DNA. Therefore, it is highly desirable to develop truly quantitative methods that do not depend upon gels, blots and probes. While Southern blot may indicate homo-plasmy (within limits of detection), it is not suitable for determining genome copy number. The real-time qPCR fills this void and provides yet another tool for the molecular characterization of transplastomic lines.

Our goal was to develop a new method for quantitative analysis of homoplasmy or heteroplasmy. Today, real-time PCR instruments are affordable and very common in research laboratories. Furthermore, real-time PCR can be used to complement qualitative data provided by blots. Direct comparison of the *mt*-1 transgene with the endogenous 16S *rrn* gene, both present in the inverted repeat region of the plastid genome, reveals the difference between homoplasmic and heteroplasmic transplastomic lines. This is also the first time that a study has been able to show the levels of heteroplasmy in plants and how these levels affect transgene expression. In the future, studies targeting the understanding of the effect of varying degrees of heteroplasmy in transgene expression, and even the ratio of transgene to wild-type genome in subsequent generations that are not germinated in antibiotic selection media, can be performed using real-time PCR. Our study demonstrates that there are differences in mRNA expression and mercury resistance between a homoplasmic line and a heteroplasmic line with 28% of the genomes lacking the transgene. The use of real-time PCR technology in this study has further advanced our understanding of chloroplast transgenic technology by providing a more accurate and quantitative description of transgene level and homoplasmy.

Transplastomic lines were also characterized for transgene transcript abundance. Total RNA from mt1 transgenic and untransformed plants was extracted, quantified, normalized and reverse-transcribed to produce the complementary DNA (cDNA). The cDNA was analysed by quantitative real-time PCR. Results showed high abundance of *mt1* mRNA in transgenic lines 1 and 3 in comparison with the untransformed plants where *mt1* mRNA was not detected (Figure 2d). Transgene transcript level for mt1 transgenic lines 1 and 3 were 183 000 and 70 300 copies per ng total RNA, respectively (Figure 2d). Transcript levels for the 16S *rrn* gene in transgenic lines 1 and 3 were 4.43×10^5 and 4.40×10^5, respectively

(Figure 2d). When we compared the transcription of the *mt1* transgene against the transcription of 16S *rrn* gene, which is one of the most transcribed genes in the plant cell and the most stable RNA in the chloroplast (Kim *et al*., 1993), we were able to observe that the *mt1* transcript levels in transgenic line 1 equaled 41% of the transcript level for the16S *rrn* gene, while the *mt1* transcript levels in transgenic line 3 equaled 16% of the transcript level for the 16S *rrn* gene. These results demonstrated that chloroplast expression of transgene can lead to high levels of RNA expression even when homoplasmy is not achieved. We also observed that a moderate increase in homoplasmy can lead to a larger increase in transgene expression. This is supported by the observation that mt1 transgenic line 1, which was 24% more homoplasmic than mt1 transgenic line 3, showed a 2.6-fold increase in transcript abundance. In the past, it has been shown that transcript levels of transgenes can be even higher than chloroplast endogenous genes. For example, when human serum albumin (HSA) was expressed through the chloroplast genome, transcript accumulation was twice the level of the chloroplast endogenous psbA gene because transgene was integrated into the inverted repeat region (Fernandez-San Millan *et al*., 2003). Others have shown that transcript accumulation in transplastomic lines can be more than 150-fold higher than in nuclear transgenic plants (Dhingra *et al*., 2004; Lee *et al*., 2003).

Mercury resistance bioassays

Mercury (Hg) bioassays were performed by amending tissue culture media (RMOP) with $HgCl_2$ (Hg) to a final concentration of 0, 10, 15 and 20 μM. Same size leaf tissue discs were obtained from mt1 transplastomic lines 1 and 3 and untransformed plants and then placed in media containing Hg. The tissues were maintained in this condition for periods of 5 and 10 days. Transplastomic lines expressing the *mt1* gene were highly resistant to 10 μM Hg, while the untransformed plant discs were completely dead (Figure 3b). Measurements of chlorophyll content showed that the mt1 transplastomic lines had higher chlorophyll content than untransformed plants, which had negligible levels (Figure 3c). In the past, chlorophyll content measurements have been used as a fast and reliable method to assess chloroplast integrity and metabolic function (Ruiz *et al*., 2003). Transplastomic lines growing in 10 μM Hg had much higher chlorophyll content per miligram fresh weight than the same transplastomic plants growing in media without mercury (Figure 3c). Furthermore, the mt1 transplastomic line 1 cultured at 15 and 20 μM Hg maintained chlorophyll levels similar to the tissue samples growing without Hg (Figure 3c). Increased chlorophyll content in Hg-resistant transgenic or transplastomic plants growing in the presence of Hg has been previously reported, but the mechanism of this phenotype is not yet understood (Heaton *et al*., 1998; Rugh *et al*., 1996; Ruiz and Daniell, 2009; Ruiz, *et al*., 2003). These results showed that the mt1 transplastomic lines had high resistance to the toxic effects of mercury, while untransformed plants were sensitive to the lower level of Hg. The mt1 transplastomic line 1 was resistant to Hg concentrations up to 20 μM, while the resistance of transplastomic line 3 was diminished at this concentration. These results clearly indicated that the resistance of the transgenic plants is affected by the number of transgene per cell, heteroplasmy level and transgene expression. We observed that even after 10 days, leaf tissue in the presence of 10 μM Hg from mt1 transgenic lines had chlorophyll levels similar to tissue placed in media without mercury. The mt1 transplastomic line 1 maintained elevated chlorophyll content, even in the presence of 15 and 20 μM Hg.

The bioremediation capability of the mt1 transgenic lines was characterized by quantifying Hg accumulation in leaves cultured in the presence of 10, 15 and 20 μM Hg by cold vapour atomic absorption spectrometry (CVAAS). It has been proposed that the resistance of mt1 transplastomic lines to Hg is attributed to the Hg-scavenging effect of the protein metallothionein, which in turns protects the chloroplast from toxicity. This mechanism of resistance also provides bioremediation by extraction and accumulation of mercury inside the chloroplasts to high concentration in an inert form. It was expected that if effective bioremediation was taking place, high mercury accumulation would be detected in leaves. The results showed that mt1 transplastomic lines had high mercury accumulation that was 3.5 and 4.3 times higher than untransformed leaves in 10 μM Hg, while maintaining higher chlorophyll levels (Figure 3d). Similar analysis performed for tissue grown in 15 and 20 μM Hg showed that mt1 transplastomic line 1 accumulated 4.5 and 2.3 times more Hg than the untransformed plant, respectively. The mt1 transplastomic line 3 showed Hg accumulation that was 2.3 and 1.5 times higher than untransformed leaves when cultured in the presence of 15 and 20 μM Hg, respectively (Figure 3d). These results show that the mt1 transplastomic lines bioremediate mercury from the culture media by chelation, accumulating up to 148 ng of Hg while maintaining the cellular integrity (Figure 3d). From these results, it is also clear that the transplastomic line with the highest transgene copy number had the highest resistance to mercury and higher accumulation.

To further evaluate the resistance of the mt1 transplastomic lines, seedlings from line 1 and untransformed plants were germinated and grown in the media containing 0, 10 and 15 μM of Hg for 30 days. After 30 days, pictures were taken, fresh weight and chlorophyll content measured, and mercury accumulation was quantified. The results showed that the transplastomic plants expressing the *mt1* gene were more resistant to mercury than untransformed plants (Figure 4). The mt1 transplastomic line had longer roots and larger and greener shoots than untransformed plants at each concentration tested, but especially at 10 μM Hg (Figure 4b,d). Also, every mt1 transgenic seedling was able to sustain growth at concentrations of 10 and 15 μM Hg (Figure 4b–d). Finally, the mt1 transplastomic seedlings had 3-fold and 2.5-fold higher chlorophyll content per mg fresh weight than untransformed seedlings at 10 and 15 μM Hg, respectively, indicating that the chloroplast was protected from the toxic effects of mercury (Figure 4e). Untransformed seedlings growing at 10 μM Hg failed to develop a root system and appeared chlorotic, while at 15 μM Hg the seedlings completely lacked chlorophyll and did not develop (Figure 4a,c). The lack of root growth or growth of the roots away from the contaminant is a plant protection mechanism to cope with this type of abiotic stress (Heaton *et al*., 2005). According to Godbold and Huttermann (1986), mercury-induced root damage may have serious consequences for nutrient and water supply to above-ground plant parts and should be taken into account when assessing the effect of mercury on the physiology of leaves. Protection of the transgenic root by the heterologous metallothionein was possible because of active proplastids and because the transgene was under the control of the constitutive 16S *rrn* promoter and translational enhancer element g10 5′UTR. The lack of chlorophyll at 15 μM Hg in untransformed seedlings is also a clear sign of Hg toxicity in chloroplasts that had lost photosynthetic capacity (Figure 4a,c).

Finally, we measured the accumulation of mercury by transgenic and untransformed seedlings growing at 15 μM Hg by CVAAS in different tissues. By characterizing the accumulation of mercury in the different plant tissues, we can obtain insights into the mechanistics of phytoextraction, phytotranslocation and phytoaccumulation. The mt1 transgenic plants detoxify and accumulate mercury by the action of metallothionein, which acts as a scavenger of the toxic ion. Accumulation of the toxic ion in an inert form protects chloroplast metabolism. The results showed that the leaf was the tissue with the highest Hg accumulation under the tested conditions followed by accumulation in the stem and finally in the root that had the lowest accumulation (Figure 4f). The transgenic seedlings accumulated twice more Hg in the leaves, 2.5 times more Hg in the stem and 4.5 times more Hg in the roots than the untransformed seedlings (Figure 4f). The tissue with the highest accumulation was the leaf with a total of 106 ng of Hg in the transgenic seedlings and 54 ng of Hg in the untransformed. The transgenic and untransformed seedling stem had 12 and 4.8 ng Hg, respectively. The roots had the lowest accumulation of all tissues with a total of 3.7 and 0.8 ng for transgenic and untransformed, respectively. Although higher-than-expected Hg concentrations were observed in the stem and leaves of untransformed seedling, this seems to be caused by direct absorption of Hg when the tissues of the bleached and shrivelled plants got in contact with the culture media containing mercury. Because transplastomic plants were green and grew normally, the stem and leaves were not in contact with the media, indicating that the accumulation in those organs was because of Hg translocation from the root. These results indicated that at least under these *in vitro* culture conditions, most of the Hg accumulation occurs in the above-ground tissues, contrary to previous reports that showed that mercury is mostly accumulated in the root (Suszcynsky and Shann, 1995; Heaton *et al*., 2003; Kim *et al*., 2005). The increased accumulation in the aboveground tissue in the mt1 transgenic plants can be explained by the action of metallothioneins. While previous phytoremediation approaches expressed mercuric ion reductase, which reduces ionic mercury to elemental mercury, which is then volatilized from the plant tissues, our mt1 transgenic plants chelate and accumulate mercury inside the chloroplasts (Hussein *et al*., 2007; Ruiz and Daniell, 2009).

Conclusions

In this report, we have shown that the expression of the *mt1* gene by chloroplast genetic engineering provides resistance to the toxic effects of mercury while allowing high mercury accumulation within plant tissues without adverse effects. Higher chlorophyll content and lack of chlorosis, more biomass and longer root and shoot systems show that the mt1 transgenic plants were more resistant to mercury than untransformed plants (up to 20 μM). Previous reports had shown that when chelator genes ppk and merP were expressed in plants via the nuclear genome, they conferred resistance up to 10 μM of mercury (Hsieh *et al*., 2009; Nagata *et al*., 2006a,b). This indicates that our transgenic seedlings were twice more resistant than previously reported for nuclear transgenic plants, although the levels are not comparable because they used different chelators. In this study, we chose to place seeds in mercury-contaminated culture media to demonstrate that transplastomic plants can germinate and develop in the presence of mercury. Seedlings are more susceptible to the

effects of mercury because of lower transgene expression than mature transplastomic plants owing to fewer chloroplasts or genomes.

This study shows that transplastomic plants not only grew better but also actually accumulated several folds more mercury in their tissues than untransformed plants. This is also the first report of the use of a metallothionein for mercury phytoremediation and the first instance where a chelator protein has been expressed through chloroplast genetic engineering to enhance phytoremediation. By expressing the mouse metallothionein gene (*mt1*) in the tobacco chloroplasts, we have shown once more that the chloroplast transformation system is efficient at expressing functional heterologous proteins for phytoremediation. A phytoremediation system that accumulates mercury inside the plant tissue in an inert form is a preferred approach because mercury is not volatilized into the atmosphere and transplastomic plants can be collected from the contaminated site once they have accumulated mercury. Also, mercury accumulated within the transplastomic plants can be recycled for industrial applications. This reduces the need for mercury ore and increases the cost-effectiveness of the phytoremediation system.

Finally, real-time PCR has been used for the first time to quantify transgene copy number, transcript abundance and homoplasmy in transplastomic plants. While Southern blots are used as the gold standard in the transplastomic field to establish homoplasmy, it is very hard to argue that this is a quantitative tool. For the quantitation of proteins, ELISA is used as the gold standard, whereas densitometric analysis using Western blots is not used. The rationale for not using Western blots for quantitation is the number of limitations including <100% transfer from gels to membranes, variation in transfer based on protein size within the same gel, saturation of binding of the antibody probe, escape of proteins through the membrane based on pore size and duration of transfer. All these limitations apply to Southern blots in addition to denaturation conditions and binding of only single-stranded DNA to membranes. Therefore, it is highly desirable to develop truly quantitative methods that do not depend upon gels, blots and probes. While Southern blot could indicate homoplasmy (within limits of detection), it is not suitable for determining genome copy number. The real-time qPCR fills this void and provides yet another tool for the molecular characterization of transplastomic lines.

Experimental procedures

Chloroplast transformation vector construction

The *mt*-1 gene was PCR-amplified from the plasmid pCMV-SPORT10 that contains the mouse mt1 cDNA and cloned into pBlueScript vector. This vector was obtained from the American Type Culture Collection; ATCC no. MGC47147; gi: BC036990. The gene-specific PCR primers were designed with flanking *NdeI* and *BamHI* restriction enzymes to facilitate downstream cloning (Table 1). The *mt*-1 gene was cloned in frame from the g10 5′UTR, which is known to enhance translation in chloroplasts. Then, the g10-mt1 construct was subcloned into a pBSK vector containing the chloroplast *rps*16 gene terminator. Finally, we cloned the g10-mt-1-rpsT construct into the pLD-5′UTR-merB-3′UTR vector producing the final chloroplast transformation vector pLD-g10-mt1-rpsT-5′UTR-merB-3′UTR (Figure 1a).

Chloroplast transformation and selection of transgenic plants

The delivery of the pLD-g10-mt1-rps-5′UTR-merB-3′UTR vector to the chloroplast genome by particle bombardment and the subsequent selection of the transgenic tobacco (*N. tabacum* var Havana 263) lines were performed essentially as previously described (Daniell *et al*., 2004; Ruiz and Daniell, 2005). Briefly, tobacco leaves were bombarded using the biolistic device PDS-1000/He (Bio Rad, Hercules, CA). After bombardment, leaves were placed in RMOP, supplied with 500 μg/mL spectinomycin for two rounds of selection on plates, and subsequently moved to jars on Murashige and Skoog medium with 500 μg/mL spectinomycin. Finally, transgenic plants were transferred to high nutrient soil and grown in controlled growth chambers at 27 °C in a 16-h light/8-h dark photoperiod. Seeds were collected from the transgenic plants.

PCR and Real-time PCR analysis of putative transgenic plants

Plant genomic DNA was extracted from 100 mg of leaf tissue from transgenic and untransformed tobacco plants using Dneasy Plant Mini kit (Qiagen, Valencia, CA) and then used as templates for PCR and real-time PCRs. PCR primer pairs 16S-FW/aadA-RV and gene-specific primers (Figure 1a) were used to confirm site-specific integration of the gene construct into the chloroplast genome. The real-time PCR analysis was performed using the MJ Mini-Opticon Real-time PCR (BioRad, Hercules, CA) with a two-step amplification programme with post-amplification melt curve analysis. Gene-specific real-time PCR primers and synthetic oligo-nucleotide standard were developed (Table 1). The synthetic oligo was serial-diluted and used as standards for quantification purposes. Real-time PCR sample reactions were produced by preparing a master mix containing the gene-specific primers, BioRad SYBR Green SuperMix and water.

Gene copy number per cell calculation

In order to estimate the transgene copy level per cell for each of the transgenic lines, a mathematical approximation was produced by converting the genome size to cell number. By multiplying the haploid tobacco genome size of 4.5×10^9 bp (Tobacco Genome Initiative Project ID 29347) by the average molecular mass of a double-stranded DNA basepair (660 g/mol), we concluded that one genome mole equals 2.97×10^{12} g. If the genome molar mass is divided by Avogadro's number, we get that a single genome molecule mass equals 4.93×10^{-12} g or 4.93×10^{-3} ng. Then, the amount of DNA in a single cell was calculated by multiplying the single genome molecular mass by four to account for the tetraploid genome of *N. tabacum*, producing a cell value of 1.972×10^{-2} ng. In this case, 1 ng of total DNA will equal to 50.5 cells. Because our real-time PCR quantification calculations are in transgene copies per nanogram, we can now substitute 1 ng by 50.5 cells, and this will provide the transgene copies per cells.

Quantitative RT-PCR for transgene expression analysis

Quantitative RT-PCR was performed to test efficiency of transcription of the transgenes. Total RNA from transgenic and untransformed plants was isolated from 100 mg of frozen leaf tissue by using the Rneasy Plant Total RNA Isolation Kit (Qiagen, Valencia, CA). The RNA samples were treated with DNAse I at a concentration of 100 μg/mL. One microgram

of RNA was reverse-transcribed using the AccuScript cDNA Kit (Stratagene, La Jolla, CA), and the cDNA was then analysed by quantitative real-time PCR as previously described to quantify the transcript levels of the genes of interests.

Leaf discs Hg bioassay

From green healthy leaves of transgenic and untransformed tobacco plants growing in soil, 9-mm-diameter leaf discs were obtained. The leaf discs were placed in RMOP amended $HgCl_2$ to a final concentration of 0, 10, 15 and 20 μM. The leaf discs were incubated for 5 and 10 days under a photoperiod of 16 h of light. The effect of mercury was assessed by quantifying the levels of chlorophyll and fresh tissue weight from triplicate leaf discs per transgenic line and untransformed. Finally, triplicate samples were used to measure Hg accumulation inside the leaf tissues by CVAAS. Chlorophyll was extracted by immersing the leaf discs in 80% (v/v) chilled acetone, and the extract absorbance was then measured.

Seedlings Hg Bioassay

Seeds of untransformed and mt1 transgenic lines were surface-sterilized in 10% (v/v) hypochloride containing 0.1% (v/v) Tween 20. Seeds were kept on a rocking platform for 10 min and then rinsed five times with sterile water. Sterilized seeds were transferred to plates containing Murashige and Skoog medium (Murashige and Skoog, 1962) without sucrose with 0.3% (w/v) phytoagar, pH 5.7. MS media plates were amended with $HgCl_2$ to a final concentration of 0, 10 and 15 μM. Plates were incubated in the dark at 4 °C for 3 day and then maintained at 27 °C with a photoperiod of 16 hrs of light. Thirty days after seed plating, the seedlings were collected and their fresh weight, chlorophyll content and Hg accumulation determined.

CVAAS analysis

Tissue samples were washed three times with ultrapure water to remove any mercury attached to the tissue surface. The tissue samples were then acid-digested by stepwise additions of 70% (v/v) nitric acid, 30% (v/v) hydrogen peroxide and concentrated HCl at 95 C in an adaptation of EPA method 3010A. Blanks and spiked control samples treated as described earlier were used as external quality controls. A standard curve was generated by performing serial dilution of a certified 5000 ppm Hg[II] standard. Triplicate samples were analysed via CVAAS with a continuous flow Vapor Generation Accessory, as described in the PerkinElmer Operation Manual. The study was made in triplicate reactions.

Acknowledgements

Research reported in this article was supported by grants from National Science Foundation NSF-CBET-0755649 and NASA-PRSGC 2006–2008 to O.N.R. Authors acknowledge valuable comments provided by both anonymous reviewers to significantly improve this manuscript.

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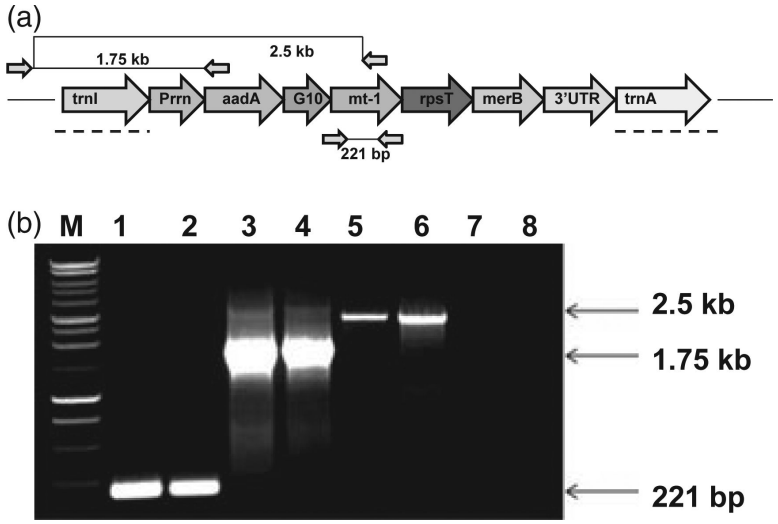

Figure 1.

Confirmation of *mt1* gene integration by PCR. (a) Schematic representation of the transformed chloroplast genome showing PCR primer landing sites and amplicon sizes used for integration analysis. Dashed line indicates the boundaries of the chloroplast homologous recombination sequences. (b) PCR analysis using primer pairs shown in (a) to demonstrate site-specific integration (1.75, 2.5 kb) and the presence of the transgene (221 bp). DNA used was obtained from mt1 transgenic lines 1 and 3 grown in soil. M: marker; 1, 3, 5: mt1 transgenic line 1. 2, 4, 6: mt1 transgenic line 3. 7, 8: untransformed tobacco plant.

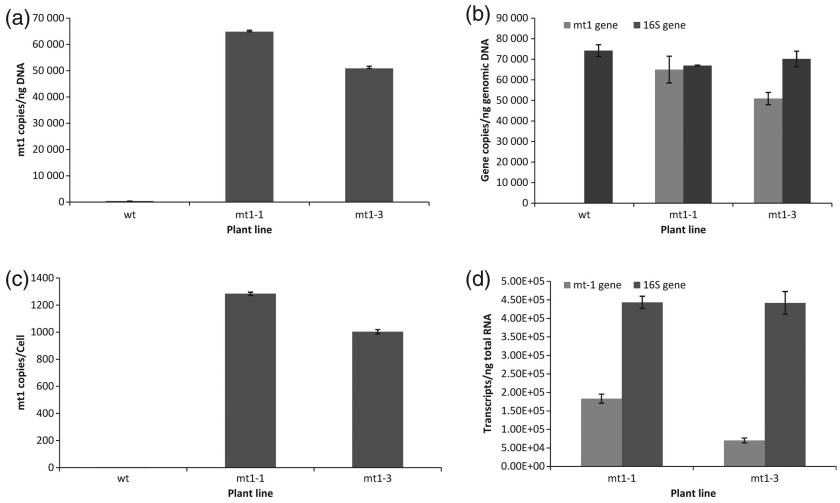

Figure 2.

Quantitative real-time PCR analysis. (a) *mt1* transgene copies per ng of total DNA. (b) % homoplasmy was calculated by comparing the transgene copy level to the copy level of the endogenous 16S rrn gene. (c) *mt-1* transgene copies per plant cell. (d) quantitative RT-PCR analysis of *mt-1* and 16S *rrn* mRNA. Total DNA and RNA were extracted from mature tobacco plants grown in soil. Wt: untransformed plant (growing in soil); mt1 transgenic lines 1 (mt1-1) and 3 (mt1-3) shown. Every sample was tested in triplicates ($n = 3$).

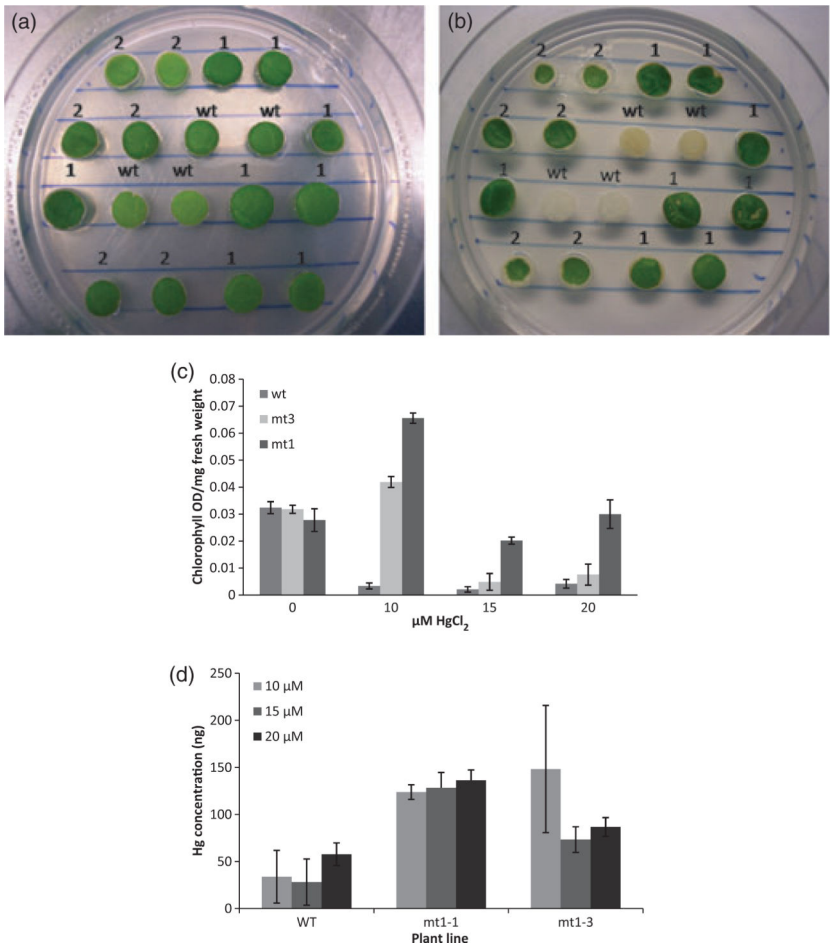

Figure 3.

Leaf discs Hg bioassays. (a and b) effect of 0 and 10 μM $HgCl_2$, respectively, on cellular integrity of *mt1* transgenic and untrans-formed plant leaf tissue. (c) effect of $HgCl_2$ concentration on chlorophyll content. (d) Leaf tissue Hg accumulation was quantified by cold vapour atomic absorption spectrometry. Same size leaf tissue discs were obtained from untrans-formed plant (wt), mt1 transgenic line 1 (1) and mt1 transgenic line 3 (2), placed in regeneration media of plants media with Hg and incubated for 5 days in a 16-h light photoperiod. Analyses were performed in triplicates.

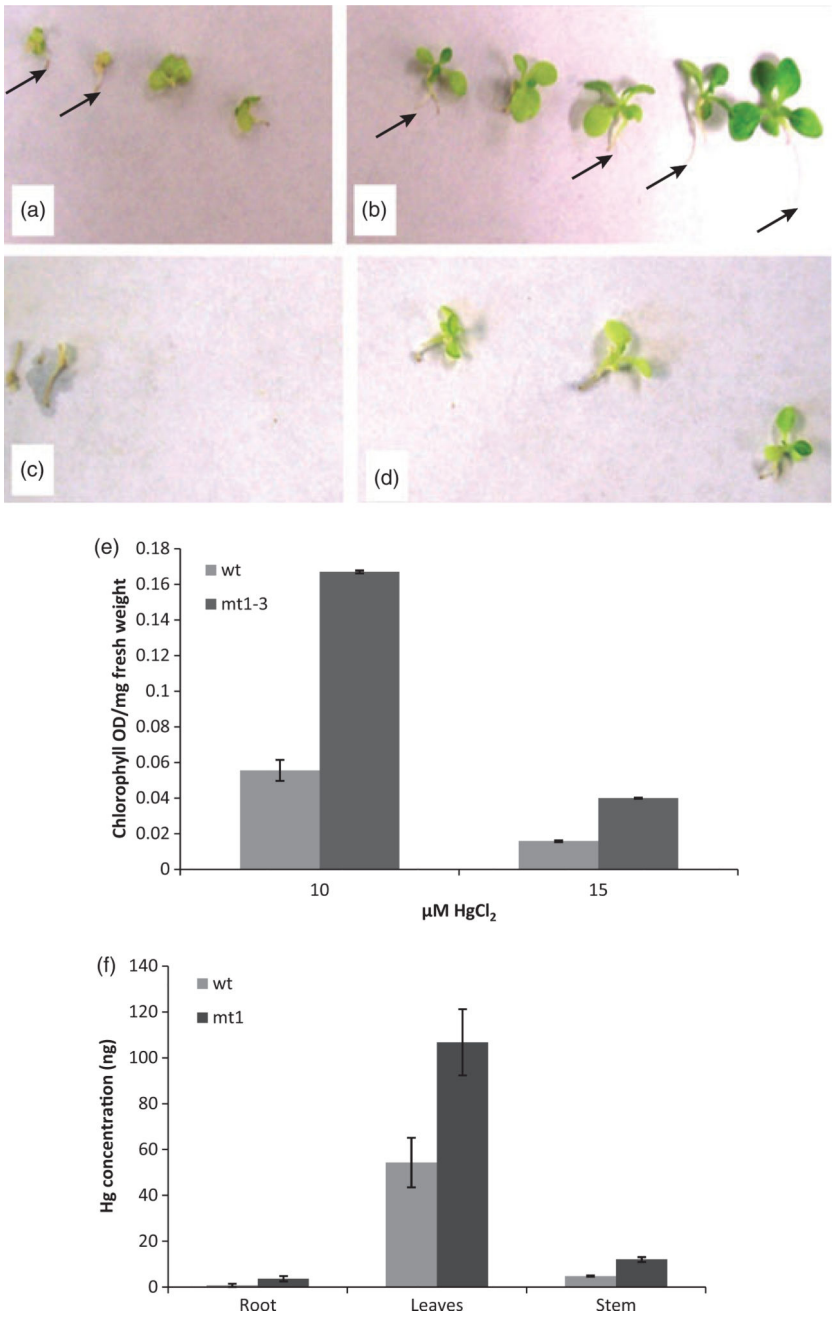

Figure 4.

Seedlings Hg bioassay. (a–d) effect of (a–b) 10 and (c–d) 15 µM $HgCl_2$ on mt1 transgenic line 1 (b and d) and untransformed plant (a and c) seedlings. (e) effect of $HgCl_2$ concentration on seedlings chlorophyll content. (f) Quantification of Hg accumulation in root, stem and leaves by cold vapour atomic absorption spec-trometry. Seedlings were grown in MS media with Hg for 30 days in a 16-h light photoperiod. Black arrows indicate the root growth.

Table 1

Transgene-specific PCR primer sequences, real-time PCR assay primers and synthetic oligo standard

Name	Sequence
FW-mtl	5′-CAGATCTCGGACCCGGGCATATGATGGACCCCAACTGCTCCT-3′
RV-mtl	5′-TTCGTCACAGGATCCTCAGGCACAGCA-3′
FW-g10	5′-GATATCCCCGGGGACTCACTATAGGGA-3′
RV-g10	5′-CATATGTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGAGGG-3′
FW-*mt1* real time primer	5′-GGCGGCTCCTGCACTT-3′
RV-*mt1* real time primer	5′-CAGGAGGTG CACTTGCAGTTCT-3′
mt1 standard oligo	5′-GGCGGCTCCTGCACTTGCACCAGGATATCCGCCTGCAAGAACTGCAAGTGCACCTCCTG-3′