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Sustainable production of myoglobin meat protein in plant chloroplasts

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Alternative routes for producing animal proteins are crucial for reducing the reliance on traditional livestock farming, which contributes significantly to greenhouse gas emissions, deforestation, and water consumption. Myoglobin (Mb) is an important oxygen-binding hemoprotein found in vertebrate muscle which enhances the nutritional and sensorial properties of meat. Due to its unique functionality, Mb has been heterologously expressed in a variety of organisms, although only transient expression in *Nicotiana benthamiana* has been reported for higher plants. In this study, we used chloroplast transformation technology to express porcine Mb in higher plants (tobacco, a non-edible model plant, and lettuce, an edible host) and bovine Mb in the green alga *Chlamydomonas reinhardtii*. Mb accumulation was estimated by immunoblotting and found to be much higher in tobacco (2.7% total soluble protein (TSP)) and lettuce (1.5% TSP) than *Chlamydomonas reinhardtii* (<0.25% TSP). The expression in tobacco chloroplasts is also superior to tobacco nuclear expression (using either the cauliflower mosaic virus 35S promoter or the *Arabidopsis thaliana* ubiquitin promoter). Total heme levels were elevated in myoglobin-producing mutants compared with control plants, although porcine Mb purified from tobacco leaves exhibited approximately 35% heme-binding (compared with 80% heme-binding in *E. coli*-expressed Mb), despite being correctly folded, suggesting that heme availability might be a bottleneck. Overall, our work describes the first report of stable Mb production in higher plants and its effect on photosynthesis and heme levels. This provides a foundation for future plant-made animal proteins for food applications.

KEYWORDS

Chlamydomonas, chloroplast, heme, lettuce, myoglobin, tobacco, transplastomic

1 Introduction

Traditional animal agriculture is a resource-intensive and ethically contentious practice, which contributes significantly to greenhouse gas emissions, deforestation, and biodiversity loss (Koneswaran and Nierenberg, 2008; Anomaly, 2015). In contrast, alternative protein sources like plant-based proteins can provide a more sustainable and scalable solution to meet the increasing demand for protein-rich foods (National Academies of Sciences, Engineering, and Medicine, 2023), especially in a world where food security is increasingly strained by global population growth, climate change, and crop diseases (Lurie-Luke, 2024).

Nutritionally important meat proteins can also be produced by alternative methods, whilst maintaining similar nutritional profiles and functional properties to traditional meat (Carlsson et al., 2020). One such protein is myoglobin (Mb), a 17-kDa oxygen-storage hemoprotein found in cardiac and skeletal muscle of vertebrates, which is composed of a single polypeptide chain, folded into eight alpha helices, that binds a single *b*-type heme (Kendrew et al., 1958). The central iron cation of the heme can make six coordinate bonds: four to the nitrogen atoms of the heme's porphyrin ring, one to the proximal histidine group of the myoglobin chain (H93), and one to a ligand such as oxygen (Suman and Joseph, 2013). The ligand bound at this position determines the perceived colour of muscle, and therefore meat; for example, oxygen ("oxymyoglobin") gives meat a bright-red colour, whereas absence of ligand ("deoxymyoglobin") gives meat a purple-red colour (Suman and Joseph, 2013).

Heme is also an important source of bioavailable iron since it is more easily taken up by the body than non-heme iron, which is much more abundant in plants than heme iron (Fairweather-Tait, 2023). Mb also plays a key role in imparting a "metallic" and umami flavour to meat (Suman and Joseph, 2013). For these reasons, Mb is a particularly interesting product in conferring meat-like properties to meat alternatives; for example, the plant-based food company "Impossible Foods" has recombinantly expressed a soy root nodule phytohemoglobin known as leghemoglobin in the yeast *Komagataella pastoris* for incorporation into their alternative meat products (Santo et al., 2020). Other heterotrophs used for Mb expression include *Escherichia coli* (Varadarajan et al., 1985; Cutruzzola et al., 1996; Meng et al., 2023) and *Saccharomyces cerevisiae* (Shimada et al., 1989; Xue et al., 2023); however, heterotrophic protein production is currently limited in terms of scalability and reliance on expensive and energy-intensive bioreactors and feedstocks (Jareonsin and Pumas, 2021).

In the case of plants, Mb expression has been reported in the leaves of *Nicotiana benthamiana* (a relative of cultivated tobacco) by transient expression using a viral vector delivered by *Agrobacterium tumefaciens* (Carlsson et al., 2020). Although constructs were designed for both cytosol-targeted and chloroplast-targeted human Mb, chloroplast-targeted Mb was abandoned at an early stage due to protein size heterogeneity observed by SDS-PAGE. The cytosol-targeted Mb was purified and demonstrated both heme incorporation and protein functionality. However, transient expression is constrained in scalability for sustainable food protein production. As for stable expression, chloroplast

transformation of plants offers several advantages over nuclear transformation, mainly high transgene expression thanks to the prokaryotic ancestry of the chloroplast, high copy number, and lack of gene silencing mechanisms (Adem et al., 2017). Other advantages include limited environmental dispersion of the transgene thanks to the maternal inheritance of the chloroplast and the lack of a positional effect on gene expression since integration occurs by homologous recombination into a specific site in the chloroplast genome (Adem et al., 2017).

The chloroplast is an interesting site for Mb expression as it is also the site of heme (iron-containing compound of the porphyrin class) production, Mb's prosthetic group (Chen et al., 2024). This pathway, known as the tetrapyrrole pathway, is one shared between the four classes of tetrapyrroles found in plants: heme, chlorophyll, siroheme, and phytychromobilin (Tanaka and Tanaka, 2007). There are also important feedback mechanisms in this pathway which regulate tetrapyrrole production (Tanaka and Tanaka, 2007).

Regarding the transplastomic expression of Mb, there has been one report of chloroplast transformation: the unicellular alga *Chlamydomonas reinhardtii*, specifically the cell wall-less strain CC-5168 (*psbH* mutant TN72), was transformed to express N-terminally FLAG-tagged bovine Mb with the aim of culturing the Mb-enriched biomass as a meat alternative (Phadnis and Prakash, 2024). Mb accumulated to ~0.234 mg/g of dry cell weight in 2-L cultures, still significantly suboptimal compared with animal meat (e.g., 6–7 mg/g for beef) (Phadnis and Prakash, 2024). Despite plant chloroplasts generally having higher transgene expression than algal chloroplasts (Rasala et al., 2011), there are currently no reports in the literature of stable transformation of higher plants for Mb accumulation.

Here, we report the production of Mb via chloroplast transformation of the model plant *Nicotiana tabacum* (tobacco), appreciated for its high transgene expression and ease of transformation (Daniell et al., 2002; Castiglia et al., 2016), and of *Lactuca sativa* (lettuce), a cultivated crop that is also amenable to chloroplast transformation (Kanamoto et al., 2006; van Eerde et al., 2019). As far as the authors are aware, this is the first report of heterologous hemoprotein expression in lettuce. For comparison, we also expressed Mb in these plants by nuclear transformation without specific subcellular targeting and in the chloroplast of *Chlamydomonas reinhardtii*. Porcine and bovine Mb were expressed due to their relevance to the food industry; porcine Mb specifically has a lower susceptibility to autooxidation compared with other orthologs (Suman and Joseph, 2013). Levels of Mb accumulation were quantified, and purified Mb was assessed for heme binding, oxidative damage, and protein folding. The impacts of expressing a heme-binding protein on the photosynthetic performance and on the levels of heme *in planta* were also assessed.

2 Materials and methods

2.1 Plant and algal growth conditions

Sterilised seeds of *N. tabacum* cv. Petit Havana (provided by Dr. Marta Hojka) and *L. sativa* line sK23 (provided by Kyomei Ltd.)

were sown on media containing Murashige and Skoog (MS) salts (Skoog and Miller, 1957) (Sigma) with 3% (w/v) sucrose and 5.8 g/L agar (pH 5.8), as described by Ruhlman (Ruhlman, 2014). Tissue culture conditions were 23 °C at a photon flux of ~40 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (16 h light, 8 h dark). Once large enough, plants were transferred to soil, covered with a transparent cover for 4–5 days to allow for humidity adaptation, and grown in controlled environmental conditions under a diurnal cycle of 16 h light at 23 °C, 50% humidity, and 8 h darkness at 20 °C, 65% humidity. *C. reinhardtii* wild-type strain cc1690 was provided by the Chlamydomonas Culture Collection (University of Minnesota). Strains were grown on 2% (w/v) agar Tris acetate phosphate (Gorman and Levine, 1965) (TAP) plates at 23 °C at a photon flux of ~25 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (16 h light, 8 h dark). Seed tests were carried out on either MS, MS with spectinomycin (500 mg/L for tobacco, 50 mg/L for lettuce), or MS with spectinomycin and streptomycin (both 500 mg/L for tobacco, both 50 mg/L for lettuce).

2.2 Vector design and assembly

All novel plasmids constructed in this work were assembled using Golden Gate assembly (Engler et al., 2008). Codon-optimised myoglobin sequences of *Bos taurus* (*Bt Mb*) and *Sus scrofa* (*Ss Mb*) for tobacco and lettuce chloroplast were based on amino acid sequences retrieved from UniProt (accession numbers P02192 and P02189, respectively). Parental and novel plasmids are listed in the supporting information (Supplementary Table 1).

The myoglobin tobacco vector was constructed from tobacco chloroplast expression vector pKM014, provided by Kyomei Ltd. To construct pKM014, the codon-optimised *Bt Mb* gene (Supplementary Table 2) for tobacco chloroplast expression was first inserted into the *E. coli* expression vector pBSU0 (Neupert et al., 2008), replacing the *GFP* gene at NcoI and HindIII sites, to generate pKM013. The use of U0 as a 5' UTR for the chloroplast is novel; 5' UTRs used for *E. coli* have previously performed well for chloroplast transformation (Ruiz et al., 2011; Wang et al., 2023). The resulting expression cassette was excised as a SacI/HindIII fragment and ligated into the plastid transformation vector pKP9 (Zhou et al., 2008), yielding pKM014. The pKM014 backbone was amplified by PCR using Q5 Polymerase (New England Biolabs) with AG122 and AG123 (Supplementary Table 3). The porcine *Mb* gene sequence was optimised for tobacco chloroplast expression using OPTIMIZER (Puigbò et al., 2007) with the Kazusa database (Nakamura et al., 2000) (Supplementary Table 2). The generated sequence was commercially synthesised (Integrated DNA Technologies) with flanking PaqCI sites and assembled in the pKM014 backbone using PaqCI-mediated Golden Gate cloning to produce vector pAG108.

The myoglobin lettuce vector was constructed from lettuce chloroplast expression vector pKM058, provided by Kyomei Ltd. To construct pKM058, a modified version of pKP9, designated pKM023, was created by replacing the tobacco *trnM-trnG* region with homologous lettuce sequences from the lettuce plastid genome (NC_007578). To enable Golden Gate cloning, undesired Type IIS restriction sites were removed via point mutations, and the BpiI-BsaI cassette from pStA212 (Taylor et al., 2019) was inserted

between the HindIII and SacI sites. The entire pKM023 vector was synthesised by GenScript. The codon-optimised bovine *Mb* gene for lettuce chloroplasts was synthesised and cloned into pBSU0 to generate gKM011. Its expression cassette was then inserted into pKM023 between HindIII and SacI sites to construct pKM058. The pKM058 backbone was amplified by PCR using Q5 Polymerase with AG156 and AG157 (Supplementary Table 3). The porcine *Mb* gene sequence was optimised for lettuce chloroplast expression using the same approach (Supplementary Table 2). The native *psbA* promoter and 5' UTR from lettuce (1541–1671 from GenBank: AP007232.1) followed by the chloroplast codon-optimised porcine *Mb* coding sequence was commercially synthesised (Integrated DNA Technologies) with flanking PaqCI sites and assembled in the pKM058 backbone using PaqCI-mediated Golden Gate cloning to produce vector pAG115.

Porcine *Mb* was also codon optimised for plant nuclear expression (Supplementary Table 2) by the same method and commercially synthesised (Integrated DNA Technologies) with flanking BsaI restriction sites. Nuclear transformation vectors for tobacco were assembled by BsaI-mediated Golden Gate using the plasmids listed: pICH51266 (CaMV35S promoter) or pICSL12015 (*Arabidopsis thaliana* ubiquitin 10 promoter), pICSL60004, and pJK403 (provided by Dr. Jiorgos Kourelis, Imperial College London) with the commercially synthesised nuclear *Mb* gene. The *Chlamydomonas* chloroplast transformation vector pAG102 was assembled by BsaI-mediated Golden Gate using the plasmids listed: pAG101 (provided by Prof. Peter Nixon, Imperial College London), pSS117, pHJ198, pHJ205, and pHJ192 (provided by Prof. Saul Purton, UCL). To build the *E. coli* vector expressing His-tagged porcine *Mb* (pAG112), AG134 and AG135 (Supplementary Table 3) were used to amplify the porcine *Mb* gene from pAG108 with Q5 Polymerase, which was assembled into plasmid pH463 (provided by Dr. Henry Taunt, Imperial College London) by SapI-mediated Golden Gate.

The final transformation vectors were transformed into competent DH5- α *E. coli* cells (New England Biolabs) and purified using the QIAfilter Plasmid Midi Kit (QIAGEN) according to manufacturer instructions. Whole plasmid sequencing (Full Circle Labs, Ltd.) was performed for sequence confirmation.

2.3 Biolistic transformation

Leaves from 6-week-old tobacco and 3-week-old lettuce were used for biolistic transformation, as described previously (Maliga et al., 2021). Briefly, tobacco (1,100 psi, 9 cm distance, abaxial side up) and lettuce (1,200 psi, 9 cm distance, adaxial side up) leaves were bombarded with 0.6- μm gold microcarriers using a PDS-1000/He Biolistic Particle Delivery System (Bio-Rad) on regenerative media (MS supplemented with 3% (w/v) sucrose, 1 mg/L 6-benzylaminopurine (BAP), 1 mg/L thiamine, 0.1 mg/L 1-naphthaleneacetic acid (NAA), 0.1 g/L *myo*-inositol, and 5.8 g/L agar, pH 5.8, for tobacco; MS supplemented with 3% (w/v) sucrose, 0.2 mg/L BAP, 0.1 mg/L NAA, 0.5 mg/L polyvinylpyrrolidone, 0.1 g/L *myo*-inositol, and 5.8 g/L agar, pH 5.8, for lettuce). After 2 days of incubation in the dark at 23 °C, the leaves were cut into explants and placed on regenerative media supplemented with selection (500 mg/L

spectinomycin for tobacco; 50 mg/L spectinomycin for lettuce) until shoots appeared. Shoots were regenerated several times on selection media and then transferred to MS supplemented with the appropriate selection. For *C. reinhardtii*, cells were inoculated into 50 mL liquid TAP medium and cultured over several days at 23 °C with shaking (140 rpm), until the optical density at 750 nm (OD_{750}) reached 0.4–0.7. 40 mL of culture was harvested by centrifugation ($4,000 \times g$, 20 min, 22 °C) and cells were concentrated 20-fold. 200 μ L of cells was spread onto 2% (w/v) agar TAP plates supplemented with 150 mg/L spectinomycin. The cells were bombarded as described previously (Taunt et al., 2023) and incubated at 23 °C at a photon flux of $\sim 5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (16 h light, 8 h dark) for 24 h followed by $\sim 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (16 h light, 8 h dark) until visible colonies formed. These colonies were restreaked under increasing selection pressure: TAP supplemented with 300 mg/L spectinomycin and subsequently TAP with 500 mg/L spectinomycin and 50 mg/L streptomycin.

2.4 Agrobacterium-mediated transformation

300–500 ng of plasmid DNA and 50 μ L of electrocompetent *Agrobacterium tumefaciens* AGL0 cells (provided by Dr. Karen Sarkisyan, Imperial College London) were mixed; electroporation was performed by the MicroPulser Electroporator (Bio-Rad) on setting “Agr”. After adding 400 μ L LB (Sezonov et al., 2007), transformed cells were incubated for 1 h at 28 °C with shaking (200 rpm) and then plated onto LB supplemented with 50 mg/L kanamycin, 50 mg/L streptomycin, and 25 mg/L rifampicin. The plates were incubated at 28 °C for 2–3 days until colonies appeared. Colonies were inoculated into 20 mL of liquid MS and incubated at 28 °C with shaking (200 rpm) until OD_{600} reached 0.6.

Leaves from 10-day-old tobacco seedlings were cut in half and incubated in the *Agrobacterium* culture for 20 min (Mitiouchkina et al., 2020). Explants were placed onto regenerative media (as for biolistics) in the dark at 23 °C for 2 days and then transferred to regenerative media supplemented with 75 mg/L kanamycin and 500 mg/L cefotaxime. Shoots were then transferred to MS supplemented with 100 mg/L kanamycin and 300 mg/L cefotaxime.

2.5 Genotyping

2.5.1 Genotyping by Southern blot

Total DNA was extracted from leaf tissue using the GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific). DNA samples (3 μ g for lettuce, 5 μ g for tobacco) were digested with the restriction enzyme BglII (New England Biolabs) and separated by gel electrophoresis in a 0.8% agarose/Tris-acetate-EDTA (TAE) gel. Denaturation, neutralisation, and capillary transfer to a positively charged nylon membrane (Roche) were performed as described in previous literature (Southern blotting: Capillary transfer of DNA to membranes, 2004). The 541-bp tobacco probe template from the *psaB* gene was amplified from the tobacco genomic DNA by PCR using AG208 and AG209 (Supplementary Table 3) with Q5 Polymerase and purified by running the PCR product on a 1% agarose/TAE gel and using the GeneJET Gel Extraction Kit (Thermo Scientific) according to the manufacturer’s instructions.

The 542-bp lettuce probe template from the *psaB* gene was amplified from the lettuce genomic DNA using the same approach with AG145 and AG212 (Supplementary Table 3) and purified using the GeneJET PCR Purification Kit (Thermo Scientific) according to the manufacturer’s instructions. Probe synthesis, hybridisation, and detection were performed using the DIG-High Prime DNA Labelling and Detection Starter Kit II (Roche) according to the manufacturer’s instructions using positively charged nylon membranes (Roche).

2.5.2 Genotyping by sequencing

Total DNA was extracted from young leaves of tobacco and lettuce plants and sequenced by Wuhan Benagen Technology Company Ltd. Library preparation followed the manufacturer’s short-read protocol: genomic DNA was sheared to ~ 350 bp with ultrasonic fragmentation, end-repaired, A-tailed, ligated to Illumina adapters, purified, and PCR-enriched. Libraries were quantified with a Qubit 2.0 fluorometer, sized on an Agilent 2100 Bioanalyzer, and validated by qPCR. Paired-end sequencing (2×150 bp) was performed on an Illumina NovaSeq 6000 S4 flow-cell, generating >10 -Gb clean data per sample. Raw reads were quality-trimmed (Phred ≥ 30) and aligned in Geneious Prime v.2023.2.1 (Biomatters Ltd.) to the reference chloroplast genomes of *Nicotiana tabacum* (NC_001879) and *Lactuca sativa* (NC_007578) using default settings. Reads-to-reference pairwise identity was calculated as the mean across all mapped reads of (number of matched bases) $\div$ (total aligned positions, including mismatches and gap columns) $\times 100\%$. Consensus sequences were generated using a default threshold of 60% (bases matching at least 60% of total adjusted chromatogram quality). Consensus and variant sequences were visualised and counted in Geneious Prime.

2.5.3 Genotyping by PCR

Genomic DNA from *Chlamydomonas* was extracted by diluting cells in 50 μ L of 5% (w/v) Chelex 100 resin (Bio-Rad). The sample was then vortexed, boiled for 10 min at 95 °C, and centrifuged ($17,000 \times g$, 1 min, 22 °C). 1 μ L of supernatant was used as template per 20- μ L PCR reaction alongside HT492, HT493, and HT494 (Supplementary Table 3) with Phire Plant Direct PCR Master Mix (Thermo Scientific) according to the manufacturer’s instructions; products were run on a 1% agarose/TAE gel.

2.6 SDS-PAGE and Western blotting

Leaf tissue (100 mg) from one plant per line grown in controlled environmental conditions was lysed using the TissueLyser II (QIAGEN) under liquid nitrogen. 200 μ L of native protein extraction buffer (PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4), 5 mM ascorbic acid, 2% (w/v) polyvinylpyrrolidone, 1 cOmplete™ EDTA-free Protease Inhibitor Cocktail tablet (Roche)/50 mL) was then added to the tissue. The sample was thawed on ice and further homogenised by vortexing at 4 °C for 1 min. The lysate was then centrifuged ($15,000 \times g$, 10 min, 4 °C).

Cultures of *C. reinhardtii* strains (2 L) were grown in liquid TAP until OD_{750} reached 1. Cells were centrifuged ($4,000 \times g$,

20 min, 22 °C) and resuspended in 50 mL buffer A (200 mM NaCl, 50 mM Tris-HCl, pH 8.5 buffer, 1 cOmplete™ Protease Inhibitor Cocktail (Roche)/50 mL). The resuspended cells were passed through the precooled Continuous Flow Cell Disruptor (Constant Systems) at 16 kpsi. The lysate was quantified by Bradford assay (Sigma) using bovine serum albumin (BSA) as a standard. The lysate was then ultracentrifuged using the Type 45 Ti Fixed-Angle Rotor (Optima XPN Ultracentrifuge, Beckman Coulter, 35–000 rpm, 40 min, 4 °C). The insoluble fraction was solubilised in lysis buffer (0.1 M NaOH, 0.05 M EDTA, 2% SDS (w/v)) using a paintbrush and quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) with BSA as a standard.

Chlamydomonas samples were quantified using a bovine Mb standard (Worthington Biochemical Corporation), and plant samples were quantified using a porcine Mb standard previously purified from *E. coli*. Protein samples were separated by electrophoresis in 10%–20% Novex™ Tris-Tricine Mini Protein Gels or 4%–20% Novex™ Tris-Glycine Mini Protein Gels (Thermo Scientific). Two gels were run in parallel: after running, one gel was stained overnight in Quick Coomassie Stain (Protein Ark). The other gel was transferred onto a nitrocellulose membrane. Mb was detected with 1:2,000 Rabbit Anti-Sheep Mb primary antibody (ab231725; Abcam) followed by 1:10,000 secondary Goat Anti-Rabbit IgG HRP Affinity Purified antibody (HAF008; Bio-Techne). SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) was applied to the membrane for visualisation. Densitometry was carried out using FIJI (Schindelin et al., 2012). The membrane was stained using Ponceau Staining Solution (5% (v/v) glacial acetic acid, 0.1% (w/v) Ponceau S) to visualise the loading control.

2.7 Protein purification

2.7.1 Mb purification from tobacco

Mb from tobacco was purified by crushing 20 g of the mature leaves (from several 7-week-old plants grown in controlled environmental conditions) into a fine powder with a mortar and pestle in liquid nitrogen. The crushed leaves were then resuspended in 50 mL of buffer B (1mM DTT, 50 mM Tris-HCl, pH 8.5 supplemented with 1 cOmplete™ EDTA-free Protease Inhibitor Cocktail tablet (Roche)/50 mL). The lysate was ultracentrifuged using the Type 45 Ti Fixed-Angle Rotor (Optima XPN Ultracentrifuge, Beckman Coulter, 20–000 rpm, 20 min, 4 °C). The supernatant was filtered through a 0.2-μm syringe filter (Sartorius), and the protein concentration was measured by Bradford assay (Sigma) using BSA as a standard.

For anion exchange chromatography, the Mono Q™ 5/50 GL column (Cytiva) was used, operated by the ÄKTA pure™ chromatography system (Cytiva) at 4 °C. The column was equilibrated with buffer C (50 mM Tris-HCl, pH 8.5). Once the sample was loaded on the system, the column was washed with 10 column volumes of buffer C. An elution gradient of 0–100 mM NaCl over 40 column volumes was applied to the column, as 0.5 mL fractions were collected. The flowthrough was concentrated with 3K molecular weight cut-off (MWCO) MilliporeSigma™ Amicon™ Ultra-15 Centrifugal Filter Units (Sigma) to a final volume of 1 mL and

buffer exchanged into buffer A. Mb was purified by size exclusion chromatography using the HiLoad 16/600 Superdex 75 pg column (Cytiva), operated by the ÄKTA pure™ chromatography system (Cytiva) at 4 °C. The column was equilibrated with buffer A. Mb eluted from the column within one column volume of buffer A, as determined by the 280- and 490-nm absorbance readings. All purified proteins were stored by adding 20% glycerol (v/v) final volume, aliquoted, and flash frozen in liquid nitrogen prior to storage at –80 °C.

2.7.2 Mb purification from *E. coli*

For *E. coli*, KRX cells (Promega) were transformed with pAG112 according to the manufacturer's instructions. Colonies were inoculated into 10–40 mL liquid LB overnight, supplemented with the appropriate antibiotic, and then scaled up to 1–4 L in Terrific Broth (TB) and incubated at 37 °C (200 rpm) until OD₆₀₀ reached 0.8–1. Protein production was induced by adding 1 mL of 1 M isopropyl β-D-thiogalactopyranoside (IPTG) and 5 mL of 1.22 M L-rhamnose per litre of culture and incubating overnight at 18 °C. The cells were harvested by centrifugation (4,000 × g, 30 min, 22 °C), resuspended in 50 mL of buffer A, and then passed twice through the pre-cooled Continuous Flow Cell Disruptor at 32 kpsi. The lysate was ultracentrifuged using the Type 45 Ti Fixed-Angle Rotor (Optima XPN Ultracentrifuge, Beckman Coulter, 30,000 rpm, 40 min, 4 °C). The ultracentrifugation supernatant was added to 4 mL of equilibrated Super Ni-NTA agarose resin (Protein Ark) and incubated overnight at 4 °C with rotation. The lysate–resin mixture was then centrifuged (1,000 × g, 5 min, 4 °C). 10 mL of wash (200 mM NaCl, 50 mM Tris-HCl, 10 mM imidazole, pH 8.5) was added, shaken, and centrifuged (1,000 × g, 5 min, 4 °C). This step was repeated twice more.

This process was repeated twice with 10 mL of elution solution 1 (200 mM NaCl, 50 mM Tris-HCl, 300 mM imidazole, pH 8.5) and once with 10 mL of elution solution 3 (200 mM NaCl, 50 mM Tris-HCl, 500 mM imidazole, pH 8.5). The elution fractions were pooled and concentrated (MWCO 3K) down to 10 mL. At this stage, the protein concentration was measured by Bradford assay (Sigma) using BSA as a standard. 60 units of thrombin (MP Biomedicals) was added per mg of protein. This was incubated at 37 °C overnight with shaking (200 rpm). To capture remaining His-tagged proteins, the digestion mixture was incubated with equilibrated Ni-NTA agarose resin for 1 h at 4 °C with rotation. The lysate–resin mixture was then centrifuged (1,000 × g, 5 min, 4 °C). The supernatant was concentrated (MWCO 3K) to 1 mL.

Mb was purified by size exclusion chromatography using the HiLoad 16/600 Superdex 75 pg column (Cytiva) and operated by the ÄKTA pure™ chromatography system (Cytiva) at 4 °C. The column was equilibrated with buffer A. Mb eluted from the column within one column volume of buffer A, as determined by the 280- and 490-nm absorbance readings.

2.8 Protein analysis

Spectrophotometry was measured using the Cary 60 UV-Vis Spectrophotometer (Agilent Technologies) over 250–700 nm in a 1-cm quartz cuvette. Mb was analysed by LC-MS using the 1290

Infinity II UHPLC (Agilent Technologies) coupled with AdvanceBio 6545XT LC/QTOF (Agilent Technologies). The protein sample was first buffer exchanged into LC-MS-grade H₂O at 4 °C (MWCO 3K). The gradient (0.1% (v/v) formic acid in water as A and 0.1% (v/v) formic acid in acetonitrile as B) was 0%-20% B 0–1 min, 20%-100% B 1–3 min, 100% B 3–4 min, and then 0% B 4–5 min. Data were acquired using Agilent MassHunter for LC/QTOF 12.0 and deconvoluted by Agilent BioConfirm 12.0. The circular dichroism (CD) spectra were measured using the Chirascan V100 CD spectrometer (Applied Photophysics). The masses were analysed by searching for masses using the expected amino acid sequence, with and without the N-terminal methionine.

A sample of ~0.1 mg/mL in 100 mM phosphate buffer (75 mM Na₂HPO₄, 25 mM NaH₂PO₄, pH 7) was measured in a 1-mm quartz cuvette. The average of three spectrum scans was calculated. The pyridine hemochromagen assay was carried out as described previously (Barr and Guo, 2015); calculations were done using the reduced pyridine hemochromagen spectrum with its extinction coefficient of 34.7 mM⁻¹ cm⁻¹ at 557 nm (Paul et al., 1953) for heme *b*.

2.9 Plant physiology analyses

All plant physiology analyses were measured using the fifth leaf from the top of 8-week-old tobacco plants grown in controlled environmental conditions; the averages and standard deviations were plotted from three biological replicates (except dry weight). Statistical significance was calculated Welch's t-test ($p < 0.05$) unless otherwise stated. Photosynthetic parameters were measured using the DUAL-PAM-100 instrument (Heinz Walz GmbH) at 22 °C when the first flower of the plant was about to open. After 30 min of dark adaptation, the maximum quantum efficiency of photosystem II (PSII) in the dark-adapted state (F_v/F_m) was measured. Light response curves were measured for parameters of ETR(II), Y(II), and Y(NPQ). The measuring times at each actinic light intensity were 150 s under light-limited and 60 s under light-saturated conditions. Statistical significance was calculated using two-way repeated-measures ANOVA (genotype $\times$ light intensity, $p < 0.05$). Chlorophyll concentrations were determined as described previously (Espinosa et al., 2012) using the Cary 60 UV-Vis Spectrophotometer (Agilent Technologies) at 663 and 645 nm; chlorophyll *a* and *b* concentrations were calculated as described previously (Lichtenthaler and Wellburn, 1983).

Heme measurement was adapted from Espinosa et al. (2012); the leaf sample (100 mg) was powdered under liquid nitrogen and extracted in 300 μ L of 80% (v/v) acetone containing 20% (v/v) 1.6 M HCl. After 5 min of incubation at room temperature, the sample was centrifuged (17,000 $\times$ g, 10 min, 22 °C). Supernatants were diluted 100-fold with ddH₂O. 10 μ L of the diluted samples was mixed with 40 μ L of apo-HRP (BBI solutions; 250 nM final concentration in 100 mM Tris-HCl, pH 8.4) and incubated at 30 min on a shaker (50 rpm). 50 μ L of Thermo Scientific SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) was added, and after 5 min of incubation, the chemiluminescence signal was quantified using a white 96-well plate (Greiner Bio-One) and the CLARIOstar Plus (BMG Labtech)

microplate reader with integration time 0.5 s. The values were compared with known bovine hemin (Sigma) concentrations; 1 mM stock was prepared in DMSO and then diluted with ddH₂O.

The dry weight of tobacco and lettuce leaves was determined by weighing leaf samples and incubating them in a 65 °C oven, reweighing every 2–3 h until constant mass. Averages and standard deviations were calculated from five biological replicates. Plant pictures were taken with an iPhone 16 (Apple).

3 Results

3.1 Generation of transplastomic tobacco, lettuce, and *Chlamydomonas* Mb-expressing lines

Vectors for myoglobin expression were designed for each organism. For tobacco, the pAG108 construct was based on pKP9 (Oey et al., 2009), which is used as an empty vector control inserting into the *psaB* locus (Figure 1a). The ribosomal RNA operon promoter from tobacco (Prn) was chosen as a strong promoter (Verhounig et al., 2010) and bacterial U0 sequence as the synthetic 5' UTR (Neupert et al., 2008) for porcine Mb expression (Figure 1a). Equivalent endogenous *psaB* insertion sequences were used for the lettuce vector pAG115, and the *psbA* promoter and 5' UTR were used to drive porcine Mb expression (Ruhlman et al., 2010) (Figure 2a). For the *Chlamydomonas* pAG102 vector, the 16S rRNA promoter and *psaA* 5' UTR were used for bovine Mb expression (Taunt et al., 2023), and insertion was targeted to the *psbA* locus (Supplementary Figure 1a).

The constructs were introduced into plastids by biolistic bombardment (Ruhlman, 2014; Maliga et al., 2021). For *Chlamydomonas*, several colonies were obtained, whereas for both tobacco and lettuce, only one mutant plant line was obtained. Antibiotic-resistant lines were subjected to several rounds of selective regeneration. For lettuce and tobacco, Southern blotting analysis (Figures 1b, 2b) and genome sequencing (Supplementary Figures 2a–d) confirmed successful transformation and that the mutant plants were homoplasmic. While previous studies examine homoplasmy using only Southern blotting (Ruhlman et al., 2010; Fatima et al., 2025), here genome sequencing was used as a complementary tool to achieve higher resolution without relying on the availability of restriction sites. Similar approaches are already well-established for mitochondrial DNA (Mok et al., 2024). For *Chlamydomonas*, homoplasmy was confirmed by PCR analysis (Supplementary Figure 1b).

Tobacco and lettuce transformants had normal morphology (Figures 1c, 2c) and were able to produce fertile flowers (Figures 1d, 2d) and functional seeds which survived when sowed on selection, demonstrating the stable nature of the transformation (Figures 1e, 2e). Tobacco and lettuce seed assays revealed that the progenies of the transplastomic lines showed no segregation on spectinomycin and streptomycin-containing media as expected for chloroplast transformants; of the 200 seeds sown, Nt-pAG108 seeds had a $93 \pm 4.4\%$ germination efficiency and a 0% bleaching efficiency as

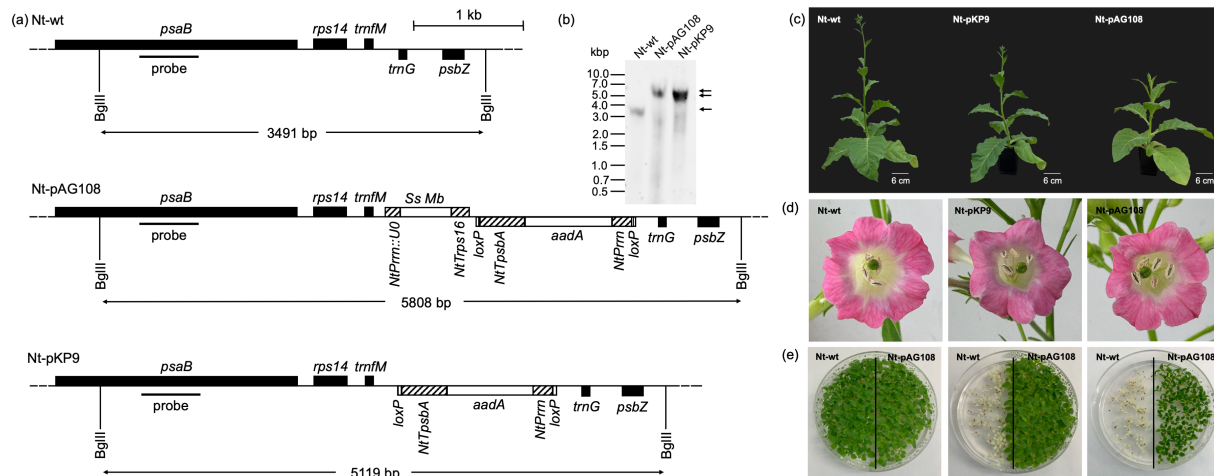


FIGURE 1

Generation of tobacco plastid transformants with vectors pKP9 and pAG108. (a) Map of the target region within the tobacco plastid genome and the design of vectors pKP9 and pAG108 used for tobacco plastid transformation. In both vectors, the transgenes are targeted to the intergenic region between *trnM* and *trnG*. The selectable marker gene *aadA* is driven by the ribosomal RNA operon promoter (NtPrn) followed by the 3' UTR from the plastid *psbA* gene (NtTpsbA), with flanking *loxP* sites for downstream marker removal (Lutz et al., 2006). The porcine myoglobin (Mb) gene (*Ss Mb*) is driven by the NtPrn promoter followed by a synthetic UO 5' UTR (NtPrn::UO) followed by the 3' UTR from the small ribosomal subunit (NtTrps16). (b) Southern blot of total genomic DNA of wild-type (Nt-wt), empty vector control (Nt-pKP9), and Mb-expressing mutant (Nt-pAG108) after digestion by BglII and hybridisation using a DNA probe shown in (a). (c) Growth phenotype of Nt-wt (left), Nt-pKP9 (middle), and Nt-pAG108 (right) 40 days after transfer to soil. (d) Flowers of Nt-wt (left), Nt-pKP9 (middle), and Nt-pAG108 (right) 60 days after transfer to soil. (e) Inheritance assay of wild-type and transplastomic tobacco plants. Seeds from Nt-wt and Nt-pAG108 plants were germinated on medium lacking selection (left), containing spectinomycin (middle) or spectinomycin and streptomycin (right).

opposed to Nt-wt seeds which had a $96 \pm 1.6\%$ germination efficiency and a 100% bleaching efficiency (Supplementary Figure 3a). Ls-pAG115 seeds had an $81.5 \pm 2.0\%$ germination efficiency and a 0% bleaching efficiency, as opposed to Ls-wt seeds which had an $85.5 \pm 1.5\%$ germination efficiency and a 100% bleaching efficiency (Supplementary Figure 3b). When grown in soil under controlled conditions, the transplastomic tobacco lines Nt-KP9 and Nt-pAG108 show a growth retardation phenotype compared with Nt-wt, but no growth stunting phenotype (Supplementary Figure 4).

3.2 Confirmation and quantification of Mb accumulation in transplastomic lines

Analysis of the total soluble protein (TSP) fraction from leaves by SDS-PAGE and Coomassie Blue staining revealed an additional band at 17 kDa in both tobacco and lettuce transplastomic plants (Figure 3a) that was also detected by immunoblotting (Figure 3b). Using the standard curve from known Mb standards (Supplementary Figure 5), the porcine Mb protein accumulation was estimated by densitometry (Ruhlman et al., 2007; Davoodi-

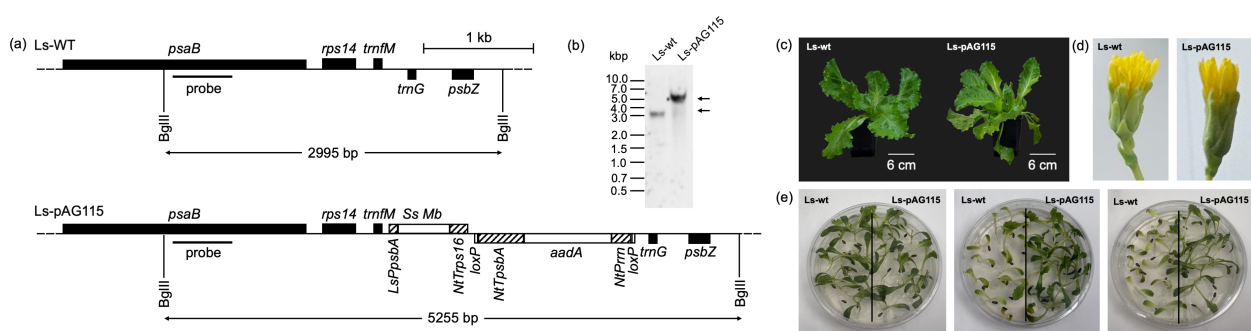


FIGURE 2

Generation of lettuce plastid transformants with vector pAG115. (a) Map of the target region within the lettuce plastid genome and the design of vector pAG115 used for lettuce plastid transformation. The transgenes are targeted to the intergenic region between *trnM* and *trnG*. The selectable marker gene *aadA* is driven by the chimeric tobacco ribosomal RNA operon promoter (NtPrn) followed by the 3' UTR from the tobacco plastid *psbA* gene (NtTpsbA), with flanking *loxP* sites for downstream marker removal. The porcine myoglobin (Mb) gene (*Ss Mb*) is driven by the endogenous lettuce *psbA* promoter and 5' UTR (LsPpsbA) followed by the 3' UTR from the tobacco small ribosomal subunit (NtTrps16). (b) Southern blot of total genomic DNA of wild-type (Ls-wt) and Mb-expressing mutant (Ls-pAG115) after digestion by BglII and hybridisation using a DNA probe shown in (a). (c) Growth phenotype of Ls-wt (left) and Ls-pAG115 (right) 65 days after transfer to soil. (d) Flowers of Ls-wt (left) and Ls-pAG115 (right) 100 days after transfer to soil. (e) Inheritance assay of wild-type and transplastomic lettuce plants. Seeds from Ls-wt and Ls-pAG115 plants were germinated on medium lacking selection (left), containing spectinomycin (middle) or spectinomycin and streptomycin (right).

Semiromi et al., 2010; Gray et al., 2011) and found to be around 2.8% of TSP in tobacco and around 1.5% of TSP in lettuce. To demonstrate reproducibility, porcine Mb protein was quantified by densitometry in the same Nt-pAG108 tobacco transformant from another immunoblot (Supplementary Figure 6), with an estimated quantification of 2.6% TSP.

As a comparison, nuclear transformation of tobacco using the cauliflower mosaic virus 35S promoter (Schnurr and Guerra, 2000) and the *Arabidopsis thaliana* ubiquitin 10 promoter (Grefen et al., 2010) was also performed (Supplementary Figures 7a, 8a). Of the 37 nuclear transformant plants analysed, porcine Mb accumulation was consistently lower (minimum 3-fold) in the leaves of nuclear transformed plants compared with transplastomic plants (Supplementary Figures 7b, 8b). Mb accumulation in *C. reinhardtii* was estimated to be <0.25% TSP, much lower than both tobacco and lettuce (Supplementary Figure 1c). A doublet band is observed in *C. reinhardtii*, likely due to a degradation product of Mb (Supplementary Figure 1c).

3.3 Porcine Mb purification and analysis

Due to its higher protein accumulation, porcine Mb was purified from tobacco leaves by anion-exchange chromatography followed by size-exclusion chromatography to yield a highly pure sample (Figure 4a). LC-MS analysis of the purified porcine Mb protein (full-length expected size of 17,085 Da) showed a single prominent peak with a mass of 16,953 Da, which corresponds exactly to the removal of the initiator methionine (Figure 4b), a common post-translational modification (PTM) in the chloroplast (Apel et al., 2010; Gisby et al., 2011). There are also two secondary peaks, at 17,085 Da and 17,161 Da. The 17,085-Da peak likely relates to Mb with its initiator methionine (predicted mass 17,085 Da), whereas the 17,161 Da peak may be due to a contaminant protein or PTMs. From the LC-MS spectrum, there is very little evidence of oxidative damage of

the protein, which could be a risk due to the oxidative environment of the chloroplast (Foyer and Hanke, 2022). LC-MS also confirms the presence of heme *b* in the sample, expected at 616 Da (Bowman and Bren, 2008); no other types of heme were observed. An absorbance band was observed at 409 nm (Figure 4c), which is indicative of the presence of metmyoglobin (where Mb heme iron is oxidised to Fe^{3+}), as opposed to other forms of myoglobin such as oxymyoglobin where the peak is observed at 418–420 nm (Millar et al., 1996). However, the ratio between the 409- and 280-nm peak was around 1.5, which is less than the ratio of *E. coli*-expressed Mb ratio of 4. This suggests that there is a significant portion of apo-Mb (Mb without heme bound) present in the sample.

To further investigate this, the pyridine hemochromatogen assay (Barr and Guo, 2015) was used to quantify how much heme was present in the sample (Supplementary Figure 9). By using an extinction coefficient of $34.7 \text{ mM}^{-1} \text{ cm}^{-1}$ at 557 nm (Paul et al., 1953) and relating this to the protein concentration determined by Bradford assay using BSA as a standard, the percentage of holo-Mb (Mb with heme bound) was estimated to be around 35%. By comparison, the percentage of heme-binding in *E. coli*-expressed Mb was 80%. The circular dichroism spectrum showed a typical alpha helix pattern as expected for proper folding of Mb (Almeida Ribeiro et al., 2003); the same pattern was observed for *E. coli*-expressed Mb (Figure 4d).

3.4 Physiology measurements of transplastomic Mb-expressing plants

To study the impact of constitutive Mb production on the physiology of the transplastomic tobacco mutants, photosynthetic parameters were measured using a pulse-amplitude-modulation (PAM) fluorometer (Figures 5a–c). Of the parameters measured (effective quantum yield of photosystem II, Y(II) ; electron transport rate through photosystem II, ETR(II) ; quantum yield of non-

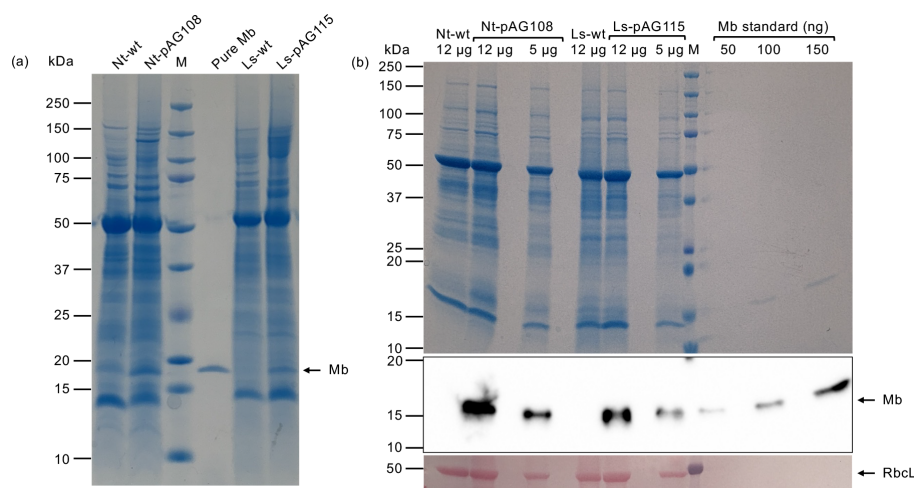


FIGURE 3

Mb quantification in tobacco and lettuce transformants. (a) SDS-PAGE of total soluble protein from wild-type plants and Mb-expressing mutants. Pure Mb was included as a size control. (b) Immunoblot analysis of tobacco and lettuce wild-type plants and Mb-expressing mutants. Immunoblot of total soluble proteins (middle) using an α -Mb antibody confirms Mb expression and Mb standards (50, 100, and 150 ng) allow for quantification of Mb expression by densitometry in tobacco and lettuce as a proportion of TSP. The replicate stained SDS-PAGE gel (top) and Ponceau S staining of the membrane (bottom) show even loading.

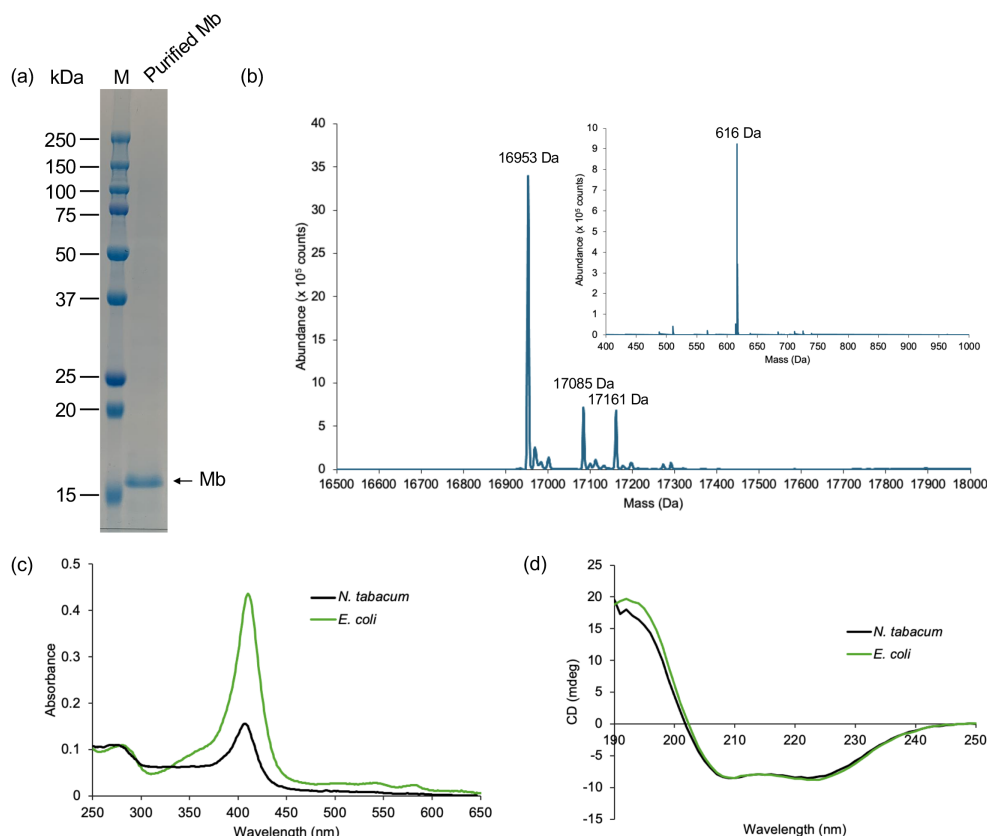


FIGURE 4

Purification and analysis of porcine Mb from transplastomic tobacco leaves. (a) Confirmation of Mb purification from tobacco leaves. Purified Mb was analysed by SDS-PAGE to confirm purity and approximate size of the purified product. (b) Mass spectrometric analysis of purified Mb. Purified porcine Mb from transplastomic tobacco leaves was analysed by LC-MS. Heme from the same sample was also analysed. (c) Absorbance of purified Mb. Absorbance of tobacco-expressed porcine Mb was measured from 250 to 650 nm (black); *E. coli*-expressed Mb was included for comparison (green). (d) Circular dichroism of purified Mb. The circular dichroism of tobacco-expressed porcine Mb was measured from 190 to 250 nm (black); *E. coli*-expressed Mb was included for comparison (green).

photochemical quenching, $Y(NPQ)$), an insignificant difference was observed between Nt-wt, Nt-pKP9, and Nt-pAG108, suggesting that Mb expression did not significantly compromise photosynthetic processes. Total chlorophyll was also measured; Nt-pKP9 (1.98 ± 0.04 mg/g FW) and Nt-pAG108 (1.95 ± 0.05 mg/g FW) showed a significant increase compared with Nt-wt (1.55 ± 0.04 mg/g FW). Interestingly, whilst the chlorophyll *a* to chlorophyll *b* ratio appears equivalent between Nt-wt (3.59 ± 0.15) and Nt-pKP9 (3.56 ± 0.22), there was a slight decrease for Nt-pAG108 (3.18 ± 0.06). Total heme levels determined using the established apo-horseradish peroxidase assay were observed to be similar between Nt-wt and Nt-pKP9; however, Nt-pAG108 showed a twofold increase in total heme levels (Figure 5d).

4 Discussion

In this work, we have produced Mb in the chloroplast of higher plants. The porcine Mb purified from tobacco leaves shows similar physical properties to other mammalian Mb proteins in terms of size, folding, and heme binding. LC-MS reveals that the predominant Mb form lacks the initiator methionine, a common PTM for Mb that also

occurs in mammals such as pigs (Rousseaux et al., 1976; Smerdon et al., 1990). It has been previously demonstrated that removal of the initiator methionine causes only minor changes to the global fold and little to no change to the heme pocket (Phillips et al., 1990). LC-MS also suggests there is little oxidative damage to the protein. Two other minor LC-MS peaks were observed, one relating to Mb with the initiator methionine retained and another unidentified peak, potentially relating to a contaminant protein or post-translational modification. The mass shift does not relate to any single common chloroplast PTM (Lehtimäki et al., 2015); however, PTMs can have an important impact on Mb's tertiary structure, for example cysteine S-nitrosation increases the heme's affinity for oxygen (Helbo et al., 2014). CD confirms correct folding for Mb as an alpha helical protein (Almeida Ribeiro et al., 2003). While the results indicate that the recombinant Mb adopts a structure consistent with correctly folded Mb, additional studies would help determine the extent to which its functional properties match those of native Mb. Future work should include biochemical characterisation of ligand binding, assessment of redox behaviour, and evaluation of colour and flavour performance in food-relevant systems (Carlsson et al., 2020).

As a yield of fresh weight (FW), the Mb yield translated to an estimate of 94 ± 12 mg/kg for tobacco (assuming an average of 2.7% TSP) and 48 ± 2.6 mg/kg for lettuce (assuming 1.5% TSP). As for

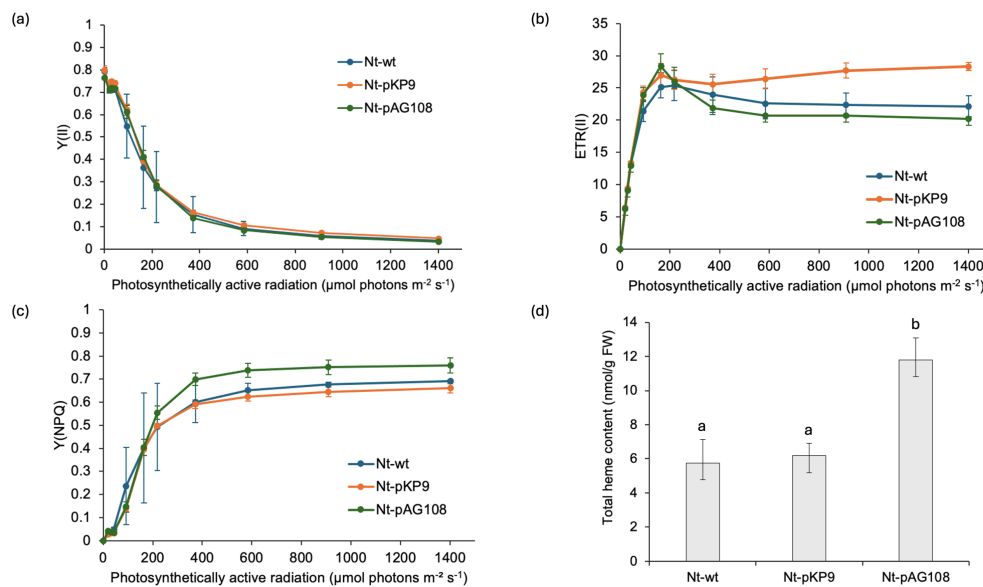


FIGURE 5

Photosynthetic and heme measurements in transplastomic tobacco leaves. (a–c) Photosynthetic parameters of wild-type and transplastomic tobacco. Effective quantum yield of photosystem II ($Y(II)$), electron transport rate through photosystem II ($ETR(II)$), and quantum yield of non-photochemical quenching ($Y(NPQ)$) are measured for Nt-wt (blue), Nt-pKP9 (orange), and Nt-pAG108 (green) as a function of increasing light intensity. Error bars represent standard deviations. (d) Total heme of wild-type and transplastomic tobacco. Total heme content is measured for Nt-wt, Nt-pKP9, and Nt-pAG108. Error bars represent standard deviations and letters represent groups that differ significantly based on Welch's t-test ($p < 0.05$).

yield of dry weight (DW), this corresponded to 800 ± 110 mg/kg for tobacco and 810 ± 60 mg/kg for lettuce. Since quantification was performed on a limited number of biological samples in the present study, the reported quantification values should be considered approximate estimates of protein accumulation. Furthermore, antibody-based quantification may be influenced by factors like epitope accessibility; independent quantification approaches would therefore be valuable for validating recombinant Mb accumulation levels in future studies. Despite the lower accumulation in lettuce as a function of total soluble protein, its higher water content means that the dry weight Mb yields between the two plants are similar. This is a higher accumulation than previously reported for the *C. reinhardtii* chloroplast (44 mg/kg FW, 234 mg/kg of dry cell weight) (Phadnis and Prakash, 2024) but still lower than the purification yield from transient expression in *N. benthamiana* (~ 210 mg/kg FW from agroinfiltration, 60–80 mg/kg FW from agrospray) (Carlsson et al., 2020).

C. reinhardtii exhibited very low levels of bovine Mb protein accumulation ($<0.25\%$ TSP; <0.1 mg/L), and the doublet band in the immunoblot indicated an unexpected lower band, likely due to Mb degradation. These values were lower than those reported in previous literature (0.257 mg/L) (Phadnis and Prakash, 2024), likely due to the difference in strain or regulatory regions used. The data in this previous report did not show a doublet band in the immunoblot, likely due to the use of an anti-FLAG antibody as opposed to the anti-Mb antibody used here. It should be noted that, in the work presented here, different Mb orthologs were evaluated in the plant and algal expression systems: intrinsic differences between the orthologs may also influence protein accumulation, stability, or heme incorporation. Therefore, the present study demonstrates the feasibility of recombinant Mb production in

each host but does not permit a direct quantitative comparison of expression performance across platforms. Future work using a single Mb ortholog across all hosts would allow a more rigorous assessment of host-dependent expression characteristics.

As a comparison with edible animal meat, bovine Mb yield amounts to 8.10 mg/g (8100 mg/kg) dry tissue from psoas major muscle (used for tenderloin/filet mignon) and 11.16 mg/g (11160 mg/kg) dry tissue from longissimus dorsi muscle (used for ribeye steak) (Rickansrud and Henrickson, 1967). Thus, the difference in dry weight Mb accumulation is around 10-fold higher in animal muscle compared with the plant yields described in this report. Despite this, plant cultivation is far more resource efficient than livestock production (Poore and Nemecek, 2018); consequently, plant-derived Mb could achieve protein yields per hectare that rival—or even potentially exceed—those of animal agriculture, whilst also benefiting from substantially lower water use and greenhouse gas emissions (Poore and Nemecek, 2018; Merlo et al., 2024). A dedicated techno-economic analysis (TEA), which is beyond the scope of the present study, could define the exact range of Mb expression level that would be competitive for large-scale, sustainable production. It should also be noted that detailed analyses were conducted using a single independent transplastomic line. Although plastid transformation employs site-specific homologous recombination and usually results in consistent transgene expression amongst transformant lines (Daniell et al., 2002), phenotypic variation arising from tissue culture, regeneration, or somaclonal variation cannot be entirely excluded (Basso et al., 2020). Validation of the observed phenotype in additional independently derived transplastomic lines would further strengthen the generality of these conclusions.

Nuclear accumulation of Mb protein in tobacco using the strong constitutive promoters from the cauliflower mosaic virus

35S or the *A. thaliana* ubiquitin 10 promoter was consistently lower than chloroplast expression in tobacco. Previous work in the literature comparing nuclear and tobacco expression of human somatotropin (hST) protein in tobacco has observed a similar result (Staub et al., 2000). Total heme levels were elevated two-fold in Nt-pAG108, which can be partially accounted for by the expression of Mb; from 35% heme-bound Mb with a yield of 94 mg/kg FW, at least an extra 1.93 $\mu\text{mol/kg}$ FW of heme would be expected (5.5 $\mu\text{mol/kg}$ FW for 100% heme-bound Mb), whereas an increase of 6 $\mu\text{mol/kg}$ FW of heme is observed. Nevertheless, only 35% of the Mb was isolated with bound heme. Similarly, 20% heme binding was observed for porcine Mb expressed in *S. cerevisiae* (Yu et al., 2023).

In the present study, incomplete holo-Mb formation could be due to the expression of an exogenous hemoprotein such as Mb exceeding the capacity of the endogenous heme biosynthetic pathway (Culbertson and Olson, 2010). Competition with chlorophyll biosynthesis for shared tetrapyrrole precursors and inefficient heme insertion into apo-Mb may further contribute to the low heme occupancy. The limitation in heme supply may not only reduce holo-Mb formation but could also constrain overall Mb accumulation, as heme availability is important for the stability and proper folding of the protein (Culbertson and Olson, 2010). Heme accumulation strategies to improve Mb heme loading include engineering plant lines for heme accumulation, for example by overexpressing the terminal enzyme of heme *b* biosynthesis ferrochelatase 1 (FC1) (Page et al., 2020). Alternatively, supplementation of heme precursors, like aminolevulinic acid (ALA) (Wen and Grimm, 2024), could also improve the Mb heme loading by increasing the flux of the chloroplast tetrapyrrole pathway (Tanaka and Tanaka, 2007). The relatively low heme occupancy would most likely compromise downstream applications of plant-produced Mb, due to heme's important contributions to the colour, taste, and nutritional properties of Mb (Suman and Joseph, 2013). Therefore, improving heme availability and incorporation is likely to be an important step towards realizing the full functional potential of plant-produced Mb. As mentioned, heme accumulation could increase Mb yield; other strategies for Mb accumulation could include the use of different regulatory regions and insertion sites, or inducible expression systems (Xu et al., 2024).

There exists a complicated regulatory network for tetrapyrrole synthesis in plants, where heme is considered the primary plastid signal that modulates nuclear gene expression, particularly for the induction of photosynthesis-associated nuclear genes (PhANGs) (Nagahatenna et al., 2015). Heme is also involved in many other processes in plants, such as the mitochondrial respiratory and chloroplast photosynthetic electron transport chains (Fang et al., 2024), and has roles in other contexts such as buffering oxygen in symbiotic nitrogen-fixing plants (Ott et al., 2005). Despite the complex relationship between heme and other plant processes, photosynthetic parameters (Y(II), ETR, and Y(NPQ)) were similar across the three genotypes, suggesting that heme binding to Mb did not impact photosynthetic processes. This is the first evidence that hemoprotein expression in tobacco chloroplasts had no drastic impact on growth or on photosynthetic performance, in part because heme levels were upregulated. While a previous publication described growth defects and reduced photosynthetic fitness in transplastomic tobacco expressing membrane-bound cytochrome

P450 enzymes (Gnanasekaran et al., 2016), it was unclear from this previous work whether these effects were due to changes in heme levels, or the nature of the protein expressed. Other work has also shown that reduced heme levels in the homozygous null ferrochelatase 2 (FC2) T-DNA insertion mutant *fc2-2* (Espinosa et al., 2016) led to a reduction in the overall chlorophyll content and chlorophyll *a* to *b* ratio, and impaired NPQ and Y(II).

Overexpression of hemoproteins in plants can have a variety of effects, depending on the protein: for example, heterologous expression of chloroplast-targeted *Vitreoscilla* hemoglobin in *Hyoscyamus niger* showed no significant difference in growth between control and transgenic plants (Guo et al., 2018), whereas the ectopic expression of chloroplast-targeted soybean leghemoglobin in potato displayed a dwarf phenotype, possibly due to gibberellin limitation (Bonna et al., 2008). Although a growth delay was observed in this work for the tobacco plant expressing Mb (Nt-pAG108) compared with Nt-wt, a similar effect was also identified in the control plant (Nt-pKP9), which would suggest that the transgene insertion site may not be completely neutral.

As for chlorophyll, Nt-pKP9 and Nt-pAG108 plants showed a significant increase compared with Nt-wt, which could also be related to the choice of insertion site. The chlorophyll *a* to chlorophyll *b* ratio appears equivalent between Nt-wt and Nt-pKP9, yet there was a slight decrease for Nt-pAG108. In *Arabidopsis*, knockdown of the heme-binding protein AtHBP5 resulted in a 25% increase in heme content, yet the chlorophyll content and chlorophyll *a* to *b* ratio remain unchanged (Lee et al., 2012). One limitation of the work presented here is that the detailed physiological analyses, including photosynthetic performance and total heme quantification, were conducted only in tobacco, the primary system for characterisation. Although no obvious phenotypic differences were observed in lettuce expressing Mb, we cannot exclude effects on plant physiology and metabolism. Given the relevance of lettuce as a potential food-production host, future studies should assess plant physiology in transformant lettuce and determine the effects of recombinant Mb accumulation.

Aside from heme's practical role in plants, heme is also an important functional molecule in the human diet, meaning Mb can be useful as an additive for plant-based food; in fact, there has been much commercial interest regarding animal protein expression in plants, although very few reports have been published in the literature (Tusé et al., 2024). With increasing interest and concern about the effects of animal agriculture, the work presented here is particularly timely; successful expression of Mb in plants represents a significant step forwards in the development of plant-based ingredients that can replicate the sensory and nutritional qualities of animal-derived products. For recombinant protein production, plants offer several advantages over other organisms; they are highly scalable, are sustainable, and carry no human pathogens (Desai et al., 2010). Compared with plants, animal agriculture is an intrinsically inefficient process: the energy conversion of plant foods to foods of animal origin is estimated to be around 10:1 (Sabaté and Soret, 2014). Using non-allergenic edible crop hosts such as lettuce allows for less stringent purification protocols and regulation hurdles (Desai et al., 2010), as the biomass can be incorporated directly into food matrices with minimal or no purification; this offers potential advantages for scalability and cost. Regulatory

frameworks with ethical, religious, and environmental considerations for the novel area of plant-produced animal proteins are still evolving and are yet to be defined by regulatory bodies (Bobo, 2024; Tusé et al., 2024).

Microbial systems, on the other hand, currently benefit from more established industrial fermentation infrastructure, more predictable product yields, and more streamlined regulatory pathways in certain jurisdictions (Gnaim et al., 2026). Plant-based protein production systems, including chloroplast-based platforms, are still nascent compared with well-established microbial expression systems, which benefit from decades of optimisation, industrial scale-up, and regulatory experience (Gnaim et al., 2026). While the threshold for the commercialisation of recombinant protein production in plants is usually around 0.1%–1% TSP (Twyman et al., 2003), the expression levels required for commercial deployment are highly dependent on the target application, production system, and downstream processing strategy. For Mb, commercial viability will depend not only on total protein accumulation but also on the yield of functional holo-Mb. Consequently, further improvements in both expression level and heme incorporation are likely to enhance the translational potential of the platform.

In summary, this work aligns with current consumer trends and regulatory shifts towards sustainability by advancing protein production in scalable, plant-based platforms. The promise of chloroplast transformation delivers overall in terms of higher protein expression than nuclear expression for Mb, but expression could be further enhanced by exploring heme optimisation strategies, different regulatory regions and growth conditions, and inducible expression systems.

5 Conclusions

In conclusion, the meat protein Mb has been successfully expressed in the chloroplast of *C. reinhardtii* (<0.25% TSP), tobacco (2.7% TSP), and lettuce (1.5% TSP). This is the first known report of heterologous hemoprotein expression in lettuce. The latter two are the first reports in the literature of stable Mb protein accumulation in higher plants. In *C. reinhardtii*, a doublet band is observed in immunoblots, probably due to protein degradation or cleavage. There is little evidence of oxidative damage from Mb expression in the tobacco chloroplast. Fresh weight yields (94 ± 12 mg/kg for tobacco and 48 ± 2.6 mg/kg for lettuce) and dry weight yields (800 ± 110 mg/kg for tobacco, 810 ± 60 mg/kg for lettuce) are promising but still require further optimisation to match animal muscle.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: ENA (EBI). Accession for the study is: PRJEB120971 <https://www.ebi.ac.uk/ena/browser/view/PRJEB120971>.

Author contributions

AG: Investigation, Visualization, Writing – original draft, Writing – review & editing. YL: Investigation, Visualization, Writing – original draft. MF: Investigation, Writing – review & editing. JPW: Validation, Writing – review & editing. SS: Methodology, Formal analysis, Visualization, Writing – original draft. KM: Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing. PN: Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

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Conflict of interest

Authors KM and MF were employed by the company Kyomei Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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