In-Planta studies of Magnesium Chelatase

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Thesis declaration

This thesis contains no material that has been accepted for the award of any higher degree or diploma at any university or institution and to the best of my knowledge contains no material previously published or written by another person, except where due references is made in the text of the thesis.

Abstract

Tetrapyrrole biosynthesis is a key metabolic process in plants, not only because it leads to the production of heme, an essential co-factor for many enzymes, as well as chlorophylls, pigments necessary to the photosynthetic process, but also because this pathway has been implicated in retrograde signaling, a process by which the chloroplast exerts control over nuclear genes with products localized in the plastid. To date there has been extensive research in the characterization and activity of the enzymes involved in the tetrapyrrole biosynthesis, as well as regulation of, and flux through the pathway.

In this field, studies utilizing plants generally employ seedlings and/or the use of herbicides such as norflurazon or lincomycin. However, interpreting the data from these studies can be ambiguous, because the use of herbicides and the process of seedling development both result in large pleiotropic effects. There has been relatively little work done to investigate magnesium chelatase in mature plants, after transition to autotrophic growth. The primary aim of the work presented in this thesis is to investigate the mechanism of magnesium chelatase activity in mature plants. This has been done *in planta* using *Agrobacterium* mediated transient over-expression of magnesium chelatase subunits CHLI, CHLD and CHLH.

Over-expression of CHLD leads to severe chlorosis in *Nicotiana benthamiana* leaf tissue, comparable to that seen when a mutant of CHLI is over-expressed. Based on over-expression of CHLD deletion mutants, the chlorotic phenotype is attributable to the N-terminal half of the protein. CHLD induced chlorosis is almost completely rescued upon co-infiltration with CHLI, and interaction of the

two subunits appears to involve a region of the C-terminal half of CHLD homologous to the Integrin I domain. Upon co-infiltration of CHLI with a CHLD construct mutagenized at the metal ion-dependent adhesion site (MIDAS), chlorosis is still observed.

Enzyme assays of tissue over-expressing individual subunits of magnesium chelatase demonstrate that any perturbation of the correct stoichiometry of the enzyme impairs activity. Moreover, in tissue over-expressing CHLD, which ultimately results in chlorosis, activity is reduced to 39% that of the control (empty vector) on a per chloroplast basis. This level is raised to 83% in tissues where CHLD is co-infiltrated with CHLI.

Proteomic analysis of infiltrated tissues using isobaric tag for relative and absolute quantitation (iTRAQ) shows that leaf tissue over-expressing CHLD (prior to the onset of chlorosis) results in the over-expression of a large range of photosynthetic apparatus proteins. Included in this list are light harvesting and reaction center proteins of photosystem II, cytochrome *f* and cytochrome b_6, as well as most of the subunits on the chloroplast ATP synthase. These data suggest that the plant does not co-ordinate gene expression of the photosynthetic apparatus to chlorophyll biosynthesis. Instead, production of photosynthetic machinery seems to be co-ordinated with that of the D-subunit of magnesium chelatase.

Although only over-expression of wild type CHLD results in chlorosis, whereas CHLI and CHLH over-expression elicit wild type phenotypes, over-expression of any subunit with a GFP-tag (CHLI, CHLD and CHLH) results in chlorosis. Confocal microscopy shows localization of the CHLH-GFP fusion-protein to the

nucleus, in contrast to the CHLI-GFP fusion, which is readily imported and diffusely distributed within the chloroplast. A single infiltration of GFP-tagged CHLD shows GFP-fluorescence in discrete punctate spots localized near, or at, the surface of the plastid membrane. However, co-expression of CHLD-GFP with HA-tagged CHLI results in correct import and diffuse localization of the GFP-tagged CHLD protein. These results suggest that while magnesium chelatase I is able to enter the plastid independently, correct import/localization of CHLD is dependent upon the presence of CHLI.

Abbreviations

5’ RACE	5’ rapid amplification of cDNA ends
6xHis-tag	hexahistidyl-tag
A	Adenosine
AA	amino acid
ABA	abscisic acid
ALA	δ-aminolevulinic acid; 5-aminolevulinic acid
ATP	adenosine triphosphate
BLAST	basic local alignment search tool
cDNA	copy deoxyribonucleic acid
CHLD	magnesium chelatase D-subunit
CHLH	magnesium chelatase H-subunit
CHLI	magnesium chelatase I-subunit
Chlide	chlorophyllide
CPK	creatine phosphokinase
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsDNA	double-stranded deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
ESI-MS	electrospray ionization mass spectrometry
FLP	FLU-like protein
FMNH2	tetrahydrobiopterin dihydroriboflavin mononucleotide
FPLC	fast protein liquid chromatography
GFP	green fluorescent protein
GluTR	glutamyl tRNA reductase
GSA	glutamate-1-semialdehyde
GSA-AT	glutamate-1-semialdehyde aminotransferase
GUN	genomes uncoupled mutants
GUS	*ß*-glucuronidase
HO	heme oxygenase
IPTG	isopropyl-β-D-thiogalactopyranoside
iTRAQ	isobaric tags for relative and absolute quantitation
LB	Luria-Bertani
l-FLP	FLU-like protein, long form
LHCII	light harvesting chlorophyll a/b-binding protein
MALDI	matrix assisted laser desorption ionization
MCS	multiple cloning site
MIDAS	metal ion-dependent adhesion site
mRNA	messenger ribonucleic acid
Mg-chelatase	magnesium chelatase
Mg-Proto	magnesium protoporphyrin IX
NF	norflurazon

OD	optical density
OEP	outer envelope protein
Pchlide	protochlorophyllide
PBS	phosphate-buffered saline
PCMB	*p*-chloromercuri benzoate – membrane permeable
PCMBS	*p*-chloromercuri benzene sulfonate – membrane impermeable
PCR	polymerase chain reaction
PGE	plastid gene expression
pI	isoelectric point
Proto	protoporphyrin IX
RBCS	ribulose 1,5-bisphosphate carboxylase small subunit
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec	secretory pathway of chloroplastic import
s-FLP	FLU-like protein, short form
ssDNA	single-stranded deoxyribonucleic acid
TAP	tobacco acid pyrophosphatase
Tat	twin argenine translocase pathway of chloroplastic import
TCEP	tris-(2 carboxyethyl) phosphine
TEAB	triethylammoniumbicarbonate
TFA	trifluoroacetic acid; trifluoroacetate
TIC	translocon at the inner envelope of chloroplasts
Tm	melting temperature
TOC	translocon at the outer envelope of chloroplasts
Top 10 cells	Invitrogen brand *Escherichia coli* cells
TP	transit peptide
TPRs	tetratricopeptide repeats (protein)
TOF	time of flight
Tris	tris(hydroxymethyl)methylamine
tRNA	transfer ribonucleic acid
$tRNA^{Glu}$	glutamyl-tRNA
UTR	un-translated region
UV	ultraviolet
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

Note: Terms for wild type genes, mutant genes and proteins are represented according to the conventions outlined by the *Arabidopsis thaliana* database (www.arabidopsis.org/nomenclature/namerule.jsp). Namely, that wild type alleles are written in italicized capital letters (*ABC*), mutant alleles are written in italicized lower case letters (*abc*), and the protein products of genes are written in capital letters without italics (ABC).